Opioid-induced AS160 Phosphorylation Through TrkA Transactivated PI3K/Akt Signaling Pathway Implies Neurite Outgrowth

Andreas Blaschke, Anika Heiss and Daniela A. Eisinger
Department of Veterinary Science, Ludwig-Maximilians-University Munich, Institute of Pharmacology, Toxicology and Pharmacy, Koeniginstrasse 16, 80539 Munich, Germany

ABSTRACT

Background:  Opioids have been revealed to promote survival of neuroblastoma x glioma (NG108-15) cells by modulating CSK3β, a down-stream component of PKB/Akt signalling pathway. In this neuronal cell line, we examined here whether opioids may also have an impact on the recently discovered 160 kDa Akt substrate AS160, a Rab GTPase implicated in glucose uptake in non-neuronal cells. Results:  Western blot analysis showed that AS160 is endogenously expressed in NG108-15 cells and cell incubation with the opioids [D-Pen²] Enkephalin and etorphine resulted in a dose-dependent and sustained phosphorylation of AS160 at Thrreonin-642. This effect was blocked by the opioid receptor antagonist naloxone, the PI3K inhibitors LY249005 and wortmannin and the Akt inhibitor VIII. Moreover, inhibition of tyrosine kinase activity of TrkA receptors by the chemical compound AG879 prevented AS160 phosphorylation. In contrast, cell exposure to the PKC inhibitors G06850 and Ro-31-8425 and the IGF-1 receptor inhibitors AG1024 and I-OMeA538 failed to affect opioid-induced AS160 phosphorylation. Confocal microscopy of transfected NG108-15 cells revealed that AS160 regulation by the opioids correlated with translocation of EGFP-tagged glucose transporter GLUT4 from cytoplasm to the cell membrane. Using cells lacking AS160 as a result of transcriptional gene silencing by small interfering RNA, further experiments showed that opioids failed to bring about tubulin polymerization and neurite outgrowth under this condition. Conclusion:  Stimulation of opioid receptors in NG108-15 cells results in AS160 phosphorylation by PI3K/Akt dependent pathway involving TrkA transactivation. The signaling mechanism enables an opiodergic control of AS160 function which correlates with GLUT4 accumulation at the plasma membrane of transfected NG108-15 cells. The finding that absence of AS160 interfered with opioid effects on microtubule stabilization and cellular morphology let us suggest that regulation of AS160 participate in processes underlying opioid-induced neuronal differentiation.

Key words: AS160, opioids, PI3K/Akt, TrkA transactivation, neurite outgrowth, tubulin polymerization

INTRODUCTION

Opioids are well known for their analgesic property, but they exert numerous additional effects through the regulation of diverse cellular signalling pathways. In neuroblastoma x glioma (NG105-15) cells, opioids were previously revealed to promote cell survival. This effect was found to rely on activation of protein kinase B (PKB), also known as Akt which results in modulation of CSK3β activity and inhibition of caspase-3 activation.

By the phosphorylation of different down-stream effector proteins, PKB/Akt is involved in cellular mechanisms regulating cell growth, proliferation and metabolism. Recently, a novel 160-kDa substrate of Akt (AS160) has been identified in 3T3-L1 adipocytes.

AS160, also known as TBC1D4, belongs to the family of Rab GTPase-activating proteins (RabGAPs). Phosphorylation by PKB/Akt is predicted to inhibit the GAP activity of AS160 resulting in active GTP-bound Rab proteins. Rabs control specific intracellular trafficking mechanisms such as translocation of the glucose transporter GLUT4 from cytosolic compartments to plasma membrane in response to insulin. In 3T3-L1 adipocytes, this process is tightly regulated via AS160 phosphorylation, indicating central role of AS160 in glucose uptake. Similarly, AS160 phosphorylation by PKB/Akt also accounts for vasopressin-induced translocation of aquaporin-2 in kidney collecting duct cells.

Besides vasopressin receptors, particular GTP-binding Protein Coupled Receptors (GPCRs) may also act on AS160. Yuasa et al. demonstrated that stimulation of α1b adrenergic receptors and B2...
bradykinin receptors in transfected Chinese ovary hamster (CHO) cells or L6 myotubes promotes AS160 phosphorylation. In contrast to vasopressin receptors, these receptors control AS160 by AMP-activated protein kinase (AMPK), an alternative kinase for AS160 phosphorylation.

G-protein coupled δ-opioid receptors (DOR) have been revealed to stimulate both PI3K-dependent PKB/Akt signaling pathway and AMPK. Although, these findings let suppose a regulation of AS160 by opioids, it has not been documented so far. Thus the present study aimed to examine the phosphorylation of AS160 by opioids and the participating signaling components in neuroblastoma×glioma hybrid (NG108-15) cells, a well known cell system for exploring the signaling of endogenously expressed DOR. In response to AS160 knockdown by small interfering RNA, this cell line also gained insights in potential physiological role of opioidergic AS160 regulation.

MATERIALS AND METHODS

Cell culture and transfection: Mouse neuroblastoma×rat glioma hybrid (NG108-15) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum, hypoxanthine, aminopterin and thymidine at 37°C in a humidified atmosphere and 5% CO₂ as previously described. For transient GLUT4-EGFP expression, cells were transfected with pcDNA3 encoding EGFP tagged GLUT4 (kindly donated by Dr. J. Pessin) by using Metafectene (Biontex Laboratories, Martinsried, Germany). Transfection with AS160 siRNAs or nonspecific control siRNA (60 pmol; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was performed with 4 µL Metafectene S1 (Biontex Laboratories) according to the manufacturer’s recommendations.

Cell treatment: Naïve or transfected NG108-15 cells were seeded onto 12-well plates and allowed to grow overnight. To induce basal AS160 phosphorylation, cells were washed and incubated in glucose-free Krebs-Ringer-HEPES buffer pH 7.4 (123 mM NaCl, 1.3 mM CaCl₂, 5 mM KCl, 100 mM HEPES, 0.2% bovine serum albumin) for 1 h at 37°C before further experiments. Cell treatments comprised incubation with D-Pen²⁵⁸⁶-enkephalin (DPDPE) or etorphine for the times and concentrations indicated in the figure legends, challenge to naloxone 5 min before opioid exposure and pre-incubation with LY294002 and wortmannin for 15 min, or with Akt inhibitor VIII, G06850 or Ro-31-8425 for 30 min. In some experiments, cells were pre-treated with Bromo-5-t-butyl-4-hydroxybenzylidenemalonitrile (AG1024; Merck Chemicals Ltd., Nottingham, UK), α-Cyano-(3-methoxy-4-hydroxy-5-iodocinnamoyl)-(3',4'-dihydroxyphenyl)ketone (I-CmeAG538; Sigma Aldrich, Taufkirchen, Germany) and α-Cyano-(3,5-di-t-butyl-4-hydroxy)thiocinnamamide (AG879; Sigma Aldrich) 30 min before opioid incubation. All reactions were stopped by medium aspiration and subsequent cell solubilization using 150 µL Læmmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% phenol red). The cell lysates were centrifuged (10 min; 15,000×g) before the supernatants were heated for 5 min to 95°C.

Western blot analysis: Cell lysates were separated by 8% (AS160) or 10% (tubulin) SDS-polyacrylamide gel electrophoresis and transferred to PVDF blotting membranes (Pall, Erlangen, Germany). The membranes were blocked for 1 h in Rotiblock® solution (Carl Roth, Karlsruhe, Germany) and incubated overnight at 4°C with antibodies raised against AS160 and phospho-(Thr³⁹⁷)AS160 (Cell Signaling Technology, Danvers, USA), α-tubulin and β-tubulin (Sigma Aldrich). Membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBS/T) and subsequently incubated for 1 h with adequate secondary antibodies coupled to horseradish peroxidase (Promega, Mannheim, Germany). Immuno-reactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences ECL, GE Healthcare Lifescience, Munich, Germany) and quantified by video densitometry.

GLUT4 localization study: NG108-15 cells grown on 22 mm coverslips were transfected with EGFP-tagged GLUT4. On the next day, medium was replaced by glucose-free Krebs-Ringer-HEPES buffer for 1 h. Afterwards cells were treated with naloxone, LY294002 or Akt inhibitor VIII alone or together with DPDPE and etorphine, fixed with 4% paraformaldehyde in phosphate-buffered saline and mounted on glass slides. Distribution of EGFP-tagged GLUT4 was then analyzed by confocal microscopy (Carl Zeiss, Jena, Germany).

Isolation of tubulin polymers: Microtubules were prepared as previously described by Plouffe et al. NG108-15 cells were grown in 100-mm Petri dishes in the presence of forskolin, DPDPE or etorphine. After incubation for 6 h and 12 h, medium was replaced by PM2G buffer containing 1 µM Taxol. Cells were sedimented by centrifugation (1,000×g, 5 min) and resulting pellet was re-suspended in 1.5 ml PM2G buffer containing 1% Nonidet P-40. After incubation for 15 min at 37°C, cell suspension were centrifuged (1,000×g, 5 min), supernatant was removed and the remaining pellet containing microtubules was...
solubilised in sample buffer (125mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol). Samples were heated for 5 min at 95°C and centrifuged at 10,000×g for 5 min. For tubulin analysis, supernatant was finally subjected by gel electrophoresis.

Cell morphology: NG108-15 cells transfected with control or AS160 siRNA were incubated in glucose-free Krebs-Ringer-HEPES without or in presence of DPDPE and etorphine for 12 h at 37°C. Subsequently, cells were examined for morphology under phase contrast microscope.

Statistical analysis: The data are presented as the Means ± SD and were tested for statistical significance by an unpaired Student’s t-test.

RESULTS
Time- and dose-dependent phosphorylation of AS160 by opioids: To find out whether opioids act on AS160, NG108-15 cells were incubated with DPDPE and etorphine for 15 min and analyzed for Thr642-phosphorylated AS160. Revealed by Immunoblot, non-treated controls already exhibited low abundance of phosphorylated AS160 which increased upon opioid exposure in a dose-dependent manner (Fig. 1a). The EC_{50} values calculated for AS160 phosphorylation by DPDPE and etorphine were 17.6 nM and 12.8 nM, respectively (Fig. 1c). AS160 phosphorylation reached maximum by 1 μM DPDPE and 0.1 μM etorphine, so that these opioid concentrations were used during the entire study.

To examine the kinetic of opioid-induced AS160 phosphorylation, NG108-15 cells were incubated with DPDPE or etorphine for different time periods. Both opioids initiated AS160 phosphorylation already within 5 min (Fig. 1b). The maximum effect was reached after 15 min and lasted for up to 1 h (Fig. 1d). Total amount of AS160 protein remained largely unchanged during the incubation periods, indicating that opioid incubation of NG108-15 cells brings about sustained phosphorylation of AS160.

The next experiment was conducted to find out whether opioids trigger AS160 phosphorylation through activation of endogenous DORs. For this, AS160 phosphorylation was analysed in presence of the opioid receptor antagonist naloxone. Incubation of NG108-15 cells with various concentrations of naloxone alone did not affect basal phosphorylation status and total expression level of AS160 (Fig. 1e). However, naloxone interfered with AS160 phosphorylation by DPDPE and etorphine with an IC_{50} of 7.9 and 4.1 μM (Fig. 1f), respectively. Thus, stimulation of DORs seems to account for AS160 phosphorylation by the opioids.

PI3K activity is involved in opioid-induced AS160 phosphorylation: Phosphorylation of AS160 by DORs may originate from AMPK or PKB/Akt stimulated by phosphoinositol-3-kinase (PI3K). To differentiate between these kinases, AS160 was examined in presence of the PI3K inhibitors LY294002 and wortmannin. As shown in Fig. 2a, both inhibitors impaired opioid-induced AS160 phosphorylation in dose-dependent manner. Whereas LY294002 interfered with DPDPE- and etorphine-mediated AS160 phosphorylation with IC_{50} of 149.9 and 46.4 nM, wortmannin blocked the opioid effect with IC_{50} of 14 and 5.6 nM (Fig. 2b), respectively. These findings let suggest that AS160 phosphorylation comes from PI3K-dependent signaling pathway triggered by DOR stimulation.

PKB/Akt, not PKC accounts for AS160 phosphorylation: Two alternative PI3K dependent signalling pathways exist for Thr642 phosphorylation of AS160. One includes activation of PKB/Akt, the other belongs to Protein Kinase C (PKC). To find out which of these kinases participate in AS160 phosphorylation, NG108-15 cells were pre-treated with inhibitors for Akt1/2 or PKC and examined for AS160 regulation in presence of DPDPE and etorphine. As revealed by Western blots (Fig. 3a) and densitometric analysis (Fig. 3b), cell exposure to the Akt1/2 inhibitor abolished both basal and opioid-induced AS160 phosphorylation. In contrast, the PKC inhibitors Ro 31-8245 and G06850 had no effect. Together these findings indicate that PI3K-stimulated PKB/Akt activity is responsible for opioidergic AS160 regulation.

Transactivation of TrkA receptors leads to opioidergic AS160 phosphorylation: In NG108-15 cell, DORs are coupled to the PI3K/Akt signalling pathway through transactivation of IGF-1 and TrkA receptor tyrosine kinases. To confirm these signalling elements for the AS160 phosphorylation pathway, opioid-induced AS160 regulation was examined in presence of inhibitors for these Receptor Tyrosine Kinases (RTKs). Inhibition of IGF-1 receptor by AG1024 abolished constitutive phosphorylation of AS160 in naive cells, but did not affect phosphorylation by DPDPE or etorphine (Fig. 4a, b). In addition, blockade of IGF-1 receptor by I-OMeAG538 had no effect on AS160 phosphorylation. In contrast, inhibition of TrkA receptor activity by AG879 prevented both basal and opioid-triggered AS160 phosphorylation (Fig. 4a, b). Thus, regulation of AS160 by DORs seems to primarily require TrkA receptor activity.

GLUT4 translocation by opioids: In muscle cells and adipocytes, the PI3K-PKB/Akt-AS160 signalling cascade is major regulator of GLUT4 trafficking from
Fig. 1(a-f): AS160 phosphorylation in opioid-exposed NG108-15 cells. (a, b) Dose- and time-dependent regulation of AS160. NG108-15 cells were incubated with (a) different concentrations of DADLE or etorphine for 15 min (b), or with maximum effective doses of the opioids for individual time periods. Controls (cn) remained untreated. Incubation was stopped by addition of sample buffer and cell lysates thus obtained were subjected to immunoblotting using phospho-AS160 (pAS160) and pan-AS160 (AS160) antibodies. (c, d) Densitometry analyses of phosphorylated and total AS160 immunobands of (n = 3–4) independent experiments. In each experiment, ratio of pAS 160 to total AS 160 was determined and normalized to the maximum opioid effect assigned an arbitrary value of 1. Values shown are Mean±SD and (e, f) Effect of naloxone on opioid-induced AS160 phosphorylation. Cells were exposed to different concentrations of naloxone in absence or presence of 1 μM DADLE or 0.1 μM etorphine for 15 min. Shown are representative blots and densitometry analysis of pAS160/AS160 ratio of three independent experiments.
Fig. 2(a-b): Phosphorylation of AS160 requires PI3K activity (a) NG108-15 cells were pre-treated with different concentrations of LY294002 or wortmannin for 15 min followed by incubation with DPDPE or etorphine for another 15 min. Cells free from PI3K inhibitors served as controls (cn). Cell lysates were analysed for total abundance (AS160) and phosphorylation status (pAS 160) of AS 160 by Western blotting. Blots shown are representative for three independent experiments and (b) Densiometry analysis of pAS 160/AS 160 ratio of (n = 3) independent experiments. In each experiment, the pAS 160/AS 160 ratio of cells solely exposed to opioids was assigned an arbitrary value of 1. Values are Mean ± SD. 

Cytosol to plasma membrane. To find out whether opioid-induced AS160 phosphorylation also correlates with GLUT4 translocation, NG108-15 cells were transfected with EGFP-tagged GLUT4 and distribution of the transporter protein was captured by confocal microscopy (Fig. 5). In untreated cell, GLUT4 is distributed throughout the cytoplasm with a strong nucleus-associated accumulation. The nuclei by themselves were free of fluorescence. Cells exposed to DPDPE and etorphine exhibited an accumulation of GLUT4 in the plasma membrane, whereby cell boundary appeared clearly. Nuclei-associated GLUT4 pool, however, largely remained unaffected by opioid treatment. Naloxone exposure prevented GLUT4 appearance in the plasma membrane of opioid-treated cells. In similar, disseminated GLUT4 distribution remained in cells incubated with LY294002 and AKT1/2 inhibitor (Fig. 5). These findings let suggest the activation of DOR-associated PI3K-dependent PKB/Akt activity correlates with translocation of GLUT4 from cytoplasm to cytosol.

Role of AS160 in opioid-induced tubulin polymerization and neurite outgrowth: GLUT4 trafficking requires microtubule network formed by polymerization of tubulin heterodimers. Tubulin polymerization is facilitated by different proteins of the "microtubule-organizing centres", also containing AS160 substrate Rab1111. We were thus interested whether opioids may exert any effects on microtubule through AS160 regulation. For that purpose, AS160 was knocked-down by siRNA transfection of NG108-15 cells (Fig. 6a), further assayed for tubulin polymerization after exposure to opioids or forskolin serving as positive
Fig. 3(a-b): Phosphorylation of AS160 depends on Akt1/2, not PKC activation (a) NG108-15 cells were pre-treated with 1 μM Akt1/2 inhibitor, 1 μM RO-31-8425 (RO-31) or 1 μM GO6800 (GO68) for 30 min and then incubated with 1 μM DPDPE (DP) and 0.1 μM etorphine (etor) for 15 min. Controls (cn) were treated with opioids alone. Phosphorylation (pAS160) and total expression of AS160 (AS160) was determined by immunoblotting. Blots shown are representative results out of three independent experiments and (b) By means of densitometric analysis, pAS 160/AS 160 ratio of (n = 3) independent experiments was assessed and normalized to DPDPE effect in controls (arbitrary value of 1). Values are Mean±SD

Fig. 4(a-b): AS160 phosphorylation involves transactivation of TrkA receptors in NG108-15 cells (a) NG108-15 cells were pre-treated with 10 μM AG1024, 100 μM I-OMeAG538 or 100 μM AG879 for 30 min and then challenged with 1 μM DPDPE (DP) or 0.1 μM etorphine (etor) for 15 min. Cells treated with opioids alone served as control (cn). Cell lysates were examined for phosphorylated (pAS160) and total expression of AS160 (AS160) by Western blotting. Blots shown are representative for (n = 3) independent experiments and (b) Immunobands were quantified by densitometry, assessed as pAS160/AS160 ratio and normalized to DPDPE effect in controls (arbitrary value of 1). Values are Mean±SD from (n = 3) independent experiments.
Fig. 5: GLUT4 translocation in opioid treated NG108-15 cells, NG108-15 cells transfected with EGFP-tagged GLUT4 were incubated with 1 µM DPDPE or 0.1 µM etorphine for 30 min in presence of 10 µM naloxone, 10 µM LY294002 or 1 µM Akt1/2 inhibitor. Controls remained untreated (cn). Cells were fixed and examined for EGFP-tagged GLUT4 localization by confocal microscopy. Shown are images representative for three independent experiments. Plasma membrane localization is indicated by arrowheads (a) cn, (b) DPDPE and (c) Etorphine

Fig. 6(a-d): Continue
In NG108-15 cells transfected with control siRNA, forskolin treatment increased the amount of tubulin polymers compared to untreated controls (Fig. 6b, c). In similar, opioid treatment for 6 h induced an increase of tubulin polymers by 30-40%, reaching forskolin-like effect after incubation for 12 h. In NG108-15 cells lacking AS160, forskolin and the opioids failed to alter level of tubulin polymers. These findings let us suggest that tubulin polymerization by opioids as well as forskolin implies AS160.

Increase of tubulin polymers in NG108-15 cells may be associated with neurite outgrowth. We thus further examined whether opioid treatment may induce any morphological changes by AS160-dependent mechanism. As captured by phase-contrast microscopy, naïve NG108-15 cells have round and spherical cell bodies (Fig. 6d). After a 12 h-treatment with DPDPE or etorphine, most of the cells exhibited neurites and extensions. NG108-15 cells transfected with AS160 siRNA, however, remained in round shape despite opioid exposure. Thus opioid-induced neurite outgrowth seems to involve AS160 in NG108-15 cells.

**DISCUSSION**

AS160 is one prominent PKB/Akt substrate which plays a central role in insulin-induced GLUT4 translocation and thus glucose homeostasis. Although the RabGTPase is widely expressed including neuronal cells, its regulation and function by non-insulin stimuli is largely unknown. In the present study we show that opioids induce phosphorylation of AS160 and analysed the underlying signaling pathway. By using AS160-knockout cells we also show that AS160 regulation seems to be involved in the induction of neurite outgrowth in opioid-treated NG108-15 cells.

Opioids are known for many different cellular effects that originate from stimulation of opioid receptors. However, alkaloid opiates such as
etorphine may also modulate cellular responses in receptor-independent manner. Phosphorylation of AS160 in presence of DPDPE and etorphine occurred dose-dependently with EC50 values that are similar binding affinities reported for the opioids to DORs. In addition, AS160 phosphorylation was blocked by the opioid receptor antagonist naloxone in dose-dependent manner with EC50 values comparable to naloxone binding affinity evaluated for DORs. Together these findings indicate that AS160 phosphorylation by opioids originates from signaling pathway that is coupled to endogenously expressed DORs in NG108-15 cells. Although, AS160 is named “Akt substrate”, several protein kinases and signaling pathways may lead to Thr642 phosphorylation of AS160 such as AMP-activated protein kinase (AMPK) or PKB/Akt and PKC, each activated by PI3K. A previous study revealed that DORs expressed in transfected CHO cells may activate AMPK by Gα11-dependent pathway, but failed to do so in SH-SY5Y neuroblastoma cells and primary olfactory bulb neurons. In neuronal cell including NG108-15 cells, opioid receptors are mainly coupled to G proteins inhibiting adenyl cyclase activity and cAMP synthesis. As AMPK activity requires cAMP, opioids rather trigger AS160 phosphorylation by alternative, AMPK-independent kinases. Indeed, opioid-induced AS160 phosphorylation was impaired by the selective PI3K inhibitors LY294002 and wortmannin. These compounds interfered with AS160 phosphorylation with potencies similar to that reported for PI3K inhibition, which strongly indicates PI3K-dependent signaling pathway for AS160 modification. Potential PI3K downstream targets involved in AS160 phosphorylation are PKB/Akt or classical and novel isoforms of PKC. As inhibition of these PKC isoforms by G06850 and Ro-31-8245 did not prevent AS160 regulation by the opioids, AS160 seems to represent a substrate of the typical PI3K/Akt signaling pathway in NG108-15 cells. This notion is further supported by the finding that inhibition of the PKB/Akt isoforms 1 and 2 by the Akt inhibitor VIII abolished opioidergic AS160 regulation. Akt1, also known as PKBα, is ubiquitously expressed and implicated in regulation of cell survival and proliferation by modulating gene expression. In contrast, PKBβ/Akt2 is widely accepted to play a central role in glucose homeostasis by regulating GLUT4 translocation upon AS160 phosphorylation. These findings let us thus suggest that AS160 regulation by opioids comes from Akt2 stimulation in NG108-15 cells. We have previously shown that PKB/Akt stimulation by opioids occurred from transactivation of IGF-1 and TrkA receptors in NG108-15 cells. Therefore, it was surprising that solely TrkA receptors participate in AS160 phosphorylation by the PI3K/Akt signaling pathway. This observation might indicate that TrkA and IGF-1 receptor signaling targets different PKB/Akt isoforms, in that IGF-1 receptors signal to the neuroprotective Akt1 and TrkA receptors to the AS160-regulating Akt2. This notion is supported by the finding that Akt1 is predominantly activated in response to IGF-1 receptor stimulation, whereas TrkA stimulation may activate all PKB/Akt isoforms equally. The modulation of AS160 by TrkA-transactivated PI3K/Akt pathway further questions the kinetic seen for AS160 phosphorylation. Activation of PKB/Akt by opioids was shown to be transient, whereas phosphorylation of the Akt substrate is long-lasting effect. Phosphorylation status of proteins is controlled by processes of phosphorylation and de-phosphorylation. Here PKB/Akt was identified to be mainly responsible for the opioid-induced AS160 phosphorylation, so that continuation of Thr642 phosphorylation by other kinases such as AMPK or PKC is thus rather unlikely. Alternatively, opioids may affect activity of phosphatases required for AS160 de-phosphorylation. Recently, Protein Phosphatase 1 (PPP1) was uncovered to de-phosphorylate AS160. In this context it is from interest that opioids may stimulate KEP1, a powerful PPP1 inhibitor in neuronal cells. Thus inhibition of de-phosphorylation might account for sustained AS160 phosphorylation seen upon opioid exposure. It was shown that sustained AS160 phosphorylation by insulin coincided with trafficking of GLUT4 to the plasma membrane. In similar, this effect was also observed for GLUT4 expressing NG108-15 cells treated with DPDPE or etorphine. The opioids provoked translocation of GLUT4 in dependence of PI3K and PKB/Akt which let suggest that also sustained AS160 phosphorylation is associated with this process. However, direct correlation of GLUT4 translocation with opioid-induced AS160 phosphorylation must be seen critical, as a central role of PI3K and PKB/Akt activity has been demonstrated for GLUT4 translocation in adipocytes without implication of AS160 regulation. Thus alternative, yet not further characterized substrates of the PI3K/Akt pathway may be involved in GLUT4 translocation as well, so that the impact of AS160 phosphorylation in opioid-induced GLUT4 translocation has to be demonstrated by separate experiments using a phosphorylation deficient AS160 mutant. Translocation of GLUT4 by opioids was visualized in an artificial cell system, so that the physiological function of AS160 phosphorylation by opioids in NG108-15 cells still remained to be examined. The control of GLUT4 trafficking by AS160 is related to its GAP activity on Rab proteins, especially Rab8 and 11. Whereas Rab8 is associated to endosomes, Rab11 is found
at microtubule-organizing centres that regulate microtubule network. GLUT4 translocation requires organized cytoskeleton, so that AS160 might promote trafficking by stabilizing microtubule, comprising polymers of tubulins. Interestingly, the DOR agonist [D-Ala²,D-Leu⁵]Enkephalin has been recently shown to bring about tubulin polymerization in neuronal progenitor cells by yet unknown signalling pathway. This observation prompted us to examine whether regulation of AS160 plays a potential role in the opioid effect. Indeed, long-term treatment of NG108-15 cells with DADLE or etorphine was accompanied with tubulin polymerization and our knock-out studies revealed that this process requires presence of AS160. In fibroblasts, PI3K/Akt signaling was already identified to account for microtubule stabilization which might thus also implicate AS160 as a prominent down-stream regulator in this process. Considering additionally the control function of AS160 on the microtubule organizer Rab11, participation of AS160 in tubulin polymerization is thus still conceivable. Future investigations focusing on AS160 and Rab11 regulation by PI3K/Akt signaling may bring further insights and thus understanding of microtubule dynamics induced by opioids as well as other extracellular signals and receptors.

Tubulin polymerization induced by forskolin which was used as positive control, also depends on AS160. This observation might be surprising, as forskolin exert completely different cellular effects than the opioids. For instance, forskolin enhances and opioids inhibit cAMP production. In contrast to opioids, tubulin polymerization is long known. cAMP-triggered forskolin effect in its effect on AS160, however, a completely new aspect. In similar to the opioids, forskolin might control AS160 via, PI3K-dependent PKB/Akt signalling which may be stimulated by Epa, a cAMP-dependent RapGEFs. Otherwise, cAMP-triggered activation of AMPK might also account for AS160 regulation by forskolin. Which of these potential signaling pathways is implicated in AS160-dependent tubulin polymerization by forskolin in NG108-15 cells, has to be examined in a separate study.

An increase of tubulin polymerization upon chronic opioid challenge may display cellular reorganization or remodelling which is reflected in cell morphology. In NG108-15 cells, such remodelling processes are accompanied by neurite outgrowth which requires enlargement of the plasma membrane by the exocytosis of specific vesicles. As Rab proteins are typical coordinators of vesicle trafficking, it is thus not surprising that also AS160 substrates Rab 8 and 11 were identified to play a central role in neurite outgrowth. Evidence for AS160 involvement in neurite outgrowth, however, is still missing. Although, changes in neuronal cell morphology is often observed upon long-term opioid exposure, the underlying mechanism and signalling components are also largely unknown. Neurite outgrowth induced by opioids was revealed by our study to require presence of AS160 which gains a new insight in function of the Akt substrate. Moreover this observation let suppose TrkA-mediated PI3K/Akt signaling pathway for opioid-induced neurite outgrowth. Indeed, several recent findings indicate a possible involvement of PI3K/Akt and TrkA receptors in organizing cell morphology. For example, transactivation of TrkA receptors by G-protein coupled angiotensin receptors promotes neurite outgrowth of NG108-15 cells. Furthermore, PI3K-dependent PKB/Akt signaling is presumed as key mediator of neurite outgrowth in neuronal cells. In addition, the AS160 substrate Rab 8 has a strong influence on reorganization of microtubule and is thus considered as regulator of cell shape during cell differentiation. Considering all these observations, regulation of AS160 might be thus involved in opioid-induced neuronal differentiation which is discussed to be responsible for chronic opioid effects such as tolerance and dependence. It is now from great interest to determine whether regulation of RabGAP AS160 by TrkA-dependent PI3K/Akt signalling pathway might also participate in adaptive cell responses to chronic opioid exposure.

ACKNOWLEDGMENT
The authors thank Dr. J. Pessin (Albert Einstein College of Medicine, N.Y., USA) for donating the pcDNA3 plasmid encoding EGFP-tagged GLUT4.

REFERENCES


