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δ-Opioid receptors stimulate ERK1/2 activity in NG108-15 hybrid cells by integrin-mediated transactivation of TrkA receptors

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Abstract **This study demonstrates that activation of δ-opioid receptors (DORs) in neuroblastoma × glioma (NG108-15) hybrid cells by [D-Ala^2, D-Leu^5]enkephalin (DADLE) and etorphine significantly enhances cell adhesion to fibronectin-coated wells. This effect is blocked by both naloxone and integrin binding RGDT peptides. In addition, cell adhesion turned out to be a prerequisite for DOR-stimulated transactivation of Tropomyosin-related kinase A (TrkA) and extracellular signal-regulated kinases 1/2 (ERK1/2). Because inhibition of TrkA activation by AG879 completely blocked DOR- and integrin-mediated ERK1/2 signaling, the present results indicate that in NG108-15 cells DOR-stimulated ERK1/2 activation is mediated by integrin-induced transactivation of TrkA.**

Keywords: Cell adhesion; δ-Opioid receptor; Integrin; Extracellular signal-regulated protein kinase; Tropomyosin-related kinase A

1. Introduction

δ-Opioid receptors (DORs) belong to the family of G protein-coupled receptors that bring about their action by stimulation of inhibitory G proteins [1]. Acute DOR activation results in attenuation of neuronal excitability, which is mediated by inhibition of adenylyl cyclase activity, voltage-dependent calcium channels or activation of inwardly rectifying potassium conductances [2]. Besides these classical effector systems, a number of studies indicate that DORs may also couple G proteins of the $G_{q/11}$, $G_{12/13}$ and G_{14} family, which regulate more complex intracellular signaling pathways, such as the extracellular signal-regulated kinases 1/2 (ERK1/2) or the PI3K/Akt cascade [3]. These signaling pathways are thought to contribute to long-term opioid effects, such as tolerance/dependence [4], impaired learning and memory behavior [5] or increased neuroprotection and cell survival [6,7].

Another non-classical aspect of opioid action has been recently identified for peripheral DORs. Pello et al. [8] demonstrated that incubation of monocytes with the DOR agonist [D-Ala^2, D-Leu^5]enkephalin (DADLE) triggers cell adherence to the extracellular matrix (ECM) protein fibronectin (FN), an effect that is mediated by activation of integrins. Integrins are a large family of heterodimeric, transmembrane cell-surface glycoproteins originally identified as mediators of cell adhesion and migration [9]. Besides their regulatory effect on the rearrangement of the cytoskeleton, certain integrin dimers may also influence cell proliferation and growth by activation of mitogenic signal transduction pathways [10]. Although DOR-stimulated cell adhesion is thought to play a critical role in leukocyte trafficking [8], the intracellular pathways mediating this integrin-stimulated processes are still unknown. Because integrins regulate opioid receptor signaling in trigeminal ganglion neurons [11] and modulate G protein-coupled receptor signaling to ERK1/2 [12], the present study was performed in order to investigate whether DOR activation also results in stimulation of cell adhesion and regulation of intracellular ERK1/2 signaling in neuronal cells.

2. Materials and methods

2.1. Materials

An original stock of NG108-15 hybrid cells was obtained from the American Type Culture Collection (ATCC). Ligands and chemical inhibitors were from the following companies and institutions: DADLE, Bachem; etorphine, National Institutes of Health; naloxone, RGDT peptides AG879, AG1478, BPIQ-I, Merck/Calbiochem. All primary antibodies were purchased from New England Biolabs, horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Promega.

2.2. Cell culture

NG108-15 cells were cultured as described in Dulbeccós modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine, at 37 °C in a humidified atmosphere of 5% CO_2 in air [13].

2.3. Cell adhesion assay

This was done according to the procedure described for monocytes [8]. Briefly, NG108-15 hybrid cells grown in log phase were detached, washed and resuspended into serum-free culture medium. Cells (1×10^5) were seeded onto FN (0.5 ng/well)-coated 96-well plates and allowed to adhere for 1 h at RT either in the absence (control) or presence of DADLE (1 μM) and etorphine (0.1 μM). Non-specific binding was blocked by preincubation of the plates with 2% (m/v) BSA for 30 min at RT. In some experiments, cells were pretreated with naloxone (10 μM, 10 min) or RGDT peptides (1 mM, 60 min). Incubations were stopped by bottom-up centrifugation of the plates ($30 \times g$, 5 min) and subsequent fixation of adherent cells with 4% (m/v) paraformaldehyde solution. Cells were stained with 0.1% (m/v) crystal violet, solubilised with Triton-X-100 and color reaction was photometrically measured at $\lambda = 570$ nm in a Tecan® Spectra multiplate reader. Each experiment was done in triplicate determination.

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Abbreviations: DADLE, [D-Ala^2, D-Leu^5]enkephalin; DOR, δ-opioid receptor; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; FN, fibronectin; NG108-15 cells, neuroblastoma × glioma (NG108-15) hybrid cells; TrkA, tropomyosin-related kinase A

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2.4. ERK1/2 and tropomyosin-related kinase A (TrkA) activation

Subconfluent cells grown on 12-well plates were serum starved for 2 h before experiments were conducted. In some experiments, integrin (RGDT peptides, 1 mM) as well as receptor tyrosine kinase inhibitors (AG1478, 5 μM; BPIQ-I, 10 μM; AG879, 100 μM) were added during the preincubation period for 30 and 15 min, respectively. Cells were stimulated for 5 min at 37 °C in the presence of various opioids at the concentrations indicated. Reactions were stopped by aspiration of the medium and the addition of 150 μl of ice-cold Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% phenol red).

2.5. Western blot

Solubilized proteins were resolved by electrophoresis over 10% (m/v) SDS–polyacrylamide gels, transferred to PVDF-membranes and subjected to immunoblot detection as described [14]. Overall abundance of ERK1/2 was evaluated using a pan-reactive ERK1/2 antibody, whereas activated ERK1/2 was determined by a phospho-specific Thr202/Tyr204 antibody (1:1000 each). Activated TrkA was determined by a phospho-Tyr490 specific antibody (1:1000). The blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:15000) and subsequently developed using enhanced chemiluminescence (Amersham). The intensity of immunoreactive bands was evaluated using a Herolab E.A.S.Y. RH-5 video densitometer.

3. Results and discussion

3.1. δ-Opioid receptors stimulate NG108-15 cell adhesion

To investigate a possible involvement of integrins in DOR-stimulated ERK1/2 activity, the effect of opioids on cell adhesion, a typical integrin-mediated process, was determined in NG108-15 hybrid cells. This cell line has a long history in investigating acute and chronic opioid actions and carries high levels of endogenous α5/β1 integrins that associate with ganglioside GM1 thereby modulating DOR activity [15]. In experiments similar to that previously conducted with monocytes [8], NG108-15 cells were seeded onto fibronectin (FN)-coated wells and incubated for 5 min either in the absence or presence of DADLE (1 μM) to allow cell adhesion. As shown in Fig. 1A, opioid treatment substantially increased the number of cells bound to the integrin ligand FN. In addition, DADLE treatment also resulted in the formation of spines in some NG108-15 cells (Fig. 1A, arrows). Quantification of adherent cells verified that DADLE treatment indeed significantly stimulates cell adhesion by about 5-fold as compared to untreated controls (Fig. 1B). A similar effect was observed for the opioid alkaloid etorphine (100 nM, 5 min), indicating that stimulation of cell adhesion is not restricted to peptide agonists (Fig. 1B). Opioid-stimulated NG108-15 cell adhesion was abolished by coincubation of the cells with both the competitive opioid receptor antagonist naloxone (10 μM, 10 min) as well as integrin blocking RGDT peptides (1 mM, 12 h). These findings suggest that opioids possess the capacity to stimulate cell adhesion not only in monocytes [8], but also in cells of neuronal origin. In addition, they also indicate that opioid-induced binding of NG108-15 cells to FN is mediated by α5/β1 integrins, two integrin isoforms abundant in neuronal cells and responsible for dendritic spine formation in hippocampal neurons [16,17]. Because activation of integrins may result in cell proliferation [9], we next investigated whether DOR-stimulated cell adhesion is associated with activation of the ERK1/2 signaling pathway.

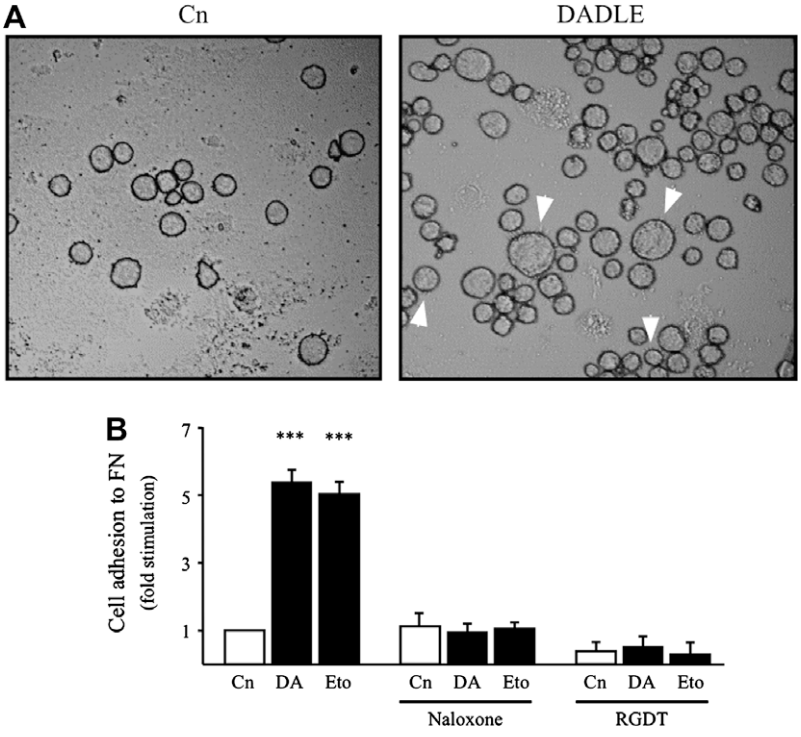

Fig. 1. DOR activation enhances NG108-15 cell adhesion. NG108-15 cells were plated onto FN-coated wells and incubated either in the absence (Cn) or presence of DADLE (1 μM) and etorphine (100 nM). Incubations were stopped by bottom-up centrifugation before cell adhesion was determined. (A) Microscopic images taken from control and DADLE-treated cells. Arrows indicate the formation of spines in some of the opioid exposed cells. (B) Quantification of adherent cells by crystal violet staining. Cell binding to FN is stimulated by both DADLE and etorphine treatment by about 5-fold. This opioid effect is completely blocked by coincubation with 10 μM naloxone or pretreatment with 1 mM RGDT peptides. The results shown are the means ± S.E.M. values from three independent experiments. ***Significantly different at $P < .001$.

3.2. DOR-stimulated cell adhesion is accompanied by ERK1/2 activation

Exposure of NG108-15 cells to DADLE and etorphine results in activation of ERK1/2, as demonstrated by an activation-specific antibody recognizing phosphorylated Thr202/Tyr204 residues (Fig. 2). To investigate whether integrin-mediated cell adhesion also results in ERK1/2 activation, NG108-15 cells were pretreated with RGDT peptides and subsequently measured for DOR-stimulated ERK1/2 activity. Most interestingly, blockade of cell adhesion to FN by integrin binding RGDT peptides completely prevented opioid-induced ERK1/2 signaling. A similar blockade of ERK1/2 signaling was also observed when the DOR-mediated effect was investigated in suspension culture (Fig. 2). These findings are in line with the observation that integrin-mediated cell adhesion is associated with mitogenic signaling [18] and suggest that integrins represent an essential prerequisite for DOR-stimulated ERK1/2 activation.

3.3. Cell adhesion stimulates ERK1/2 signaling by transactivation of TrkA receptors

Previous studies revealed that ERK1/2 activation by integrin-mediated cell adhesion involves transactivation of epidermal growth factor (EGF) receptors in epithelial cells [19]. Therefore, we investigated whether a similar signaling pathway might also account for DOR-stimulated, integrin-dependent ERK1/2 activation in NG108-15 cells. Although in stably transfected HEK293 cells DOR-stimulated ERK1/2 signaling is mediated by transactivation of EGF receptors [13], pre-incubation of NG108-15 cells with the selective EGF receptor blockers AG1478 [20] and BPIQ-I [21] failed to attenuate DADLE- and etorphine-stimulated ERK1/2 activation (Fig. 3). This finding demonstrates that in NG108-15 cells integrin-mediated stimulation of the Ras/Raf/ERK1/2 cascade is independent of EGF receptors. It also implicates that HER2/NEU receptors, which might enhance EGFR signaling by formation of functional heterodimers with EGF receptors, are not involved in opioid-stimulated ERK1/2 signaling [22].

The TrkA receptor represents another member of the superfamily of receptor tyrosine kinases that has been previously shown to link distinct G protein-coupled receptors to the ERK1/2 signaling pathway [23]. Therefore, we tested the effect

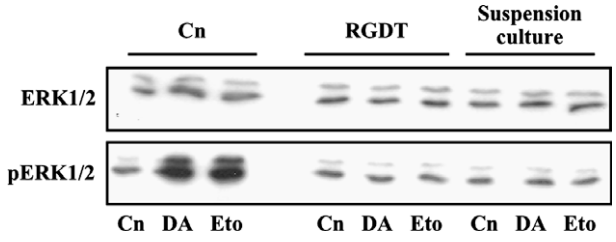

Fig. 2. DOR-stimulated ERK1/2 activation is integrin dependent. NG108-15 cells were stimulated for 5 min with DADLE (1 μM; DA) and etorphine (100 nM; Eto) before ERK1/2 activation was determined by Western blot using a phospho-specific antibody (bottom panel). Equal protein load was verified by incubation with an overall reactive ERK1/2 antibody (top panel). Short-term DOR activation with DADLE and etorphine results in strong ERK1/2 phosphorylation, an effect that is completely blocked by preincubation of the cells with RGDT peptides or determination of ERK1/2 activity in suspension culture. The blots shown are representative for at least three independent experiments yielding similar results.

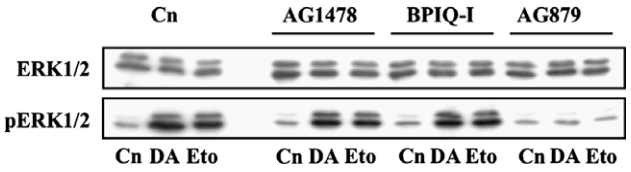

Fig. 3. DOR-stimulated ERK1/2 activation is mediated by transactivation of TrkA. NG108-15 cells were pretreated with inhibitors for the EGF receptor (AG1478, 5 μM; BPIQ-I, 10 μM) or TrkA (AG879, 100 μM) before DADLE- (1 μM; DA) and etorphine (100 nM; Eto)-stimulated ERK1/2 activation was determined. Inhibition of TrkA, but not of the EGF receptor, completely blocks DOR-stimulated ERK1/2 activity. The blots shown are representative for at least three independent experiments yielding similar results.

of the specific TrkA inhibitor AG879 [21] on DOR-mediated ERK1/2 activation. Indeed, preincubation of the cells with AG879 completely prevented-opioid induced ERK1/2 phosphorylation (Fig. 3).

3.4. DOR-mediated transactivation of TrkA is dependent on integrin function

Because cell adhesion results in activation of integrins [18] and facilitates DOR-stimulated ERK1/2 signaling, we finally examined whether integrins are involved in DOR-mediated transactivation of the TrkA/ERK1/2 signaling module. For this, the role of cell adhesion in DADLE and etorphine stimulated TrkA activity was determined. As shown in Fig. 4, opioid treatment results in phosphorylation of TrkA at Tyr490, which is indicative for receptor autophosphorylation and, hence, activation [24]. This effect is blocked by integrin binding RGDT peptides and is absent in cells kept in suspension culture during opioid stimulation. These results indicate that DOR-mediated cell adhesion results in transactivation of TrkA. Such a mechanism is supported by the possibility that integrin binding to extracellular matrix proteins leads to transactivation of TrkA in a src kinase dependent manner [25].

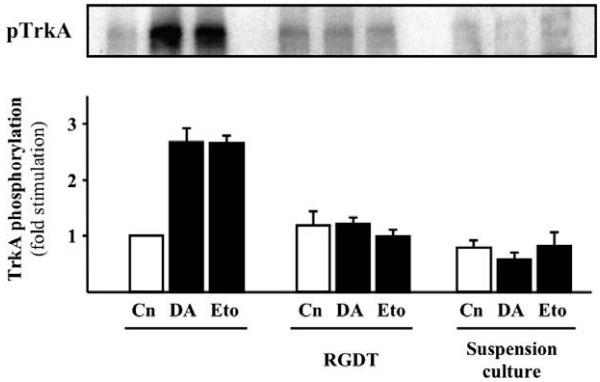

Fig. 4. DOR stimulation results in integrin-dependent TrkA phosphorylation. NG108-15 cells were cultured for 5 min either in the absence (Cn) or presence of DADLE (DA; 1 μM) and etorphine (Eto; 100 nM) before cells were lysed and TrkA phosphorylation was determined by Western blot using a phospho-Tyr490 specific TrkA antibody. Short-term opioid treatment strongly stimulates TrkA autophosphorylation, an effect that is absent in cells pretreated with RGDT peptides measured in suspension culture (susp.). The data shown are the means ± S.E.M. values from three independent experiments.

4. Conclusion

This study provides evidence that in neuroblastoma × glioma NG108-15 hybrid cells DOR-stimulated mitogenic signaling involves cell adhesion and is transduced by integrin-mediated transactivation of the TrkA/ERK1/2 cascade. Integrins are transmembrane "adhesion receptors" that usually control cell behavior by linking mechanical extracellular stimuli to intracellular signal transduction pathways and the cytoskeleton [9]. Here we demonstrate that the DOR, a member of the family of G protein-coupled receptors, utilize these signaling components to bring about activation of the ERK1/2 cascade. In the central nervous system, long-term modulation of integrin activity plays a critical role in synaptic plasticity [26], spatial memory [27] as well as neural survival and regeneration [28]. Because long-term potentiation and memory consolidation have also been associated with continuous activation of the ERK1/2 signaling pathway [29], the present findings might contribute to chronic opioid-induced synaptic plasticity [30], improved memory and learning [5] as well as the development of tolerance/dependence [4]. In this respect, transactivation of TrkA receptors by opioid-induced release of Trk agonists [31] could explain the neuroprotective properties demonstrated for some δ-opioids [6] and might contribute to the development of opioid tolerance/dependence [32]. To confirm the role of the integrin/TrkA/ERK1/2 signaling pathway in chronic opioid-induced neuronal plasticity, additional experiments performed on primary neurons using opioid ligands known for their addictive propensity (e.g. morphine) and additional TrkA inhibitors (e.g. TrkA-blocking antibodies [33]) will be required.

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