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Phosphorylation of adenylyl cyclase VI upon chronic δ-opioid receptor stimulation

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Abstract

An immunoprecipitation method was used to measure [^{32}P]phosphate incorporation into the adenylyl cyclase VI protein in Chinese Hamster Ovary (CHO) cells stably expressing the human δ-opioid receptor. Chronic SNC 80 ((+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl-benzamide) (1 μM, 24 h) treatment increased the incorporation of [^{32}P] into a 200 kDa protein band 2.5-fold after gel electrophoresis. The increase in phosphorylation of adenylyl cyclase VI was antagonized by naltrindole (1 μM) and the immunoprecipitation was prevented by the saturation of the antibody with the blocking peptide.

Keywords: Opioid receptor; Adenylyl cyclase VI; Phosphorylation

Chronic activation of receptors coupled to the inhibition of adenylyl cyclase often induces an increase in the activity of this enzyme after the removal of the agonist, called adenylyl cyclase superactivation. Adenylyl cyclase superactivation was first described for the opioid receptor–adenylyl cyclase system in NG108-15 cells (Sharma et al., 1975). Later, it was observed, in other cell systems and in brain areas thought to be involved in opiate addiction. The mechanism of adenylyl cyclase superactivation is poorly understood. Recently, it was shown (Avidor-Reiss et al., 1997) that the opioid induced adenylyl cyclase superactivation is isoenzyme specific. The most abundant adenylyl cyclase isoenzyme in brain regions implicated in drug addiction (corpus striatum and nucleus accumbens) is adenylyl cyclase V (Mons and Cooper, 1995).

Adenylyl cyclase superactivation is not restricted to cells of central nervous system origin. The construction of recombinant cell lines expressing only the receptor of interest therefore, provides useful model systems to study the biochemical mechanisms of adenylyl cyclase superactivation. We have shown (Malatynska et al., 1995) that chronic (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl-benzamide (SNC 80) treatment of Chinese Hamster Ovary (CHO) cells expressing the human δ-opioid receptor (hDOR/CHO) leads to a robust adenylyl cyclase superactivation. Subsequently, we have reported (Varga et al., 1998) that the CHO cells express AC VI and AC VII isoenzymes. The recombinant hDOR/CHO cell line therefore, is a useful tool to study the regulation of the adenylyl cyclase V/VI family of isoenzymes by chronic δ-opioid agonist treatment.

Avidor-Reiss et al. (1996) have demonstrated that adenylyl cyclase V superactivation is mediated by the G-protein βγ subunits. The G-protein βγ subunits, however, do not regulate directly the adenylyl cyclase V/VI family of isoenzymes (Ishikawa and Homcy, 1997). The cellular mediator conveying the G-protein βγ regulation to adenylyl cyclase V/VI is presently unknown. The G-protein βγ subunits directly and indirectly regulate the activity of several protein kinases (Inglese et al., 1995). On the other hand, the regulation of adenylyl cyclase activity by direct phosphorylation of the enzyme is well documented

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(Ishikawa and Homcy, 1997). Therefore, we tested whether long term δ-opioid receptor agonist treatment leads to the phosphorylation of adenylyl cyclase VI.

hDOR/CHO cells (10 cm plate, 90% confluency) were incubated in phosphate free Dulbecco's Modified Eagle Medium (DMEM) + 5% dialyzed fetal calf serum for 45 min and labeled with [^{32}P]orthophosphate (NEN, 3000 Ci/mmol, 200 μCi/ml) for 1 h. After the metabolic labeling, the incubation continued in the presence of: (a) [^{32}P] containing medium alone (control); (b–c) 1 μM SNC 80 (2 plates); and (d) 1 μM SNC 80 + 1 μM naltrindole. After 24-h incubation, the cells were washed two times with phosphate buffered saline and scraped into ice cold homogenization buffer (50 mM Tris, 250 mM sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaF and 10 mM Na-pyrophosphate supplemented with 10 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO), 100 nM Na-orthovanadate and 10 nM okadaic acid immediately before use). The cells were centrifuged at 25,000 rpm for 30 min and the pellet resuspended in 1 ml radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% Igepal CA-630, 0.5% Triton X-100, 0.2% digitonin, 5 mM EDTA, 10 mM NaF, 10 mM β-glycerol-phosphate with 10 μl/ml protease inhibitor cocktail, 10 nM okadaic acid and 100 nM Na-orthovanadate added immediately before use). The solution was incubated on ice for 3 h and centrifuged at 40,000 rpm for 30 min. The lysate was precleared by incubation in the presence of 1 μg preimmune rabbit immunoglobulin G and 10 μl protein A-agarose for 1 h and centrifuged at 3000 rpm. The precleared lysate was incubated overnight with adenylyl cyclase V/VI specific antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). For the lysate in plate c (peptide antagonism), the antibody was preincubated with 50 μl blocking peptide (Santa Cruz Biotechnologies) for 4 h. A 10 μl protein A-agarose was added and the mixture incubated on ice with gentle rocking for 3 h, centrifuged (3000 rpm, 5 min) and washed three times with 10 min incubations in RIPA wash buffer (RIPA buffer with detergent concentrations reduced to 0.075% Triton X-100, 0.05% Igepal CA-630 and 0.1% digitonin) in the presence of protease and phosphatase inhibitors as before. The immunoprecipitate was eluted from the final pellet by incubating with 10 μl glycine–Cl buffer, pH = 2.3. The mixture was neutralized with 5 μl neutralization buffer and boiled with 15 μl 2 × Laemmli sample buffer for 5 min. The immunoprecipitate was resolved on a 7.5% denaturing polyacrylamide gel. The gel was stained with Coomassie blue, destained, dried and subjected to autoradiography. Two protein bands of approximately 130 and 200 kDa were obtained by both protein staining and autoradiography. The 200 kDa band is presumably the glycosylated form of the adenylyl cyclase VI protein (deduced MW = 130 kDa), because only the 130 kDa band is apparent after *N*-aminoglycosidase F treatment of the immunoprecipitate (data not shown). The protein content of the Coomassie stained 200 kDa band and the [^{32}P] incorporation (autography film) were quantitated using the Arcus II scanning densitometer with the Documax OneDScan software for PC (Scanalytics, Billerica, MA).

Fig. 1 shows the histogram of the density ratio of [^{32}P] incorporation and Coomassie stain in arbitrary units. As seen on the figure, adenylyl cyclase VI is constitutively phophorylated in the CHO cells (Fig. 1a). Chronic (24 h) SNC 80 (1 μM) treatment of the hDOR/CHO cells leads to a 2.5 fold increase in [^{32}P] incorporation into the adenylyl cyclase VI protein. The immunoprecipitation of the labeled protein was prevented by saturation of the antibody with the blocking peptide. The increase in phosphorylation was antagonized by the selective δ-opioid receptor antagonist, naltrindole. The phosphorylation of adenylyl cyclase VI was SNC 80 dose- and incubation time-dependent (data not shown).

In summary, we have shown that chronic δ-opioid receptor agonist (SNC 80) treatment of the CHO cells stably expressing the human δ-opioid receptor leads to an increase in the [^{32}P] incorporation into the adenylyl cyclase VI protein. The regulation of different adenylyl cyclase isoenzymes by protein kinase A, protein kinase C and calcium/calmodulin kinase phosphorylation and by calcineurin dephosphorylation have been shown (Ishikawa and Homcy, 1997). Further investigations are under way to determine which kinase(s) or phosphatase(s) are involved in the chronic SNC 80 treatment mediated phosphorylation of adenylyl cyclase VI in the CHO cells and to determine whether the phosphorylation of adenylyl cyclase VI is the cause of adenylyl cyclase superactivation after chronic inhibitory receptor stimulation in the CHO cells.

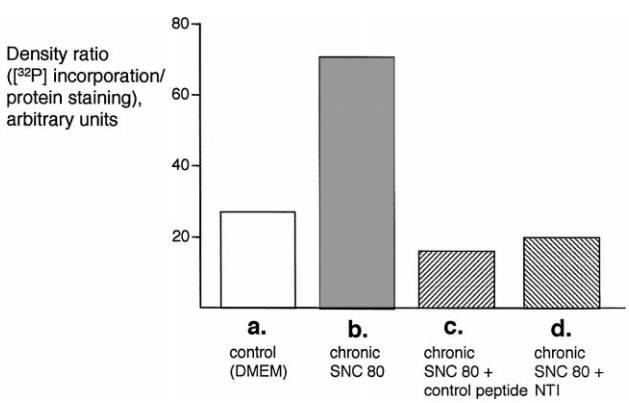

Fig. 1. Phosphorylation of adenylyl cyclase VI in hDOR/CHO cells upon chronic δ-opioid agonist (SNC 80) treatment. hDOR/CHO cells were pretreated with 200 μCi/ml [^{32}P]orthophosphate in phosphate free DMEM with (a) media alone; (b and c) 1 μM SNC 80; (d) 1 μM SNC 80 and 1 μM naltrindole, for 24 h. The cell lysate was treated with an adenylyl cyclase V/VI specific antibody and protein A-agarose. In (c), the antibody was saturated with the control peptide. The immunoprecipitate was resolved on a 7.5% denaturing polyacrylamide gel. The results are shown as the density ratio of [^{32}P]incorporation and protein amount as determined by scanning densitometry of the autoradiography film and the Coomassie stained gel, respectively.

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