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Calcium/calmodulin-dependent protein kinase II supports morphine antinociceptive tolerance by phosphorylation of glycosylated phosducin-like protein

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Abstract

The long isoform of the phosducin-like protein (PhLPl) is widely expressed in the brain and it is thought to influence G-protein signalling by regulating the activity of Gβγ dimers. We show that in the mature nervous system, PhLPl exists as both a 38 kDa non-glycosylated isoform and as glycosylated isoforms of about 45, 100 and 150 kDa. Additionally, neural PhLPl is subject to serine phosphorylation, which augments upon the activation of Mu-opioid receptors (MORs), as does its association with Gβγ subunits and 14-3-3 proteins. While the intracerebroventricular (icv) administration of morphine to mice rapidly reduced the association of MORs with G proteins, it increased the serine phosphorylation of these receptors. Moreover, activated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) accumulated in the MOR environment and phosphorylated PhLPl was seen to co-precipitate with these opioid receptors. Opioid-induced phosphorylation of PhLPl was impaired by inhibiting the activity of CaMKII and, in these circumstances, the association of PhLPl with Gβγ dimers and 14-3-3 proteins was diminished. Furthermore, these events were coupled with the recovery of G protein regulation by the MORs, while there was a decrease in serine phosphorylation of these receptors and morphine antinociceptive tolerance diminished. It seems that CaMKII phosphorylation of PhLPl stabilizes the PhLPl·Gβγ complex by promoting its binding to 14-3-3 proteins. When this complex fails to bind to 14-3-3 proteins, the association of PhLPl with Gβγ is probably disrupted by GαGDP subunits and the MORs recover control on G proteins.

Keywords: Opioid receptors; Phosducin-like protein; CaMKII; Morphine tolerance; G-proteins; Gβγ dimers

1. Introduction

The Mu-opioid receptor (MOR) that belongs to the family of G protein-coupled receptors (GPCR) regulates in the cell membrane heterotrimeric Gαβγ proteins that, upon the action of agonists, segregate into activated GαGTP subunits and free Gβγ dimers. Both these elements regulate intracellular effectors, thereby propagating the signals originated by extracellular messengers. In the nervous system, the MOR is the main target for opioids to alleviate high intensity inflammatory pain. It is known that opioids that induce the endocytosis and recycling of MORs promote little desensitization to their effects (Koch et al., 2001; Zhang et al., 1998). Thus, morphine induces a high degree of tolerance because it provokes little phosphorylation and internalization of the MORs (Finn and Whistler, 2001). Interestingly, neural cells have developed specific mechanisms to control MOR function when the agonists are poor inducers of receptor internalization, and these involve the regulation of Gα subunits of the MOR-regulated G proteins. As a result, tolerance to morphine develops through the stable transfer of part of the MOR-activated Gα subunits to RGS proteins of the R7 and Rz subfamilies (Garzón et al., 2005a; Rodríguez-Muñoz et al., 2007a). This is observed before this

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agonist provokes the phosphorylation and recycling of the MORs (Rodríguez-Muñoz et al., 2007b).

The free Gβγ dimers generated by the action of morphine on the MORs could also be a target of this regulation. Indeed, a series of studies in cell lines, isolated neurons and in mice indicate that endogenous phosducin-like protein long (PhLPl) binds free Gβγ dimers that originate through the effects of GPCR agonists (Garzón et al., 2002; McLaughlin et al., 2002; Partridge et al., 2006). However, little is known about how this interaction could affect morphine-induced tolerance. At least two forms of PhLP have been identified to date, the so-called long PhLPl and the short PhLPs, which differ in the length of their N terminus, containing 297 and 218 residues, respectively (Miles et al., 1993). PhLPl binds in vitro with high affinity to Gβγ dimers, blocking their action on cellular effectors (Thibault et al., 1997; Schröder and Lohse, 1996; Schulz et al., 1996; Humrich et al., 2003). The PhLP present in the nervous system is related to the retinal phosducin, and by analogy to the role displayed by phosducin on rhodopsin signalling, it was inferred that PhLPl could bind directly to Gβγ dimers and thus regulate the activity of GPCRs. The association between phosducin and Gtβγ dimers is disrupted by cAMP-dependent protein kinase (PKA), then facilitating the binding of GtαGDP subunits to the Gtβγ dimer, which now releases the phosphorylated phosducin (Lee et al., 1990; Yoshida et al., 1994; Wilkins et al., 1996). The PhLPl is a poor PKA substrate but is rapidly phosphorylated by casein kinase 2 (CK2) near the N terminus (Schröder and Lohse, 1996; Thibault et al., 1997). However, PhLPl and CK2-phosphorylated PhLPl display comparable affinity for Gβγ dimers (Humrich et al., 2005) which brings into question how the CK2 phosphorylation/dephosphorylation of PhLPl may regulate this complex, as described for retinal phosducin and Gtβγ.

The little information available regarding the signals that trigger the association of PhLPl with Gβγ dimers or those responsible for the dissociation of this complex has reinforced the idea that PhLPl only works as a molecular chaperone for Gβγ dimer assembly, whereby it would form a ternary complex with the nascent Gβ chain and the cytosolic chaperoning complex (CCT) (Lukov et al., 2005, 2006). However, the accumulated evidence indicates that PhLPl regulates GPCR signalling by directly binding to agonist-generated free Gβγ dimers, and interestingly, the binding of retinal phosducin to the phosphoserine-binding protein 14-3-3 requires phosphorylation by CaMKII (Thulin et al., 1999), and this kinase also promotes the phosphorylation of PhLPl (Garzón et al., 2002). A series of reports have connected morphine-induced tolerance with the activation of CaMKII (Fan et al., 1999; Lu et al., 2000; Tang et al., 2006a). It is therefore possible that binding of PhLPl to Gβγ dimers and 14-3-3 proteins is regulated in the cell membrane by a mechanism in which CaMKII could play a relevant role. We have addressed this issue by pull-down studies in neuronal synaptosomes from periaqueductal grey matter (PAG), a neural structure relevant in the analgesia of opioids when given by the intracerebroventricular (icv) route. The analgesic tolerance that develops after a single icv dose of morphine was correlated with the activation of CaMKII as well as with changes in the association of MORs with G proteins and of PhLPl with Gβγ dimers and 14-3-3 proteins. Our results indicate that in neural membranes, PhLPl exists both in non-glycosylated and glycosylated forms. The activation of opioid receptors greatly increased the CaMKII-mediated serine phosphorylation of PhLPl and the binding of the glycosylated isoforms of this protein to Gβγ dimers. This action facilitated the desensitization of MORs to the analgesic action of morphine by reducing the pool of regulated G proteins, whilst it increased the serine phosphorylation of the MORs. The existence of a common pathway in which activated CaMKII and PhLPl operate to desensitize MORs is therefore suggested.

2. Methods

2.1. Preparation of membranes from neural cells

Experimental tissue was obtained from male albino CD1 mice (Charles River, Barcelona, Spain) weighing 22–27 g. Mice were sacrificed by cervical dislocation and, the periaqueductal grey matter (PAG) was removed. About 1 mm of tissue around the aqueduct was collected from 2 mm thick coronal sections (MP-600 micropunch, Activational Systems Inc.). In immunoprecipitation studies, a typical assay used the structures from about six to eight mice, e.g. a post-morphine interval. The tissues were washed and pooled in ice-cold 25 mM Tris–HCl (pH 7.5), 1 mM EGTA and 0.32 M sucrose supplemented with a phosphatase inhibitor mixture (Sigma # P2850), H89 (Sigma, B1427) and a protease inhibitor cocktail (Sigma, P8340) that contained 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatin A, transepoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin and aprotinin. The PAG synaptosome rich fraction was then obtained. The tissue was homogenized for 15 s in a Polytron (PT 10–35, Kinematica, Kriens-Luzern, Switzerland) at a setting of 3 in 10 volumes of the buffer. The homogenate was centrifuged for 10 min at 1000*g* to remove the nuclear fraction (Sorvall RC5C, rotor SS-34, Newton, CT, USA). Subsequently, the supernatant (S1) was centrifuged for 20 min at 20,000 *g* to obtain the crude synaptosomal pellet (P2). This pellet was diluted in Tris buffer supplemented with a mixture of protease inhibitors (0.2 mM phenylmethylsulphonyl fluoride, 2 μg/mL leupeptin and 0.5 μg/mL aprotinin) and used for the immunoprecipitation assays. The supernatant (S2) was centrifuged at 105,000*g* for 1 h to obtain the crude microsomal pellet (P3) (Beckman XL-70 ultracentrifuge, rotor Type 70 ti). The S3 supernatant was concentrated in Amicon Ultra-4 centrifugal filter devices (nominal molecular weight limit NMWL of 10,000 #UFC8 01024, Millipore Iberica S.A., Madrid, Spain), and it was then used to study the possible presence of internalized MORs.

2.2. Two-dimensional electrophoresis

About 100 μg of P2 membranes were solubilized in a buffer containing 40 mM Tris–HCl pH 7.7, SDS 1%. The solubilized proteins were cleaned using PlusOne 2-D Clean-Up kit (GE Healthcare, 80-6484-5) according to the manufacturer's recommendations. The protein pellets were resuspended in a rehydratation solution consisting of 5 M urea (Bio-Rad, 161-0730), 2 M thiourea (Sigma–Aldrich, T-7875), 2% CHAPS (Sigma–Aldrich, C-3023), 2% caprylyl sulfobetaine (SB 3–10; Sigma–Aldrich, D-4266), 40 mM Tris–HCl pH 7.7 (Bio-Rad, 161-0716), 0.2% Bio-Lyte corresponding to the range of the IPG strip and 2 mM reducing agent tributyl phosphine (Bio-Rad, 163-2101) and after remaining for 1 h at room temperature the protein solution was centrifuged in centrifugal filter devices of 0.22 μm porus size (Ultrafree-MC #UFC30GV, Millipore Iberica S.A.).

Samples were resolved on IPG Ready Strips [7 cm pH 4–7 linear gradient (Bio-Rad, 163-2001) or 3–10 non-linear gradient (Bio-Rad 163-2002)], that were actively rehydrated at 50 V for 16 h at ambient temperature under oil in the Isoelectric focusing (IEF) tray of the IEF cell. IEF was carried out