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DOPAMINE D_1 RECEPTOR-DEPENDENT MODIFICATIONS IN THE DOPAMINE AND cAMP-REGULATED PHOSPHOPROTEIN OF M_r 32 kDa PHOSPHORYLATION PATTERN IN STRIATAL AREAS OF MORPHINE-SENSITIZED RATS

S. SCHEGGI,[1] A. CROCIANI,[1] M. G. DE MONTIS, A. TAGLIAMONTE AND C. GAMBARANA*

Department of Neuroscience, Pharmacology Unit, University of Siena, Italy

Abstract—Morphine sensitization is a model of latent, functionally inducible increase in dopamine D_1 receptor–mediated transmission, which may be unmasked by an external stimulus. Morphine-sensitized rats present dopamine D_1 receptor–dependent stereotypies upon morphine challenge and resilience to unavoidable stress-induced behavioral deficits. This tonic increase in dopamine D_1 dopaminergic transmission is counter-adaptive to an enhanced μ-opioid receptor-dependent signaling in striatal areas. Control and sensitized rats show a similar dopamine and cAMP-regulated phosphoprotein of M_r 32 kDa (DARPP-32) phosphorylation pattern in striatal areas. Acute morphine administration induced an early increase and delayed decrease in phosphothreonine (Thr)34 DARPP-32 levels accompanied by a delayed increase in phospho-Thr75 DARPP-32 levels in the nucleus accumbens and caudate-putamen of sensitized rats, while it had no effects in control animals. The administration of a selective dopamine D_1 receptor antagonist (SCH 23390) before morphine challenge prevented the behavioral and neurochemical modifications in sensitized rats. 6-Methyl-2-(phenylethynyl)-pyridine, a selective metabotropic glutamate receptor 5 ($mGluR_5$) antagonist, administered 1 h after morphine challenge, prevented the delayed phosphorylation changes, but it had no effect when administered before challenge. Moreover, the DARPP-32 phosphorylation pattern in the caudate-putamen of sensitized rats after unavoidable stress exposure was studied. The stress-induced neurochemical modifications and their sensitivity to receptor antagonists were similar to those observed after acute morphine administration. In conclusion, these results suggest that in the experimental conditions used an increase in dopamine output in striatal areas is followed by a complex neurochemical pattern, in which the initial stimulation of dopamine D_1 receptors triggers a sequence of signaling events that lead to an $mGluR_5$-mediated increase in phospho-Thr75 DARPP-32 levels. Since DARPP-32 phosphorylated in Thr75 inhibits cAMP-dependent protein kinase (PKA) activity, the final result is a decrease in the dopamine D_1 receptor–dependent phosphorylation events.

Key words: caudate putamen, nucleus accumbens, metabotropic glutamate receptor 5, $MGluR_5$, μ-opioid receptor, unavoidable stress.

Behavioral sensitization is a condition in which repeated passive- or self-administration of a drug elicits increasing behavioral effects and neurochemical modifications. The condition of morphine sensitization appears to be associated with a persistent state of inducible functional increase in dopamine D_1 receptor transmission. Accordingly, the stereotypies expressed by sensitized rats upon morphine challenge are selectively antagonized by the administration of a dopamine D_1receptor antagonist (Pollock and Kornetsky, 1989; Scheggi et al., 2000). Moreover, morphine-sensitized rats are resilient to the development of behavioral sequelae after unavoidable stress exposure, an effect also dependent on dopamine D_1 receptor activation (Scheggi et al., 2000). The tonic increase in dopamine D_1 receptor responsiveness in the striatal areas of sensitized rats appears to be counter-adaptive to the enhanced μ-opioid receptor-dependent signaling underlying the condition of morphine sensitization, as outlined by studies at the receptor level or downstream in the transduction pathway (Viganò et al., 2003; Yao et al., 2005). μ-Opioid receptors in striatal areas are negatively coupled to adenylyl cyclase activity and appear to be preferentially expressed in the medium spiny neurons that express dopamine D_1 receptors (Wang et al., 1997; Noble and Cox, 1995). Thus, repeated morphine administration prevalently affects these neurons, which counterreact by increasing the expression of $G\alpha_{s/olf}$ and decreasing that of $G\alpha_i$ proteins (Van Vliet et al., 1993), i.e. by strengthening dopamine D_1 receptor-stimulated cAMP-dependent protein kinase (PKA) activity. In this context, we hypothesized that a prevalent, functional dopamine D_1 receptor-sustained transmission would result in a prevalent, PKA-mediated phosphorylation pattern of the dopamine and cAMP-regulated phosphoprotein of M_r 32 kDa (DARPP-32). DARPP-32 is a major target protein for PKA and its function is determined by the phosphorylation state. Dopamine, by means of the D_1 receptor–mediated activation of PKA, increases the phosphorylation of DARPP-32 at threonine-34 (Thr-34), thereby converting DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP-1)(Hemmings et al., 1984). Phosphorylation at Thr-75, dependent on glutamate transmission through the activation of a complex signaling cascade (Nishi et al., 2005), converts DARPP-32 into a PKA inhibitor (Bibb et al., 1999).

[1] These authors contributed equally to this work.

*Corresponding author. Tel: +39-0577234116; fax: +39-0577234208.
E-mail address: gambarana@unisi.it (C. Gambarana).

Abbreviations: ANOVA, analysis of variance; CPu, caudate-putamen; DARPP-32, dopamine and cAMP-regulated phosphoprotein of M_r 32 kDa; $mGluR_5$, metabotropic glutamate receptor 5; MPEP, 6-methyl-2-(phenylethynyl)-pyridine; NAc, nucleus accumbens; PKA, cAMP-dependent protein kinase; PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A; PP-2B, protein phosphatase-2B; Thr, threonine.

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In morphine-sensitized rats, the basal DARPP-32 phosphorylation pattern in the nucleus accumbens (NAc) and caudate–putamen (CPu) is not modified compared to control rats (Scheggi et al., 2004). Acute morphine administration increases striatal extraneuronal dopamine levels in control and sensitized rats (Cadoni and Di Chiara, 1999). Moreover, in sensitized rats it induces a decrease in phospho-Thr34 DARPP-32 levels associated with an increase in phospho-Thr75 DARPP-32 levels 2 h after the challenge, while it has no effect in control animals (Scheggi et al., 2004).

The aim of this study was to further examine the modifications in the phosphorylation pattern of DARPP-32 and some other PKA substrates, the $GluR_1$ and NR_1 subunits of AMPA and NMDA receptors, respectively, elicited by a morphine challenge in morphine-sensitized rats and to investigate the mechanisms underlying these changes. In addition, since stress resistance in morphine-sensitized rats is sustained by a dopamine D_1 receptor-mediated mechanism (Scheggi et al., 2000), we also studied in the striatal areas of control and sensitized rats the possible modifications in DARPP-32 phosphorylation pattern induced by unavoidable stress exposure.

EXPERIMENTAL PROCEDURES

Animals

Experiments were carried out on male Sprague–Dawley rats (Charles River, Calco, Italy) that were allowed at least 1 week of habituation to the animal colony and that weighed 200–225 g when the experimental procedures began. Animals were housed five per cage, were kept in an environment maintained at a constant temperature and humidity with free access to food and water, and they were moved to a different cage or apparatus only for the time required for behavioral manipulation. A reverse 12-h light/dark cycle (7:00 AM lights off, 7:00 PM lights on) was used. Experiments were carried out from 9:00 AM to 5:00 PM under a red light and controlled noise conditions in a testing room separated from and adjacent to the main animal room, under the same conditions of temperature and humidity. The procedures used were in accordance with the European legislation on the use and care of laboratory animals (EEC Council Directive 86/609), the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the University of Siena Ethics Committee. All efforts were made to minimize the number of animals used and their suffering.

Induction of morphine sensitization

Sensitization was induced by administering morphine (10 mg/kg/day s.c.) for 7 days as described (Scheggi et al., 2004). At the end of the 7-day protocol rats were left undisturbed for 10 days before the assessment of behavioral sensitization development: rats were tested for their response to a morphine challenge (5 mg/kg s.c.) and locomotor activity and stereotypies were recorded for 30 min. Locomotor activity was determined in motility cages that detected horizontal activity and rearings (Imetronic, Pessac, France). Stereotypies were scored by an experimenter blind to the experimental conditions, according to a published rating scale (Scheggi et al., 2000). A cumulative score reflecting stereotypy intensity during the 30 min observation period was assigned to each rat.

Unavoidable stress exposure

Rats were exposed to a 50 min unavoidable stress session. Each rat was completely immobilized by a flexible wire-net; an electrode was applied to the distal third of the tail and about 80 electric shocks (1 mA×5 s, one every 30 s) were administered (Gambarana et al., 2001).

Immunoblotting

Rats were sacrificed, heads briefly immersed (5–6 s) in liquid nitrogen, and brains rapidly removed and cut into 1 mm slices using an ice-cold metal brain matrix (ASI Instruments, Inc., Warren, MI, USA). The CPu and NAc were quickly dissected out from slice 3, approximately 2.7 to 1.7 mm from the bregma, that had been identified using the atlas of the rat brain (Paxinos and Watson, 1998) as corresponding to plates 10–12. Tissues were then flash-frozen in liquid nitrogen and stored at −80 °C until assayed. Frozen tissue samples were prepared by solubilization in boiling 1% sodium dodecyl sulfate (SDS) and 50 mM NaF. Proteins (30 μg) were separated by electrophoresis on precast 10% bis–Tris polyacrylamide gels (XT-Criterion, Bio-Rad Laboratories, Hercules, CA, USA), with XT-MES or XT-MOPS (Bio-Rad Laboratories) as the running buffer for DARPP-32 or AMPA and NMDA receptor subunits, respectively, and then transferred to nitrocellulose membranes. Small aliquots of the homogenate were retained for protein determination by a modified Lowry protein assay method (DC protein assay, Bio-Rad Laboratories). Immunoblotting was carried out with phosphorylation-state-specific antibodies against phospho-Thr34 DARPP-32 and phospho-Thr75 DARPP-32 (Cell Signaling Technology, Beverly, MA, USA), phospho-Ser845 $GluR_1$ and phospho-Ser897 NR_1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or antibodies that are not phosphorylation-state-specific against total DARPP-32 (Cell Signaling Technology), total $GluR_1$, or NR_1 (Santa Cruz Biotechnology). Antibody binding was detected using a chemiluminescence detection system (Pierce Biotechnology Inc., Rockford, IL, USA) and quantified with the Versa Doc 1000 Imaging System (Bio-Rad Laboratories). For each experiment, samples containing the same amount of total proteins from rats in each experimental group were run on the same immunoblots and then analyzed together. To control for equal loading, blots were reprobed with the respective non-phosphorylation-state-specific antibody. Values obtained from experimental groups were calculated as the percentage of their respective control group values.

Statistical analysis

Statistical analyses were performed on commercially available software (Prism 4.0a, GraphPad Software Inc., San Diego, CA, USA) by one-way analysis of variance (ANOVA) followed by the Dunnett's or Bonferroni's test, when $P<0.05$. The unpaired *t*-test was used to compare two experimental groups.

Drugs

Morphine, SCH 23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride), 6-methyl-2-(phenylethynyl)-pyridine (MPEP), and naloxone ((5α)-4,5-epoxy-3,14-dihidroxy-17-(2-propenyl)morphinan-6-one) were dissolved in deionized/distilled water and injected in a volume of 1 ml/kg rat body weight. Rats in the different control groups received the corresponding volume of saline by the appropriate route of administration. Morphine was purchased from SALARS (Como, Italy). All other drugs and chemicals were purchased from commercial sources.