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Research Report

Chronic morphine administration enhances the expression of Kv1.5 and Kv1.6 voltage-gated K^+ channels in rat spinal cord

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Abstract

Prolonged opiate administration leads to the development of tolerance and dependence. These phenomena are accompanied by selective regulation of distinct cellular proteins and mRNAs, including ionic channels. Acute opiate administration differentially affects voltage-dependent K^+ currents. Whereas, opiate activation of K^+ channels is well established opioid-induced inhibition of K^+ conductance has also been studied. In this study, we focused on the effect of chronic morphine exposure on voltage-dependent Shaker-related Kv1.5 and Kv1.6 K^+ channel gene expression and on Kv1.5 protein levels in the rat spinal cord. Several experimental approaches including in-situ hybridization, RNAse protection, reverse transcriptase–polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry were employed. We found that motor neurons are highly enriched in Kv1.5 and Kv1.6 mRNA and in Kv1.5 channel protein. Moreover, we found significant increases in the amount of mRNA encoding for these two K^+ channels and in Kv1.5 channel protein in the spinal cord of morphine-treated rats, compared with controls. For example, quantitative in-situ hybridization, revealed a 2.1 ± 0.15- and 2.3 ± 0.5-fold increase in Kv1.5 and Kv1.6 channel mRNA levels, respectively. Similar results were obtained by semiquantitative RT-PCR analyses. Kv1.5 protein level was increased by 1.9-fold in the spinal cord of morphine-treated rats. Our results suggest that Kv1.5 and Kv1.6 Shaker K^+ channels play an important role in regulating motor activity and that increases in mRNA and protein levels of the spinal cord K^+ channels after chronic morphine exposure could be viewed as a cellular adaptation which compensates for a persistent opioid-induced inhibition of K^+ channel activity. These alterations may account, in part, for the cellular events leading to opiate tolerance and dependence.

Keywords: Opioid receptor; Opiate tolerance; Hybridization, in-situ; Spinal cord; K^+ channel

1. Introduction

Voltage-gated K^+ channels play a pivotal role in regulating neuronal excitability [28]. The diversity of firing patterns displayed by neurons is reflected by a variety of voltage-gated K^+ currents that differ in their pharmacological and biophysical properties [53,57]. Recently, this heterogeneity of K^+ channels has been confirmed at the molecular level (for reviews see [32,33,54,58]). Studies of the molecular structure of voltage-gated K^+ channels reveal the existence of two different classes. The first class comprises Shaker-like K^+ channels, which give rise to delayed rectifier and transient A-type K^+ currents. Members of this superfamily of polypeptide molecules (55–100 kDa) consist of a conserved core region and variable cytoplasmic flanking COOH and NH_2 sequences [53]. The second broad group includes channels of the inward-rectifier type and also G protein-coupled and ATP-dependent K^+ channels with two hydrophobic segments and a linker region (H5), homologous to those of the Shaker-type K^+ channels [15,35]. Differential expression of the voltage-gated K^+ channels has been demonstrated in various areas of the central nervous system (CNS) employing in-situ hybridization and immunohistochemical procedures. Several of the K^+ channels, such as Kv1.1, Kv1.2, Kv1.4, Kv3.3 and Kv4.2, are localized in a cell-specific manner and are distributed in defined cellular regions [17,24,30,31,60,63,68,69]. The regional preference of various K^+ channels suggests that specific location is essential for normal neuronal function.

Opioid receptors were found to modulate K^+ channel activity in various areas of the nervous system, thus regu-

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lating neuronal firing patterns. Most frequently, opiates were found to activate K^+ channels. Studies of rat locus coeruleus neurons have indicated that activation of μ-receptors leads to opening of inward rectifier K^+ channels, resulting in membrane hyperpolarization and inhibition of firing [11,16,52]. Various other reports have demonstrated that μ- and δ-opioid receptor stimulation leads to K^+ channel activation in different brain regions including hypothalamus, submucous plexus and hippocampus, as well as in dorsal root ganglia (DRG) [2,37,64,70,72]. The involvement of κ-opioid receptors in the activation of K^+ channels has recently been shown in substantia gelatinosa and in hippocampal CA3 pyramidal neurons [26,45]. Interestingly, low concentrations of opioid agonists (in the nanomolar range) have been found to inhibit delayed-rectifier K^+ channels in DRG cell cultures and in F11 neuroblastoma × DRG hybrid cells [20,21]. This phenomenon indicates that opioid receptors can elicit excitatory effects on sensory neurons. It has been suggested that these excitatory effects are achieved by coupling of the channel to opioid receptor-activated G_i proteins [14]. Excitatory effects of opiates were also reported by DiChiara and North [16] in the nucleus accumbens and in the ventral tegmental area, as well as by Nicoll et al. [49] in GABAergic pathways. Similarly, it was recently shown that κ-opioid receptors inhibit voltage-dependent K^+ currents in the CATH.a cell line, originally derived from the brainstem [9].

Prolonged exposure to opioids results in tolerance and dependence. These phenomena are accompanied by various cellular adaptations, including alterations in gene expression [48]. A large body of evidence has accumulated pointing to changes in the levels of various proteins and mRNAs as a possible compensatory mechanism for tolerance and dependence. It has been demonstrated that chronic administration of opiates causes an increase in adenylate cyclase activity in several cell culture models (e.g., NG108-15 neuroblastoma × glioma hybrid cells) or Chinese hamster ovary (CHO) cells transfected with opioid receptors and in various brain regions [8,12,48,59]. In agreement with these results, it has been reported that opiate exposure increases the level of type VIII adenylate cyclase mRNA in several rat brain areas [43] and enhances the activity of cAMP-dependent protein kinase in locus coeruleus [48]. The levels of the α-subunit of G_i protein were found to either increase or decrease following opiate treatment in different regions of the CNS and in various neuronal cell cultures [3,48,66]. Up-regulation of β-adrenergic receptor kinase (βARK) [61] and of cAMP responsive element binding protein (CREB) [71] in locus coeruleus, as well as of early immediate genes (*fos-B* and *jun-D*) in the spinal cord (SC) [62] were also reported. Recently, we have found elevated levels of synapsin I mRNA in the locus coeruleus, amygdala, pontine central gray and SC of chronically morphine-treated rats [44]. In view of the role played by delayed-rectifier K^+ currents in mediating opiate excitatory action, we examined whether changes in Shaker K^+ channel expression could be part of the cellular compensatory mechanisms which occur following opiate tolerance. In this work, we focused on Kv1.5 and Kv1.6, two closely related K^+ channels of the Shaker-like subfamily which are found in brain tissues (for review see [53]). When expressed in *Xenopus* oocytes, these channel isoforms give rise to delayed-rectifier K^+ currents [7,53]. Here, we report that chronic morphine exposure increases the mRNA and protein levels of two Shaker-related voltage-gated K^+ channels, Kv1.5 and Kv1.6, in the rat SC.

2. Materials and methods

2.1. Materials

Rabbit antirat Kv1.5 antibody was raised against the specific C-terminal region downstream of the S6 transmembrane segment of Kv1.5. Antibodies were raised against a Kv1.5 fusion protein which was generated by polymerase chain reaction (PCR) amplification of a 299-bp fragment of the Kv1.5 cDNA coding region, corresponding to the C-terminus of the channel protein (amino acids 504–602). This PCR fragment was subcloned into the gluthatione-S-transferase fusion protein expression vector pGEX-3X (Pharmacia, Uppsala, Sweden). The fusion protein was injected into New Zealand rabbits. Anti-pGEX-Kv1.5 antibodies were affinity-purified from crude antiserum on nitrocellulose strips containing the purified Kv1.5 protein. Alkaline phosphatase-conjugated goat anti-rabbit and horseradish peroxidase-conjugated streptavidin were obtained from Jackson Laboratories (West Grove, PA). All restriction enzymes were supplied by Promega (Madison, WI). Radioactive nucleotides were purchased from Amersham (Buckinghamshire, UK). In-situ hybridization detection reagents and RNA transcription kit were from Boehringer–Mannheim (Germany). All other chemicals were from Sigma (St. Louis, MO).

2.2. Animals and tissue preparation

Adult male Wistar rats (150–200 g) were used in this study. Slow-release morphine pellets (75 mg each, NIDA, Rockville, MD) were transplanted subcutaneously, two on the first day and two on the next day. Control rats were implanted with placebo pellets using the same surgical procedure. Tolerance state was determined by the hot plate assay and dependence was assessed by naloxone injection. On the fifth day, animals were decapitated and the SC were removed (for RNAse protection assay and immunoblotting). Alternatively, animals were perfused transcardially with 250 ml of saline flush followed by 250 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) (for in-situ hybridization and immunohistochemistry). SCs

were removed and post-fixed in 4% paraformaldehyde for additional 48 h at room temperature. Paraffin blocks were prepared by standard methods. Coronal sections of 5 μm were cut and mounted on poly-L-lysine (Sigma, St. Louis, MO)-coated slides, which were stored at room temperature in a dust-free box.

2.3. Synthesis of cRNA probes

Bluescript SK⁺ plasmid containing 342 bp of the Kv1.5 cDNA (positions 350–691) was cut using *Xba*I (for antisense orientation) or *Xho*I (for sense orientation). Bluescript SK⁺ plasmid containing 475 bp of the Kv1.6 cDNA (positions 2072–2546) was linearized using *Xho*I (antisense) or *Xba*I (sense). cRNAs were transcribed in vitro with either T7 RNA polymerase (Kv1.5 antisense and Kv1.6 sense) or T3 RNA polymerase (Kv1.5 sense and Kv1.6 antisense) in the presence of digoxygenin-labeled UTP (Boehringer–Mannheim) for in-situ hybridization experiments, or in the presence of α-[^{32}P]UTP (4000 Ci/mmol) (Amersham, Buckinghamshire, UK) for RNAse protection assay.

2.4. In-situ hybridization

SC sections were deparaffined, rinsed twice in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), then treated with proteinase K (25 μg/ml) for 30 min at 37°C. Each section was prehybridized with 100 μl of prehybridization buffer (50% formamide, 4 × SSC, 2 × Denhardt's solution, 500 μg/ml salmon sperm DNA and 0.1% SDS) for 1–3 h at room temperature. For hybridization, 25 ng of Kv1.5 or Kv1.6 digoxygenin-labeled antisense RNA probes were applied in 30 μl of hybridization solution (equivalent to the prehybridization buffer with the addition of 10% dextran sulfate) for each section. Slides were coverslipped with Parafilm and incubated at 42°C for 16 h in a humid chamber. Post-hybridization washes were as follows: 4 × SSC (two changes, 15 min each) followed by RNAse A treatment (50 μg/ml) for 30 min at 37°C, 2 × SSC + 0.1% SDS, 2 × SSC (twice), 1 × SSC (twice), for 10 min each. Following a blocking step (using the blocking reagent supplied with Boehringer's DIG detection kit), sections were incubated for 3–5 h at room temperature with anti-digoxygenin antibody conjugated to alkaline phosphatase (diluted 1:400 in blocking solution). Color was developed overnight using a mixture of 5-bromo-4-chloro-3-indolylphosphate (0.17 mg/ml) and nitroblue tetrazolium salt (0.34 mg/ml). Reaction was stopped by washing with 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA followed by ascending concentrations of alcohol, by xylene and by Permount® mounting. Morphine-treated and control sections from parallel SC areas were obtained under the same conditions and were hybridized, stained and viewed in parallel.

The in-situ hybridization signals were viewed and photographed by bright-field microscopy. In order to ensure the specificity of the probes, we performed the following controls on adjacent sections: hybridization with Kv1.5 or Kv1.6 digoxygenin-labeled sense RNA probes, hybridization in the absence of RNA, and RNAse A treatment of the section prior to hybridization with the antisense probe.

2.5. Semiquantitative RT–PCR analysis

First-strand cDNA synthesis was carried out under the following conditions: 1–5 μg of total RNA was mixed on ice with 0.5 μg oligo(dT) primer (Not I-d(T)$_{18}$, Pharmacia, Uppsala, Sweden) and adjusted to a volume of 9 μl with the diethylpyrocarbonate (DEPC)-treated water. The mixture was incubated for 10 min at 70°C and chilled on ice. Six microliters of first-strand reaction mix buffer, providing final concentrations of 45 mM Tris-HCl, pH 8.3, 68 mM KCl, 15 mM dithiothreitol (DTT), 9 mM $MgCl_2$, 1.8 mM of dNTP, 0.08 mg/ml BSA, 100 U of RNasin, and 50 U of FPLC pure murine RT (Pharmacia, Uppsala, Sweden) was then added and the mixture (final volume of 15 μl) incubated for 1 h at 37°C. After the first-strand cDNA synthesis was completed, the reverse transcriptase (RT) was inactivated by heating the reaction to 95°C for 5 min. Five microliters of the cDNA synthesis reaction mixture were used for PCR, which was carried out in a final volume of 50 μl, containing final concentrations of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.2 mM each dNTP, 1 μM of each upstream and downstream primers, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI). The PCR reaction was cycled as followed: 1-min denaturation at 95°C, 1.5-min annealing at 50°C, 1-min extension at 72°C, for 30 cycles. The last cycle was performed with a 7-min extension at 72°C. The following PCR primers were used: Kv1.5 sense-5′-CATCGGGAGACAGACCAC-3′; Kv1.5 antisense-5′-TTACAAATCTGTTTCCCG-3′; Kv1.6 sense-5′-CACTACTTCTACCACCGA-3′; Kv1.6 antisense-5′-TCAAACCTCGGTGAGCAT-3′.

A semiquantitative PCR was carried out to quantify the input mRNA of the control and treated rat SC mRNA. The co-amplification of an internal control housekeeping mouse S16 ribosomal protein mRNA was performed using an upstream primer (MS16 sense-5′-AGGAGCGATTTGCTGGTG-3′) and a downstream primer (MS16 antisense-CAGGGCCTTTGAGATGGA-3′), which amplify a 102-bp cDNA fragment. An equal aliquot of each PCR product was removed at cycles 28, 29, 30 and 31, and analyzed by 1.2% agarose gel electrophoresis. To evaluate the possibility of PCR contamination, we also performed the reaction with both primers in the absence of cDNA template and/or RT. To eliminate possible contamination by genomic DNA, we performed the reaction containing all PCR reagents and RNA templates without RT.

2.6. RNAse protection assay

Total RNA was extracted from SC by the method of Chomczynski and Sacchi [13]. α-[^{32}P]UTP-labeled antisense cRNA probes were transcribed in vitro (see above) using either T7 RNA polymerase (for Kv1.5) or T3 RNA polymerase (for Kv1.6). A mixture of 20 μg of total RNA and 2.5×10^5 c.p.m. of the riboprobe were applied to 30 μl of RNAse protection buffer (80% formamide, 40 mM PIPES buffer, pH 6.4, 1 mM EDTA). Following denaturation at 85°C for 15 min, the reaction was allowed to hybridize at 50°C overnight. After hybridization, the samples were treated with RNAse reaction mixture (containing 300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 μg/ml RNAse T1, 40 μg/ml RNAse A) for 1 h at 30°C. Reaction was terminated by the addition of 0.5% (final) SDS and 0.27 mg/ml (final) proteinase K, and the incubation was allowed to proceed for additional 30 min at 37°C. Following phenol extraction and ethanol precipitation, samples were resuspended, denatured (85°C, 15 min) and loaded on 6% acrylamide–7 M urea gel. The dried gels were exposed to Kodak XAR5 films for 3 days at −70°C.

2.7. Immunohistochemistry

Sections were deparaffined and incubated for 30 min at room temperature in PBS containing 0.1% Triton X-100. The sections then underwent a 30-min blocking step in PBS supplemented with 0.5% bovine serum albumin and 2% calf serum prior to application of rabbit anti-rat Kv1.5 antibody (diluted 1:250, room temperature, overnight). Following three washes (15 min each), goat anti-rabbit conjugated to alkaline phosphatase (diluted 1:200) was applied to the sections for 2 h at room temperature. Color was developed (for 20 min) and sections were dehydrated and mounted as described for the in-situ hybridization procedure. The immunohistochemical signals were viewed and photographed by bright-field microscopy. Controls were performed by the omission of either the first or the second antibody. Pre-adsorption of affinity-purified antibodies with GST-Kv1.5 fusion protein (10 μg/ml) prevented immunostaining of the cells (not shown).

2.8. Western blot analysis

SC tissues were homogenized in 1% SDS and boiled for 10 min. Protein concentration was determined by the method of Lowry et al. [38], with bovine serum albumin as a standard. Aliquots of 50 μg protein were electrophoresed on SDS–10% polyacrylamide gels and blotted onto nitrocellulose papers. Blots were incubated overnight with rabbit anti-Kv1.5 antibody (diluted 1:500), then for 2 h with biotinylated goat anti-rabbit IgG (1:3000), and for 1 h with horseradish peroxidase-conjugated streptavidin (1:1000). Blots were then stained for peroxidase activity with 4-chloronaphtol and hydrogen peroxide [46]. Pre-immune serum, as well as pre-adsorbed antibodies with GST–Kv1.5 fusion protein did not reveal immunoreactive bands (not shown).

2.9. Quantitation and statistical analysis

Quantitative analysis of the in-situ hybridization of Kv1.5 and Kv1.6 mRNAs was performed with the aid of a computerized video-imaging system using the public domain NIH Image program. For each of the regions studied, the areas and the mean optical densities (OD) of the K^+ channel mRNA signals were determined. The OD values of background levels were subtracted from the signal mean OD values. The data for Kv1.5 and Kv1.6 mRNAs are presented as mean OD and are expressed as fold over control (mean fold ± S.E.M.). Data were analyzed by two tailed unpaired t test. Sections were taken from three control and three morphine-treated rats. At least three experiments were performed for each condition. The number of sections examined is denoted by *n*.

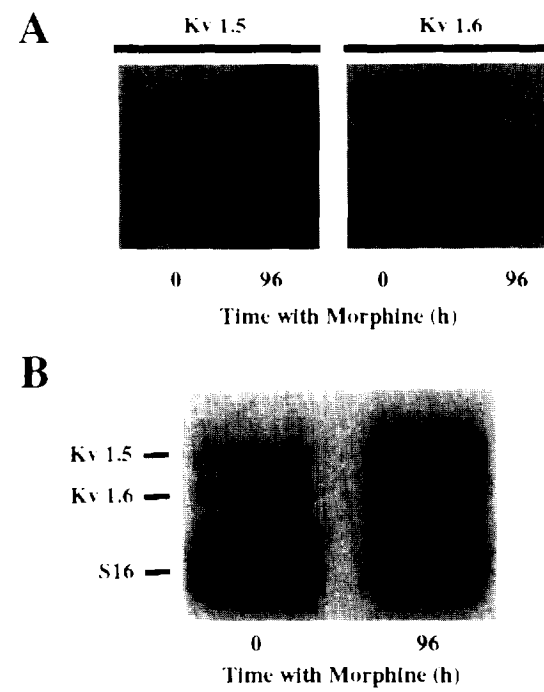

Fig. 1. RNAse protection and RT-PCR analyses of Kv1.5 and Kv1.6 in SC of control and chronic morphine-treated rats. A: RNAse protection analyses of Kv1.5 and Kv1.6 K^+ channels were performed by hybridization of specific α-[^{32}P]UTP-labeled antisense probes with total RNA extracted from SCs of control and morphine-treated (96 h) rats. This representative experiment illustrates morphine regulation of the mRNA for both K^+ channels. In morphine-treated SC, Kv1.5 was increased by 4.3-fold over control rats and Kv1.6 by 3.3-fold. B: mRNA expression level of Kv1.5 and Kv1.6 channel subtypes has been determined by semiquantitative RT-PCR experiments. The two upper bands (273 and 206 bp fragments) correspond to the amplified Kv1.5 and Kv1.6 channel fragments, respectively. The lower band (102 bp) corresponds to the S16 ribosomal PCR fragment used as an internal standard.

3. Results

In order to assess the effects of opiates on K^+ channel mRNAs and protein in rat SC, we performed RNAse protection, semiquantitative RT–PCR, in-situ hybridization, immunohistochemistry and Western blot experiments using a chronic regime of morphine treatment in comparison to control rats (which have received placebo pellets). Defined matched SC areas were compared for their content of Kv1.5 and Kv1.6 mRNA and protein with or without treatment. The distribution and changes in the amounts of mRNA and protein were quantitated using video-computerized imaging. The probes used in this study recognize specific sequences of the tested channel mRNA, as was confirmed by RNAse protection assay analysis of RNA extracted from rat SC (see Fig. 1A). Specificity of the cRNA probes was also determined by conducting control experiments in which sense RNA probes were hybridized in parallel on adjacent sections or in which RNAse A treatment was performed prior to the antisense hybridization. In both experimental approaches, no specific signals were detected (see Fig. 2B).

3.1. Effect of morphine on the expression of Kv1.5 and Kv1.6 mRNAs in SC

We examined the patterns of distribution of the mRNAs of the two K^+ channels in coronal sections of several subregions of the SC (cervical, dorsal and lumbar). The mRNAs of both channels were similarly distributed and

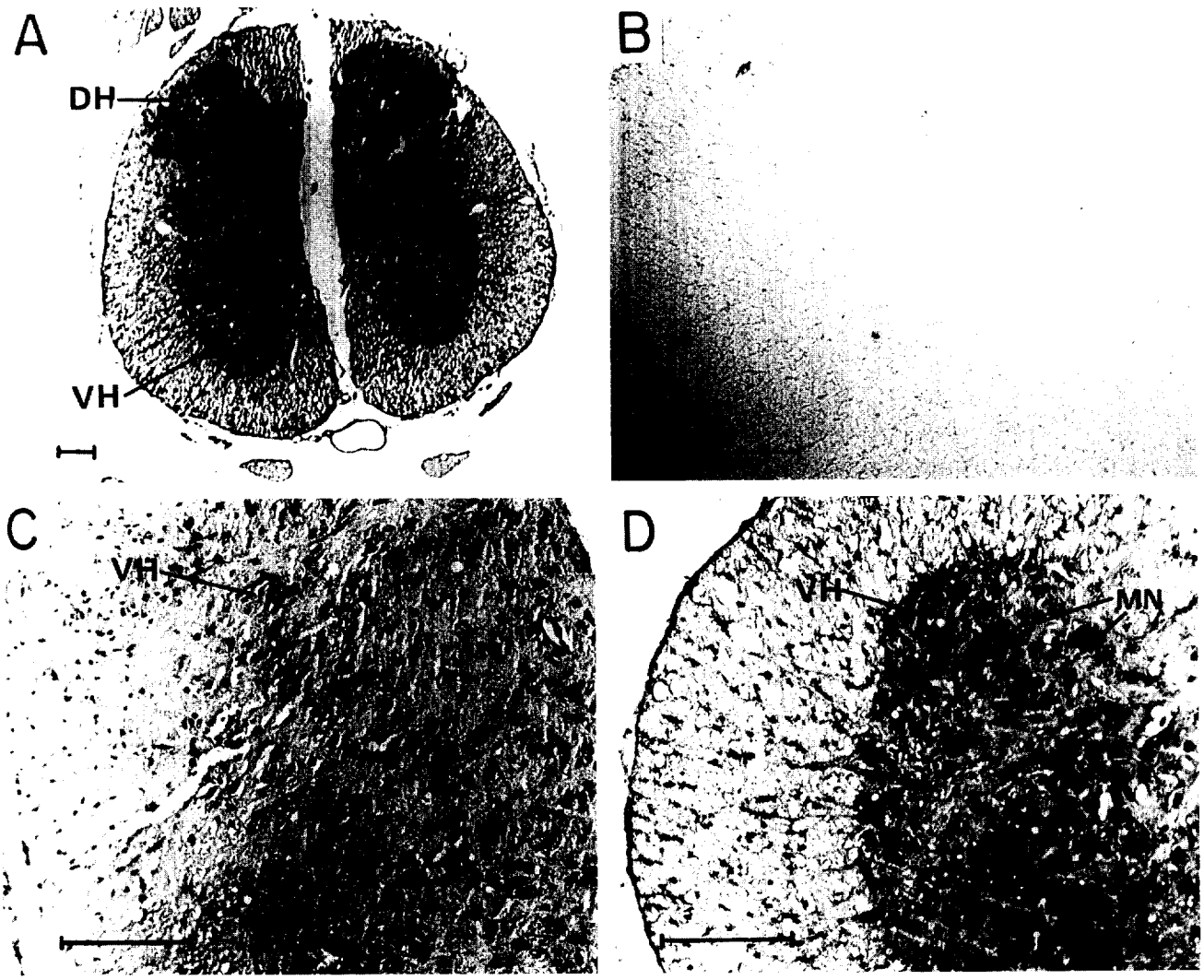

Fig. 2. Localization of Kv1.6 mRNA in the SCs of control and chronic morphine-treated rats. A: Overview of a section showing Kv1.6 mRNA signals from morphine-treated SC. B: Representative section which was hybridized with sense Kv1.6 cRNA. Hybridization with Kv1.5 sense cRNA probe showed the same result. C: Micrograph of the ventral horn of a control section showing Kv1.6 antisense hybridization signals. D: Micrograph of the ventral horn from a morphine-treated rat showing intense hybridization signals of Kv1.6 in the large cells of the motor neurons. Kv1.6 mRNA levels were increased by 2.3 ± 0.5-fold in the SC of treated rats compared with control ($n = 27$ for control, $n = 24$ for treated, $P < 0.001$). DH, dorsal horn; MN, motor neurons; VH, ventral horn. Bars: 250 μm.

were predominantly located in the gray matter in the laminae of the dorsal and ventral horns, which include the cell bodies of motor and sensory neurons. The localization of high-intensity signals of the mRNAs to the large cells of the ventral horn may reflect K^+ channel expression in motor neurons of the SC.

Chronic morphine treatment of 96 h resulted in a 2.1 ± 0.15-fold increase in Kv1.5 mRNA ($n = 29$ for control, $n = 26$ for treated, $P < 0.001$) and in a 2.3 ± 0.50-fold increase in Kv1.6 mRNA ($n = 27$ for control, $n = 24$ for treated, $P < 0.001$) in cell bodies in all three subregions described above (Figs. 2 and 3). The increases in mean OD (from 50.3 ± 4.7 to 113.8 ± 9.4 for Kv1.5 and from 50.9 ± 6.6 to 116.3 ± 5.5 for Kv1.6) seem to represent an elevation of the mRNA content in the affected cells rather than a major change in the number of cells that express K^+ channel mRNA. However, a small increase in the number of such cells cannot be totally ruled out. RNAse protection assay clearly demonstrated marked increases in the levels of the mRNAs of both channels following 96 h of continuous morphine exposure. 4.3- and 3.3-fold increases over controls were observed for Kv1.5 and Kv1.6, respectively (Fig. 1A). In agreement with the RNAse protection data, a semiquantitative PCR analysis (Fig. 1B)

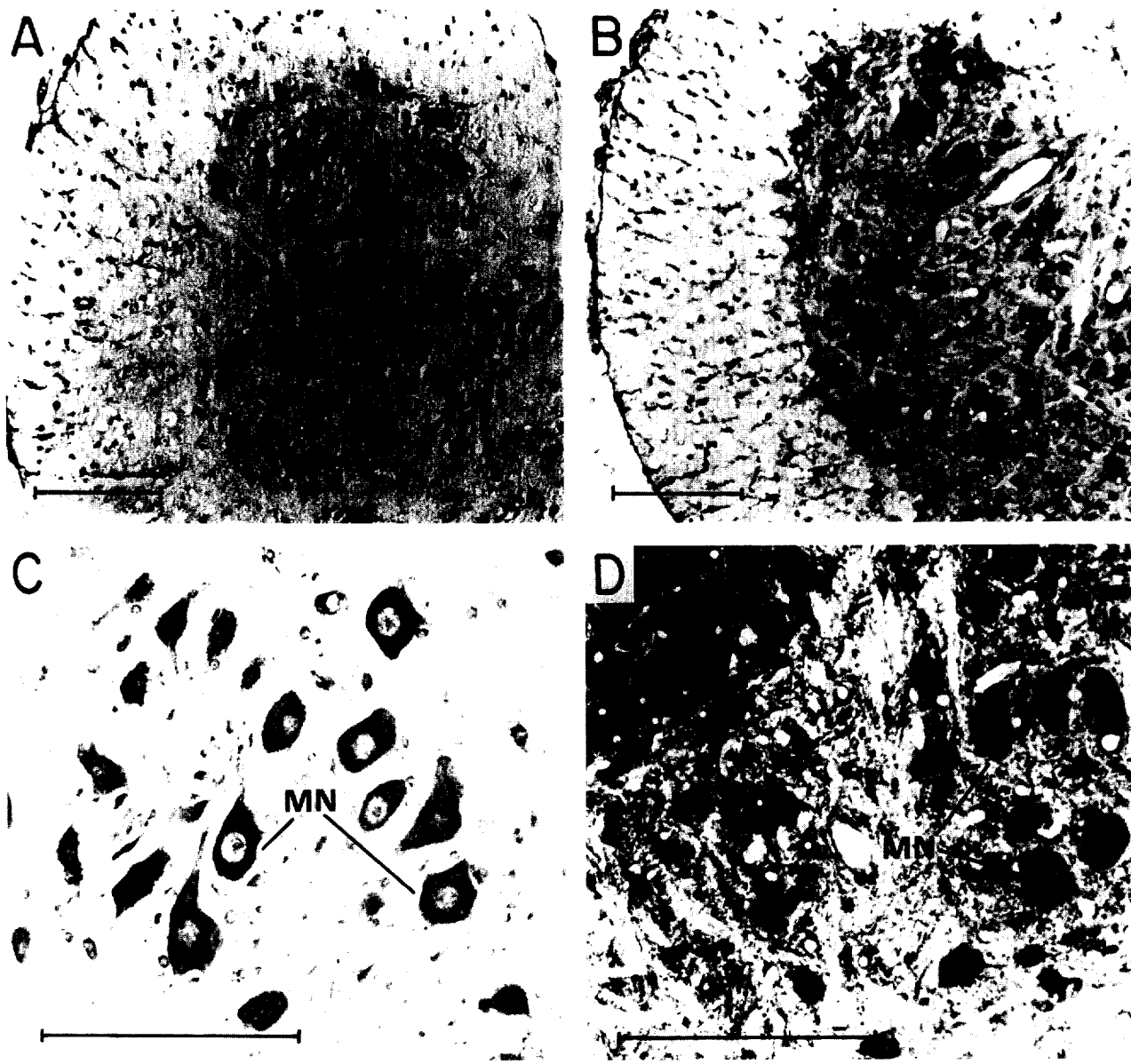

Fig. 3. A: Micrograph of the ventral horn of a control section showing Kv1.5 antisense hybridization signals. B: Micrograph of the ventral horn from a morphine-treated rat showing intense hybridization signals of Kv1.5 in the large cells of the motor neurons. C: High magnification micrograph showing Kv1.5 mRNA hybridization signals in the motor neurons in control, and (D) in morphine-treated SCs. Kv1.5 mRNA levels were increased by 2.1 ± 0.15-fold in the SC of treated rats compared with control ($n = 29$ for control, $n = 26$ for treated, $P < 0.001$). DH, dorsal horn; MN, motor neurons; VH, ventral horn. Bars: 250 μm.

showed that the levels of mRNAs encoding Kv1.5 and Kv1.6 were increased by 3.3- and 2.94-fold, respectively, in morphine-treated animals when compared to control rats. No changes were observed in the levels of the internal control S16 ribosomal protein.

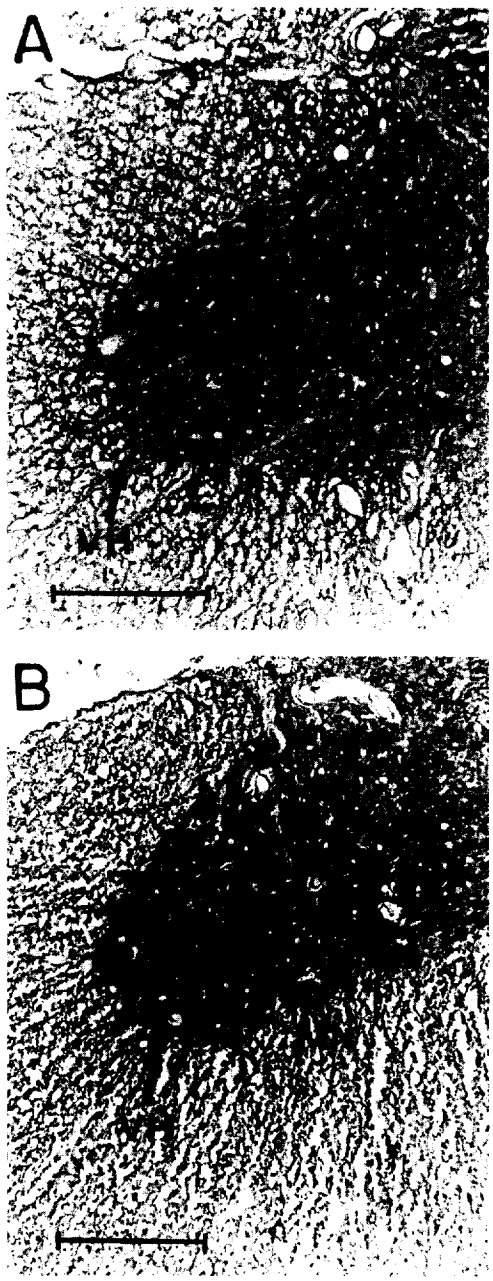

Fig. 4. Localization of Kv1.5 protein in the SCs of control and chronic morphine-treated rats. Immunohistochemistry was performed in parallel with in-situ hybridization using adjacent sections obtained from the same animal. The signals of Kv1.5 were obtained using alkaline phosphatase-conjugated goat anti-rabbit. The distribution of the protein is comparable with that of the mRNA. Most of the signal is located in the giant cells of the ventral horn. A: Representative section showing Kv1.5 protein distribution in the ventral horn of control SC ($n = 15$). B: Representative section of Kv1.5 protein in SC of morphine-treated SC ($n = 18$). No signals were obtained with the omission of the first or second antibodies. DH, dorsal horn; VH, ventral horn; MN, motor neurons. Bars: 250 μm.

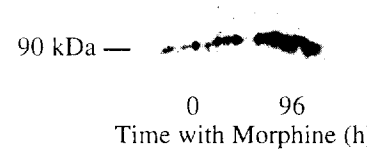

Fig. 5. Western blot analysis of Kv1.5 in SC of control and chronic morphine-treated rats. Samples of 50 μg protein taken from control and chronic morphine-treated (96 h) rats were separated by SDS-PAGE and immunoblotted using rabbit anti-rat Kv1.5. This representative experiment illustrates the increase in Kv1.5 levels following chronic morphine treatment (1.9-fold over control).

3.2. Effect of morphine on SC Kv1.5 protein level and distribution

To evaluate whether the distribution of Kv1.5 protein corresponds to its mRNA localization, we performed immunohistochemical experiments on neighboring sections of rat SC. Employing specific rabbit anti-Kv1.5 antibody conjugated to alkaline phosphatase, we were able to localize Kv1.5 protein to cell bodies of various cell types of the rat SC. It can clearly be seen that Kv1.5 protein is highly expressed in the large motor neuron cells. To a lesser extent, Kv1.5 protein was also found in other cell types of the ventral and dorsal horns (Fig. 4A). These results are compatible with the pattern of distribution obtained for the Kv1.5 mRNA. To study the effect of chronic morphine on Kv1.5 protein, we conducted immunohistochemical experiments on SC sections taken from the same morphine-treated animals that were used for the in-situ hybridization experiments. As shown in Fig. 4B, the Kv1.5 protein is expressed at higher levels in sections obtained from morphine-treated SCs than in control section. It is clearly demonstrated that an elevation in protein is found in the giant cells of the ventral horn (probably motor neurons).

In order to confirm the data obtained by immunohistochemistry and to compare the amount of Kv1.5 protein in SC of treated animals with controls, we conducted immunoblot analysis of Kv1.5 protein in treated and untreated rats. Results of the immunoblot show that 4 days of morphine administration causes an elevation (1.9-fold) of SC Kv1.5 protein as compared with untreated controls (Fig. 5).

4. Discussion

The studies described above were aimed at assessing the role of voltage-gated K^+ channels in the complex events leading to the development of opiate dependence. We have focused on the adaptive changes in the expression of Kv1.5 and Kv1.6 voltage-gated K^+ channels in SC following chronic opiate treatment, since it was shown that these two channels are modulated by chronic opiate treatment in the striatum [39]. It is well documented that the SC

is rich in opioid receptors, especially of the μ- and κ-subtypes [6,41], and plays a crucial role in mediating opiate analgesia and opiate addictive effects. We found that chronic opiate exposure is accompanied by increases in levels of the mRNAs for these two K^+ channels, as well as by a concomitant increase in the amount of Kv1.5 protein.

Several K^+ channels were found in SC cells and in motor neurons, and were shown to affect motor activity. Robitaille et al. [56] reported that Ca^{2+}-gated K^+ channels are clustered in the presynaptic membrane of the neuromuscular junction facing the postsynaptic membrane. Wall and Dale [67] showed that the reduction in K^+ current amplitudes produce specific perturbations in the motor pattern. It has been demonstrated by intracellular recordings from the motor neurons of the ventral roots that Ca^{2+}-dependent K^+ channels are associated with termination of locomotor bursts [19]. Fisher and Nistri [22] suggested that suppression of the K^+ current by thyrotropin releasing hormone (TRH) can explain the excitatory effect of TRH on rat SC motor neurons. Here, we show that delayed-rectifier K^+ channels Kv1.5 and Kv1.6 are present in the motor neurons of the SC.

Opioid receptors can be classified into three main types referred to as μ, δ and κ (for review see [55]). The three opioid receptors are members of the seven transmembrane G protein-coupled receptor superfamily and are homologous to one another at both the amino acid and the nucleic acid levels [10,23]. Recent reports suggest differential anatomical distribution of the three opioid receptors in the CNS which implies distinct functional roles [41,42]. Agonist activation of opioid receptors leads to inhibition of adenylate cyclase activity and of voltage-dependent Ca^{2+} channels [4,5,12,25,59]. Biochemical and pharmacological studies have shown that chronic opiate exposure leads to the development of tolerance to most of the acute actions of opiates. Sharma et al. [59] were the first to report opposing effects of acute and chronic opiate exposure on cAMP levels in cultured neuroblastoma × glioma hybrid cells. Since then, many other reports have pointed to alterations in opiate signal transduction components as possible mechanisms underlying tolerance phenomena [3,18,27,43,44,46–48,61,65,66,71].

In various cell systems, it has been shown that opioid receptors exert stimulatory or inhibitory effects on K^+ channels [9,20,45,50]. It is well established that opioid receptors could exert their inhibitory effects on neuronal firing via activation of inward-rectifying K^+ channels [1]. Stimulation of μ- and δ-opioid receptor types leads to increased K^+ conductance in postsynaptic neurons examined in vitro [50,51]. κ-Opioid receptors have been found to increase K^+ conductance in guinea pig substantia gelatinosa neurons [26], and to inhibit K^+ conductance in CATH.a cells [9]. Relatively, little is known about the ionic mechanisms underlying the opiate excitatory effects.

Moore et al. [45] reported opposing effects of κ- and δ-agonists on CA3 hippocampal K^+ conductances. Opioid agonist excitatory effects have been also described for sensory DRG, F11 neuroblastoma × DRG hybrid cells [20,21]. In these neuronal cells, opioid agonists were shown to decrease voltage-dependent K^+ currents. Interestingly, Kv1.5 and Kv1.6 are delayed-rectifier K^+ channels whose biophysical properties appear to be similar to those found for the K^+ channels that were described in F11 cells. No information is yet available regarding the nature of this K^+ channel(s) and its modulation by acute and chronic opiate exposure. It is reasonable to assume that some of these K^+ channels are under excitatory opiate regulation, leading to decreased K^+ currents.

Mackler and Eberwine [39] have also observed alterations in K^+ channel mRNA expression following chronic morphine treatment. They reported down-regulation of Kv1.5 and Kv1.6 mRNA in the striatum of morphine-treated rats and in δ-agonist-treated NG108-15 cells. These apparent differences between their results (using striatum) and ours (using SC) could arise for several reasons. (1) Various regions of the CNS may by differentially influenced by opiate treatment; indeed, region-specific alterations for other proteins were observed and discussed in Nestler et al. [48]. (2) These variations could be due to opioid-receptor subtype-specific effects. Rat striatum contains mainly δ-receptors [41,42], whereas SC contains mainly κ- and μ-receptors and very few δ-receptors [6,40]. (3) Opiates could have opposing effects in various tissues, for example, in hippocampal CA3 pyramidal neurons, opiates display opposing effects on the same voltage-dependent K^+ channel (M current), with the direction of the response depending on which receptor subtype (κ or δ) is activated [45]. Excitatory effects of opioid agonists can be achieved by inhibiting GABAergic pathways resulting in increased firing rates, as was reported by Nicoll et al. [49]. It has also been shown that injection of opioid peptides into the nucleus accumbens and ventral tegmental area stimulates locomotor activity [16,34]. In a way, opioid receptor agonists may depress K^+ channel activity by a mechanism very similar to that underlying muscarinic inhibition of the same kind of delayed-rectifier K^+ channel, through tyrosine kinase phosphorylation [29]. The possibility that opiates decrease Kv1.5 and Kv1.6 currents in the SC is supported by Kuschinsky et al. [36], who showed morphine excitatory effects on rat SC motor neurons. Hence, following continuous opiate exposure, up-regulation of Kv1.5 and Kv1.6 (which were predominantly located in the motor neurons) will tend to compensate for the persistent K^+ current inhibition.

In conclusion, several cellular adaptations were shown to accompany chronic exposure to opiate drugs. This study provides evidence that continuous exposure to morphine evokes increases in Kv1.5 and Kv1.6 mRNAs and in the amount of Kv1.5 protein in rat SC. We suggest that alterations in the expression of K^+ channels are part of the molecular basis that underlies opiate dependence.

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