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Expression of μ-, δ-, and κ-Opioid Receptor-Like Immunoreactivities in Rat Dorsal Root Ganglia after Carrageenan-Induced Inflammation

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Recently, antisera that recognize unique epitopes of the cloned μ-, δ-, and κ-opioid receptors (MOR, DOR, KOR, respectively) have been developed. In the present study MOR-, DOR-, and KOR-like immunoreactivities (LIs) were examined in rat dorsal root ganglia (DRGs, L4-5) after injection of carrageenan (CAR) into the hindpaw. In normal control rats, 20.9%, 13.5%, and 9% of the DRG neurons contained MOR- , DOR-, KOR-LI, respectively. A marked upregulation in MOR-LI was observed in DRG neurons 1 and 3 d after inflammation. In contrast, CAR induced a distinct downregulation in DOR- and KOR-LIs. MOR-, DOR-, and KOR-LIs were preferentially localized in small DRG neurons. MOR-LI was often located in patches in the cytoplasm, and in some cells close to the somatic plasmalemma. However, DOR- and KOR-LIs mainly showed a diffuse staining pattern within cytoplasm. Two or even all three receptors could sometimes be found to coexist in DRG neurons. In the spinal cord, these receptors were mainly confined to the superficial dorsal horn, with a somewhat diffuse staining which was strong for MOR-LI, and weak for KOR-LI. DOR-LI had a distinctly punctate, varicose distribution. CAG induced-alterations in opioid receptor staining in spinal cord were much less pronounced than those in the DRGs with a small increase in MOR-LI and a slight decrease in DOR-LI ipsilaterally. There was an accumulation of all three types of receptors in the sciatic nerve both proximal and distal to the ligation site as early as 2 hr, indicating both antero- and retrograde transport of multiple opioid receptors. However, DOR-LI accumulation was stronger than that of MOR- and KOR-LIs. Taken together, these results suggest that all three opioid receptors are involved in the response to inflammation and that they may play different roles in this pathological state. The coexistence of MOR, DOR, and KOR in at least some primary sensory neurons provides a substrate for functional interactions between these receptors.

[Key words: multiple opioid receptors, pain, primary sensory neurons, spinal cord, coexistence, axonal transport]

Multiple receptor sites recognizing exogenous opiate drugs and endogenous opioid peptides have been defined based on pharmacological, behavioral and receptor binding experiments. Thus, there are μ, δ, and κ receptors, which display high affinities for morphine, enkephalin, and dynorphin, respectively (Martin et al., 1976; Lord et al., 1977; Chang and Cuatrecasas, 1979; Smith and Simon, 1980; Corbett et al., 1993; Simon and Gioannini, 1993). These receptors are involved in regulation of, for example, nociception, respiration, cardiovascular functions, gastrointestinal motility, and mood (Basbaum and Fields, 1984; Pasternak, 1988; Herz, 1993). Recently a selective loss of δ opioid analgesia and binding has been demonstrated after intrathecal administration of antisense oligonuclotides to a δ opioid receptor (Standifier et al., 1994). Using ligand binding autoradiography, all three types of opioid receptors have been demonstrated in the superficial spinal cord (Atweh and Kuhar, 1977; Iadarola et al., 1988; Besse et al., 1990; Stevens et al., 1991b; see also Mansour et al., 1988), a region of the dorsal horn enriched in opioid peptides, and a known site of termination of peptide containing and nociceptive primary afferents (Hökfelt et al., 1977; Besson and Chaouch, 1987; Willis and Coggeshall, 1991; Dado et al., 1993; Arvidsson et al., 1995b). In autoradiographic ligand binding studies Ninkovic et al. (1981, 1982) demonstrated presence of opiate receptors on primary sensory neurons. Transection of the sciatic nerve or dorsal root afferents results in a marked decrease in all three types of opioid binding sites within the dorsal horn, suggesting a partly presynaptic localization of the receptors (Fields et al., 1980; Besse et al., 1990). A comparable decrease is observed after capsaicin treatment (Gamse et al., 1979; Besson and Chaouch, 1987). However, ligand binding autoradiography is generally limited by a low resolution mostly not allowing unequivocal identification of cellular and of subcellular structures. With the recent cloning of the δ (Evans et al., 1992; Kieffer et al., 1992; Bzdega et al., 1993), μ (Thompson et al., 1993; Wang et al., 1993; Chen et al., 1993a), and κ (Chen et al., 1993b; Meng et al., 1993; Minami et al., 1993; Yasuda et al., 1993) receptors, it has become possible not only to study opioid receptor expression using *in situ* hybridization (Mansour et al., 1993; Schäfer et al., 1993; Mansour et al., 1994a–c), but one can now generate antibodies to these receptors. In fact, such antibodies have recently been used in immunohistochemical studies to localize opioid receptor protein at the

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cellular level (Dado et al., 1993; Arvidsson et al., 1995a–c; Hiller et al., 1995).

A number of studies have shown that injection of irritative chemicals in the hindpaw of the rat can produce an intense inflammation characterized by erythema, edema, and hyperthermia limited to the injected paw (Iadarola et al., 1988; Dubner and Ruda, 1992; Ji et al., 1994). Such inflammatory tissues are characterized by an increased sensitivity to mechanical stimuli (hyperalgesia) (Dubner and Ruda, 1992; Levine et al., 1993). It has been shown that peripheral inflammation induces a marked upregulation of dynorphin and enkephalin at both mRNA and peptide levels in the dorsal horn (Millan, 1986; Ruda et al., 1988; Weihe et al., 1988; Noguchi et al., 1989; Dubner and Ruda, 1992; Ji et al., 1994). Inflammation also induces c-fos expression in the dorsal horn and this can be blocked by morphine (Presley et al., 1990). Moreover, an extensive body of research has addressed the question of plasticity of opioid receptor binding at the spinal level after inflammation or neuropathic pain (Millan et al., 1986, 1988; Iadarola et al., 1988; Stevens et al., 1991a; Besse et al., 1992a,b; Kar et al., 1994).

In the present study we have used immunohistochemistry and recently developed antibodies against three opioid receptors, the μ receptor (MOR-), the δ receptor (DOR-), and the κ receptor (KOR) to study their expression in primary sensory neurons after unilateral peripheral inflammation. These opioid receptors were also studied in the spinal cord as was their transport in the sciatic nerve. Moreover, since the different types of opioid receptors may be functionally coupled (see Holaday et al., 1985; Rothman et al., 1993), attempts were made to analyze a possible colocalization of three opioid receptors.

Materials and Methods

Induction of inflammation. Adult male Sprague–Dawley rats weighing 190–220 gm were anesthetized with sodium pentobarbital (40–60 mg/kg,i.p), and 200 μl of a 4% carrageenan solution (Sigma, dissolved in saline) were injected into the plantar surface of the left hindpaw. Animals were kept in cages (three or four animals in a cage) at an ambient temperature of 20–25°C under a 12 hr/12 hr light/dark cycle and had free access to food and water. The experiments have been approved by "Stockholm norra försöksdjursetiska kommitté" and attention was paid to the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983).

Antisera. Antibodies were raised against peptides corresponding to amino acids 384–398 (NHQLENLEAETAPLP, code no. 551; Arvidsson et al., 1995b), 3–17 (LVPSARAELOSSPLV, code no. 442; Dado et al., 1993; Arvidsson et al., 1995b), and 366–380 (DPASMRDVGGMNKPV; Arvidsson et al., 1995c) of the predicted sequence of MOR1 (Chen et al., 1993a; Thompson et al., 1993), DOR (Evans et al., 1992; Kieffer et al., 1992), and KOR (Chen et al., 1993b; Meng et al., 1993), respectively. All peptides were conjugated to bovine thyroglobulin, and used to immunize rabbits as described.

Immunohistochemistry. One day or 3 d after injection of CAR , both inflammatory and control rats were deeply anesthetized with sodium pentobarbital (120 mg/kg, i.p) and transcardially perfused with 60 ml warm saline, followed by 400 ml 4% paraformaldehyde with 0.4% picric acid in 0.16 M phosphate buffer solution (pH 7.2) (Pease, 1962; Zamboni and De Martino, 1967). The L5 DRGs and L4-5 segments of the spinal cord were removed, postfixed in the same fixative for 90 min, and then placed in 10% sucrose solution at 4°C overnight. Tissues were embedded in OCT compound (Miles, USA), cut coronally in a cryostat at 4 μm (to study coexistence) or at 14 μm thickness and mounted onto gelatin coated slides. The sections were placed in a humid chamber and processed for immunohistochemistry according to both ABC method (Hsu et al., 1981) or indirect immunofluorescence staining (see Coons, 1958), by incubating the sections overnight at 4°C in the following primary antisera: MOR (1:4000), DOR (1:1000), or KOR (1:2000). The antisera were diluted in PBS containing 0.3% Triton X-100 (Hartman et al., 1972), 0.01% sodium azide, and 0.02% Bacitracin. For ABC staining, the sections were then incubated for 1 hr at 37°C with the biotinylated secondary antibody (1:200) and subsequently with ABC complex (1:100; ABC Kit, Vector Labs, Burlingame, CA). Finally the reaction product was visualized with 0.05% DAB/0.003% hydrogen peroxide in 0.1 M acetate buffer (pH = 6) containing 2% ammonium nickel sulfate for 5 min, and then rinsed in acetate buffer, air dried, dehydrated, and coverslipped. For immunofluorescence, the sections were incubated at 37°C for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antisera (1:80; Boehringer Mannheim Scandinavia, Stockholm, Sweden). Finally the sections were mounted in a mixture of glycerol and PBS (9:1) containing 0.1% paraphenylenediamine (Johnson and de C Nogeira Araujo, 1981; Platt and Michael, 1983) in order to retard fading and examined in a Nikon Microphot-FX microscope. FITC-induced fluorescence was analyzed with a Nikon B-1E filter cube (excitation at 480 ± 10 nm with a bandpass emission filter passing 520–550 nm). The specificity of the immunostaining was tested by preabsorbing the antibody with synthetic peptides 10^{-6} M; (Peninsula, Belmont, CA), or by replacing the primary antisera with normal serum.

For laser scanning confocal microscopy analysis a BIO-RAD MRC-600 laser scanning confocal imaging system equipped with a krypton/argon mixed gas laser was used. The K1 channel was used. FITC staining was detected with exciter 488DF10 (488 nm line). The images were printed using a Tektronix Phaser IICD digital colour printer.

For analysis of axonal transport, the left sciatic nerve was ligated at mid thigh level. The animals were allowed to survive 2, 6, 12, 24, and 48 hr (two rats for each time point). After perfusion the portion (10 mm) of the sciatic nerve surrounding the ligation was removed, cut, and processed for immunofluorescence staining.

Quantification. The total number of MOR-, DOR-, and KOR-immunoreactive (IR) neuronal profiles was divided by the total number of profiles in each DRG section, and the percentage of immunoreative neuronal profiles was calculated. Percentages from four sections from one animal were averaged as the percentage for that animal. Three rats were used for anlysis of each group.

The size of immunoreactive neuronal profiles in the DRGs and the density of fiber networks in the dorsal horn were measured on a Macintosh IIx computer (Apple Computer Inc., Cupertino, CA), equipped with a Quick Capture frame grabber board (Data Translation, Marlboro, MA) and a Dage-MTI 72 CCD series camera (DAGE-MTI, Michigan City, IN) connected with a Nikon microscope. Image processing was performed with NIH-IMAGE software (courtesy Dr. W. Rasband, NIMH). The size of 200 neuronal profiles each containing MOR-, DOR-, or KOR-LI of DRGs from both control and inflamed animals were measured. In spinal cord, the relative density levels of immunostaining were measured in the medial half of the superficial layers (laminae I and II) of the dorsal horn. Each image was digitized with 256 gray levels for each picture element. Eighteen sections of the spinal cord from rats subjected to 1 (n = 3) and 3 (n = 3) d of inflammation were analyzed.

Statistics. All data were assessed using the two-tailed t test for group comparison and ANOVA for multiple comparison. The criterion for statistical significance was $P < 0.05$.

Results

Carrageenan-induced inflammation

Intraplantar injection of 200 μl CAR (4%) led to a visibly obvious oedema and erythema, indicative of inflammation. The circumference of the injected paw gradually increased to a mean value of 152.7 ± 6.0 and 167.8 ± 7.0% (mean ± SEM, n = 8, $P < 0.001$, paired t test) at 1 d and 3 d, respectively, when the paw circumference of the noninjected side (right side) was set as 100%. No marked oedema was observed in the right paw.

Opioid receptors in DRGs

In control (normal) rats, many DRG neurons (20.9%) were MOR-positive. These neurons were mainly small with profiles ranging in size from 169–1170 μm^2, the majority lying between 300–500 μm^2 (Figs. 1*b*, 2*A*, 3*A*). Confocal analysis showed that the MOR-LI was localized close to the somatic plasmalemma, as well as in patches in the cytoplasm (Fig. 4*a*). However, in some neurons the association of MOR-LI with the plasmalemma

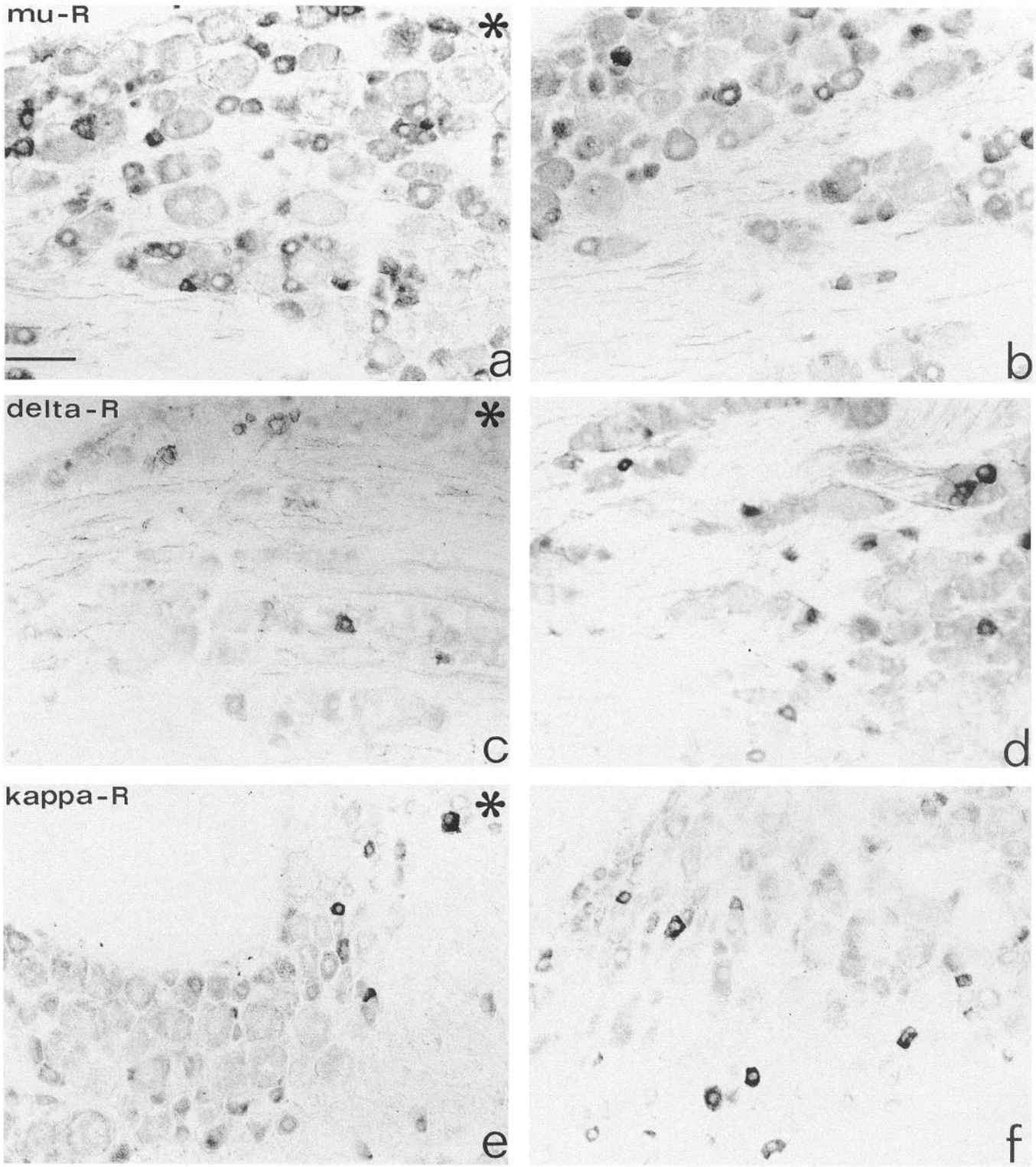

Figure 1. *a–f*, Bright-field micrographs after ABC immunostaining showing the distribution of μ (*a, b*), δ (*c, d*), and κ (*e, f*) opioid receptor-LIs in neurons on the ipsilateral (*a, c, e*) and contralateral (*b, d, f*) dorsal root ganglia (L5) in rats 1 d after carrageenan injection into the left hindpaw. *Asterisks* indicate ipsilateral side. All three types of opioid receptors were preferentially localized in small DRG neurons. Inflammation induced an upregulation of μ- (*a, b*), but a downregulation of δ- (*c, d*) and κ- (*e, f*) opioid receptor-LIs. Scale bar, 100 μm. All micrographs have the same magnification.

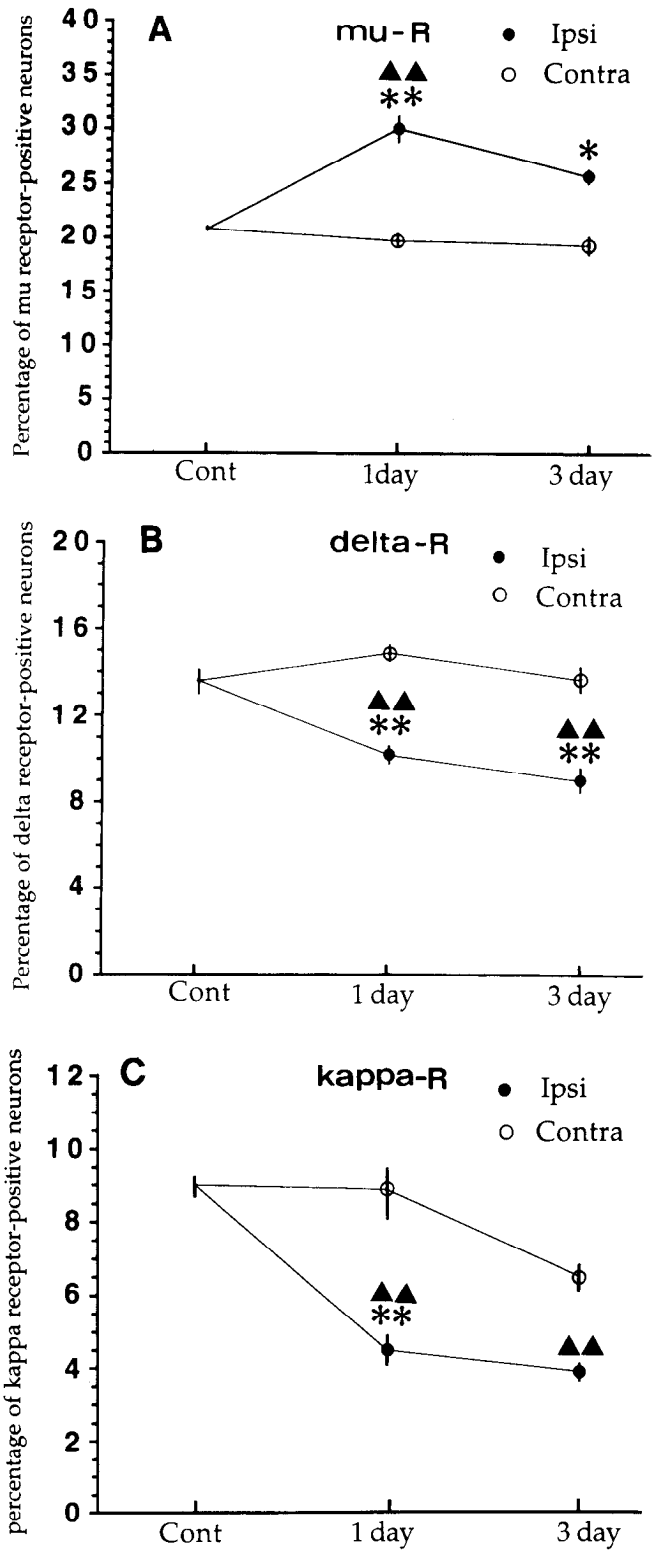

Figure 2. *A–C*, Changes in the percentage of μ (*A*), δ (*B*), and κ (*C*) opioid receptor-IR neurons in dorsal root ganglia (L5) of ipsilateral (*Ipsi*) and contralateral (*Contra*) side in control (*Cont*) rats and in rats 1 and 3 d after carrageenan injection. Carrageenan produced a marked upregulation in μ (*A*), and a downregulation in δ (*B*) and κ (*C*) opioid receptor LIs. The data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ as compared to the contralateral side; ▲, $p < 0.05$; ▲▲, $p < 0.01$, as compared to the controls (normal) (ANOVA followed by Fisher PLSD test).

was less pronounced, and the immunoreactivity was mainly seen in the cytoplasm (Fig. 4*b*). CAR induced a distinct increase in the number of MOR-positive neurons (Fig. 1*a,b*). There was a 52% ($P < 0.01$) and 33% ($P < 0.05$) increase in the percentage of MOR-IR neurons in the ipsilateral DRGs 1 d and 3 d after the CAR-injection, respectively, as compared to the contralateral side (Fig. 2*A*). A significant elevation of MOR-LI could also be observed when compared to DRGs from the normal rats (Fig. 2*A*). No change was detected on the contralateral side (Fig. 2*A*).

Fewer DRG neurons, that is 13.5% contained DOR-LI in normal control rats. They were mainly small, with profiles ranging in size from 148–1024 μm², with the majority between 300 and 600 μm² (Figs. 1*d*, 2*B*, 3*B*). DOR-LI was only found diffusely in the cytoplasm, but not close to the somtic plasmalemma (Fig. 4*c*). In contrast to the upregulation of MOR-LI, CAR induced a marked decrease in the number of DOR-positive neurons (Fig. 1*c,d*). There was a 32% ($P < 0.01$) and a 34% ($P < 0.01$) decrease in the percentage of DOR-IR neurons in the ipsilateral DRGs 1 day and 3 d after the injection, respectively, as compared to the contralateral side (Fig. 2*B*). No change was detected on the contralateral side (Fig. 2*B*).

Nine percent of the DRG neurons contained KOR-LI in normal contral rats, that is less than either MOR- or DOR-IR cells. They were also mainly of the small category. The neuronal profiles ranged in size from 149–932 μm², with the majority between 300 and 500 μm² (Figs. 1*f*, 2*C*). Just as DOR-LI, the KOR-LI was seen diffusely in the cytoplasm of cell bodies, not adjacant to the somatic plasmalemma (Fig. 4*d*). Peripheral inflammation produced a marked decrease in the number of KOR-positive neurons (Fig. 1*e,f*). There was a 49% ($P < 0.01$) and a 40% ($P < 0.001$) decrease in the percentage of KOR-IR neurons in the ipsilateral DRGs 1 d and 3 d after the injection, respectively, as compared to the contralateral side (Fig. 2*C*). A significant decrease in KOR-LI could also be observed when compared to control DRGs (Fig. 2*C*). There was a nonsignificant decrease on the contralateral side at 3 d (Fig. 2*C*).

Inflammation did not alter the size of MOR-, DOR-, or KOR-IR neurons; thus they were still in the small size range (Fig. 3*A–C*). There was no evidence that inflammation affected the intracellular distribution of the three receptors (data not shown).

Colocalization of opioid receptors

Coexistence of the three opiate receptor-LIs was examined in adjacent, 4 μm thick DRG sections from normal rats. As shown in Figure 5, not only MOR- and DOR-LIs were localized in the same neurons, but also DOR- and KOR-LIs, as well as KOR- and MOR-LIs coexisted. In fact all three types of opioid receptors coexisted in some neurons.

Opioid receptors in spinal cord

Immunohistochemistry demonstrated a dense staining for three types of receptors in the superfical layers (laminae I-II) of the dorsal horn (Fig. 6*a–c*). However each receptor had a different staining intensity and appearance, as well as a unique distribution. MOR-LI was the strongest, especially in lamina II, and the MOR-IR structures appeared most densly packed resulting in a somewhat diffuse appearance (Fig. 6*a*). A dense DOR-IR fiber network was distributed in a parallel fashion in the laminae I-II. However, the DOR-IR structures had a distinctly varicose appearance, very much resembling, for example, peptide-IR nerve fibers. DOR-LI plexuses were also seen in deeper lamine (Fig. 6*b*). KOR-LI was quite weak, but distinct in the lateral part of

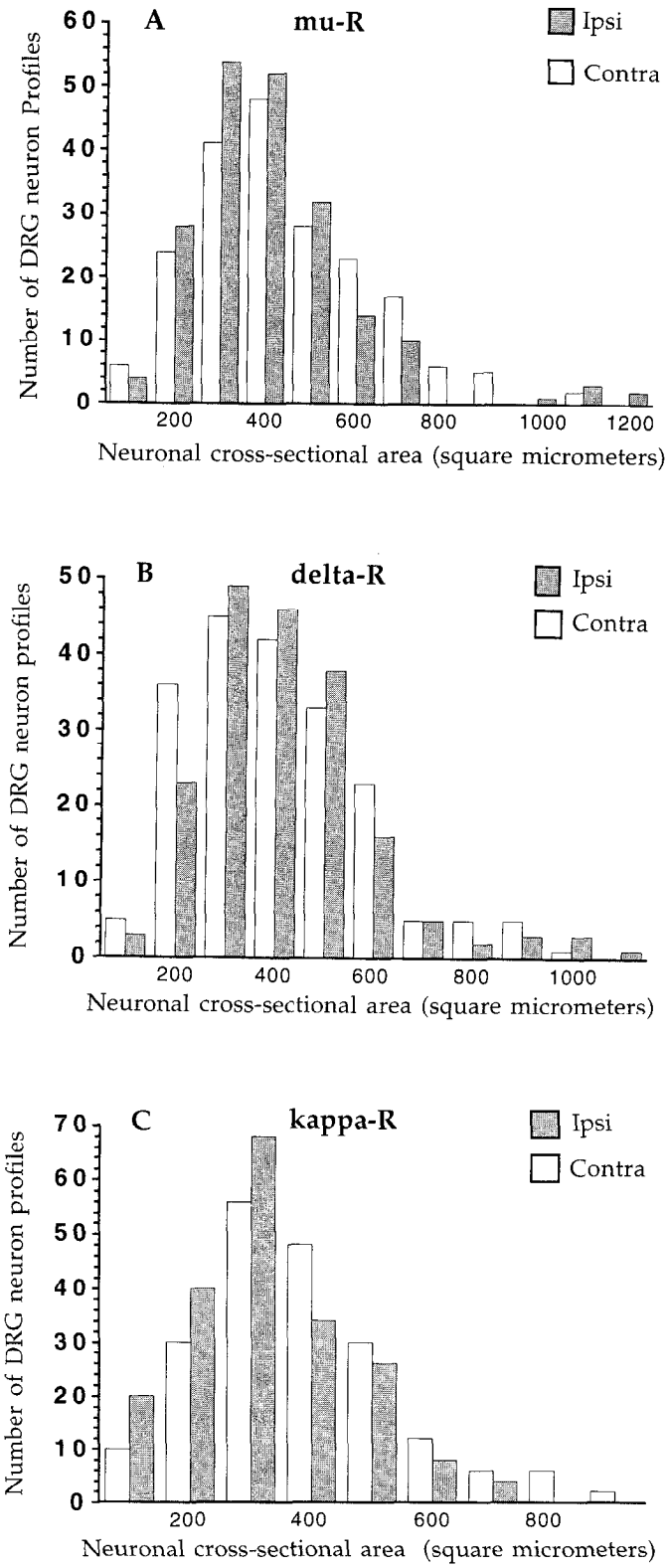

Figure 3. *A–C*, Size range of μ (*A*), δ (*B*), and κ (*C*) opioid receptor-IR neuron profiles in control (Cont) and ipsilateral DRGs (L5) of rats one day after carrageenan injection. Two hundred cells were measured for each group. There were no significant changes in size ranges of cells expressing these receptors after inflammation. The opioid receptors were predominantly distributed in the range of small DRG neurons.

lamina I (Fig. 6*c*). After inflammation no apparent change in MOR-, DOR-, or KOR-LI staining patterns or intensities could be seen in the microscope. However, quantitative evaluation of the staining intensity in the medial part of the ipsilateral superficial dorsal horn revealed a moderate increase (21.9% and 13.1%) in MOR-LI and a small decrease in DOR-LI (3.1% and 8.3%) 1 and 3 d after inflammation, respectively, as compared to the contralateral side (Table 1). KOR-LI in spinal cord was too weak to allow acceptable measurements.

Transport of opioid receptors

As early as 2 hr after ligation a distinct accumulation of MOR-, DOR-, and KOR-LIs was detected in the proximal part of the sciatic nerve (Fig. 7*a,c,e*). The accumulation in the distal part, although weaker, could also be observed after two hours (Fig. 7*a,c,e*). The staining for all three receptors then increased and the strongest accumulation was seen at 24 hr, when also the staining distal to the ligation site was most pronounced (Fig. 7*b,d,f*). Forty-eight hours after ligation the accumulations for the three receptors were weaker, especially for the MORs and KORs (data not shown). At all time intervals the DOR-LI was stronger than MOR- and KOR-LIs, both in the proximal and distal parts (Fig. 7).

Discussion

Taking advantage of the recent cloning of the three opioid receptors (for references, see introductory section), antibodies have been raised against these receptors and used in immunohistochemical studies (Dado et al., 1993; Arvidsson et al., 1995a–c; Hiller et al., 1995), These new data are complementary to previous ligand binding results presented in numerous papers (see Mansour et al., 1988, and introductory section) based on the original autoradiographic methodology described by (Atweh and Kuhar, 1977). In particular the cellular resolution of the immunohistochemical approach provides a more detailed picture of the receptor protein distribution, for example, with regard to distribution in cell bodies versus processes and to presynaptic versus postsynaptic localization. Moreover, specific antisera may, to some extent, overcome the shortcoming of the fact that a ligand can bind to several receptors albeit with different affinity. Also the immunohistochemcial approach may suffer from shortcomings, for example lack of specificity of the antisera. However, the antibodies used are well characterized using a number of criteria (Arvidsson et al., 1995a–c).

Our results show that all three opioid receptors are expressed in DRG neurons, mainly of the small type (see also Dado et al., 1993; Arvidsson et al., 1995b), and that peripheral inflammation differentially regulates the expression of these three types of opioid receptors. This is especially obvious at the DRG level. Moreover, the three opioid receptors were found to coexist in several primary sensory neurons. Finally, although all three opioid receptors to some extent were transported along the sciatic nerve, only the δ receptor protein showed a dramatic accumulation central to a crush.

Primary sensory neurons

In the present study MOR-, DOR-, and KOR-LIs were detected in ~20%, ~15%, and ~10%, respectively, of all DRG neurons in normal rats. Size measurement indicated that all these receptors were predominantly localized in small neurons, that is neurons that give rise to C- or Aδ-fibers (see Willis and Coggeshall, 1991). This result is consistent with that of Dado et al. (1993)

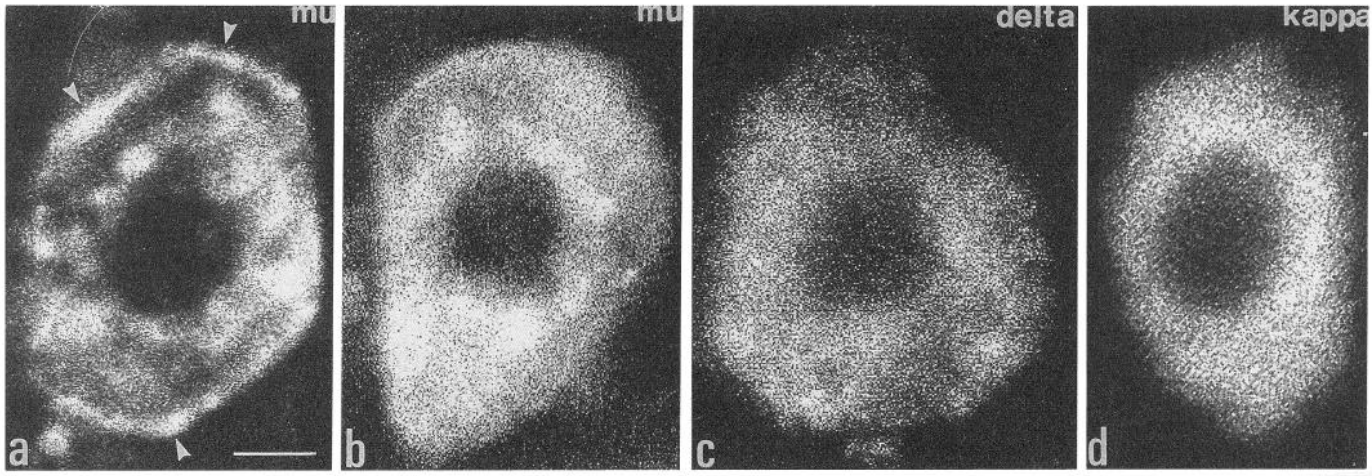

Figure 4. *a–d*, Immunofluorescence micrographs obtained by confocal microscopy of rat DRGs after incubation with μ (*a, b*), δ (*c*), or κ (*d*) opioid receptor antisera. The μ receptor-LI was localized on plasmalemma (*arrowheads in a*) and in patches in the cytoplasm (*a, b*). δ and κ receptors were diffusely distributed within the cytoplasm (*c, d*). Scale bar, 5 μm.

on DORs and with that of Arvidsson et al. (1995b) showing that MOR-positive DRG neurons were mainly negative for RT 97, a marker for large, myelinated primary afferents (Lawson and Waddell, 1991). It is also in agreement with a previous capsaicin study indicating that opioid receptors are preferentially localized on thin afferent fibers (Gamse et al., 1979). However, these results are in some contrast to a recent *in situ* hybridization study (Mansour et al., 1994a) showing presence of MOR mRNA mainly in large and medium sized ganglion cells and DOR mRNA in a few large neurons. The relative frequencies seen may however be similar, as was the expression of KOR, since both studies found it to be in small DRG neurons.

A strong immunostaining for MOR-LI was often seen along the somatic plasmalemma of DRG neurons, suggesting that some MORs are incorporated in the cell membrane of ganglionic cell bodies. In contrast, no such membrane association could be observed for DORs or KORs, which were exclusively found in the cytoplasm. Electron microscopic analysis will be required to define to which subcellular structures the three opioid receptors are confined. It is possible that some MORs are confined to the soma of DRG neurons. A similar situation has been found for the neuropeptide Y1 receptor which is distinctly localized in the somatic plasmalemma and does not seem to be transported centrifugally (Zhang et al., 1994a). The question then arises what the source(s) of the endogenous ligand(s) for the somatic MORs in the DRGs might be? It is well known that proopiomelanocortin, a precursor for β-endorphin, is produced in the pituitary gland (Eipper and Mains, 1980) and that chromaffin cells in the adrenal medulla can synthesize enkephalins (Schultzberg et al., 1978; Viveros et al., 1979). It is possible that these blood borne opioid peptides activate MORs on DRG neuronal cell bodies and that this could contribute to the phenomenon of stress-induced analgesia (Lewis et al., 1982).

In contrast, DOR-LI was only cytoplasmic in the DRG soma

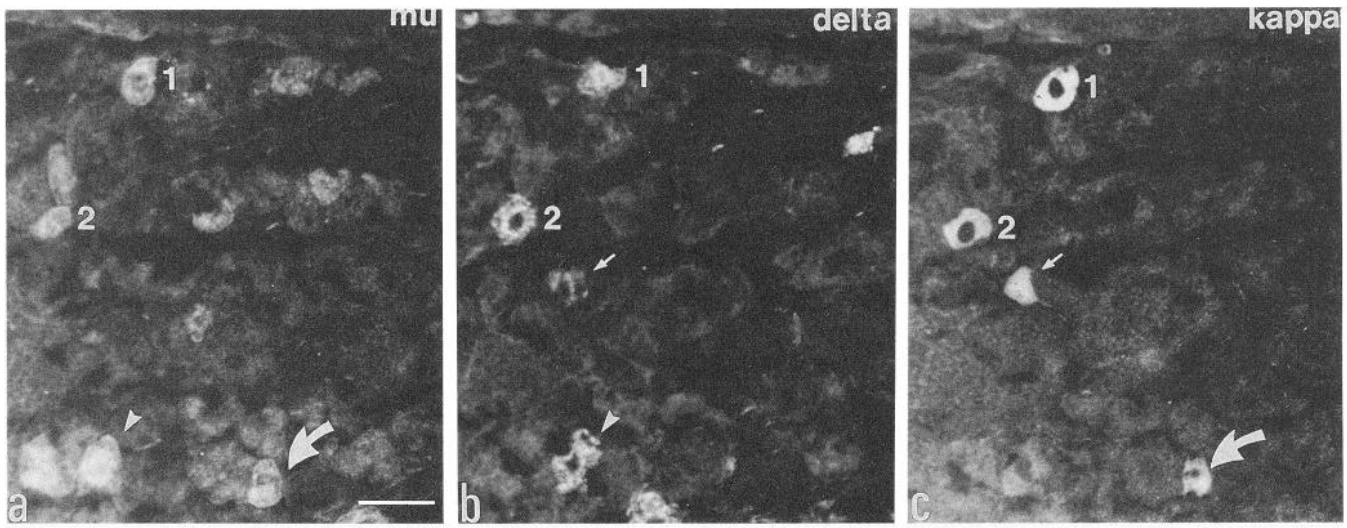

Figure 5. *a–c*, Immunofluorescence micrographs of three adjacent, 4 μm thick sections from a DRG (L5) after incubation with μ- (*a*), δ- (*b*), and κ- (*c*) opioid receptor antisera. Note coexistence between μ and δ (*a, b; arrowheads*), δ and κ (*b, c; small arrows*), κ and μ (*a, c; large arrows*) opioid receptors. Neurons 1 and 2 contain all three types of opioid receptors. Note that the order of serial sections is $b \rightarrow c \rightarrow a$. This sequence is used to maintain the same order for the receptor as in the article in general. Scale bar, 50 μm.

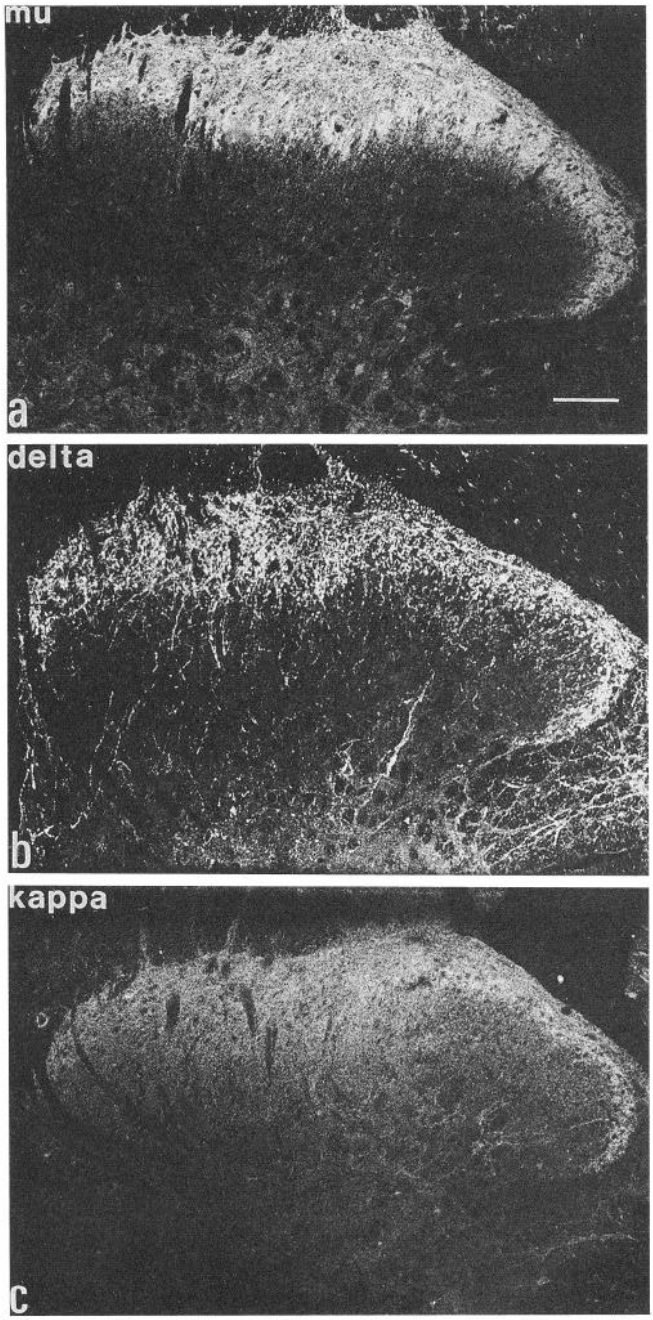

Figure 6. *a–c*, Immunofluorescence micrographs showing the distribution of μ- (*a*), δ- (*b*), and κ- (*c*) opioid receptor-like immunoreactiviry (LI) in the dorsal horn (L4-5). The staining of the three receptor immunoreactivities was predominantly observed in the superficial layers (laminae I–II). The heaviest labeling was seen for μ receptor, especially in lamina II (*a*). A dense fiber network containing δ receptor was observed in both laminae I and II, with single fibers in deeper laminae (*b*). The κ receptor staining was compartatively weak (*c*). Scale bar, 100 μm.

and accumulated strongly central to the crush, and was also present in high levels in CGRP-positive primary afferent fibers in the dorsal horn (Dado et al., 1993). Thus, DORs may be preferentially functionally active in the dorsal horn of the spinal cord, that is they represent presynaptic receptors localized on primary afferents. To what extent KOR is targeted to the axonal compartment of neurons to function at a presynaptic site remains

Table 1. Relative intensity of μ- and δ-opioid receptor-like immunoreactivity (MOR-LI and DOR-LI) in ipsilateral (Ipsi) and contralateral spinal dorsal horns (L4-L5) 1 and 3 d after unilateral injection of carrageenan into the hindpaw

	Ipsi	% Change
MOR-LI (1 d)	121.9 ± 4.5	21.9%
MOR-LI (3 d)	113.1 ± 7.7	13.1%
DOR-LI (1 d)	96.9 ± 11.7	−3.1%
DOR-LI (3 d)	91.7 ± 15.2	−8.3%

The intensity in the contralateral side was set as 100%. Measurements were done in the medial half of the superficial layers (laminae I–II) of the dorsal horn that innervated by the hindpaw. Results are presented as the mean ± SEM from three rats. For each rat counts from three to four sections were averaged. All the changes ware significant ($P < 0.01$) if paired t test is used but not significant ($P > 0.05$) with the unpaired t test.

to be studied. It seems to be transported somewhat better than MORs, and it could not be detected in association with the cell soma plasmalemma, suggesting a function in the dorsal horn. The KOR staining is somewhat difficult to evaluate, since this antiserum clearly is not as powerful as the MOR and DOR antisera.

An interesting finding in the present study is that three opioid receptors are differentially regulated in DRG neurons following inflammation. CAG induced a distinct increase in MOR-LI but a decrease in DOR- and KOR-LIs 1 d and 3 d after inflammation. The changes in receptor levels after inflammation were mainly confined to the ipsilateral side, and therefore the contralateral DRG could be used as a control together with the control rats. The time points 1 and 3 d were chosen to detect the CAG-induced changes in the present study, since not only the edema and the hyperalgesia are dramatic at this stage (Iadarola et al., 1988), but inflammation-induced changes in synthesis of neuropetides and their receptors are also peaking at these time intervals (Iadarola et al., 1988; Ji et al., 1994).

Our results indicate that inflammation does not alter the size category of neurons that express opioid receptors. This is in agreement with our previous results for NPY (Y1) receptor mRNA (Ji et al., 1994), and galanin mRNA (Ji et al., unpublished data), whereas in contrast there is a distinct shift in expression of SP (Noguchi et al., 1994), NPY (Y1) receptor (Zhang et al., 1994a), and bFGF mRNA (Ji et al., unpublished results) from small to medium sized or large neurons after sciatic nerve transection (axotomy), a process which may also result in neuropathic pain (Wall et al., 1979; Wall and Devor, 1981).

Spinal cord

All three opioid receptors were found in high concentrations distributed in the superficial dorsal horn, with the highest density for the μ receptor. The rank order of staining density, that is MOR>DOR>KOR is in agreement with receptor autoradiographic studies (Morris and Herz, 1987; Besse et al., 1990; Stevens et al., 1991a,b). This may be related to electrophysiological studies showing that the analgesic effects of μ agonists are more pronounced than those produced by other agonists (Dickenson et al., 1987). In comparison to the receptor alterations in the DRGs, the inflammation-induced changes in the spinal cord appeared much less distinct. Thus, a slight increase in MOR-LI and a small decrease in DOR-LI were observed in the medial part of ipsilateral dorsal horn. These changes in spinal cord parallel those seen in the DRGs. An increase in MOR after inflam-

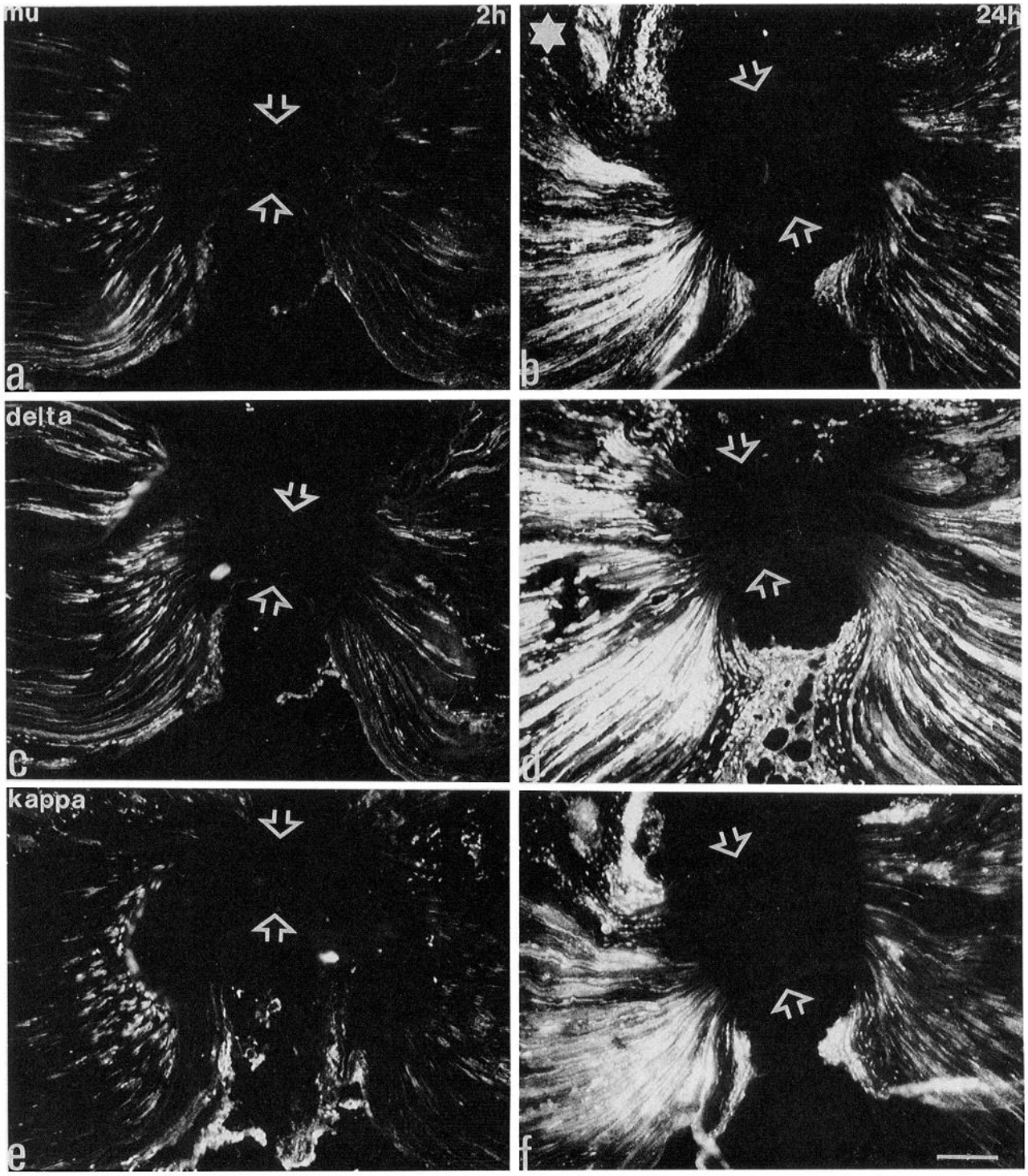

Figure 7. *a–f*, Immunofluorescence micrographs of sections of the sciatic nerve stained with μ-, δ-, and κ-opioid receptor antibodies 2 hr (*2h, a, c, e*) and 24 hr (*24h, b, d, f*) after the sciatic nerve ligation. MOR- (*a, b*), DOR- (*c, d*), and KOR-LIs (*e, f*) were seen in many nerve fibers in the proximal and distal portion of the nerve as early as 2 hr, and was very strong at 24 hr. DOR-LI is comparatively stronger than that of MOR- and KOR-LI, especially in the distal part. The *arrows* indicate the ligation sites. The proximal side (*star*) is to the left in the micrographs. Scale bar, 100 μm.

mation has also been shown in binding experiments (Stevens et al., 1991a; Besse et al., 1992a,b), and may at least partly explain the enhanced potency of exogenous μ opiates on analgesia after injection of CAG into the paw. This has been shown both in behavioral (Hylden et al., 1991) and electrophysiological (Stanfa et al., 1992) studies. A decrease in DORs has also been revealed in some binding studies (Stevens et al., 1991a; Besse et al., 1992b). However, other authors demonstrated no change in opioid binding in rats with unilateral hind paw inflammation (Iadarola et al., 1988; Millan et al., 1988).

The upregulation of dynorphin and enkephalin in arthritic rat spinal cord (Millan, 1986; Iadarola et al., 1988; Ruda et al.,

1988; Weihe et al., 1988) favors a role for these two peptides in the modulation of sensory input during inflammation. It is at present not clear whether the increased endogenous content of enkephalin and dynorphin in the dorsal horn in fact reflects an increase in opioids available for release and activation of their receptors, or an intracellular accumulation perhaps due to decreased release. In fact, spontaneous and evoked release of met-enkephalin-LI from the spinal dorsal horn of arthritic rats has been reported to be decreased both *in vitro* (Cesselin et al., 1984) and *in vivo* (Bourgoin et al., 1988). Thus, further work is essential to elucidate the precise role of the individual opioid receptor types during inflammatory pain. Interestingly, the present results may have some clinical implications, since earlier studies in humans showed that chronic neurogenic pain is associated with an altered proportion of opioid peptides in the cerebrospinal fluid (Terenius, 1984).

Transport of opioid receptor and peripheral effects

After ligation MOR-, DOR-, and KOR-LIs accumulated in nerve fibers both in the central and the distal portion of the sciatic nerve, providing evidence that all three types of opioid receptors are transported both anterogradely and retrogradely along the sciatic nerve. This corresponds well with the initial binding study of Young et al. (Young et al., 1980) on the transport of opioid receptor in the vagus nerve. Also, ^{125}I-β-endorphin binding sites accumulate both proximally and distally to a ligature placed on the sciatic nerve, indicating bidirectional axonal transport (Hassan et al., 1993). Also in the dorsal roots, opioid receptors accumulate around ligatures (Zarbin et al., 1990) and crushes (Zhang et al., 1994b). The present study provide a time course for both anterograde and retrograde accumulation of the three opioid receptors. Thus, an accumulation of opioid receptors could be found as early as 2 hr after ligation, whereby DOR accumulation was stronger than that of MORs and KORs, especially in the distal part. This suggests that the DOR is the main type of the three opioid receptors undergoing axonal transport. At 48 hr there was a less pronounced accumulation of all three receptor immunoreactivities, perhaps reflecting the down-regulation of DOR- and MOR-LIs in DRG neurons after axotomy (Zhang at al., unpublished data).

Local inflammation induced an enhanced axonal transport of ^{125}I β-endorphin binding sites in rat sciatic nerve and accumulation in paw tissue (Hassan et al., 1993). Peripherally administered opioids produce powerful antinociception in the inflamed, but not in the noninflamed paw (Levine and Taiwo, 1989; Stein et al., 1989). Opioid receptors were detected on small-diameter cutaneous nerves of noninflamed and inflamed paws (Stein et al., 1990a,b). After inflammation increased amounts of opioid peptides in immune cells was shown to infiltrate the inflamed paw and interact with receptors on sensory nerves to inhibit nociceptive responses in inflammation (Stein et al., 1990a,b). Thus, through axonal transport, the CAG-induced alterations of three opioid receptors in DRG neurons are likely to play roles in regulating peripheral opioid effects under inflammatory situations.

Coexistence of opioid receptors

A main finding of the present study is that opioid receptors coexist in some primary sensory neurons. This is in agreement with findings that a subset of MOR-IR axon terminals in the superficial dorsal horn also stain for DOR-LI (Arvidsson et al., 1995b). The present study in DRG neurons demonstrated not only the coexistence between MOR and DOR, but also between DOR and KOR, and KOR and MOR. Some neurons even contained all three types of opioid receptors. A functional interaction between opioid receptors was first suggested by Vaught and Takemori (1979) who showed that (Leu^5)enkephalin given intracerebroventricularly at doses which did not produce antinociception potentiated the antinociceptive potency of morphine. In contrast, Lee et al. (1980) demonstrated that (Met ^{5})enkephalin, in nonanalgesic doses, antagonized the antinociceptive effects of morphine. Morever, it was shown that intrathecal perfusion with the μ agonist DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol) signifcantly cnhanced the spontaneous release of substance P, whereas the κ againsct U 50488 produced no change in the peptide outflow. However, intrathecal perfusion with DAGO plus U 50488 caused a significant decrease in substance P release (Collin et al., 1992). A δ-mu receptor complex has been suggested to coexist based on a diverse and complex pharmacology (see Holaday et al., 1985; Rothman et al., 1993). The further elucidation of coexistence of opioid receptors is important to improve our understanding of the functional coupling among opioid receptors.

Concluding remarks

By using recently developed antibodies against three types of opioid receptors and immunohistochemistry, we have compared similarities and differences among these receptors with special reference to DRGs. Similarities include that (1) all three types of receptors are preferentially localized in small DRG neurons, and that inflammation did not affect the size category of neuronal profiles containg these receptors, (2) all three receptors immunoreactivities can be found diffusely in the cytoplasma of DRG neurons, (3) they coexist to a certain extent with each other in DRG neurons, (4) the expression of all three is altered following inflammation, (5) in the dorsal horn of the spinal cord they are mainly distributed in the superficial layers, and (6) they are transported along the sciatic nerve both anterogradely and retrogradely. The main differences are as follows: (a) They occur in different numbers, whereby ~20%, 15%, and 10% of the DRG neurons contain μ, δ, and κ receptor, respectively; (2) after inflammation their expression is changed in different directions, with an upregulation of MOR-LI and downregulation of DOR- and KOR-LIs; (3) MOR-LI in the superficial dorsal horn is very strong, whereas the staining intensity is moderate for DOR-LI, and weak for KOR-LI. However, DOR-LI has a distinct varicose/dotted appearance, whereas MOR- and KOR-LIs are more diffuse; (4) DOR- and KOR-LI are only observed in the cytoplasm of DRG neurons, whereas MOR-LI could also be found on the plasma membrane; and (5) after ligation accumulation of DOR-LI is most pronounced, indicating that DOR to a larger extent than the other two types of receptors undergoes axonal transport.

Taken together, our results provide anatomical evidence for roles of multiple opioid receptors in the regulation of CAG-induced hyperalgesia accompanying peripheral inflammation. The differential regulation of three opioid receptors after CAG injection indicates that they play different roles during inflammation. The colocalization of opioid receptors provides a structural basis for a functional interaction between these receptors.

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