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Inflammation Enhances μ-Opioid Receptor Transcription and Expression in Mice Intestine

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ABSTRACT

Opioid receptors (ORs) and their mRNA are present in the central and peripheral nervous systems of mammals and in different peripheral tissues, including the gut. Using a model of croton oil-induced (CO) intestinal inflammation in mice, we have shown a 6-fold increase in the potency of the antitransit and antisecretory effects of μ-OR agonists, mediated by peripheral ORs. We postulate that the enhanced effects are mediated by an increase in the expression of intestinal OR. We used jejunum (stripped of the mucosal layer) from mice with CO-induced intestinal inflammation and, as control subjects, saline-treated animals (SS). We evaluated the quantity of μ-OR mRNA determined by a competitive reverse-transcriptase polymerase chain reaction; the levels of μ-OR protein by Western blot immunoassay, and the localization and number of cells expressing μ-OR using immunohistochemistry. The results show a significant increase of μ-OR mRNA (7.7-fold) and receptor protein (3-fold) during intestinal inflammation. Inflammation also induced a 64.3% increase in the number of neurons expressing μ-OR immunoreactivity in the myenteric plexus but not in the submucosal plexus. Our results show that intestinal inflammation enhances the transcription and translation of μ-OR mRNA, thus explaining the increased potency of μ-opioids during inflammation.

The molecular characterization of ORs has enabled us to study with precision the mechanisms involved in synthesis regulation and their anatomical distribution (Mansour et al., 1995; Raynor et al., 1996). Opioid receptors and their mRNA are present in the brain and spinal cord and also in the gastrointestinal tract, with particular abundance in the myenteric and submucosal plexuses (Bagnol et al., 1997), and in lower densities in enterocytes (Lang et al., 1996). In the gut, ORs participate in the inhibitory modulation of gastrointestinal functions, such as motility and secretion.

We have shown previously that during intestinal inflammation, the potency of μ- and δ-OR agonists on the inhibition of intestinal transit and permeability is significantly increased (between 10- and 6-fold) by a peripheral mechanism (Puig and Pol, 1998; Valle et al., 2001). We hypothesized that ORs present in the gut could be "sensitized" or "up-regulated" in response to injury, thus modulating intestinal functions and local inflammation. The morphological configuration of the gut, which contains both neuronal cell bodies (intramural ganglia) and sensory nerve endings, permits us to study possible changes in the expression of OR-protein induced by inflammation. However, the myenteric and submucosal plexuses that express OR represent a small proportion of the total weight of the gut, thus increasing the complexity of the quantification of these receptor proteins. The aim of the present study was to determine whether the enhanced potency of μ-OR agonists observed during inflammation could be related to an increase in the transcription of μ-OR mRNA and/or in the expression of μ-OR in the gut.

Our laboratory has described and validated a model of intestinal inflammation in mice, induced by the intragastric administration of CO (Puig and Pol, 1998). In the present investigation, we have used this model to determine μ-OR mRNA and receptor protein using quantitative PCR and Western immunoassay, respectively. Moreover, the localization and density of cells expressing μ-OR in the jejunum has been evaluated by immunohistochemistry using anti μ-OR antibodies. In all instances, mice receiving intragastric saline (SS) served as noninflamed control animals.

Materials and Methods

Animals. Male Swiss CD-1 mice weighting 25 to 30 g were used in all experiments. The study protocol was approved by the local Committee of Animal Use and Care of our Institution, in accordance with

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ABBREVIATIONS: OR, opioid receptor; PCR, polymerase chain reaction; SS, saline; RT, reverse transcriptase; bp, base pair(s); CO, croton oil; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

the International Association for the Study of Pain guidelines on ethical standards for investigations in animals. Mice were housed under 12-h/12-h light/dark conditions in a room with controlled temperature (22°C) and humidity (66%). Animals had free access to food and water and were used after a minimum of four days acclimatization to the housing conditions. All experiments were conducted between 9:00 AM and 5:00 PM.

Induction of Inflammation. Intestinal inflammation was induced by the intragastric administration of two 0.05-ml doses of the irritant agent CO administered 24 h apart; control animals received the same volume of intragastric SS. Before the administration of CO or SS, animals were fasted for 18 h except for free access to water. Morphological changes induced by CO have been reported previously by our group (Puig and Pol, 1998) and were established by optical microscopy. In brief, a clear disruption of the mucosa with a massive infiltration of lymphocytes within the submucosa was observed in animals treated with CO, but not in SS animals. The greatest morphological inflammatory changes after treatment with CO were observed in the jejunum from control animals and animals with intestinal inflammation.

Tissue Isolation and Total RNA Extraction. Jejunums from animals with and without inflammation were excised, cut open lengthwise, and washed in ice-cold physiological saline. Because OR in the gut are localized mainly in the myenteric and submucosal plexuses, the mucosa was removed under a stereomicroscope, and RNA was extracted from the smooth muscle layer and inherent nervous plexuses. Total RNA was extracted with RNAzol (Molecular Research Center, Inc., Cincinnati, OH) and the amount of the purified RNA (A_{260}/A_{280} ratio was ≥ 1.9) was determined by spectrophotometry.

Semiquantitative and Competitive Reverse-Transcriptase Polymerase Chain Reaction. The expression of μ-OR mRNA in the jejunum from control animals and animals with intestinal inflammation was initially assessed by semiquantitative reverse-transcriptase (RT)-PCR, with β-actin as an internal standard. We used jejunal samples from three animals for each control and inflamed animal. Two primers that anneal to exon 1 (bases 284–304; 5′-ACCTGGCTCCTGGCTCAACTT-3′; sense) and exon 2 (bases 832–852; 5′-TGGACCCCTGCCTGTATTTTG-3′; antisense) of the gene receptor sequence were used to detect μ-OR expression (Buzas and Cox, 1997). For semiquantitative assessment, primers of the mouse housekeeping gene β-actin were used with the following sense (5′-TCATGAAGTGTGACGTTGACATCCGT-3′) and antisense primers (5′-CCTAGAAGCATTTGCGGTGCACGATG-3′).

Because of the exponential nature of the PCR technique, a direct quantification of specific mRNA using this approach was not possible, and we performed a competitive PCR that permits an accurate quantification of μ-OR mRNA (Buzas and Cox, 1997). Tissues from three control animals and three animals with intestinal inflammation were used. For the competitive PCR, we used a nonhomologous competitor or mimic that contains the same primer sequence as the μ-OR mRNA. The same primers that anneal to bases 284 to 304 and 832 to 852 of the μ-OR gene receptor sequence were used. The mimic was prepared by successive PCR amplifications using sense (5′-**ACC TGG CTC CTG GCT CAA CTT** TCA TGA AGT GTG ACG TTG ACA TCC GT-3′) and antisense primers (5′-**TGG ACC CCT GCC TGT ATT TTG** CCT AGA ACG ATT TGC GGT GCA CGA TG-3′), containing the upstream and downstream primers for the μ-OR sequence (first 21 bases in bold letters) and linked to a DNA fragment strand. The PCR product was purified using a Nucleo Trap PCR kit (CLONTECH Laboratories, Inc., Palo Alto, CA) and the amplified μ-OR fragment (568 bp) and mimic (327 bp) were obtained.

In both assays (semiquantitative and competitive PCRs), a reverse transcriptase using 1 μg of total RNA, with 100 U of Moloney murine leukemia virus-RT (Invitrogen, Barcelona, Spain) was performed in a final volume of 20 μl. The reaction mixture contained 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl, and 10 mM dihiothreitol, 50 U of RNase inhibitor, 1.5 mM each dNTP, and 3 μM random hexamers (Amersham Pharmacia Biotech, Barcelona, Spain). Forty microliters of PCR master mix [30 pmol of each primer, 2.5 U of *Taq* polymerase (Invitrogen), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 2.5 mM $MgCl_2$] was dispensed into tubes containing: 20 μl of cDNA for semiquantitative PCR or 15 μl of cDNA and 5 μl of mimic for competitive PCR. Negative controls were performed for both assays, in which all of the components were included except RT to show that the PCR products were a result of RNA and not of genomic DNA amplification. PCR using a Perkin-Elmer 2400 Thermal Cycler was initially set at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C. The last primer extension step was at 72°C for 7 min.

Products were separated on a 1.5% (semiquantitative) and 3% (quantitative) agarose gel (Sigma Chemical Co., St. Louis, MO) and visualized under UV light. The image was digitized using a Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA) and the intensity of the bands determined using the Diversity database program. During digitalization, the color saturation was checked to ensure that the image was not oversaturated. In the semiquantitative PCR, the results were expressed as the ratio of band intensity of the target to that of the housekeeping gene. For the quantification of results obtained in the competitive PCR, the ratio of the amplified competitor to target products was graphed as a function of the quantity of competitor added to the PCR reaction (log scales). From the regression lines, the X-intercepts (log ratio = 0) were determined.

Preparation of Membranes from Brain and Jejunum. Brain from control animals and jejunal preparations (without the mucosal layer) from CO and SS-treated animals were minced with scissors and homogenized with a homogenizer (Ultra-Turax, T8.01; Ika-Werke, Staufen, Germany) in ice-cold buffer containing: 50 mM Tris-HCl and 0.32 M sucrose, pH 7.5, at 4°C (Garzón et al., 1995). The homogenate was centrifuged at 1,000*g* at 4°C for 10 min. The pellet was discarded and the supernatant was centrifuged at 20,000*g* for 20 min; the new pellet was resuspended in buffer and centrifuged again at 20,000*g* for an additional 20 min. The final pellet was diluted in Tris buffer to a final protein concentration of 3 μg/μl using the method of Bradford (1976).

Western Immunoassay. Membranes from SS- (brain and jejunum) and CO- (jejunum) treated animals, were solubilized in a buffer containing 62.5 mM Tris-HCl, 2.3% SDS, 10% glycerol, and 5% β-mercaptoethanol, adjusted to pH 6.8. After 3-h incubation at room temperature, the samples were boiled for 5 min and stored at −20°C until use. Approximately 80 μg (jejunums) and 60 μg (brain) of total protein/lane were used to perform the SDS-PAGE (14% acrylamide gel) at 100 V, during 3 h (Amersham Pharmacia Biotech, San Francisco, CA). The resolved proteins were transferred (Mini-Trans-Blot electrophoretic transfer cell; Bio-Rad) to nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) by the application of 100 V (200–300 mA) for 2 h. Membranes were first blocked with nonfat dry milk in PBS overnight at 4°C; then they were incubated with anti-μ-OR antibody (Chemicon Intl, Temecula, CA) diluted 1:500 in 1% PBS-bovine serum albumin, for 1 h at room temperature and overnight at 4°C. After removal of the antibody, membranes were washed with PBS and then incubated with a universal secondary antibody conjugated with biotin at a 1:200 dilution (AB600; The Binding Site Limited, Birmingham, UK) for 1 h at room temperature. The secondary antiserum was removed and the membranes washed again and incubated in streptavidin peroxidase (IC019; The Binding Site Limited) for 1 h at room temperature. A substrate solution containing 0.05% of 3–3′-diaminobenzidine and 100 μl of hydrogen peroxidase in PBS was then added. Negative controls were performed for the Western blot assay, in which all components were included except the first antibody. Immunoblots were quantified by digitizing the images and measuring the integrated density of the immunoreactive bands by the Diversity database program.

Immunohistochemistry. Five days after CO or SS, animals were perfused transcardially under deep anesthesia with periodate-lysine-paraformaldehyde fixative. The jejunum was removed, post-

fixed in 4% paraformaldehyde (24 h), included in paraffin, and sectioned (3 μm). After antigen retrieval in heated citrated buffer, tissues were incubated with one of the two specific antibodies: 1) a polyclonal rabbit anti-μ-OR antibody against the μ-OR 3rd extracellular loop (1:3000; Chemicon) or 2) a monoclonal mouse anti-synaptophysin antibody (1:20; DAKO, Carpinteria, CA). In all instances, tissues were incubated with a second antibody combined with Envision reagent (DAKO, Carpinteria, CA) and diaminobenzidine was used as a chromogen. The number of cells containing μ-OR per millimeter were assessed in 10 animals per group (SS or CO), four sections per animal, using optical microscopy (BX-40; Olympus, Tokyo, Japan).

Statistics. Data from the immunohistochemical evaluation are expressed as a group mean ± S.E.M. Statistical analysis for significant differences between the two groups were obtained by Student's t test. A value of $p < 0.05$ was considered significant.

Results

Semiquantitative PCR. The expression of μ-OR mRNA levels was initially estimated with a semiquantitative PCR. The cDNA obtained from control and inflamed jejunums (n = 3 mice each) was amplified with the μ-OR primers and the housekeeping gene (β-actin). Figure 1 shows the separation of the DNA products by gel electrophoresis of a representative experiment, using specific primers for μ-OR (568 bp) and β-actin (285 bp). The size marker is shown in the first lane, whereas lanes 2 and 3 illustrate the PCR products from control and inflamed tissues, respectively. No bands were detected in PCR reactions containing only RNA that was not reverse-transcribed (data not shown). The results obtained from three different experiments showed that the intensities of the β-actin PCR products were equivalent for the control and inflamed tissues, indicating the same amounts of total RNA were present in both groups of study. The analysis of the ratios of the band intensities of the μ-OR and β-actin in controls (ratio, 0.26 ± 0.06) and inflamed tissues (ratio, 0.80 ± 0.08) demonstrates a significant increase of the μ-OR mRNA levels during intestinal inflammation ($p < 0.001$; Student's t test).

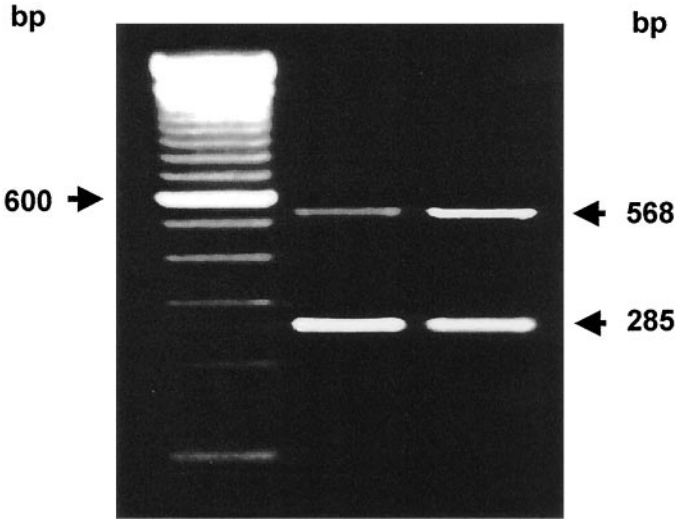

Fig. 1. Gel electrophoresis of μ-OR mRNA levels in jejunum by semiquantitative PCR. The figure shows the PCR products of a representative experiment from a control animal (lane 2) and an animal with inflammation (lane 3). The size marker is shown in first lane giving bands of 100-bp spacing. The band of 568 bp is a product of the μ-OR, and the band of 285 bp is β-actin mRNA. Inflammation produced a significant increase in the expression of μ-OR mRNA.

Competitive PCR. Quantification of μ-OR mRNA levels in the jejunum was performed with a competitive PCR using a nonhomologous competitor (mimic). We generated a mimic (327 bp) containing the same primer sequences of the μ-OR mRNA, which competed with a cDNA fragment (568 bp) of these receptors during PCR. Figure 2A shows the separation of the DNA products by gel electrophoresis of a representative experiment, where μ-OR cDNA is coamplified in the presence of the mimic; the upper gel shows the PCR products obtained in controls, and the lower gel those obtained from animals with intestinal inflammation. In both gels, the size marker is shown in the first lane, and lanes 2 to 6 illustrate the PCR products obtained with different dilutions of the mimic. No bands were detected in PCR reactions containing only RNA that was not reverse-transcribed (data not shown). RT-PCR was performed in six animals (three controls and three with intestinal inflammation) and the mean values were used to quantify the results. The number of molecules of

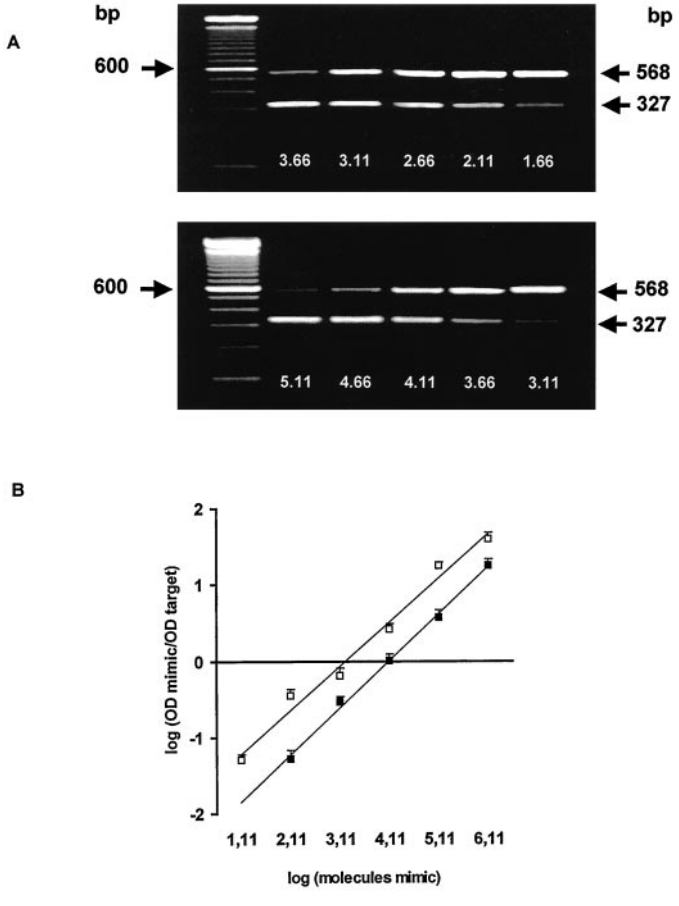

Fig. 2. A, competitive PCR products of μ-OR mRNA levels in jejunum from a representative experiment. Gel electrophoresis of RT-PCR products from control (top) and inflamed animals (bottom). In both gels, the size marker is shown in the first lane, and lanes 2 to 6 illustrate the products obtained with different dilutions of the mimic. The log of the number of mimic molecules added to the PCR reaction, is indicated in each lane. B, quantitative analysis of results obtained by competitive PCR. The mean ratios of the absorbance values of competitor to target are plotted as a function of the number of molecules of mimic present in the each reaction (log/log scales). We have represented the regression lines obtained from control animals (□) and during inflammation (■) from three independent experiments; vertical bars are the S.E.M. During inflammation, μ-OR mRNA levels increased by approximately 7.7-fold.

mimic used in each lane were plotted against the mean log values of the fraction of the absorbance values of mimic/target (Fig. 2B), and the number of cDNA molecules present in 1 μg of total RNA was determined by linear regression analysis ($r^2 > 0.98$ for each line). The results show that during inflammation, the μ-OR mRNA levels increased approximately 7.7 times compared with the control samples (1285.5 ± 66.2 molecules/μg of total mRNA in control samples and 9972.5 ± 1020.8 in inflamed tissue; $p < 0.01$, Student's t test).

Immunoblotting. Western blot analysis was performed to compare the amount of μ-OR protein in jejunum membranes obtained from control animals and animals with intestinal inflammation. The experiments were repeated three times in control animals ($n = 3$) and in animals with intestinal inflammation ($n = 3$). Western blots were also performed using brain plasma membranes from control animals ($n = 3$). Figure 3 shows the results of a representative immunoblot experiment, obtained from samples of jejunum (control in lane 2; inflamed in lane 3) and brain (lane 4); lanes 1 and 5 are the molecular mass marker and a control sample in which the primary antibody was eliminated, respectively. The resulting immunoblots show similar bands in all tissues (lanes 2–4), with molecular masses of approximately 43, 51, and 58 kDa; the band at 51 kDa is clearly predominant. The brain lane exhibits similar but more pronounced bands, even in the presence of a lower amount of protein (see *Materials and Methods*). When analyzing the data obtained from the jejunum by densitometry, we could demonstrate significant increases in μ-OR immunoreactivity in all bands compared with control samples. Values obtained from the 51-kDa band established a 306.6 ± 13.8% increase over the mean values obtained from control animals ($p < 0.001$, Student's t test).

In all three experimental conditions, immunoreactivity was completely abolished when the staining was performed in the absence of first or secondary antibody and also without streptavidin peroxidase. The results obtained from a sample without the first antibody have been included in Fig. 3.

Immunohistochemistry. Immunoreactivity for μ-OR was present in the longitudinal and circular muscle nerve fibers, and in the myenteric and submucosal plexuses. In the submucosal plexus, μ-OR immunoreactive fibers were predominantly distributed around the crypts. Figure 4, A and B, illustrate the immunoreactive cells for μ-OR in the myenteric plexus from control and inflamed tissues, respectively; the results show that during inflammation, the number of neurons expressing μ-OR in the myenteric plexus increased a 64.3% compared with saline controls (2.30 ± 0.24 neurons per millimeter in control tissue and 3.78 ± 0.28 in inflamed

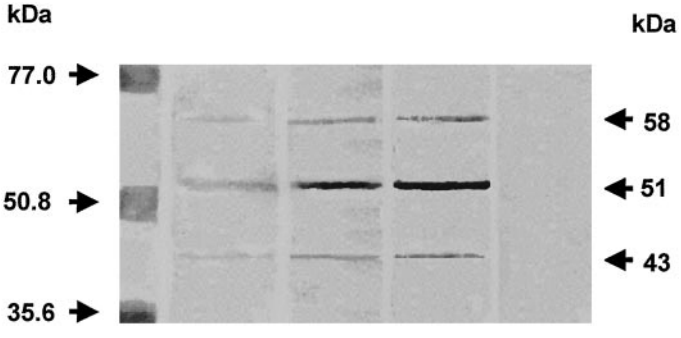

Fig. 3. Immunoblots of a representative experiment with anti μ-OR antibody, of plasma membranes from control jejunum (lane 2), inflamed jejunum (lane 3), and brain (lane 4). Lane 1 is the molecular mass marker and lane 5 is a control in which the primary antibody was eliminated. Equal amounts of SDS solubilized protein (80 μg/lane) from control and inflamed jejunums and 60 μg/lane from brain were loaded into the gels. Immunoreactive bands of about 43, 51, and 58 kDa were detected in all samples. In the inflamed jejunum, the analysis of the data by densitometry demonstrated an increased μ-OR immunoreactivity (approximately 3 times) compared with control tissues. The immunoreactivity was abolished completely when the staining was performed in the absence of the first antibody (lane 5). The molecular mass standards (MW) used were: phosphorylase B (106.0 kDa), bovine serum albumin (77.0 kDa), ovalbumin (50.8 kDa), carbonic anhydrase (35.6 kDa), soybean trypsin inhibitor (28.1 kDa), and lysozyme (20.9 kDa) (Bio-Rad). All data were reproducible for three independent experiments.

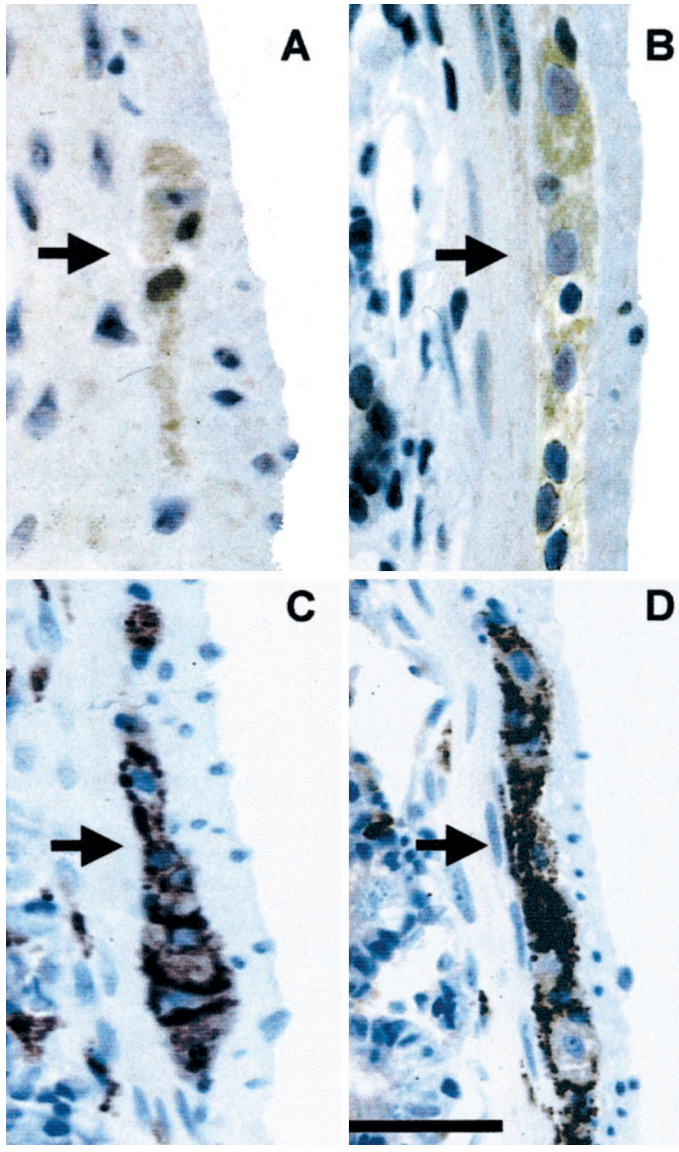

Fig. 4. Immunohistochemistry experiments with μ-OR polyclonal (top) and synaptophysin monoclonal antibodies (bottom). The figure shows a representative experiment of transverse sections of jejunum in control animals (A and C) and during inflammation (B and D). An increase in the number of cells expressing μ-OR (arrows) was observed in the myenteric plexus of inflamed jejunum (B) compared with controls (A). The pattern of distribution of synaptophysin (arrows) in control animals (C) and in animals with intestinal inflammation (D) was very similar to μ-OR immunoreactivity. Calibration bar, 25 μm. Experiments were repeated 10 times ($n = 10$ animals for control and inflammation) and at least four different tissue sections were obtained from each animal.

tissue; $p < 0.001$, Student's t test). The number of immunostained cells in the submucosal plexus was unaltered in the presence of inflammation (2.13 ± 0.1 neurons per mm in control tissue and 2.34 ± 0.1 in inflamed tissue). Moreover, a similar number of neurons expressing μ receptor-like proteins was found in both plexuses in control tissues. Thus, these experiments show that the presence of inflammation induces an increase in the number of immunoreactive cells expressing μ-OR in the myenteric (but not the submucosal) plexus of the gut.

The type of cells expressing μ-OR was further assessed using an anti-synaptophysin antibody, a vesicular protein present at the axonal terminal of the synapse (Hachisuka et al., 1999). In Fig. 4, we show the pattern of distribution of synaptophysin in control and inflamed tissues (Fig. 4, C and D, respectively). The pattern of distribution of the neuronal marker is similar to that of the μ-OR in both control (Fig. 4, A and C) and inflamed tissues (Fig. 4, B and D), thus suggesting that μ-OR are expressed in neurons.

Discussion

The present study shows that intestinal inflammation induces an increase in the levels of μ-OR mRNA (7.7 times) and μ-OR protein (3 times) in the jejunum. We also show that inflammation induced a 64.3% increase in the number of immunoreactive neurons expressing μ-OR in the myenteric plexus, whereas no inflammation-induced changes were observed in the submucosal plexus. These results suggest that during inflammation, an increased number of newly synthesized μ-OR could mediate the enhanced effects of opioids observed in behavioral studies.

Our results demonstrate for the first time that inflammation increases the levels of mRNA and the expression of μ-OR in a peripheral tissue. Our results differ from those reported by other investigators using a model of inflammation of the rat paw. In the paw model, a low density of μ-OR was shown to be present in sensory terminals (Stein, 1995); peripheral inflammation increased the number of μ-OR in the subcutaneous tissue (Hassan et al., 1993), the dorsal root ganglia (Ji et al., 1995), and the spinal cord (Maekawa et al., 1995). However, μ-OR mRNA levels were unaltered in the cell bodies of the primary afferent neurons sited in the dorsal root ganglia (Schäfer et al., 1995). These experiments demonstrate that inflammation of the rat paw enhances the axonal transport (but not the actual synthesis) of a pre-existing neuronal *pool* of OR proteins that are later expressed at the peripheral (and central) terminals of primary sensory neurons. Thus, during skin inflammation, post-transcriptional changes could explain the increased expression of ORs in the periphery, whereas in the gut, an increased synthesis of μ-OR (transcription) seems to occur.

Structural differences between the paw and the intestine could explain the distinct mechanisms involved in the enhanced effects of opioids during peripheral inflammation. In the gut, constitutive ORs are present in variable concentrations throughout the intestinal tract, where they probably modulate intestinal function (motility, secretion, other) under physiological conditions. The cell bodies of the plexuses that form the intrinsic innervation synthesize μ-OR (Wittert et al., 1996; Fickel et al., 1997) and thus a turnover of intestinal μ-OR is likely to occur. Intestinal inflammation enhances synthesis and expression of these receptors that, when activated by endogenous opioids, would contribute to decrease intestinal motility, secretion, and probably local edema.

Our results show that inflammation induces a significant increase in the μ-OR immunoreactivity (approximately 3-fold) compared with the control samples. These results correlate with those obtained by PCR, supporting the idea that during intestinal inflammation, the expression of μ-OR is increased, thus enhancing the potency of μ-opioids inhibiting intestinal functions. The different ratios of increase (control versus inflamed) observed for mRNA (7.7 times) and protein (3 times) levels could be related to the type of sample used for each assay. In the Western blot experiments, a preparation of membranes was used, and the receptor protein present in the membrane but not in the cytoplasm of the cells was determined; however, in the competitive PCR, jejunal preparations of the gut (except the mucosal layer) were used. Western blot experiments from brain and jejunum samples showed an immunoreactive band of 43 kDa that coincided with the predicted molecular mass of the cloned μ-OR, and two other bands of higher molecular mass (51 and 58 kDa), which could represent different degrees of protein glycosylation. In our experiments, samples from brain and jejunum present immunoreactive bands that have similar molecular masses, thus confirming the specificity of the antibody; the lower intensity of the bands in the jejunum compared with brain membranes, is probably related to the different in μ-OR densities in these tissues. Although unusual, the presence of a nonglycosylated tight band at 43 kDa has been previously reported in the brain (Garzón et al., 1995); in the present investigation, it was obtained in all samples evaluated (Fig. 3). In the brain, other investigators have observed similar glycosylated bands than in the jejunum, with the same (Cho et al., 1986; Ueda et al., 1988) and slightly higher (Schoffelmeer et al., 1989; Gioannini et al., 1993; Liu-Chen et al., 1993; Sánchez-Blázquez et al., 1997) molecular masses. Thus, there is no universal agreement regarding the precise localization and number of bands that appear in the immunoblots for μ-OR in the brain; however, all the studies seem to concur that the various bands represent different degrees of glycosylation of the μ-OR. The irregular mobility exhibited by the glycoproteins in SDS-PAGE could be related to the acrylamide concentration and the buffer systems used in the different studies (Garzón et al., 1995; Sánchez-Blázquez et al., 1997).

An interesting finding of the present study is that during inflammation, μ-ORs are up-regulated in myenteric (but not in submucosal) neurons, which mainly control intestinal motility. Thus, the predominant role of the μ-OR could be to modulate the intestinal transit that is usually increased during inflammation (Pol et al., 1994). The irritant used in our study to induce inflammation (CO) produced a clear disruption of the mucosa and an infiltration of lymphocytes in the submucosa demonstrated by optical microscopy (Puig and Pol, 1998). Then, inflammatory changes within the mucosa may affect motility by activation of a population of neurons innervating the mucosa that have their cell bodies in the myenteric plexus (Goyal and Hirano, 1996). In the control (SS) experiments, μ-OR immunoreactivity was similar on neurons of the myenteric and submucosal plexuses, providing further insight on the localization of μ-OR in mice intes-

tine. This is in contrast to the rat, in which μ-OR immunoreactive cells are more abundant in the submucosal plexus than in the myenteric plexus (Bagnol et al., 1997).

Our experiments show that in the gut, the expression of the μ-OR is significantly increased in the presence of CO induced inflammation, a fact that correlates closely with the enhanced effects of μ-OR agonists on gastrointestinal transit and permeability in vivo. In our model, the effects of the irritant (CO) are closely linked to the inflammatory reaction; thus, a direct effect of the CO itself as an inductor of the μ-OR gene, although unlikely, cannot be excluded. To demonstrate that inflammation is responsible for the enhanced effects of opioids in the gut, we tested the effects of μ-OR agonists in animals treated with castor oil, an agent that enhances transit and permeability in a manner similar to CO but does not induce intestinal inflammation. The results demonstrated that during inflammation (CO), the effects of morphine increased approximately 10 times (Puig and Pol, 1998), whereas if no inflammation was present (castor oil), the effects of morphine remained unaltered (Pol et al., 1996). These experiments demonstrate that inflammation is required or necessary to increase the effects of opioids, and support our working hypotheses that the μ-OR gen is induced by factor/s or mediators related to the inflammatory process. However, based on the present experiments, a potential role of CO as a possible factor that could induce μ-OR synthesis cannot be excluded.

The precise mechanisms implicated in the increased expression of μ-OR during inflammation are not yet elucidated. Inflammation stimulates the production and/or release of a number of endogenous chemicals, including prostanoids, cytokines, kinins, neurotrophins, histamine, serotonin, etc. (Dray and Bevan, 1993). Some of these agents can activate sensory neurons directly (bradykinin, serotonin, histamine, etc.) or indirectly (prostaglandins, cytokines, etc.). Recent studies have demonstrated that cytokines may regulate μ-OR gene expression (in astrocytes and endothelial cells) by interaction with the transcription factor nuclease interleukin-6, present in the promoter DNA sequence of the μ-OR (Ruzicka and Akil, 1997; Vidal et al., 1998). Other studies have shown that the expression of proteins, whose genes have the activator protein-1 binding sites in their promoter region (such as μ-OR), are increased after induction of Fos expression (Rauscher et al., 1998). In our study, increases in the μ-OR number are associated with histological changes of the mucosa (inflammatory response) with a significant increase in lymphocyte/macrophages infiltration. The mechanisms responsible for the increased expression of μ-OR gene during intestinal inflammation remain unknown, but they are under investigation in our laboratory.

In conclusion, the present report shows that intestinal inflammation induces a significant increase in the levels of μ-OR mRNA and receptor-protein, located in the myenteric plexus; the results suggest that the enhanced antitransit effects of μ-opioid agonists during inflammation could be explained by an increase in the local synthesis and expression of the receptor protein.

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