Endocrine, *vol. 13, no. 1, 11–15, August 2000* 0969–711X/00/13:1:11–15/$11.25

Interleukin-6 Regulation of Kappa Opioid Receptor Gene Expression in Primary Sertoli Cells

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Three classes of opioid receptors—mu, delta, and kappa—mediate physiological and pharmacological functions of the endogenous opioid peptides and exogenous opioid compounds in the central nervous system (CNS), as well as in peripheral tissues including the immune system. Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis, we show that freshly isolated and highly purified somatic (Sertoli and Leydig) and specific germ (spermatogonia, pachytene spermatocytes, round, and elongating spermatids) cells of the rat testis differentially express the mRNAs for these opioid receptor genes. Furthermore, to identify a functional mechanism for cytokine regulation of testicular opioid receptor gene expression, we employed primary Sertoli cells as a model system. In a semiquantitative PCR analysis using the S16 ribosomal RNA gene as an internal control, we show that interleukin-6 reduces kappa opioid receptor mRNA levels from 6 to 24 h of treatment in primary Sertoli cells. This regulation requires new RNA and protein synthesis and is partially mediated by the protein kinase A pathway. These findings are consistent with a role for the cytokine and opioid signaling pathways in Sertoli cellular function and the interaction that exists between the opioid and the immune systems in the CNS.

Key Words: Opioid receptor, subtypes; Sertoli; Leydig; male germ cells; protein kinase A.

Introduction

Three major classes of opioid receptors—mu, delta, and kappa (MOR, DOR, and KOR, respectively)—mediate overlapping and distinct pharmacology in pain relief, motor activity, gastrointestinal motility, respiratory activity, and stress through interaction with the endogenous opioid peptides, endorphins, enkephalins, and dynorphins *(1,2)*. Although these receptors are widely and differentially expressed in the central nervous system (CNS), several observations indicate the localization and functions of the opioid systems in nonneuronal and peripheral tissues, including the reciprocal interactions between the immune and the neuroendocrine systems that modulate growth, proliferation, and differentiation of immune cells *(3–6)*. In astrocytes, the expression of both opioid and opioid receptor gene expression are differentially altered by cytokines, such as interleukin-1β (IL-1β), indicating opioid-immune interactions in the CNS as well *(7,8)*.

Although many cytokines perform an essential role in regulating host immune responses in hematopoietic and inflammatory cells, they also serve as proliferative and differentiation factors in other cell types. IL-6 binds to specific cell-surface receptor in many cell types and induces the homodimerization of its receptor (IL-6R) to bring about rapid intracellular tyrosine phosphorylation of the gp130-IL-6R complex, Janus Kinase (JAK)-1, JAK-2, and TYK-2, as well as signal transducer and activator of transcription (STAT)-1 and STAT-3 proteins *(9–11)*. The phosphorylated STAT proteins then translocate to the nucleus and, by binding to DNA response elements, regulate specific gene expression, including that of the immediate early genes *jun*B and *c-fos (12–16)*.

Recent studies from our laboratory have elucidated the regulation of testicular IL-6 and IL-6R and its immediate early signaling pathways, including the activation of JAK/STAT and activator protein-1 (AP-1) proteins as well as the induction of *c-fos* and *jun*B gene expression using primary Sertoli cells *(15–18)*. The opioid peptide messages are expressed and regulated in the Sertoli cells by paracrine factors including follick-stimulating hormone (FSH) as well as germ cell factors *(19–21)*. In addition, β-endorphin has been shown to regulate Sertoli cell function *(22)*. In the present study, we establish the expression of the mRNAs for the three major opioid receptor genes in both somatic and germ cells and identify a mechanism for IL-6 regulation of KOR transcription using primary Sertoli cells as a model system.

Received November 29, 1999; Revised January 14, 2000; Accepted January 25, 2000.
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Results

We employed reverse transcriptase polymerase chain reaction (RT-PCR) analysis to establish the distribution of the three opioid receptor mRNAs in specific freshly isolated and purified cell types of the testes, including rat immature and adult somatic cells (Fig. 1, lanes 4–7) and germ cells at different developmental stages (Fig. 1, lanes 8–11). RT-PCR analyses of total RNA showed the expression of the three receptors in freshly isolated Sertoli cells and in each of the four distinct germ cells, of which all are located within the seminiferous tubules. In contrast, the steroidogenic Leydig cells only expressed DOR and KOR mRNAs (Fig. 1, lanes 4–11), although these cells previously have been shown to express the three endogenous opioid peptides, proenkephalin and prodynorphin, as well as proopiomelanocortin *(20,21)*. Our findings suggest that the MOR is expressed only in the seminiferous tubule and is a target for interstitially produced MOR agonists. A set of primers for an internal control, S16 mRNA, showed comparable RNA levels in the samples from the different cell preparations (Fig. 1). RT-PCR analysis of total RNA from rat brain and liver were used as positive (MOR, DOR, KOR) and negative (MOR, KOR) controls, respectively.

Because the KOR gene was the predominant opioid receptor gene expressed in cultured Sertoli cells (data not shown) and is the receptor subtype likely to be the target of testicular dynorphins and cytokines, we next focused on its regulation. We used a semiquantitative RT-PCR analysis to investigate the steady-state levels of KOR mRNA after IL-6 treatment of primary Sertoli cells (Fig. 2A). Sertoli cells were treated with IL-6 (2.5 n*M*) for various times ranging from 45 min to 24 h. PhosphorImager analyses of the resulting PCR products demonstrated that IL-6 decreased KOR mRNA levels in a time-dependent manner from 6 to 24 h of treatment (Fig. 2B).

To identify the mechanisms responsible for reduction KOR mRNA by IL-6, the protein synthesis inhibitor, cyclohexamide (CHX), and the transcription inhibitor, actinomycin D (ACT-D), were employed (Fig. 3). A 12-h treatment of the Sertoli cells with CHX (5 μg/mL) alone induced KOR message by two- to threefold; pretreatment with CHX for 30 min before a 12-h addition of IL-6 did not significantly reduce KOR mRNA levels. The absolute reduction in KOR mRNA induced by IL-6 treatment was similar in the presence or absence of CHX. However, in the absence of CHX, the reduction was about 70% of its control value whereas in the presence of CHX this reduction was about 22% of its control value. The addition of the transcription inhibitor, ACT-D (5 μg/mL), inhibited the steady-state levels of KOR mRNA by 50%; no further reduction by IL-6 was observed after 12 h of treatment (Fig. 3). Taken together, these data indicate that new RNA and protein syntheses are both required for KOR mRNA regulation.

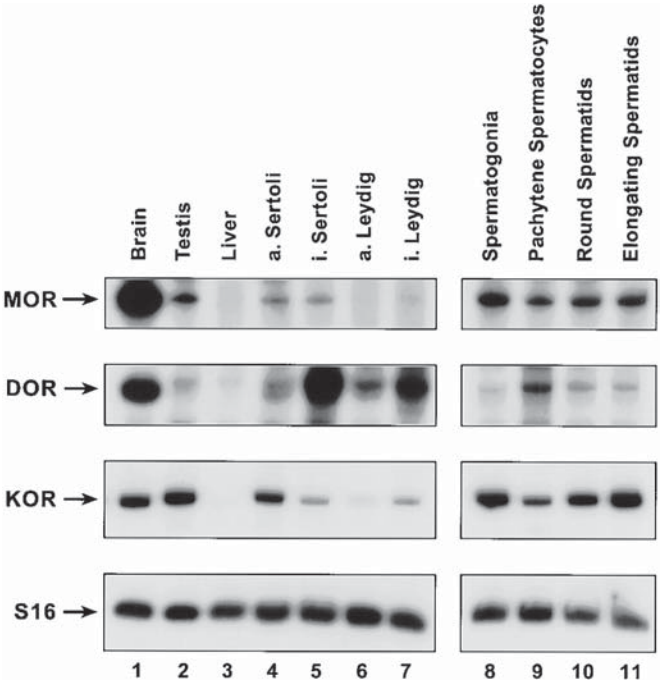

Fig. 1. Distribution of the three opioid receptor mRNAs in rat brain, liver, and testicular cells as determined by RT-PCR analysis. RT-PCR products from somatic (lanes 4–7) or specific germ cells (lanes 8–11) were analyzed using primers for MOR, DOR, KOR, or S16. For the Sertoli and Leydig cell samples, a = those freshly isolated and purified from adult and i = those from the testes of immature rats.

To assess whether the protein kinase A (PKA) pathway is involved in the IL-6 reduction of KOR transcription, we treated the Sertoli cells with forskolin (10 μ*M*) in the presence or absence of IL-6. Figure 4 shows that although both forskolin and IL-6 decreased KOR mRNA levels independently, the combination of forskolin and IL-6 was not additive and did not further reduce KOR mRNA levels.

Discussion

Despite the detailed analysis of testicular opioid gene expression *(20,21)*, little information is available on testicular opioid receptor expression or regulation, in part owing to a low abundance of these opioid receptor mRNAs *(23,24)*. In the present study, using a semiquantitative RT-PCR analysis, we showed that the somatic and germ cells of the testis differentially express the three opioid receptor mRNAs. Furthermore, we showed that a cytokine, IL-6, decreases KOR mRNA levels in primary Sertoli cells in a time-dependent manner. This reduction in KOR mRNA level is dependent on new transcription and new protein synthesis, findings consistent with requirements for both transcriptional and translational modifications.

Few studies indicate alterations in opioid receptor gene expression. Within the CNS, one target of cytokines is the

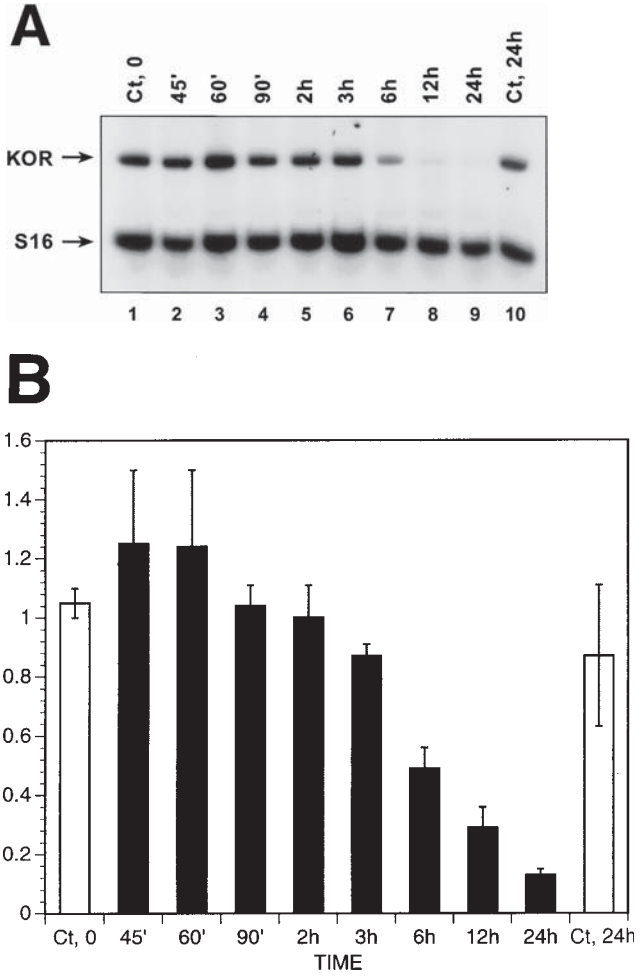

Fig. 2. Semiquantitative RT-PCR analysis of KOR regulation by IL-6 in Sertoli cells. Primary Sertoli cells were treated with IL-6 (2.5 n*M*) from 45 min to 24 h. Total RNA samples from each duplicate plate were subjected to RT-PCR analysis using primers for KOR and S16 simultaneously in the same reaction tube. **(A)** Representative polyacrylamide gel electrophoresis of the PCR products from time-matched experimental samples from the same Sertoli cell primary culture. **(B)** PhosphorImager analysis of PCR products from repeated experiments show the mean (±SEM) in KOR mRNA/S16 mRNA levels as normalized to control values. Ct, control.

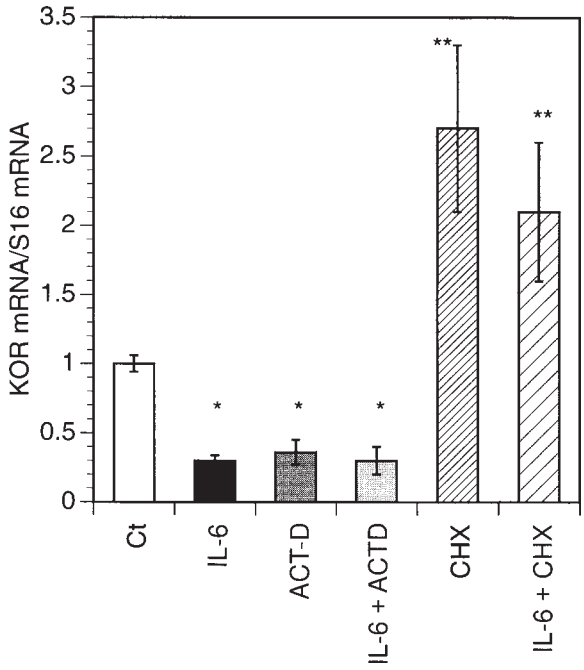

Fig. 3. Effects of ACT-D and CHX on IL-6 regulation of KOR gene expression. Sertoli cells were pretreated with ACT-D (5 μg/mL) for 15 min or with CHX (5 μg/mL) for 30 min before vehicle or IL-6 were added for 12 h. PhosphorImager analyses of PCR reactions were used to measure KOR and S16 mRNA levels. The mean (± SEM) in KOR mRNA/S16 mRNA levels as normalized to control values are shown. * and **, a statistically significant difference compared to control (Ct) value.

astrocyte, a cell also expresses the opioid receptors. IL-1β differentially decreases astrocyte KOR mRNA levels while increasing MOR mRNA levels in a brain region-specific manner by as-yet unknown mechanisms *(7)*. Although IL-1β treatment also increases *c-fos* transcription in astrocytes *(25)*, it is not known whether immediate early genes directly regulate opioid receptor gene expression. In the Sertoli cell, we previously showed that IL-6, by activating STAT-3/STAT-1 proteins, induces *c-fos/jun*B transcription and increases AP-1 DNA binding *(15,16)*. Because AP-1 factors can activate or inhibit targeted gene transcription depending on the composition of *c-fos* and *jun* family members within the complex *(26–28)*, it is plausible that IL-6 inhibition of KOR transcription is mediated by activation of AP-1 proteins. The protein synthesis inhibitor, CHX, has been demonstrated to induce DOR gene expression in the neuroblastoma cell line, NG108-15 *(29)*. We have now shown similar a upregulation of KOR mRNA levels in primary Sertoli cells by CHX. Because it is well known that protein synthesis inhibitors induce the expression of *c-fos* by preventing the translation of inhibitor proteins (as well as *c-fos* itself, i.e., autofeedback mechanism), our findings provide further evidence consistent with an AP-1 regulation of the opioid receptors *(30,31)*.

A second signaling pathway that regulates opioid-receptor gene expression is the PKA pathway. In several neuroblastoma cell lines, cyclic adenosine monophosphate (cAMP)-PKA alterations regulate both DOR mRNA and protein levels. In NG108-15 cells, compounds that directly increase intracellular cAMP levels (forskolin and dibutyryl cAMP) also reduce the levels of DOR mRNA, whereas the PKA inhibitor (Rp)-cAMPS increases DOR mRNA levels *(32,33)*. The data from our studies using primary Sertoli cells, indicate that forskolin decreases KOR mRNA levels as well, whereas the supplement of forskolin to IL-6 is not additive. Taken together, these studies show that, in part, IL-6 regulation of KOR may involve activation of PKA.

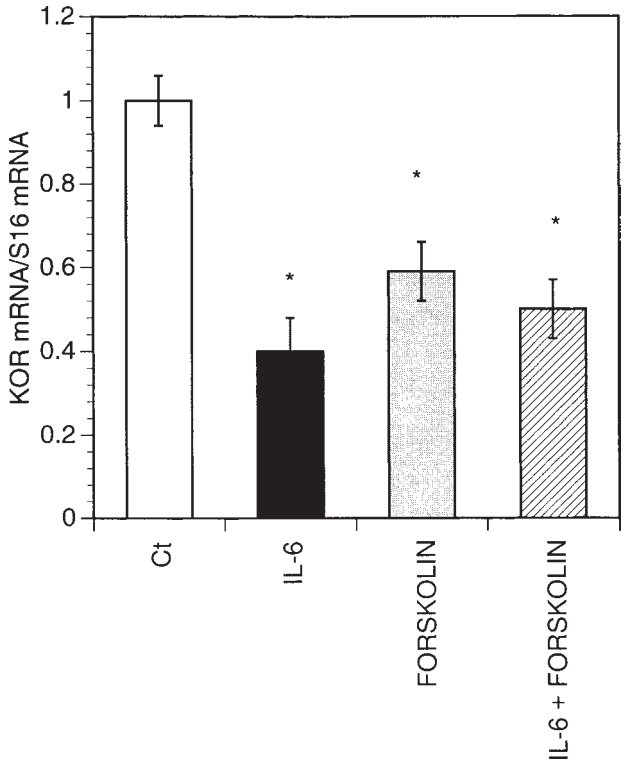

Fig. 4. Effects of forskolin on IL-6 regulation of KOR gene expression. Sertoli cells were treated with IL-6, forskolin, or a combination of both for 18 h and total RNA was analyzed by RT-PCR using KOR and S16 primers. Phos-phorImager analysis of PCR products from repeated experiments shows the mean (± SEM) in KOR mRNA/S16 mRNA levels as normalized to control values. *, a statistically significant difference compared to control (Ct) value.

Activation of PKA decreases the expression of few genes. In a pre-B cell line, forskolin induces a rapid downregulation of *c-myc* mRNA levels by inhibition of the initiation of transcription *(34)* and in rat C6 glioma cells, forskolin downregulates β_2-adrenergic receptor binding sites and transcription of receptor mRNA *(35)*. Alternatively, compounds that increase cAMP levels and PKA activity induce the expression of many genes including the endogenous opioid genes, proenkephalin, prodynorphin, and proopiomelanocortin, by phosphorylating the transcription activator, CREB, or the AP-1 proteins *(34,36–38)*. Because PKA activation (mediated by forskolin or FSH) is known to induce *c-fos*, *jun*B, IL-6, IL-6R, and proenkephalin gene expression while inhibiting *c-jun* transcription in Sertoli cells *(17–20,39,40)*, the PKA and AP-1 signaling pathways may coordinately regulate KOR gene expression.

In summary, we have demonstrated the testicular cell-specific expression of the three opioid receptor mRNAs and furthermore show the regulation of KOR by IL-6 in Sertoli cells. Because regulation at the mRNA level is only one component of a multilayered receptor control system, further investigation is required to fully characterize functional receptor protein alterations.

Materials and Methods

Primary Testicular Cell Preparations

Specific rat somatic (adult, 60-d-old, and immature 18-d-old, Sertoli and Leydig) and germ (spermatogonia, pachytene spermatocytes, round and elongating spermatids) cells were isolated as previously described *(17,18,41,42)*. Primary rat Sertoli cells were isolated and purified from the testes of 18-d-old Sprague-Dawley rats (Charles River, Kingston, NY). Sertoli cells were maintained at 34°C at a density of 1×10^7 cells per 100-mm polystyrene dish in phenol red-, serum-, and endotoxin-free DME/F-12 medium (Irvine Scientific, Santa Ana, CA) as described previously *(15,16)*. The medium was supplemented with 2.5 μg/mL bovine insulin (Sigma, St. Louis, MO), 1 μg/mL transferrin (Calbiochem, La Jolla, CA), and 10 μg/mL bacitracin (Sigma). On d 3 in vitro, following the addition of specific factors, RNA was isolated at the indicated times. Duplicate or triplicate culture dishes were used for each drug treatment and primary cultures were repeated at least twice. mIL-6 (R & D System, Minneapolis, MN) was dissolved in 0.1% BSA as 100X (5 μg/mL) stock solutions with the final culture medium concentration of 2.5 n*M*. Matched aliquots of 0.1% BSA were used as the vehicle blank in control cultures. Cyclohexamide (CHX) was dissolved in water and ACT-D (Calbiochem, La Jolla, CA) was dissolved in absolute ethanol as 1000X stock solutions. Forskolin (Calbiochem) was dissolved in absolute ethanol (50 m*M*) with a final concentration of 10 μ*M* in the medium.

Procedures involving the use of animals strictly followed the *NIH Guidelines for the Care and Use of Laboratory Animals*.

Analysis of mRNA Levels

Total RNA was extracted using Trizol reagent (Life Sciences, Gaithersburg, MD). One microgram of total RNA was then reversed transcribed using GeneAmp kit (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's directions. Two microliters of the RT reaction products were next used in a 50-mL PCR reaction that contained 1.5 μCi of ^{32}P-dCTP (3000 Ci/mmol, Amersham, Arlington Heights, IL) to visualize the products. PCR amplification of cDNAs for each of the receptors was performed using 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C using rat opioid receptor primers as follows:

1. MOR: 5'-ATCTACATTTTCAACCTTGCT-3' and 5'-CAGGAAGGCGTAAAGAACT-3 (705-base long).
2. DOR: 5'-ATCTTCACGCTCACCATGAT-3' and 5'-CG GTCCTTCTCCTTGGAGCC-3' (356-base long).
3. KOR: 5'-CCGCTGTCTACTCTGTGGTGT-3' and 5'-ATGTTGATGATCTTTGCTTTC-3' (352-base long).

These primers were designed from different exons to distinguish between amplification of cDNA and genomic DNA *(3)*. The PCR products were confirmed by sequencing analysis. The amplification of the S16 ribosomal gene was used as an internal control with the following primers: 5'-AAGTCTTCGGACGCAAGAAA-3' and 5'-GACAA GACGAAGACCCGTT-3' *(43)*. For semiquantitative PCR, S16 and KOR products were simultaneously coamplified in one reaction tube as described in a primer-dropping method *(44)*. Ten microliters of PCR products were analyzed on a 5% polyacrylamide gel, auto-radiographed, and quantitated by PhosphorImager analysis (Storm™ system with Image Quant™ software, Molecular Dynamics, Sunnyvale, CA). The mean (±SEM) of the KOR mRNA per S16 mRNA values for experimental samples normalized to that of controls are shown for duplicate or triplicate samples from two to four primary experiments. For statistical analyses, a one-way analysis of variance was performed, and subsequent comparisons of pairs were made using the Student–Newman–Keuls test at the 0.05 level of significance.

Acknowledgments

We wish to express appreciation for the primary testicular cell preparations by Lyann Mitchell, and the technical assistance of Desiree Policarpio and Arash Akhavan. We are also grateful for editorial assistance by Jean Schweis and illustrations by Evan Read. DNA sequencing analysis was provided by The Rockefeller University DNA Technology Center, which is supported in part by National Institutes of Health (NIH) shared instrumentation grants and by funds provided by the US Army and Navy for purchase of equipment. These studies were supported by NIH RO1 HD-29428.

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