Regulation of Mu Opioid Receptor mRNA Levels by Activation of Protein Kinase C in Human SH-SY5Y Neuroblastoma Cells

Elizabeth K. Gies, B.A.,† Dawn M. Peters, B.A.,† Carol R. Gelb, M.A.,† Kathleen M. Knag,‡ Robert A. Peterfreund, M.D., Ph.D.§

Background: The mu opioid receptor (MuOR) is a member of the superfamily of G protein-coupled receptors that mediates the analgesic actions of endogenous opioid peptides and the narcotic alkaloid derivatives of morphine. Activation and translocation of protein kinase C (PKC) by N-methyl-D-aspartate receptor stimulation correlates with resistance to opioid drugs in experimental states of neuropathic pain, but the cellular mechanisms of resistance have not been identified. One possibility is that PKC activation regulates MuOR mRNA expression and thus the ability to generate functional receptors. Using a human neuroblastoma cell line, the authors tested the hypothesis that phorbol ester activation of PKC regulates MuOR mRNA levels.

Methods: SH-SY5Y cells were maintained in a continuous monolayer culture and treated with phorbol esters or other agents before extraction of total cellular RNA. Slot blot hybridization was used to measure the level of MuOR mRNA using \(^{32}P\)-labeled MuOR cDNA probes under high-stringency conditions. Autoradiograms were analyzed by scanning and densitometry.

Results: MuOR mRNA levels decreased in a dose- and time-dependent manner after tetradecanoyl phorbol acetate (TPA) was administered to activate PKC. The nadir, a level of approximately 50% of control, was at 2–8 h, followed by gradual recovery. The actions of TPA were blocked by pretreatment with the selective PKC inhibitor bisindolylmaleimide, but not by inhibition of protein synthesis with cycloheximide or anisomycin. The combination of TPA treatment and transcription inhibition with actinomycin D was associated with a transient increase in MuOR mRNA.

Conclusions: Mu opioid receptor mRNA levels are regulated by activation of PKC in a neuronal model. Protein kinase C effects which decrease MuOR mRNA levels appear largely independent of new protein synthesis, and cytoxicity does not account for the findings. Plasticity of MuOR gene expression may contribute to variations in clinical responses to opioid analgesics in clinical states such as neuropathic pain. (Keywords: Cell lines; SH-SY5Y neuroblastoma cells. Gene expression: mRNA. Receptors: mu opioid receptor. Signal transduction: protein kinase C; phorbol esters.)

THE mu opioid receptor (MuOR) subtype is a clinically important target for agents commonly used in anesthesia and analgesia, including morphine and fentanyl, and is also a site of action for some commonly abused drugs. N-methyl-D-aspartate (NMDA) receptor activation decreases opioid receptor responsiveness to analgesic opioids in states of neuropathic pain\(^1\) and in opioid tolerance.\(^2,3\) The mechanisms underlying these NMDA receptor effects are unknown. Activation and translocation of protein kinase C (PKC) after NMDA receptor stimulation correlates with resistance to opioid drugs.\(^1\) Phosphorylation of the opioid receptor protein attributed to PKC activation has been demonstrated.\(^4,5\) A mechanism contributing to NMDA regulation of opioid receptor function thus could be PKC-mediated opioid receptor phosphorylation. However, PKC activation has multiple cellular effects, including regulation of gene expression.\(^6,7\) Consequently, PKC activated by NMDA receptors in neuropathic pain states could exert its effects at the nucleic acid level to regulate the expression of MuOR mRNA and the subsequent production of receptor protein.

* Medical Student, University of Connecticut School of Medicine.
† Research Technician.
‡ Summer Intern.
§ Assistant Professor of Anesthesia, Harvard Medical School.

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Address reprint requests to Dr. Robert A. Peterfreund, Department of Anesthesia and Critical Care, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114-2696. Address electronic mail to: Peterfreund@Helix.MGH.Harvard.edu

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The existence of a discrete MuOR subtype was confirmed by molecular cloning of a cDNA sequence encoding a protein whose predicted amino acid sequence is consistent with its assignment to the G protein-coupled receptor superfamily. In transfection studies, the expressed cDNA exhibited ligand-binding and signal-transduction properties expected for the MuOR. The availability of this cDNA provides sequence information and the reagent essential for molecular studies of the relation between opioid responsiveness and the structure and expression of the MuOR gene.

Several studies have shown regulation of MuOR mRNA levels with in vivo or in vitro experimental systems. Among the identified regulatory influences are interleukin-1β, cocaine, opioid antagonists, and butyrophenones. Effects were tissue or brain region specific, and also dependent on time of exposure. Mu opioid receptor mRNA levels remained unchanged in other studies, despite alterations in receptor ligand binding. The expression of opioid receptor transcripts by both neurons and glial cells in animal experiments and in mixed cell culture models complicates the interpretation of the mRNA results and the analysis of regulatory mechanisms. Effects of PKC activation on MuOR mRNA have not been studied before.

SH-SY5Y human neuroblastoma cells express functional MuOR receptors that are sensitive to fentanyl, morphine, and naloxone. We found that SH-SY5Y cells express detectable levels of MuOR mRNA. We show that activation of PKC by the phorbol diester tetradecanoyl phorbol acetate (TPA) decreases MuOR mRNA in SH-SY5Y cells, an effect that is apparently mediated by existing proteins. In addition, we provide evidence for a transient increase in MuOR mRNA when TPA is administered in combination with transcription inhibitors. Thus MuOR mRNA levels are subject to multiple regulatory influences after PKC activation. Our findings could be relevant to understanding mechanisms of cellular responsiveness to opioids in states of drug tolerance or chemical dependence and in patients under anesthesia or being treated for pain.

Materials and Methods

Reagents
Trizol reagent and cell culture media and supplies were from Gibco-BRL (Grand Island, NY). Chloroquin and fetal bovine serum were from Sigma Chemical Company (St. Louis, MO). Nusserum was from Fisher Scientific (Pittsburgh, PA). Bisindolylmaleimide was from Calbiochem (La Jolla, CA). All other drugs were from Sigma. DEAE-dextran and an oligolabeling kit using the Klenow fragment of DNA polymerase were obtained from Pharmacia (Uppsala, Sweden). Plasmid preparation kits and restriction endonucleases were obtained from Promega (Madison, WI). Sequenase II and a DNA sequencing kit were from Amersham Life Science (Arlington Heights, IL). Radiolabeled nucleotides were purchased from Dupont/ New England Nuclear (Boston, MA). All other reagents were of molecular biology grade.

Cell Culture
The experimental model for these studies was the undifferentiated SH-SY5Y human neuroblastoma cell line. Cells were grown at 37°C in a 5% carbon dioxide incubator in media consisting of fetal bovine serum, 10% (vol/vol), in Dulbecco's modified Eagle medium (DMEM) with penicillin and streptomycin. Cells were passaged by trypsinization. Seeding density was 1 × 10^6 cells per 100-mm plate. The monolayer was subconfluent after 1 week, when experimental treatments were administered. COS-7 cells were used as the hosts for transfection and expression of receptor cDNAs in control studies. These cells were also grown at 37°C in an atmosphere of 5% carbon dioxide in DMEM supplemented with fetal bovine serum, 10% (vol/vol), penicillin, and streptomycin. Cells were passaged by trypsinization and seeded at a density of 5 × 10^6 cells per 150-mm plate for transfection the next day.

RNA Studies
The experimental endpoint was the measurement of MuOR mRNA levels in extracts from SH-SY5Y cells. The cells received fresh media 1 hr before experimental treatment. After treatment, cells were washed once with ice-cold phosphate-buffered saline and collected by scraping in a small volume of that solution. Samples were pelleted by centrifugation for 2 min at 14,000g, frozen in liquid nitrogen, and stored at −80°C. Frozen cell pellets were thawed on ice for 5 min, and total cellular RNA was isolated by extraction with the Trizol reagent. RNA concentrations were determined by ultraviolet absorbency at 260 nm. The integrity of the RNA was checked by electrophoresis of an aliquot of each sample on an ethidium-stained agarose gel and inspection of the 28S and 18S ribosomal RNA bands.

To measure the MuOR mRNA concentrations by nuclease acid hybridization, samples of total RNA (20 μg) were lyophilized and resuspended in a solution of form-
aldehyde, formamide, water, and 3-[N-morpholino]propanesulfonic acid (MOPS). Samples were heated for 15 min at 65°C. An equal volume of TE (Tris-HCl, 10 mm [pH 7.5] and 1 mm EDTA) with formamide containing-RNA loading dye was added to each tube. Each sample was equally divided between two adjacent wells of a Schleicher and Schuell model SRC 072/0 slot-blot apparatus and transferred to a GeneScreen nitrocellulose membrane (DuPont-New England Nuclear), which served as the solid support for the RNA samples in subsequent hybridization steps. The RNA was fixed by ultraviolet light to the membrane with a Stratallinker (Stratagene). Membranes were immediately placed into prehybridization solution consisting of 50% (vol/vol) formamide, 1% (vol/vol) sodium dodecyl sulfate, 1 m NaCl with 10% (wt/vol) dextran and 10% Denhardt’s solution buffered with Tris-HCl and supplemented with 100–200 μg/ml boiled, sheared salmon sperm DNA at 42°C for 1–18 h. Membranes were hybridized with 32P-labeled cDNA probes in the same solution for 16–24 h. After hybridization, membranes were washed twice for 5 min in 2 × SSC at room temperature, followed by two washes in 2 × SSC with 1% sodium dodecyl sulfate at 65°C (for 30 min each), and two washes in 0.2 × SSC at room temperature (for 30 min each). SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0. Membranes were sealed in bags and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at −80°C with one intensifying screen for 1–72 h.

3H-Leucine Incorporation Studies

Incorporation of the labeled amino acid into protein was used to assess the efficacy of protein synthesis inhibitors on new protein synthesis. The procedure was a modification of a previous protocol.19 Cells were seeded at 1 × 106 cells/well in six-well plates and studied at 48 h. After washing with leucine-free DMEM, the cells were preincubated in a medium of leucine-free DMEM supplemented with fetal bovine serum, 10% (vol/vol) for 1 h. Cells were pretreated with protein synthesis inhibitors, TPA, or excess cold leucine (0.210 g/l, twice the normal media concentration of l-leucine) before the addition of 3H-leucine at 5 μCi/well. After an incubation period of 4 h, cells were lysed with a dilute solution of phosphate-buffered saline (1:1 with water) supplemented with Nonidet (Sigma Chemical Co.) P 40 (0.1% [vol/vol]). Trichloroacetic acid was added to a final concentration of 10% (vol/vol) and the samples were chilled on ice for 30 min, heated to 85°C for 90 s, and cooled. An aliquot was vacuum filtered through Whatman GF/C filters (Whatman International, Kent, England), which were then washed four times with trichloroacetic acid (10% vol/vol), dried, and counted after the addition of Liquiscint (National Diagnostics, Atlanta, GA). The results reported are representative of two similar experiments. Six samples per treatment group were included in each experiment.

Transfections

Transfection assays were designed to carry out control studies on the specificity of the radiolabeled hybridization probes used for mRNA measurements. Confluent COS-7 cells in 150-mm plates were transfected using the DEAE-dextran/chloroquin method. A DNA medium consisting of DMEM, NuSerum (10% [vol/vol]), and Tris-HCl (1 m) was prepared. After washing the cells with DMEM, 4.5 ml DNA medium with plasmid DNA (11.25 μg) was added to the plates. After 1 min, DNA medium with 1.6 mg/ml DEAE-dextran was added and the plates were incubated at 37°C for 3.5 h. The dextran and DNA-containing medium was aspirated, and a solution of dimethylsulfoxide (10% [vol/vol]) in phosphate-buffered saline was applied to the plates for 1.5 min. The plates were incubated in culture medium containing 51.6 mg/ml chloroquin for an additional 3.5 h and then returned to growth medium. Cells were harvested after 48 h by scraping into phosphate-buffered saline, pelleted by centrifugation at 14,000g, and frozen in liquid nitrogen. Cell pellets were stored at −80°C until the RNA was isolated using the Trizol method. RNA was analyzed by the slot-blot technique as described previously.

Preparation of Hybridization Probes

A cDNA for the human MuOR (GenBank accession number L25119), consisting of 1,200 basepairs of coding sequence flanked by 238 basepairs of 5'UT and 172 basepairs of 3'UT cloned into pcDNA3 (Invitrogen, San Diego, CA) was provided by Dr. Lei Yu, Indiana University School of Medicine. A cDNA for the human Kappa opioid receptor (GenBank accession number L37362, KOR) was provided by Dr. Lee-Yuan Liu-Chen, Temple University School of Medicine.20 The KOR cDNA comprises 378 basepairs of 5'UT, 1,140 basepairs of coding sequence, and 89 basepairs of 3'UT cDNA cloned into pBK-CMV (Stratagene). The cDNA for the human delta opioid receptor (DOR, GenBank accession number U07882) was provided by Dr. Henry Yamamura, University of Arizona Health Sciences Center.21 This cDNA contains 233 basepairs of 5'UT, 1,116 basepairs of cod-

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ing region, and 423 basepairs of 3'UT. Originally supplied in a Bluescript vector, the DOR cDNA was recloned into pcDNA3 for transfection.

The hybridization probe templates for the opioid receptors consisted of the complete cDNAs, which were separated from plasmid backbones by enzyme digestion followed by gel electrophoresis in low melting point agarose. The nucleotide sequence homologies for the probe fragments were determined with the GAP subroutine of the Genetics Computer Group. The MuOR and DOR sequences were 54% homologous. The MuOR and the KOR sequences were 58% homologous. A human actin cDNA template was from Clontech (Palo Alto, CA). cDNA probes were labeled by the random priming method with the large fragment of DNA polymerase I (Klenow fragment) to a specific activity of $0.5 - 1 \times 10^6$ cpm/µg. Labeled fragments were purified over a gel filtration column (NICK column, Pharmacia Biotech).

**Analysis of Autoradiograms**

Autoradiograms were scanned into the program Adobe Photoshop 3.0 using a Nikon Model A × 1200 scanner (Melville, NY) and then analyzed with the program NIH Image (version 1.59; National Institutes of Health, Bethesda, MD). The autoradiographic signals from individual samples were marked and the densities recorded. Film background was subtracted from each sample. The resulting density data were analyzed using the program Instant 2.0 (GraphPad, Sorrento Valley, CA) as described in the figure legends.

**Results**

**Evaluation of the MuOR cDNA Probe**

The MuOR cDNA probe hybridized to RNA from SH-SY5Y cells in Northern blot analysis (not shown) and in slot-blot analysis. Because of the large size of the MuOR transcript and the possibility of inconsistent transfer of the mRNA from gel to membrane in Northern blotting, further experiments were done with the slot-blot technique.

To investigate the specificity of the probe, three sets of transfection experiments were performed. First, COS cells were transiently transfected with the MuOR cDNA or pcDNA3. Slot-blot samples were prepared by diluting RNA from MuOR-transfected cells with RNA from the pcDNA3 transfection such that a total of 10 µg RNA were loaded per well (figs. 1A, 1B). The hybridization results suggest that the autoradiographic signal intensities increase with increasing amounts of MuOR RNA in the sample. Second, hybridization of the MuOR probe to RNA from several sources was assessed. A strong hybridization signal was detected for RNA from MuOR-transfected COS cells, but there were only faint or absent signals with RNA from control cell lines and control cell lines transfected with the cDNAs for other, nonopioid, G-protein-coupled receptors (fig. 2). Third, cross-hybridization with two other opioid receptor subtypes was assessed. COS cells were transfected.
### Regulation of Mu OR mRNA Levels

As the first step in testing the hypothesis that PKC activation by phorbol esters regulates Mu OR mRNA levels, SH-SY5Y cells were treated with TPA (100 nm final concentration). A time-dependent decrease in Mu OR mRNA hybridization was observed (figs. 4A, 4B). The nadir was between 2 and 8 h of continuous treatment, after which there was a rise in the mRNA detected. The response to TPA depended on the dose of TPA applied to the cells. The median effective concentration to reduce Mu OR mRNA levels was 1 - 10 nm (figs. 5A, 5B). The maximum reduction in Mu OR mRNA was about 50% of control values. The levels of β-actin mRNA were unchanged for TPA incubations lasting 24 h (data not shown), and mRNA for the A2a adenosine receptor increased with TPA in a time-dependent manner in 24 h (R. Peterfreund et al., Eur J Pharmacol 1997; in press).

### Fig. 2

The μ opioid receptor (MuOR) probe exhibits minimal cross-hybridization with control RNA samples. Slot blots were prepared with RNA from several transfected or untransformed cell lines. The A2b adenosine receptor (A2bR) and the melatonin 1 receptor (MelR) are two other members of the G-protein–coupled receptor superfamily. Blots were first hybridized to the MuOR probe (left). The exposure depicted for the COS-MuOR sample was 1 h, overlaid on an exposure lasting 24 h for all other samples. The positive signal for the COS MuOR sample was grossly overexposed at 24 h. To confirm that similar amounts of RNA were loaded in each well, the blot was rehybridized (without stripping) to a cDNA probe for the human β-actin (right). All exposures were at ~80°C with one intensifying screen.

### Fig. 3

Cross-hybridization of Mu OR and DOR probes. COS cells were transiently transfected with the indicated cDNAs or no DNA (mock) and duplicate slot blots were prepared with 10 µg RNA per lane. One blot was hybridized to the DOR probe and one to the Mu OR probe as indicated at the top of the figure. Two exposures are depicted for each blot, 1 h and 16 h for the long exposure, the sample identical to the probe was removed to prevent spillover of the autoradiographic signal. A similar result was obtained in experiments to assess cross-hybridization of the Mu OR and KOR (not shown).

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<th>PROBE</th>
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<th>Actin</th>
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<td>CHO-A2bR</td>
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<td>3T3-MeIR</td>
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with either the MuOR, DOR, or KOR cDNAs, and parallel blots were hybridized to probes from each opioid receptor subtype. The hybridization data suggest that Mu OR and KOR exhibit less than 6% cross-reactivity (not shown). Cross-hybridization for the Mu OR and DOR is < 8.8% (fig. 3). Although SH-SY5Y cells are reported to express functional DORs only faint hybridization signals after long exposures were detected when SH-SY5Y RNA was hybridized to a DOR probe. The data are consistent with hybridization of the Mu OR cDNA probe proportional to the abundance of Mu OR mRNA in an experimental sample.
specificity of the regulatory effects of tetradecanoyl phorbol acetate on mu opioid receptor mRNA

To determine whether TPA-associated reductions of MuOR mRNA levels result from nonspecific effects of phorbol compounds, the actions of two other phorbol derivatives were assessed. 4β phorbol and 4-O-MeTPA are reported to be relatively inactive in other biological assays, including our measurements of the regulation of adenosine A2a receptor mRNA in SH-SY5Y cells (R. Peterfreund et al., Eur J Pharmacol 1997; in press). When treated with these phorbol compounds (100 nM final concentration), SH-SY5Y cells did not exhibit reduced levels of MuOR mRNA. Paradoxically, the level of mRNA was slightly but significantly elevated when cells received the long-chain derivative 4-O-MeTPA (figs. 6A, 6B).

If the actions of TPA are mediated by protein kinase C, then PKC inhibitors should prevent the decrease in MuOR mRNA. Pretreatment of SH-SY5Y cells with the selective PKC inhibitor bisindolylmaleimide (1 μM) prevented the reductions in MuOR mRNA associated with the subsequent application of TPA (figs. 7A, 7B). There was little effect of bisindolylmaleimide alone. In other experiments (data not...
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shown), cells were initially incubated with TPA for 16 h, followed by an additional 4 h with ethanol vehicle control or a repeated dose of TPA. Compared with cells receiving an initial TPA incubation followed by an ethanol vehicle treatment, MuOR mRNA levels did not decrease in cells receiving a second application of TPA. This result supports the possibility of desensitization of PKC to prolonged incu-

Fig. 6. Bisindolylmaleimide blocks tetradecanoylephorbol acetate (TPA)-associated reductions in mu opioid receptor (MuOR) mRNA. SH-SY5Y cells were pretreated with bisindo-

lymaleimide (1 μM final concentration, BIS) for 20 min before the administration of TPA (100 nm) or ethanol vehi-
cle control. Cells were harvested after 4 h. (A) Representative autoradiograms from one experiment. The experimental
treatments are indicated. (B) Graph representing the levels of MuOR mRNA in SH-SY5Y cells after experimental treatment as indicated. Data from three independent experiments (n = 15–22 per data point) were normalized to the control value, pooled, and plotted as the mean ± SE. **Indicates different from control, at P = 0.01, with other values not significantly different from control as determined by analysis of variance with Dunn's test for multiple comparisons.

Fig. 7. Effects of phorbol analogs on mu opioid receptor (MuOR) mRNA. SH-SY5Y cells were treated with ethanol vehicle control, tetradecanoylephorbol acetate or one of the inactive analogs 4-O-MeTPA or 4β phorbol (all at 100 nm final concentration). Cells were harvested after 4 h. (A) Representative autoradiograms from one experiment. The experimental treatments are indicated. (B) Graph representing levels of MuOR mRNA in SH-SY5Y cells after experimental treatment as indicated. Data from two independent experiments (n = 15–16 per data point) were normalized to the control value, pooled, and plotted as the mean ± SE. **Indicates different from control, at P = 0.01, with other values not significantly different from control as determined by analysis of variance with Dunn's multiple comparisons test.

Protein Synthesis Inhibitors and Mu Opioid Receptor mRNA Levels

Protein kinase C activation has multiple cellular effects, including transcriptional activation with production of new proteins. To investigate the role of new protein synthesis in the regulation of MuOR mRNA by PKC activation, cells were incubated with TPA in the presence or absence of cycloheximide (100 μm final concentration) or anisomycin.
Fig. 8. Tetradecanoyl phorbol acetate (TPA) effects are not blocked by protein synthesis inhibitors. SH-SYSY cells were treated with ethanol vehicle control, TPA (100 nM final concentration), or the combinations of agents as indicated. Cycloheximide (CHX, 100 μM final concentration) or anisomycin (ANI, 5 μg/ml) were administered 15–20 min before TPA. Cells were harvested after 4 h of TPA treatment. (A) Representative autoradiograms from one experiment. The experimental treatments are indicated. (B) Graph representing the levels of MuOR mRNA in SH-SYSY cells after experimental treatment as indicated. Data from two independent experiments with anisomycin (n = 16 per data point) and five independent experiments with cycloheximide (n = 40–42 per data point) were normalized to the control value, pooled, and plotted as the mean ± SE. “Indicates different from control, at P = 0.01. + + Indicates different from cycloheximide alone, at P = 0.01; + indicates different from anisomycin alone, at P ≤ 0.05 as determined by analysis of variance with Dunn’s test for multiple comparisons.

The efficacy of these treatments to inhibit protein synthesis was demonstrated by measuring the incorporation of 3H-leucine into trichloroacetic acid precipitable protein. Label incorporation was inhibited >86% by cycloheximide and >89% by anisomycin (table 1), which corresponds closely to the results of other studies.27 Cycloheximide alone had no effect on MuOR mRNA levels. The response to TPA was preserved in the presence of cycloheximide. Anisomycin, when administered alone, reduced MuOR levels significantly. However, coadministration of TPA and anisomycin resulted in a further significant reduction of mRNA for the MuOR. The TPA-associated reductions were 40–50% in the control, cycloheximide-pretreated, and anisomycin-pretreated groups. The results suggest that little or no new protein synthesis is required for the reduction in MuOR mRNA resulting from PKC activation with TPA.

Transcription Inhibition and Mu Opioid Receptor mRNA Levels

Regulation of levels of a particular mRNA transcript could result from changes in transcription rates, altered stability of the transcript (mRNA half-life), or a combination of both mechanisms. To begin to investigate these possibilities, measurement of the MuOR mRNA half-life was attempted. SH-SYSY cells were incubated in the presence of standard concentrations of the transcription inhibitors actinomycin D (5 μg/ml) or dichlorobenzimidazole riboside (25 μg/ml) to block new RNA synthesis. Mu opioid receptor mRNA levels were measured as a function of time after adding TPA to activate PKC or ethanol vehicle control (fig. 9A, 9B). We predicted that linear decreases in MuOR mRNA levels would be observed. The half-life of the transcript would be proportional to the slope of the decay curve. In the presence of vehicle alone, SH-SYSY cells treated with transcription inhibitors exhibited a time-dependent decrease in MuOR mRNA levels, which reached an apparent plateau. However, when the transcription inhibitors were administered in combination with TPA, there was an initial increase in MuOR mRNA levels compared with administration of the transcription inhibitor alone (figs. 9A, 9B). The MuOR mRNA levels in TPA-treated cells then converged with the levels obtained in vehicle-treated cells. Because of the nonlinear response profiles, the half-life (mRNA decay rate) of the MuOR mRNA could not be measured. The data suggest that activation of PKC influences MuOR mRNA levels by

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<th>Treatment</th>
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<tr>
<td>Control</td>
<td>100.0 ± 5.2</td>
</tr>
<tr>
<td>Cycloheximide (100 μM)</td>
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</tr>
<tr>
<td>Anisomycin (5 μg/ml)</td>
<td>10.7 ± 0.6</td>
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<tr>
<td>TPA (100 nM)</td>
<td>115.0 ± 5.7</td>
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<td>TPA + cycloheximide</td>
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Dishes of cells were incubated with or without the protein synthesis inhibitors cycloheximide or anisomycin, treated with TPA as indicated, and harvested. Data are normalized to the control value and expressed as the percent incorporation ± SE after subtracting the background level (labeled precipitate in the presence of excess cold leucine, 2.7% of the control incorporation absolute value). The control uptake was 4.4% of the applied labeled substrate (5 μCi/well).
eliciting complex combinations of regulatory mechanisms that increase or decrease the transcript’s expression. Degradation of the MuOR transcript is likely to account for the decline of MuOR mRNA levels in cells treated to activate PKC.

Discussion

With a MuOR cDNA hybridization probe in slot-blot analysis, RNA extracted from SH-SY5Y human neuroblastoma cells exhibited a detectable autoradiographic signal. In control experiments, the probe’s hybridization signal intensity increased with increasing amounts of MuOR RNA in the sample, with low cross-reactivity with other RNA species, including the homologous KOR and DOR transcripts. We conclude that the probe specifically detects MuOR mRNA in proportion to the amount of MuOR transcript applied to the wells.

Incubation of SH-SY5Y cells with the phorbol ester TPA was associated with a dose- and time-dependent decrease in MuOR mRNA levels. Five reasons suggest that it is unlikely that this decrease results from a non-specific, toxic effect to the cells. First, we have shown that mRNA levels for actin are unchanged with a similar treatment protocol, and levels of the mRNA for the A2a adenosine receptor increase over the same time period (R. Peterfreund et al., Eur J Pharmacol 1997; in press). Second, incubation with similar concentrations of phorbol esters for periods greater than 24 h has been used in experiments examining the differentiation of SH-SY5Y cells.26-30 Third, incubation with the two chemically similar, but biologically less active,26,25 compounds 4β Phorbol and 4-O-Me TPA was not associated with reductions in MuOR mRNA. Fourth, the effects of TPA administration waned with time, even with repeated administration of the TPA. Fifth, the effects associated with TPA could be blocked by the selective PKC inhibitor bisindolylmaleimide. The reductions in MuOR transcript levels are therefore likely attributable to a biological activity of phorbol esters to activate PKC. The findings add the MuOR to the list of members of the G protein-coupled receptor superfamily whose transcript levels are regulated by phorbol ester activation of PKC.31-35

Activation of PKC initiates a cascade of phosphorylation events that can include activation of MAP kinase and other cellular enzymes.6 Activation of the cascade can result in the regulation of gene expression with consequent new protein synthesis. Co-administration of the translation inhibitors cycloheximide or anisomycin with TPA did not block TPA-associated reductions in MuOR mRNA levels. Instead, the approximately 50% reductions in MuOR mRNA levels were still observed. It is possible that low-level translation of a protein involved in MuOR mRNA regulation persists in SH-SY5Y.

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cells despite the presence of anisomycin and cycloheximide at standard concentrations used to inhibit translation and the substantial reduction in 3H-leucine uptake that we found under these conditions. However, the actions of TPA to reduce MuOR mRNA levels in this experimental system appear largely independent of new protein synthesis. Phosphorylation of preexisting proteins by PKC likely contributes to the mechanism through which TPA affects MuOR transcripts.

Nucleic acid sequence analysis of the putative promoter region of the rat and mouse MuOR genes suggests the possible presence of several previously characterized sites for transcription factor binding, including a glucocorticoid receptor binding site, a cyclic adenosine monophosphate response element, and an AP1 site for the binding of Fos and Jun. Interpretation of these findings is confounded by uncertainty about the precise location of the transcription start site(s), which may lie as close as 230 basepairs or 268–291 basepairs to the translational start site, or several hundred bases further upstream. Kraus et al. constructed fusion genes consisting of putative regulatory sequences from the MuOR promoter region combined with the choline acetyl transferase gene coding sequence as the reporter gene expression. These fusion gene constructs were then transfected into SH-SY5Y cells. Enhanced expression of the fusion gene was observed in response to phorbol esters, consistent with a functional AP1 site responsive to PKC activation. Fusion gene expression also increased when cyclic adenosine monophosphate levels were stimulated by the administration of forskolin, consistent with a functional cyclic adenosine monophosphate response element. Whether these regulatory influences actually modulate native MuOR mRNA expression was not determined.

Based on the functional data obtained by Kraus et al. with reporter-promoter constructs expressed in SH-SY5Y cells, increases in MuOR mRNA levels would have been expected after PKC activation. In our experiments, early elevation of MuOR mRNA levels was seen when combinations of TPA and transcription inhibitors were administered, compared with incubations with transcription inhibitors alone. This response was detected within 1 h of incubation but was not sustained. Instead, we found a late decrease in MuOR mRNA, which indicates degradation of the transcript. Transcriptional control of endogenous, intact genes may differ from the transcriptional activity of gene fragments studied in the transfection constructs. Furthermore, nonmammalian reporter transcripts, such as the bacterial choline acetyl transferase gene, may resist regulatory influences acting on native transcripts. Our results are consistent with the concept that influences about net cellular effects of gene regulation based on data from promoter-reporter constructs in transfection studies must be evaluated cautiously.

We observed a transient increase in MuOR mRNA levels when PKC was activated in the presence of transcription inhibitors. mRNA levels for other genes increase after treatment with transcription inhibitors. Most notably, thyrotropin-releasing hormone receptor transcripts increase in the GH3/GC1 cell after incubation with actinomycin D or dichloroindazolreo riboside in concentrations identical to those that we used here. Such responses may represent a type of superinduction, although the precise mechanism of elevations in mRNA levels remains uncertain. In the present case, one possibility is that inhibition of the transcription of labile RNAs in SH-SY5Y cells unMASKS an early action of PKC activation, which stabilizes existing transcripts, increases transcription, or both. This early effect is apparently countered when the activation of preexisting proteins by PKC later results in the delayed loss of MuOR mRNA. We propose that two or more mechanisms are involved in regulating MuOR mRNA levels in the SH-SY5Y neuronal model; one mechanism elevates whereas the other reduces MuOR mRNA levels in response to PKC activation.

In addition to the effects on mRNA levels demonstrated in this study, TPA may influence the function or levels of MuORs at the protein level. In the SH-SY5Y model, TPA treatment for 4 days was associated with an increase in Bmax for the MuOR without a change in the apparent affinity. The results of shorter incubations were not reported. The effect of TPA is complex in that cells at a high passage number exhibited an opposite response. In an oocyte expression system, MuOR-activated potassium currents were reduced after TPA was administered. The interpretation of this finding was that activation of PKC could influence MuOR-K+ channel coupling, but in this report experiments with PKC inhibitors were not described. The precise target(s) of PKC in the oocyte expression system remain undetermined, whereas TPA treatment was associated with phosphorylation of the MuOR expressed in Chinese hamster ovary cells. The published results obtained at the protein level, combined with the data from this study of mRNA, indicate the possibility of a multi-
faceted role for PKC in regulating cellular responses to MuOR ligands.

The present findings demonstrate regulation of mRNA for the MuOR in a neuronal cell line. Because receptor binding of an appropriate agonist is associated with activation of phospholipase C and subsequent activation of PKC in some models of MuOR signal transduction, our findings are consistent with the possibility of negative autoregulation of MuOR mRNA levels after activation of the MuOR. Alternatively, activation of another receptor subtype coupled to PKC, such as the NMDA receptor, could influence the expression of the MuOR. Additional studies are needed to address the implications of alterations in MuOR mRNA levels for expression of MuOR protein and for responses to opioid agonists. Nevertheless, these findings suggest that the level of expression of the MuOR mRNA is not static; rather it is subject to regulation by PKC activation. Such regulation may contribute to altered responsiveness to opioid agents in pain states or chronic opioid use.

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