Stimulation of the Calcitonin Gene-Related Peptide Enhancer by Mitogen-Activated Protein Kinases and Repression by an Antimigraine Drug in Trigeminal Ganglia Neurons

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Calcitonin gene-related peptide (CGRP) is involved in the underlying pathophysiology of all vascular headaches, including migraines. Elevated levels of CGRP during migraine are restored to normal coincident with headache relief after treatment with the antimigraine drug sumatriptan. We have used primary cultures of trigeminal neurons under conditions simulating migraine pathology and therapy to study the mechanisms controlling the CGRP promoter. Using reporter genes in transient transfection assays, we demonstrate that an 18 bp enhancer containing a helix–loop–helix element is both necessary and sufficient for full promoter activity. NGF treatment and cotransfection with an upstream activator of the extracellular signal-regulated MAP kinases (MAPKs) activated the enhancer. Treatment with sumatriptan repressed NGF- and MAPK-stimulated CGRP promoter activity. Repression was also observed using a synthetic MAPK-responsive reporter gene. Sumatriptan regulation of CGRP gene expression did not couple to a G_i/G_o pathway, but rather caused a prolonged increase in intracellular calcium. The importance of the prolonged calcium signal in repression of MAPK activity was demonstrated by using the ionophore ionomycin to mimic sumatriptan action. We propose that activation of MAPK pathways may increase CGRP gene expression during migraine, and that sumatriptan can diametrically oppose that activation via a prolonged elevation of intracellular calcium.

Key words: **CGRP; trigeminal ganglia; MAPK; calcium; migraine; gene expression**

Introduction

Calcitonin gene-related peptide (CGRP) is a multifunctional regulatory neuropeptide that is believed to play an important role in the underlying pathology of vascular headaches (Van Rossum et al., 1997). Serum levels of CGRP are elevated in patients during migraine and cluster headaches (Goadsby and Edvinsson 1993, 1994; Edvinsson and Goadsby, 1994; Fanciullacci et al., 1995). In the neurovascular model of migraine, trigeminal ganglia nerves are activated and release CGRP and other neuropeptides that mediate neurogenic inflammation within the meninges (Moskowitz, 1993; Buzzi et al., 1995; Williamson and Hargreaves, 2001). Interestingly, the wave of cortical-spreading depression during the migraine aura can activate the trigeminal ganglia (Bolay et al., 2002). Additional evidence for a role of trigeminal CGRP in migraine comes from clinical studies in which the serotonergic drug sumatriptan lowers CGRP levels in the jugular outflow, coincident with the relief of headache pain (Goadsby and Edvinsson, 1991). Because migraine can last for up to 3 d, we reasoned that there might be a sustained increase in CGRP synthesis during neurogenic inflammation. Likewise, although the fast action of sumatriptan in patients is consistent with the inhibition of secretion, we reasoned that there might also be a coordinate regulation at the level of transcription.

CGRP transcription is controlled by a cell-specific enhancer that contains a basic helix–loop–helix (bHLH) site and an adjacent octamer-binding motif (Peleg et al., 1990; Ball et al., 1992; Tverberg and Russo, 1993; Lanigan and Russo, 1997). This distal enhancer was identified using thyroid C-cell lines, which have neuronal-like properties (Russo et al., 1992, 1996). The promoter also contains a complex proximal element that is cAMP- and ras-responsive (de Bustros et al., 1986, 1992; Monia et al., 1995; Thiagalingam et al., 1996). It has been well established that CGRP levels are elevated by nerve growth factor (NGF) (Lindsay and Harmar, 1989; Verge et al., 1995; Patel et al., 2000; Shadiack et al., 2001; Supowit et al., 2001), and that NGF action appears to involve the cAMP/ras element and an undefined region (Watson et al., 1995; Freeland et al., 2000). We have shown that the cell-specific CGRP enhancer is stimulated by depolarization and strongly activated (>10-fold) by a constitutively active mitogen-activated protein kinase (MAPK) kinase (MEK1) in the CA77 thyroid C-cell line (Durham and Russo, 1998, 2000). Activation of serotonin type 1 (5-HT_1) receptors repressed basal and MEK1-stimulated enhancer activity in the CA77 cells (Durham and Russo, 1998). However, although the CA77 cells are a good model, the question remained as to whether this regulation occurs in normal trigeminal neurons. To address this question, we have used primary cultures of rat trigeminal ganglia that we have shown previously to be responsive to sumatriptan and other 5-HT_1 agonists that inhibit CGRP secretion (Durham and Russo, 1999).

In the present study, we demonstrate that NGF and MAPK

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stimulation of CGRP promoter activity requires a single HLH site in the enhancer. These stimulatory effects were greatly repressed by sumatriptan treatment via a pertussis-toxin-insensitive pathway. Furthermore, the inhibitory effect of sumatriptan on promoter activity can be mimicked by a prolonged elevation in intracellular calcium. These results demonstrate that CGRP gene expression is stimulated by MAPKs and repressed by the antimigraine drug sumatriptan in trigeminal ganglia neurons.

Materials and Methods

Cell culture. Trigeminal ganglia cultures were based on our previous protocol (Durham et al., 1997). Ganglia isolated from ~20–24 3- to 4-d-old Sprague Dawley rats were washed in 10 ml of cold plating medium [25 mM HEPES, pH 7.2–7.4, DMEM (high glucose)] and collected at 100 × *g* for 2–3 min. The ganglia were resuspended in 10 ml of plating medium containing 10 mg/ml dispase II (Invitrogen, Gaithersburg, MD) and 1 U/ml RQ1 DNase (Promega, Madison, WI) and then split into two 15 ml tubes (5 ml each) for 30 min at 37°C. The cell suspension was collected by centrifugation at 100 × *g* for 3 min. The pellets were resuspended and further dissociated in 5 ml of plating medium by vigorous trituration (~15 times) using a 5 ml pipette. After allowing larger fragments to settle (~1 min), the suspensions from each tube were combined into a new 15 ml tube. The remaining large fragments were pooled and triturated ~15 times in 5 ml of plating medium. After the trituration, any remaining fragments were mechanically removed by gently swirling a Pasteur pipette in the suspension. The suspension was combined with the cells obtained after the first trituration step (15 ml total) and centrifuged at 100 × *g* for 4 min. The cell pellet was resuspended in 6 ml L15 (Leibovitz) medium containing 10% fetal bovine serum, 50 mM glucose, 250 μM ascorbic acid, 8 μM glutathione, 2 mM glutamine, and 10 ng/ml mouse 2.5 S NGF (Alomone Labs, Jerusalem, Israel) at 37°C at ambient CO_2 levels. For transfection studies, the cells were plated on 12 well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) coated with poly-D-lysine (molecular weight, 30,000–70,000; Sigma, St. Louis, MO). Cultures were noticeably healthier on Becton Dickinson Falcon dishes compared with those of other manufacturers. Penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml; Sigma) were added to the L15 medium unless otherwise noted. For some studies, the cultures were enriched for neuronal cells by density-gradient centrifugation. After the dissociation step, the cell pellet was resuspended in 3 ml of plating medium containing 1 mg/ml bovine serum albumin. The cells were carefully layered onto 6 ml of plating medium containing 10 mg/ml bovine serum albumin in a 15 ml conical tube, and then centrifuged at 100 × *g* for 3 min. The cell pellet was resuspended in L15 medium and plated as described above. The culture medium was changed after 24 hr and every other day thereafter. Bradykinin was purchased from Sigma; sumatriptan succinate (GlaxoSmithKline Pharmaceuticals, Harlow, UK) was obtained from the University of Iowa Pharmacy; and ionomycin, PD98059, U0124, and U0126 were supplied by Calbiochem-Novabiochem (La Jolla, CA). Pertussis toxin (100 ng/ml; Invitrogen) treatments were for 20 hr before the experiment. In all studies, cells were treated with equivalent amounts of vehicle.

Immunohistochemistry. Transfected cultures were briefly rinsed in PBS and fixed in 100% methanol for 10 min at −20°C. The fixed cells were incubated for 30 min in PBS with 10% fetal bovine serum and then costained for 1 hr with rabbit anti-rat CGRP polyclonal antibodies (1:1000 dilution; gift from I. Dickerson, University of Miami, Miami, FL) and a mouse anti-β-galactosidase monoclonal antibody (1:1000 dilution; Promega). Secondary antibodies were rhodamine red X-conjugated donkey anti-rabbit IgG (1:100; The Jackson Laboratory, Bar Harbor, ME) and FITC-conjugated donkey anti-mouse IgG (1:1000; The Jackson Laboratory).

Transfection of trigeminal cultures. All of the CGRP and herpes simplex thymidine kinase (TK) luciferase reporter plasmids and the cytomegalovirus (CMV) β-galactosidase reporter plasmid have been described previously (Tverberg and Russo, 1993; Lanigan and Russo, 1997). The 1250 bp CGRP promoter–luciferase reporter contains sequences from the *Kpn*I site (−1250) to the *Sau*3A site (+21) in exon 1. A *Bam*HI linker (CGGATCCG) was inserted at the *Pvu*II site (−1038 bp) of the 1250 bp CGRP luciferase gene (Tverberg and Russo, 1993). The 18 bp cell-specific enhancer [HLH and octamer-binding sites (HO)] and HO+A reporters contain four tandem repeats in the sense orientation. All plasmids were sequenced to confirm the insertions. The plasmids containing CMV–MEK1 (S218/222E, Δ32–51), the Elk-1 activation domain fused to the Gal4 DNA-binding domain, and the luciferase reporter with gal1 sites have been described previously (Durham and Russo, 1998).

Trigeminal ganglia cultures were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Approximately 3–5 × 10^4 cells (three to four ganglia) per well (12 well dish) were transfected 1 hr after plating with 1–2 μg of CGRP–luciferase reporter, 0.5 μg of gal1–luciferase reporter, 0.5 μg of Gal4–Elk-1, and/or 0.5 μg of CMV–MEK1 plasmid DNAs. The amount of DNA was kept constant by the addition of the empty expression vector CMV-5 (Durham and Russo, 1998). The DNA and Lipofectamine 2000 reagent (ratio, 1 μg:3 μl) were incubated together for 20 min in L15 medium (without supplements). The DNA–Lipofectamine complex was then added to the trigeminal cultures maintained in the fully supplemented L15 medium (including serum, 10 ng/ml NGF and +/− antibiotics, and an antifungal agent) and incubated for ~24 hr before assaying for reporter activity. Two hours before harvesting, 60 mM KCl, 10 μM bradykinin, and 100 ng/ml NGF were added. In some experiments, the cells were preincubated for 30 min with sumatriptan, ionomycin, or the MAPK inhibitors PD98059, U0126, or U0124 before the addition of NGF. Cells transfected with MEK1 were incubated overnight in serum-containing medium with sumatriptan or ionomycin and harvested 20 hr later. Luciferase was measured using the Luciferase Assay System (Promega) and was reported as relative light units per 20 μg of protein. Protein concentrations were determined by Bradford assays (Bio-Rad Laboratories, Hercules, CA). In all experiments, transfection efficiencies were normalized to CMV–β-galactosidase activity that was measured using Galacto-Light reagents (Tropix, Bedford, MA). No difference in cell viability (trypan blue staining) was observed after transfection with the different plasmids or treatments. The normalized luciferase activities are reported as means with SEs per 20 μg of protein. Each experimental condition was repeated in at least three independent experiments done in duplicate. Statistical analyses were done using Student's *t* test.

Calcium measurements. Intracellular calcium levels in cultured trigeminal neurons were measured using a video microscope digital image analysis system (Photon Technology International Inc., South Brunswick, NJ) as described previously (Durham et al., 1997). Briefly, dissociated trigeminal ganglia cells grown on laminin-coated 25 mm glass coverslips were maintained in complete L15 serum-containing medium 24 hr before the start of the calcium imaging procedure. Cells were incubated in L15 containing 0.2% bovine serum albumin and 1 μM fura-2 AM (Molecular Probes, Eugene, OR) for 30 min at 37°C in ambient CO_2. After washing the cells twice with HEPES-buffered saline, pH 7.4 (Durham and Russo, 1999), the cells were incubated for 30 min in L15 medium with insulin transferrin selenium (Sigma) (for sumatriptan studies) or L15 media with 10% serum (for ionomycin studies) before measurement using a Nikon (Tokyo, Japan) Diaphot microscope. Basal calcium levels were measured for a minimum of 120 sec before treatment with sumatriptan or ionomycin. An equal volume of the appropriate medium containing sumatriptan or ionomycin (at two times the final concentration) was added directly to the cells, and the values were recorded every 20 sec, usually for 10–15 min, on a heated stage at 37°C. Calcium levels in individual trigeminal neurons as identified by morphological characteristics and response to depolarization with 60 mM KCl were determined using a 50 × 50 pixel area in the center of each cell.

Results

CGRP promoter activity in primary trigeminal ganglia neurons

A 1250 bp rat CGRP promoter–β-galactosidase reporter plasmid was used to detect transiently transfected neurons in primary trigeminal ganglia cultures (Fig. 1). This promoter includes an 18 bp cell-specific enhancer that is necessary and sufficient for cell-

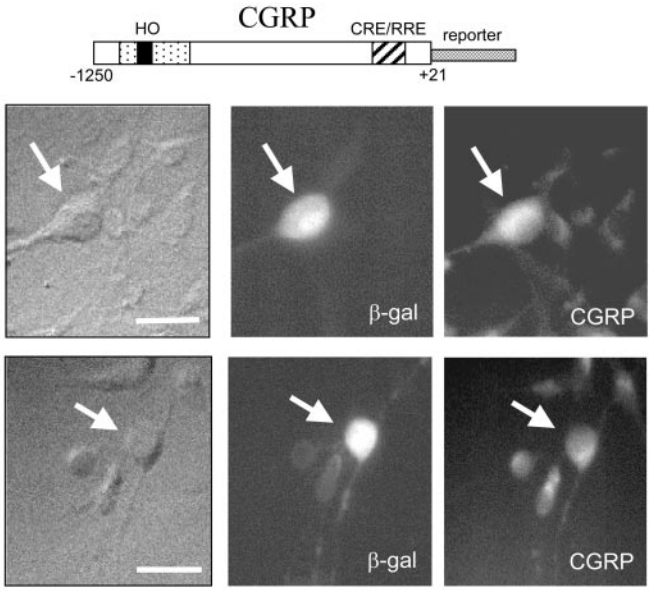

Figure 1. Expression of a CGRP promoter–β-galactosidase reporter gene in trigeminal ganglia cultures. A schematic of the 1250 bp rat CGRP promoter fragment showing the proximal cAMP-response element (*CRE*) and ras-response element (*RRE*) responsive regions (*striped*) and the distal enhancer that contains both cell-specific 18 bp HO (*solid*) and non-cell-specific (*spotted*) elements is shown at the *top*. Trigeminal cultures were transfected with the 1250 bp CGRP–β-galactosidase reporter gene and then analyzed 24 hr after transfection. The cells were visualized by Nomarski optics (*left*) and double stained for β-galactosidase (*β-gal; middle*) and CGRP (*right*) immunoreactivity. The cell body of a neuronal cell is identified by an *arrow* in each set of photographs. Scale bars, 50 μm.

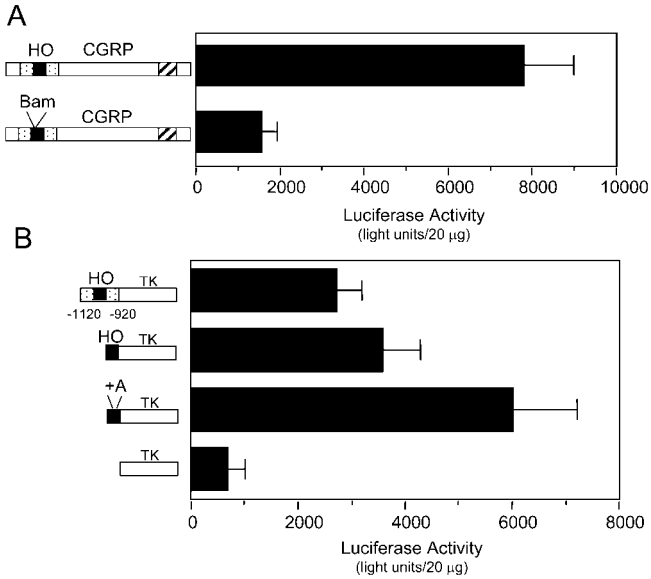

Figure 2. Mapping of basal CGRP enhancer activity in trigeminal neurons. *A*, The 1250 bp CGRP promoter–luciferase reporter gene was transfected into primary trigeminal cultures, and luciferase activity was measured after 24 hr. Insertion of a *Bam*HI linker into the HLH motif in the HO enhancer greatly reduced reporter activity. *B*, Reporter genes containing the minimal TK promoter with either a single 200 bp region containing the HO enhancer or a multimer of the 18 bp HO enhancer yielded luciferase activity greater than that of the TK promoter alone. Insertion of a single adenosine between the HLH and octamer motif (+A) did not reduce the activity of the multimerized HO element. The normalized means with SEs from at least three independent experiments are shown.

specific expression in rat and human thyroid C-cell lines (Tverberg and Russo, 1993; Lanigan and Russo, 1997). The expression of β-galactosidase and CGRP in the transfected cultures was determined by counting the number of positive cells from several fields from each of the dishes with a 10× objective (total magnification, 100×). The β-galactosidase reporter was usually detected in 5–10% (25 of 279) of cells present in the day 1 cultures (Fig. 1). Of the cells counted from several dishes, most (>90%; 23 of 25) of the β-galactosidase-positive cells were identified as neuronal based on morphology (round cell body of 20–50 μm) and colocalization with CGRP. Identification of neuronal cells based on the presence of neurites was not reliable, because many of the CGRP-containing neurons did not exhibit visible processes until day 2 in culture. A serendipitous consequence of the transfection protocol was the enrichment of neuronal cells (>90%; 136 of 148; $n = 6$ dishes). This enrichment is likely a result of toxicity to replicating cells when antibiotics are present in the media during transfection using Lipofectamine 2000. The percentage of neuronal cells in the absence of antibiotics was much lower (~40%; 39 of 98; $n = 3$ dishes). These data demonstrate that the 1250 bp CGRP promoter is sufficient to direct reporter gene expression in trigeminal neurons.

To test whether the cell-specific enhancer that had been identified in neuronal-like cell lines was active in trigeminal neurons, we used a series of CGRP promoter–luciferase reporters. CGRP promoter activity was greatly diminished by insertion of an 8 bp *Bam*HI linker into the HLH motif of the HO enhancer (Fig. 2*A*). A reporter with the TK promoter and the CGRP 5′ region from −1120 to −920, which contains the HO element and flanking non-cell-specific elements, had threefold to fourfold greater activity than the TK promoter alone (Fig. 2*B*). A similar increase over TK was observed with a reporter containing four copies of the HO element. These results are in agreement with previous cell-line studies. However, a mutation in the octamer-binding motif that had been shown previously to block enhancer activity almost completely in thyroid C-cell lines (Durham et al., 1997) did not inhibit enhancer activity, but rather, slightly stimulated activity in the trigeminal cultures (Fig. 2*B*). This mutation is an insertion of an adenosine adjacent to the HLH motif, which generates a consensus octamer site. These data indicate that although the role of the octamer site is not clear, the HLH site of the HO enhancer is required for basal enhancer activity in trigeminal neurons.

Stimulation of CGRP promoter activity by depolarization, proinflammatory agents, and MAPKs

The effect of trigeminal neuron activation on CGRP promoter activity was investigated using several paradigms. Treatment with KCl to mimic neuronal depolarization caused a more than twofold increase in promoter activity (Fig. 3*A*). A similar effect on promoter activity was observed after treatment with bradykinin, a pronociceptive agent that causes inflammation in peripheral tissues (Davis and Dostrovsky, 1988; Kai, 1993). CGRP promoter activity was markedly stimulated (almost fivefold) by the proinflammatory agent NGF that is involved in chronic pain conditions and elevated during headaches (Bennet, 2001; Sarchielli et al., 2001). These results demonstrate that CGRP promoter activity is stimulated in cultured trigeminal neurons by factors known to activate sensory neurons and promote inflammation.

The mechanism of NGF activation of the CGRP enhancer was then determined. We focused on the MAPK pathway, because the NGF receptor can activate MAPKs in other systems (Ahn et al., 1992; Pang et al., 1995) and we have shown that the enhancer is MAPK-responsive in the CA77 cells (Durham et al., 1998, 2000).

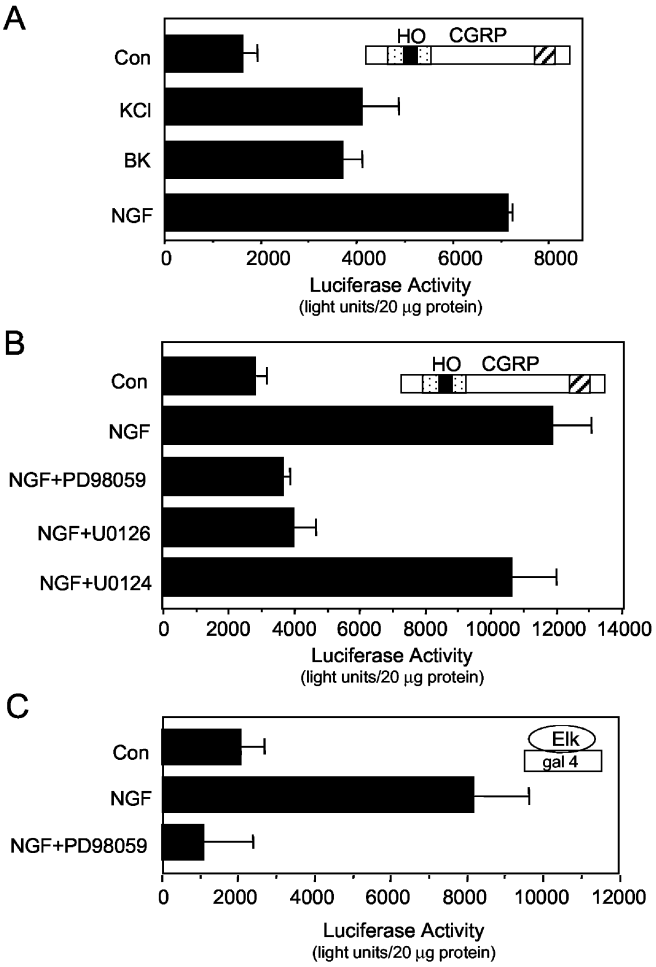

Figure 3. NGF stimulation of CGRP promoter activity via ERK MAPK. *A*, Trigeminal cultures transfected with the 1250 bp CGRP reporter were untreated [controls (*Con*)] or treated with 60 mM KCl, 10 μM bradykinin (*BK*), or 100 ng/ml NGF for 2 hr before harvest. *B*, Trigeminal cultures transfected with the 1250 bp CGRP reporter were treated with NGF in the absence or presence of the MEK inhibitors PD98059 (10 μM), U0126 (1 μM), or the inactive control U0124 (10 μM) for 2 hr before harvest. *C*, The effect of 100 ng/ml NGF and PD98059 on Elk-1 reporter gene activity is shown. The normalized means with SEs from at least four independent experiments for each *panel* are shown.

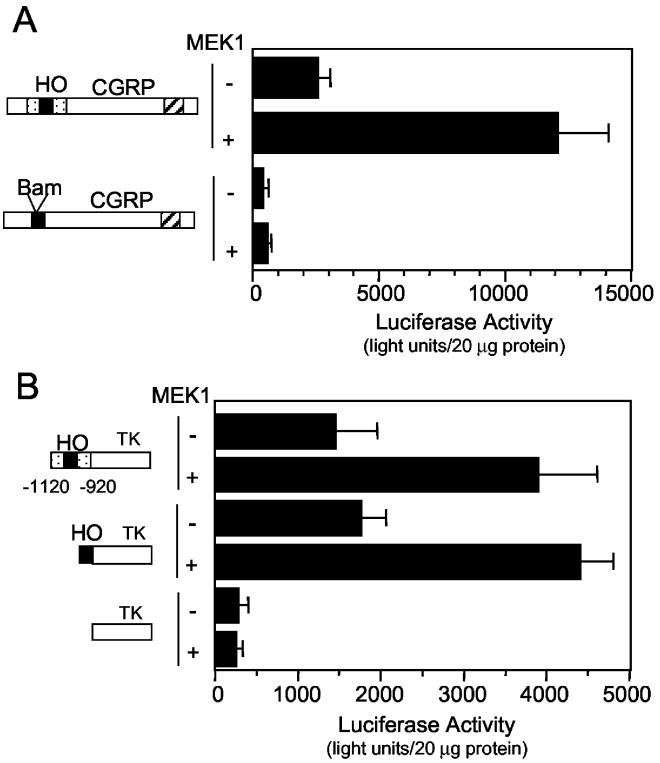

Figure 4. MEK1 stimulation of the CGRP HO enhancer. *A*, Trigeminal cultures were cotransfected with the 1250 bp CGRP–luciferase reporter or the 1250 bp reporter with the HLH mutation (*Bam* insertion) with or without a CMV–MEK1 expression vector. The activity of the 1250 bp promoter, but not the mutant promoter, was increased by MEK1. *B*, Activation of the CGRP HO enhancer within the 200 bp region or as a multimerized 18 bp element linked to the TK promoter by cotransfected MEK1. The normalized means with SEs from at least three independent experiments are shown.

CGRP promoter activity was measured from cultures cotreated with NGF and two different inhibitors (PD98059 and U0126) of MEK1 and MEK 2, which selectively activate only the extracellular signal-regulated MAPKs ERK1 and ERK2. Treatment with the MEK1/2 inhibitors greatly diminished the stimulatory effect of NGF (Fig. 3*B*). As a control, no appreciable change in promoter activity was observed with the inactive analog U0124. NGF activation of MAPK pathways was confirmed by an independent test using a synthetic reporter gene that is activated by the known MAPK-responsive transcription factor Elk-1. NGF stimulated Elk-1 reporter activity approximately fourfold (Fig. 3*C*). As a control, PD98059 cotreatment blocked the NGF stimulation. These data establish that NGF activation of an ERK MAPK pathway is required for the activation of the CGRP promoter in cultured trigeminal neurons.

Because NGF can activate multiple signaling pathways, we then tested whether ERK MAPK activation is sufficient to activate the CGRP promoter. Trigeminal cultures were cotransfected with the CGRP promoter reporter plasmid and a plasmid encoding a constitutively active MEK1. Coexpression of activated MEK1 increased CGRP promoter activity almost fivefold over control (Fig. 4*A*).

To identify the target sequence of the MEK1-activated signaling pathway, we tested the effect of MEK1 on the 1250 bp CGRP promoter fragment containing the mutated enhancer (Fig. 4*A*). Mutation of the HLH site of the HO enhancer almost eliminated the stimulatory effect of MEK1. In addition to being required for MAPK responsiveness, the HO enhancer was also sufficient to mediate the effect of MEK1. MEK1 caused a severalfold stimulation of two reporters containing either the −1120 to −960 bp enhancer fragment or a multimer of the 18 bp HO enhancer, whereas no effect was seen on the control TK promoter activity (Fig. 4*B*). These data demonstrate that MEK1 stimulation of the CGRP promoter is mediated via the HLH site in the HO enhancer.

Repression of stimulated CGRP promoter activity by sumatriptan

Having shown that the HO enhancer is stimulated by MAPKs, we then determined whether treatment with the antimigraine drug sumatriptan could repress this activation. As seen in Figure 5*A*, increasing concentrations of sumatriptan correlated with greater repression of the MAPK-activated enhancer. At 0.1 μM, CGRP activity was reduced to ~70% of the MEK1-stimulated activity. Repression to 27% of the MEK1 value was observed at 10 μM sumatriptan. These results are in agreement with the dose dependence of sumatriptan repression of stimulated CGRP release from cultured trigeminal neurons (Durham and Russo, 1999).

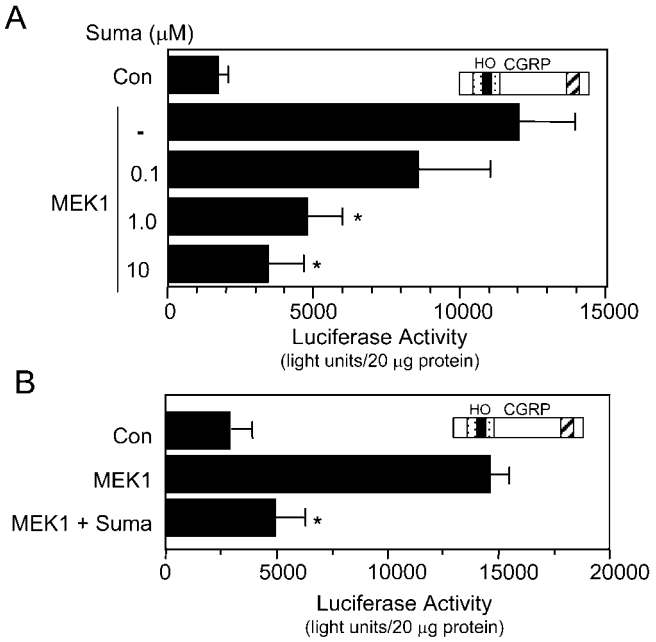

Figure 5. Sumatriptan (*Suma*) repression of MEK1 stimulation of the CGRP reporter genes. *A*, Trigeminal cultures transfected with only the 1250 bp CGRP–luciferase reporter gene or co-transfected with CMV–MEK1 expression vector were either untreated (−) or treated overnight with the indicated amounts of sumatriptan, which lowered reporter activity. *B*, Trigeminal cultures were treated as in *A*, except for incubation overnight in antibiotic- and antifungicide-free media. The cultures were treated with 10 μM sumatriptan. The normalized means with SEs from at least three independent experiments are shown. *$p < 0.01$ compared with MEK1-stimulated values. *Con*, Controls.

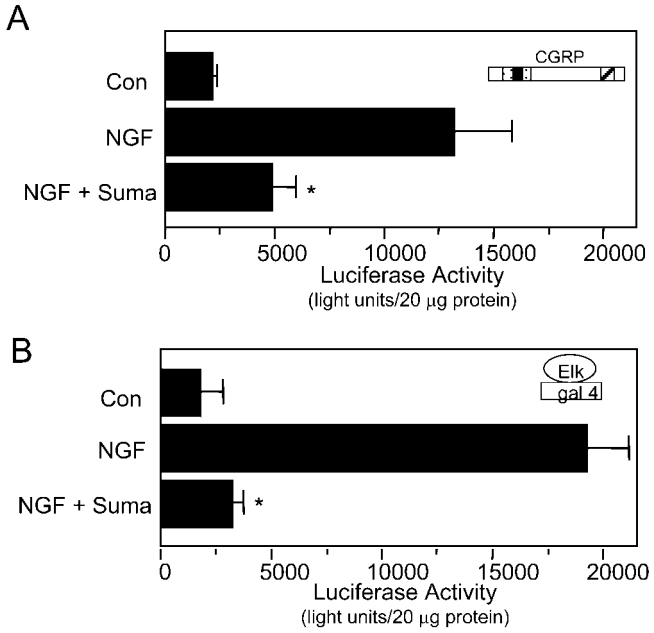

Figure 6. Sumatriptan (*Suma*) repression of NGF stimulation of the CGRP and Elk-1 reporter genes. *A*, Trigeminal cultures transfected with the 1250 bp CGRP–luciferase reporter gene were either untreated, treated with NGF for 2 hr, or pretreated with 10 μM sumatriptan for 30 min before NGF addition for 2 hr before harvest. *B*, Elk-1 reporter activity in cultures treated with NGF and sumatriptan as in *A* was repressed by sumatriptan treatment. The normalized means with SEs from at least three independent experiments are shown. *$p < 0.01$ compared with stimulated values. *Con*, Controls.

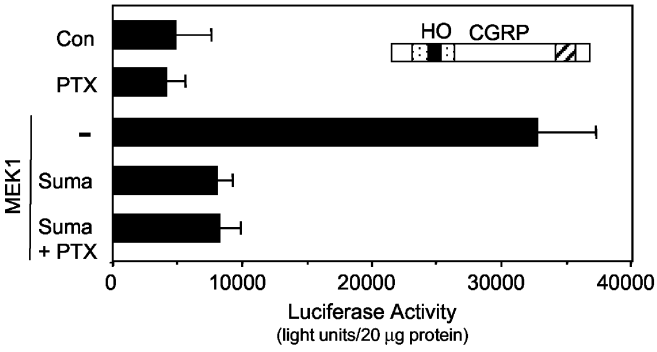

Figure 7. Pertussis toxin treatment does not block the effect of sumatriptan (*Suma*). Trigeminal cultures transfected with the CGRP reporter gene and MEK1 expression vector were treated with sumatriptan overnight alone or with 100 ng/ml pertussis toxin (*PTX*). The luciferase activities are the normalized means with SEs from at least three independent experiments. *Con*, Controls.

Typically, cultures were transfected and incubated overnight in media containing antibiotics, which as noted previously, resulted in an enrichment for neurons. As a control, we also tested promoter activity in the absence of antibiotics and amphotericin after enrichment for neurons by density centrifugation. MEK1 stimulation and repression by sumatriptan of CGRP promoter activity was very similar for these enriched cultures in the absence of antibiotics and antifungal agents (Fig. 5*B*).

Consistent with the repression of MEK1 activation, 10 μM sumatriptan also repressed NGF stimulation of the CGRP promoter (Fig. 6*A*). To determine whether sumatriptan caused a general repression of MAPK responsiveness, the effect on Elk-1 transactivation activity was investigated. Sumatriptan treatment markedly decreased NGF stimulation of the MAPK-responsive Elk-1 transactivation domain (Fig. 6*B*).

Sumatriptan repression is pertussis-toxin-insensitive

To begin to understand the mechanism by which sumatriptan represses MAPK-stimulated CGRP gene expression, transfected trigeminal cultures were treated with pertussis toxin before sumatriptan treatment. Sumatriptan has a high affinity for the 5-HT_1 class of receptors that have been reported to inhibit adenylate cyclase via the pertussis-toxin-sensitive G_i/G_o proteins (Boess and Martin, 1994). However, pertussis toxin did not block sumatriptan-mediated repression of the CGRP promoter in cultured trigeminal neurons (Fig. 7). This finding is in agreement with our previous observations that trigeminal neuron 5-HT_1 receptors are apparently not pertussis-toxin-sensitive and do not decrease intracellular cAMP levels (Durham and Russo, 1999).

Sumatriptan causes prolonged elevation of intracellular calcium

We have previously observed a prolonged increase in calcium after sumatriptan treatment of trigeminal neurons (Durham et al., 1997; Durham and Russo, 1999). Because the culture conditions used in this study differ from previous investigations, we first confirmed that sumatriptan caused a steady and prolonged increase in intracellular calcium levels (Fig. 8*A*). As seen previously, there was a lag and considerable heterogeneity in the responses. Approximately one-half of the neurons responded with increased calcium levels that reached a plateau of ~200 nM after 5 min (Fig. 8*B*). The percentage of cells shown to be sumatriptan-responsive correlates with our observation that 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors are expressed at varying levels in approxi-

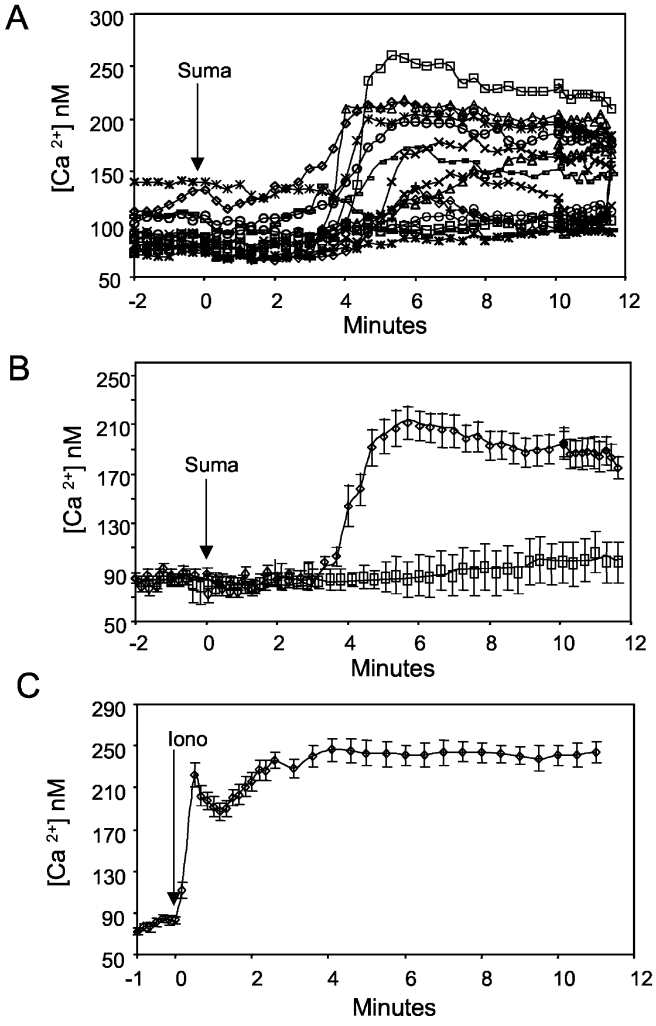

Figure 8. Sumatriptan (*Suma*) and ionomycin (*Iono*) cause prolonged elevation of intracellular calcium in trigeminal neurons. Calcium concentrations [Ca^{2+}] from day 2 cultures were measured using fura-2 and a microscopic digital imaging system. *A*, The calcium concentrations of individual neurons in response to 10 μM sumatriptan are shown ($n = 14$ cells from 2 independent experiments). *B*, The means and SEs in calcium levels are shown for sumatriptan-responsive cells (designated as >150 nM calcium response; $n = 8$) and the nonresponsive neurons ($n = 6$) from *A*. *C*, All neurons responded to 1 μM ionomycin. The means and SEs are shown ($n = 34$ cells from 3 independent experiments).

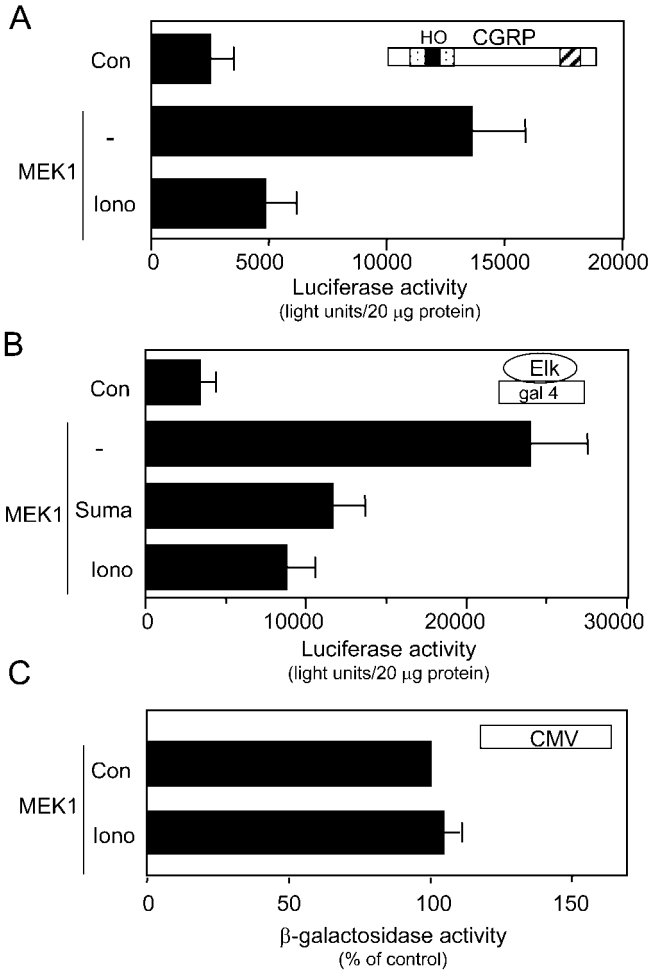

Figure 9. Ionomycin treatment represses MEK1 stimulation of CGRP and Elk-1 reporter genes. *A*, Cells were cotransfected with the 1250 bp CGRP reporter and CMV–MEK1 expression vector and then treated overnight with 1 μM ionomycin (*Iono*). *B*, Repression of Elk-1 reporter gene activity by treatment overnight with 10 μM sumatriptan and 1 μM ionomycin. The luciferase activities are the normalized means with SEs from at least three independent experiments. *C*, As a control, the CMV–β-galactosidase reporter activities were not affected by the overnight ionomycin treatment. Activities from the ionomycin-treated cultures were normalized to the control dishes that were set at 100% for each experiment ($n = 6$). The mean and SE is shown. *Con*, Controls.

mately one-half of the neurons in our cultures (data not shown). The heterogeneous expression of 5-HT_1 receptors is in agreement with the results of Hou et al. (2001) from studies on human trigeminal ganglia. The sumatriptan-mediated elevation in intracellular calcium could be maintained for as long as 2 hr, the longest time point examined (data not shown). Although the duration of the calcium increase was similar to that in our previous reports, the amplitude of the increase was less. The difference may be attributable to the effect of transfection on the cells and/or the shorter culture time (2 vs 4 d). For comparison, treatment of the cultures with the calcium ionophore ionomycin (1 μM) caused an elevation of calcium in all neuronal cells that had a faster onset, but was otherwise similar in amplitude and duration to that observed using sumatriptan (Fig. 8*C*).

Elevated calcium levels are sufficient to repress the CGRP promoter

To test directly whether the sustained calcium elevation is responsible for sumatriptan repression of the CGRP promoter, trigeminal cultures were treated with ionomycin to mimic the calcium increase caused by sumatriptan. Overnight, ionomycin treatment repressed MEK1-stimulated CGRP promoter activity to ~35% of the activity of MEK1 alone (Fig. 9*A*). The degree of repression observed with ionomycin was similar to that seen with sumatriptan (Fig. 5*A*). It was essential that the treatment was done in the presence of serum to avoid cell toxicity that was otherwise seen within minutes of ionomycin application.

As with sumatriptan, we tested whether the ionomycin treatment repressed the synthetic MAPK-responsive reporter gene. Treatment with ionomycin markedly decreased MEK1 stimulation of the Elk reporter gene, similar to that seen with sumatriptan treatment (Fig. 9*B*). As a control, ionomycin treatment did not affect the cotransfected CMV–β-galactosidase reporter gene (Fig. 9*C*). These data demonstrate that a prolonged increase in intracellular calcium is sufficient to repress MAPK-responsive genes.

Discussion

In this study, we have shown that CGRP gene expression in trigeminal ganglia neurons is upregulated by proinflammatory mediators and repressed by an antimigraine drug. Migraine is a painful neurological disorder that afflicts 16% of the general population (Stewart et al., 1994; Ferrari, 1998). Although the specific cause remains unknown, current theories suggest that the initiation of migraine involves a primary CNS dysfunction with subsequent activation of the trigeminovascular system (Burnstein, 2001; Buzzi, 2001; Bolay et al., 2002). Activation of trigeminal neurons is known to elevate CGRP levels during migraine (Edvinsson and Goadsby, 1994; Williamson and Hargreaves, 2001). The ability of acute antimigraine drugs such as sumatriptan to return serum CGRP levels to normal coincident with alleviation of pain is suggestive that CGRP is involved in the underlying pathology of migraine (Goadsby and Edvinsson, 1991). More recently, evidence in support of a causative role for CGRP in migraine was demonstrated by an *in vivo* study in which the administration of CGRP was shown to cause headache and migraine in migraineurs (Lassen et al., 2001).

Because an untreated migraine can persist for up to 72 hr, it seems likely that increased CGRP synthesis would be required to maintain elevated levels. Our results indicate that proinflammatory agents, such as NGF, can stimulate CGRP promoter activity in trigeminal neurons. These observations are consistent with the proposal that CGRP release during migraine results in the production and/or release of agents that escalate and sustain the inflammatory response (Williamson and Hargreaves, 2001). It is interesting to note that both NGF and CGRP levels appear to be elevated in the CSF of patients with chronic daily headache (Sarchielli et al., 2001). Our findings are in overall agreement with previous studies showing that NGF stimulates CGRP expression (Lindsay and Harmar, 1989; Verge et al., 1995; Watson et al., 1995; Freeland et al., 2000; Patel et al., 2000; Shadiack et al., 2001; Supowit et al., 2001). We have extended those studies by showing that the CGRP HO enhancer is an NGF-responsive target in trigeminal neurons. Previous results from dorsal root ganglia and PC12 cells have demonstrated a requirement for the downstream cAMP/ras-responsive element for activation by NGF (Watson et al., 1995; Freeland et al., 2000), and our findings do not rule out a role for this element in NGF responsiveness. Instead, we suggest that it may act with the HO element and be important for proinflammatory signals mediated by other MAPKs, because in our system the cAMP/ras element could be activated by a constitutively active MEK kinase 1 (amino acids 380–672) (data not shown). In this regard, there is a recent report that both the bHLH protein upstream stimulatory factor-1 (USF-1)/USF-2 and cAMP-responsive element binding protein contribute to KCl-induced activation of the BDNF promoter (Tabuchi et al., 2002). Together, these results suggest that at least two elements are activated by NGF and MAPK pathways, which provide a positive feedback mechanism for maintaining a prolonged elevation of CGRP during migraine (Fig. 10).

Within the HO enhancer, the target of the ERK MAPK pathway appears to be a bHLH transcription factor. The bHLH factors are well documented as key regulators of neuronal phenotype (Ma et al., 1998; Cau et al., 2002). Within the past year, bHLH factors have also been shown to be directly regulated by MAPK pathways (Bain et al., 2001; Galibert et al., 2001). For example, USF-1 is phosphorylated and activated by proinflammatory agents via MAPK p38 (Galibert et al., 2001). A heterodimer of USF-1/USF-2 can bind the HO HLH site *in vitro* (Lanigan and Russo, 1997). Whether USF-1 binds the HO site in trigeminal neurons or if the p38 pathway can stimulate the enhancer remains to be tested. The bHLH requirement for basal and MAPK-stimulated enhancer activity is in agreement with our results from the neuronal-like CA77 thyroid C-cell line (Durham et al., 1997). However, one difference appears to be the requirement for an adjacent octamer-binding protein. A mutation in the octamer-binding site (18 bp+A) that virtually eliminated activity in CA77 cells (Durham et al., 1997), had no deleterious effect on enhancer activity in the trigeminal cultures. Thus although the HLH site is clearly important, the octamer motif is either recognized by a different protein or is not required in trigeminal neurons.

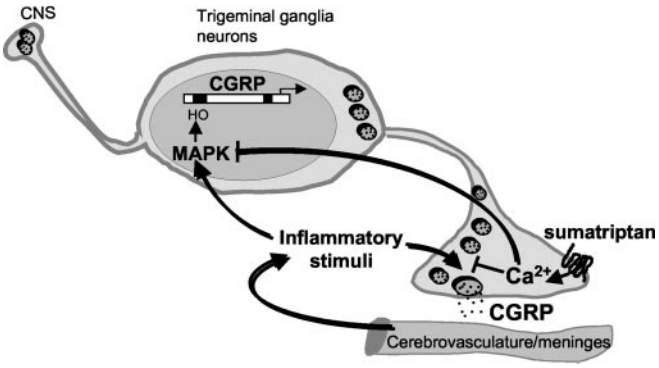

Figure 10. Model of CGRP regulation in trigeminal ganglia neurons under conditions simulating migraine pathology and therapy. Activation of trigeminal nerves leads to the initial release of CGRP and other neuropeptides that release proinflammatory mediators. These stimuli further augment CGRP synthesis and secretion via MAPKs. The antimigraine drug sumatriptan can cause a prolonged elevation of intracellular calcium that blocks the MAPK activation of CGRP synthesis and release.

A key finding of this study is that sumatriptan can repress the activation of the CGRP promoter by increasing intracellular calcium levels (Fig. 10). We reported previously that activation of 5-HT_1 receptors by sumatriptan causes a markedly prolonged increase in intracellular calcium in trigeminal neurons (Durham et al., 1997; Durham and Russo, 1999). We have now demonstrated a causal role for that prolonged calcium elevation. Using a calcium ionophore, we were able to mimic the amplitude and duration of the calcium increase observed after sumatriptan treatment. The prolonged calcium elevation was sufficient to repress MAPK-stimulated CGRP promoter activity. Interestingly, there is evidence that bHLH binding can be inhibited by elevated calcium in response to receptor activation or ionomycin (Corneliussen et al., 1994; Onions et al., 2000). Thus, calcium-induced repression of CGRP gene expression may involve changes in the phosphorylation state of bHLH transcription factors that act at the HO enhancer.

The regulation of CGRP has important implications for migraine pathology and therapy. In addition to the transcriptional regulation in this study, we have shown previously that depolarization and proinflammatory agents cause a marked increase in CGRP release that can be repressed by sumatriptan (Durham and Russo, 1999). Thus, it appears that trigeminal neurons are able to regulate coordinately both the synthesis and secretion of CGRP in response to stimulatory and inhibitory signals. The release of CGRP was repressed within 1 hr after sumatriptan treatment, which is in agreement with the reported time of sumatriptan action in patients. The concentration of sumatriptan that is required to repress either CGRP secretion or transcription is 5–50 times greater than the estimated sumatriptan levels in the plasma

of patients (0.2 μM) (Fowler et al., 1991). It is interesting that Eltorp et al. (2000) also found that high sumatriptan doses (10–50 μM) were required to repress CGRP release in a study using an *in situ* preparation of dura mater and trigeminal nerves. Possible explanations for these relatively high dose requirements may be that receptor levels or localization are different in culture and/or the stimulation paradigm is stronger *in vitro*. In either case, the inhibition of transcription is unlikely to affect CGRP levels in perivascular fibers in the clinical time frame of sumatriptan action. Instead, we speculate that a transcriptional mechanism could help maintain lower CGRP levels in the face of stimulatory signals. The finding that MAPKs stimulate the CGRP enhancer in primary trigeminal neurons may be particularly relevant to migraine pathology, because many proinflammatory agents implicated in migraines are known to activate MAPK pathways (Durham and Russo, 2002). We suggest that targeted and selective delivery of inhibitors of MAPK pathways to trigeminal neurons may provide a novel approach for dampening CGRP levels.

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