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Two Alphaherpesvirus Latency-Associated Gene Products Influence Calcitonin Gene-Related Peptide Levels in Rat Trigeminal Neurons.

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Abstract

Herpes simplex virus type-1 (HSV-1) initially infects mucoepithelial tissues of the eye and the orofacial region. Subsequently, the virus is retrogradely transported through the axons of the trigeminal sensory neurons. HSV-1 establishes a life-long latent infection in these neurons, during which the transcription of the viral genome is silent, except for the sequences encoding the latency associated transcript (LAT). To determine if HSV-1 latency might affect calcitonin gene-related peptide (CGRP) expression in trigeminal sensory neurons, we transfected primary neuronal cultures of trigeminal ganglia from rat embryos with plasmids expressing LAT. In the presence of Bone Morphogenetic Protein-7 (BMP7), CGRP was expressed in 49% of sensory neurons. However, this percentage was reduced to 19% in neurons transfected with LAT expressing plasmids. We also found that transfection of the IE63 gene of varicella-zoster virus (VZV) reduced the percentage of trigeminal neurons containing CGRP. However, the observed effect of IE63 in contrast to that of LAT, was completely reversed by treatment of cultures with MgCl₂, which indicates that the effect of IE63 was due to increased release of CGRP from trigeminal neurons. We provide here the first evidence that HSV-1 LAT decreases the level of CGRP in trigeminal neurons. These effects may be important for reducing the neuroinflammatory response, thus protecting host neuronal cells during HSV-1 latency in trigeminal neurons. In contrast, increased release of CGRP in the presence of IE63 protein may contribute to the neuralgias associated with VZV infection.

Introduction

Herpes simplex virus type-1 (HSV-1) infection is widespread, affecting two thirds of the human population (Schillinger et al., 2004). HSV-1 is a member of the alphaherpesvirus family that shares the ability to establish life-long latency in neurons (Cook et al., 1974). HSV-1 infects the orofacial region and produces a self-limiting illness that usually resolves in one or two weeks following primary infection. However, HSV-1 establishes latency in the sensory neurons that supply the infected areas. The primary host cells for HSV-1 latency are the sensory neurons of the trigeminal ganglia (Bastian et al., 1972). During HSV-1 latency in neurons, the only significantly expressed viral RNA is the latency associated transcript (LAT) (Stevens, 1978). The most stable transcript of LAT is a 2.0 kb intron that is expressed exclusively in the nuclei

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of latently infected neurons (Wagner et al., 1988). The role of LAT in HSV-1 latency and reactivation, and its effects on the host cells have been extensively studied. Previous work has shown that LAT can promote survival of neurons and plays a role in viral reactivation from latency (Perng et al., 2000; Thompson and Sawtell, 1997). Varicella-zoster virus (VZV) is another member of the alphaherpesvirus family that establishes latency in dorsal root ganglia (DRG) and to a lesser extent trigeminal neurons (LaGuardia et al., 1999). There is evidence that the expression of the IE63 protein of VZV also promotes neuronal survival (Hood et al., 2006). However, there is currently no evidence that either LAT or IE63 affect other functional properties of the latently infected neurons. In this study, we investigated the effects of LAT and IE63 on the expression of a key neuropeptide expressed in trigeminal neurons, the calcitonin gene-related peptide (CGRP).

CGRP is a 37 amino-acid peptide that is involved in a large number of biological processes including vasodilatation, edema, immunomodulation, and sensitization of sensory nerve terminals (Brain and Cox, 2006). Previous studies have shown that CGRP can modulate the immune response through inhibition of antigen presentation cell activity in Langerhans cells, and this mechanism has been found to be involved in ultraviolet radiation-induced immunosuppression (Hosoi et al., 1993; Gillardon et al., 1995). CGRP is an active player in neurogenic inflammation, and it is believed that CGRP is involved in the pathophysiology of migraine and other facial pains (Richardson and Vasko, 2002). Trigeminal ganglia are the primary source of the CGRP released in the cerebral vasculature (Durham et al., 2004).

In this study, we examined the hypothesis that the expression of HSV-1 LAT and VZV IE63 may alter CGRP expression in cultured trigeminal neurons. Our data show for the first time that HSV-1 LAT decreases CGRP expression in trigeminal neurons. This finding contributes to our understanding of how HSV-1 latency alters the functional properties of the host neurons. Our findings also show that the IE63 protein of VZV increases the release of CGRP from trigeminal neurons. This finding may explain some of the clinical features associated with VZV reactivation such as post-herpetic neuralgia, and why these clinical features are different from those associated with HSV-1 latency.

Materials and Methods

Materials

Human recombinant bone morphogenetic protein-7 (BMP7) was generously provided by Curis (Cambridge, MA). Magnesium Chloride (MgCl_2) was purchased from Sigma (Saint Louis, MO).

Plasmids

Plasmids containing a LAT 2.2 kb insert (119311-121571 bp of HSV-1 strain 17+) and expressing the full length 2.0 kb LAT intron as a lariat and truncations thereof were generous gifts from Dr. Lawrence Feldman (UCLA, LA, CA). The plasmid expressing the VZVIE63 protein has been previously described (Zuranski et al., 2005). The plasmid encoding enhanced green fluorescence protein (EGFP-C1) was purchased from Clontech (Clontech, Palo Alto, CA).

Cell Cultures

Trigeminal ganglia were dissected from embryos (E19) of Holtzman rats (Harlan Sprague Dawley, Rockford, IL). The cells were dissociated after treatment with trypsin (2.5 mg/ml) and collagenase (1mg/ml) for 35 minutes. Cells were plated on poly-D-lysine-coated (200 $\mu\text{g}/\text{ml}$) coverslips and maintained on serum-free medium containing β -nerve growth factor (NGF: 100 ng/ml). One day later, the cultures were treated with medium containing 1 μM of the

antimitotic drug cytosine- β -D-arabinofuranoside (ara-c) (Sigma Chemical Corp, St Louis, MO) for 2 days to eliminate non-neuronal cells, and then the culture were allowed to recover for two days before transfection. Dorsal root ganglia were dissected from embryos (E14) of Holtzman rats (Harlan Sprague Dawley, Rockford, IL), and then were dissociated and plated as described above. The cultures were maintained on serum-free media supplemented with NGF (100 ng/ml). One day later, the cultures were treated with medium containing ara-c (1 μ M) for two days, and then the cultures allowed to recover for two days before transfection.

Transfection

Cells were cotransfected with a plasmid encoding the enhanced green fluorescence protein (pEGFP-C1, Clontech, Palo Alto, CA) and the expression vector LTF900 containing the LAT 2.2 kb insert, or one of a set of four expression vectors (LTF901, LTF902, LTF903 and LTF904) containing different deletion mutants of LAT. In control experiments, cells were cotransfected with pEGFP-C1 and the pCDNA3.1 vector (Invitrogen, CA). Transfection was performed using Lipofectamine 2000 (Invitrogen, CA). Briefly, cells were treated with 150 μ l of DMEM containing 1.6 μ g of plasmid DNA and 6 μ g of lipofectamine for six hours. Subsequently, cells were washed and allowed to recover for 2 days.

In situ hybridization

Antisense and sense riboprobes for LAT were prepared as follows: a fragment of LAT (119615–120426 bp of HSV-1 strain 17+) was amplified by PCR, and then it was subcloned into the PCR II-TOPO vector (Invitrogen, CA). Digoxigenin-labeled riboprobes were generated by in vitro transcription according to the manufacturer's instructions (Roche, Indianapolis, ID). Cultures were fixed with 4% formaldehyde for 1 hour and washed twice with 2X SSC. Cultures were treated with proteinase K (5 μ g/ml) for 10 minutes then washed twice with 2X SSC. Cells were incubated at 50° C for 3 hours with a pre-hybridization solution which contained 50 % formamide, 4X SSC, 1 mM EDTA, 1X Denhardt's solution, 10% dextran sulfate, yeast tRNA (250 μ g/ml) and denatured salmon sperm DNA (250 μ g/ml). Cells were hybridized by incubation at 50° C for 16 hours with 2 μ g/ml of probe in hybridization solution. The LAT signal was detected using anti-digoxigenin Fab fragment conjugated to rhodamine (Roche, Indianapolis, ID) and visualized by fluorescence microscopy.

Immunocytochemistry

Expression of CGRP in cultured neurons was detected by immunostaining. Cells were fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.1% Triton X-100 for 4 minutes. This was followed by incubation with 5% bovine serum albumin for 2 hours at room temperature. Cells were reacted with rabbit polyclonal antibodies to CGRP (Sigma, Saint Louis, MO) overnight, and then with anti-rabbit rhodamine conjugated secondary antibodies (Boehringer Mannheim, West Grove, PA), and visualized by fluorescence microscopy.

Assessment of cell survival

Neuronal death was induced by withdrawal of NGF from the medium through washing the cultures twice with DMEM containing bovine serum albumin (500 μ g/ml) and then maintaining the cells on DMEM for 14–16 hours. Dead cells were detected through the recognition of nuclear morphology by incubating the cells with Hoechst stain (Bisbenzimidazole, Sigma, St. Louis, MO) for 20 minutes.

Statistical analysis

All of the experiments reported were repeated at least 3 times, and 40 or more neurons were counted per group in each experiment. Data in the text are presented as mean $\pm$ SEM. Statistical

significance was determined by a one-way ANOVA test followed by Tukey's *post hoc* test, or a two-tailed unpaired t-test.

Results

Bone morphogenetic protein-7 induces CGRP expression in trigeminal neurons

Cultured trigeminal sensory neurons obtained from rat embryos expressed CGRP in approximately 30 % of neurons when maintained on a serum-free medium (Figure 1). TGF- β family members were found to induce CGRP expression in sensory neurons in previous studies (Hall et al., 2001). We found that treatment of cultured trigeminal neurons with Bone morphogenetic protein-7 (BMP7), a member of the TGF- β family involved in the development of both the central and the peripheral nervous system, results in a more than 50% increase in the percentage of neurons expressing CGRP (Figure 1E). Further, the effect of BMP7 on CGRP expression in trigeminal neurons was found to be dose-dependent (Figure 1F). This novel finding indicates that BMP7 has a role in the regulation of CGRP expression in trigeminal neurons.

HSV-1 LAT decreases CGRP-immunoreactivity in trigeminal neurons

HSV-1 LAT is abundantly expressed in the nuclei of the latently infected trigeminal neurons (Wagner et al., 1988). To study the effect of LAT on CGRP expression in cultured primary trigeminal neurons, cells were transfected with plasmids expressing the stable 2.0 kb LAT. We treated the cultures with BMP7 to increase the expression of CGRP following LAT expression in trigeminal neurons. Thus, we were able to study the effects of LAT on both basal and induced CGRP expression. We detected LAT exclusively in the nuclei of transfected neurons (Figure 2), which is similar to findings concerning LAT expression in trigeminal neurons latently infected with HSV-1 in vivo (Wagner et al., 1988). To detect individual neurons transfected with LAT, cultured trigeminal neurons were cotransfected with plasmids containing LAT and EGFP, which was used as a marker for identification of transfected neurons. In our model, 88% of neurons expressing EGFP also expressed LAT. Thus, we used EGFP as a surrogate marker for LAT expression in cultured trigeminal neurons. Cultured trigeminal neurons were cotransfected with plasmids expressing EGFP and plasmids expressing LAT, or an empty vector containing the pCMV IE promoter. For cells treated with BMP7, the protein was present in the media for 6 days prior to immunostaining for CGRP. Expression of LAT in trigeminal neurons did not change the percentage of neurons expressing CGRP under basal conditions, but prevented the BMP7-induced increase in neurons expressing CGRP (Figure 2). There was no significant difference in the percentage of neurons expressing CGRP between untransfected neurons and neurons transfected with plasmids containing an empty vector (Figure 2), indicating that the transfection process itself had no effect on CGRP expression.

LAT increases the survival of trigeminal neurons

To exclude the possibility that LAT expression decreased the viability of trigeminal neurons and this in turn resulted in inhibition of CGRP expression, the effect of LAT on the survival of trigeminal neurons after deprivation of nerve growth factor (NGF) was examined. In agreement with previous reports that LAT increases neuronal survival (Perng et al., 2000), we found that expression of LAT increased the percentage of surviving trigeminal neurons after induction of neuronal death through NGF deprivation (Figure 3). Thus the inhibition of CGRP expression is due to a mechanism that does not involve decreased viability of trigeminal neurons.

LAT deletion mutants do not decrease CGRP-immunoreactivity in trigeminal neurons

To determine the region(s) of the 2.0 kb LAT that is/are responsible for the inhibition of CGRP expression, plasmids expressing 5' deletion mutants of the LAT were utilized (Figure 4A). None of the deletion mutants decreased CGRP expression in trigeminal neurons (Figure 4B). This finding indicates that the entire 2.0 kb LAT intron is required for the inhibition of CGRP expression.

IE63 of VZV decreases CGRP immunoreactivity in trigeminal neurons

VZV is another alpha herpes virus that latently infects neurons. Although the primary reservoir for VZV latency is in DRG neurons, VZV latency can also occur in trigeminal neurons (Cohrs et al., 2004). During VZV latency, a number of its lytic phase genes have been reported to be transcriptionally active. The most frequently detected of these genes is the product of the viral open reading frame 63, commonly designated as IE63 (Debrus et al., 1995). We wanted to know whether inhibition of CGRP expression is an effect that is shared by other viral gene products associated with alphaherpesvirus latency, in addition to the LAT of HSV-1. Trigeminal neurons were transfected with a plasmid expressing IE63, and cultures were treated with BMP7 before cells were immunostained for CGRP. Expression of IE63 in trigeminal neurons reduced the percentage of neurons expressing CGRP by approximately 50% (Figure 5) in BMP7-treated cultures, when compared with untransfected neurons or neurons transfected with an empty vector.

Neither LAT nor IE63 decrease CGRP-immunoreactivity in DRG neurons

HSV-1 usually infects the orofacial region where the virus becomes latent in the trigeminal neurons. HSV-1 can also infect the genital region although to a much lesser extent than the orofacial region (Jones, 2003). After genital infection, HSV-1 latency occurs in DRG neurons (Quinn et al., 2000). We wished to determine if LAT decreases CGRP immunoreactivity in DRG neurons as well as in trigeminal neurons. As shown in Figure 6, we found that LAT expression in DRG neurons did not significantly decrease CGRP immunoreactivity in comparison with neurons transfected with an empty vector. Similarly, we found that IE63 of VZV did not decrease CGRP immunoreactivity in DRG neurons. These findings indicate that the effects of LAT and IE63 on CGRP expression are specific to trigeminal neurons and do not extend to DRG neurons.

MgCl₂ reverses the effect of IE63 but not the effect of LAT on CGRP immunoreactivity in trigeminal neurons

We next compared the effects of LAT to those of IE63 on CGRP expression in trigeminal neurons. We found that both LAT and IE63 similarly decreased the percentage of neurons containing CGRP (Figure 7). The observed decrease in CGRP signal in trigeminal neurons could be due to either a decrease in CGRP synthesis or an increase in CGRP release. The release of neuropeptides is dependent on external calcium entering the neuronal cells through non-selective cation channels and voltage-gated calcium channels (Szallasi and Blumberg, 1999). To examine the possibility that the decrease in CGRP signal was due to an increase in CGRP release from trigeminal neurons in the presence of either LAT or IE63, we treated cultures with MgCl₂. MgCl₂ blocks calcium entry into cells and prevents the increase in intracellular calcium levels (Zhang et al., 1996), and ultimately inhibits CGRP release. The effect of IE63 was completely reversed after treatment of cultures with MgCl₂, while that of LAT was not affected (Figure 7). This finding indicates that the IE63-induced reduction of CGRP immunoreactivity in trigeminal neurons was due to increased release of CGRP from neurons but this was not the case for LAT reduction of CGRP immunoreactivity.

Discussion

The prevalence of HSV-1 infection increases with age, and approximately two thirds of the total U.S. human population have serological evidence of HSV-1 infection (Schillinger et al., 2004). HSV-1 causes the majority of the orofacial herpes infections and 25% of the genital herpes infections (Jones, 2003). As a result, the primary host cells for HSV-1 latency are the sensory neurons of the trigeminal ganglia, although HSV-1 latency can occur, to a lesser extent, in the neurons of the dorsal root ganglia (Quinn et al., 2000). Previous studies have shown that HSV-1 infects both CGRP-reactive and CGRP-negative trigeminal neurons following corneal inoculation of mice with HSV-1 (LaVail et al., 1991; Margolis et al., 1992; LaVail et al., 1993).

VZV- another alphaherpesvirus - causes varicella during the primary infection, and becomes latent primarily in the neurons of the dorsal root ganglia, and to a lesser extent in the trigeminal ganglia. VZV can reactivate producing herpes zoster. We hypothesized that HSV-1 and VZV latency may alter numerous properties of the host neurons. There is evidence from previous studies that LAT promotes neuronal survival in a variety of neuronal cell types (Perng et al., 2000; Thompson and Sawtell, 1997). A recent study has shown that the VZV IE63 protein decreases apoptosis in rat DRG neurons (Hood et al., 2006). Further, recent data from our laboratories indicate that the presence of LAT has effects on axonal regeneration and dendritic growth, whereas VZV IE63 does not (unpublished data). In this work, we examined the question whether HSV-1 LAT and the VZV IE63 protein alter the functional properties of trigeminal and DRG neurons, specifically the expression of neuropeptides.

CGRP is expressed and stored in the sensory neurons of the trigeminal ganglia and the dorsal root ganglia (Hall et al., 2001). Sensory nerve fibers originating from trigeminal ganglia and the dorsal root ganglia not only carry sensation from innervated tissues, but also release neuropeptides from their nerve terminals (Van Rossum et al., 1997). The release of CGRP from the sensory fiber terminals of trigeminal neurons produces vasodilatation in the cerebral blood vessels, and this in turn contributes to the pathogenesis of migraine and headaches (Limmroth et al., 2001; Goadsby et al., 1991). CGRP also stimulates extravasation from blood vessels, activates immune cells, and sensitizes sensory nerve fibers (Maggioni et al., 1995). These effects suggest that CGRP has a role in the modulation of immune response and the pathogenesis of neuroinflammatory response and pain.

CGRP is expressed in approximately half of the trigeminal neurons in vivo (Quartu et al., 1992). In our primary cultures of trigeminal neurons, CGRP was expressed in 20 to 30 % of neurons, about half of the percentage observed in vivo. This may be due to the lack of factors that are required to stimulate CGRP expression. We took advantage of previous findings that TGF- β family members induce CGRP expression in sensory neurons (Hall et al., 2001). Our findings that treatment of trigeminal neuronal cultures with BMP7, a member of that family, induced CGRP expression to a level comparable to that observed in vivo are in agreement with the previous studies that indicate a role for BMP7 in the development and differentiation of trigeminal neurons (Zhang et al., 1998). We used this information and added BMP7 to induce CGRP expression in trigeminal neurons after transfection with LAT expressing plasmids. Inhibition of CGRP expression would result in a reduction in the CGRP available for release by neurons latently infected with HSV-1, consequently reducing inflammation and the immune response in the vicinity of the neurons. These effects may be important for protecting the latently infected neurons, so the virus can maintain its latency for long periods of time.

The failure of LAT to inhibit CGRP in DRG neurons was unexpected and interesting. This may explain the reason why the HSV-1 primary location for latency is the trigeminal ganglia. HSV-1 LAT appears able to change the profile of CGRP expression in trigeminal neurons, and

this will reduce the inflammation in the area of the latently infected neurons, resulting in protection of the neurons from the inflammatory response, making them suitable for the long term latency of the virus. On the other hand, LAT is unable to reduce CGRP expression in DRG neurons, consequently the inflammatory response is not reduced and the DRG neurons would be less suitable hosts for HSV-1 latency. Finally, LAT did not decrease basal levels of CGRP before treatment with BMP7, but inhibited the induction of CGRP by BMP7, and this suggests that LAT may block the effects of BMP7 on trigeminal neurons.

VZV is another member of the alphaherpes virus subfamily that latently infects sensory neurons. Although there are a number of genes that are reported to be transcriptionally active during the latency of VZV, IE63 is the most frequently identified viral protein expressed in human DRG autopsy material (Mahalingam et al., 1996). Our findings show that trigeminal neurons expressing IE63 have a lower percentage of CGRP-expressing neurons, and this effect was reversed after blockade of the release of neuropeptides by treatment of cultures with $MgCl_2$. This indicates that IE63 increases the release of CGRP from trigeminal neurons. The increased release of CGRP can lead to vasodilatation and sensitization of sensory nerve fibers and increased pain sensation. This may explain the observation that post-herpetic neuralgia is much more common and severe in VZV reactivation than in HSV-1 reactivation. Our findings may provide a mechanism that explains the observation that chronic allodynia developed following VZV infection but not HSV-1 infection in rats (Dalziel et al., 2004). Since IE63 is also expressed during the immediate early phase of productive infection (Debrus et al., 1995), the increase of CGRP release observed here may occur during reactivation.

In Summary, we provide evidence for the first time that the HSV-1 LAT alters CGRP expression in trigeminal neurons but not in DRG neurons. Our findings may help to explain different mechanisms involved in the latency of HSV-1, and this may lead to a potential therapeutic intervention of HSV-1 latency. We also shed light on a possible mechanism for the neuralgia associated with VZV infection.

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E

F

Figure 1. BMP7 induced CGRP in trigeminal neurons

Cultured trigeminal neurons were treated with ara-C (1 μ M) for 48 hours to reduce the number of non-neuronal cells. Cultures were then treated with culture medium with or without BMP7 (30 ng/ml) for 6 days. Cells were fixed and then immunostained for CGRP. A–D, phase contrast (A, C) and fluorescence (B, D) micrographs of trigeminal neurons treated with BMP7 (C, D) or with culture medium only (A, B). E, treatment of trigeminal neurons with BMP7 increases the percentage of CGRP-immunoreactive (CGRP-IR) neurons by more than 50 %. F, dose-response curve of BMP7 effect on CGRP-immunoreactivity in trigeminal neurons (* p < 0.05).

Figure 2. HSV-1 LAT decreased CGRP-immunoreactivity in trigeminal neurons

Cultured trigeminal neurons were co-transfected with plasmids containing EGFP and LAT or the pCDNA3.1 vector. Two days later, cultures were treated with medium with or without BMP7 (30 ng/ml) for 6 days, then cells were fixed and immunostained for CGRP. Phase contrast (A) and Fluorescence (B) micrographs of two trigeminal neurons, one of them was transfected and LAT was detected by in situ hybridization in the nucleus of that neuron. C–F, trigeminal neurons (indicated by white arrows) transfected with EGFP and pCDNA3.1 containing plasmids (C) were CGRP-IR (D) and neurons transfected with EGFP and LAT containing plasmids (E) were negative for CGRP. G, LAT decreased the percentage of CGRP-IR neurons (* $p < 0.05$).

Figure 3. HSV-1 LAT increased survival of cultured trigeminal neurons

Cultured trigeminal neurons were co-transfected with plasmids containing EGFP and LAT or the pCDNA3.1 vector. Two days later, cultures were deprived of NGF for 16 hours then fixed and stained with Hoechst stain. A–D, micrographs show a trigeminal neuron co-transfected with EGFP and pCDNA3.1 vector (A) and its nucleus was stained by Hoechst stain (B), while another trigeminal neuron was co-transfected with EGFP and LAT (C) and its nucleus did not stain with Hoechst stain (D). LAT transfected neurons had a higher percentage of live neurons than pCDNA3.1 transfected neurons (* $p < 0.05$).

Figure 4. LAT deletion mutants did not decrease CGRP-immunoreactivity in trigeminal neurons Cultured trigeminal neurons were co-transfected with plasmids containing EGFP and LAT or pCDNA3.1 or one of four deletion mutants of LAT (A). Two days later, cells were treated with medium with or without BMP7 (30 ng/ml) for 6 days, then fixed and immunostained for CGRP. B, LAT deletion mutants did not decrease the percentage of CGRP-IR neurons (* $p < 0.05$).

Figure 5. VZV IE63 decreased the percentage of CGRP immunoreactivity in trigeminal neurons
Cultured trigeminal neurons were co-transfected with plasmids containing EGFP and IE63 or the pCDNA3.1 vector. Two days later, cells were treated with medium containing BMP7 (30 ng/ml) for 6 days and then fixed and immunostained for CGRP. IE63 decreased the percentage of CGRP-IR neurons by approximately 50% (* $p < 0.05$).

Figure 6. Neither LAT nor IE63 decreased CGRP immunoreactivity in DRG neurons

Cultured DRG neurons were treated with ara-C (1 μ M) for 2 days to eliminate non-neuronal cells. Cells were allowed to recover for two days, and then they were co-transfected with plasmids containing EGFP and LAT or IE63 or the pCDNA3.1 vector. Two days later, cells were treated with medium containing BMP7 (30 ng/ml) for 6 days, and then cells were fixed and immunostained for CGRP. A–C, a cluster of three DRG neurons (A), and one of them was transfected with EGFP and LAT plasmids (B) and was also immunoreactive to CGRP (C). Neither LAT (D) nor IE63 (E) decreased the percentage of CGRP-IR DRG neurons.

Figure 7. Magnesium chloride (MgCl₂) reversed the effect of IE63 but not the effect of LAT on CGRP immunoreactivity in trigeminal neurons

Cultured trigeminal neurons were co-transfected with plasmids containing EGFP and LAT or IE63 or the pCDNA3.1 vector. Cells were treated with medium containing BMP7 (30 ng/ml) or BMP7 (30 ng/ml) and MgCl₂ (3 mM) for 6 days. Cells were fixed and immunostained for CGRP. The VZV IE63 reduction of the percentage of CGRP-IR neurons was reversed by MgCl₂. The LAT reduction of CGRP-IR neurons was not affected by MgCl₂ (**p* < 0.05).