Pergamon

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PII: S0306-4522(02)00261-0

Neuroscience Vol. 114, No. 1, pp. 55–67, 2002
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0306-4522/02 $22.00+0.00

SYNERGISTIC EFFECTS OF CORTICOSTERONE AND KAINIC ACID ON NEURITE OUTGROWTH IN AXOTOMIZED DORSAL ROOT GANGLION

S. Y. TSAI,[a,b1] P. Y. CHIU,[b1] C. P. YANG[a] and Y. H. LEE[a*]

[a]Department of Physiology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan

[b]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan

Abstract—Corticosterone is the main adrenal glucocorticoids induced by stress in rats. Therapeutic use of high concentration of synthetic glucocorticoids in clinical treatment of spinal cord injury suggests that pharmacological action of glucocorticoids might be beneficial for nerve repair. In this article we cultured axotomized rat dorsal root ganglion neurons to investigate the effects of corticosterone and a glutamate receptor agonist kainic acid on neurite outgrowth. Our results revealed a synergistic effect of corticosterone and kainic acid in promoting neurite outgrowth when applied as early as one and two days *in vitro*, but not effective at three and four days *in vitro*. In addition, applied corticosterone and kainic acid were neurotoxic at three and four days *in vitro* but not at one and two days *in vitro*. The minimal concentrations of corticosterone and kainic acid to be effective were 10 μM and 1 mM, respectively. The neurotrophic effect of corticosterone and kainic acid was attenuated by the receptor tyrosine kinase A (TrkA) inhibitor AG-879. Western blot analysis and immunocytochemical studies revealed an increase of expressions of both TrkA and growth-associated protein GAP-43 in dorsal root ganglion neurons with combined treatment of corticosterone and kainic acid. Immunocytochemistry showed that corticosterone+kainic acid increase nerve growth factor immunoreactivity in dorsal root ganglion neurites and enhance GAP-43 immunointensity in dorsal root ganglion neurons. These results suggest that the neurotrophic effect of glucocorticoids on axonal regeneration might require facilitation of excitatory stimulation at an early stage of nerve injury, and nerve growth factor may mediate a growth signaling to accomplish the effect. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: nerve growth factor, GAP-43, nerve growth factor receptor.

Peripheral nerves are frequently susceptible to mechanical ruptures such as compression and transection, and their functional repair requires axonal regeneration to allow target tissues to be reinnervated. Messengers required for axonal regeneration are believed to be neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3). NGF in particular is known to be an important retrograde signal in axotomized peripheral nerves to maintain the morphologic, biochemical, and electrophysiological properties of the injured neurons (Rich et al., 1987; Gold et al., 1991). NGF binds to its high-affinity receptors TrkA (tyrosine kinase A), which is expressed at the terminal of injured axons or growth cones of growing neurites, and the ligand–receptor complex is internalized and transported back to the soma to influence transcription of growth-related genes in the nucleus (Riccio et al., 1997).

Glucocorticoids, especially cortisol in humans and corticosterone (CORT) in rats and mice, have varied effects on survival and growth of central and peripheral neurons. Deprivation of glucocorticoids by adrenalectomy results in dramatic loss of granule cells in dentate gyrus (Sloviter et al., 1989; Gould, 1994; Cameron and Gould, 1994). It was also suggested that glucocorticoids might modulate neuronal plasticity of hippocampal neurons by regulating neurotrophin expression (Chao et al., 1998). Synthetic glucocorticoids, such as methylprednisolone, have been proven to be effective in improving neurological function after acute spinal cord injury (ASCI) in both humans and experimental animals (Bracken et al., 1990; Chen et al., 1996; Oudega et al., 1999; Harat and Kochanowski, 1999). Glucocorticoids bind to the intracellular glucocorticoid receptor, which in turn binds to the glucocorticoid responsive element to exert ligand-activated transcriptional regulation (for review see Slater et al., 1986). The level of plasma glucocorticoids and glucocorticoid receptor expression in the spinal cord

[1] These authors contributed equally to this work.

*Corresponding author. Tel.: +886-2-27361661 ext. 3188; fax: +886-2-23781073.

E-mail address: hsuan@tmu.edu.tw (Y. H. Lee).

Abbreviations: AG-879, 3,5-di-*tert*-butyl-4-hydroxybenzyl-isodene-cyanthioacetamide; AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate; ANOVA, analysis of variance; ASCI, acute spinal cord injury; AX-DRG, axotomized dorsal root ganglion; BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; CORT, corticosterone; DAB, diaminobenzidine; DIV, days *in vitro*; EDTA, ethylenediaminetetra-acetate; FBS, fetal bovine serum; GAP-43, growth-associated protein 43; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid); HHBSS, HEPES Hanks balanced saline solution; HRP, horseradish peroxidase; KA, kainic acid; MK-801, dizocilpine maleate; NGF, nerve growth factor; NT-3, neurotrophin 3; PBS, phosphate-buffered saline; TrkA, receptor tyrosine kinase A.

were found both elevated after spinal cord injury (Campagnolo et al., 1999; Yan et al., 1999). Functional proteins up-regulated by glucocorticoids after spinal cord injury include p75 NTR, a low-affinity neurotrophin receptor, glutamate binding sites, and the growth-related enzyme ornithine decarboxylase (Gonzalez et al., 1995, 1999). These observations imply that glucocorticoids may facilitate functional recovery of nerve injury by regulating both neurotrophin signaling and neurotransmission settings. However, information regarding the mechanism of this assumption is still limited. In addition, a major concern regarding the adverse effects of glucocorticoids to ASCI, such as higher incidence of severe sepsis and pneumonia in 48-h steroid treatment (Nesathurai, 1998), has made the glucocorticoids in ASCI a more debatable clinical application.

The kainate (or KA) receptor is the most abundant glutamate receptor subtype expressed in dorsal root ganglion (DRG) neurons (Bettler et al., 1990; Sato et al., 1993; Petralia et al., 1994.). It has been demonstrated that primary afferent c-fibers in the spinal cord are depolarized by KA, but not by quisqualate, a common agonist for both α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and KA receptors (reviewed in Henley, 1994). KA receptors are distributed in both the central branch and the peripheral branch of the axotomized (AX)-DRG axon, and have been suggested to modulate transmitter release and pain sensation, respectively (Agrawal and Evans, 1986; Ault and Hildebrand, 1993). KA is better known as a potent neurotoxin to central neurons, and CORT has been reported to enhance KA neurotoxicity in hippocampal neurons (Elliott and Sapolsky, 1993; Sapolsky, 1986). DRG neurons, where KA receptor activity often confronts stress-induced CORT, may hence be subjected to the synergistic effect of these two neuroactive substances.

In the present study, we demonstrate a synergistic effect of CORT and KA on neurite outgrowth of axotomized rat DRG neurons. The mechanism of this effect, with special focus on the expressions of NGF, TrkA, and a growth-associated protein GAP-43 was investigated. In parallel, the possible toxicity of CORT and KA in DRG neurons was also examined.

EXPERIMENTAL PROCEDURES

Primary culture of DRG neurons

Primary culture of the rat DRG neurons was prepared as described previously (Sotelo et al., 1991) with minor modification. In brief, adult male Sprague–Dawley rats (National Institute of Experimental Animal Research, Taipei, Taiwan) weighing around 250 g were killed following overdose anesthetization with 60 mg/kg body weight of sodium pentobarbital. Approximately 35–40 DRGs from cervical to sacral levels were dissected from the spinal column, chopped with blade, and dissociated with 0.15% collagenase in F12 medium by being shaken in a 37°C water bath for 40 min. The dissociated tissue was spun down at 1000 r.p.m. for 5 min, washed twice with HEPES Hanks Balanced Saline Solution (HHBSS), and further dissociated with 0.25% trypsin in HHBSS in 37°C water bath for 15 min. The digestion was stopped by adding 0.05% trypsin inhibitor, and the cell suspension was fractionated by 30% Percoll and centrifuged at 1500 r.p.m. for 5 min. The pellet, which was enriched with DRG neurons, was washed twice with F12 medium containing 10% fetal bovine serum (FBS) and plated onto poly-L-lysine-coated 35 mm culture dishes and cultured in F12/10% FBS medium in a humidified tissue culture incubator with 5% CO_2 and maintained at 37°C. The plating day was counted as the zero day *in vitro* (0 DIV). F12 medium, HHBSS, collagenase, trypsin inhibitor, 30% Percoll, and FBS were obtained from Life Technology (Gaithersburg, MD, USA). Animal experiments were carried out according to the NIH Guidelines for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Determination of neurite outgrowth

Cultured DRG neurons at 1 DIV were treated with 10 μM CORT (dissolved in F12/10% FBS) for 16–24 h, followed by 1 mM KA [dissolved in Earle's balanced saline solution (EBSS)] treatment. Vehicle solutions of CORT and KA were added to the control groups. Neurite length was measured every day from 1 DIV to 6 DIV by randomly selecting neurites and visualizing their length with a microruler. About 20 to 30 neurites in a single batch were measured, and at least five batches of experimental data were collected for statistical analysis. For the dose dependency study, CORT at 0.1, 1, and 10 μM, and KA at 1, 50, and 1000 μM were used. CORT and KA were obtained from Sigma (St. Louis, MO, USA).

Quantitation of cell death

The degree of neuronal death was evaluated by microscopic observation of trypan blue uptake in the dying neurons. Cultured DRG neurons at 6 DIV were stained with 0.4% trypan blue in phosphate-buffered saline (PBS) for 5 min, followed by three washes with PBS. Cells with or without blue stain were then counted separately from five selected fields under the microscope. The death rate of DRG neurons was presented as the percentage of blue-stained neurons out of total neurons in each microscopic field.

Immunocytochemistry

Cultured DRG neurons at 4 DIV were washed three times with a control salt solution (120 mM NaCl, 5.4 mM KCl, 8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.4, and 15 mM glucose), followed by incubation with fixative solution (4% formaldehyde, 1% H_2O_2, and 0.1% NaN_3 in 20 mM PBS, pH 7.5) for 30 min at room temperature. Fixed cells were rinsed with 20 mM PBS containing 0.2% Tween 20 (PBST), and incubated sequentially with blocking solution, primary antibody, biotinylated secondary antibody, and avidin–horseradish peroxidase (HRP) reagent as described in the instructions of Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The anti-calcitonin gene-related peptide antibody (anti-CGRP rabbit antiserum, Sigma), the anti-GluR2/3 polyclonal antibody, the anti-GluR5,6,7 monoclonal antibody, the anti-GAP-43 polyclonal antibody, the anti-NGF antibody (Chemicon, Temecula, CA, USA), and the anti-TrkA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were properly diluted for primary antibody incubation. Color development of the immunoreactive cells was performed using diaminobenzidine tetrahydrochloride (DAB) in a HRP substrate DAB kit (Vector Laboratories, Burlingame, CA, USA). Immunomicrograph was pictured using Olympus DP50 digital camera (Olympus, Tokyo, Japan). The relative optical density was analyzed by image analysis program (Beta 4.0.2 of Scion image, USA).

Western blot analysis

Cultured DRG neurons at 6 DIV with designated treatment were harvested with ice-cold homogenizing medium (50 mM Tris–HCl containing 1 mM EDTA, 1 mM sodium orthovana-

date, 10 μg/ml trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin, pH 7.4). Cells were sonicated and centrifuged at $100\,000 \times g$ for 30 min to obtain the crude membrane fraction in the pellet. To determine the expression of TrkA and growth-associated protein 43 (GAP-43), 20 μg of the pellet protein was separated onto 7% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to Hybond ECL nitrocellulose membrane (Amersham), and probed with the polyclonal anti-TrkA or polyclonal anti-GAP 43 antibodies overnight at 4°C. The immune complex was further probed with HRP-conjugated goat anti-rabbit IgG, visualized by HRP-reactive chemiluminescence reagents (Pierce, Rockford, IL, USA), and developed on autoradiographic film (Kodak BioMax film, Eastman Kodak Co., Rochester, NY, USA). The relative density of the protein band in the western blot was further analyzed with an electrophoresis image analysis system (Eastman Kodak Co., Rochester, NY, USA).

RESULTS

Expression of the AMPA receptor and the KA receptor in AX-DRG neurons

Cultured DRG neurons were first characterized for their identity as sensory neurons by the expression of

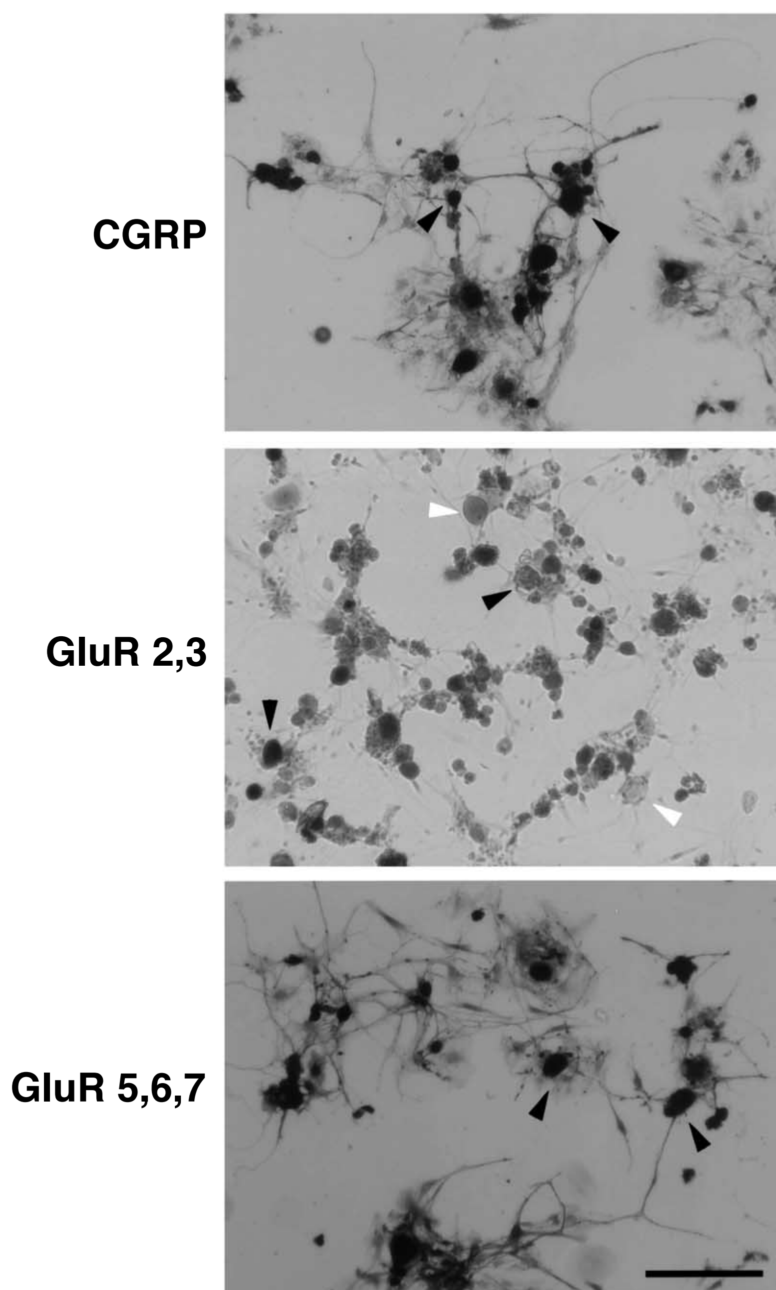

Fig. 1. Immunocytochemistry of CGRP, the AMPA receptor subunits GluR2/3, and the KA receptor subunit GluR5,6,7 in cultured DRG neurons. AX-DRG neurons at 4 DIV were immunostained with anti-CGRP, anti-GluR2,3 (for the AMPA receptor), and anti-GluR5,6,7 (for the KA receptor) antibodies, and visualized using HRP–DAB color development method. Positively stained neurons (black arrowhead) and negatively stained neurons (white arrowhead) are indicated. Scale bar = 200 μm.

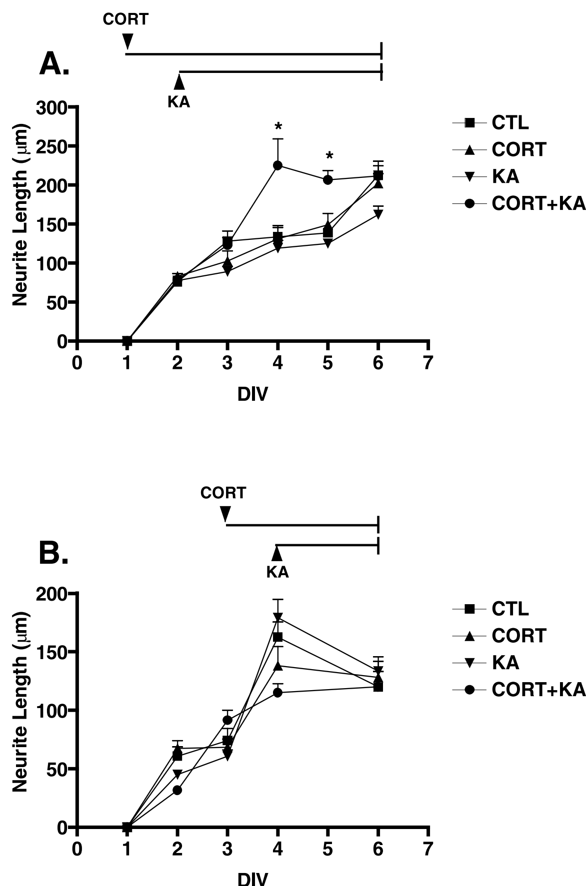

Fig. 2. Effect of CORT and KA on neurite extension in AX-DRG. Cultured DRG neurons were pretreated with CORT (10 μM) at 1 DIV (A) or 3 DIV (B), followed by application of KA (1 mM) at 2 DIV (A) or 4 DIV (B). Neurite length of DRG neurons was measured once a day from 1 DIV to 6 DIV under the microscope with a microruler. The data was expressed as the mean ± S.E.M. ($n = 100$–200). $^*P < 0.05$ as compared with the other three groups by one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparison posttest.

CGRP. Figure 1 shows that cultured cells with neuronal morphology were all CGRP-positive. These CGRP-positive neurons have various sizes ranging from 10 to 60 μm in soma diameter, coincide with the previously reported DRG sizes in the immunohistochemical studies (Zhou and Rush, 1996). Furthermore, immunocytochemistry shows that the KA receptor subunits GluR5/6/7 were expressed in 95% of DRG neurons, whereas the AMPA receptor subunits GluR2/3 were only expressed in 43% of DRG neurons. Furthermore, the expression of GluR5/6/7 appears abundant in the cytoplasmic compartment of both cell body and growing neurites, whereas the expression of GluR2/3 was more restricted to the surface of cell body (patched staining) with no signal appeared along neurites. This unique subcellular distribution of the KA receptor might result from its association to organelles or simply its cytosolic pool for rapid up-regulation of the KA receptor to cell surface (Martin et al., 1993; Trudeau and Castellucci, 1995) for axonal growth. In addition, the expression of GluR5/6/7 appeared much more abundant than the expression of GluR2/3, implying that response of cultured DRG neurons to their common agonist KA could be predominantly mediated by the KA receptor.

Effect of CORT and KA on neurite outgrowth in DRG neurons

To investigate the neurite outgrowth rate of AX-DRG neurons in culture, CORT (10 μM) and KA (1 mM) were sequentially applied at 1 and 2 DIV, respectively. The reason that CORT was applied prior to KA is based upon the hypothesis that CORT may prime the gene expression necessary for KA to stimulate neurite outgrowth. Figure 2A shows that the growing phase of DRG neurites was from 1 to 4 DIV; after 4 DIV most neurites stopped lengthening upon reaching their target cells. A maximal synergy of CORT and KA on neurite outgrowth was observed at 4 DIV, which was two days after the KA treatment. Either CORT or KA treatments had no influence on the basal level of neurite growth rate. If the application time of CORT and KA was postponed to 3 and 4 DIV, respectively, their synergistic effect did not appear (Fig. 2B). These results suggest that the neurotrophic effect of CORT and KA seemed restricted to the early stage of axonal injury.

Since concentrations of CORT and KA used above are relatively high as compared with their physiological concentration or concentration for receptor binding, we further examined this neurotrophic effect by applying lower concentration of CORT and KA with several combinations in culture. Figure 3A shows that when the CORT concentration was fixed at 10 μM, KA showed facilitation of neurite growth only at 1 mM, but not at 50 and 1 μM. However, when the KA concentration was fixed at 1 mM, CORT only at 10 μM but not 1 μM or 100 nM was effective in promoting neurite growth (Fig. 3B). As further illustrated by the phase contrast photomicrographs in Fig. 3C, these results suggest that high concentrations of CORT and KA are indeed the most effective condition for facilitating early axonal regeneration.

Neurotoxicity of CORT and KA in AX-DRG neurons

The high dosages of CORT and KA required for promoting neurite outgrowth of DRG neurons raised a concern toward their possible neurotoxicity. Therefore, we further analyzed the survival rate of DRG neurons subjected to CORT and KA treatment. Figure 4 shows that application of 10 μM CORT at 3 DIV and 1 mM KA at 4 DIV did increase the neuronal death rate, but no synergistic or additive effect was observed in the co-treatment condition. However, when CORT and KA were, respectively, applied at 1 and 2 DIV, the time effective for promoting neurite outgrowth, no detrimental effect was observed. These results suggest that CORT and KA, when applied within the time window effective for promoting axonal regeneration, were not toxic to DRG neurons. As neurite growth reached completion, CORT and KA may become neurotoxic.

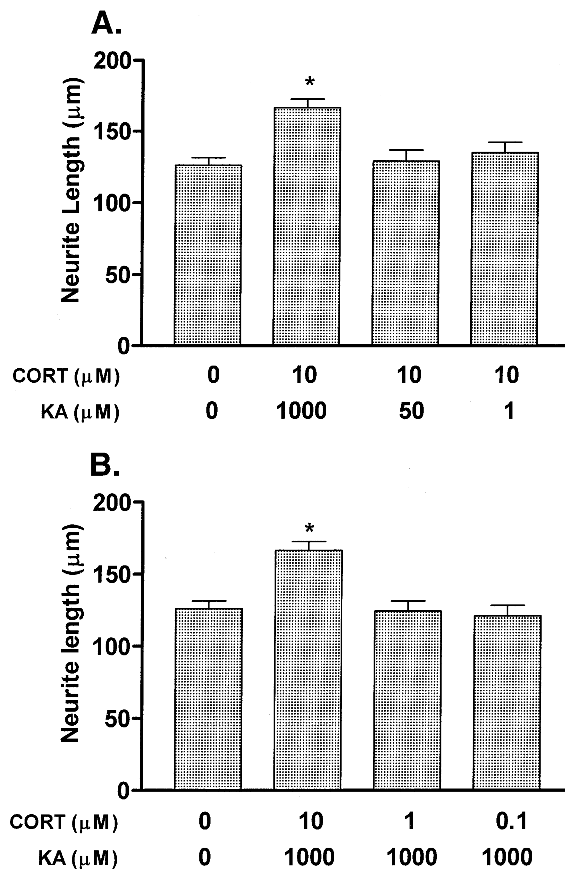

Fig. 3. Concentration dependency of CORT+KA-promoted neurite growth rate of cultured AX-DRG neurons. Cultured DRG neurons were pretreated with CORT at 1 DIV, followed by application of KA at 2 DIV. Neurite length of DRG neurons at 4 DIV was measured under the microscope with a microruler. The data was expressed as the means ± S.E.M. (n = 9–12). (A) The concentration of CORT was fixed at 10 μM, and concentrations of KA used were 1 μM, 50 μM, and 1 mM. (B) The concentration of KA was fixed at 1 mM, and concentrations of CORT used were 0.1 μM, 1 μM, and 10 μM. At least three batches of experiment were performed with similar results. The data represented mean ± S.E.M. *$P < 0.05$ as compared with the other three groups by one-way ANOVA with Newman–Keuls multiple comparison posttest. (C) Phase contrast photomicrographs; 1: control; 2: CORT(1 μM)+KA(1 mM); 3: CORT(0.1 μM)+KA(1 mM); 4: CORT(10 μM)+ KA(1 mM); 5: CORT(10 μM)+KA(50 μM); 6: CORT(10 μM)+KA(1 μM). Scale bar = 50 μm.

Blockade of NGF receptor activity attenuated CORT+KA-enhanced neurite outgrowth

To examine if the synergistic effect of CORT and KA on neurite outgrowth of AX-DRG is via enhancement of neurotrophic factor signaling, we used 3,5-di-*tert*-butyl-4-hydroxybenzyl-isodenecyanthioacetamide (AG-879) to block the autophosphorylation activity of the high-affinity NGF receptor TrkA (Levitzki and Gazit, 1995; Lee et al., 2000). As shown in Figs. 5 and 3 μM AG-879, with no toxicity and also no significant effect on the basal level of the neurite growth rate (Fig. 5A, C), significantly reduced the CORT and CORT+KA-enhanced neurite growth rates to near the basal level (Fig. 5B, D). Photomicrographs also showed that neurite length at 4 DIV was apparently shorter with less arborization in the AG-879-treated group (Fig. 5E). These results suggest that CORT+KA may activate NGF signaling to promote neurite outgrowth. In addition, we used immunocytochemistry to examine the source of endogenous NGF in the DRG neurons. Figure 7A shows that NGF immunoreactivity was strongly present in all DRG soma of both the control and the CORT+KA groups, whereas the NGF immunostaining on neurites was stronger in the CORT+KA group than staining in the control group.

Effects of CORT and KA on expression of TrkA and GAP-43 in DRG neurons

Growth of neurites often results from increased expression of growth-associated proteins, such as TrkA for retrograde signaling to the nucleus upon receiving NGF, and GAP-43 for membrane fusion to extend the growing neurites. In western blot analysis as shown in Fig. 6, both trkA and GAP-43 expression were lightly elevated by either CORT or KA. A profound increase of GAP-43 expression was observed in the CORT+KA group with up to four-fold over the control group. Whereas the CORT+KA increased TrkA expression was only two-fold over the control.

We further examine the cellular expression level of TrkA and GAP-43 in 4 DIV DRG neurons using immunocytochemical staining (Fig. 7). Both control and

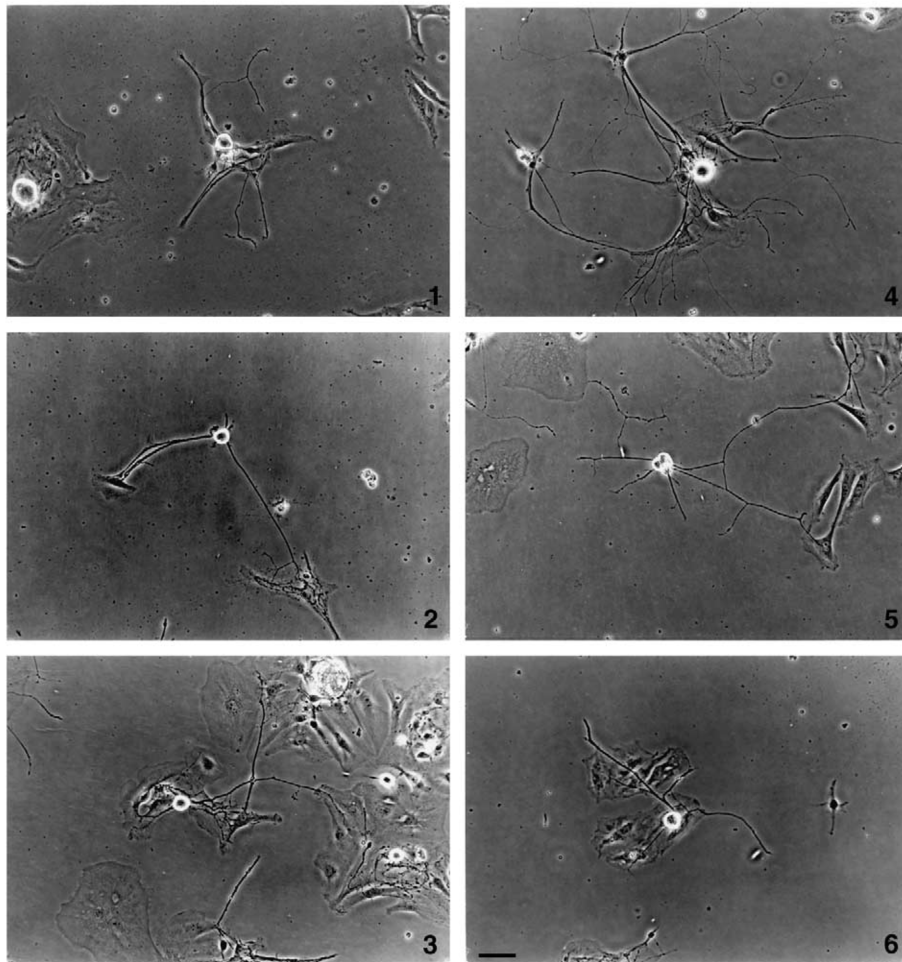

Fig. 3 (*Continued*).

CORT+KA groups expressed the similar immunoreactivity of TrkA. However, the GAP-43 immunoreactivity in the CORT+KA group neurons was apparently stronger than the control group (Fig. 7B). Figure 7C shows further quantitation of GAP-43 immunoreactivity in terms of staining density as well as percentage of positive-stained neurons, and both parameters are significantly higher in the CORT+KA group as compared with the control group. These results suggest that GAP-43, but not TrkA, was up regulated by CORT+KA synergy.

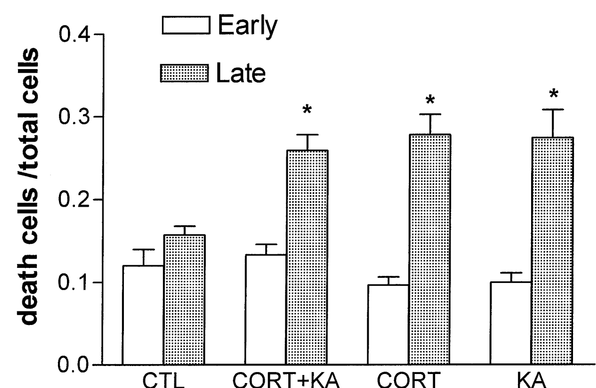

Fig. 4. Neurotoxicity of CORT and KA in DRG neurons. Cultured DRG neurons were subjected to CORT (10 μM) and KA(1 mM) treatment: CORT at 1 DIV and KA at 2 DIV was termed early treatment; and CORT at 3 DIV and KA at 4 DIV was termed late treatment. Cells were stained with 0.4% trypan blue for necrotic cells, and both blue-stained neurons and unstained neurons were counted under each microscopic visual field. Neuronal death rate was expressed as the ratio of dead neurons (stained neurons) to total neurons (stained+unstained neurons). The data was expressed as the means ± S.E.M. ($10 < n < 15$). At least four batches of experiment were performed with similar results. *$P < 0.05$, as compared with the control group (unpaired t-test).

DISCUSSION

The present study demonstrates that CORT can act with KA synergistically to facilitate neurite outgrowth of DRG neurons. The possible mechanism of this action seems to be via activation of the NGF receptors TrkA and increase of GAP-43 expressions for neurite extension. However, the neurotrophic activity of CORT+KA was limited within a time window as early as 24–48 h after axotomy (1–2 DIV). After that both CORT and

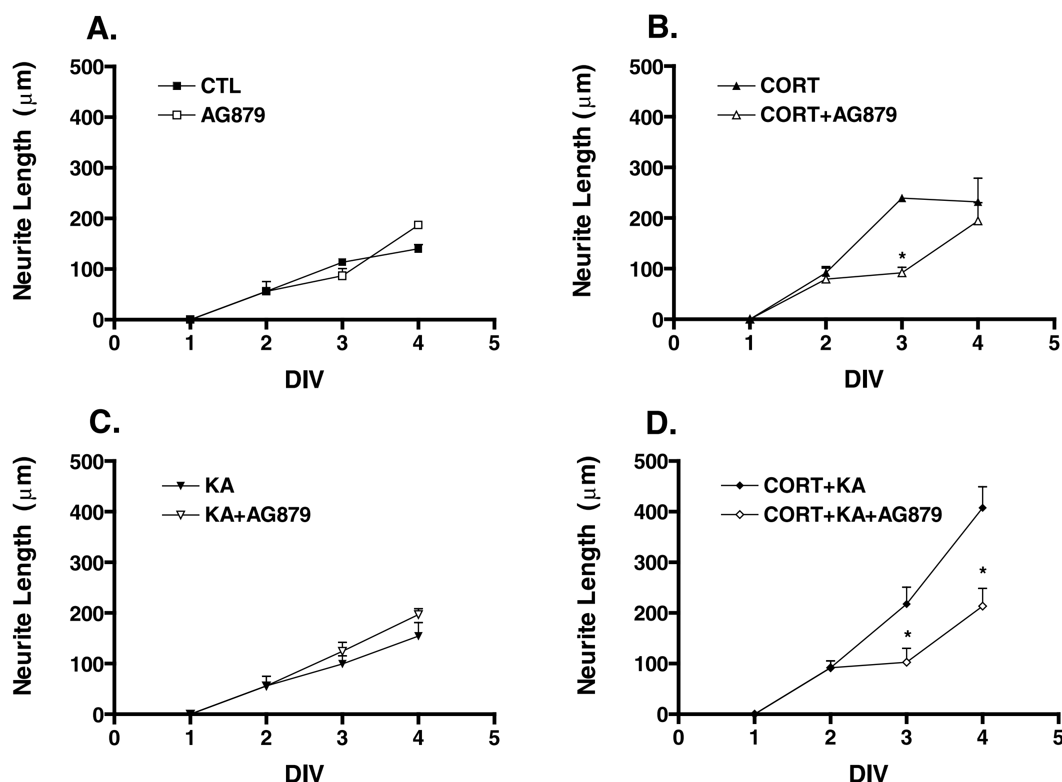

Fig. 5. Effects of TrkA inhibitor AG-879 on neurite outgrowth of AX-DRG neurons. Cultured DRG neurons at 1 DIV were pretreated with CORT (10 μM), 2 DIV with AG879 (3 μM) for 30 min followed by KA (1 mM) treatment. A–D show effects of AG-879 on control, CORT, KA, and CORT+KA-treated groups, respectively. The data was expressed as the mean ± S.E.M. ($n = 8$–15). One-way ANOVA indicated that not all groups had the same mean ($P < 0.01$). *$P < 0.05$ as compared with their respective control by Newman–Keuls multiple comparison posttest. E shows phase contrast photomicrographs at 4 DIV for each condition; 1: control (CTL); 2: CORT only; 3: KA only; 4: CORT+KA; 5: AG-879 only; 6: CORT+AG-879; 7: KA+AG-879; 8: CORT+AG-879+KA. Scale bar = 50 μm.

KA not only became neurotoxic, but also could not further speed up the neurite growth rate. Possible roles of glucocorticoids and glutamate receptors in nerve repair are discussed as follows.

Most studies regarding the neuronal effect of CORT to date have observed its own neurotoxicity and its potentiation of KA neurotoxicity to central neurons. However, CORT seems to be not as toxic to the peripheral neurons as to the central neurons, although in other studies dexamethasone was found to reduce the content of functional neuropeptides such as substance P and CGRP in DRG neurons (Smith et al., 1991). Similar observations were also reported in the hippocampal CA2 region and in differentiating human neuroblastoma cell lines, in which CORT and dexamethasone increased the expression of NT-3 and TrkA, respectively (Chao et al., 1998; Glick et al., 2000). However, it has also been reported that CORT decreased expressions of neurotrophins and GAP-43 in spinal motor neurons and hippocampal CA1 neurons (Chao et al., 1998; Gonzalez Deniselle et al., 1999). The contradiction of these observations may result from the differential physiological states of the evaluated neurons. Glucocorticoids seem to be toxic in healthy neurons with a well-established neuronal network, but turn out to be neurotrophic to neurons with damage or to those stimulated by differentiating factors. This assumption seems to be true when applied to our study, in which CORT+KA was able to promote neurite extension of AX-DRG while neurites were budding out toward every direction to look for their possible targets, but became toxic once the direction of a growing neurite was determined. Clinical therapy using a synthetic glucocorticoid MP for spinal cord injury also had a limited therapeutic window within 8 h after injury (Bracken et al., 1990; Bracken and Holford, 1993). MP therapy more than 8 h after injury was also found detrimental to neurological recovery. Although this observation was thought to be due to the anti-inflammatory or antioxidant effects of MP (Ildan et al., 1995; Koc et al., 1999), our study provides another insight into the possible action of glucocorticoids in neuronal regeneration. However, MP therapy and our study both require supraphysiological concentration of glucocorticoids to exert the neurotrophic effects implicating that a low-affinity glucocorticoid binding site is involved. One of the potential candidates of low-affinity glucocorticoid binding site is on the GABA-A receptor, which was demonstrated to be allosterically modulated by higher than 5 μM of CORT in rat hippocampal neurons and frog primary afferent neurons (Orchinik et al., 2001; Ariyoshi and Akasu, 1986). Since the modulation of GABA receptor activity may increase neuronal response to excitatory input, it is conceivable that high CORT concentration may facilitate activity-dependent neurotrophic gene

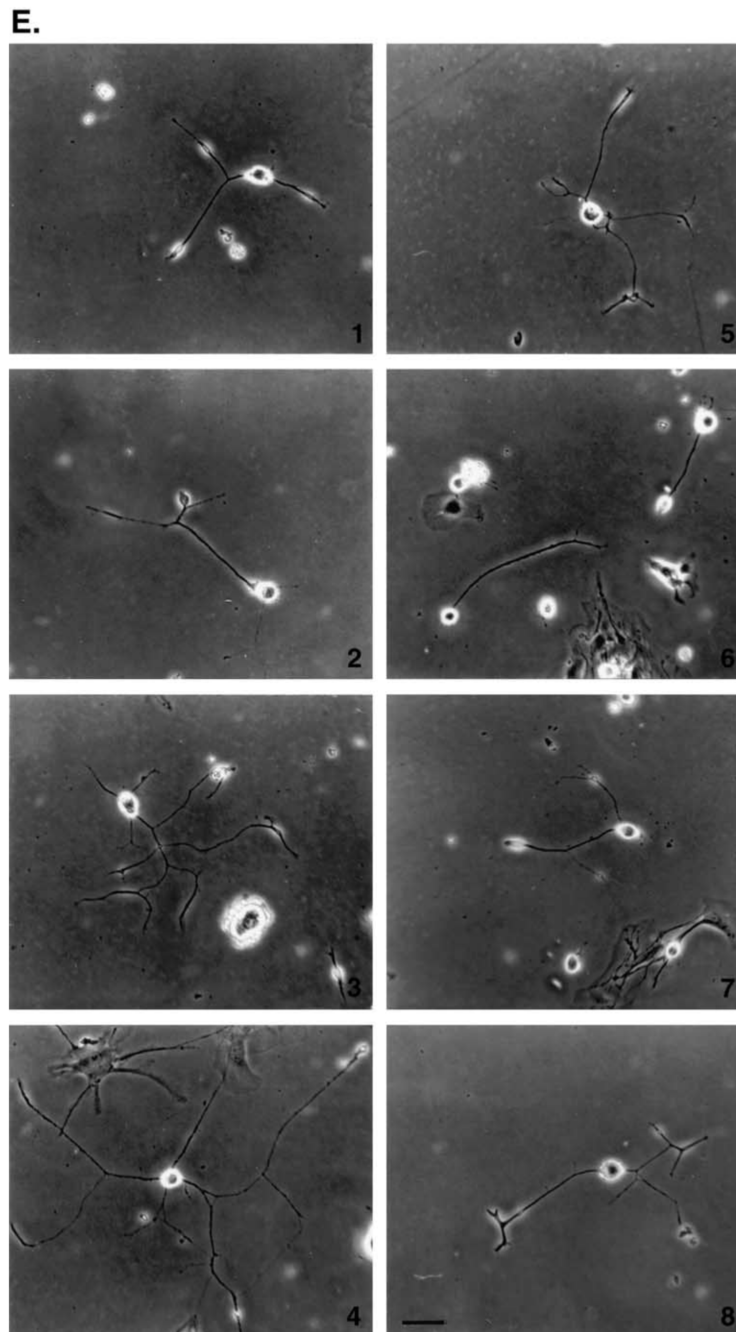

Fig. 5 (*Continued*).

expression by modulating inhibitory tones. Further investigations are needed for this hypothesis. Other postulations, such as the membrane effect of glucocorticoids, might also deal with the Kd value of glucocorticoid binding site similar to the intracellular glucocorticoid receptor in nanomolar range (Joëls, 2001). Whether the low- and the high-affinity glucocorticoid binding proteins both contribute to the neurotrophic activity of CORT remains to be elucidated.

Furthermore, CORT may also contribute to increased reactivity of DRG neurons to KA. It has been reported that CORT caused a 40-fold increase of the KA-elicited pair-pulse response profile of mouse hippocampal neurons (Talmi et al., 1995). The reason for this phenomenon may come from the increased KA receptor expressions by CORT treatment (Joëls et al., 1996). However, *in vivo* studies CORT was also found to increase glutamate accumulation in the hippocampus (Stein-Behrens et al., 1994; Venero and Borrell, 1999). This might be the case in our study, since we demonstrated that HAA, a glutamate transporter inhibitor, can also increase expressions of TrkA and GAP-43 in DRG neurons (unpublished data). Therefore, CORT treatment may result in activation of all types of glutamate recep-

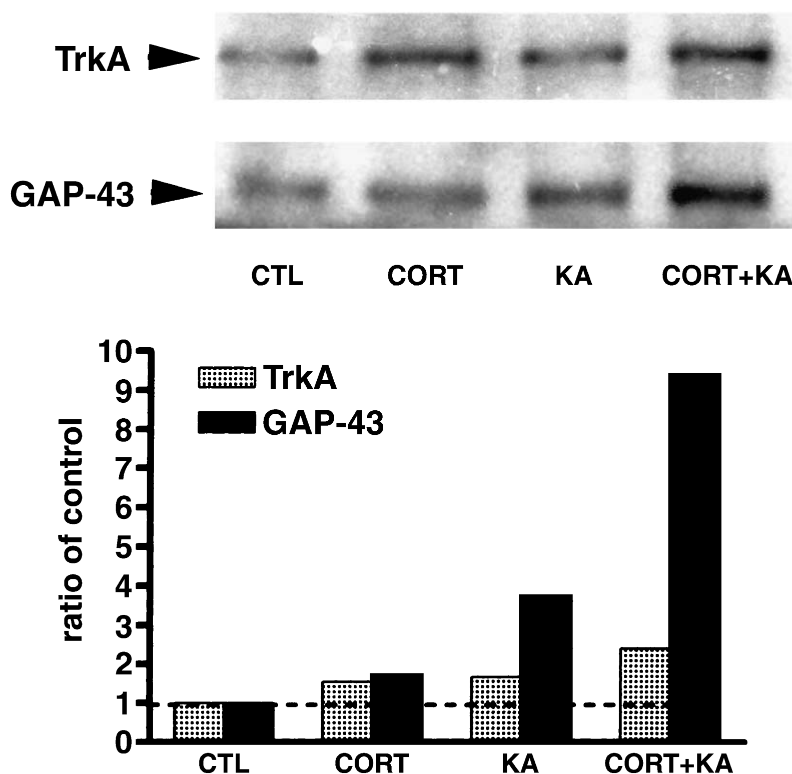

Fig. 6. Western blot analysis of TrkA and GAP-43 expressions in cultured DRG neurons. DRG were pretreated with CORT (10 μM) at 3 DIV, followed by KA (1 mM) treatment at 4 DIV. (A) Neurons were harvested at 6 DIV, and the crude plasma membrane fraction was western blotted with anti-TrkA polyclonal antibody and anti-GAP-43 monoclonal antibody. (B) Immunoblots were analyzed by densitometry, and values were given as the ratio of control (CTL) values. The same experiment was performed in four different batches of cultured DRG neurons with similar results.

tors present in the DRG neurons by increasing extracellular glutamate.

The effect of KA on neurite outgrowth in the present study was never apparent unless DRG neurons were pretreated with CORT. In fact, immunocytochemical studies show that cultured DRG neurons may receive glutamatergic input predominantly via both the AMPA receptor and the KA receptor. In vivo, DRG neurons are primary afferent neurons receiving sensory input from the peripheral sensory receptors and chemical influence from constituents in the cerebrospinal fluid, where glutamate may originate. High concentrations of glutamate present in cerebrospinal fluid may chronically desensitize the glutamate receptors on DRG neurons, and in turn increase the effective concentration of KA required to activate AMPA/KA receptors. It is interesting to note that a dense cytosolic localization of KA receptors in DRG neurons implies that the expression and recycling of the KA receptor could be actively processed. The physiological importance of such a cytosolic distribution of membrane receptors remains to be elucidated.

In addition, KA is also an effective inhibitor for glutamate transporters EAAT1 and EAAT2 (Wadiche et al., 1995; O'Kane et al., 1999). Inhibition of glutamate transporters may increase glutamate concentration in the extracellular space, which will facilitate activation of glutamate receptors. Therefore, it is conceivable that the activation of glutamate receptors, without a neurotrophic effect by itself, may provide a depolarizing condition required for NGF-mediated neurite growth induced by CORT. It should be noted that the lack of KA neurotoxicity in this study coincides with the observation in other studies that in DRG neurons, KA is not toxic to either cell bodies or axons (Wolf and Keilhoff,

Fig. 7. Immunocytochemistry of NGF, TrkA and GAP-43 in DRG neurons. Cultured DRG neurons at 4 DIV were immunostained with anti-NGF, anti-TrkA polyclonal antibodies (A), and anti-GAP-43 monoclonal antibody (B). Controls (CTL) were vehicle-treated culture, and CORT+KA were CORT (10 μM at 1 DIV)+KA (1 mM at 2 DIV)-treated culture. Immunoreactivity signal was amplified using avidin–biotin system, and was visualized using HRP–DAB color development. Positive (black arrowheads) and negative (white arrowheads) immunoreactivities in the DRG soma were as indicated. Scale bar = 50 μm. (C) Analysis of GAP-43 immunoreactivity in DRG neurons was shown as the percent of GAP-43-positive (stained neurons) to total neurons (top). The relative density of GAP-43-positive neurons was obtained by ratio of their net intensity to the GAP-43-negative neurons (bottom). The data was expressed as the means ± S.E.M. (n = 50–100). *$P < 0.05$; **$P < 0.01$, as compared with the control group (unpaired t-test).

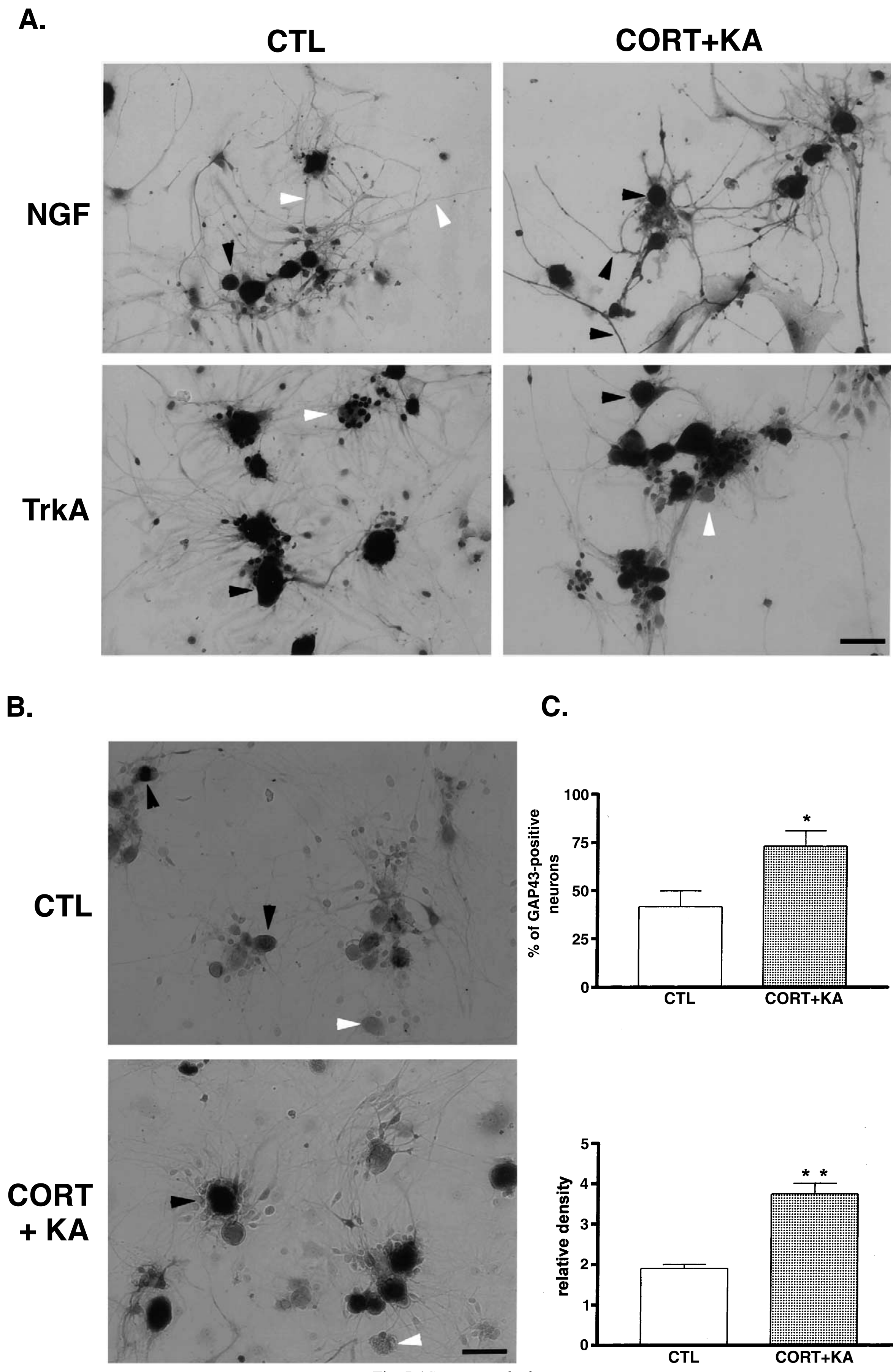

Fig. 7 (*Caption overleaf*).

1983; Agrawal and Evans, 1986). In fact, mild KA toxicity in 4 DIV DRG neurons observed here was attenuated by the NMDA receptor channel blocker MK-801 (unpublished data). This phenomenon could be due to the fact that cultured DRG neurons may form synapses to each other so that the KA-stimulated glutamate release can activate the NMDA receptor. However, DRG neurons do not form synapse to each other *in vivo*, and there is no neuron presynaptic to DRG to release glutamate to the NMDA receptor. Therefore KA may be even less toxic to DRG neurons *in vivo* than *in vitro*.

One of the most profound features of the synergistic effect of CORT and KA is the induction of GAP-43 expression. GAP-43 is located in growth cones of developing neurons or injured neurites, and is frequently used as a marker for neurite outgrowth (for review see Oestreicher et al., 1997). Although the expression of GAP-43 was found increased by NGF and KA under both *in vitro* and *in vivo* conditions (Meberg et al., 1993; Levi-Montalcini, 1987), little is known about the transcription factor that directly promotes GAP-43 gene transcription (Eggen et al., 1994; Nedivi et al., 1992; Starr et al., 1994). However, our results seem to have better coincidence with the stabilization mechanism of the GAP-43 transcript, which is mediated by the protein kinase C and NGF signaling pathways (Perrone-Bizzozero et al., 1993; Cao et al., 1997). Although other neurotrophins, such as BDNF or NT-3, may also contribute to the up-regulation of GAP-43 (Klocker et al., 2001), the most abundant neurotrophin receptors in DRG neurons appears to be the NGF receptor TrkA and p75 NTR as compared with TrkB and TrkC (Josephson et al., 2001). In addition, the promoting effect of BDNF and NT3 on axonal regeneration of AX-DRG has been proved far less than NGF (Bloch et al., 2001, Kimpinski et al., 1997). Therefore, it is conceivable that GAP-43 may have better chance to be up-regulated by NGF than by other neurotrophins in DRG neurons. Furthermore, how CORT and KA synergy affect phosphorylation and dephosphorylation of GAP-43, a key determinant for GAP-43-mediated neuritogenesis, will be worth investigating.

In summary, our studies have demonstrated that administration of CORT to axotomized sensory neurons has a beneficial effect on the neurite growth rate, especially with KA treatment. Although neurite outgrowth in culture is not exactly the same as axonal regeneration in the *in vivo* condition, a profound induction of GAP-43 by CORT or CORT+KA treatment strongly supports the idea that the same treatment may facilitate the repair of injured peripheral nerves. This study also provides a clue for judging the effective time to apply glucocorticoids in peripheral nerve injury. Finally, the role of KA, or other excitatory amino acids, in nerve regeneration remains to be carefully examined, especially with respect to their receptor specificity to avoid possible neurotoxicity.

Acknowledgements—This work was in partial fulfillment for a Ph.D. degree for S.Y.T. This work was supported by grants from the Taipei Medical University Seed Fund (TMC88-Y05-A104) and the National Science Council in Taiwan (NSC 89-2320-B-038-011). The authors wish to thank Dr. Mao-Tsun Lin at Yang Ming University (Taipei, Taiwan) and Dr. Jang-Yen Wu at the University of Kansas for their generous critiques of this work.

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(*Accepted* 10 *May* 2002)