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Role of PKA in the Anti-Thy-1 Antibody-Induced Neurite Outgrowth of Dorsal Root Ganglionic Neurons

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Abstract Thy-1 is highly expressed in the mammalian nervous system. Our previous study showed that addition of anti-Thy-1 antibody to cultured dorsal root ganglionic (DRG) neurons promotes neurite outgrowth. In this study, we identified a novel signaling pathway mediating this event. Treatment with function-blocking anti-Thy-1 antibodies enhanced neurite outgrowth of DRG neurons in terms of total neurite length, longest neurite length, and total neurite branching points. To elucidate the possible signal transduction pathway involved, activation of kinases was evaluated by Western blotting. Transient phosphorylation of protein kinase A (PKA) and mitogen-activated kinase kinase (MEK) was induced after 15 min of anti-Thy-1 antibody treatment. Pretreatment with a PKA inhibitor (PKI) or an MEK inhibitor, PD98059, significantly decreased the neurite outgrowth response triggered by anti-Thy-1 antibody, indicating the involvement of both kinases. In addition, anti-Thy-1 antibody treatment also induced transient phosphorylation of cyclic AMP-response element-binding protein (CREB) and this effect was also blocked by a PKI or PD98059. Furthermore, the fact that PKI abolished anti-Thy-1 antibody-induced MEK phosphorylation showed that PKA acts upstream of the MEK-CREB cascade. In summary, the PKA-MEK-CREB pathway is a new pathway involved in the neurite outgrowth-promoting effect of anti-Thy-1 antibody. J. Cell. Biochem. 101: 566–575, 2007.

Key words: Thy-1; anti-Thy-1 antibody; neurite outgrowth; DRG neuron; PKA; MEK; CREB

Thy-1, a glycosylphosphatidylinositol (GPI)-anchored protein, is expressed in the neurons of the striatum, hippocampus, neocortex, cerebellum, and spinal cord in the rat brain [Seki et al., 2002]. In mouse vestibular ganglion neurons, Thy-1 is not expressed on the axonal surface until axon growth is complete, suggesting an inhibitory function of Thy-1 on axonal growth [Xue et al., 1991]. By interacting with β3 integrin on astrocytes, Thy-1 inhibits neurite outgrowth on astrocyte feeder layers and in astrocyte-rich areas of the injured brain [Tiveron et al., 1992; Leyton et al., 2001; Avalos et al., 2002, 2004]. Inhibition of neurite outgrowth can be prevented by the addition of soluble Thy-1 or of anti-Thy-1 antibodies [Leifer et al., 1984; Lipton et al., 1992; Mahanthappa and Patterson, 1992]. Thy-1 is localized to the non-caveolar microdomains of the plasma membrane and its GPI anchor plays a key role in blocking neurite outgrowth [Tiveron et al., 1994; Lang et al., 1998; Deininger et al., 2003b; Stuermer and Plattner, 2005].

Since it does not have a transmembrane domain, the question of how Thy-1 in lipid rafts triggers the signaling cascade has received

Abbreviations used: CREB, cyclic AMP-response element-binding protein; DRG, dorsal root ganglion; ERK, extracellular signal-related kinase; GPI, glycosylphosphatidylinositol; JAK, Janus tyrosine kinase; MEK, mitogen-activated kinase kinase; NF-L, neurofilament subunit L; NGF, nerve growth factor; PBS, phosphate-buffered saline; PKI, protein kinase A inhibitor; PKA, protein kinase A; TBS, Tris-buffered saline.

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much attention over the past 5 years. Several signaling pathways have been proposed for the effect of anti-Thy-1 antibody on neuritogenesis. The reaction of membranous Thy-1 with anti-Thy-1 antibodies results in an increased interaction between Thy-1 and c-fyn, but a decrease in c-fyn kinase activity, in lipid rafts in chick brain cells [Thomas and Samelson, 1992; Henke et al., 1997]. Thy-1 signaling may also regulate neurite extension by other pathways. In PC12 cells, neurite extension triggered by anti-Thy-1 antibodies is mediated by calcium influx through N- and L-type calcium channels, and thus is calcium- and calmodulin-dependent kinase-dependent [Doherty et al., 1993]. In addition, this neurite outgrowth effect is Gαi-dependent, since the response is blocked by pertussis toxin.

The precise function of Thy-1 in the neurite growth of dorsal root ganglionic (DRG) neurons remains to be explored. Previously, we showed that Thy-1 expression in the dorsal root ganglia is dramatically decreased 2 days after sciatic nerve crush and returns to normal levels 1 week after crush, temporally matching the functional recovery of the pain withdraw reflex, placing reflex, and locomotor functions [Chen et al., 2005]. Furthermore, during postnatal development, Thy-1 expression is closely associated with the functional maturation of DRG neurons [Chen et al., 2005]. Incubation of DRG neurons with anti-Thy-1 antibodies stimulates neurite outgrowth [Chen et al., 2005]. All these data provide support for an inhibitory role of Thy-1 on neuritogenesis of DRG neurons, although the detailed mechanism remains unknown. Several signaling pathways are responsible for nerve regeneration in DRG neurons. Intracellular calcium mobilization is critical for neurite outgrowth in DRG neurons [Kocsis et al., 1994]. Neural cell adhesion molecules activate the extracellular signal-related kinase (ERK) and protein kinase A (PKA) pathways in DRG neurons, with subsequent phosphorylation of the transcriptional factor, cyclic AMP-responsive element binding protein (CREB), and the expression of downstream genes [Schmid et al., 1999; Kolkova et al., 2000; Jessen et al., 2001]. Furthermore, the PKA-CREB and JAK2-STAT3 pathways are reported to regulate the regeneration of the CNS branch of DRG neurons after conditioning peripheral crush [Qiu et al., 2005; Teng and Tang, 2006]. Whether these signaling pathways are engaged in Thy-1-mediated inhibition of neurite outgrowth in DRG neurons deserves further study.

This study aimed to elucidate the signaling pathways activated when anti-Thy-1 antibodies bind to Thy-1. We applied anti-Thy-1 antibodies to primary DRG neuronal cultures and characterized the role of PKA in the enhanced neurite outgrowth response. We also examined events downstream of PKA which might be related to anti-Thy-1 antibody-induced neuritogenesis.

MATERIALS AND METHODS

Cell Culture

Postnatal Day 2 Wistar rats of both sexes were purchased from the Facility for Animal Research of the National Taiwan University. Animal care and procedures were performed to the standards set down in the "Guide for the Care and the Use of Laboratory Animals," published by U.S. National Institutes of Health (NIH publication N0. 85–23, revised 1985). The animals were anesthetized with ether and DRG were collected in Hank's balanced saline solution (HBSS) containing 10 mM sodium pyruvate and 10 mM HEPES. The cells were dissociated by digestion for 30 min at 37°C with 0.05% trypsin (Gibco, Grand Island, NY) and 0.05% type I collagenase (Sigma, St. Louis, MO) in HBSS solution and trituration, harvested by low-speed centrifugation, and washed in L-15 Leibovitz medium (containing 1.176 g/L of $NaHCO_3$, 1% penicillin, and streptomycin) (Gibco). The cells were preplated for 1 h to remove fibroblasts, then the cell suspension was replated on coverslips at 100 cells/mm^2 in 35-mm dishes or directly in 35-mm dishes at 400 cells/mm^2 in L-15 Leibovitz media at 37°C in an atmosphere of 95% air and 5% CO_2.

Antibody Treatment

Ascites containing mouse anti-Thy-1 antibody (1:20 dilution in culture medium) [Jeng et al., 1998] was applied to Day 2 cultures for the indicated time. Normal mouse serum (NMS) was used as the control.

Inhibitors

The PKA inhibitor (PKI) and the mitogen-activated kinase kinase (MEK) inhibitor, PD98059, were purchased from Biomol (Plymouth Meeting, PA).

Immunocytochemistry

DRG neurons on coverslips were briefly washed in cold D-phosphate buffered saline (D-PBS) (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 2.16 g/L $Na_2HPO_4{\cdot}7H_2O$, 0.1 g/L $MgCl_2$, 0.1 g/L $CaCl_2$, pH 7.4), then in cold methanol, and fixed in cold methanol at −20°C for 20 min. After washing in D-PBS for 15 min, the cells were permeabilized and blocked for 1 h at room temperature in blocking buffer (450 mM NaCl, 20 mM Na_2HPO_4, pH 7.4, 0.05% Triton X-100, and 5% normal goat serum). The neurons were then incubated overnight at 4°C with mouse anti-NF-L antibody (Sigma Chemical Co., St. Lewis, MO; 1:100 in blocking buffer), then for 1 h at 37°C with biotinylated goat anti-mouse IgG antibody (Vectastain ABC Elite kit, Vector Labs, Burlingame, CA) diluted 1:50 in blocking buffer. After washes in Tris buffer (50 mM Tris, pH 7.4), the cells were incubated for 1 h at room temperature with avidin–biotin–peroxidase complex (ABC reagent, Vector) diluted 1:100 in Tris buffer, washed with Tris buffer, and reacted with an Vector SG substrate kit for peroxidase (SK-4700) (3% chromogen, 3% H_2O_2 in PBS). After a buffer wash and serial dehydration in ethanols and xylene, the coverslips were mounted with permount and images taken on a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkocheu), equipped with a Nikon DIX digital camera (Nikon, Tokyo, Japan).

Western Blotting

After treatments, the cultured DRG neurons were homogenized in ice-cold lysis buffer (10 mM EGTA, 2 mM $MgCl_2$, 0.15% Triton X-100, 60 mM PIPES, 25 mM HEPES, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin A, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $NaVO_4$, pH 6.9) and sonicated. The protein concentration was measured using a Bio-Rad protein kit (Bio-Rad Lab, Hercules, CA). An equal volume of 2× reducing SDS sample buffer was added to the lysates and boiled at 95°C for 5 min, then protein samples (50 μg protein/gel lane) were separated on 10% polyacrylamide-SDS gels and electrotransferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). The membrane was then blocked using Tris-buffered saline (TBS: 50 mM Tris-base, 150 mM NaCl, pH 8.2) containing 5% bovine serum albumin, 0.1% Tween 20, and incubated overnight at 4°C with primary antibodies [rabbit anti-p-VASP (1:1,000)], mouse anti-phosphorylated-MEK1/2 (1:500), or rabbit anti-Ser-133-phosphorylated-CREB (1:500) (all from Cell Signaling Technology, Beverly, MA), or mouse anti-cytochrome c (1:1,000; Promega Corporation, Madison, WI). Following washes with TBST (TBS containing 0.1% Tween 20), alkaline phosphatase-conjugated secondary antibodies (7,500 dilution in TBST; Promega) were added, and bound antibodies visualized using an enzyme-substrate reaction (substrate: 3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-base, pH 9.5).

Quantification

Immunostained sections were photographed at 20× magnification, and the images converted into 256 gray scale images. The total neurite length, longest neurite length, and total tip number of neurite branches were measured from the somata using PC-based image analyzer software (Image Pro 3.0 Plus, Media Cybernetics, Silver Spring, MD).

Quantification of bands on Western blotting was performed using Gel Pro 3.1 (Media Cybernetics). The Western blots used for statistical analysis were repeated three times for each group. Student's *t*-test was used to evaluate statistical differences between the means for different groups, a *P*-value of <0.05 being considered significant.

RESULTS

Effect of a PKA Inhibitor on Anti-Thy-1 Antibody-Induced Neuritogenesis

Small and large DRG neurons treated with NMS for 6 h had a typical bipolar shape (Fig. 1A,B). Application of anti-Thy-1 antibodies to the DRG culture for 6 h not only increased the neurite length, but also increased the complexity of neurite branching (Fig. 1C,D). The neuritogenic effect was more obvious with large neurons (Fig. 1C) than small neurons (Fig. 1D). Neural survival was not affected by either of the above treatments.

The cAMP-PKA pathway is suggested to be one of the pathways regulating neuronal

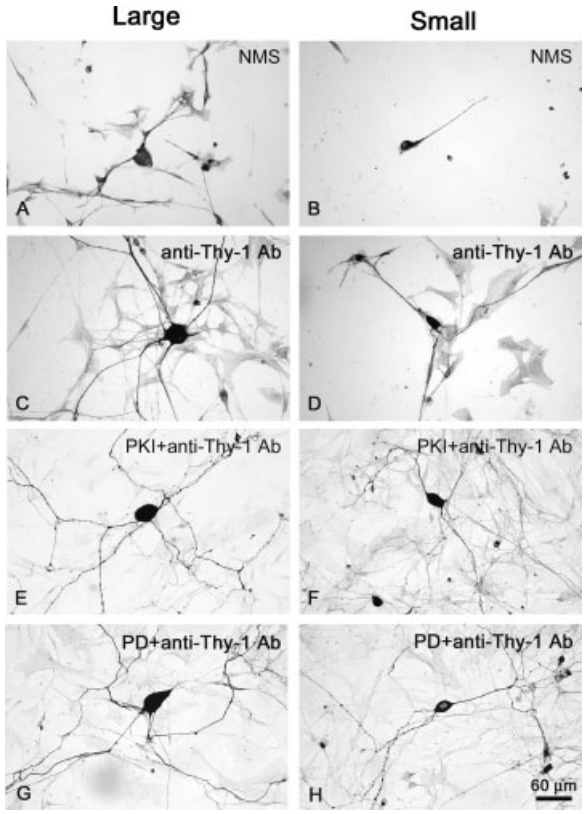

Fig. 1. Effects of a PKA inhibitor on the neurite outgrowth induced by anti-Thy-1 antibody. DRG neurons were treated for 6 h with normal mouse serum (NMS) (**A**–**B**) or anti-Thy-1 antibody (**C**–**D**) or for 30 min with 3 μM PKI (**E**–**F**) or 30 μM PD98059 (**G**, **H**), then for 6 h with anti-Thy-1 antibody before processing for NF-L immunostaining. A, C, E, G: Large neurons; (B, D, F, H), small neurons. Bar = 60 μm.

functions, including neuritogenesis and synaptic plasticity [Ma et al., 2001; Lonze and Ginty, 2002; Lonze et al., 2002a; Tojima et al., 2003]. In addition, activation of the ERK signaling pathway has been shown to induce neuritogenesis in PC12 cells [Charles et al., 2003]. To examine the possible involvement of PKA and MEK in the response evoked by anti-Thy-1 antibody, we pretreated cultured DRG neurons for 30 min with the PKI (3 μM) or MEK inhibitor, PD98059 (30 μM), prior to incubation for 6 h with anti-Thy-1 antibody and found that this prevented anti-Thy-1 antibody-induced neuritogenesis (Fig. 1E–H). Quantification of the inhibitory effect on neurite extension and branching complexity in both small and large neurons is shown in Figure 2. This result suggests that PKA and MEK are involved in the regulation of neuritogenesis.

Activation of Various Kinases and CREB After Anti-Thy-1 Antibody Treatment

We next investigated whether PKA and MEK were activated by anti-Thy-1 antibody treatment. Vasodilator-stimulated phosphoprotein (VASP) is a substrate for cAMP-dependent kinase [Profirovic et al., 2005] and its level of phosphorylation is proportional to the degree of PKA activation. In this study, we used the level of phosphorylation of VASP to represent the activation status of PKA. Treatment with anti-Thy-1 antibody induced rapid phosphorylation of PKA and MEK. Maximal activation of these two kinases occurred after 15 min of treatment (Fig. 3), and activation higher than basal values was seen for at least 60 min. Since CREB-mediated gene transcription is required for various neuronal functions, such as differentiation and neuroplasticity [Lonze and Ginty, 2002], we next examined whether CREB was activated by anti-Thy-1 antibody treatment. Ser-133 on CREB is located within the kinase inducible domain and its phosphorylation favors the binding of CREB to its coactivator, CREB binding protein [Chrivia et al., 1993]. Treatment with anti-Thy-1 antibody resulted in phosphorylation at Ser-133 of CREB with a similar time course to the activation of PKA and MEK (Fig. 3).

PKA is the Upstream Regulator of MEK

MEK and PKA can regulate the expression of certain genes via CREB phosphorylation [Tojima and Ito, 2004; Nagase et al., 2005]. We therefore used specific inhibitors to block either MEK or PKA to identify the possible upstream regulator responsible for CREB phosphorylation. Inhibition of PKA by PKI decreased the CREB phosphorylation induced by anti-Thy-1 antibody treatment (Fig. 4A), as did inhibition of MEK by PD98059 (Fig. 4B). Previous studies have concluded that PKA and ERK signaling act independently at the initial stage of neuritogenesis, but converge during later stages of neuronal development [Vogt Weisenhorn et al., 2001; Tojima et al., 2003]. We therefore examined whether there was a link between the PKA and MEK signaling pathways in the processes following anti-Thy-1 antibody treatment and found that pretreatment with 3 μM PKI also prevented the anti-Thy-1 antibody-induced phosphorylation of MEK (Fig. 5), indicating that PKA regulates the activation of MEK.

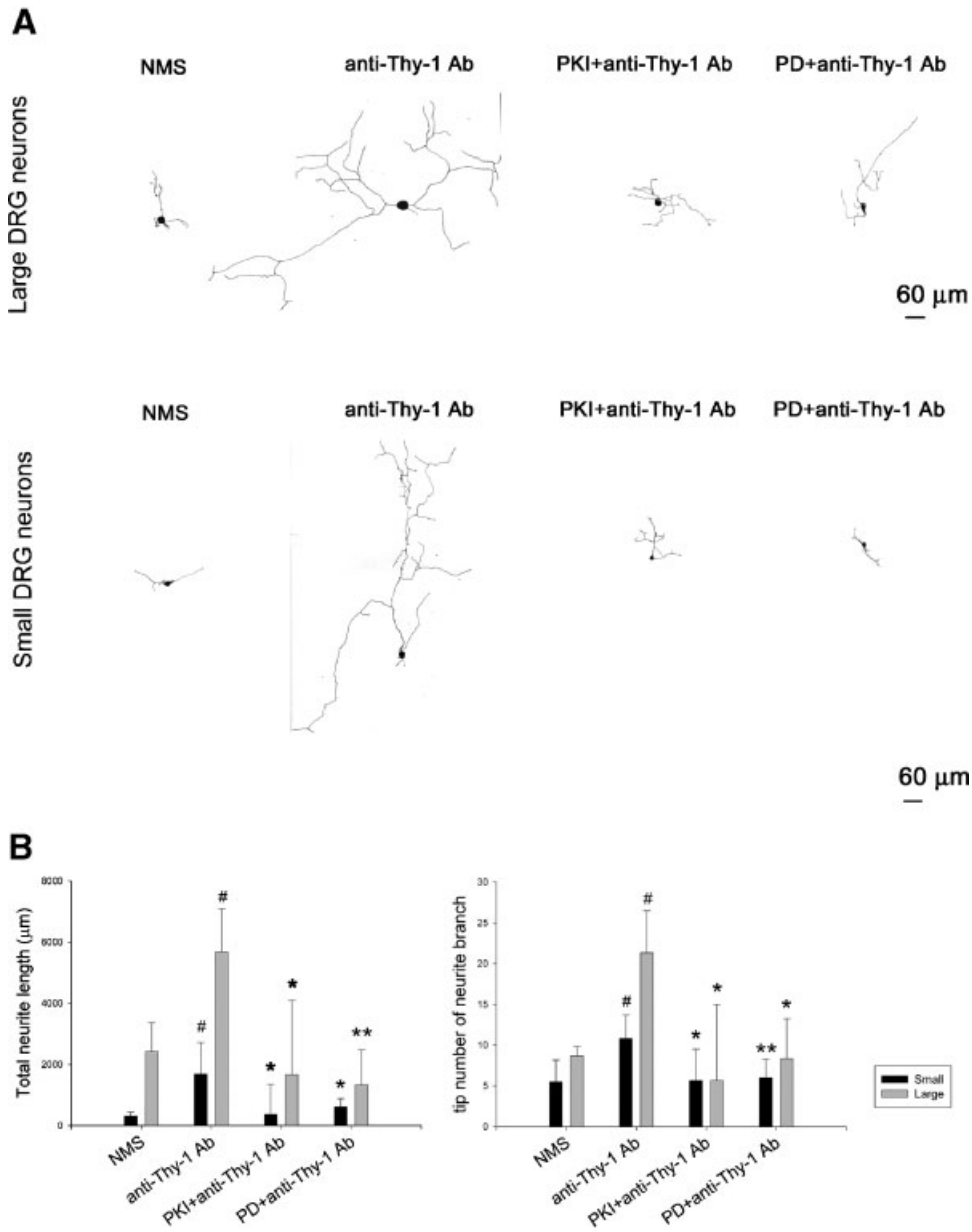

Fig. 2. Quantitative analyses of the effect of a PKA inhibitor and an MEK inhibitor on the anti-Thy-1 antibody-induced neurite outgrowth of DRG neurons. Cells were treated as above with anti-Thy-1 antibody with or without prior incubation with PKI or PD98059. **A**: Neuronal morphology drawn with a camera lucida. **B**: quantitative data for total neurite length and tip number of neurite branches. n = 6; $^{**}P < 0.01$; $^{*}P < 0.05$, compared to the anti-Thy-1 antibody-treated group. $^{\#}P < 0.05$, compared to the NMS group.

DISCUSSION

This study showed that PKA was involved in the anti-Thy-1 antibody-induced neurotrophic response in DRG neurons. We also observed that inhibition of PKA abolished the MEK phosphorylation induced by anti-Thy-1 antibody treatment, indicating the upstream role of PKA. CREB served as a downstream target of MEK activation and possibly turns on the new gene expression necessary for neurite outgrowth triggered by anti-Thy-1 antibodies.

How cross-linking of Thy-1 by anti-Thy-1 antibodies triggers signaling transduction has received attention in several studies. In teleost retinal ganglionic cell axons, Thy-1 interacts with reggie-1 and -2 in lipid raft microdomains, and its expression is upregulated during nerve

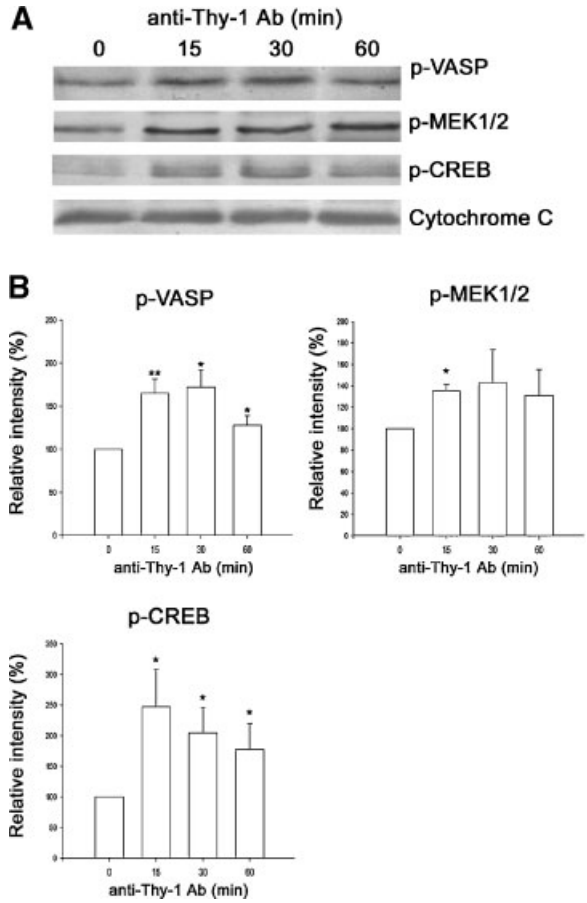

Fig. 3. Phosphorylation of VASP, MEK, and CREB induced by anti-Thy-1 antibody treatment. DRG neurons were untreated (0 time) or incubated with anti-Thy-1 antibody for 15, 30, or 60 min, then the cell homogenates were analyzed on Western blots for phosphorylated VASP, phosphorylated MEK, or phosphorylated CREB. Cytochrome c, loading control. **A**: A typical blot; (**B**) summarized results for densitometric scans of triplicate blots. $^*P < 0.05$, compared to the untreated controls, n = 3.

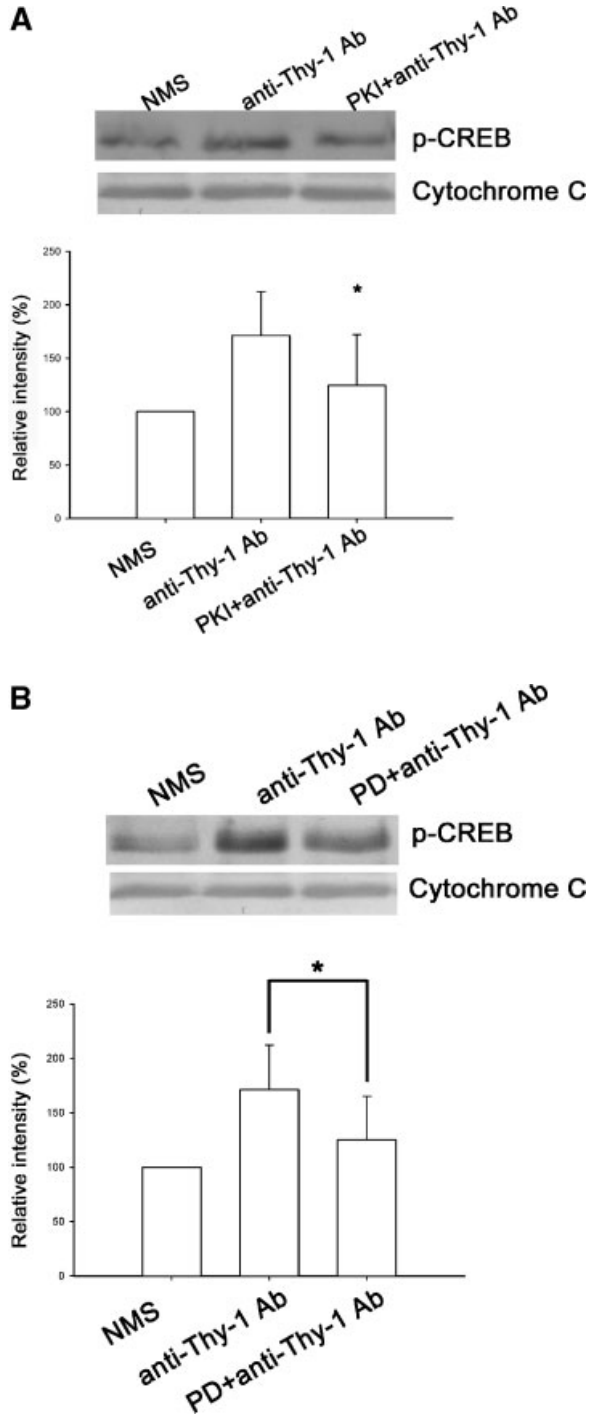

Fig. 4. Inhibition of anti-Thy-1 antibody-induced CREB phosphorylation by PKI and PD98059. DRG neurons were treated for 15 min with normal mouse serum (NMS) or anti-Thy-1 antibody, or for 30 min with 3 μM PKI (**A**) or 30 μM PD98059 (**B**), then for 15 min with anti-Thy-1 antibody and the cell homogenates analyzed for phosphorylated CREB. Cytochrome c, loading control. A typical blot (**upper panel**) and densitometric scans of triplicate blots (**lower panel**) are shown.$^*P < 0.05$, compared to the anti-Thy-1 antibody-treated group, n = 3.

regeneration [Deininger et al., 2003a]. When Thy-1 molecules are cross-linked by anti-Thy-1 antibody, they selectively copatch with reggie-1, -2, ganglioside GM1, the T-cell receptor complex, and fyn in membranous microdomains of neurons and astrocytes [Stuermer et al., 2001]. In membrane complexes isolated from chicken forebrain neurons, Thy-1 molecules are associated in a signaling complex with c-fyn and the heterotrimeric Gαi subfamily members, and activation of this signaling complex by anti-Thy-1 monoclonal antibody elicits a decrease in the total phosphoprotein profile and tyrosine kinase activity [Henke et al., 1997]. In contrast, the interaction of the tyrosine kinase, fyn kinase, with GPI-anchored molecules, such as

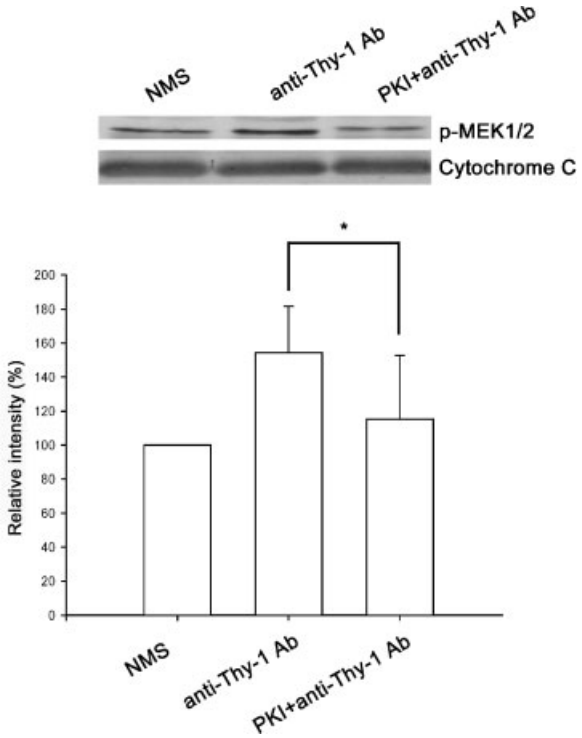

Fig. 5. Effect of PKI on the anti-Thy-1 antibody-induced activation of MEK. DRG neurons were treated as in Figure 4, then the cell homogenates were analyzed for phosphorylated MEK. Cytochrome c, loading control. A typical blot (**upper panel**) and densitometric scans of triplicate blots (**lower panel**) are shown. n = 3; $^*P < 0.05$, compared to the anti-Thy-1 antibody-treated group.

Thy-1, in the cell membrane of oligodendrocytes facilitates fyn kinase activation during myelination [Kramer et al., 1999]. Whether fyn is activated or inhibited by anti-Thy-1 antibody treatment, therefore, varies depending on the cell type. Whether the interaction between Thy-1 and fyn results in PKA activation in DRG neurons requires further studies. PKA might be activated by the route discussed below. Cross-linking of Thy-1 molecules by anti-Thy-1 antibody triggers calcium influx through L- and N-type calcium channels in PC12 cells [Doherty et al., 1993], and increases intracellular calcium levels in T lymphocytes [Kroczek et al., 1986a,b; Ledbetter et al., 1987]. The calcium influx can activate adenylate cyclase, resulting in activation of PKA, then CREB [Poser and Storm, 2001]. Ca^{2+}-calmodulin can activate CaMKI, CaMKII, and CaMKIV, any of which is capable of phosphorylating CREB [Curtis and Finkbeiner, 1999]. However, this seems unlikely in the current model, since pretreatment with the CaMK inhibitor, KN-93, did not affect anti-Thy-1 antibody-induced CREB phosphorylation (data not shown).

In rat DRG neurons, the signaling pathways responsible for regeneration and axon growth are different, depending on the age of the experimental animals and the type of stimulation used. MEK and phosphatidylinositol-3 kinase mediate axon growth from both naïve and nerve growth factor (NGF)-stimulated embryonic neurons, whereas only Janus tyrosine kinase (JAK) mediates the axon regeneration response in adult DRG neurons after a conditioned lesion [Liu and Snider, 2001]. Inhibition of PKC activity stimulates the regeneration of dorsal column axons beyond the lesion site in adult rats [Sivasankaran et al., 2004]. On the other hand, cytokine-stimulated regeneration of the central branches of DRG neurons after conditional lesion depends on the JAK2-signal transducer and activator of transcription 3 (STAT 3) pathway, while NGF-induced regeneration is mediated by CREB [Teng and Tang, 2006]. We here provide evidence that PKA-MEK is one of the pathways mediating the neurotrophic response of DRG neurons to anti-Thy-1 antibody. ERK is known to regulate neuronal differentiation and neuronal outgrowth [Kaplan and Miller, 2000]. Activation of the ERK pathway induced by NGF leads to phosphorylation of CREB at Ser-133 in PC12 cells [Kita et al., 1998] or to the direct activation of BDNF promoter IV in DRG neurons [Park et al., 2006]. Our data showed that the downstream target of MEK-ERK was CREB.

By means of G-protein-coupled receptors, neurotransmitters and neuropeptides can activate the cAMP-PKA-CREB cascade. CREB appears to be required for NGF-mediated neuronal induction [Batistatou et al., 1992; Ahn et al., 1998]. CREB binds to several separate sequences within the c-fos promoter [Ginty et al., 1994; Bonni et al., 1995], and its activation turns on transcription of the bcl-1 gene, which favors the survival of DRG neurons [Lonze et al., 2002b]. Dominant-negative inhibitors of CREB family members attenuate the outgrowth of cortical neuron dendrites [Redmond et al., 2002]. Lonze and Ginty [2002] reported a list of downstream target genes induced by CREB activation; these include neurofilament subunit L (NF-L), brain-derived neurotrophic factor, and NGF. In the DRG, the

morphine-induced increase in immunoreactivities of calcitonin, calcitonin-related peptide, and substance P in DRG neurons and primary sensory afferents is dependent on CREB phosphorylation, and ERK acts upstream of CREB [Ma et al., 2001]. Activation of the PKA-CREB cascade by pituitary adenylate-activating peptide results in neurite outgrowth of F11 neuroblastoma/DRG hybrid cells [McIlvain et al., 2006]. In addition, estrogen-induced CREB phosphorylation upregulates the expression of spinophillin, a spine marker, and favors synapse formation in hippocampal neurons [Lee et al., 2004]. The neurotrophic response to anti-Thy-1 antibody treatment might be mediated by the turning on these relevant genes via CREB or ERK activation.

In conclusion, the effect of anti-Thy-1 antibodies is mediated by activation of the PKA signaling pathway. Our data show that activation of the MEK-ERK-CREB pathway is a downstream event after PKA activation. CREB phosphorylation may lead to CREB-mediated expression of genes for neurite extension. These results provide new insights into the neurite-promoting effect of anti-Thy-1 antibody and application of anti-Thy-1 antibody could be used as a strategy to promote nerve regeneration in the injured peripheral nervous system.

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