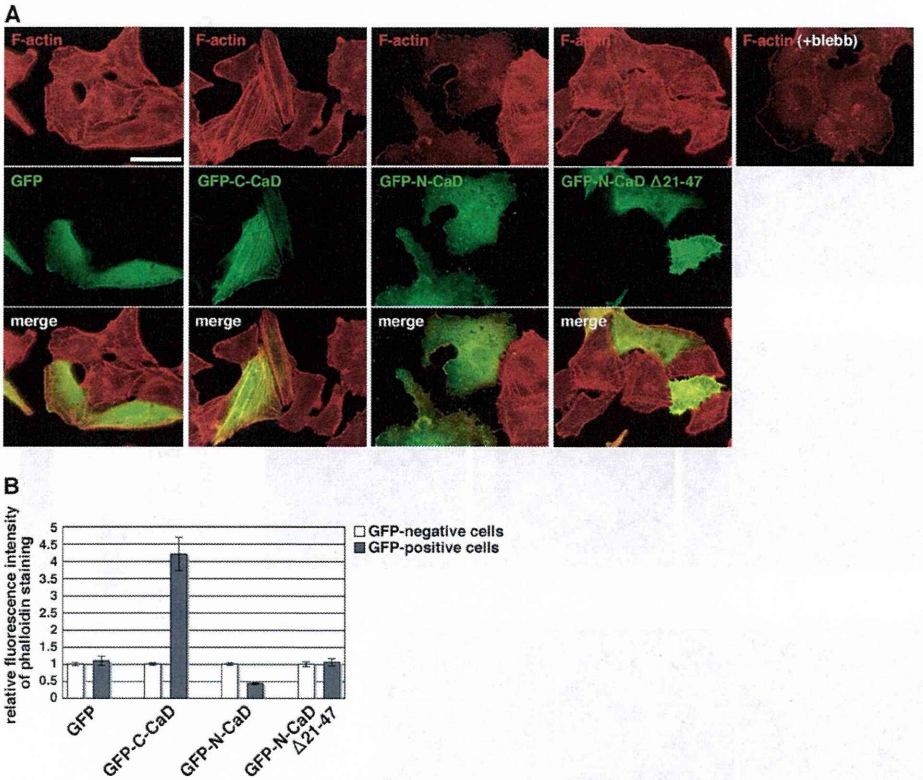

FIGURE 5. **CaD fragments ectopically expressed in A549 cells.** A549 cells transfected with GFP, GFP-C-CaD, GFP-N-CaD, or GFP-N-CaD Δ21–47 were fixed and stained with anti-GFP antibody (*green*) and phalloidin (F-actin, *red*). At right, cells were treated with 10 μM blebbistatin (blebb) for 30 min and then fixed and stained with phalloidin. Bar, 100 μm. *B*, quantification of fluorescence intensity of phalloidin staining in GFP-positive and GFP-negative cells, respectively.

an actomyosin component and enhances axon extension through direct interaction with non-muscle myosin II.

N-CaD Exhibits the Same Effect as Blebbistatin—To determine the significance of CaD interaction with myosin, N-CaD or C-CaD was transfected into A549 cells. CaD has been reported to stabilize actin filaments via its C-terminal F-actin-binding domains, causing thick actin fibers to form (12, 26). In A549 cells, C-CaD strongly induced thick actin fiber formation (Fig. 5, *A* and *B*). On the other hand, cells expressing N-CaD showed significant actin fiber loss and a flat cell shape with prominent lamellipodia (Fig. 5, *A* and *B*). These effects were completely lost in A549 cells expressing an N-CaD Δ21–47 fragment lacking the 27-amino acid myosin-binding sequence (Fig. 5, *A* and *B*). Further, these morphological changes were very similar to those found in cells treated with the myosin II-inhibitor blebbistatin (Fig. 5*A*). These results suggest that CaD binds to myosin at its N terminus, and that it inhibits myosin II function independently of its C-terminal F-actin-binding domains.

CaD Changes Growth Cone Morphology and Myosin II Localization—To determine the function of CaD in growth cones, we observed growth cone morphology and myosin II localization in the hippocampal neurons expressing CaD fragments (Fig. 6). N-CaD inhibited lamellipodia expansion, whereas C-CaD enhanced filopodia formation in growth cones. Full-length CaD induced both lamellipodia retraction and filopodia formation. N-CaD Δ21–47 had no effect on growth cone morphology.

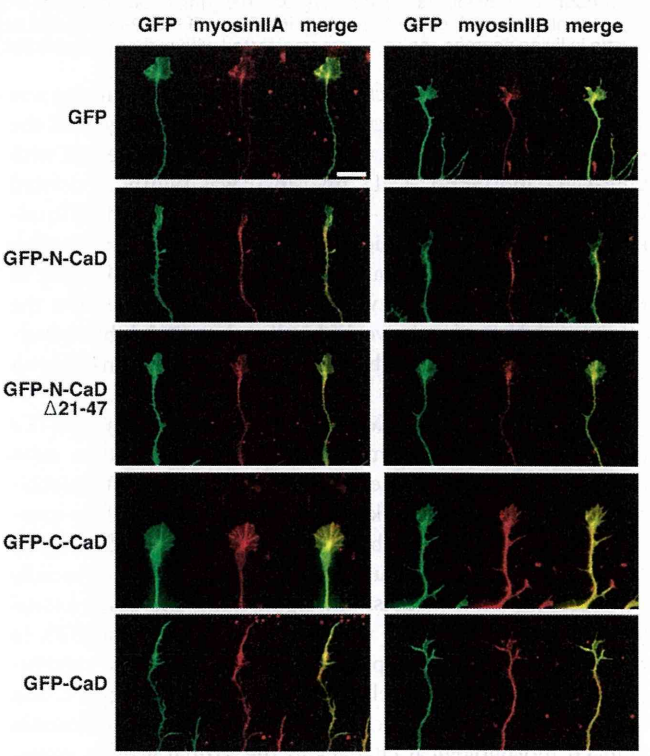

FIGURE 6. **The effect of CaD fragments on growth cone morphology and myosin II localization.** Hippocampal neurons transfected with GFP, GFP-C-CaD, GFP-N-CaD, GFP-N-CaD Δ21–47, or GFP-CaD were fixed and stained with anti-GFP (*green*) and anti-myosin IIA or IIB (*red*) antibodies. Bar, 10 μm.

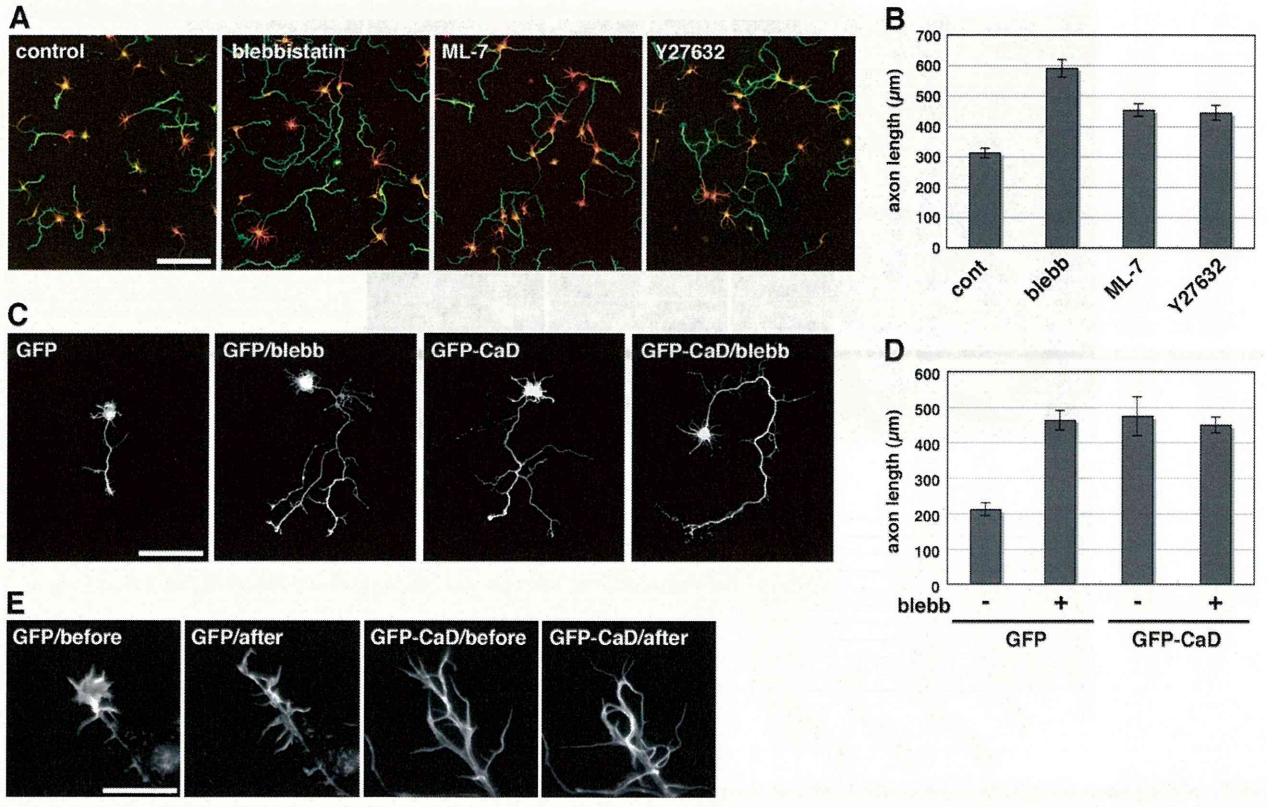

FIGURE 7. **Myosin II functions in CaD-enhanced axon extension.** *A*, hippocampal neurons were cultured with 10 μM blebbistatin, 50 μM ML-7, or 10 μM Y27632 for 3 days, and then fixed and stained with anti-tau1 (*green*) and anti-MAP2 (*red*) antibodies (bar, 400 μm.); *B*, quantification of axonal length in these neurons. Data are means ± S.E. of values from at least 70 cells. *C*, primary cultured hippocampal neurons transfected with GFP or GFP-CaD and incubated with or without 10 μM blebbistatin for 3 days (bar, 100 μm); *D*, quantification of axonal length in these neurons. Data are means ± S.E. from four independent experiments. *E*, growth cone morphology in GFP- or GFP-CaD-transfected neurons before or after treatment with 10 μM blebbistatin for 30 min. To visualize F-actin in living neurons, the cells were transfected with mcherry-LifeAct. Bar, 20 μm.

In GFP-N-CaD-transfected neurons, myosin II staining was slightly diffuse, but distinctly strong in the basal region of the lamellipodia-poor growth cones. In the cells transfected with GFP-CaD and GFP-C-CaD, myosin II was tightly associated with filopodia. N-CaD Δ21–47 had no effect on myosin II localization. These results indicate that C-terminal actin binding domains enhances actin bundling in growth cones, leading to filopodia formation, with which myosin II associates. On the other hand, N-terminal myosin-binding domain inhibits lamellipodia formation in growth cone, but scarcely have an effect on the myosin II localization.

CaD Enhances Axon Extension by Inhibiting Myosin—To examine how inhibiting myosin function would affect axon extension, hippocampal neurons were incubated with blebbistatin, myosin light chain kinase inhibitor ML-7, or Rho-associated protein kinase inhibitor Y27632, drugs that directly or indirectly inhibit myosin function. All of these drugs, especially the direct inhibitor blebbistatin, significantly increased axonal length compared with the vehicle control (Fig. 7, *A* and *B*). In GFP-CaD-transfected hippocampal neurons, however, blebbistatin did not further accelerate axon extension (Fig. 7, *C* and *D*). Coupled with its effects on axon extension, blebbistatin induced morphological changes in the axonal growth cones, inducing a switch from lamellipodial to filopodia-like protrusions (Fig. 7*E*). In the GFP-CaD-transfected neurons, axonal growth cones displayed a filopodia-like morphology without expanded lamellipodia, and their morphology was not affected by blebbistatin treatment (Fig. 7*E*). These findings indicate that blebbistatin and CaD enhance axon extension via the same pathway, through which myosin II function is inhibited.

DISCUSSION

CaD is a ubiquitous regulator of the actin cytoskeleton. Most of CaD functional domains that bind F-actin, tropomyosin, and calmodulin are located in its C terminus, and the C-terminal fragment can inhibit myosin ATPase activity and stabilize actin filaments (8, 12, 26–28). CaD N-terminal region also has a myosin-binding sequence, through which CaD binds to smooth and skeletal muscle heavy meromyosins (23). This binding domain is probably involved in tethering myosin to actin filaments (22, 29), but the significance of myosin binding to CaD had been unclear. In our present study, we clearly demonstrated that CaD enhances axon extension through direct interaction with non-muscle myosin II via its N-terminal myosin-binding sequence. N-CaD, which lacks the all C-terminal functional domains, exhibited the same effect on axon extension as full-length CaD (Fig. 3, *C* and *D*), indicating that axon extension does not depend on the CaD-mediated physical bridge between myosin and actin.

In addition to axon extension, CaD induced formation of the filopodia-like protrusions from soma and axon branches (Figs. 1*F* and 3*E*). CaD also enhanced filopodia formation in growth

cones (Fig. 6). C terminus of CaD, but not N terminus, was required for both functions. C terminus contains some actin binding domains, which are necessary for stabilization of actin bundles (7, 8), and the filopodia-like protrusion were composed of concentrated actin filaments (Fig. 1*H*). These indicate that actin stabilization by the C-terminal domains facilitates formation of these filopodial protrusions independently of N-terminal myosin binding domain.

Results of our experiments using myosin II ATPase inhibitor blebbistatin strongly suggest an inhibitory effect of N-CaD on myosin II function in hippocampal neurons and non-neuronal A549 cells (Figs. 5 and 7). However, previous *in vitro* study showed that the CaD^{1-597} fragment, which lacks the C-terminal actin-binding domains, does not inhibit actin-activated myosin ATPase activity (29). Velaz *et al.* (22) reported that CaD inhibits actin-activated myosin ATPase activity via its C-terminal F-actin-binding domains, by preventing the myosin head from binding to actin *in vitro* (21). Considering the discrepancy between these *in vitro* studies and our *in vivo* study, we propose that N-CaD inhibits myosin II function by unknown mechanisms, which may include interacting with or recruiting additional myosin-inhibitory factors. Further investigations are required to clarify how N-CaD inhibits myosin II function.

Growing evidences indicate that myosin II function is important for axon outgrowth and axon guidance (30–35). However, the molecular mechanism underlying actomyosin-mediated axon extension has not been fully evaluated. An early study by Letourneau *et al.* (36) clearly demonstrated that both "push" by microtubules and "pull" by actomyosin in the growth cone play central roles in axon extension. Actually, actin destabilization by cytochalasin D or ADF/cofilin and myosin II inhibition by blebbistatin enhance axon extension (Refs. 5, 25 and the present study). CaD may inhibit the traction force generated by the actomyosin contraction, thereby augmenting the pushing force from microtubule extension.

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