Process formation results from the imbalance between motor-mediated forces
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INTRODUCTION
The development of axons and dendrites is critical for the establishment of synaptic connections between neurons in order to form functional networks but the mechanisms that support formation of these processes in developing neurons are not completely understood. Several reports have suggested that neurite outgrowth implicates the interaction between microfilaments and microtubules. These cytoskeletal components produce opposing forces: tension produced by microfilaments of the cell cortex (Bray et al., 1986; Zheng et al., 1991; Edson et al., 1993) and compression generated by microtubule bundles in the cytoplasm (Letourneau et al., 1987; Dennerll et al., 1989; Edson et al., 1993). However, the molecular basis for the generation of such forces needs to be investigated. The cytoplasm is rich in molecular motor proteins such as dynein and myosin, which appear to be important in generating forces on microtubules and microfilaments (Carminati and Stearns, 1997; Busson et al., 1998; Evans et al., 1998; Inoue et al., 1998). The first evidence of motor driven forces in neurite outgrowth has emerged from recent studies by Ahmad and collaborators (Baas and Ahmad, 2001). Indeed, the formation of neurites seems to depend on the forces generated upon the microtubules by the motor protein dynein (Ahmad et al., 1998). Moreover, Ahmad and colleagues have shown that the inhibition of the motor activity of dynein causes the axon to retract and that this effect is prevented if myosins are inhibited (Ahmad et al., 2000).

From these observations, we postulate that process formation results from the imbalance between tensile and compressive forces mediated by myosins and dyneins, respectively. To test this hypothesis, we transfected non-neuronal cells that do not develop processes in control conditions with acidic calponin (ac.CaP), an actin-binding protein known to inhibit the actomyosin activity (Gimona and Small, 1996; Winder and Walsh, 1996; Winder et al., 1998) and/or dynamitin, which when present at abnormally high levels results in an immediate cessation of dynein activity (Echeverri et al., 1996; Wittmann and Hyman, 1999). We report that the overexpression of ac.CaP induces the formation of cell processes and that this effect is blocked by the overexpression of dynamitin. Our results provide evidence that the formation of processes, previously shown to be induced by a depolymerisation of microfilaments (Letourneau et al., 1987; Edson et al., 1993; Ferhat et al., 1996b; Ferhat et al., 1998; Meberg and Bamburg, 2000), is also produced by an inhibition of myosin-mediated forces. Our observations strongly support the idea that the formation of processes is a result of antagonistic forces. In addition, consistent with recent observations on primary neurons (Ahmad et al., 2000), our results indicate that these antagonistic forces are generated by motor proteins: tensile forces generated by myosins and compressive forces generated by dyneins. We conclude that the imbalance between myosin and dynein-mediated forces determines whether a neurite forms and elongates or whether it retracts.

MATERIALS AND METHODS
RNA purification and reverse transcription
Total RNA was prepared from the hippocampi of newborn or adult rats by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). 1 μg of total RNA samples was reverse transcribed using ThermoScript RT-PCR System from Gibco BRL. The cDNA synthesis reactions were performed at 25°C for 10 minutes and at 55°C for 60 minutes. To terminate the reactions samples were incubated for 5 minutes at 85°C. To remove the RNA prior to PCR, the cDNA samples were treated with 1 μl of RNase H at 37°C for 20 minutes. Finally, the samples were diluted five times with water.

PCR analysis of amplified PCR products and cloning
cDNAs encoding the full length of ac.CaP and dynamitin were
amplified by PCR from P0 and adult rat hippocampal RNA, respectively, using the ThermoScript RT-PCR System, with forward primers containing an EcoRI and reverse primers containing a BamHI restriction sites. Ac.CaP primers were EcoRI calp: tatataagtc AGCCCATGACCCACTTAACACAGGCCTCT and BamHI calp: atatatgatcctc GTAATCGATGCCCTGGTCTGTCAC. Dynamitin primers were EcoRI dyn: tatataagtcagga ATGCCGGAGCC- TAAATACG and BamHI dyn: tatataagtgctc CTTTCCAGC- TTCTCATCAGCTTC. All the primers were selected in the rat cDNA sequences (Ferhat et al., 1996a; Echeverri et al., 1996).

PCR reactions were carried out in a programmable heating block (GeneAmp PCR System 9700). Following the gel analysis, the PCR products were purified using Qiagen spin columns. Finally, a PCR fragment encoding the full length ac.CaP protein was inserted into the mammalian expression vectors pDsRed1-N1 and pEGFP-N1 (Clontech) and the ones encoding the full length dynamitin and ac.CaPΔ proteins were inserted into pEGFP-N1. The vectors pDsRed1-N1 and pEGFP-N1 encode for Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP), respectively. The obtained constructs were subsequently fully sequenced in order to verify the integrity of the fusion protein (ac.CaP-RFP/GFP, dynamitin-GFP and ac.CaPΔ-GFP).

**Cell line and transfection**

Human embryonic kidney (HEK 293) cells from the American Type Tissue Culture Collection (ATCC) were grown in MEM (minimum essential medium, Gibco BRL), supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 2 mM glutamine (Gibco BRL), 100 IU/ml penicillin and 100 mg/ml streptomycin (Sigma). Transfections were performed using Lipofectamine Plus according to the manufacturer's protocol (Gibco BRL). Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2) for 20 minutes at room temperature (RT).

Quantitative analyses of the number of transfected cells were performed using a fluorescence microscope with a 20x objective. Twenty fields per coverslip per experiment (n=7) were analysed. Data were expressed as the mean percentage of total cells per experiment±s.e.m.

**Immunofluorescence**

Fixed cells were permeabilized with 0.5% Triton X-100 for 10 minutes and incubated overnight at 4°C with mouse monoclonal antibody directed against β-tubulin (1/100, Sigma). Tubulin staining was revealed with the fluorescein (FITC)-conjugated goat anti-mouse (1/200, Jackson Immunoresearch). Before the actin staining, the cells were permeabilized with 0.1% Triton X-100 for 5 minutes and exposed for 2 hours at RT to 0.5 unit per coverslip of Texas Red-X phalloidin (Molecular Probes). Cells were then mounted with Fluoromount G (Southern Biotechnology Associates) and analysed using Zeiss LSM-410 laser scanning microscope.

**RESULTS**

**Overexpression of acidic calponin induces process formation in non-neuronal cells**

Fig. 1 shows the effect of overexpression of ac.CaP on the cellular morphology of HEK 293 cells. Cells transfected with RFP used as control (Fig. 1A) displayed a diffuse staining pattern in the cytoplasm as well as in the nucleus, but the staining in the latter was stronger. Furthermore, these cells did not show any morphological changes compared with untransfected cells (data not shown). In contrast to RFP, HEK 293 transfected with ac.CaP-RFP displayed striking morphological changes (observed in 38±3%) characterised by the formation of several processes (Fig. 1B,C) frequently branched (Fig. 1C). The shape of the cell body was rather round and its size was significantly reduced. In addition, ac.CaP-RFP was absent from the nucleus but it appeared to form thick twisted bundles within the cytoplasm, from which filaments radiate to project into the extensions (Fig. 1B,C). Similar observations were obtained using ac.CaP-GFP (Fig. 2) as well as on CHO-K1 cells (data not shown). In order to assess that ac.CaP-RFP/GFP exerts its effect via its interaction with the actomyosin system, we generated a mutant ac.CaP-GFP without actin-binding domains (amino-acid residues 142-272). As with RFP, cells transfected with ac.CaPΔ-GFP displayed a diffuse staining pattern in the cytoplasm as well as in the nucleus. In contrast to ac.CaP-GFP, ac.CaPΔ-GFP, which do not have the actin-binding domains, did not exhibit any morphological changes (Fig. 1D). This observation reinforces the idea that ac.CaP acts through the inhibition of the actomyosin system to induce processes in non neuronal cells.

**Acidic calponin induces a reorganisation of microfilament arrays**

It has been shown that ac.CaP binds microfilaments (Applegate et al., 1994). To verify that overexpressed ac.CaP-GFP still binds to microfilaments, we visualised them using Texas Red-X phalloidin. In this set of experiments we chose ac.CaP-GFP instead of ac.CaP-RFP because of a better visualisation of microfilaments staining using Texas Red-X phalloidin. These studies also allowed the evaluation of the effects of ac.CaP-GFP on the microfilaments organisation.

In control cells, Texas Red-X phalloidin detected the presence of actin stress fibres that cross over the cytoplasm and...
bundled actin filaments beneath the plasma membrane (Fig. 2A). In all transfected cells (Fig. 2B-D), ac.CaP-GFP (Fig. 2C) was mainly co-distributed with microfilaments (Fig. 2B,D, yellow) suggesting that the majority of ac.CaP-GFP effectively bound microfilaments. Furthermore, our results show that in the transfected cells there was a striking microfilament reorganisation (Fig. 2B,D): twisted bundles of microfilaments were observed in the cell body. A few actin bundles that originate from this central core projected within the extensions. In addition, these cells had lost both actin stress fibres and cortical actin bundles.

Acidic calponin induces a reorganisation of microtubule arrays

Using β-tubulin antibodies, we investigated the effects of ac.CaP-RFP on microtubule organisation (Fig. 3). In control cells (Fig. 3A-C), microtubules emerging from a centrosomal organising centre next to the nucleus radiated through the cytoplasm as individual filaments (Fig. 3B) until they were stopped by the presence of cortical actin filaments (Fig. 3A,C). By contrast, ac.CaP-RFP-transfected cells (Fig. 3D) displayed a clear-cut reorganisation of microtubules (Fig. 3E). Thick bundles of microtubules were observed within the cell bodies and extensions. In all transfected cells, the ac.CaP-RFP (in red) was not co-localised with microtubules (in green) but rather was adjacent (Fig. 3F). In addition, the ac.CaP/microfilaments bundles were detached from the cell cortex and localised in the core of the cytoplasm so that microtubules distributed at the cell periphery (Fig. 3F). We also noticed that in many cases, ac.CaP filaments/microfilaments (Fig. 3G,I, red) and microtubules (Fig. 3H,I, green) clearly form ‘curves’ within the processes (see arrows). This wavy pattern of microfilaments and microtubules might be a consequence of the forces generated by motor proteins and bone by these two cytoskeletal elements.

Process formation induced by acidic calponin depends on dynein activity

There is much evidence showing that dynein motor may generate forces between microtubules and microfilaments in mitotic cells and postmitotic neurons (Baas and Ahmad, 2000). In mitotic cells, it has been suggested that dynein-mediated forces are required for positioning microtubules and centrosomes during interphase anaphase (Koonce et al., 1999; Ma et al., 1999) and separating duplicated centrosomes during prophase and anaphase (Carminati and Stearns, 1997; Busson et al., 1998; Inoue et al., 1998; Gonczy et al., 1999). In postmitotic neurons, these same forces are essential for the transport of microtubules from the centrosome into the axon and this transport is required in axon outgrowth (Ahmad et al., 1998). Here we tested the hypothesis that process formation induced by the overexpression of ac.CaP depends on the dynein activity. If this is correct, the effects of ac.CaP should be blocked by the inhibition of dynein activity. For this purpose, we simultaneously overexpressed ac.CaP-RFP and dynamitin-GFP in HEK 293 cells. Indeed, when present at abnormally high levels, dynamitin is known to inhibit dynein activity. As we have shown above, the overexpression of ac.CaP-RFP induced the formation of several processes (Fig. 4A,C, red). By contrast, dynamitin-GFP-transfected cells did not show any significant morphological change and never displayed extensions (Fig. 4B,C, green). As described by Burkhardt et al., dynamitin-GFP protein was diffusely distributed in the cytoplasm and was absent from the nucleus (Fig. 4B) (Burkhardt et al., 1997). Double-transfected cells overexpressing both ac.CaP-RFP (Fig. 4D) and dynamitin-GFP (Fig. 4E) did not develop significant processes (Fig. 4F). The size of double-transfected cells was significantly reduced (Fig. 4F) compared with cells expressing either ac.CaP-RFP or dynamitin-GFP (Fig. 4C). Similar observations were obtained using CHO-K1 cells (data not shown). We cannot completely exclude that dynamitin effects are related to the alteration of the Golgi apparatus (Burkhardt et al., 1997; Ahmad et al., 1998). However, our data, in agreement with previous observations (Ahmad et al., 2000), strongly support the notion that dynein-mediated forces between microtubules and microfilaments play a key role in process formation.

DISCUSSION

The present data show that the overexpression of ac.CaP in non-neuronal cells induces the formation of processes and that this effect depends on dynein activity.

Previous data have demonstrated that the formation of neurites and their elongation are induced by the depolymerisation of microfilaments using destabilising agents such as cytochalasins (Marsh and Letourneau, 1984; Bentley and Toroiyan-Raymond, 1986; Letourneau et al., 1987; Ferhat et al., 1996b; Ferhat et al., 1998). Consistent with these observations, it has more recently been reported that the depletion of microfilaments by the overexpression of endogenous factors such as actin depolymerising factor (ADF) also promotes neurite elongation (Meberg and Bamberg, 2000). By contrast, our results suggest that process formation induced by ac.CaP did not require a depolymerisation mechanism. Several lines of evidence reinforce this idea. First, cytochalasin treatment of control HEK 293 cells does not induce process formation (Ferhat et al., 1996b). Second, the main in vitro effect of calponin family is to inhibit the actomyosin activity (Gimona and Small, 1996; Winder and Walsh, 1996; Winder et al., 1998). Third, biochemical studies have shown that calponins also stimulate actin polymerisation, and bundling and stabilisation of F-actin filaments (Kake et al., 1995; Kolakowski et al., 1995). In agreement with these in vitro effects our present data show that ac.CaP-transfected cells display a more bundled organisation of actin filaments compared with control cells. Thus we suggest that the formation of processes induced by ac.CaP is due to the inhibition of actomyosin-mediated forces rather than to the depletion of the microfilaments array. Based on all these observations we propose that, to generate processes, cells can use two independent mechanisms in parallel: disassembly of microfilaments and inhibition of actomyosin activity.

In non-neuronal cells, the microtubules radiate through the cytoplasm as individual filaments until they are stopped by a dense network of actin filaments of the cell cortex. This cortical cytoskeleton has been suggested to act as a tensile envelope maintaining the shape of the cell (Bray and White, 1988; Janmey, 1991). In addition, physical studies indicated that actin filaments are more resistant to deformation than microtubules (Janmey et al., 1991). By contrast, when ac.CaP is
overexpressed in HEK 293 cells, the organisation of these cytoskeleton components is strikingly altered. Indeed, in these cells, microtubules were localised at the cell periphery while microfilaments were detached from the cell cortex and accumulated within the cytoplasm. Therefore, the microfilament reorganisation observed in ac.CaP cells facilitates the microtubule bundles to pull out processes. One possible explanation of the microfilament reorganisation relates to the inability of myosins to generate forces required for the transport of microfilaments into the periphery. Indeed, it is now well established that motor proteins, known to convert the chemical energy released by nucleotide hydrolysis directly into movement, are capable not only of transporting organelles along microtubules or microfilaments but also of transporting and organising the cytoskeleton components themselves. Specifically, it has been shown that myosins are involved in the organisation and transport of microfilaments (Evans et al., 1998; Wu et al., 2000). Thus, the processes induced by ac.CaP may be mediated by the attenuation of myosin forces generated on microfilament arrays.

Our data show that the overexpression of ac.CaP is associated with the formation of processes containing microtubules. These processes cannot be the result of the assembly properties of microtubules since their formation depends on the dynein activity. It has been suggested that dynein drives microtubules down to the axon by generating forces upon the microfilament array (Ahmad et al., 1998; Baas, 1999; Baas and Ahmad, 2001). Thus we propose that the processes induced by the ac.CaP are mediated by the organisation and the transport of microtubules by dynein-mediated forces. This strongly supports the idea that the formation of processes is a result of antagonistic forces (Letourneau et al., 1987; Bray et al., 1986; Dennettl et al., 1989; Zheng et al., 1991; Edson et al., 1993) that are generated by motor proteins (Ahmad et al., 2000; Baas and Ahmad, 2001): tensile forces generated by myosins and compressive forces generated by dyneins. We propose that it is the imbalance between these forces that determines whether a process forms and elongates or whether it retracts. According to this model, a decrease in the tensile forces generated by
Motor proteins in process formation


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REFERENCES


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