GAP43 stimulates inositol trisphosphate-mediated calcium release in response to hypotonicity

Marco Caprini[1,2], Ana Gomis[2], Hugo Cabedo[1,2,3], Rosa Planells-Cases[1], Carlos Belmonte[2], Félix Viana[2] and Antonio Ferrer-Montiel[1,4]

[1]Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, 03202 Alicante, [2]Instituto de Neurociencias-CSIC, Universidad Miguel Hernández, 03550 San Juan de Alicante and [3]Unidad de Investigación, Hospital Universitario de San Juan, 03550 Alicante, Spain

[4]Corresponding author
e-mail: aferrer@umh.es

M.Caprini and A.Gomis contributed equally to this work

The identification of osmo/mechanosensory proteins in mammalian sensory neurons is still elusive. We have used an expression cloning approach to screen a human dorsal root ganglion cDNA library to look for proteins that respond to hypotonicity by raising the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ). We report the unexpected identification of GAP43 (also known as neuromodulin or B50), a membrane-anchored neuronal protein implicated in axonal growth and synaptic plasticity, as an osmosensory protein that augments [Ca²⁺]ᵢ in response to hypotonicity. Palmitoylation of GAP43 plays an important role in the protein osmosensitivity. Depletion of intracellular stores or inhibition of phospholipase C (PLC) activity abrogates hypotonicity-evoked, GAP43-mediated [Ca²⁺]ᵢ elevations. Notably, hypotonicity promoted the selective association of GAP43 with the PLC-δ_1 isoform, and a concomitant increase in inositol-1,4,5-trisphosphate (IP₃) formation. Collectively, these findings indicate that hypo-osmotic activation of GAP43 induces Ca²⁺ release from IP₃-sensitive intracellular stores. The osmosensitivity of GAP43 furnishes a mechanistic framework that links axon elongation with phosphoinositide metabolism, spontaneous triggering of cytosolic Ca²⁺ transients and the regulation of actin dynamics and motility at the growth cone in response to temporal and local mechanical forces.
Keywords: actin cytoskeleton/dorsal root ganglion/mechanotransduction/phosphoinositides/PLC

Introduction

Mechanotransduction enables living organisms to detect touch, vibration, movement and mechanical pain (Hamill and Martinac, 2001). Detection of mechanical and osmotic stimuli is essential for a variety of cellular properties such as regulation of cell volume, shape and motility (Bourque and Oliet, 1997; García-Añoveros and Corey, 1997; Hamill and Martinac, 2001). Molecularly, transduction of mechanical forces into electrochemical signals that underlie the cellular response is carried out by a widely expressed, specialized class of membrane proteins known as mechanosensitive ion channels (MSCs) (Bourque and Oliet, 1997; Gillespie and Walker, 2001). Several putative MSCs have been cloned recently. Members of the epithelial sodium channel/degenerin family and of the TRP channel family, in particular the TRPV subfamily of Ca²⁺-permeable channels, have been associated with responses to mechanical and osmotic stimuli in neuronal and non-neuronal cells in *Drosophila melanogaster*, *Caenorhabditis elegans* and mammals (Colbert *et al*., 1997; García-Añoveros and Corey, 1997; Suzuki *et al*., 1999; Liedtke *et al*., 2000; Strotmann *et al*., 2000; Walker *et al*., 2000). Mutations in these channels may produce important dysfunctions in touch, hearing and olfaction, as well as impaired proprioception and, possibly, mechanical pain (Walker *et al*., 2000; Hamill and Martinac, 2001).

Gating of MSCs in specialized mechanosensory cells appears to be regulated by extracellular and intracellular attachments to proteins that pull the channel open (García-Añoveros and Corey, 1997). In *C.elegans*, several genes have been cloned and found to encode proteins that are assembled to form with MSCs a 'mechanosensory apparatus' for the detection of mechanical forces. This type of arrangement has also been postulated for mechanotransduction in specialized hair cells of vertebrates (Hamill and Martinac, 2001). Mechanosensitivity is present in the peripheral nerve endings of various classes of mammalian primary sensory neurons. These exhibit a wide spectrum of sensitivities to stretch, ranging from displacement by a few microns to injurious mechanical forces. Variability in threshold is associated with different subpopulations of neurons endowed with characteristic electrophysiological properties, that respond to hypotonicity-induced membrane stretch with Ca²⁺ responses differing markedly in kinetics and amplitude (Viana *et al*., 2001). However, it is not established whether differences in mechanical threshold among mechanosensitive neurons are due to the presence of distinct molecular entities in their mechanosensory apparatus (Hart *et al*., 1999). Thus, the identification of osmo/mechanosensory proteins in mammalian sensory neurons remains elusive.

Here, we have used an expression cloning strategy to screen a human dorsal root ganglion (DRG)-derived cDNA library to look for proteins that respond to hypoosmotic stimuli by raising the intracellular calcium concentration ([Ca²⁺]ᵢ). Unexpectedly, the screen identified GAP43, a hydrophilic, membrane-anchored neuronal protein implicated in axonal growth and synaptic plasticity (Aigner and Caroni, 1993, 1995; Dent and Meiri, 1998; Mueller, 1999), as an osmoreceptive protein that augments the [Ca²⁺]ᵢ in response to hypotonicity. Membrane anchoring of GAP43 was critical for the osmosensitive

activity. Analysis of the mechanism of action indicates that $[Ca^{2+}]_i$ augmentation was due to activation of the inositol-1,4,5-trisphospate (IP$_3$) signaling pathway that leads to Ca^{2+} release from IP$_3$-sensitive intracellular stores. It is noteworthy that hypotonicity stimulated the formation of IP$_3$ and favored the association of GAP43 with phospholipase C-δ_1 (PLC-δ_1). Our findings imply that this previously unrecognized osmosensitive activity of GAP43 may underlie its biological function in axonal growth and synaptic plasticity.

Results

Identification of GAP43 as an osmosensory protein

To identify osmosensory proteins, we screened a DRG cDNA library arranged in 50 pools each containing ~3000 clones. Each pool was transiently transfected into human embryonic kidney-derived (HEK293) cells for functional screening. The functional osmosensitive assay consisted of measuring intracellular Ca^{2+} elevations in transfected cells upon exposure to a 30% hypo-osmotic solution (217 mOsm/l). For this task, transfected cells were loaded with the calcium-sensitive dye Fura-2 AM and micro-

Fig. 1. Expression cloning of GAP43 protein using Ca^{2+} imaging. HEK293 cells transiently transfected with clones from a human DRG library were subjected to microscopic fluorescent calcium imaging in isotonic (left) and 30% hypotonic conditions (right). Non-transfected cells exhibited no response to the osmotic–mechanical stimulus (**A** and **B**). Cells transfected with pool 10 show a marked increase in cytoplasmic calcium (**C** and **D**). This pool was subdivided and re-assayed iteratively until a single positive clone (GAP43) was isolated (**E** and **F**). Elevated relative Ca^{2+} concentrations are indicated by an increased ratio of Fura-2 emission at 340 versus 380 nm excitation wavelenght (see calibrated colored bar). (**G**) Modular organization of GAP43. Palmitoylation occurs at Cys3 and Cys4. The protein displays a positively charged segment (amino acids 39–53) known as the ED domain.

scopically examined for hypotonicity-evoked changes in fluorescence. As illustrated in Figure 1A and B, untransfected HEK293 cells (control) did not elicit $[Ca^{2+}]_i$ changes under either isotonic or hypotonic conditions. Similar results were obtained with cells transfected with the empty plasmid (data not shown). In contrast, cells transfected with cDNA from pool 10 of the library exhibited a significant hypotonicity-evoked increment in cytosolic Ca^{2+} (Figure 1D). Iterative subdivision and re-assay of the subsequent pools led to the identification of an individual clone containing an 800 bp cDNA insert that conferred osmosensitivity to HEK293 cells (Figure 1F). The open reading frame of this cDNA encoded a protein of 238 amino acids with a predicted mol. wt of 24.8 kDa. A homology BLAST search in non-redundant protein databases identified this protein as GAP43 (also known as neuromodulin or B-50), a neuronal protein implicated in axonal growth and synaptic plasticity (Aigner and Caroni, 1993, 1995; Dent and Meiri, 1998; Mueller, 1999; Bomze et al., 2001; Hulo et al., 2002). GAP43 is a hydrophilic protein anchored to the cell membrane through palmitoylation of two key cysteines located at the N-terminus (Figure 1G). In addition, the protein has a basic domain [known as the effector domain (ED), Figure 1G] that is involved in its interaction with phosphoinositides and calmodulin, and contains a phosphorylation site for protein kinase C (PKC; Caroni, 2001).

GAP43-dependent, hypo-osmotic activation of calcium release

Because GAP43 is not an integral membrane protein, we next investigated whether the hypotonicity-induced, GAP43-mediated $[Ca^{2+}]_i$ increase is due to an influx of extracellular Ca^{2+} or its release from intracellular stores. In the presence of extracellular Ca^{2+}, hypo-osmotically triggered $[Ca^{2+}]_i$ changes appeared ~2 min after external medium change. At variance with exposure to hypotonicity, application of a 30% hyper-osmotic stimulus did not elicit Ca^{2+} responses in GAP43-expressing cells (GAP43+ cells) (data not shown). Hypo-osmotic responses typically had a rapid rise and decay, with a modest plateau phase in some cells (Figure 2A). Virtually all cells responded once to the hypotonic stimulus, although the latency time of the $[Ca^{2+}]_i$ increase was variable from cell to cell. Removal of the external Ca^{2+} ($0Ca^{2+}$) did not prevent these osmotically sensitive transient increases in cytosolic Ca^{2+} (Figure 2B), suggesting that hypotonicity releases Ca^{2+} from intracellular stores. To substantiate this tenet further, we evaluated whether depletion of the endoplasmic reticulum (ER) with thapsigargin affected hypotonicity-induced $[Ca^{2+}]_i$ increases in GAP43+ cells. As illustrated in Figure 2C, treatment with 2 µM thapsigargin for 1 h substantially reduced the osmotically evoked $[Ca]_i$ increase in GAP43+ cells in the presence of extracellular Ca^{2+} ($[Ca^{2+}]_I = 3.8 \pm 1.0$ nM, $n = 150$). Similar results were obtained in thapsigargin-treated GAP43+ cells that were hypo-osmotically stimulated in $0Ca^{2+}$ (data not shown). Furthermore, carbachol (Cch)-induced Ca^{2+} release, prior to the hypo-osmotic stimulus, completely abolished hypotonic Ca^{2+} responses in GAP43+ cells (Figure 2D). Taken together, these results demonstrate that the hypotonicity-induced, GAP43-mediated $[Ca^{2+}]_i$ increment is due to release from the ER.
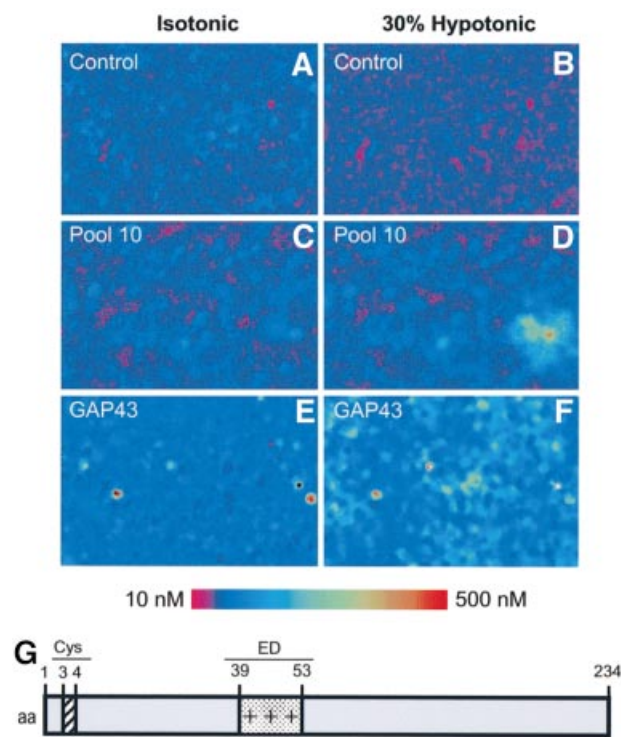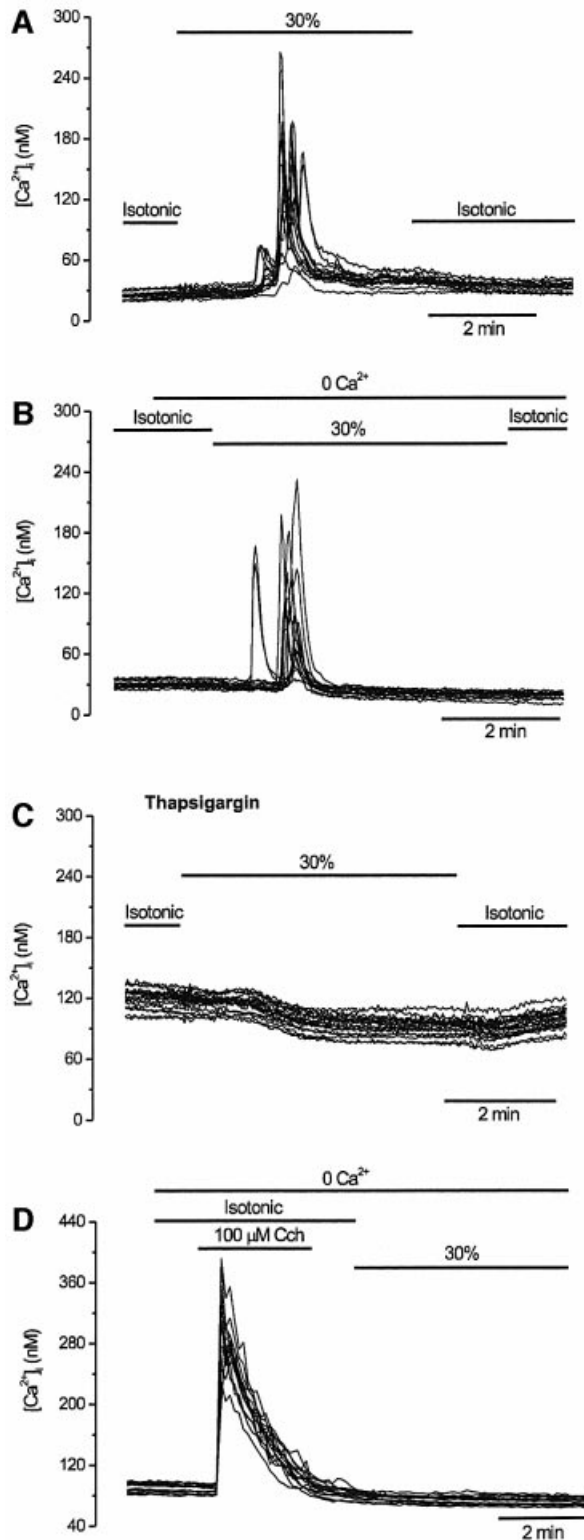
To characterize the osmosensitive release of Ca^{2+} in GAP43^{+} cells further, we examined the effects of different levels of hypo-osmolarity to obtain a dose–response relationship. As depicted in Figure 3A and C, the percentage of untransfected HEK293 cells (control) that responded to low-to-moderate levels of hypotonicity (15–45%) increasing the $[Ca^{2+}]_i$ was marginal. This lack of response was not due to emptiness of the ER nor to deficiencies in the signaling pathways, since Ca^{2+} release could be readily evoked by the muscarinic agonist carbachol (Figure 3A). Furthermore, untransfected cells produced significant Ca^{2+} responses when intense hypo-osmotic stimuli such as 70% were used (Figure 3C). In contrast, $[Ca^{2+}]_i$ increases in GAP43^{+} cells were evoked by hypo-osmotic solutions as low as 15% (Figure 3B). Both the mean amplitude of the Ca^{2+} transients and the number of responsive GAP43^{+} cells increased as a function of the hypotonicity of the extracellular solution (Figure 3B, inset and Figure 3C). Note that the magnitude of the $[Ca^{2+}]_i$ elevation was significantly higher at strong hypotonicity (Figure 3B, inset). Collectively, these results indicate that GAP43 endows the cells with osmosensitivity, presumably by decreasing their threshold for signaling downstream of osmosensation.

The palmitoylation sites of GAP43 play an important role in hypotonicity-activated Ca^{2+} release

GAP43 is a protein anchored to the membrane through palmitoylation of cysteine residues at the N-terminus (Arni *et al.*, 1998). In addition, this neuronal protein strongly segregates into lipid rafts (Laux *et al.*, 2000). Thus, we questioned whether anchoring to the cellular membrane and interaction with membrane rafts modulated the osmosensory activity of GAP43. To address this issue, both palmitoylation sites were mutated, giving rise to the single mutants C3A and C4A, and the double mutant C3A/C4A. Mutant proteins were expressed in HEK293 cells to evaluate hypotonicity-induced Ca^{2+} release, membrane location and lipid raft interaction. Functionally, replacement of either C3 or C4 or both by alanine drastically reduced the number of transfected cells responding to hypotonicity, as illustrated for the C3A/C4A double mutant (Figure 4A). Quantitative analysis showed that the number of mutant-transfected cells responding to 30% hypotonicity for C3A ($13 \pm 6\%$, mean $\pm$ SD, n (number of cells) = 150), C4A ($8 \pm 3\%$, $n = 230$) and C3A/C4A ($14 \pm 5\%$, $n = 150$) was not statistically different from that of untransfected HEK293 cells ($7 \pm 6\%$, $n = 250$) (Figure 4B). Biochemical analysis of the mutant proteins revealed that whereas incorporation of alanine residues at positions C3 or C4 did not abolish partitioning into membranes of mutated GAP43, simultaneous mutation of both cysteine residues (C3A/C4A) fully prevented membrane insertion of the protein, as evidenced by its absence in the low speed cellular pellets (Figure 4C, panel 1). However, mutation of either cysteine or both residues

Fig. 2. Swelling-activated Ca^{2+} signals involve release from intracellular stores. GAP43^{+} cells were loaded with Fura2-AM to record intracellular calcium signals and activated as indicated. Each panel shows superimposed traces of 15 individual cells recorded in the same experiment. (**A**) Extracellular Ca^{2+} (2.4 mM) was present throughout the experiment. After 1 min in standard isotonic solution, the tonicity of the solution was reduced to 70% for 5 min. (**B**) The external Ca^{2+} was removed from the medium (and 0.1 mM EGTA was added) 1 min before the start of the 30% hypotonic solution. (**C**) Responses evoked in GAP43^{+} cells incubated for 60 min in 2 μM thapsigargin to empty intracellular Ca^{2+} stores. Identical conditions to those in (A) were used. (**D**) Pre-treatment of GAP43^{+} cells with 100 μM carbachol in Ca^{2+}-free solution abolished the subsequent response to 30% hypotonicity.

notably impaired the segregation of the protein into membrane rafts, as suggested by the virtual absence of the three mutant species in the low density fractions of
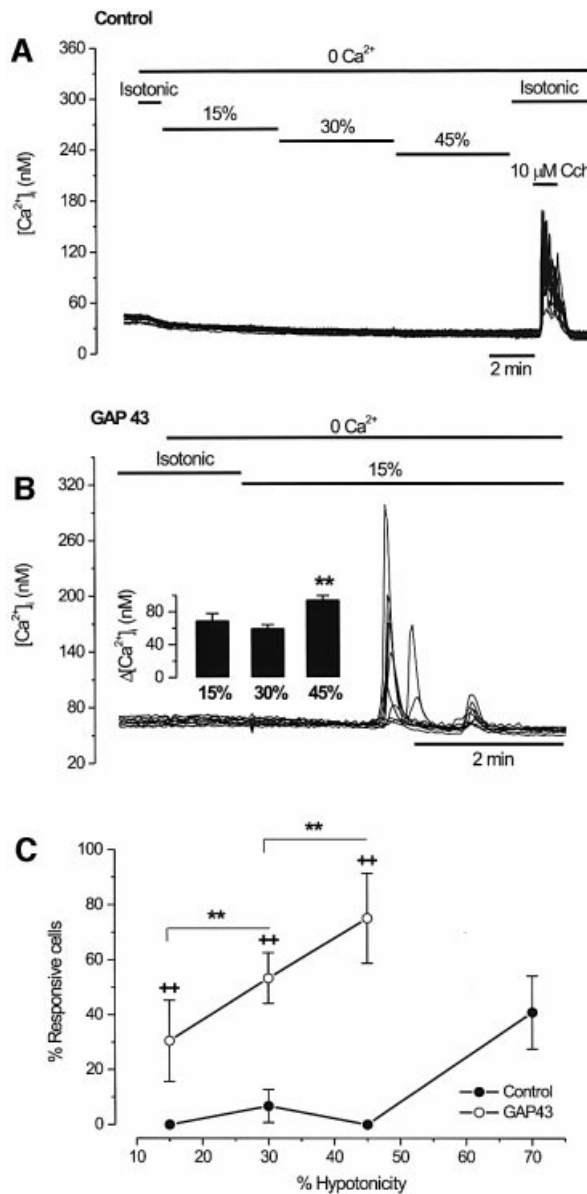
Triton X-100 flotation gradients (Figure 4C, panel 2). Taken together, these findings suggest that GAP43 osmosensory activity required the interaction of the protein with plasma membranes, primarily its association with membrane rafts.
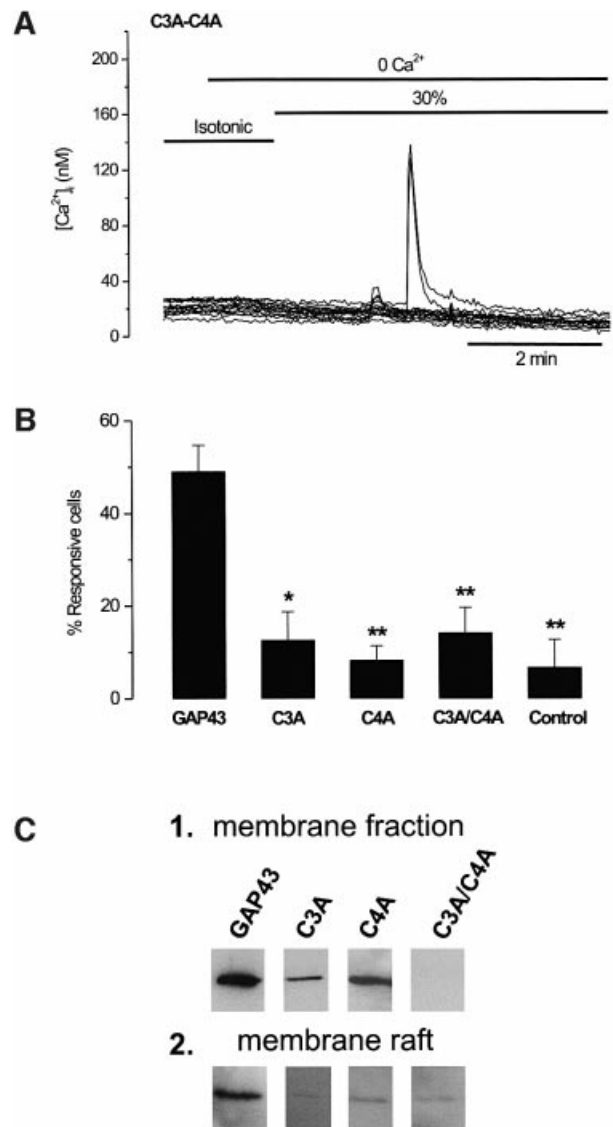

Fig. 3. Swelling-activated intracellular Ca^{2+} release is dose dependent. HEK293 cells were loaded with Fura2-AM to record intracellular calcium signals, and activated as indicated. (**A**) Untransfected HEK293 cells (control) were placed in a Ca^{2+}-free (0.1 mM EGTA) isotonic solution and stimulated with solutions of increasing hypotonicity (15, 30 and 45%) for 5 min. Note the absence of response to the osmotic stimulus and the robust response to muscarinic stimulation with 10 μM carbachol. (**B**) In GAP43$^+$ cells, a mild hypotonic stimulus (15%) in a Ca^{2+}-free solution evokes robust [Ca^{2+}]$_i$ signals. The inset shows the mean amplitude of [Ca^{2+}]$_i$ elevations for different degrees of hypotonicity. Cells were tested with a single level of hypotonicity. The mean amplitude of the [Ca^{2+}]$_i$ signals was larger for the strongest hypotonic stimulus. **$P < 0.05$ Mann–Whitney rank sum test with N (number of cells) $\geqslant 150$, and n (number of experiments) $\geqslant 3$. (**C**) The percentage of swelling-responsive cells as a function of extracellular hypotonicity. For each experiment, 50 cells in the field were marked at random and scored for a response if the [Ca^{2+}]$_i$ was >15 nM. Data are given as mean ± SEM with $N \geqslant 150$ cells, and $n \geqslant 3$. (++) and (**) denote the $P < 0.001$ significance (Z-test) for the hypotonic responses of GAP43-transfected versus untransfected cells, and for the different hypoosmolarities tested in GAP43$^+$ cells, respectively.

Fig. 4. The palmitoylation sites of GAP43 are important for the protein osmosensitivity. (**A**) [Ca^{2+}]$_i$ response in GAP43 C3A/C4A mutant-transfected cells during a 30% hypotonic stimulation in a Ca^{2+}-free solution. (**B**) Bar histogram showing the percentage of cells hypotonic-responsive to a stimulus protocol similar to that in (A) in wild-type GAP43$^+$ cells and after single and double mutations of the palmitoylation sites. Control denotes untransfected HEK293 cells. For each experiment, 50 cells in the field were marked at random and scored for a response if the [Ca^{2+}]$_i$ was >15 nM. Data are given as mean ± SEM with $N \geqslant 150$ cells, and $n \geqslant 3$. **$P < 0.001$ and *$P < 0.005$ significance (Z-test) of GAP43 mutants versus wild type. (**C**) Membrane fractions (1) and membrane rafts (2) from a cell expressing the wild-type GAP43 protein and mutant species. Membrane fractions denote crude plasma membranes. Lipid rafts were obtained from detergent-insoluble complexes in Triton X-100 flotation gradients. The figure depicts the top layer of the Optiprep discontinuous gradient. Crude membranes and rafts were separated by SDS–PAGE and analyzed by western immunoblot using an anti-GAP43 monoclonal antibody.

Integrity of the cortical cytoskeleton is not necessary for GAP43-dependent, hypotonicity-activated Ca²⁺ release

We next investigated the mechanism involved in GAP43-dependent, hypotonicity-induced release of Ca^{2+}. Because GAP43 can bind actin filaments and promote actin cytoskeleton assembly (Qin *et al*, 1997; Caroni, 2001), we evaluated whether modulation of the actin cytoskeleton
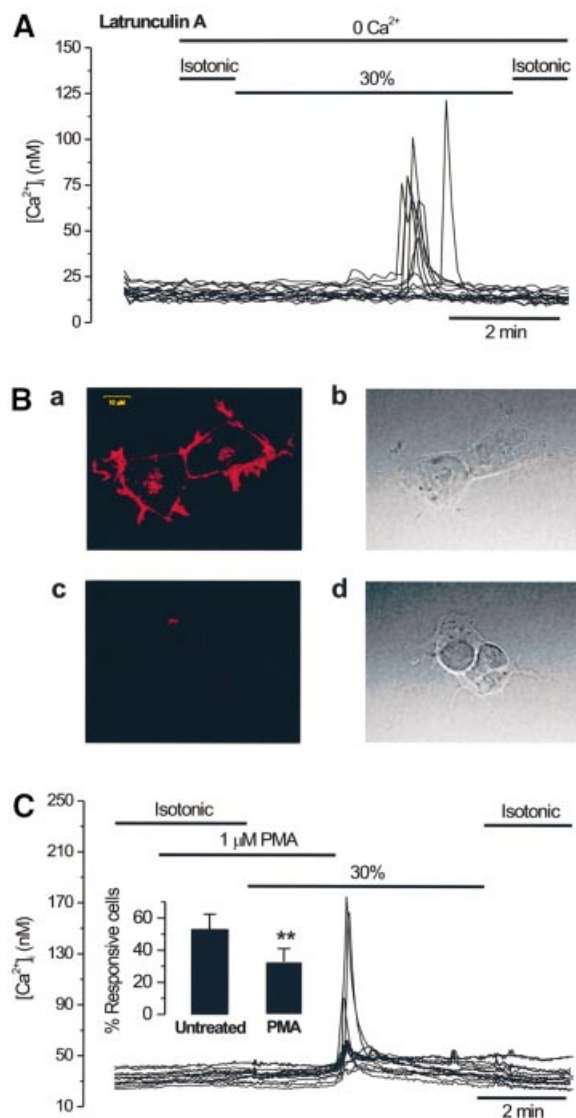

Fig. 5. GAP43-dependent, hypotonicity-activated Ca²⁺ release is not mediated by alterations in the actin cytoskeleton but is inhibited by PKC activation. (**A**) $[Ca^{2+}]_i$ response in GAP43⁺ cells during a 30% hypotonic stimulation in a Ca^{2+}-free solution, after incubating the cells with 1 μM latrunculin A for 35 min. (**B**) Latrunculin A disrupts the cortical cytoskeleton. Panels a and c display confocal images of phalloidin–rhodamine staining of untreated and latrunculin A-treated (1 μM, 35 min) HEK293 cells. Panels b and c depict light-transmitted images of the cells exhibited in panels a and c. The calibration bar for all images is shown in panel a. (**C**) Pre-incubation with 1 μM PMA for 2 min reduces the number of GAP43⁺ cells responding to hypotonicity in Ca^{2+}-free external medium. Inset: the percentage of untreated and PMA-treated GAP43⁺ cells that responded to hypotonicity. For each experiment, 50 cells in the field were marked at random and scored for a response if the $[Ca^{2+}]_i$ was >15 nM. Data are given as mean ± SEM with $N \geqslant 150$ cells, and $n \geqslant 3$. **$P < 0.001$ significance (Z-test).

was involved in the hypotonicity-evoked Ca^{2+} release. As illustrated in Figure 5A, incubation of GAP43⁺ cells with 1 μM of the actin-severing toxin latrunculin A for 35 min did not alter the percentage of cells that release Ca^{2+} in response to hypotonicity: $52 \pm 12\%$ ($n = 150$) for toxin-treated versus 53 ± 9 ($n = 450$) for untreated cells. A 10-fold increment in the toxin concentration resulted in a modest decrease in the number of cells responding ($39 \pm 8\%$, $n = 300$), but not in the magnitude of the intracellular Ca^{2+} increase. Under these experimental conditions, latrunculin A disrupted the cortical actin cytoskeleton. As illustrated in Figure 5B (panel a), the phalloidin–rhodamine staining pattern in HEK293 cells shows an F-actin network primarily concentrated at the cell cortex. Note the uniform fluorescent labeling of a narrow region beneath the plasma membrane. In marked contrast, latrunculin A-treated HEK293 cells were round and exhibited a more diffuse phalloidin labeling, with significant discontinuities in the periphery (Figure 5B, panels c and d). Therefore, the finding that disruption of the F-actin network does not markedly affect hypotonicity-induced Ca^{2+} release in GAP43⁺ cells suggests that an intact cortical actin cytoskeleton plays a marginal role in the osmosensory activity of GAP43. Nonetheless, we cannot rule out a contribution of the actin cytoskeleton downstream of Ca^{2+} release.

Activation of PKC signaling modulates GAP43-mediated hypotonic Ca²⁺ increases

Because GAP43 is a well known substrate of PKC (Widmer and Caroni, 1993; Qin *et al*., 1997), we next investigated whether activation of this protein kinase modulated the osmosensitivity of GAP43. Figure 5C shows that incubation of GAP43⁺ cells with 1 μM phorbol-12-myristate-13-acetate (PMA) reduced the number of cells that responded to hypotonicity-evoked $[Ca^{2+}]_i$ to $33 \pm 8\%$ ($n = 250$) without altering the amplitude nor the shape of the response. This result indicates a 40% reduction with respect to non-treated GAP43⁺ cells ($53 \pm 9\%$, $n = 450$) (Figure 5C, inset), and suggests that activation of PKC signaling modulates the cellular osmosensitivity, presumably by direct phosphorylation of GAP43.

GAP43-dependent, hypotonicity-evoked Ca²⁺ release requires activation of PLC

Cumulative evidence shows that GAP43 co-distributes with phosphatidylinositol-4,5-bisphosphate $[PI(4,5)P_2]$ in membrane rafts (Laux *et al*., 2000). $PI(4,5)P_2$ can be hydrolyzed by PLC to IP_3 which, in turn, can release Ca^{2+} from the ER. Thus, to determine whether formation of IP_3 mediates the hypotonicity-induced Ca^{2+} release in GAP43⁺ cells, we inhibited PLC activity with the aminosteroid U-73122 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione). As depicted in Figure 6A, treatment of GAP43⁺ cells with 3 μM U-73122 resulted in complete abrogation of $[Ca^{2+}]_i$ responses triggered by the hypo-osmotic challenge (Figure 6B). Surprisingly, in these cells, Ca^{2+} release evoked by carbachol was not fully blocked by 3 μM U-73122 treatment (Figure 6A). Quantitative analysis indicates that 3 μM U-73122 inhibited only 40% of 10 μM carbachol-evoked $[Ca^{2+}]_i$ responses in HEK293 cells (data not

shown). This result contrasts with the strong U-73122 blockade efficacy of muscarinic Ca^{2+} release in HEK293
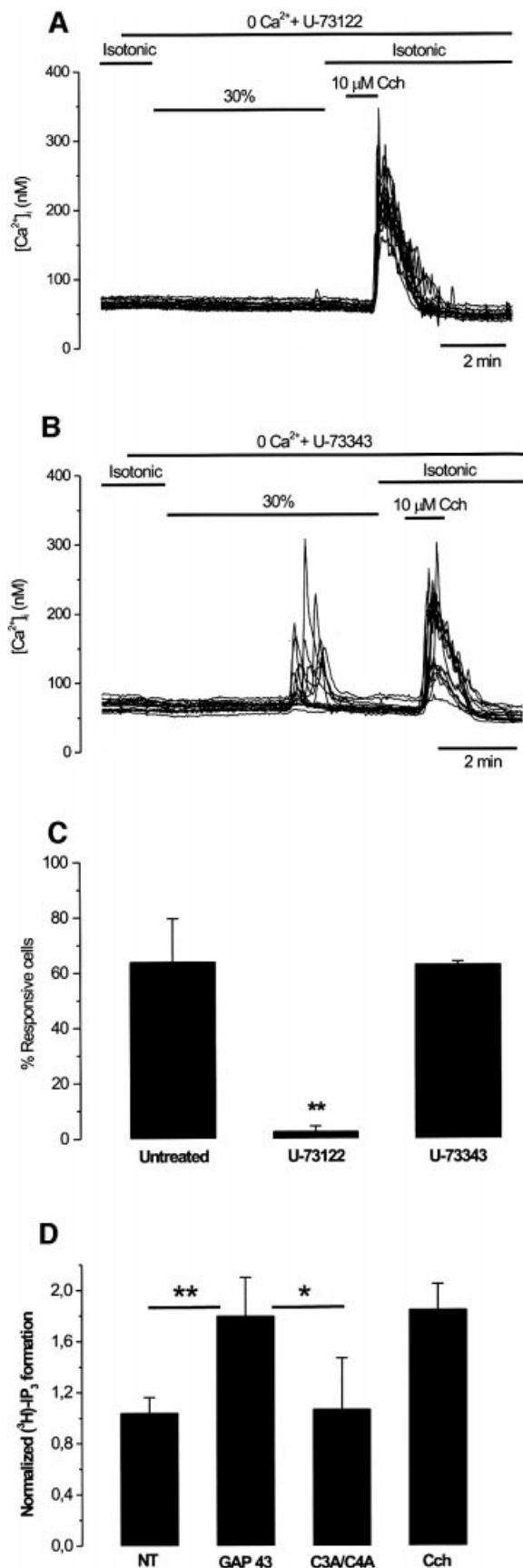
cells reported by Short *et al.* (2000), suggesting the existence of molecular differences between individual HEK293 cells. Nonetheless, significant variability of the muscarinic response to U-73122 inhibition has also been noticed by other groups in different cellular systems (Muto *et al.*, 1997; Cruzblanca *et al.* 1998; Suh and Hille, 2002).

At variance with U-73122, incubation of GAP43$^+$ cells with 3 μM U-73343 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione), an analog of U-73122 that does not inhibit PLC activity, did not affect the hypotonicity-activated [Ca^{2+}]$_i$ increases in GAP43$^+$ cells (Figure 6B). A quantitative summary of the results is shown in Figure 6C. Taken together, these data suggest that hypotonicity-evoked [Ca^{2+}]$_i$ elevations of GAP43$^+$ cells require the synthesis of IP$_3$ which, in turn, releases Ca^{2+} from IP$_3$-sensitive stores.

To substantiate this tenet further, we determined the levels of IP$_3$ in response to hypotonicity. As depicted in Figure 6D, the 30% hypotonic stimulus provoked an ~2-fold increase in the formation of IP$_3$ in cells heterologously expressing GAP43. This increase was similar to that elicited by carbachol. No increment in the IP$_3$ level was evoked in cells expressing the C3A/C4A GAP43 double mutant. Therefore, GAP43 appears to transduce hypo-osmotic stimuli into IP$_3$ formation.

Hypo-osmotic stress favors the association of GAP43 with PLC-δ_1

How does hypo-osmotic activation of GAP43 stimulate IP$_3$ production? We hypothesized that GAP43 may regulate PLC activity by interaction with a member of this enzyme family (Rhee, 2001). To test this hypothesis, we used an immunological approach aimed at determining whether complexes containing GAP43 and PLC can be immunopurified with a specific anti-GAP43 monoclonal antibody (mAb). The co-immunopurification of PLC isoforms was then evaluated by western immunoblotting using specific anti-PLC mAbs against isoforms β_1, γ_1 and δ_1. Figure 7A, panels b–d shows that the PLC-γ_1 and PLC-δ_1, but not the PLC-β_1 isoform were endogenously expressed in HEK293 cells. As shown, whole-cell extracts (WC lanes) exhibit an immunoreactive band of 140 kDa that matches that of PLC-γ_1 (Figure 7A, panel b), and a band of 68 kDa that correspond to a known proteolytic

Fig. 6. GAP43-dependent, hypotonicity-activated Ca^{2+} release involves IP$_3$ signaling. (**A**) Pre-incubation for 60 min with the PLC inhibitor U-73122 (3 μM) suppressed the hypotonicity-activated [Ca^{2+}]$_i$ response. Note that the response to 10 μM carbacol was unaffected. (**B**) Pre-incubation for 60 min with the inactive control compound U-73343 (3 μM) did not prevent the hypotonicity-activated [Ca^{2+}]$_i$ response. (**C**) Analysis of the effect of aminosteroid on the percentage of hypotonic-responsive (30% hypotonic stimulus) GAP43$^+$ cells. For each experiment, 50 cells in the field were marked at random and scored for a response if the [Ca^{2+}]$_i$ was >15 nM. Data are given as mean ± SEM with $N \geqslant 150$ cells, and $n \geqslant 3$. **$P < 0.001$ significance (Z-test) for non-treated versus U-73122-treated cells. (**D**) Hypotonicity induces the synthesis of IP$_3$ in GAP43$^+$ cells. The amount of [^{3}H]IP$_3$ was measured in cells labeled with [2-^{3}H]*myo*-inositol in isotonic and hypotonic external conditions. Normalized values refer to the ratio of [^{3}H]IP$_3$ after the stimuli (hypotonicity or carbachol) with respect to that before (isotonic). NT denotes non-transfected. Values are given as mean ± SD, with $n \geqslant 3$. **$P < 0.01$ and *$P < 0.05$ significance (Student's *t*-test) for GAP43 versus NT and GAP43 versus C3A/C4A double mutant, respectively.

fragment of PLC-δ_1 (Feng *et al.*, 1996) (Figure 7A, panel c). The absence of PLC-β_1 immunoreactivity (Figure 7A, panel d) could not be attributed to failure of the antibody since this enzyme isoform was readily detected in mouse brain extracts (Figure 7C). Under isotonic conditions, neither PLC isoform γ_1 nor δ_1 co-immunopurified with GAP43 (Figure 7A, panels a–c, IP lanes). This lack of GAP43–PLC association may be due to the very low affinity of both proteins. Thus, to address this issue, we attempted to cross-link both proteins using the cell-permeable, thiol-cleavable homobifunctional cross-linking reagent dithiobis(succinimidyl) propionate (DSP). Incubation of GAP43$^+$ cells with 1 mM DSP did not result in co-immunopurification of any PLC isoform using the anti-GAP43 antibody (data not shown), thus supporting the tenet that in isotonic conditions, GAP43 does not interact with these PLC isoforms. As a control for cross-linking activity, the well-known GAP43–calmodulin complex was covalently linked, as evidenced by the co-immunoprecipitation of calmodulin using the anti-GAP43 antibody in GAP43$^+$ cells (data not shown).

We next questioned if the interaction/association between PLCs and GAP43 may be favored by exposing the cells to a hypo-osmotic stimulus (Figure 7B). For this purpose, untransfected and GAP43-transfected cells were challenged with a 30% hypotonic stimulus for 5 min, followed by immunoprecipitation with anti-GAP43 mAb. Figure 7B (bottom) shows that PLC-γ_1 did not co-immunoprecipitate with GAP43. Similar results were obtained for this PLC isoform in the presence of the cross-linker DSP, further supporting the conclusion that PLC-γ_1 does not associate with GAP43 under hypo-

osmotic conditions (data not shown). Moreover, since tyrosine phosphorylation is a necessary condition for PLC-γ recruitment and activation, we evaluated whether inhibition of the src-family of tyrosine kinases abrogated GAP43-mediated osmosensitivity. Our findings show that treatment with 1 μM PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), a potent and specific inhibitor of the src family of tyrosine kinases, for 35 min did not alter the GAP43-stimulated release of Ca^{2+} in response to hypotonicity (see Supplementary data available at *The EMBO Journal* Online).

In marked contrast, hypotonicity clearly induced the association of GAP43 with PLC-δ_1, as demonstrated by the co-immunopurification of this enzyme isoform with GAP43 when an anti-GAP43 antibody was used (Figure 7B, bottom), thus indicating that both proteins associate when cells are under hypo-osmotic stress. Mutation of either one or both palmitoylation sites in GAP43 fully abolished the interaction between GAP43 and PLC-δ_1 (Figure 7D). Taken together, these findings suggest that the hypotonicity-induced association of GAP43 and PLC-δ_1 is a molecular event that underlies hypotonic Ca^{2+} release in GAP43$^+$ cells.

Discussion

Transduction of mechanical and osmotic stimuli is essential for a diversity of cellular processes (Hamill and Martinac, 2001). Thus a significant effort is being devoted to defining the molecular components implicated in mechano- and osmosensation, particularly those located in the plasma membrane that transduce external stimuli.
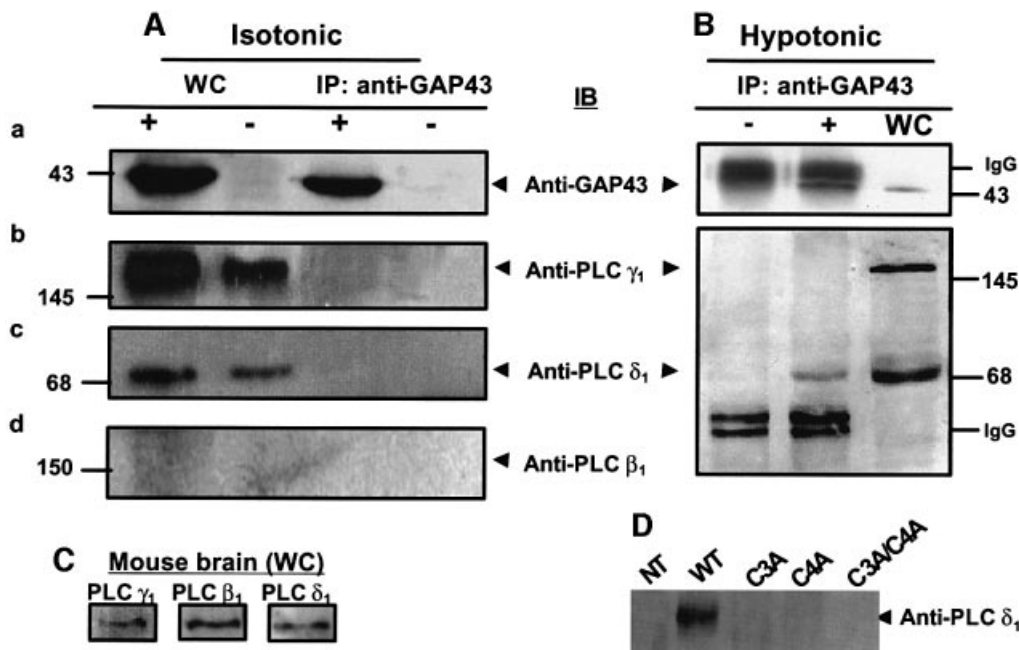

Fig. 7. Hypotonicity induces the selective association between GAP43 and PLC-δ_1. Whole-cell (WC) extracts from GAP43$^+$ cells (+) and untransfected cells (–) in isotonic (**A**) or 30% hypotonic media (**B**) were immunoprobed with specific antibodies against GAP43 (panel a), PLC-γ_1 (panel b), PLC-δ_1 (panel c) and PLC-β_1 (panel d), or immunoprecipitated with anti-GAP43 (IP) and thereafter immunoprobed with the displayed antibodies (IB). (**C**) Mouse brain extracts immunoblotted with anti-PLC isoform-specific antibodies. (**D**) Co-immunoprecipitation of PLC-δ_1 with GAP43 mutant species. Proteins were separated by 10 or 12% SDS–PAGE and electrotransferred onto cellulose membranes. Immunobands were revealed with ECL plus. Numbers indicate molecular weight markers in kDa.

We have screened a cDNA library from DRG to search for osmoreceptors. The mechanically insensitive HEK293 cell line was used to express recombinantly the cDNA clones contained in the library. The salient contribution of this study is the identification of GAP43, a 238 amino acid, hydrophilic, neuronal protein that plays a central role in axonal growth and synaptic plasticity (Aigner and Caroni, 1993, 1995; Dent and Meiri, 1998; Mueller, 1999; Bomze et al., 2001; Hulo et al., 2002), as an osmosensory protein that releases Ca^{2+} in response to hypotonicity, but not to hypertonicity. GAP43 appears to lower the threshold for signaling downstream of osmosensation, as suggested by the response of GAP43$^+$ cells to low-to-moderate hypotonic stimuli in comparison with the drastic hypotonicity required by untransfected HEK293 cells.

Structurally, GAP43 is a cytosolic protein anchored to the plasmalemma through palmitoylation of two cysteine residues at the N-terminus (Widmer and Caroni, 1993; Gamby et al., 1996; Qin et at, 1997; Laux et al. 2000). We found that the protein osmosensory activity was fully compromised by mutation of either palmitoylation site. This functional impairment was not correlated with the ability of the protein to integrate in the plasmalemma, since both single mutants still partitioned into the cell membrane. In contrast, however, we observed that removal of a single or both palmitoylation sites markedly prevented the segregation of the protein into membrane rafts. Therefore, palmitoylation of GAP43 is required for its osmosensitivity, primarily because of its importance for the segregation of the protein into membrane rafts. This conclusion is supported further by the finding that treatment with PMA, a phorbol ester that activates PKC, reduces GAP43 osmosensitivity, presumably as a result of GAP43 phosphorylation. PKC-mediated phosphorylation of GAP43 at Ser42 in the basic ED domain, or incorporation of negatively charged residues at this position, has been shown to weaken notably the binding of GAP43 to phosphoinositides (Laux et al. 2000). As a result, GAP43 interaction with lipid rafts is perturbed, affecting axonal growth and guidance (Laux et al., 2000; Caroni, 2001).

Mechanistically, hypotonicity-induced augmentation in [Ca^{2+}]$_i$ is due to release from the ER, as evidenced by its independence from external Ca^{2+} and abrogation after treatment with thapsigargin. A signaling pathway for this activity may involve alteration of the cortical actin cytoskeleton by GAP43 that could couple hypotonicity to Ca^{2+} release in GAP43$^+$ cells. This is plausible since GAP43 is known to interact with cytoskeletal proteins including actin (Qin et al., 1997; Caroni, 2001). However, the low sensitivity of GAP43 osmosensory activity to actin cytoskeleton disruption by the toxin latrunculin A argues against a major role for this transduction pathway.

Because GAP43 interacts with phosphoinositides (Laux et al. 2000), an alternative mechanism is the transduction of hypo-osmolarity to Ca^{2+} release through IP$_3$ signaling. The finding that hypotonicity induced an ~2-fold increment in the level of IP$_3$, along with the observation that inhibition of PLC activity completely abolished hypo-osmolarity-induced Ca^{2+} release in GAP43$^+$ cells, substantiates this notion. It is noteworthy that hypotonicity promoted the selective assembly of GAP43 with the PLC-δ_1 isoform, an event that could modulate the activity of the enzyme. Since GAP43 sequesters PI(4,5)P$_2$ into lipid rafts,

hypotonic stimulation could enhance the local recruitment of PLC-δ_1 to these plasmalemma microdomains of PI(4,5)P$_2$, thus facilitating its hydrolysis and formation of IP$_3$. This tenet is consistent with the finding that segregation of GAP43 into membrane rafts modulates its osmosensory activity.

A wealth of data demonstrate that GAP43 is implicated in morphogenic activity and anatomical plasticity in the nervous system, specially during development and axonal nerve regeneration. For instance, transgenic animals that overexpress the protein exhibit spontaneous and ectopic nerve sprouting, while GAP43 knockout mice display abnormalities in axon guidance and topography in the barrel cortex (Maier et al., 1999). All these findings suggest that GAP43 participates in signal transduction pathways in the growth cone that modulate the rate, extent and track of axons (Dent and Meiri 1998; Shen et al., 2002). In support of this notion, GAP43 interacts in a mutually exclusive way with several second messengers in nerve terminals, including calmodulin, PKC, the α-subunit of the GTP-binding protein Go or Gi proteins, rabaptin-5 and PI(4,5)P$_2$ (Widmer and Caroni, 1993; Chao et al., 1996; Gamby et al., 1996; Qin et al., 1997; Nakamura et al., 1998; Neve et al., 1998; Laux et al., 2000; Tejero-Diaz et al., 2000). The relevance of these interactions is still under intense investigation, although there is a strong argument for a critical role in modulating actin dynamics and motility (Caroni, 2001).

A central question arises as to what could be the physiological relevance of the osmosensory activity of GAP43. Recently, a unifying model for the regulation of the actin cytoskeleton through modulation of PI(4,5)P$_2$ rafts by GAP43-like proteins has emerged (Laux et al., 2000; Caroni, 2001). In this model, GAP43-mediated local sequestration of PI(4,5)P$_2$ into lipid rafts releases the inhibitory activity that the phosphoinositide exerts on actin-regulating proteins such as profilin, cofilin and gelsolin (Janmey, 1998; Laux et al., 2000). As a result, actin dynamics are augmented, cytoskeleton stability is decreased and growth cones are elongated. Our finding that GAP43 modulates PI(4,5)P$_2$ metabolism in response to osmotic stress is compatible with this model, and provides further insights into the mechanism by which this neuronal protein may modulate actin-based structures, as well as axon elongation and guidance. Hence, in isotonic conditions, the masking of PI(4,5)P$_2$ by GAP43 would promote actin cortex dynamics and, therefore, growth cone extension (Laux et al. 2000; Caroni 2001). Osmo/mechanical stimuli arising from growth or track changes would favor the association of PLC-δ_1 and GAP43 in membrane rafts, facilitating the formation of IP$_3$ which, in turn, would release Ca^{2+} from IP$_3$-sensitive stores. An increment in [Ca^{2+}]$_i$ would inhibit actin dynamics, thus promoting actin polymerization. Phosphorylation of GAP43 by PKC may also contribute by modulating its interaction with PI(4,5)P$_2$ (Caroni, 2001), as well as the protein osmosensitivity. Consequently, the actin cytoskeleton would be stabilized and axonal growth slowed or, even, collapsed. In support of this notion, growth cones generate Ca^{2+} transients with a frequency that is inversely proportional to the rate of axon outgrowth (Goldberg and Grabham, 1999; Gomez and Spitzer, 1999). Suppression of these cytosolic Ca^{2+} waves accelerates axonal exten-

sion, whereas its stimulation notably slows growth cones (Gomez and Spitzer, 1999). In this context, the osmotically induced, GAP43-mediated transient elevation of $[Ca^{2+}]_i$ may be used by growing axons to modulate temporally and locally the rate and trajectories of neuritic extension. Hence, the question that emerges is: do changes in osmotic/mechanical tension play a role in regulating axonal development? Cumulative evidence is making a case for regulatory effects of mechanical tension in axonal development (Zheng *et al.*, 1991; Heidemann and Buxbaum, 1994; Chada *et al.*, 1997). For instance, axonal retraction and guidance may involve active force generation by the neurite shaft (Chada *et al.*, 1997). Recently, it has been reported that endoneural tubes provide strictly mechanical guidance which, together with chemical cues, may contribute to axonal growth during peripheral regeneration (Fournier and Strittmatter, 2002; Nguyen *et al.*, 2002). These findings, taken together with ours, support the tenet that both mechanical and molecular stimuli are involved in growth cone and axonal guidance. The osmosensitivity of GAP43 furnishes a mechanistic framework that links axon elongation to the spontaneous triggering of cytosolic Ca^{2+} transients in the growth cone, the metabolism of phosphoinositides, regulation of cytoskeleton stability and the generation of regulatory osmo/mechanical forces.

In conclusion, we have reported that GAP43 is an osmosensory protein that triggers transient elevations of $[Ca^{2+}]_i$ in response to mild to moderate hypotonicity. The protein lowers the threshold for signaling downstream of osmosensation. The mechanism involves the activation of the IP_3-sensitive Ca^{2+} release channel, which appears to result from the specific association of GAP43 and PLC-δ_1 that may facilitate IP_3 formation. The use of an expression cloning approach and a recombinant system has unveiled an unsuspected function of GAP43 that may play an important role in modulating the rate, extent and trajectory of growing axons. Further experimental work, however, is necessary to understand the neuronal mechanism of GAP43 osmosensitivity and its relationship to axonal generation and synaptic plasticity.

Materials and methods

Expression cloning and Ca²⁺ imaging
A human DRG cDNA library (Invitrogen) was used. Independent bacterial clones (4.4×10^6), with an average insert size of 1.3 kb, were divided into 50 pools each containing ~3000 c.f.u. HEK293 cells were transiently transfected with 2 µg of plasmid DNA from individual pools using Lipofectamine 2000 (Invitrogen). The day prior to the transfection, HEK293 cells were trypsinized and replated in a 24-well plate, on coverslips treated with poly-D-lysine or laminin for 30 min, at a density of $2.5–3.0 \times 10^5$ cells per dish and maintained in supplemented Dulbecco's modified Eag;le's medium (DMEM). At 48 h post-transfection, cells were incubated with 5–10 µM Fura-2 AM with 0.02% pluronic acid (Molecular probes) for 1 h at 37°C for ratiometric calcium imaging (Viana *et al.*, 2001). Briefly, cells were perfused continuously (2–4 ml/min) in the recording chamber with isotonic solution warmed at 27 ± 1°C (66 mM NaCl, 3 mM KCl, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES pH 7.4, 141 mM D-mannitol; 310 mOsm/kg). Solutions of different osmolarities were employed. The osmolarity of each solution was measured using a cryoscopic osmometer (Gonotec). To maintain a constant ionic composition, only the concentration of D-mannitol was reduced in all hypo-osmotic solutions. In Ca^{2+}-free solutions, 100 µM EGTA was added to chelate residual Ca^{2+}. The perfusion could be switched to one of several test solutions via a multibarrelled stopcock.

Exchange of solutions was completed in ~20–30 s. For each library pool, 10–15 microscopic fields were analyzed.

Fluorescence measurements were carried out with a Zeiss Axioskop FS upright microscope fitted with a Sensys CCD camera (Roper Scientific) through an Olympus ×20 water immersion objective. Fura-2 was excited at 340 and 380 nm with a Lambda 10-2 filter wheel (Sutter Instruments), and emitted fluorescence filtered with a 510 nm long-pass filter. Calibrated ratios were displayed on-line with AIW software (Axon Instruments). Images were acquired and stored at 0.5 Hz. Data are reported as mean $\pm$ SEM. Statistical significance (*$P < 0.05$ and **$P < 0.001$) was assessed by Z-test as indicated.

Phalloidin–rhodamine staining and confocal microscopy
HEK293 cells seeded on glass coverslips were rinsed briefly in 0.1 M phosphate-buffered saline (PBS) and fixed for 15 min in 4% paraformaldehyde in PBS. Latrunculin A-treated (1 µM, 35 min) and untreated HEK293 cells were permeabilized for 10 min with 0.2% Triton X-100 in 0.1 M PBS and washed twice for 20 min with PBS. Subsequently, cells were incubated for 30 min in 1 µM phalloidin–rhodamine (Sigma) and washed for 30 min in PBS. Staining was visualized with an upright confocal laser scanning microscope (Olympus Fluoview 300) using a 543 nm helium/neon laser.

Isolation of plasma membrane fractions from transfected cells and immunoblotting
Crude plasma membranes were prepared essentially as described (Cabedo *et al.*, 2002). Briefly, transfected cells were washed with ice-cold PBS and lysed with buffer A [2 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM HEPES pH 7.4]. Cell lysates were centrifuged at 6000 *g* at 4°C for 10 min to prepare low speed pellets, which were washed with buffer B (buffer A + 1 M NaCl), shaken for 15 min at 4°C, centrifuged, and washed with buffer A for desalting. Final pellets, prepared from equivalent amount of cells, were mixed with β-mercaptoethanol-containing SDS sample buffer, boiled and separated by SDS–PAGE. Proteins were electrotransferred onto nitrocellulose membranes, blocked with 10% fat-free skim milk in Tris-buffered saline (TBS) and incubated with an anti-GAP-43 mAb (Chemicon). Membranes were washed with TBS–Tween (0.3%), incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG and developed with the enhanced chemiluminescence system (ECL; Amersham).

Preparation of membrane rafts
Analysis of detergent-insoluble complexes in flotation gradients was performed essentially as described (Cabedo *et al.*, 2002). Briefly, ~2.5×10^6 transiently transfected cells were washed with ice-cold PBS and scraped off in buffer C (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.5% Triton X-100), passed 10 times through a 29 gauge needle, extracted at 4°C for 30 min, and brought to 35% Optiprep. A 1 ml aliquot of the extract was overlayered sequentially with 8 ml of 30% Optiprep in 0.5× buffer C and 400 µl of buffer C in an SW41 tube. After centrifugation (178 000 *g* at 4°C for 4 h), 10 fractions were collected from the top to the bottom of the gradient; 200 µl of each fraction were precipitated with trichloroacetic acid, neutralized and analyzed by immunoblotting using the anti-GAP43 antibody.

Protein immunoprecipitation and immunoblotting
Protein immunoprecipitation was carried out as described (Caprini *et al.*, 2001). HEK293 cells were washed twice in PBS, scraped off from the wells into 1 ml of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 5 mM EDTA, 0.1 mM PMS, 0.01 mM iodacetamine, 0.5% sodium deoxycholate, 1% IGEPAL, 0.1% SDS), sonicated (four times for 10 s) and centrifuged at 14 000 r.p.m. for 30 min at 4°C. The supernatant was used as a whole-cell extract or immunoprecipitated with the anti-GAP43 mAb (3 µg of mAb/mg of protein) at 4°C overnight. Immunocomplexes were captured with agarose-conjugated protein G (75 µl; Pierce), washed six times with 500 µl of RIPA at 4°C, dissociated with 75 µl of SDS–PAGE sample buffer, boiled for 5 min, and analyzed by SDS–PAGE and immunoblotting. Nitrocellulose membranes were blocked with 3% bovine serum albumin (Sigma) and probed with anti-GAP43, anti-PLC-β_1, anti-PLC-δ_1 or anti-calmodulin (Upstate Biotechnology, USA) and anti-PLC-γ_1 (Chemicon, USA) mAbs.

Intact cell chemical cross-linking experiments
HEK293 cells were washed twice in PBS and incubated with 1 mM DSP (Pierce) at 22°C for 30 min following the manufacturer's instructions. The cross-linking reaction was stopped with 20 mM Tris–HCl pH 7.4. After incubating the reaction mixture for an additional 15 min, cells were

washed twice with ice-cold PBS, scraped off the dish, and cellular pellets were resuspended in RIPA buffer. Cross-linked complexes were immunopurified with the anti-GAP43 antibody. The material bound via the cross-linking agent to the GAP43 immune complex was released by incubating the washed beads for 30 min at 37°C with elution buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 0.2 M DTT, 1.2% β-mercaptoethanol), followed by a brief centrifugation. Immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with specific antibodies as indicated.

GAP43 mutation

The MLCC motif of the GAP43 protein was mutated to MLAC (C3A), MLCA (C4A) and MLAA (C3A/C4A) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All mutations were verified by sequencing.

IP$_3$ formation

HEK293 cells (2.5×10^6) were transiently transfected with GAP43 or C3A/C4A double mutant cDNA and pre-labeled with [2-^{3}H]myo-inositol ($2 \, \mu Ci/ml$) in Medium-199 (low IP$_3$ levels, Sigma) supplemented with 5% fetal calf serum for 48 h. Cells were then challenged with isotonic or hypotonic solution or 100 μM carbachol for 5 min. Formation of IP$_3$ was terminated with 5% ice-cold trichloroacetic acid. After neutralization, samples were passed through a HiTrapQFF column (Amersham Biosciences). [^{3}H]IP$_3$ was eluted with 1.1 M ammonium formate/0.1 M formic acid. Radioactivity was counted in a β-counter.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

References

Aigner,L. and Caroni,P. (1993) Depletion of 43-kDa growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. *J. Cell Biol.*, **123**, 417–429.

Aigner,L. and Caroni,P. (1995) Absence of persistent spreading, branching and adhesion in GAP-43-depleted growth cones. *J. Cell Biol.*, **128**, 647–660.

Arni,S., Keilbaugh,S.A., Ostermeyer,A.G. and Brown,D.A. (1998) Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. *J. Biol. Chem.*, **273**, 28478–28485.

Bomze,H.M., Bulsara,K.R., Iskandar,B.J., Caroni,P. and Pate Skene,J.H. (2001) Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nature Neurosci.*, **4**, 38–43.

Bourque,C.W. and Oliet,S.H.R. (1997) Osmoreceptors in the central nervous system. *Annu. Rev. Physiol.*, **59**, 601–619.

Cabedo,H., Luna,C., Fernández,A.M., Gallar,J. and Ferrer-Montiel,A. (2002) Molecular determinants of the sensory and motor neuron-derived factor insertion into plasma membrane. *J. Biol. Chem.*, **277**, 19905–19912.

Caprini,M., Ferroni,S., Planells-Cases,R., Rueda,J., Rapisarda,C., Ferrer-Montiel,A.V. and Montal,M. (2001) Structural adaptability between the putative voltage sensor of Kv channels and the prokaryotic KcsA channel. *J. Biol. Chem.*, **276**, 21070–21076.

Caroni,P. (2001) Actin cytoskeleton regulation through modulation of PI(4,5)P$_2$ rafts. *EMBO J.*, **20**, 4332–4336.

Chada,S., Lamoureux,P., Buxbaum,R.E. and Heidemann,S.R. (1997) Cytomechanics of neurite outgrowth from chick brain neurons. *J. Cell Sci.*, **110**, 1179–1186.

Chao,S., Benowitha,L.I., Krainc,D. and Irwin,N. (1996) Use of a two-hybrid system to investigate molecular interactions of GAP-43. *Mol. Brain Res.*, **40**, 195–202.

Colbert,H.A., Smith,T.L. and Bargmann,C.I. (1997) OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation and olfactory adaptation in *Caenorhabditis elegans*. *J. Neurosci.*, **17**, 8259–8269.

Cruzblanca,H., Koh,D.S. and Hille,B. (1998) Bradykinin inhibits M current via phospholipase C and Ca^{2+} release from IP$_3$-sensitive Ca^{2+} stores in rat sympathetic neurons. *Proc. Natl Acad. Sci. USA*, **95**, 7151–7156.

Dent,E.W. and Meiri,K.F. (1998) Distribution of phosphorylated GAP-43 (neuromodulin) in growth cones directly reflects growth cone behavior. *J. Neurobiol.*, **35**, 287–299.

Feng,J.-F., Rhee,S.G. and Im,M.-J. (1996) Evidence that phospholipase δ$_1$ is the effector in the G$_h$ (transglutaminase II)-mediated signaling. *J. Biol. Chem.*, **271**, 16451–16454.

Fournier,A.E. and Strittmatter,S.M. (2002) Regenerating nerves follow the road more traveled. *Nat. Neurosci.*, **5**, 821–822.

Gamby,C., Waage,M.C., Allen,R.G. and Baizer,L. (1996) Analysis of the role of calmodulin binding and sequestration in neuromodulin (GAP-43) function. *J. Biol. Chem.*, **271**, 26698–26705.

García-Añoveros,J. and Corey,D.P. (1997) The molecules of mechanosensation. *Annu. Rev. Neurosci.*, **20**, 567–594.

Gillespie,P.G. and Walker,R.G. (2001) Molecular basis of mechanosensory transduction. *Nature*, **413**, 194–202.

Goldberg,D.J. and Grabham,P.W. (1999) Braking news: calcium at the growth cone. *Neuron*, **22**, 423–425.

Gomez,T.M. and Spitzer,N.C. (1999) *In vivo* regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature*, **397**, 350–355.

Hamill,O.P. and Martinac,B. (2001) Molecular basis of mechanotransduction in living cells. *Physiol. Rev.*, **81**, 685–740.

Hart,A.C., Kass,J., Shapiro,J.E. and Kaplan,J.M. (1999) Distinct signaling pathways mediate touch and osmosensory responses in a polymodal sensory neuron. *J. Neurosci.*, **19**, 1952–1956.

Heidemann,S.R. and Buxbaum,R.E. (1994) Mechanical tension as a regulator of axonal development. *Neurotoxicology*, **15**, 95–107.

Hulo,S., Alberi,S., Laux,T., Mueller,D. and Caroni,P. (2002) A point mutant of GAP-43 induces enhanced short-term and long term hippocampal plasticity. *Eur. J. Neurosci.*, **15**, 1976–1982.

Janmey,P.A. (1998) The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiol. Rev.*, **78**, 763–781.

Laux,T., Fukami,K., Thelen,M., Golub,T., Frey,D. and Caroni,P. (2000) GAP43, MARCKS and CAP23 modulate PI(4,5)P$_2$ at plasmalemmal rafts and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.*, **149**, 1455–1471.

Liedtke,W., Choe, Y, Martí-Renom,M.A., Bell,A.M., Denis,C.S., Sali,A., Hudspeth,A.J., Friedman,J.M. and Heller,S. (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell*, **103**, 525–535.

Maier,D.L., Shyamala,M., Donovan,S.L., Soppet,D., Tessarollo,L., McCasland,J.S. and Meiri,K.F. (1999) Disrupted cortical map and absence of cortical barrels in growth-associated protein (GAP)-43 knockout mice. *Proc. Natl Acad. Sci. USA*, **96**, 9397–9402.

Mueller,B.K. (1999) Growth cone guidance: first steps towards a deeper understanding. *Annu. Rev. Neurosci.*, **22**, 351–388.

Muto,Y., Nagao,T. and Urushidani,T. (1997) The putative phospholipase C inhibitor U73122 and its negative control, U73343, elicit unexpected effects on the rabbit parietal cell. *J. Pharmacol. Exp. Ther.*, **282**, 1379–1388.

Nakamura,F., Strittmatter,P. and Strittmatter,S.M. (1998) GAP-43 augmentation of G-protein mediated signal transduction is regulated by both phosphorylation and palmitoylation. *J. Neurochem.*, **70**, 983–992.

Neve,R.L., Coopersmith,R., Mcphie,D.L., Santeufemio,C., Pratt,K.G., Murphy,C.J. and Lynn,S.D. (1998). The neuronal growth-associated protein GAP-43 interacts with rabaptin-5 and participates in endocytosis. *J. Neurosci.*, **18**, 7757–7767.

Nguyen,Q.T., Sanes J.R. and Lichtman,J.W. (2002) Pre-existing pathways promote precise projection patterns. *Nat. Neurosci.*, **5**, 861–867.

Qin,H., Dent,E.W. and Meiri,K.F. (1997) Modulation of acting filament behavior by GAP-43 (neuromodulin) is dependent on the phosphorylation status of serine 41, the protein kinase C site. *J. Neurosci.*, **17**, 3515–3524.

Rhee,S.G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.*, **70**, 281–312.

Shen,Y., Mani,S., Donovan,S.L., Schwob,J.E. and Meiri,K.F. (2002) Growth-associated protein-43 is required for commissural axon guidance in the developing vertebrate nervous system. *J. Neurosci.*, **22**, 239–247.

Short,A.D., Winston,G.P. and Taylor,C.W. (2000) Different receptors use inositol trisphosphate to mobilize Ca^{2+} from different intracellular pools. *Biochem. J.*, **351**, 683–686.

Strotmann,R., Harteneck,C., Nunnenmacker,K., Schultz,G. and Plant,T.D. (2000) OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat. Cell Biol.*, **2**, 695–702.

Suh,B.-C. and Hille,B. (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron*, **35**, 507–520.

Suzuki,M., Sato,J., Kutsuwada,K., Ooki,G. and Imai M. (1999) Cloning of a stretch-inhibitable nonselective cation channel. *J. Biol. Chem.*, **274**, 6330–6336.

Tejero-Diez,P., Rodríguez-Sanchez,P., Martin-Cofreces,N.B. and Diez-Guerra,F.J. (2000) bFGF stimulates GAP-43 phosphorylation at ser41 and modifies its intracellular localization in cultured hippocampal neurons. *Mol. Cell. Neurosci.*, **16**, 766–780.

Viana,F., de la Peña,E., Pecson,B., Schmidt,R.F. and Belmonte,C. (2001) Swelling-activated calcium signaling in cultured mouse primary sensory neurons. *Eur. J. Neurosci.*, **13**, 722–734.

Walker,R.G., Willingham,A.T. and Zucker,C.S. (2000) A *Drosophila* mechanosensory transduction channel. *Science*, **287**, 2229–2234.

Widmer,F. and Caroni,P. (1993) Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cells spreading and morphology. *J. Cell Biol.*, **120**, 503–512.

Zheng,J., Lamoureux,P. Dennerll,S.V., Buxbaum R.E. and Heidemann,S.R. (1991) Tensile regulation of axonal elongation and initiation. *J. Neurosci.*, **11**, 1117–1125.