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Regulation of Plasma Membrane Blebbing by the Cytoskeleton

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Abstract When neuroblastoma cells are exposed to lysophosphatidic acid (LPA), they undergo a vigorous, but transient blebbing phase. The effect is sensitive to inhibition by staurosporine, KT 5926 (an inhibitor of myosin light chain kinase), and cytochalasin B, suggesting that LPA activates the phosphorylation of myosin light chain and increases the contractile activity of the actomyosin network. Cell contractions increase the intracellular pressure driving bleb formation. Calyculin, an inhibitor of protein phosphatase2A, also causes blebbing which continues as long as the drug is present, presumably by keeping myosin light chain in the phosphorylated state. Blebbing of neuroblastoma cells is regulated by the status of all three cytoskeletal systems: disassembly of microtubules by nocodazole and of intermediate filaments by acrylamide increased the number of blebbing cells. Cytochalasin B, on the other hand, prevents bleb retraction and, after prolonged incubation, bleb formation. These results are discussed in terms of a model viewing the cytoskeleton as an integrated network transmitting force throughout the cell. Bleb retraction was studied by transfecting neuroblastoma cells with a vector containing the gene for γ-cytoplasmic actin fused to the green fluorescent protein EGFP (EGFP-actin). EGFP-actin was not detected on the membranes of extending blebs, but started accumulating along the cytoplasmic surface of blebs as soon as the extension phase came to an end and retraction set in. These results confirm earlier suggestions that actin polymerization is required for bleb retraction and for the first time directly relate the two events. J. Cell. Biochem. 73:488–499, 1999. © 1999 Wiley-Liss, Inc.

Key words: blebbing; lysophosphatidic acid; cytoskeleton; actomyosin; neuroblastoma; green fluorescent protein

Blebbing—the protrusion and subsequent retraction of plasma-membrane bound blebs on the surface of cells—has been observed in both physiological and pathological situations. Mesoderm cells moving away from the primitive streak of mouse embryos bleb vigorously during mitosis, an observation made in situ. [Nakatsuji et al., 1986]. Blebbing during mitosis has also been observed in tissue culture cells [Boss, 1955; Laster and Mackenzie, 1996]. After seeding cells in culture dishes, spreading is preceded by the formation of blebs [Erickson and Trinkaus, 1976; Hoglund, 1985; Bereiter-Hahn, et al., 1990; Cunningham, 1995]. Finally, blebs have been observed at the leading edge of moving cells [Trinkaus, 1980; Grinnell, 1982; Keller et al., 1985]. Neurons differentiating in primary culture show circus movements, i.e., blebs propagating around the cell circumference [Olson, 1996]. Blebs also form when cells are damaged, notably during apoptosis; apoptotic blebs, however, might be structurally different [Laster and Mackenzie, 1996]. Although the phenomenon is widespread, so far no function could be assigned to blebs. It is not even clear whether they have a physiological function, or whether they simply accompany certain physiological or pathological states of the cell.

Very few publications have considered the mechanical aspects of bleb formation. Albrecht-Buehler [1982] has suggested that blebbing reflects the convulsive flow of liquid through the cytoplasmic matrix. Cunningham [1995] supported this theory by showing that the state of crosslinking of the actin skeleton controls the extent of blebbing in tissue culture cells observed in the initial stage of spreading. Mutant cells lacking actin–binding protein (ABP, an actin crosslinking protein), showed prolonged blebbing. On the other hand, an increase of the F-actin content reduced blebbing frequency and rates of bleb expansion. The author also presented evidence that retraction itself was caused by actin polymerization in the bleb. From these

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results it was concluded that an increase in gelation counteracts convulsive flow and prevents or decreases blebbing. That the hydrostatic pressure and not actin polymerization are responsible for bleb formation and motility in Walker carcinoma cells has recently been demonstrated by Fedier and Keller [1997].

Lysophosphatidic acid (LPA) was shown to induce the retraction of neuronal processes in rat neuroblastoma cells [Tigyi and Miledi, 1992; Jalink et al., 1993, 1994]. When observing live neuroblastoma cells, we found that LPA also causes vigorous transient blebbing. The system turned out to be a good model for studying the mechanism of blebbing. Here we report that LPA induces blebbing by stimulating actomyosin contraction that the process is regulated by the state not only of the actin, but also of the microtubule and intermediate filament systems, and we directly demonstrate, using green fluorescent protein technology, how actin filaments are involved in the process of bleb retraction.

MATERIALS AND METHODS

Materials

The cell culture media DME and L-15 were from GIBCO (Burlington, Ontario). Acrylamide was obtained from Serva (Heidelberg, Germany), taxol and KT 5926 were from Calbiochem (San Diego, CA), cytochalasin B from Fluka (Buchs, Switzerland), lipofectamine from GIBCO, YO-PRO-1 iodide from Molecular Probes (Eugene, OR) and staurosporine and calyculin were from Alexis (Gelterkinden, Switzerland). Lysophosphatidic acid (LPA), polylysine, 2,3-butanedione monoxime (BDM), and nocodazole were from Sigma (St. Louis, MO). BDM was prepared as described [Cramer and Mitchison, 1995], staurosporine, KT 5926, taxol, nocodazole, and calyculin were prepared as stock solutions in DMSO at 100µM, 5 mM, 5 mM, 4 mg/ml, and 10µM, respectively, and cytochalasin B was dissolved in ethanol at 1.9 mM. Acrylamide was dissolved in DME at 400 mM and used at 4 mM.

Cells

NB2a rat neuroblastoma cells were cultured in DME containing 10% FCS. Cells were prepared for observation on an inverted microscope by plating them onto a polylysine-coated coverslip which was glued over a hole in the bottom of a 6 cm diameter petri dish. Before experiments the cells were washed twice with serum-free medium and subsequently starved for at least 6 h in DME without serum. Starved cells developed neurite-like processes. During experiments, DME was replaced by the phosphate-buffered medium L-15.

Vectors and Transfection

Vectors containing either the enhanced green fluorescent protein EGFP alone or MAP2c fused to the aminoterminus of EGFP were a gift from Dr. A. Matus, Friedrich Miescher-Institute, Basel. The fused genes are situated behind a full-length β-actin promoter. Their properties have been described [Ludin et al., 1996]. The EGFP-γ-actin construct was created by inserting the rat γ-actin sequence into the BsrGI restriction site immediatedly preceding the stop codon of EGFP. Cells were transfected using lipofectamine as prescribed by the manufacturer; 70–80% of the cells became transfected.

Microscopy and Image Processing

An inverted microscope (Axiovert 35, Zeiss, Zürich, Switzerland) was equipped with a slow scanning CCD camera (MicroMAX, Princeton Instruments, Inc.) and the image processing software Metamorph (Universal Imaging, West Chester, PA). Time-lapse recordings were obtained at 20-sec intervals, unless otherwise indicated. In order to minimize cell damage, the paths for both fluorescent and transmitted light were blocked by a shutter between recordings. The temperature was maintained at 37°C. In some cases (e.g., Fig. 4) a SIT camera from Hamamatsu was used as previously described [Hagmann et al., 1992, 1994]. EGFP-actin density (corresponding to actin-polymerization) was quantified by measuring the brightness of the fluorescent signal with the 'Line profile' command of the NIH-Image software package. A three pixel thickness of horizontal lines, placed across images of blebs, was used to increase the signal to noise ratio by averaging across columns of data (see NIH-Image manual). The density of EGFP-actin was obtained directly from the change in area under such line profile plots, confined to the region of a bleb's membrane and is given as number of pixels comprising this area. Since the width of the lining representing the region of actin-polymerization

does not change significantly the measure is of brightness/unit area.

RESULTS

LPA Induces Transient Blebbing in Neuroblastoma Cells

When NB2a cells that had been deprived of serum for 6 h or longer were exposed to 1μM LPA, vigorous blebbing set in almost immediately (Fig. 1). Blebs formed rapidly: the distance between the base and the apex increased at a maximal rate of 0.42μm/min (standard deviation ± 0.17μm/min, n = 6). Retraction of blebs occurred more slowly, at a maximal rate of 0.23μm/min ± 0.02μm/min, n = 5). Average rates of bleb formation or retraction would be meaningless, because extension slows down until the maximal point of extension is reached, whereas the speed of retraction initially increases until the maximum is achieved. Most blebs were spherical with a straight base. However, some had irregular, tongue-like outlines, whereas others assumed the shape of long tubes (not shown). Sometimes, new blebs grew out of older ones, and this is how we think the tubes arose.

The formation of blebs was preceded by a sudden transient contraction of the cell body (Fig. 2, compare positions of the triangle at 10 and 30 sec). The contraction was real and not due to a shift of focus. It could be observed in all cells, but while most of them subsequently extruded blebs, a minority did not (Table I). Finally, as described by others [Jalink et al., 1993, 1994], processes were retracted in response to LPA.

Soluble cytosolic molecules have ready access to the interior of blebs, as is demonstrated by the fact that unpolymerised MAP2-EGFP expressed in transfected cells fills blebs as quickly as they are formed (Fig. 3). Microtubules, on the other hand, were always excluded: cells transfected with a vector containing the microtubule-binding protein MAP2c which stains microtubules [Kaech et al., 1996] never showed microtubular structures inside blebs. Blebs were also devoid of organelles. This was best seen

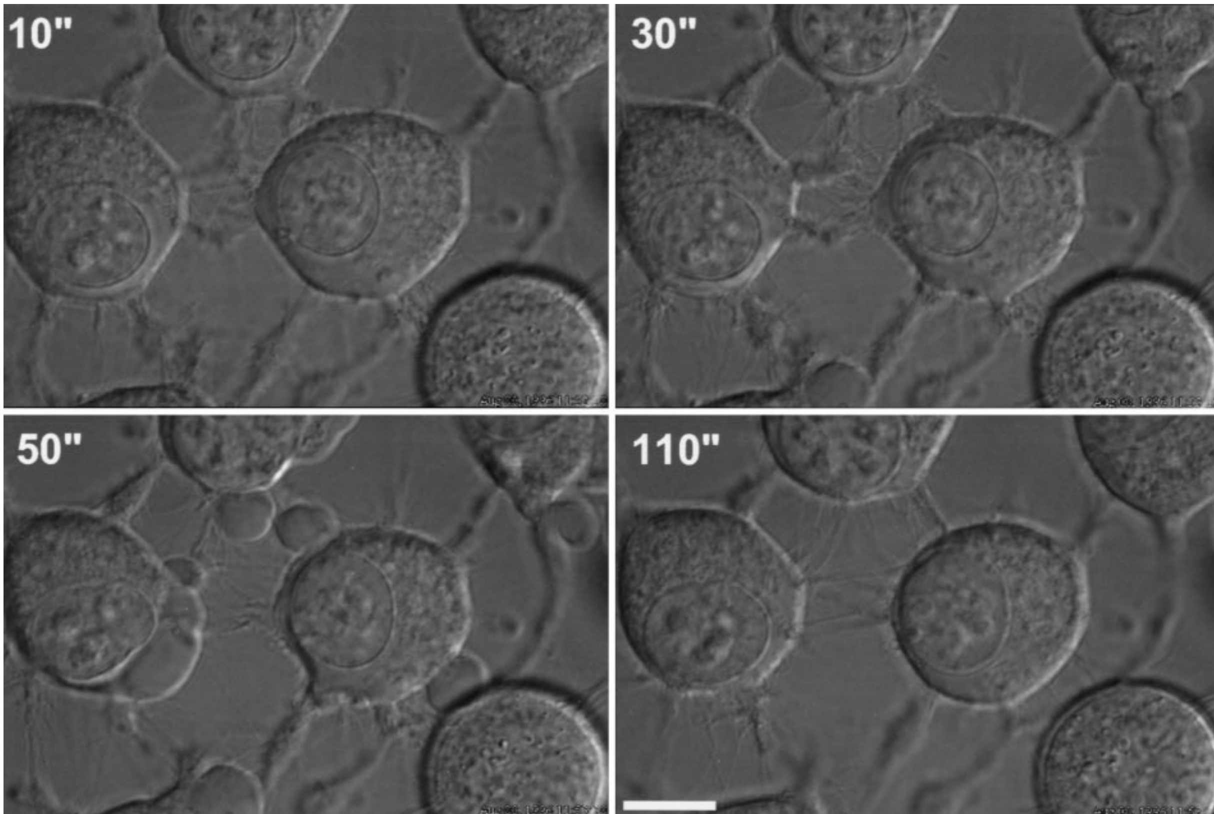

Fig. 1. DIC images of neuroblastoma cells exposed to 1μM LPA. Time is indicated in seconds after the addition of LPA. Scale bar = 5μm.

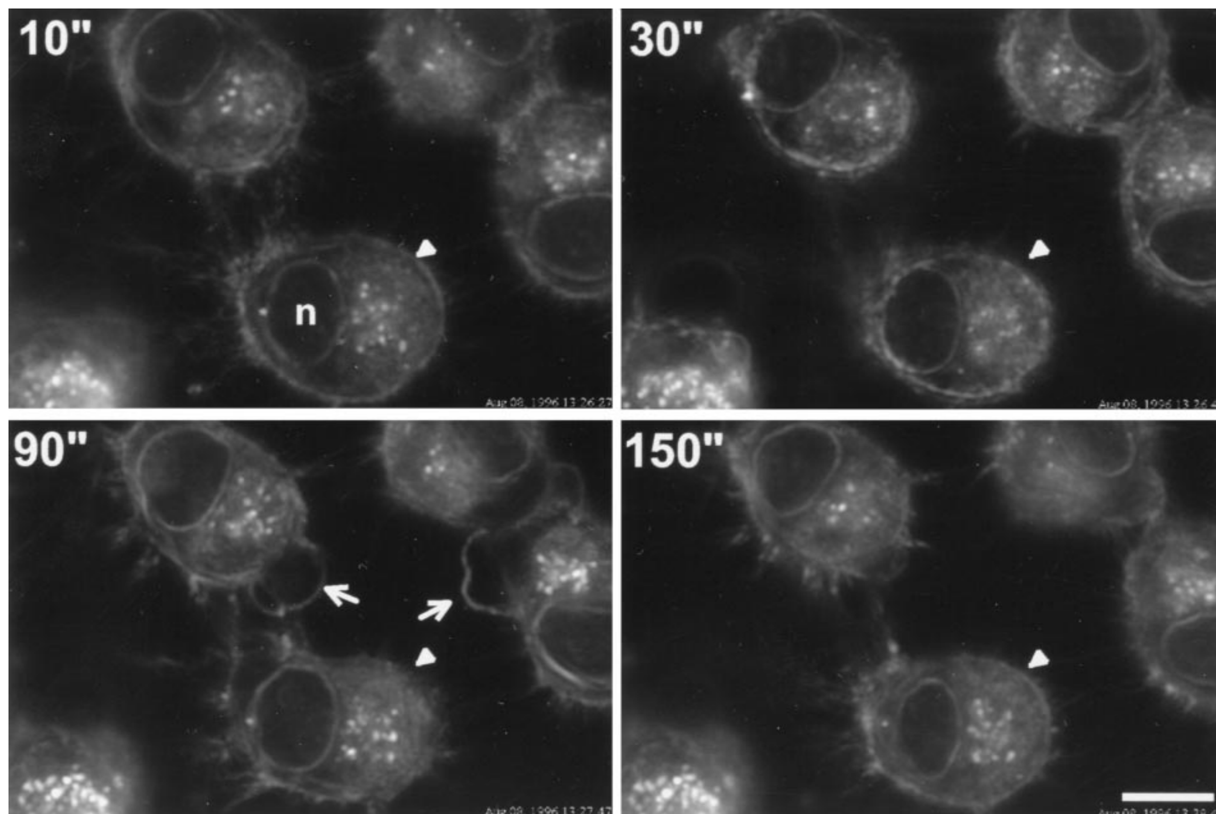

Fig. 2. Darkfield images of neuroblastoma cells stimulated with 1 μM LPA showing cell contraction, the absence of light scattering organelles in blebs and the deformation of nuclei. The arrows point at two blebs and the triangle marks the same portion in all four panels illustrating the contraction after addition of LPA. n = nucleus. Time is indicated in seconds after the addition of LPA. Scale bar = 5 μm.

using darkfield microscopy, because membraneous organelles scatter light strongly (Fig. 2). Only occasionally small vesicles were found included in blebs, but unlike cytoplasmic vesicles, those within blebs moved in a Brownian fashion, demonstrating that they were not tethered to cytoskeletal elements (not shown).

The contraction of the actomyosin system not only causes blebbing, but has also an effect on the nucleus: during the blebbing phase, the nucleus was deformed, a phenomenon most evident in dark field microscopy (Fig. 2).

In most cells, LPA-induced blebbing was transient. However, the duration of the blebbing stage was very variable between individual cells and also between experiments. Whereas sometimes blebbing stopped after only 2–3 min, other cells were still blebbing when, after 30 min, the experiment was terminated. Even prolonged blebbing did not compromise the barrier function of the plasma membrane, as judged by the fact that the fluorescent probe YO-PRO-1 iodide which enters cells before other signs of cell damage are visible, didn't accumulate inside blebbing cells (data not shown).

Blebbing is Independent of Calcium

Chelating extracellular calcium with 2 or 5 mM EGTA did not prevent blebbing, showing that the formation of blebs does not depend on the influx of calcium (Table I). Blebbing was also not affected when the intracellular calcium concentration was clamped at low levels by the cell-permeable chelator BAPTA-AM (Table I). Either, intracellular calcium is not involved in the phenomenon, or whatever low level persists after intracellular accumulation of BAPTA is sufficient, and no transient changes in Ca^{2+} concentration are required.

Blebbing is Inhibited by Staurosporine

Early in the chain of signals transmitted by LPA there is a link sensitive to staurosporine and other tyrosine inhibitors [Jalink et al., 1993; Hordijk et al., 1994]. Since staurosporine also

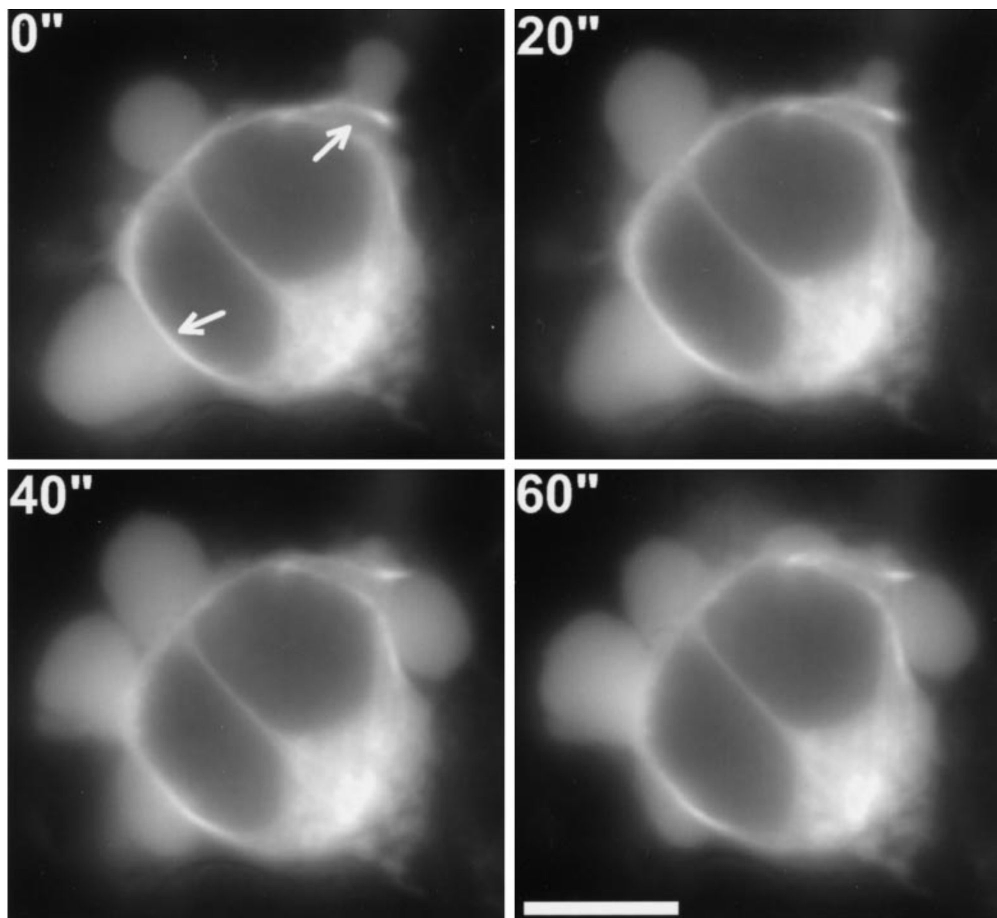

Fig. 3. Fluorescence image of a blebbing neuroblastoma cell transfected with MAP2c-EGFP and stimulated with 1 μM LPA. The cell has two nuclei. Time is indicated in seconds. Scale bar = 10μm.

blocks blebbing induced by LPA (Table I), the pathway leading to the formation of blebs is, at least initially, most likely the same as the one resulting in the retraction of neuronal processes and the activation of the MAP kinase cascade.

Bleb Formation is Regulated by the Activity of the Actin-Myosin Network

The formation of blebs is dependent on hydrodynamic pressure in the cell [Albrecht-Buehler, 1982]. This pressure is regulated by the degree of cross-linking of the actin skeleton [Cunningham, 1995], but it may also be modified by the tension generated by actomyosin ATPase. That this is the case, is shown by the following experiments.

As expected, cytochalasin B which inhibits the polymerization of actin affected blebbing induced by LPA. After incubation with 1μM cytochalasin B for 25 min, blebs formed, but did not retract (Table I), an observation previously reported by Cunningham for blebs formed during the initial stage of spreading after plating melanoma cells in tissue culture dishes [Cunningham, 1995]. After 2 h of incubation with 2μM of the drug, bleb formation was inhibited (Table I).

The sudden contraction of neuron cell bodies after addition of LPA and the rapid onset of blebbing that follows contraction suggest that not a modification of the cross-linking state of the actin skeleton, but increased tension applied to the actin network is responsible for bleb formation. Indeed, when the neuroblastoma cells were incubated with KT 5926, a specific inhibitor of myosin light chain kinase, both the initial contraction and blebbing were completely blocked (Table I). Butanedione monoxime (BMD), on the other hand, which was reported to inhibit actin-myosin interaction by inhibiting myosin ATPase activity [Higu-

TABLE I. Effect of Drugs on Number of Blebbing Cells[a]

Treatment	Before LPA (%)	After LPA (%)	n[b]
Control	<1	71.4 ± 9.1	14
EGTA (5 mM)	<1	61.1 ± 9.5	4
BAPTA (30 μM)	19.4 ± 4.8	84.5 ± 5.0	3
Staurosporine (0.5 μM)	<1	0.7 ± 1.2	3
KT 5926 (2 μM)	<1	64.2 ± 25.7	2
KT 5926 (10 μM)	<1	3.0 ± 1.7	3
BDM (20 mM)	<1	67.6 ± 6.8	3
KT5926 (2 μM) + BDM (20 mM)	<1	3.3 ± 5.8	3
Cytochalasin B (1 μM, 25 min)	<1	82.5	1
Cytochalasin B (2 μM, 2 h)	<1	34.1 ± 4.0	3
Nocodazole (10 μg/ml)	28.0 ± 11.0	74.9 ± 4.4	3
Taxol (20 μM)	<1	64.8 ± 6.1	3
Acrylamide (4 mM)	24.7 ± 2.4	100 ± 0	3

[a]Percent blebbing cells before and after stimulation with 1 μM LPA. One-hundred cells were counted per experiment. The cells were preincubated with the drugs for 30 min (BAPTA), 1 h (staurosporine, KT5926, and BDM), 25 min and 2 h (cytochalasin B), 7 h (nocodazole and taxol) and 15 h (acrylamide).
[b]Number of experiments.

chi and Takemori, 1989] and which proved to be effective in PtK2 cells [Cramer and Mitchison 1995], had no effect on blebbing up to a concentration of 20 mM and a preincubation time of 1 h (Table I). However, together with a suboptimal concentration of KT 5926, BMD blocked blebbing (Table I). Finally, the protein phosphatase 2A inhibitor calyculin induced very intensive and prolonged blebbing. Cells not only kept blebbing for as long as the experiment lasted (at least 3 h), they also carried a large number of blebs each. Often the cell body was invisible underneath the many blebs (Fig. 4).

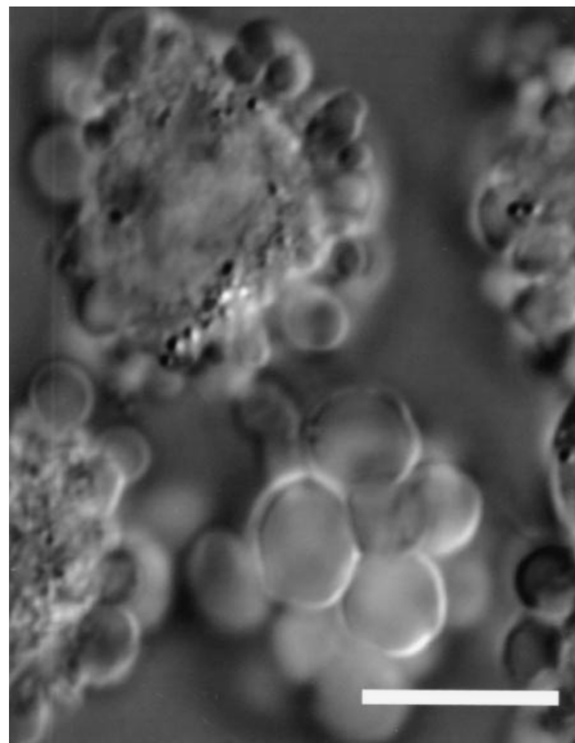

Fig. 4. DIC image of blebbing neuroblastoma cells treated with 5 μM calyculin. Scale bar = 5 μm.

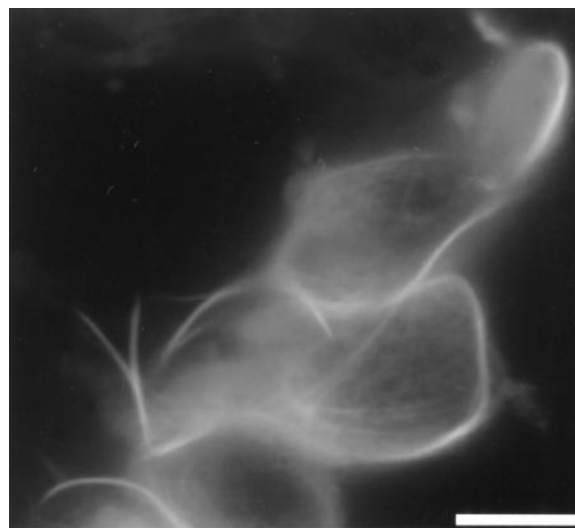

Fig. 5. Fluorescence image of neuroblastoma cells transfected with MAP2c-EGFP. Scale bar = 5 μm.

Forces Opposing Tension Generated by Actomyosin

The contractile force of the actin-myosin network is opposed by, on the one hand, attachment to the extracellular matrix, and on the other hand microtubules which serve as internal struts [Danowski, 1989; Ingber, 1993]. When microtubules were disrupted by preincubating neuroblastoma cells with nocodazole, spontaneous blebbing occurred in 28% of the cells (Table I). Addition of LPA brought the number of cells forming blebs to the same level as LPA-treated control cells (Table I). Taxol, on the other hand, which renders microtubules more flexible [Felgner et al., 1996], did not prevent blebbing induced by LPA (Table I). MAP2c binds to microtubules and stiffens them [Kaech et al., 1996].

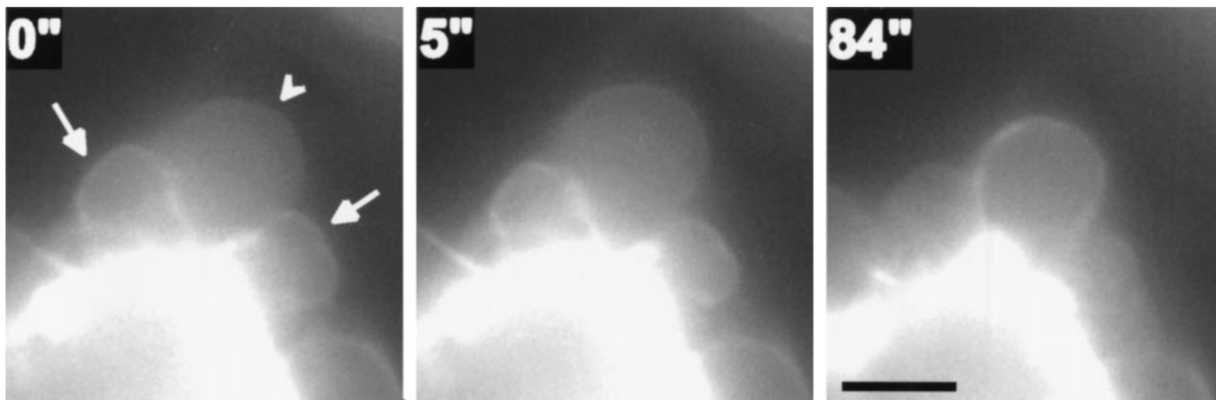

Fig. 6. Fluorescence images of a neuroblastoma cell transfected with EGFP-actin and stimulated with 5 μM calyculin. The arrows are pointing at two reacting blebs, the arrowhead at an expanding bleb. After 84 seconds, the bleb in the center is retracting, whereas the two retracting blebs have been replaced by new extending ones. The time intervals between the panels are indicated. Scale bar = 5μm.

In order to see whether overexpression of MAPs in NB2a cells which do not express MAPs themselves stiffens their microtubules sufficiently to resist blebbing, we transfected them with a MAP2c-GFP fusion protein. Observation of live transfected cells showed that microtubules bound the fusion protein, and in many cells thick bundles of microtubules hold together by Map2-GFP lined the periphery of the cell and reached into the processes (Figs. 3 and 5), as has been shown for fibroblasts [Kaech et al., 1996]. When MAP2c-GFP transfected cells were exposed to LPA, 63.1 ± 18.8% of those exhibiting bundles formed blebs, whereas 68.2 ± 18.4% of the untransfected cells in the same dish blebbed (4 experiments, 100 cells per experiment were counted). The "softening" and stabilization of microtubules by taxol or MAP2c overexpression, respectively, was thus not sufficient to overcome the LPA-induced force causing bleb formation.

The network of intermediate filaments is disrupted by acrylamide. We examined the effect disruption of intermediate filaments had on the blebbing response by incubating neuroblastoma cells overnight in 4 mM acrylamide and then exposing them to LPA. Incubation with acrylamide increased the number of cells blebbing spontaneously (Table I). After stimulation with 1μM LPA, most cells started blebbing vigorously. Contractions and blebbing appeared to be stronger than in control cells not exposed to acrylamide. While some acrylamide-treated cells went on blebbing, many ceased doing so, but entered a second round of blebbing after about 50 min (not shown).

Actin Distribution in Blebs

It has been proposed that, whereas bleb formation is driven by intracellular pressure, retraction sets in when actin polymerises along the cytoplasmic surface of a bleb [Cunningham, 1995]. We tried to correlate the two events—bleb dynamics and actin polymerization—directly by using green fluorescent protein technology. The gene for γ-actin was fused into the 3′-terminus of the EGFP gene as described in the Methods section, and neuroblastoma cells were transfected with the construct. The fusion protein was incorporated into stress fibers and ruffles of fibroblasts, indicating that it is capable of being incorporated into cellular actin polymers (not shown). Transfected cells were treated with calyculin in order to achieve prolonged blebbing phases that could be studied conveniently. During bleb extension, the rim was hardly visible by fluorescence microscopy, indicating that no or at most a very low amount of actin polymerization, below the level of detection, took place. However, once the extension phase ended and retraction set in, blebs were lined by a layer of increasing brightness (Fig. 6). When the brightness of a section of the bleb margin was quantitated and compared to the diameter of the bleb, it could be shown that appearance of the fluorescent lining correlated to a leveling off of the bleb diameter. Figure 7 is an example of six experiments all yielding qualitatively identical results. Sometimes actin polymerization occurred along a section of the bleb only. In these cases, blebs were pinched because the actin coated part contracted while the remainder of the bleb continued extending (or

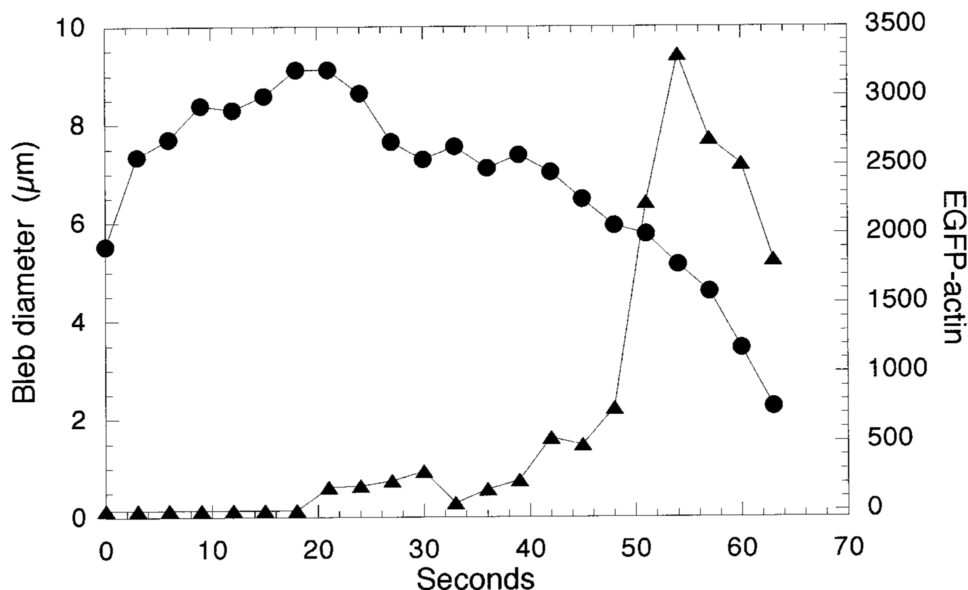

Fig. 7. Plot of bleb diameter and the density of EGFP-actin (arbitrary units) forming the inner lining of a bleb vs. time. The density of EGFP-actin was obtained directly from the change in area under a line profile plot, confined to the region of a bleb's membrane and is given a number of pixels comprising this area. The cell was stimulated with 5μM calyculin.

remained stationary; Fig. 8). After completion of the retraction phase, a "scar" of accumulated actin marked the site where the bleb had been (not shown).

DISCUSSION

Blebbing of cells has been observed in vivo and in vitro: cultured cells bleb during mitosis [Boss, 1955; Laster and Mackenzie, 1996], during the initial stages of spreading after plating them in tissue culture dishes [Forsby et al., 1985; Cunningham, 1995], and, in some cases, during locomotion [Trinkaus, 1980; Grinnell, 1982; Keller et al., 1985]. That cells undergoing mitosis can display blebbing in situ has been shown in mouse embryos [Nakatsuji et al., 1986]. Although so far no functions could be assigned to blebbing, suppression of bleb formation in Walker carcinosarcoma cells by hypertonic media also inhibited locomotion [Fedier and Keller, 1997]. On the other hand, the blebs which form in damaged or apoptotic cells seem to differ from the cases mentioned in that their blebs, sometimes called blisters, do not acquire a coat of polymerised actin on the cytoplasmic side and do not retract [Endresen et al., 1995; Laster and Mackenzie, 1996].

When neuroblastoma cells are treated with either thrombin [Jalink and Moolenaar, 1992; Suidan et al., 1992] or LPA [Jalink et al., 1993, 1994], they retract their processes and round up. Suidan et al. [1992] noticed that thrombin induced transient blebbing of NB2a neuroblastoma cells. We also observed vigorous transient blebbing in neuroblastoma cells treated with supernatants of human platelet suspensions. Boiling of the supernatant did not prevent the effect, but phospholipase treatment of the supernatant abolished it (J.H. and D.D., personal observation). We therefore concluded that LPA released from platelets is most likely the active agent. Treating neuroblastoma cells with LPA provided us with a convenient system for studying the mechanics of bleb formation and retraction. Similar responses of neuronal cells might also occur during brain haemorrhage, when platelets release thrombin, LPA and many other neurotransmitters and ligands. The phenomenon could therefore have pathological significance.

The force responsible for bleb formation is believed to be hydrodynamic pressure within the cell [Trinkaus, 1973; Dipasquale, 1975; Albrecht-Buehler, 1982; Cunningham, 1995]. As pressure increases or the cortical actin skeleton becomes weaker, nearly hemispherical blebs form which, initially, are devoid of polymerised actin. New blebs form when old ones are being retracted, and the volume changes of extending and retracting blebs are closely related [Albrecht-Buehler, 1982]. We found that all these properties also hold for LPA-induced blebbing in neuroblastoma cells.

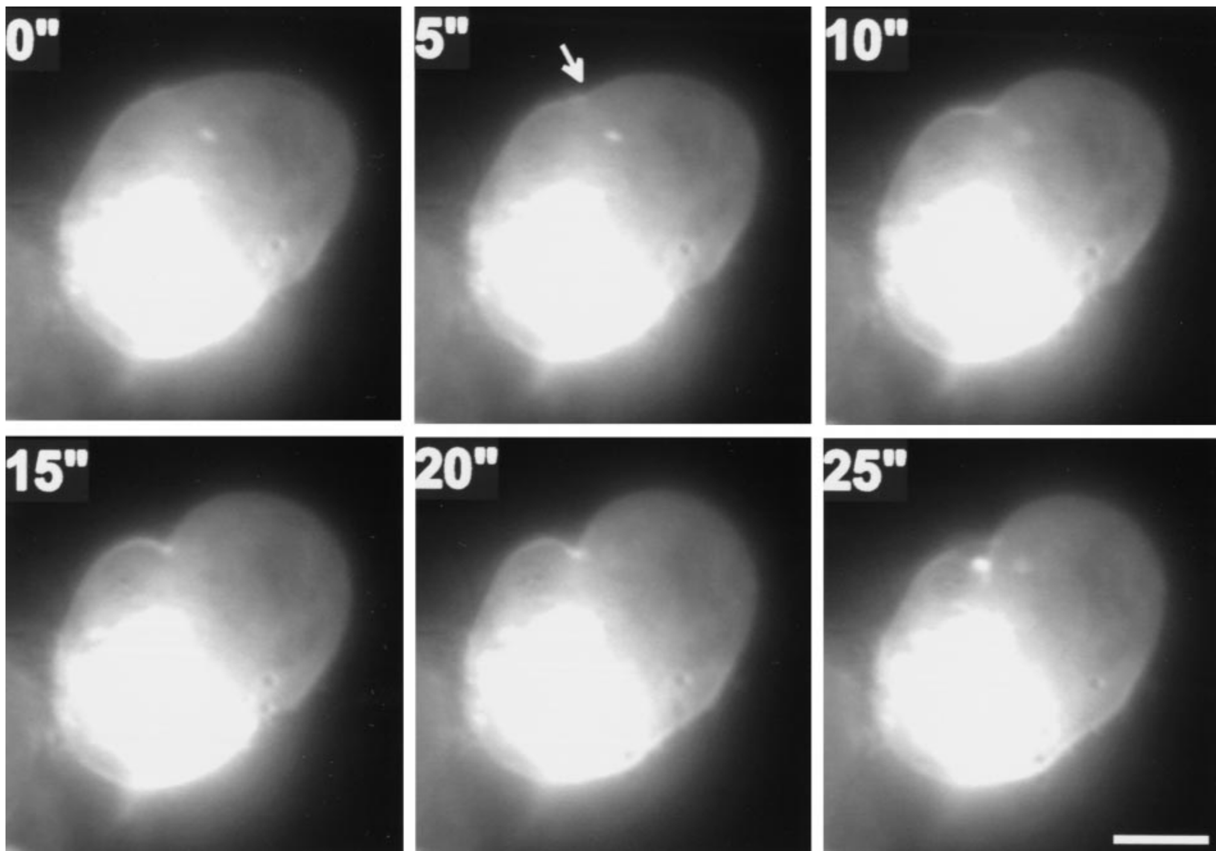

Fig. 8. Fluorescence images of a blebbing neuroblastoma cell transfected with GFP-actin illustrating pinching of a section of the bleb. The site where the pinch is forming is indicated by an arrow in panel 2. The cell was treated with 5 nM calyculin. Intervals between panels: 5 sec. Scale bar = 5 µm.

Theoretically, bleb formation might be regulated by modifying either the contractile force of the actin-myosin system or the state of actin polymerization and crosslinking. In an elegant study, Cunningham showed that in cells lacking actin-binding protein (ABP) blebbing was prolonged during the initial stages of spreading after plating [Cunningham, 1995]. Since ABP crosslinks actin filaments at right angles [Hartwig and Shevlin, 1986], this observation proves that the state of the actin network is indeed a regulatory factor. Given the sudden contraction of neuroblastoma cells that immediately precedes blebbing after addition of LPA, it was reasonable to assume that in this case an increase in contractile force, rather than a weakening of the actin skeleton, is responsible for bleb formation. LPA binds to receptors on the cell surface and triggers different G-protein-mediated signalling pathways, resulting in a number of effects such as cell proliferation, chemotaxis, early gene transcription, stress fiber formation and neurite retraction [reviewed in Durieux and Lynch, 1993; Moolenaar, 1994; Moolenaar et al., 1997]. We hypothesized that the path leading to blebbing involves, as in the case of neurite retraction, myosin light chain–phosphorylation to actomyosin contraction. We confirmed this hypothesis by testing the effect of several inhibitors on LPA-induced blebbing of neuroblastoma cells: (a) the tyrosine kinase inhibitor staurosporine completely blocked LPA-induced blebbing (Table I), in agreement with the observation that tyrosine kinases are involved in the activation of Rho which precedes MLC phosphorylation [Nobes et al., 1995]; (b) KT5926 inhibits the phosphorylation of MLC [Nakanishi et al., 1990]; it also blocks blebbing at a concentration of 10µM (Table I); (c) the inhibitor of phosphatase 2A causes vigorous blebbing (Fig. 4); (d) BDM alone, at concentrations as high as 20 mM, did not prevent blebbing. As BDM has been reported to inhibit the actin-myosin interaction by inhibiting myosin ATPase activity [Higuchi and Takemori, 1989; Cramer and Mitchison, 1995], this finding ap-

pears to contradict the hypothesis that an increase in tension due to increased MLC phosphorylation is responsible for blebbing. However, when combined with a suboptimal concentration of KT5926 which by itself didn't inhibit bleb formation, 20 mM BDM became effective (Table I). We don't know why in our case BDM is active only in combination with the kinase inhibitor.

An increase in intracellular calcium concentration was not necessary for blebbing. Both chelating extracellular calcium with EGTA and clamping intracellular calcium concentrations at very low levels with a high concentration of the cell-permeable chelator BAPTA-AM did not prevent blebbing (Table I). At first sight, this observation might contradict the notion that calcium is needed for the activity of calmodulin-dependent myosin light chain kinase. But any negative effect on that kinase might be overcome by direct phosphorylation of MLC by Rho-kinase [Amano et al., 1996].

The three cytoskeletal systems—actin filaments, microtubules, and intermediate filaments—form an integrated scaffold that is providing mechanical continuity between the cell surface and the nucleus [Ingber, 1997]. Elegant studies pioneered by Ingber and coworkers show that mechanical force applied to integrin receptors at the cell surface is transmitted through this system of cooperatively interacting cytoskeletal elements [Maniotis et al., 1997]. The properties of the combined cytoskeleton can be characterized by the Poisson ratio, a measure of the lateral contraction of the network of interacting filaments after pulling it [Maniotis et al., 1997]. When connections within the network are broken by treating the cells with nocodazole which depolymerises the microtubules or with acrylamide which affects intermediate filaments, the Poisson ratio increases. We expected that altering the Poisson ratio would also affect blebbing in LPA-treated neuroblastoma cells. The results confirmed this expectation: treatment with either nocodazole or acrylamide increased the number of spontaneously blebbing cells (Table I) and prolonged blebbing. Treating the cells with 2μM cytochalasin B for 2 h, on the other hand, had an inhibitory effect on blebbing, although by decreasing the number of actin filaments the Poisson ratio is increased [Maniotis et al., 1997]. However, this is to be expected because intact microfilaments are necessary by providing, together with myosin, the contractile apparatus. The enhancing effect that a deficiency of actin binding protein had on the blebbing phase of melanoma cells [Cunningham, 1995] could also be interpreted as a result of increasing the Poisson ratio, but the same result would be expected if the cortical actin network alone were affected. Our results show that in neuroblastoma cells blebbing is not only regulated by how strongly the cell cortex resists intracellular pressure, but by the whole cytoskeleton consisting of all three interconnected filament types.

After extending, blebs induced by LPA or by calyculin retracted again. Retraction is cytochalasin-sensitive, indicating that it must depend on actin polymerization. Inhibition of bleb retraction by Cytochalasin had been demonstrated in a different system before [Cunningham, 1995]. Cunningham showed that blebs fixed during the retraction phase had acquired a coat of polymerised actin at the cytoplasmic membrane surface, whereas extending blebs were lost during fixation and presumably lacked polymerised actin [Cunningham, 1995]. We tried to directly correlate bleb dynamics and actin accumulation by time lapse videomicroscopy of neuroblastoma cells transfected with the fluorescent fusion protein EGFP-actin. Actin tagged with GFP had been successfully used before [Westphal et al., 1997]. That our EGFP-actin fusion protein was incorporated into microfilaments is shown by the fact that, apart from a cytoplasmic pool of G-actin in the cell body, it is concentrated in filopodia extending from the growth cones and from the processes, sites where phalloidin stained microfilaments can be detected. Moreover, in cultured fibroblasts it is incorporated into stress fibers (not shown). Figures 6 and 7 demonstrate that EGFP-actin, not visible at the bleb membrane during bleb extension, starts accumulating there as soon as the extension phase ends. Two interpretations could explain this relationship: Actin polymerised at the bleb membrane might bring the extension phase to a halt and initiate the retraction phase. Alternatively, actin polymerization might be prevented by the process of bleb extension and only set in after the bleb has reached its full extension. The fact that extending blebs are not supported by a membrane skeleton and collapse during fixation might indicate that actin polymerization doesn't occur during the extension phase. However, polymerised actin accumulating as long as the surface of the bleb mem-

brane increases might be “diluted” and not be sufficiently dense to either give a fluorescence signal of EGFP-actin or resist fixation and permeabilization. Interestingly, EGFP-actin sometimes accumulated over a restricted section of a bleb only, causing that part of the bleb to retract, whereas the remaining protrusion continued extending or remained stationary (Fig. 8). We presently do not know what limits the area of bleb membrane covered by polymerised actin in these cases.

The function of blebbing has remained elusive in all systems studied so far. One possibility is that the blebs serve as initial stages for cell protrusions such as filopodia or lamellipodia and hence play a role in locomotion [Keller et al., 1985]. An alternative explanation is suggested by our observation that the nuclei of blebbing cells are being deformed extensively (Fig. 2). The nucleus is linked to the plasma membrane through an extensive network involving all three cytoskeletal systems [Maniotis et al., 1997]. This network offers a mechanism whereby signals can be rapidly transmitted from the cell surface to the nucleus without the involvement of soluble messengers acting more slowly. Deformation of the nucleus might in turn alter gene expression [Ingber et al., 1987]. Whether LPA-induced nuclear deformation of neuroblastoma cells affects gene expression remains to be investigated.

In summary we have presented a model system for transient blebbing in cells and shown the following: (i) LPA induces blebbing by activating myosin; (ii) blebbing is regulated by all three components of the cytoskeleton, i.e., microfilaments, microtubules, and intermediate filaments; and (iii) we have for the first time shown directly that accumulation of actin along the cytoplasmic surface of the bleb membrane is correlated with the phase of bleb retraction.

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