Eur. J. Biochem. **271**, 3255–3264 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04259.x

Calcium-independent cytoskeleton disassembly induced by BAPTA

Yasmina Saoudi[1], Bernard Rousseau[2], Jacques Doussière[3], Sophie Charrasse[4], Cécile Gauthier-Rouvière[4], Nathalie Morin[4], Christelle Sautet-Laugier[2], Eric Denarier[5], Robin Scaïfe[6], Charles Mioskowski[2] and Didier Job[1]

[1]*Institut National de la Santé et de la Recherche Médicale, Département Réponse et Dynamique Cellulaires, Grenoble, France;* [2]*CEA/Saclay, Service de Marquage Moléculaire et de Chimie Bio-organique, Département de Biologie Joliot-Curie, Gif sur Yvette, France;* [3]*Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Département Réponse et Dynamique Cellulaires, Grenoble, France;* [4]*Centre de Recherche de Biochimie Macromoléculaire, Centre National de la Recherche Scientifique, Montpellier, France;* [5]*McGill University, Royal Victoria Hospital, West Montreal, Canada;* [6]*Department of Pathology, University of Western Australia, Crawley, Australia*

In living organisms, Ca^{2+} signalling is central to cell physiology. The Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) has been widely used as a probe to test the role of calcium in a large variety of cell functions. Here we show that in most cell types BAPTA has a potent actin and microtubule depolymerizing activity and that this activity is completely independent of Ca^{2+} chelation. Thus, the depolymerizing effect of BAPTA is shared by a derivative (D-BAPTA) showing a dramatically reduced calcium chelating activity. Because the extraordinary depolymerizing activity of BAPTA could be due to a general depletion of cell fuel molecules such as ATP, we tested the effects of BAPTA on cellular ATP levels and on mitochondrial function. We find that BAPTA depletes ATP pools and affects mitochondrial respiration *in vitro* as well as mitochondrial shape and distribution in cells. However, these effects are unrelated to the Ca^{2+} chelating properties of BAPTA and do not account for the depolymerizing effect of BAPTA on the cell cytoskeleton. We propose that D-BAPTA should be systematically introduced in calcium signalling experiments, as controls for the known and unknown calcium independent effects of BAPTA. Additionally, the concomitant depolymerizing effect of BAPTA on both tubulin and actin assemblies is intriguing and may lead to the identification of a new control mechanism for cytoskeleton assembly.

Keywords: actin; BAPTA; calcium; cytoskeleton; microtubules.

Calcium ions are essential second messengers in eukaryotic cells. A large variety of vital cell functions such as actin-dependent motion and contraction, cell proliferation and secretion, gene expression and synaptic transmission depend on calcium concentrations [1].

Calcium chelators are widely used to probe the role of calcium signalling in cell functions [2,3]. Such chelators principally include EGTA and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) [4]. The two molecules have similar chelating units but in BAPTA the methylene links between oxygen and nitrogen are replaced by benzene rings. BAPTA is not protonated at physiological pH. The absence of a deprotonation step during calcium complexation results in a higher Ca^{2+} complexation rate for BAPTA compared to EGTA and this has been the main rational for the introduction of BAPTA in studies of calcium signalling [5]. A data base search shows that since the year of its discovery (1980), BAPTA has been used in nearly 3000 published works, spanning the entire field of cell biology [6–9]. In addition to its use for experimental work, BAPTA and its analogues may also find important therapeutic applications in diseases [10–13]. In particularly, BAPTA can attenuate neurotransmitter release in central mammalian synapses [14]. Other studies showed that the cell-permeant calcium chelator BAPTA can reduce neuronal ischemia *in vivo* [15].

The present study began when we tried to use the cell-permeant BAPTA AM (acetoxymethyl ester form) to probe the role of calcium in regulating microtubule-stabilizing proteins STOP [16] in cells. To our surprise we found that in many cell types, BAPTA AM displays a potent microtubule depolymerizing effect. We subsequently found that the depolymerizing effect of BAPTA on the cell cytoskeleton is general, also affecting actin assemblies, and that it is completely independent of its known calcium chelating properties.

Correspondence to D. Job, INSERM U366, DRDC/CS, 17 rue des Martyrs 38054 Grenoble Cedex 9, France.
Tel.: +33 04 38 78 21 48, E-mail: djob@cea.fr

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA AM, BAPTA acetoxymethyl ester; DBB, 5,5′-dibromo BAPTA; DMB, 5,5′-diméthyl BAPTA; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone.

(Received 19 April 2004, revised 15 June 2004, accepted 18 June 2004)

Methods

Reagents

BAPTA, BAPTA AM, 5,5′-dimethyl BAPTA AM (DMB AM) and EGTA AM were from Molecular Probes.

D-BAPTA AM, a BAPTA derivative lacking one acetic acid group was prepared by chemical synthesis (CEA Saclay, France). BAPTA and BAPTA derivatives were stored in 50 mM dimethyl sulfoxide. EGTA AM and BAPTA AM from several independent commercial sources were tested with similar results. The purity of D-BAPTA AM was routinely checked using MS. At least five independent batches were tested in the course of this study.

Nocodazole and cytochalasin D were from Sigma Aldrich. Paclitaxel (Taxol equivalent) and Phalloidin were from Molecular Probes.

Cell culture

The A6 *Xenopus* cell line (ATCC) was adapted in 50% L15 medium (Gibco BRL) complemented with 10% fetal bovine serum at 25 °C. RAT2 cells were grown in DMEM medium (Gibco BRL) supplemented with 10% fetal bovine serum at 37 °C in a 4% CO_2 humidified incubator.

Calcium imaging

For time-lapse calcium imaging, cells were incubated at 37 °C with 5 μM Fluo4 AM (Molecular Probes) in the absence or presence of test molecules. After 30 min, cells were washed and placed in NaCl/P_i for 30 min. Before time-lapse acquisitions, 5 μM ionomycine (Calbiochem) was added to the medium. Time-lapse sequences were collected on a Leica TCS-SP2 laser-scanning confocal microscope every 3 s for 10 min. Fluorescence intensities were quantified using Leica Confocal sofware.

Microinjection

Cells grown on glass coverslips were injected using a 5171 micromanipulator and a 5246 transjector (Eppendorf).

Immunofluorescence staining and confocal microscopy

Cells were fixed in NaCl/P_i containing 3.7% paraformaldehyde for 1 h, permeabilized for 30 min with 0.2% Triton X-100 in NaCl/P_i. Cells were sequentially incubated with a primary rat anti-tubulin mAb, YL1/2 (1 : 1000; originally a gift from J. V. Kilmartin and available from Chemicon International, Inc., Temecula, CA, USA), a secondary anti-rat mAb conjugated with cyanine 2 (1 : 1000; Jackson), and rhodamin-phalloidin (1 : 100; Molecular Probes). For simultaneous microtubules and mitochondria labelling, MitoTracker Red CMXRos-H_2 (Molecular Probes) was added (500 nM) to the medium at 37 °C. After 30 min, cells were fixed, permeabilized and immunostained as described above. Cells were visualized in Leica TCS -SP2 laser-scanning confocal microscope.

ATP determination in cell extracts

Cells were grown on plastic dishes (2×10^6 cells·mL^{-1}). The cells were treated with 50 μM EGTA AM or 50 μM BAPTA AM and its derivatives (50 μM DMB AM, 50 μM D-BAPTA AM) at 37 °C for 1 h. Then, the culture medium was removed and cells were washed in NaCl/P_i. For nucleotide extraction, cells were treated at 4 °C with perchloric acid 0.4 M (1 mL·dish^{-1}, 2 min). The cell carpet was centrifuged (100 000 ***g***, 10 min, 4 °C) in a Beckman TLA-100 rotor. The supernatants were then neutralized (KOH, 6 N) and centrifuged (100 000 ***g***, 10 min, 4 °C). The supernatants were collected and stored at −80 °C. Cell extracts were used for quantitative determination of ATP, using an ATP determination kit (Molecular Probes). All of the reagents were prepared according to the manufacturer's instructions.

Mitochondria preparation and respiration

Mitochondria were isolated from mouse livers as described previously by Hogeboom [17]. The suspensions of mitochondria (2 mg·mL^{-1}) were placed in KCl 150 mM, NaCl 10 mM, potassium phosphate 10 mM, $MgCl_2$ 6 mM pH 7.4, 230 μM O_2 in balance with atmosphere into a 1.5 mL measurement chamber. Drugs were added to the incubation buffer at 0.5 μmol·mg protein^{-1}. The oxygen consumption in the mitochondria samples was measured at 25 °C by oxygraphy using a Clark electrode polarized at 0.6 V [18]. For determination of the ADP stimulated respiration and the P : O ratio, which, in the absence of decoupling, assesses the ATPase efficiency [19,20], ADP (106 nmol) was added to the mitochondrial suspension to induce a transient increase in respiration. The P : O ratio was then calculated as the ratio of the amount of added ADP vs. the amount of oxygen consumed during the stimulated respiratory phase. For measurement of the maximal mitochondrial respiration, oxygen consumption was measured in the presence of 0.4 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma).

Results

BAPTA is a potent cytoskeleton-depolymerizing agent

We initially observed a microtubule depolymerizing effect of BAPTA, in interphase Rat2 cells. In such cells exposure to 10–50 μM BAPTA AM, a cell-permeable BAPTA that is rapidly hydrolysed to form BAPTA in cells [21], induced a rapid microtubule disassembly. Above 20 μM BAPTA AM, the disassembly was virtually complete in most cells after 30 min, and microtubules were uniformly depolymerized in cells within 60 min (Fig. 1A). We tested BAPTA AM from different sources, to detect possible chemical impurities, and found a similar effect of the drug whatever the supplier. The depolymerizing effect of BAPTA AM was observed in many cell types, including mammalian cells such as MDCK cells, mouse myoblasts, or primary cultures of mouse embryo fibroblasts (not shown) or in *Xenopus* cells (Fig. 1A). In a series of control experiments, BAPTA had no detectable effect on purified tubulin assembly when added at millimolar concentrations to tubulin solutions (data not shown). In addition, BAPTA was unable to block tubulin assembly in permeabilized cells reconstituted either with homologous cell extracts or with *Xenopus* extracts, which are known to be competent to restore microtubule dynamics in lysed cells [22] (data not shown). BAPTA AM showed a similar absence of effect to BAPTA, when added to tubulin solutions or to acellular extracts, at 50 μM (close to the maximal concentration, in

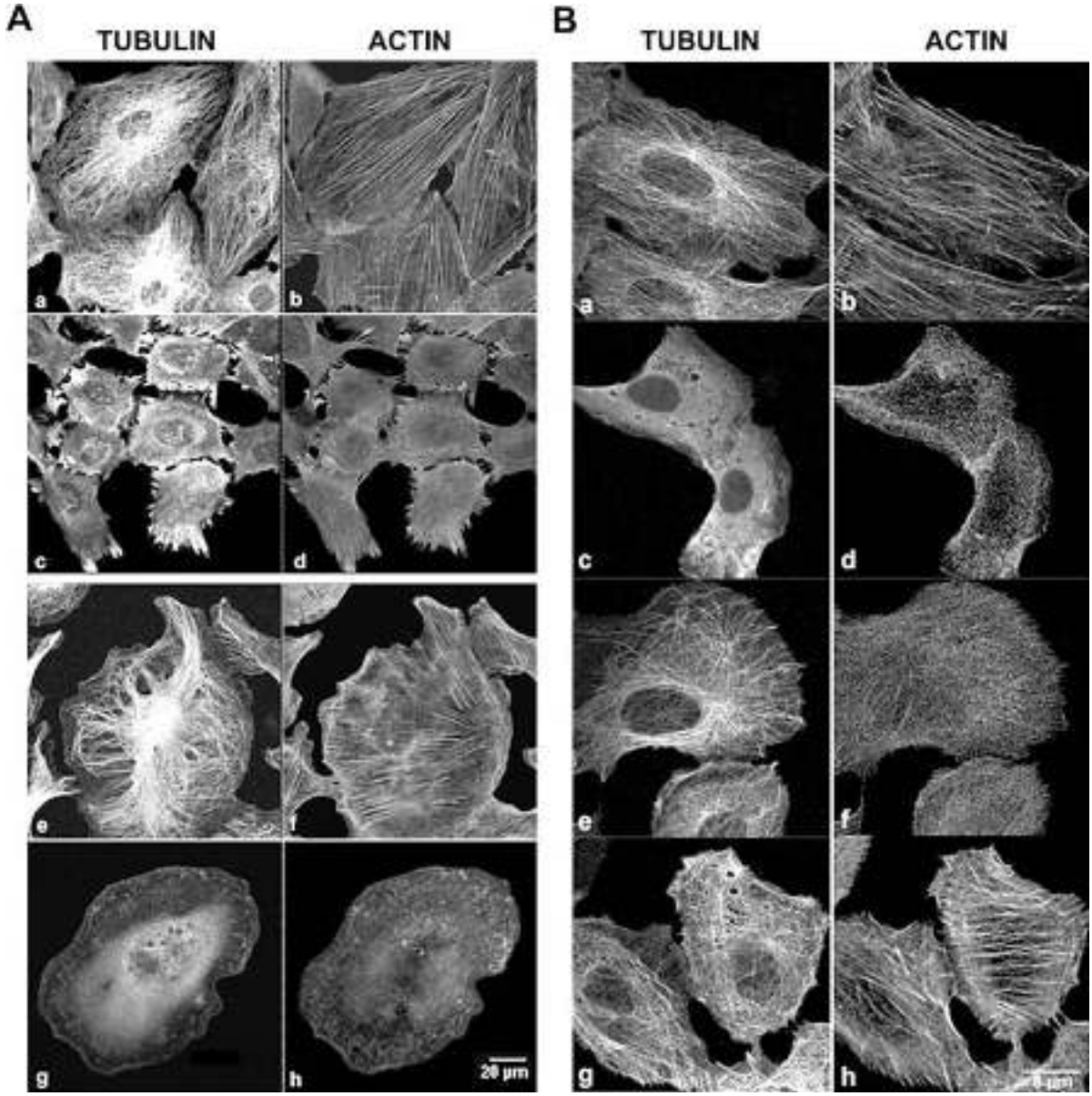

Fig. 1. BAPTA action on the cytoskeleton. (A) Immunostaining of interphasic RAT2 cells (a–d) and *Xenopus* cells (e–h) with tubulin YL1/2 antibody (a,c,e,g) or rhodamin–phalloidin (b,d,f,h). Cells were incubated in the culture medium in the absence (a,b,e,f) or presence of 50 µm BAPTA AM for 1 h at 37 °C (c,d) or at 25 °C (g,h). Scale bar, 20 µm. (B) Immunostaining of interphasic RAT2 cells (a–h) with tubulin mAb YL1/2 (a,c,e,g) and rhodamin-phalloidin (b,d,f,h). Cells were incubated with 50 µm BAPTA AM for 1 h at 37 °C (c,d). Then, cells were washed and placed in DMEM containing 10% foetal bovine serum at 37 °C for 1 h (e,f) or 2 h (g,h). Scale bar, 8 µm.

aqueous solutions). These results suggest an indirect effect of the drug on microtubule assembly, involving signalling cascades or metabolic pathways functioning in whole cells but not in acellular extracts. We then tested whether BAPTA had a specific effect on microtubules or also affected actin assemblies. Results showed drastic effects of the drug on actin assembly in Rat2 cells. Within 60 min of BAPTA AM treatment the cells retracted and had lost their normal array of stress fibres, as well as lamellipodia (Fig. 1A, b,c). BAPTA AM also induced peripheral spike-like extensions, which in videomicroscopy experiments proved to be retraction fibres, not filopodia (data not shown). Similar actin disorganization was observed in *Xenopus* cells. In these cells, actin assemblies in stress fibres, lamellipodia, and filopodia are particularly distinct and all of these three types of actin assemblies were disrupted during exposure to BAPTA AM (Fig. 1A, g,h).

Interestingly, the effect of BAPTA on the cytoskeleton was reversible. When cells were treated with BAPTA AM for 1 h and then incubated in fresh medium devoid of BAPTA AM, microtubule re-growth began at $\approx$30 min and the microtubule network was completely reorganized within 1 h. Actin assemblies re-formed somewhat later, within 2 h of BAPTA AM removal (Fig. 1B).

BAPTA effects in the presence of other cytoskeleton drugs

We tested whether microtubule or actin drugs interfered with BAPTA effects. We tested the effect of the microtubule-stabilizing drug taxol (Fig. 2A, a–d), which suppresses microtubule dynamics [23,24] and induces indirectly a rearrangement of actin filaments from stress fibres into a marginal distribution [25,26]. In Rat2 cells exposed to taxol and then treated with BAPTA AM, microtubules resisted BAPTA exposure. This indicates that BAPTA action perturbs the tubulin assembly and disassembly balance on

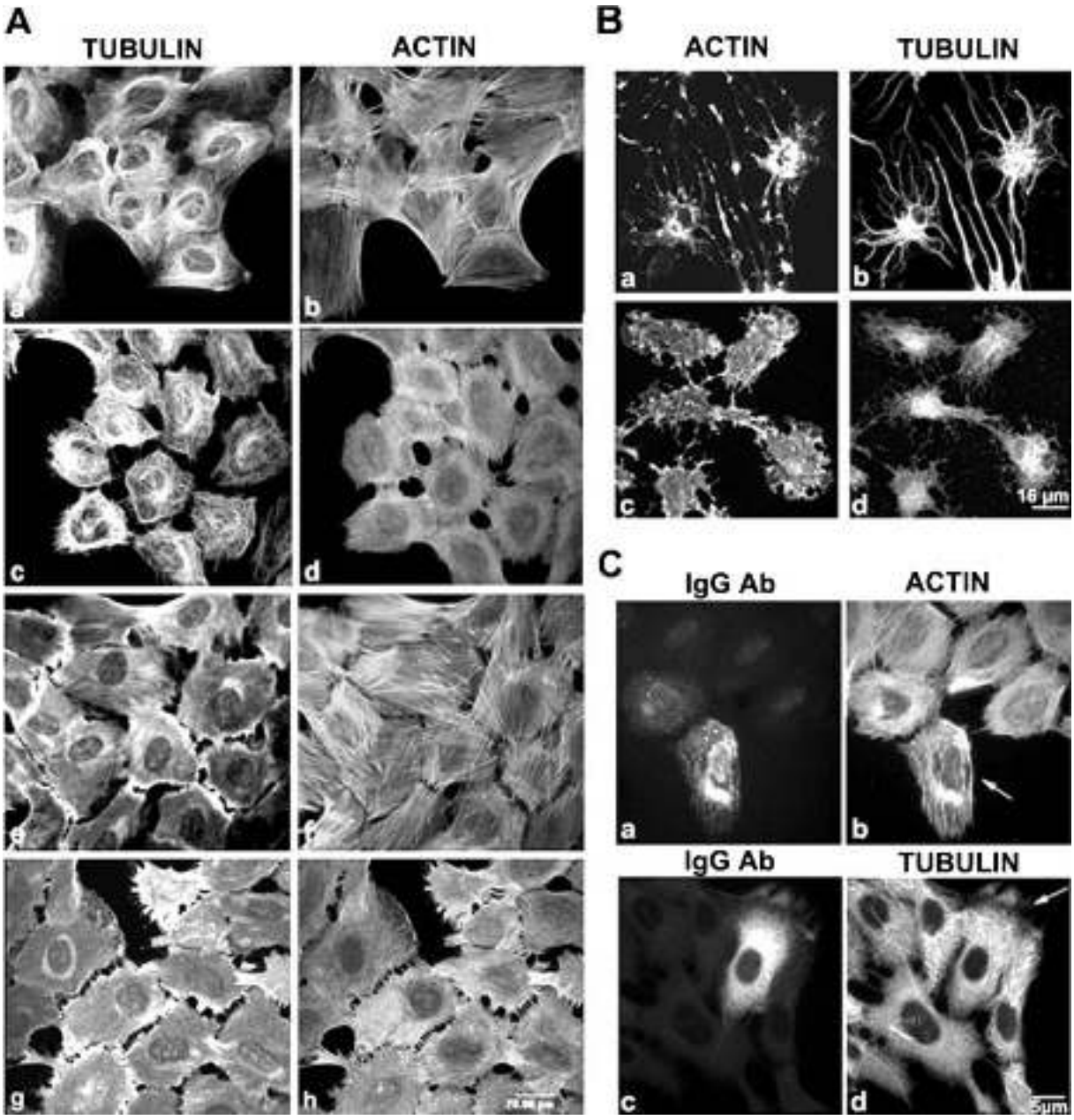

Fig. 2. Effects of BAPTA in the presence of other cytoskeleton drugs. (A) Immunostaining of interphasic RAT2 cells (a–h) with tubulin YL1/2 antibody (a,c,e,g) or rhodamin–phalloidin (b,d,f,h). (a–d) Cells were incubated with 50 µm taxol for 30 min, then incubated for 1 h at 37 °C in the presence of fresh medium containing: (a,b) 50 µm taxol alone; (c,d) a mixture of 50 µm taxol and 50 µm BAPTA AM. (e–h) Cells were incubated with 20 µm nocodazole for 30 min, then incubated for 1 h at 37 °C in the presence of fresh medium containing: (e,f) 20 µm nocodazole alone; (g,h) a mixture of 20 µm nocodazole and 50 µm BAPTA AM. Scale bar, 20 µm. (B) Immunostaining of interphasic RAT2 cells with rhodamin–phalloidin (a,c) or with tubulin YL1/2 antibody (b,d). Cells were incubated with 10 $\mu g \cdot mL^{-1}$ cytochalasin D for 30 min at 37 °C then and incubated for 1 h at 37 °C in the absence (a,b) or presence (c,d) of 50 µm BAPTA AM. Scale bar, 16 µm. (C) Immunostaining of interphasic RAT2 cells with rhodamin–phalloidin (b) or with tubulin YL1/2 antibody (d). Cells were injected with a mixture of nonreactive mouse IgGs and 100 mM phalloidin. After injection, cells were incubated with 50 µm BAPTA AM for 1 h at 37 °C. (a–d) Cells were stained with mouse IgG antibody to identify injected cells, which are indicated by arrows in b and d. Scale bar, 5 µm.

dynamic microtubules but does not disrupt the interaction between tubulin dimers that are incorporated in the microtubule wall. BAPTA is thereby similar to most known microtubule depolymerizing drugs [22]. In the same cells, BAPTA AM induced an extensive disruption of the actin cytoskeleton, showing that BAPTA effects on actin do not depend on concomitant microtubule disassembly (Fig. 2A, c,d).

When Rat2 cells were treated with the microtubule depolymerizing drug nocodazole alone (Fig. 2A, e,f), microtubules were depolymerized and stress fibres were strongly enhanced, as previously observed in other cell types [27–29]. Addition of BAPTA AM to nocodazole-treated cells still resulted in an extensive disruption of the stress fibres showing that BAPTA action could overcome the stimulation of actin polymerization induced by nocodazole (Fig. 2A, g,h).

In Rat2 cells treated with the actin depolymerizing drug cytochalasin D, microtubule arrays were severely disturbed due to global cell retraction. However, assembled polymers were readily visible in cytochalasin-treated cells not exposed to BAPTA AM whereas microtubules were fully depolymerized in cytochalasin-treated cells exposed to BAPTA AM, indicating that BAPTA effects on microtubules persist in the presence of concomitant actin disassembly (Fig. 2B).

Finally when BAPTA AM was added to cells injected with the actin-stabilizing drug phalloidin, BAPTA-induced actin disassembly was suppressed, showing that BAPTA acts on dynamic actin assemblies, but the microtubule depolymerizing effect of BAPTA was unaffected (Fig. 2C).

These data indicate that the disrupting effect of BAPTA on microtubules or actin assemblies relies on the microtubule and actin dynamics. Additionally, the assembly state of tubulin does not interfere with the effects of BAPTA on actin assembly and vice-versa.

BAPTA effects on the cytoskeleton are independent of calcium chelation

We tested EGTA and a series of BAPTA derivatives to assess the relationship between the calcium chelating activity of BAPTA and its depolymerizating activity on the cell cytoskeleton. BAPTA derivatives included calcium chelators such as 5,5′-diméthyl BAPTA (DMB) (Fig. 3A), 5,5′-difluoro BAPTA and 5,5′-dibromo BAPTA (DBB) (data not shown). For a direct test of the role of calcium chelation in the effects of BAPTA on the cytoskeleton, we designed a BAPTA AM synthesized derivative (D-BAPTA AM), in which one acetic acid group essential for the chelating activity is substituted with a methyl (Fig. 3A). We then tested the chelating activity of D-BAPTA AM in cells (Fig. 3B). For this RAT2 cells were incubated with a fluorescent calcium indicator (fluo4 AM) in the presence of BAPTA AM or its derivatives. The Ca^{2+} ionophore ionomycin was then added to create a pulse of calcium entry into the cell. The resulting variation of the intracellular Ca^{2+} concentration was recorded using fluorescence calcium imaging. In control experiments, a sharp and large increment of intracellular calcium concentration was observed (Fig. 3B, trace 1). Such a variation was largely quenched in cells exposed to BAPTA AM (Fig. 3B, trace 2). A similar quenching was observed both with DMB AM (Fig. 3B, trace 3) and with DBB AM (data not shown). In contrast, in the presence of D-BAPTA AM, the calcium increase was somewhat delayed but of similar amplitude as with BAPTA AM (Fig. 3B, trace 4), indicating a drastically reduced chelating capacity of D-BAPTA in cells, compared to BAPTA.

We tested the effect of various BAPTA derivatives on the cell cytoskeleton. Strikingly both calcium chelators DMB (Fig. 3C, g,h) and DBB (data not shown) were completely devoid of depolymerizing activity, on both microtubules and actin assemblies. In contrast, D-BAPTA which had lost its calcium chelating capacity, had depolymerizing activity identical to that of as BAPTA itself (Fig. 3C, e,f) with a similar dose–effect curve and similar reversibility (data not shown).

In a series of additional control experiments, cells were treated with a variety of drugs known to affect calcium pools, for example ionomycin or thapsigargin. Cells were also injected with peptides, mimicking myosin light chain kinase, CaM1 calmodulin binding domains to inhibit cellular calmodulin or transfected with a constitutively active form of CaM kinase II [30] prior to cell exposure to BAPTA. Such treatments did not suppress the effects of BAPTA. In particular, BAPTA was still active in cells in which all calcium pools had been pre-depleted by thapsigargin treatment in the presence of extracellular EGTA (data not shown).

Taken together these results give very strong evidence that the observed action of BAPTA on the cell cytoskeleton is unrelated to its calcium chelating properties.

BAPTA affects ATP levels and mitochondrial function

Both actin and tubulin assemblies require a permanent supply of fuel molecules (ATP and GTP, respectively) for their generation and maintenance [31,32]. An obvious possibility was that BAPTA was somehow depleting ATP pools in cells, thereby inducing a general depolymerization of the cell cytoskeleton. We tested whether ATP concentrations were lower in extracts from BAPTA-treated cells compared to controls. Indeed, the ATP concentrations in BAPTA extracts were diminished threefold compared to that of controls (Fig. 4). However, DMB and EGTA, which are devoid of cytoskeleton depolymerizing activity (Fig. 3C, a,b,g,h) had also effects on ATP concentration, indicating that the depletion of ATP pools is not sufficient to account for the depolymerizing effects of BAPTA. D-BAPTA also affected ATP concentrations, indicating that the calcium chelating activity of BAPTA is not required for depletion of ATP pools. The depletion of ATP pools observed with BAPTA suggested a poisoning effect of BAPTA on mitochondrial function. To test this possibility, mitochondrial respiration was assayed on purified mitochondria, in the presence and absence of BAPTA AM (Table 1). In the presence of BAPTA AM, the resting oxygen consumption showed no significant variation, indicating that that BAPTA has no decoupling activity and the P : O ratio was not sizeably affected, indicating a conserved ATPase efficiency. The ADP stimulated respiration was diminished by 24% in the presence of BAPTA AM and this was accounted for by a diminution of 58% of the maximal oxygen consumption measured in the presence of the uncoupler FCCP (Table 1). These results indicate a perturbation of the mitochondrial

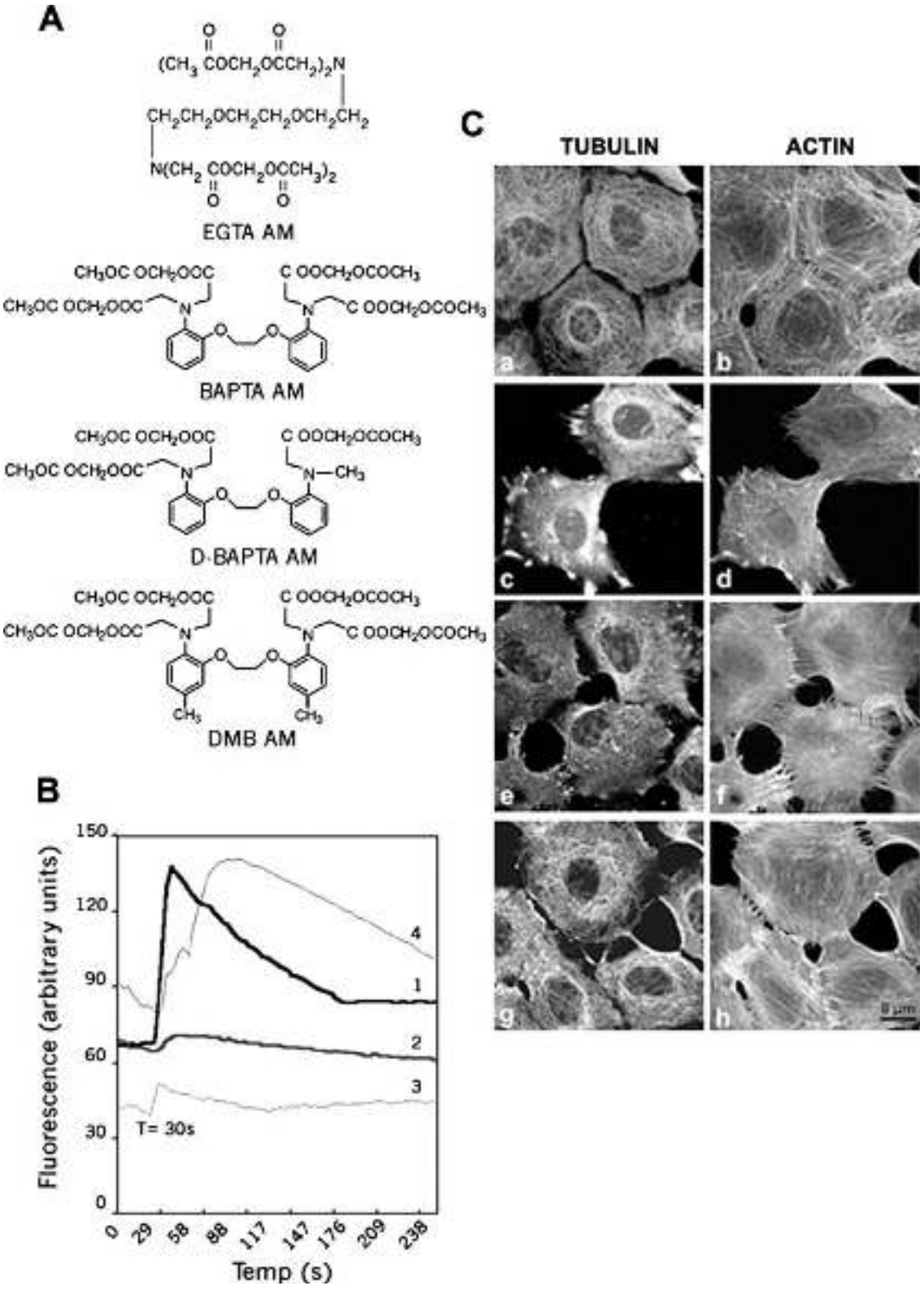

Fig. 3. Effects of BAPTA on the cytoskeleton are independent of calcium chelation. (A) Chemical structures of EGTA and of BAPTA derivatives. (B) Effects of BAPTA and its derivatives on intracellular calcium concentrations. Calcium concentrations were quantified as described in methods in absence of drugs (1) or in the presence of 50 μm BAPTA AM (2); 50 μm DMB AM (3); 50 μm D-BAPTA AM (4). (C) Effects of EGTA and of BAPTA derivatives on the cytoskeleton. Immunostaining of interphasic RAT2 cells (a–h) stained with YL1/2 antibody (a,c,e,g) or rhodamin–phalloidin (b,d,f,h). Cells were incubated with 50 μm EGTA AM (a,b); 50 μm BAPTA AM (c,d); 50 μm D-BAPTA AM (e,f); 50 μM DMB AM (g,h) for 1 h at 37 °C. Scale bar, 8 μm.

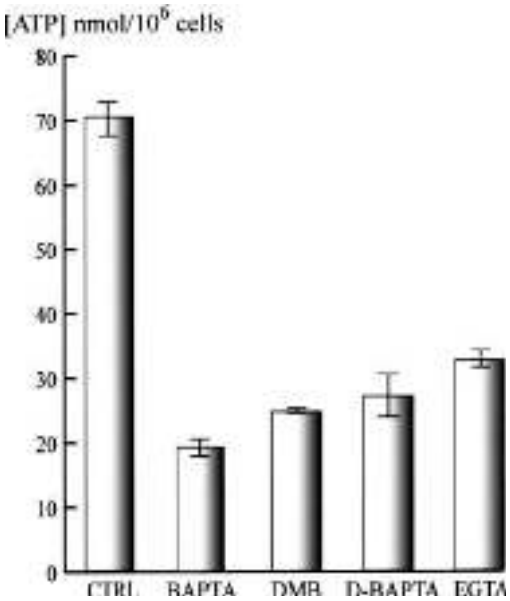

Fig. 4. BAPTA and its derivatives affect ATP pools. ATP concentrations (mean ± SEM) were measured using bioluminescent luciferin/luciferase assays in cell extracts (n = 5) from control cells, or from cells exposed for 1 h to 50 μM BAPTA AM, or to 50 μM BAPTA AM derivatives or to 50 μM EGTA AM, prior to extraction.

Table 1. Effect of BAPTA (0.5 μmol·mg^{-1}) on mitochondrial respiration. The resting state respiration, the P : O ratio, the ADP stimulated respiration and the FCCP uncoupled respiration were determined as described in Methods. For absolute respiration measurements, results are in nmol O_2 consumed·min^{-1}·mg protein^{-1}.

	Control	+ BAPTA AM	Inhibition (%)
Respiration resting rate	13	16	
P : O ratio	3.2	2.9	9.4
Respiration ADP	42	32	24
Stimulated respiration FCCP uncoupled	60	25	58.3

respiratory chain. Similar effects on mitochondrial function were observed with EGTA and with the various BAPTA derivatives (data not shown), compatible with the observed depletion of the ATP pools induced by these compounds.

BAPTA effect on mitochondrial localization and distribution

Mitochondria are normally connected to the microtubule cytoskeleton [33] and this connection may be important for microtubule assembly. Given the effect of BAPTA on mitochondrial function, we tested whether BAPTA affected mitochondria localization in cells.

Strikingly, whereas in control cells, mitochondria were distributed over the whole cytoplasm (Fig. 5a,b). In BAPTA AM-treated cells, mitochondria clustered around the nucleus and became rounded (Fig. 5d). Thus, in addition to affecting ATP levels, BAPTA affects mitochondria distribution and shape in cells. We then used BAPTA derivatives to test whether the effects of BAPTA on mitochondrial shape and localization required calcium chelation and whether these effects were related to the depolymerizing effect of BAPTA (Fig. 5). D-BAPTA, which does not chelate calcium efficiently, had similar action as BAPTA on mitochondrial shape and localization (Fig. 5e,f). Thus these effects of BAPTA apparently do not require its calcium chelating activity. Interestingly, whereas DMB (Fig. 5g) and DBB (data not shown) have no detectable effect on the cytoskeleton, both drugs had an effect similar to that of BAPTA on mitochondrial morphology and distribution (Fig. 5h). These results indicate that the effects of BAPTA on mitochondrial shape and distribution do not mediate the effects of BAPTA on the cytoskeleton. Finally, cell exposure to EGTA AM did not affect mitochondrial shape (Fig. 5h, insert) and had little effect on mitochondria distribution (Fig. 5j, insert). Thus the effects of BAPTA on mitochondrial shape and distribution seem to require the aromatic rings.

In most cells, the effects of BAPTA and D-BAPTA on the mitochondria were reversible, with a recovery time in fresh drug-free medium of ≈ 1 h (data not shown).

BAPTA and small GTPases

A definite possibility to account for the cytoskeleton depolymerizing action of BAPTA was that the drug had an inhibitory effect on small GTPases such as cdc42, Rac1 and RhoA, which are known to be centrally involved in the regulation of actin and tubulin assembly and dynamics [34–36].

In a series of experiments (data not shown) carried out to test this possibility we found a 50% decrease of the GTP bound form of these GTPases which indicated a significant perturbation of the GDP/GTP cycle of small G-proteins. However the activation of Rho GTPases by bradykinin, lysophosphatidic acid or platelet-derivated growth factor [37] or cell transfection with constitutively active forms of Cdc42, Rac1 or RhoA [38] were unable to block BAPTA action on either microtubules or actin assemblies (data not shown).

Inversely cell treatment with a Rho inactivator C3 transferase [39] or Y27632, a specific inhibitor of p160ROCK, which consistently suppresses the formation of Rho-induced stress fibres [40] did not prevent BAPTA action on microtubules. In addition cell transfections with dominant-negative p160Rock mutant KDIA [41], Rac1 (N17 mutant) and Cdc42 (N17 mutant) [38] did not inhibit the action of BAPTA on microtubules. It is therefore unlikely that these GTPases are directly involved in the cytoskeletal effects of BAPTA.

BAPTA AM and formaldehyde

The hydrolysis of BAPTA AM in cells leads to an accumulation of formaldehyde which could have dramatic cellular effects [21,42]. Our data showing dramatically different effects of a series of AM derivatives of BAPTA or of EGTA strongly suggested that formaldehyde accumulation is not responsible for the cellular effects of BAPTA AM. This was confirmed in a in a series of control experiments in which exposure of cells to 10 mM formalde-

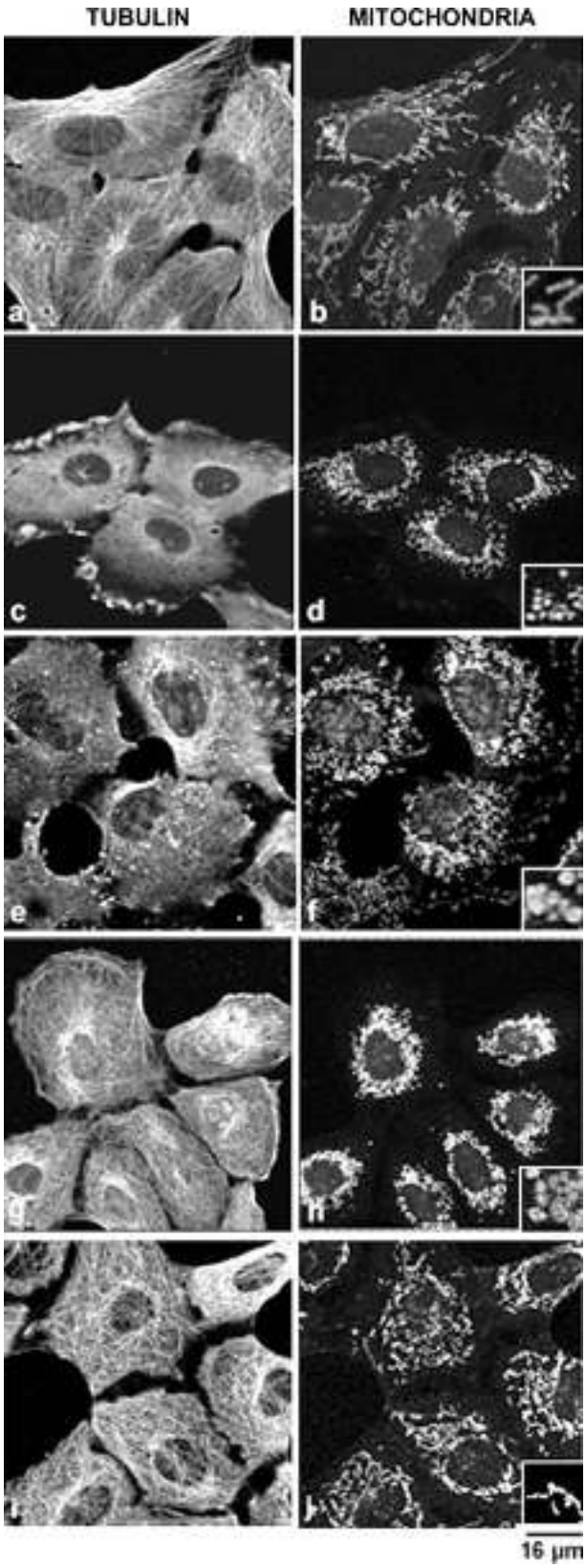

Fig. 5. Effects of BAPTA on mitochondrial distribution and shape. Immunostaining of interphasic RAT2 cells with YL1/2 antibody (a,c,e,g,i) and MitoTracker (b,d,f,h,j). Cells were incubated for 1 h at 37 °C with: (a,b) control medium (no addition); (c,d) 50 μm BAPTA AM; (e,f) 50 μm D-BAPTA AM; (g,h) 50 μm DMB AM; (i,j) 50 μm EGTA AM. Scale bar, 16 μm (inserts b,d,f,h,j): Image (×5) of mitochondrial morphology.

hyde did not induce measurable changes in the actin or microtubule cytoskeleton, whereas it did induce apparent extensive damage of mitochondria, which were not stained anymore with MitoTracker (not shown). Additional experiments carried out with known inhibitors of cellular formaldehyde effects [21] also showed a persistent effect of BAPTA on the cellular cytoskeleton.

Discussion

It is somewhat surprising that a drug as widely used as a calcium chelator as BAPTA turns out to be a potent cytoskeleton depolymerizing drug, and a mitochondrial poison, independently of its calcium chelating activity. It is also striking that BAPTA affects the two systems that were tested in the present study, the cytoskeleton and the mitochondria. BAPTA may have cellular effects on other systems or functions that have not been tested here. The mechanisms involved in the cytoskeleton depolymerizing effects of BAPTA are intriguing. BAPTA does not interact directly with tubulin or actin. Our data give a very strong indication that the calcium chelating activity of BAPTA is unrelated to its depolymerizing effects. BAPTA and BAPTA derivatives induce ATP depletion, apparently due to poisoning of mitochondrial respiration. However, ATP depletion is apparently not sufficient to account for the cytoskeleton depolymerizing effect of BAPTA. We cannot exclude that ATP depletion is necessary for such an effect, as all the compounds that we have tested affected mitochondrial respiration and ATP pools. BAPTA also affects mitochondrial shape and distribution in cells. But this effect of BAPTA is unrelated to the cytoskeleton depolymerizing effect of BAPTA. BAPTA has a depolymerizing effect on both microtubules and actin filaments. In contrast, known microtubule depolymerizing agents such as nocodazole induce an increase in actin assembly, through signalling cascades [43]. To our knowledge, there is no example, other than BAPTA, of a molecule that induces both tubulin and actin disassembly, without killing cells. It may be that the effects of BAPTA on microtubules and on actin assemblies are mechanistically independent. However both effects require the aromatic rings and remarkably the mere substitution of an aromatic hydrogen with a bromo or a methyl on these rings, is sufficient to abolish BAPTA effects on both the microtubule and the actin cytoskeletons. There is apparently a stringent structural requirement for the cytoskeletal effects of BAPTA, and this favours the possibility that common molecular targets are responsible for BAPTA effects on microtubules and on actin assemblies. Apparently, the most studied small GTPases such as RhoA, Cdc42 or Rac1 are not involved. In the future, it may be of interest to identify putative ligands that bind to BAPTA but not to DMB or to DBB. One of these ligands may turn out to be important for the regulation of cytoskeletal assembly in cells.

The cytoskeletal effects of BAPTA and the effect of BAPTA on mitochondrial shape and localization seem to arise from the presence of two aromatic rings that are not present in EGTA. The presence of such aromatic rings is a common occurrence in pharmacological compounds. Our study suggests the need to check systematically the cytoskeleton assembly state and the mitochondrial shape and distribution in any evaluation of the cellular effects of drugs containing aromatic rings.

Neither calcium chelation nor the aromatic groups of BAPTA seem to be important for mitochondrial poisoning. BAPTA effects on mitochondrial respiration and thereby on ATP levels may involve the acid chelating chains of BAPTA per se independently of calcium chelation. Perhaps the carboxylic acid groups present in BAPTA, in BAPTA derivatives, and in EGTA compete with mitochondrial substrates, such as glutamate, which are also carboxylic acids.

In conclusion, BAPTA has unexplained and unexpected calcium independent effects on the cell physiology, and this may be true, although to a lesser degree, for EGTA. BAPTA derivatives lacking one acid chain, such as D-BAPTA, share the side effects of BAPTA, while at the same time having drastically reduced calcium binding activity. D-BAPTA or the corresponding EGTA derivative could be used in calcium signalling experiments as controls for the known and unknown calcium independent effects of the two drugs.

Acknowledgements

We thank Dr M. Albrieux for help in calcium imaging, T. Lorca for providing *Xenopus* extracts, peptide myosin light chain kinase and plasmid CaMKII, C. Arnoult for advice and N. Collomb for technical assistance.

References

1. Rizzuto, R. & Pozzan, T. (2003) When calcium goes wrong: genetic alterations of a ubiquitous signaling route. *Nat. Genet.* **34**, 135–141.
2. Hepler, P.K. (1994) The role of calcium in cell division. *Cell Calcium* **16**, 322–330.
3. Means, A.R. (1994) Calcium, calmodulin and cell cycle regulation. *FEBS Lett.* **347**, 1–4.
4. Harris, R.A. & Hanrahan, J.W. (1994) Effects of EGTA on calcium signaling in airway epithelial cells. *Am. J. Physiol.* **267**, C1426–C1434.
5. Tsien, R.Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404.
6. Freichel, M., Suh, S.H., Pfeifer, A., Schweig, U., Trost, C., Weissgerber, P., Biel, M., Philipp, S., Freise, D., Droogmans, G., Hofmann, F., Flockerzi, V. & Nilius, B. (2001) Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4-/- mice. *Nat. Cell Biol.* **3**, 121–127.
7. Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M. & Poo, M. (2000) Calcium signalling in the guidance of nerve growth by netrin-1. *Nature* **403**, 93–98.
8. Li, H., Chen, A., Xing, G., Wei, M.L. & Rogawski, M.A. (2001) Kainate receptor-mediated heterosynaptic facilitation in the amygdala. *Nat. Neurosci.* **4**, 612–620.
9. Zhang, C. & Zhou, Z. (2002) Ca^{2+}-independent but voltage-dependent secretion in mammalian dorsal root ganglion neurons. *Nat. Neurosci.* **5**, 425–430.
10. Tymianski, M., Sattler, R., Bernstein, G. & Jones, O.T. (1997) Preparation, characterization and utility of a novel antibody for resolving the spatial and temporal dynamics of the calcium chelator BAPTA. *Cell Calcium* **22**, 111–120.
11. Rothman, S.M. (1983) Synaptic activity mediates death of hypoxic neurons. *Science* **220**, 536–537.
12. Rothman, S. (1984) Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J. Neurosci.* **4**, 1884–1891.
13. Choi, D.W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**, 623–634.
14. Niesen, C., Charlton, M.P. & Carlen, P.L. (1991) Postsynaptic and presynaptic effects of the calcium chelator BAPTA on synaptic transmission in rat hippocampal dentate granule neurons. *Brain Res.* **555**, 319–325.
15. Tymianski, M., Wallace, M.C., Spigelman, I., Uno, M., Carlen, P.L., Tator, C.H. & Charlton, M.P. (1993) Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury in vitro and in vivo. *Neuron* **11**, 221–235.
16. Job, D., Rauch, C.T., Fischer, E.H. & Margolis, R.L. (1982) Recycling of cold-stable microtubules: evidence that cold stability is due to substoichiometric polymer blocks. *Biochemistry* **21**, 509–515.
17. Hogeboom, G.H. (1955) Fractionation of cell components of animal tissues. *Meth. Enzymol.* **1**, 16–19.
18. Clark, L.C. Jr, Wolf, R., Granger, D. & Taylor, Z. (1953) Continuous recording of blood oxygen tensions by polarography. *J. Appl. Physiol.* **6**, 189–193.
19. Chance, B. & Williams, G.R. (1955) Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J. Biol. Chem.* **217**, 383–393.
20. Mitchell, P. & Moyle, J. (1965) Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. *Nature* **208**, 147–151.
21. Tsien, R. & Pozzan, T. (1989) Measurement of cytosolic free Ca^{2+} with quin2. *Meth. Enzymol.* **172**, 230–262.
22. Saoudi, Y., Fotedar, R., Abrieu, A., Doree, M., Wehland, J., Margolis, R.L. & Job, D. (1998) Stepwise reconstitution of interphase microtubule dynamics in permeabilized cells and comparison to dynamic mechanisms in intact cells. *J. Cell Biol.* **142**, 1519–1532.
23. Derry, W.B., Wilson, L. & Jordan, M.A. (1995) Substoichiometric binding of taxol suppresses microtubule dynamics. *Biochemistry* **34**, 2203–2211.
24. Wilson, L., Miller, H.P., Farrell, K.W., Snyder, K.B., Thompson, W.C. & Purich, D.L. (1985) Taxol stabilization of microtubules in vitro: dynamics of tubulin addition and loss at opposite microtubule ends. *Biochemistry* **24**, 5254–5262.
25. Herman, B., Langevin, M.A. & Albertini, D.F. (1983) The effects of taxol on the organization of the cytoskeleton in cultured ovarian granulosa cells. *Eur. J. Cell Biol.* **31**, 34–45.
26. Jordan, M.A. (2002) Mechanism of action of antitumor drugs that interact with microtubules and tubulin. *Curr. Med. Chem. Anti-Canc. Agents* **2**, 1–17.
27. Bershadsky, A., Chausovsky, A., Becker, E., Lyubimova, A. & Geiger, B. (1996) Involvement of microtubules in the control of adhesion-dependent signal transduction. *Curr. Biol.* **6**, 1279–1289.
28. Danowski, B.A. (1989) Fibroblast contractility and actin organization are stimulated by microtubule inhibitors. *J. Cell Sci.* **93**, 255–266.
29. Liu, B.P., Chrzanowska-Wodnicka, M. & Burridge, K. (1998) Microtubule depolymerization induces stress fibers, focal adhe-

sions, and DNA synthesis via the GTP-binding protein Rho. *Cell Adhes. Commun.* **5**, 249–255.
30. Lorca, T., Cruzalegui, F.H., Fesquet, D., Cavadore, J.C., Mery, J., Means, A. & Doree, M. (1993) Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**, 270–273.
31. Weisenberg, R.C. (1972) Microtubule formation in vitro in solutions containing low calcium concentrations. *Science* **177**, 1104–1105.
32. Weisenberg, R.C. (1981) Invited review: the role of nucleotide triphosphate in actin and tubulin assembly and function. *Cell Motil.* **1**, 485–497.
33. Heggeness, M.H., Simon, M. & Singer, S.J. (1978) Association of mitochondria with microtubules in cultured cells. *Proc. Natl Acad. Sci. USA* **75**, 3863–3866.
34. Etienne-Manneville, S. & Hall, A. (2002) Rho GTPases in cell biology. *Nature* **420**, 629–635.
35. Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science* **279**, 509–514.
36. Wittmann, T. & Waterman-Storer, C.M. (2001) Cell motility: can Rho GTPases and microtubules point the way? *J. Cell Sci.* **114**, 3795–3803.
37. Gauthier-Rouviere, C., Vignal, E., Meriane, M., Roux, P., Montcourier, P. & Fort, P. (1998) RhoG GTPase controls a pathway that independently activates Rac1 and Cdc42Hs. *Mol. Biol. Cell* **9**, 1379–1394.
38. Cau, J., Faure, S., Vigneron, S., Labbe, J.C., Delsert, C. & Morin, N. (2000) Regulation of *Xenopus* p21-activated kinase (X-PAK2) by Cdc42 and maturation-promoting factor controls *Xenopus* oocyte maturation. *J. Biol. Chem.* **275**, 2367–2375.
39. Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. & Gill, D.M. (1989) The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J.* **8**, 1087–1092.
40. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. & Narumiya, S. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990–994.
41. Ishizaki, T., Naito, M., Fujisawa, K., Maekawa, M., Watanabe, N., Saito, Y. & Narumiya, S. (1997) p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.* **404**, 118–124.
42. Tsien, R.Y. (1981) A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **290**, 527–528.
43. Enomoto, T. (1996) Microtubule disruption induces the formation of actin stress fibers and focal adhesions in cultured cells: possible involvement of the rho signal cascade. *Cell Struct. Funct.* **21**, 317–326.