Calcium storage and release properties of F-actin: evidence for the involvement of F-actin in cellular calcium signaling

Klaus Lange*, Ursula Brandt

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-14195 Berlin, Germany

Received 4 June 1996; revised version received 5 September 1996

Abstract **Preceding studies have shown that the bulk of the ATP-dependent, inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} store of hamster insulinoma (HIT) cells is located in microvilli on the cell surface. Similar results were obtained with isolated rat hepatocytes. Moreover, in vesicles of microvillar origin, passive fluxes of Ca^{2+}, ATP, and IP_3 occur through cation and anion channels, respectively, suggesting that Ca^{2+} storage is due to ATP-dependent Ca^{2+} binding to an intravesicular component. Here we demonstrate that F-actin may be a possible candidate for this function. ATP-actin monomers bind Ca^{2+} with high affinity (K_d = 2–8 nM) to their divalent cation binding sites. Polymerization of actin monomers decreases the rate constant for divalent cation exchange at this binding site by more than 3 orders of magnitude rendering bound cations nearly unavailable. F-actin-bound Ca^{2+} can be released by depolymerization and dissociation from Ca^{2+}-ADP-actin monomers (K_d = 375 nM). We now provide additional evidence for the possible involvement of actin in Ca^{2+} storage. (1) Preincubation of surface-derived Ca^{2+}-storing vesicles from HIT cells with the F-actin stabilizer, phalloidin, strongly inhibited ATP-dependent Ca^{2+} uptake, reducing the IP_3-sensitive Ca^{2+} pool by 70%. Phalloidin, when added after the loading process, affected neither the amount of stored Ca^{2+} nor IP_3 action on the store. (2) F-actin polymerized in the presence of Mg^{2+} in nominally Ca^{2+}-free buffer still contained about half of the high affinity sites occupied with Ca^{2+} (Mg/Ca-F-actin). (3) Using the fura-2 technique, we found that in the presence of ATP, Mg/Ca-F-actin incorporated free Ca^{2+} at a relatively low rate. Short pulses of ultrasound (3–10 s) strongly accelerated Ca^{2+} uptake, decreasing free Ca^{2+} from 500 nM to below 100 nM. (4) In the presence of physiological levels of Mg^{2+} (0.5 mM), sonication liberated large amounts of Ca^{2+} from Mg/Ca-F-actin. (5) Ca-F-actin released bound Ca^{2+} at a very slow rate. Short ultrasonic pulses rapidly elevated free Ca^{2+} from about 50 nM up to 500 nM. (6) Small amounts of profilin, an actin-binding protein, released Ca^{2+} both from Ca- and Mg/Ca-F-actin and also inhibited uptake of Ca^{2+} into Mg/Ca-F-actin. (7) Phalloidin completely inhibited Ca-uptake into Mg/Ca-F-actin even during ultrasonic treatment. These findings suggest that Ca^{2+} storage may occur by addition of Ca-ATP-actin monomers to reactive ends of the polymer and emptying of this store by profilin-stimulated release of Ca-ADP-actin. Thus, receptor-operated Ca^{2+} signaling, initiated by phospholipase C activation, may proceed via the well-known phosphatidylinositol phosphate-regulated profilin/gelsolin pathway of actin reorganization/depolymerization. The importance of the proposed microvillar Ca^{2+} signaling system for living cells remains to be established.**

Key words: Calcium ion store; HIT cell; Microvilli; F-actin; Profilin; Phalloidin

1. Introduction

Between 1963 and 1969, first experimental evidence was presented that divalent cation exchange at the high affinity binding site of G-actin is a rapid process compared to that of F-actin which proceeds at a more than 3000-fold slower rate [1,2]. On the other hand, the very high affinity of Ca^{2+} to ATP-G-actin (K_d = 2–8 nM) is known from the work of Gershman et al. [3] and Carlier et al. [4]. However, the notion that F-actin may function as an ATP-dependent intracellular Ca^{2+} store, in which the ATP-actin monomers represent the high-affinity Ca^{2+}-binding species and F-actin the storage form, has been generally discarded because the ionic conditions within living cells favor binding of Mg^{2+} rather than Ca^{2+}. Although polymerization of actin in vitro in the presence of 2 mM Mg^{2+} has been shown to yield F-actin containing both Ca^{2+} and Mg^{2+} [5], F-actin in vivo is generally believed to contain almost exclusively Mg^{2+}.

This view has been challenged by our recent work on the localization of ATP-dependent Ca^{2+} stores in microvilli of hamster insulinoma (HIT) cells and hepatocytes [6,7]. Microvilli-derived vesicles can be prepared using a low-force shearing technique which is essentially identical with normal cell homogenization by teflon-glass homogenizers but leaves cell bodies intact [8]. Microvillar vesicles become part of the microsomal fraction when classical homogenization and fractionation techniques are applied [6]. Accordingly, their Ca^{2+} storing properties were found to be identical with those for microsomal fractions.

We have further shown that Ca^{2+} uptake into and IP_3-induced Ca^{2+} release from these microvillar vesicles occur by passive diffusion through a La^{3+}-sensitive but IP_3-insensitive cation influx pathway. The vesicles are also permeable for ATP and IP_3 via anion channels [7,9]. Consequently, we proposed that the mechanisms for Ca^{2+} storage should be located within the vesicles. The data further suggested that the receptor-operated Ca^{2+} influx pathway may as well be a microvillar component.

The involvement of microvilli and their cytoskeletal core protein, F-actin, in Ca^{2+} storage and signaling processes was further indicated by scanning electron microscopic studies. The actions of Ca^{2+} signaling agonists such as bombesin and vasopressin as well as that of the IP_3-independent Ca^{2+} store inhibitor, thapsigargin, were accompanied by immediate shape changes of microvilli [6,10], indicating the reorganization of their internal cytoskeletal structure.

*Corresponding author. Fax: (49) (30) 8315954.

PII S0014-5793(96)01025-3

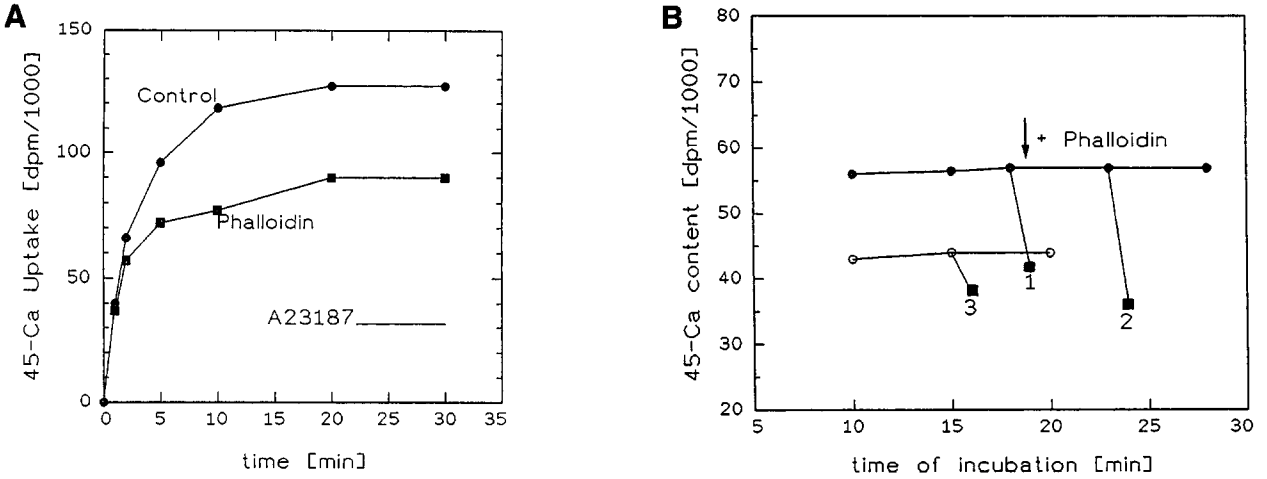

Fig. 1. A: Effect of phalloidin on ^{45}Ca uptake into HIT cell surface-derived vesicles (P3) prepared from HIT cells. The vesicle suspension was prepared as described in Section 2. 2 mM ATP (control; filled circles) and 2 mM ATP plus 10 μM phalloidin (filled squares) were added to the vesicle suspensions 5 min prior to initiating the uptake experiment by addition of ^{45}Ca. The line at the lower right side represents the A23187-insensitive part of vesicle-bound ^{45}Ca. The A23187-insensitive Ca^{2+} is not affected by the presence of phalloidin. Each point represents the mean of two determinations. The experiment was carried out at room temperature. B: Inhibition by phalloidin of the IP_3-sensitive Ca^{2+} pool of HIT cell surface-derived vesicles (P3) from HIT cells. The general experimental conditions are identical with those of A. The upper line (filled circles) shows the ^{45}Ca content of vesicles preloaded with ^{45}Ca in the absence of phalloidin. At the time point indicated with an arrow, 10 μM phalloidin was added; ^{45}Ca release from vesicles loaded in the absence of phalloidin was induced by addition of 1 μM IP3 (square number 1) and 5 min after addition of 10 μM phalloidin to the loaded vesicles (square number 2). The lower line (open circles) represents the ^{45}Ca content of vesicles pretreated with phalloidin for 5 min and then loaded with ^{45}Ca in the presence of phalloidin; IP_3-induced Ca^{2+} release is indicated by the filled square number 3. Each point represents the mean of two determinations. The time scale indicates the time elapsed after starting the ^{45}Ca uptake by addition of ^{45}Ca to the vesicle suspension.

2. Materials and methods

2.1. Materials

Phalloidin, acetone muscle powder was purchased from Sigma, and IP_3 from Boehringer.

2.2. Preparation of proteins

Actin was prepared from muscle acetone powder according to [11] and profilin from bovine spleen according to [12].

2.3. Cell culture

The hamster insulinoma cell line, HIT T15 was purchased from the American Type Culture Collection (Rockville, MD). Cells were cultivated in plastic bottles (Costar) for 1 week in Ham's F12 with 10% horse serum and 2.5% fetal calf serum supplemented with 2 mM glutamine. They were used between the 64th and the 72nd passage. Prior to the experiments, the cells were incubated for 3 h without serum.

2.4. Preparation of the cell surface-derived vesicles

Plasma membrane, microsomal and cell surface-derived membrane fractions were prepared as recently described [9]. Shortly, these fractions were prepared by gently detaching the cells from the growth substrate in ice-cold 1 mM HEPES (pH 7.4)/0.25 M saccharose/1 mM EDTA. Subsequently, the cells, suspended in the same buffer, were gently pressed through hypodermic needles of the following size: No. 1 (0.9×40 mm, 0.6 mm internal diameter) and then No. 2 (0.8 mm×38 mm, 0.5 mm internal diameter). Each step was repeated once. The resulting suspension was centrifuged at 2600×*g* for 10 min to remove the intact cells (80–90% by trypan blue exclusion). The 2600×*g* supernatant was centrifuged at 16000×*g* for 20 min and the supernatant of this step was centrifuged at 180000×*g* for 80 min yielding the pellets P2 and P3, respectively. P3 was used for ^{45}Ca uptake and release experiments.

2.5. Calcium-45 uptake into and release from cell surface-derived vesicles

Cell surface-derived vesicles were washed twice with buffer A (140 mM KCl, 2 mM Mg^{2+}, 10 mM HEPES, 49 μM EGTA, pH 7.2), sedimented at 180000×*g* in a Beckman TL 100 centrifuge (TLA 100,3) at 4°C, resuspended in buffer A to give a protein concentration of 300 μg/ml. Ca^{2+} uptake was initiated by addition of ^{45}Ca (10^{-5} μCi/ml; final Ca^{2+} concentration 10 μM; final free Ca^{2+} concentration 100–200 nM) and 2 mM ATP at room temperature. Effectors were added to each sample (100 μl) in volumes of at most 1 μl. The uptake reaction was terminated by dilution of the reaction mixture with 3 ml of washing buffer (buffer A plus 10 μM $CaCl_2$; at room

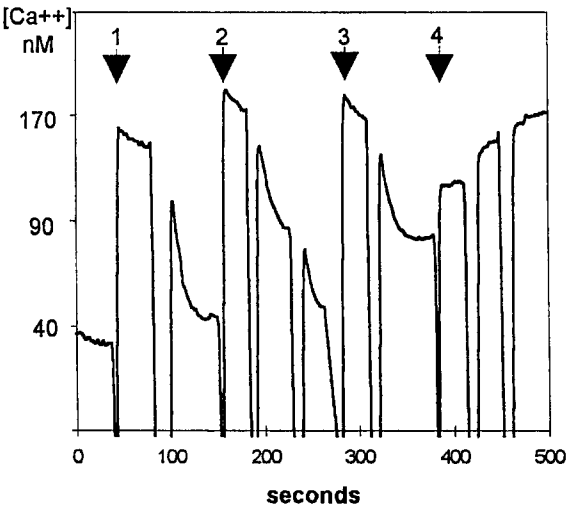

Fig. 2. Uptake of Ca^{2+} into Mg/Ca-F-actin. A solution of 30 μM Mg/Ca-F-actin in 10 mM Tris buffer (50 mM KCl, 0.2 mM ATP) was treated as described in Section 2 to reduce the concentration of free Ca^{2+} to about 40 nM. The fluorescence assay contained 75 nM fura-2. At the time points 1, 2 and 3, indicated with arrows, 0.5 μM $CaCl_2$ was added. To increase the rate of Ca^{2+} uptake, the solution was treated once or twice with ultrasound (7 pulses 0.5 s each) indicated by intervals of about 10 s each, during which the recorder track goes below zero. At the time point indicated with the arrow number 4, 1.8 μM profilin was added followed by 2 treatments with ultrasound. The experiment was carried out at 25–27°C and pH 7.4.

temperature), subsequent rapid filtration through Schleicher and Schuell cellulose nitrate filters (type BA 85) and two additional washes with the same buffer. Radioactivity remaining on the filters was determined by liquid scintillation counting. All experiments were carried out at room temperature. Vesicle suspensions were kept on ice until the beginning of the experiment. The Ca^{2+} concentration of the used buffers and solutions were controlled by the fura-2 fluorescence technique. The actual Ca^{2+} concentration of the uptake solutions was computer-calculated according to Fabiato [13].

2.6. Fura-2 assay of calcium binding to and release from F-actin

Ca-G-Actin was prepared from rabbit muscle acetone powder (Sigma) and purified to gel-electrophoretic homogeneity [10]. Actin preparation were stored at 0°C and used within one week after preparation. Ca-F-Actin was prepared by addition of 50 mM KCl to the solution of 50–75 μM Ca-G-actin in G-buffer (5 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 2 mM NaN_3, 0.2 mM dithiothreitol, pH 7.4). Mg/Ca-F-actin was prepared from Ca-G-actin (75 μM) dialyzed for 15 h against nominal Ca^{2+}-free G-buffer supplemented with 0.1 mM $MgCl_2$ by addition of 50 mM KCl. For use in the fura-2 fluorescence assay, all F-actin solutions were first diluted with divalent cation-free G-buffer to the required concentration, mostly 25 μM, supplemented with ATP to attain a final concentration of 0.2 mM ATP, and treated at room temperature once with Chelex (15% weight/volume) and twice with (10% weight/volume) diethylenetriaminopentaacetic acid coupled to polyacrylamide (DTPA-polyacrylamide; prepared according to [14]) in order to remove traces of unbound Ca^{2+} (and bulk of Mg^{2+}). Prior to use, all necessary equipment (plastic ware only) and the reagents to be used were tested in the fura-2 assay for possible Ca^{2+} contamination. Fura-2 assays were carried out at room temperature using a Shimadzu spectrofluorometer (RS 1502) equipped with a stirring device. Scaling was achieved by addition of EGTA and $CaCl_2$ in excess to the F-actin solutions at the end of the assay.

2.7. Atomic absorption spectrophotometry (AAS) of calcium and magnesium in F-actin

The calcium content of polymerized actin was determined by atomic absorption spectrophotometry (Perkin Elmer). F-actin (50–75 μM), polymerized under different conditions, was treated with Chelex-100 to remove divalent cations, and centrifuged at 400 000 × *g* for 50 min.

Table 1
Binding of Ca^{2+} and Mg^{2+} to the high affinity divalent binding sites of F-actin

No.	Concentration during polymerization (mM)		F-actin-bound divalent cations (μM)			
	Mg^{2+}	ATP	Ca^{2+}	Mg^{2+}	$Ca^{2+}+Mg^{2+}$	% Ca^{2+}
1	0.1	0.2	30.1	25.6	55.7	54
2	0.1	0.2	16.7	8.9	25.6	65
3	0.1	0.2	4.0	15.1	39.1	61
4	0.1	0.2	17.6	10.5	28.1	63
5	0.1	0.5	32.5	27.7	60.2	54
6	0.1	1.0	24.4	28.8	52.8	46
7	0.2	0.5	25.5	35.8	61.3	42
8	0.2	5.0	26.4	27.5	53.9	49
9	0.4	1.0	27.7	43.4	71.1	39
10	0.4	5.0	25.7	35.4	61.1	42
Buffer at the end of dialysis			12.0	112		

Influence of variations in Mg^{2+} and ATP concentrations during polymerization. G-actin was dialysed against nominally Ca^{2+}-free Tris buffer containing 0.2 mM ATP and 0.1 mM Mg^{2+}. The indicated concentrations of Mg^{2+} and ATP were adjusted immediately prior to polymerization with 50 mM KCl. After 2 h, the polymerizate was treated with Chelex 100 to remove free divalent cations, and F-actin was sedimented at 400 000 × *g* in a Beckmann TL-100 centrifuge. The pellets were dissolved in low ionic strength buffer free of divalent cations. Actin was used at concentrations between 50 and 75 μM.

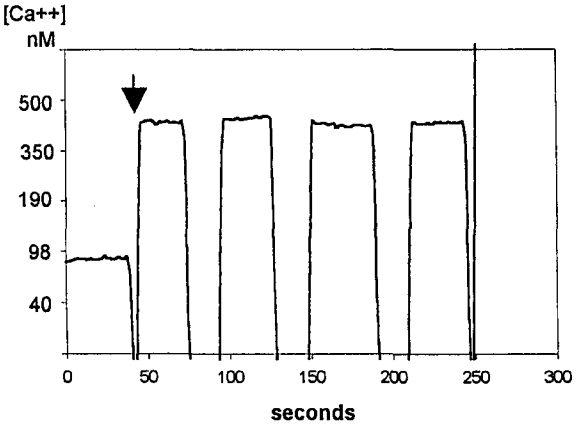

Fig. 3. Inhibition of Ca^{2+} uptake into Mg/Ca-F-actin in the presence of phalloidin. The experimental conditions were identical with those of Fig. 2. 25 μM Phalloidin was added prior to the treatment of the Mg-F-actin solution (25 μM) with ion exchangers. $CaCl_2$ (0.5 μM) was added at the time point indicated with an arrow. Treatment with pulses of ultrasound (7×0.5 s each) occurred during the time periods in which the recorder tracks goes below zero.

The sediments were dissolved in low ionic strength buffer. Their Ca^{2+} and Mg^{2+} contents were determined after dilution with La^{3+} solution and deproteinization by perchloric acid.

3. Results

The first indication for the involvement of the actin system in cellular Ca^{2+} signaling came from experiments shown in Fig. 1A. About 40% of the A23187-sensitive, ATP-dependent Ca^{2+} uptake of the HIT surfaces vesicles was inhibited by phalloidin. The phalloidin-sensitive portion of the stored vesicular Ca^{2+} comprised about 70% of the IP_3-sensitive Ca^{2+} pool (Fig. 1B). Phalloidin, added to Ca^{2+}-loaded vesicles did not affect stored Ca^{2+} nor did it inhibit the IP_3 effect on this store. In the presence of phalloidin, the amount of IP_3-released Ca^{2+} is somewhat higher than in its absence. This effect may be due to inhibition of Ca^{2+} reuptake into the store in the presence of IP_3 and is comparable with the well known enhancement by vanadate of the amount of IP_3-induced Ca^{2+} release. The inhibitory effect of phalloidin resembles that of other known F-actin-stabilizing agents such as vanadate, phosphate, AlF_4^-, and BF_3^- [15] on microsomal Ca^{2+} storage.

Using AAS, we determined the Ca^{2+} and Mg^{2+} content of F-actin, polymerized by KCl in nominal Ca^{2+}-free buffers in the presence of 0.1 to 0.4 mM Mg^{2+}. Freshly prepared Ca-G-actin was polymerized by addition of 2 mM $MgCl_2$ and 100 mM KCl. After sedimentation of F-actin, the polymer was dialysed for 18h against nominal Ca^{2+}-free low-ionic-strength buffer containing 0.1 mM Mg^{2+} and 0.2 mM ATP. The resulting G-actin was then polymerized by addition of 0.1 to 0.3 mM $MgCl_2$ and 50 mM KCl. 40–65% of the resulting F-actin subunits still bound Ca^{2+} (Table 1). Since 0.1 mM Mg^{2+} is the threshold concentration for polymerization, it is nearly impossible to get Ca^{2+}-free F-actin without using EGTA. Generally, the Ca^{2+} load of the polymer depends on the Ca^{2+} content of the monomers prior to polymerization because polymerization proceeds at a much higher rate than the exchange of bound Ca^{2+} for Mg^{2+} [4].

Fig. 2 demonstrates the uptake of added Ca^{2+} into freshly

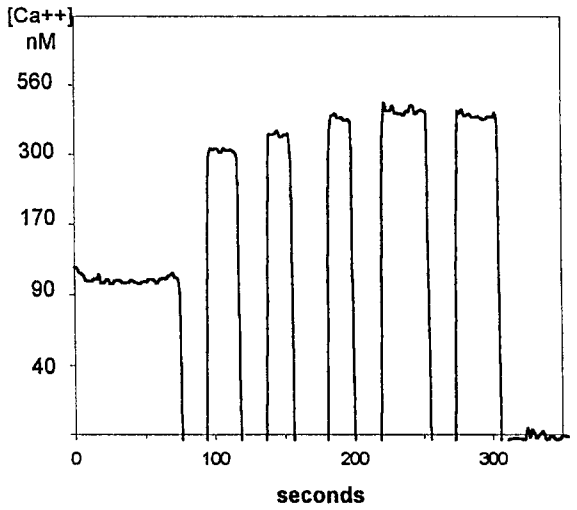

Fig. 4. Ultrasound-induced Ca^{2+} release from Ca-F-actin. Ca-F-actin (25 μM) was repeatedly treated with ultrasound (7×0.5 s each) as indicated by the sub-zero periods of the recorder track. The general experimental conditions were identical with those of Fig. 2.

prepared rabbit muscle Mg/Ca-F-actin using the fura-2 technique. G-actin was polymerized in the presence of 0.2 mM ATP and 0.1 mM Mg^{2+} in nominal Ca^{2+}-free buffer. After removal of residual Ca^{2+} and Mg^{2+} from the test solution with DTPA-polyacrylamide, a high affinity ion exchanger for divalent cations (see Section 2), basal Ca^{2+} was below 40 nM. Addition of $CaCl_2$, raising Ca^{2+} to about 200 nM, induced a slow Ca^{2+} uptake which could be dramatically increased by short pulses (3–4 s) of ultrasound. Ultrasound treatment of Mg/Ca-F-actin reduced the concentration of free Ca^{2+} to approximately 40 nM. Rate and extent of Ca^{2+} uptake into Mg/Ca-F-actin strongly depends on actin concentration (data not shown). At 6 μM Mg/Ca-F-actin, Ca^{2+} uptake could no longer be observed, suggesting that the system was working at the critical concentration of actin polymerization.

As shown in Fig. 3, phalloidin completely inhibited Ca^{2+} uptake into Mg/Ca-F-actin, consistent with the interpretation of Fig. 1A,B, that inhibition of Ca^{2+} uptake into HIT surface vesicles by phalloidin is due to its interaction with F-actin.

Ca^{2+}-loaded F-actin, prepared by polymerization of Ca-G-actin in the presence of 0.2 mM Ca^{2+} and absence of Mg^{2+}, does not release Ca^{2+} even at free Ca^{2+} concentrations as low as 30 nM. Ultrasonic treatment, however, released bound Ca^{2+} even in the presence of ATP, increasing the concentration of free Ca^{2+} up to 500 nM (Fig. 4). Rate and extent of ultrasound-induced Ca^{2+} release from Ca-G-actin also depends on the concentration of actin (data not shown). The dependence of Ca^{2+} release on ultrasound treatment and actin concentration suggests that the concentration of filament ends bearing Ca-ADP-actin monomers is an essential parameter determining the rate and amount of released Ca^{2+}.

Fig. 5 shows the effect of a small amount of profilin on Ca^{2+}-loaded F-actin. Compared to the actin concentration (25 μM), very low concentrations of profilin (0.5–3 μM) released significant amounts of Ca^{2+}. The low-dose response of profilin can be explained by its property to induce accelerated 'treadmilling'. Further details are discussed below. Profilin-induced Ca^{2+} release is non-linearly related to the profilin concentration. Above 3 μM, profilin did not further increase Ca^{2+} release from Ca-loaded F-actin (data not shown).

In the right part of Fig. 2, it is shown that profilin can induced Ca^{2+} release from Mg/Ca-F-actin that has just taken up external Ca^{2+} under the same conditions. Profilin-induced Ca^{2+} release is accelerated by ultrasonic treatment. Addition of profilin to Mg/Ca-F-actin prior to the addition of Ca^{2+}, effectively inhibited Ca^{2+} uptake during sonication (data not shown). Profilin also caused Ca^{2+} release from Mg/Ca-F-actin during sonication. Following additional sonication steps in the presence of profilin, initially bound Ca^{2+} was delivered again in a time-dependent manner (Fig. 6).

Ca^{2+} was also released from Mg/Ca-actin by sonication in the presence of 0.5–1 mM Mg^{2+} (Fig. 7). The initial spontaneous increase in Ca^{2+} most likely is due to displacement by Mg^{2+} of Ca^{2+} from actin monomers and ATP.

4. Discussion

This study presents strong evidence that F-actin may function as receptor-operated Ca^{2+} store. The highly specific F-actin-stabilizing toxin, phalloidin, effectively inhibited both the Ca^{2+} storage process of microvillar vesicles from HIT cells and that of purified F-actin. The mechanism of phalloidin action is identical with those of the known inhibitors of Ca^{2+} storage such as phosphate, vanadate and AlF_4^- which all act as F-actin stabilizer [15] and also inhibit the endo-/sarcoplasmic reticulum Ca^{2+} pump [16,17].

Another strong argument for the involvement of the actin system in Ca^{2+} storage is the finding, that both storage and release processes exactly cover the physiological concentration range between 40 and 500 nM of free Ca^{2+} which is consistently observed in basal and stimulated intact cells when influx of external Ca^{2+} is eliminated. This range is determined by the

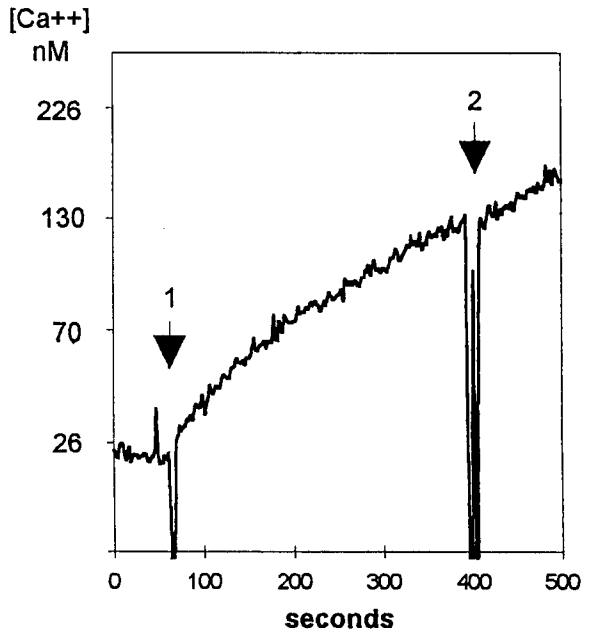

Fig. 5. Profilin-induced Ca^{2+} release from Ca-F-actin. Ca-F-actin (25 μM) was treated with cation exchangers to remove free Ca^{2+} as described in Section 2. At the time points indicated by the arrows, profilin was added giving final concentrations of 3 μM (arrow 1) and 6 μM (arrow 2).

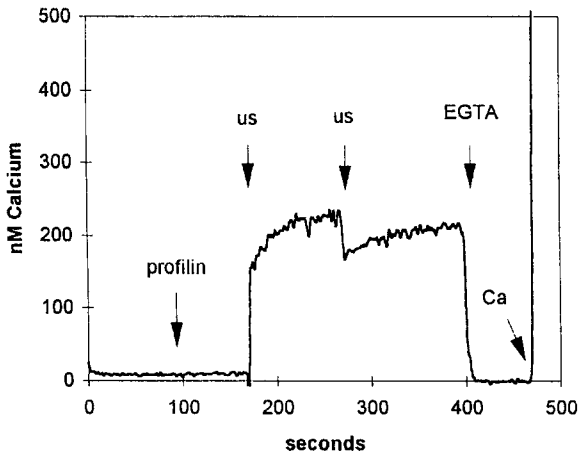

Fig. 6. Profilin-induced Ca^{2+}-release from Mg/Ca-F-actin. Mg/Ca-F-actin (25 μM) was pretreated with cation exchangers as described in Section 2. 3 μM profilin was added at the time point indicated by the first arrow. Ultrasonication (us) of 3–5 s duration initiated a rapid Ca^{2+} release.

intrinsic Ca^{2+}-binding properties of the actin system, the dissociation constants of Ca^{2+} at the ATP- and ADP-actin monomer species of 2–8 nM and 370 nM, respectively [17].

The essential process of Ca^{2+} storage into F-actin is the addition of Ca-ATP-actin monomers to an existing F-actin chain followed by hydrolysis of ATP when the monomers have become part of the polymer chain. Actin filaments at steady-state are assumed to be in a dynamic situation, oscillating between the states of slow elongation and rapid shortening at the barbed end (for review see [19,20]). Steady-state dynamics of this type, also called 'dynamic instability', are assumed to produce terminal regions of limited length (dynamic region) in which the chain subunits are rapidly exchanging with the pool of free monomers. Therefore, divalent cations can be bound to subunits of the dynamic region of F-actin under steady-state conditions. In contrast, cations bound to subunits of the middle part and pointed ends of the filaments, which are not part of the dynamic region, may reflect ionic conditions prevailing some time before the actual state.

High affinity binding of Ca^{2+} (K_d=2–8 nM) only occurs at ATP-G-actin monomers. After addition of Ca-ATP-G-actin monomers to F-actin, ATP hydrolysis and release of the formed P_i converts the actin Ca^{2+}-binding site to a potential low-affinity site, but Ca^{2+} release is still blocked as long as the ADP-actin subunit is part of the polymer chain. Only when Ca^{2+}-ADP-actin monomers were liberated from the filaments, dissociation of bound Ca^{2+} occurs. According to the high K_d of ADP-G-actin for Ca^{2+} (380 nM), this process leads to a significant increase in the concentration of free Ca^{2+}. Free Ca^{2+} can further be elevated by exchange for Mg^{2+} which has a much higher affinity to ADP-G-actin than Ca^{2+} [18].

In the present study, liberation of ADP-actin subunits was achieved by two different methods. First, filaments were mechanically shortened by ultrasonic treatment. The formation of a large number of barbed chain ends exposing endpieces of Ca-ADP-actin considerably increased the concentration of free Ca-ADP-G-actin and thus the concentration of free Ca^{2+}. Increased exchange rates of the divalent cations in F-actin during ultrasonic treatment was first described by Kasai and Oosawa [2].

The second method depends on a specific property of the actin monomer-binding protein, profilin. As first shown by Pring et al. [21], profilin-actin complexes (profilactin) bind to the barbed ends of actin filaments, thereby mediating addition of the actin monomer from the complex to the filament end followed by release of free profilin. Thus, one profilin molecule can mediate a number of cycles of monomer addition. Therefore, at the barbed end of the filament, monomer addition in the presence of profilin proceeds at the same rate and with similar affinity as in its absence [21]. However, profilin-mediated chain elongation does not occur at the pointed end of the filament. Consequently, the reduction of free actin monomers by complex formation with profilin accelerates the dissociation of ADP-monomers from the pointed ends of the filament. Subsequently, these monomers can bind again to the barbed ends of the filaments. Thus, profilin evokes a specific dynamic situation which is characterized by increased monomer release at the pointed end and enhanced monomer addition at the barbed ends. In contrast to the state of dynamic instability, this special type of dynamic state was termed 'treadmilling'. The low concentration of profilin needed for Ca^{2+} release suggests that the mechanism of profilin action may be indeed stimulated treadmilling. Treadmilling and Ca^{2+} release is further facilitated by the ability of profilin to increase by several orders of magnitude the dissociation rates of actin-bound divalent cations and nucleotides [21].

Ultrasound- as well as profilin-induced Ca^{2+} release clearly demonstrates that free Ca^{2+} can be elevated without changing the overall polymerization state of the system. Both types of induced Ca^{2+} release solely depend on increased steady-state dynamics of the polymer. The high rate of Ca^{2+} release observed under ultrasonic treatment, compared with that of the profilin-induced release, indicates that the concentration of barbed filament and critically determines the rate of Ca^{2+}

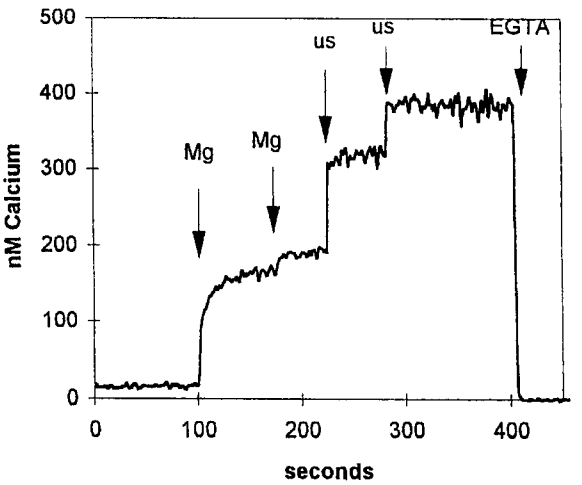

Fig. 7. Mg^{2+}-induced Ca^{2+}-release from Mg/Ca-F-actin. Mg/Ca-F-actin (25 μM) was treated with cation exchangers as described in Section 2. The general experimental conditions were identical with those of Fig. 2. Two portions of 0.5 mM Mg^{2+} each was added (arrows). At the time points indicated with arrows (us), 3–5 s periods of sonication were applied. Release of Ca^{2+} after the first addition of Mg^{2+} most likely is due to displacement of bound Ca^{2+} from actin monomers and free ATP by Mg^{2+}. A second increase of free Ca^{2+} is caused by ultrasonic treatments (us).

release. Thus, the rapid Ca^{2+} release observed in intact cells and vesicle fractions may result from the high cytosolic actin concentrations of 100–200 μM [19] compared with 25 μM in the present experiments, and the relative low average chain length of actin filaments of about 80 subunits found in cortical regions of living cells [22], compared with an average chain length of about 1000 subunits of in vitro polymerized actin [23,24]. Much longer filaments were observed in stress fibers and the contractile rings involved in cell motility [25]. In addition, a number of filament-severing proteins may be involved in the generation of the cellular Ca^{2+} signal by additionally providing new reactive ends from central regions of the filaments.

The high rate of Ca^{2+} uptake into Mg/Ca-F-actin even at very low Ca^{2+} levels can be explained by a few kinetic and equilibrium data: the relative affinity of ATP versus ADP is 200:1 for Ca-actin against only 4:1 for Mg-actin and the rate constant for ADP/ATP exchange is 100-fold higher for Ca-G-actin than for Mg-G-actin [26]. Finally, according to the different hydration properties of Ca^{2+} and Mg^{2+}, the association rate constant of ATP-G-actin for Ca^{2+} is two orders of magnitude higher than for Mg^{2+} [27]. These data indicate that, in the presence of ATP, Ca-G-actin may be the kinetically preferred species for polymerization to F-actin. Especially at high concentrations of filament ends, when diffusion is no longer a rate-limiting process, loading of the polymer with divalent cations may be governed by kinetic rather than by equilibrium properties.

The well-known connection between the phospholipase C-mediated Ca^{2+} signaling pathway with the actin-based cytoskeleton [28–31], provides the functional link between receptors and the actin system. These two systems are interconnected by a few capping, severing and monomer-binding proteins, such as gelsolin, cofilin/destrin and profilin which are bound to the inositol phosphate headgroups of clusters of membrane phosphatidylinositol phosphates (PIP/PIP_2) (reviewed [32,33]). Cleavage of these phospholipids by receptor-mediated activation of phospholipase C liberates these proteins. In addition, released IP_3 acts as amplification signal for further release of actin-binding proteins by competing with the inositol phosphate headgroups of PIP/PIP_2 clusters. The mechanistic basis of this IP_3 effect, the inhibitory action of IP_3 on the complex formation between PIP_2 and profilin has been reported by Lassing and Lindberg [28].

As recently proposed, the microvillar tip region may represent a special cellular microcompartment, named entrance compartment, which is uncoupled from the cytoplasm by a diffusion barrier of F-actin bundles [6,7,9,34,35]. The entrance compartment is connected with the extracellular medium by cation and anion channels located in the microvillar tip region. The preferential selectivity of the cation channels for Ca^{2+} (over Mg^{2+}) may provide the ionic conditions necessary for uptake of Ca^{2+} into Mg-F-actin in this compartment.

The observed close spatial arrangement of Ca^{2+} stores and cation channels within microvilli is highly compatible with the phenomenon of 'capacitative Ca^{2+} loading' as described by Putney and others. The concept of actin-based Ca^{2+} storage with its intrinsic feature of the 'diffusion barrier', also supplies a compelling explanation for the well known dependence of the Ca^{2+} influx activity on the filling state of the Ca^{2+} store. However, the importance of these findings for living cells where it is difficult to determine local concentrations of divalent cation and cytoskeletal proteins remains to be established.

Acknowledgements: This work was supported by the Bundesanstalt für Arbeitsmedizin.

References

[1] Kasai, M. and Oosawa, F. (1968) Biochim. Biophys. Acta 154, 520–528.
[2] Kasai, M. and Oosawa, F. (1969) Biochim. Biophys. Acta 172, 300–310.
[3] Gershman, L.C., Selden, L.A. and Estes, J.E. (1986) Biochem. Biophys. Res. Commun. 135, 607–614.
[4] Carlier, M.F., Pantaloni, D. and Korn, E.D. (1986) J. Biol. Chem. 261, 10778–10784.
[5] Kitazawa, T., Shuman, H. and Somlyo, A.P. (1982) J. Muscle Res. Cell Motil. 3, 437–454.
[6] Lange, K. and Brandt, U. (1993) FEBS Lett. 320, 183–188.
[7] Lange, K. and Brandt, U. (1996) Exp. Cell Res. (in press).
[8] Carothers Carraway, C.A., Cerra, R.F., Bell, P.B. and Carraway, K.L. (1982) Biochim. Biophys. Acta 600, 126–139.
[9] Lange, K. and Brandt, U. (1993) FEBS Lett. 325, 205–209.
[10] Lange, K. and Brandt, U. (1996) Activation of calcium signaling in isolated rat hepatocytes is associated with shape changes of microvilli. In preparation.
[11] Pardee, J.D. and Spudich, J.A. (1985) Methods Enzymol. 85, 167–173.
[12] Lindberg, U., Schutt, C.E., Hellsten, E., Tjäder, A.-C. and Hult, T. (1988) Biochim. Biophys. Acta 967, 3391–4000.
[13] Fabiato, A. (1979) J. Physiol. (Paris) 75, 463–505.
[14] Meyer, T., Wensel, T. and Stryer, L. (1990) Biochemistry 29, 32–37.
[15] Combeau, C. and Carlier, M.-F. (1988) J. Biol. Chem. 263, 17429–17436.
[16] Narayanan, N., Su, N. and Bedard, P. (1991) Biochim. Biophys. Acta 1070, 83–91.
[17] Troullier, A., Girardet, J.-L. and Dupont, Y. (1992) J. Biol. Chem. 267, 22821–9.
[18] Selden, L.A., Gershman, L.C., Kinosian, H.J. and Estes, J.E. (1987) FEBS Lett. 217, 89–93.
[19] Korn, E.D., Carlier, M.-F. and Pantaloni, D. (1987) Science 238, 638–644.
[20] Carlier, M.-F. (1991) J. Biol. Chem. 266, 1–4.
[21] Pring, M., Weber, A. and Bubb, M.R. (1992) Biochemistry 31, 1827–1836.
[22] Goldschmidt-Clermont, P.J., Machesky, L.M., Doberstein, S.K. and Pollard, T.D. (1991) J. Cell. Biol. 113, 1081–1089.
[23] Hartwig, J.H. and Shevlin, P. (1986) J. Cell. Biol. 103, 1007–1020.
[24] Burlacu, S., Janmey, P.A. and Borejdo, J. (1992) Am. J. Physiol. 262, C569–C677.
[25] Small, J.V. (1989) Electron Microsc. Rev. 1, 155–174.
[26] Kinosian, H.J., Selden, L.A., Estes, J.E. and Gershman, L.C. (1993) J. Biol. Chem. 268, 8683–8691.
[27] Gershman, L.C., Selden, L.A. and Estes, J.E. (1991) J. Biol. Chem. 266, 76–82.
[28] Lassing, I. and Lindberg, U. (1985) Nature 314, 472–474.
[29] Goldschmidt-Clermont, Machesky, L.M., Baldassare, J.J. and Pollard, T.D. (1990) Science 247, 1575–1578.
[30] Goldschmidt-Clermont, P.J., Kim, J.W., Machesky, L.M., Rhee, S.G. and Pollard, T.D. (1991) Science 251, 1231–1233.
[31] Janmey, P.A., Iida, K., Yin, H.L. and Stossel, T.P. (1987) J. Biol. Chem. 262, 12228–12236.
[32] Forscher, P. (1989) Trends Neurosci. 12, 468–474.
[33] Stossel, T.P. (1989) J. Biol. Chem. 264, 18261–18264.
[34] Lange, K., Brandt, U. and Zimmermann, B. (1990) J. Cell. Physiol. 142, 1–15.
[35] Lange, K. and Brandt, U. (1990) FEBS Lett. 261, 459–463.