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Activation of LFA-1 by ionomycin is independent of calpain-mediated talin cleavage

Lisa Dreolini, Fumio Takei *

Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada V5Z 1L3
Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada V6T 2B5

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Abstract

Activation of calpains by calcium flux leading to talin cleavage is thought to be an important process of LFA-1 activation by inside-out signalling. Here, we tested the effects of the calcium ionophore ionomycin and calpain inhibitor calpeptin on LFA-1-mediated adhesion of a T cell hybridoma line, cytotoxic T cells and primary resting T cells. Ionomycin activated LFA-1-mediated adhesion of all three types of T cells, and calpeptin inhibited the effects of ionomycin. However, calpeptin also inhibited activation of LFA-1 by PMA, which did not induce calcium flux. Cleavage of talin was undetectable in ionomycin-treated T cells. Furthermore, treatment with ionomycin and calpeptin induced apoptosis of T cells. Inhibitors of phosphatidyl Inositol-3 kinase inhibited activation of LFA-1 by ionomycin, but not by PMA, whereas the protein kinase C inhibitor inhibited the effects of PMA, but not ionomycin. Thus, activation of LFA-1 by ionomycin is independent of calpain-mediated talin cleavage.

Keywords: LFA-1; Cell adhesion; Talin; Calpain; Ionomycin; T cell; Calpeptin

LFA-1, a leukocyte-specific integrin, binds ICAMs to mediate cell adhesion [1]. ICAM-1 is the most prominent ligand for LFA-1 [2]. LFA-1 is critical to a number of steps in immune surveillance and the mounting of an immune response by T cells. It is involved in the firm adherence of circulating T cells to endothelial cells, which facilitates transmigration to lymph nodes and sites of inflammation [3]. LFA-1 is also involved in the formation of the immunological synapse between T cells and APC [4], which is important for the activation of T cells and effector functions including killing of target cells by cytotoxic T lymphocytes (CTLs) [5]. LFA-1 expressed on resting lymphocytes is in an inactive state to ensure that the cells freely circulate throughout the body. However, it can be rapidly converted to the active state in response to activation signals. Activation of LFA-1 is proposed to be regulated by two mechanisms: changes in conformation, resulting in an increased affinity for ligand [6], and clustering of molecules on the cell surface [7], which together with affinity changes increases the overall avidity for ligand. Although the processes of LFA-1 activation have not been fully elucidated, a number of intracellular signaling components have been described [8], and interactions between the α and the β subunits seems to control LFA-1 activation [9]. Interactions with cytoplasmic proteins may disrupt these interactions, and may regulate LFA-1 activation.

Talin is a major cytoskeletal protein that has recently been proposed to act as the final common step in integrin activation [10]. It is composed of a ~50 kDa head and a ~200 kDa tail domain. The talin head contains a predicted FERM (band four-point-one, ezrin, radixin, and moesin) domain that mediates interactions with the cytoplasmic tails of β1 [11], β2 [12], and β3 [10,13] integrins. Overexpression of recombinant talin fragments containing this FERM domain activates β2 [12] and β3 [14,15] integrins.

* Corresponding author. Address: Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada V5Z 1L3. Fax: +1 604 877 0712.
E-mail address: ftakei@bccrc.ca (F. Takei).

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RNAi experiments indicate that talin is required for activation of β1 and β3 integrins [10]. The talin N-terminal FERM domain may be masked by the C-terminal tail, as has been shown to occur with other ERM proteins [16]. Cleavage of talin by calpains *in vitro* to separate the head domain from the tail increases the affinity for β3 cytoplasmic tails [17]. Calpain I (μ-calpain) and calpain II (m-calpain) are calcium-dependent proteases that are known to cleave a number of cytoskeletal proteins including talin [18]. Treatment of T cells with the Ca^{2+} mobilizers, ionomycin, 2,5-di-*t*-butylhydroquinone, and thapsigargin activates LFA-1-mediated cell adhesion. Furthermore, activation of LFA-1 by thapsigargin-induced calcium fluxing is prevented by pre-treatment with the calpain inhibitor calpeptin [19], suggesting that activation of calpains may be responsible for LFA-1 activation. However, cleavage of talin by calpains in activated T cells has not been demonstrated.

In this study, we have tested the hypothesis that the calcium ionophore ionomycin stimulates LFA-1 through the activation of calpains and the consequential cleavage of talin to liberate the talin head domain. Unexpectedly, our results show that activation of LFA-1 by ionomycin treatment is independent of calpain-mediated talin cleavage.

Materials and methods

Mice, cells, antibodies, and reagents. C57BL/6 (B6) mice were from Jackson Laboratories (Bar Harbor, ME) and the male antigen-specific T cell receptor-transgenic RAG2-deficient C57BL/10 mice were from Taconic Farms (Tarrytown, NY). The murine T cell hybridoma line T28 has been described [20]. The human prostate cell line LNCaP was from Dr. M. Sadar (BC Cancer Research Centre, Vancouver, Canada). LNCaP was cultured in phenol red-free RPMI 1640 medium plus 10% FCS and antibiotics. Splenic resting T cells were isolated as described [21]. CTLs were generated as described [22]. On day 6 of culture, CTLs were transferred to media lacking IL-2 for 36–48 h to return cells to resting state. Mouse anti-talin mAb (clone 8d4) was from Sigma (St. Louis, MO). Goat anti-talin tail (C-20) polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-α-spectrin (non-erythroid) mAb (MAB1622) was from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated anti-mouse secondary antibody was from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Ionomycin, calpeptin, PD150606, ALLN, Calpain inhibitor III, Calpain II (rat recombinant), Ly294002, and NP-40 were from Calbiochem (EMD Biosciences, San Diego, CA). PMA (phorbol myristate acetate) and Wortmannin were from Sigma (Oakville, Ont., Canada). Bicinchoninic acid (BCA) Protein Assay kit was from Pierce (Rockford, IL). Calcein-AM and FURA-2 were from Molecular Probes/Invitrogen (Eugene, OR). Annexin V-PE Apoptosis Detection Kit was from BD Pharmingen (San Diego, CA). Protein G beads were from Amersham (Piscataway, NJ). Murine recombinant soluble ICAM-1 has been described [23].

Cell adhesion assay. LFA-1-mediated cell adhesion to immobilized ICAM-1 was assayed as described [24]. For stimulation of LFA-1, cells were pre-treated with 50 ng/ml PMA or the indicated concentrations of ionomycin for 10 min at 37 °C. For inhibitor assays, Cells were pre-incubated with the specified concentrations of inhibitors for 15–30 min at 37 °C prior to stimulation. To calculate percent inhibition, the following formula was applied:

$$\%\text{inhibition} = 100 \times \frac{\%\text{adhesion}(-\text{inhibitor}) - \%\text{adhesion}(+\text{inhibitor})}{\%\text{adhesion}(-\text{inhibitor})}$$

Talin and spectrin cleavage assay. LNCaP cells were treated as described for the spectrin cleavage assay [25]. T28 cells were washed once and resuspended to 1×10^6 cells/ml in phenol red-free RPMI 1640 medium, then stimulated with 500 ng/ml ionomycin, or 50 ng/ml PMA for 20 min at 37 °C. Cells were pre-incubated with 500 μM calpeptin at 37 °C for 15 min prior to stimulation with ionomycin as indicated. Cells were lysed as described [25], supernatants were recovered, and total protein was quantitated using the BCA Protein Assay kit. Talin was immunoprecipitated from mouse splenocytes with anti-talin tail antibody bound to protein G-coupled beads. The beads were washed, and resuspended in 10 mM Tris, pH 7.6, 2 mM $CaCl_2$. Purified calpain II was added to the beads, and the mixture was incubated at room temperature for 1 hour. The reaction was stopped by the addition of SDS–PAGE sample buffer, and the samples were boiled for 5 min. 1×10^6 cell equivalents (talin cleavage control) or 15 μg of protein per sample (talin cleavage assay) were loaded onto 5% SDS–PAGE gels and transferred to membranes for Western analysis. Membranes were probed with appropriate monoclonal antibody (10 μg/ml) and HRP-conjugated anti-mouse Ig secondary antibody, and visualized by chemiluminescence by an ECL system (Amersham Biosciences).

Apoptosis assay. T28 cells were resuspended to 10^6 cells/ml in HBSS + 2% FCS. Calpeptin was added to each condition as indicated, and cells were incubated at 37 °C for 30 min. 500 ng/ml ionomycin or 50 ng/ml PMA was added as appropriate, and the cells were incubated for another 10 min at 37 °C. The cells were kept at 4 °C from this point forward. Cells were then stained with the Annexin V-PE Apoptosis Detection Kit (BD Pharmingen). The cells were then analyzed by FACS, and the percentage of cells undergoing apoptosis was determined by the percentage of cells that were Annexin V-PE^+ and Annexin V-PE^+7-AAD^+ (double positive). To calculate the percentage apoptosis induction by inhibitors, the following equation was applied:

$$\%\text{apoptosis induction} = \%\text{apoptosis}(+\text{inhibitor}) - \%\text{apoptosis}(\text{unstimulated})$$

Results

Activation of LFA-1 by ionomycin treatment

The effects of ionomycin on LFA-1-mediated adhesion of various T cells to immobilized recombinant ICAM-1 were tested. The murine helper T cell hybridoma line T28 showed low basal level of adhesion to immobilized ICAM-1, and treatment with ionomycin significantly increased the adhesion (Fig. 1, top), which was almost completely inhibited by anti-LFA-1 mAb. CTLs, generated *in vitro* in this study, constitutively adhered to immobilized ICAM-1 (data not shown). Therefore, they were cultured for additional 2 days without IL-2 to bring them to rest. They showed low level of adhesion to ICAM-1, and ionomycin significantly enhanced the adhesion (Fig. 1, middle). LFA-1-mediated adhesion of resting T cells was only weakly activated by ionomycin (Fig. 1, bottom). High concentrations of ionomycin was toxic, resulting in reduced cell adhesion. Intracellular calcium measurement showed calcium fluxing in ionomycin-treated T cells as expected (data not shown). Treatment with PMA induced LFA-1-mediated adhesion of T cells, but it did not induce Ca^{2+}-fluxing, indicating that ionomycin and PMA activate LFA-1-mediated T cell adhesion by two separate pathways.

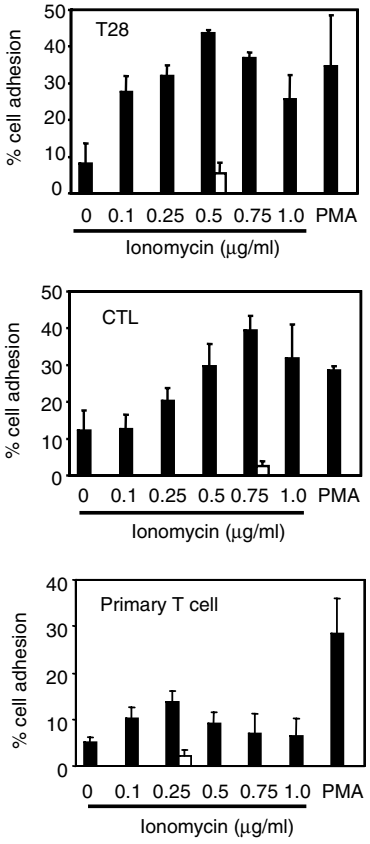

Fig. 1. Activation of LFA-1 by ionomycin-induced calcium fluxing. T28 cells (top), primary resting T cells (middle), or CTLs (bottom) were incubated with the indicated concentrations of ionomycin for 10 min at 37 °C. LFA-1 mediated adhesion to immobilized ICAM-1 was measured as described in Materials and methods. Cells stimulated with 50 ng/ml PMA were used as positive control. Anti-LFA-1 antibody (TIB213) was tested as specificity control (open bars). The results shown are the averages of at least three independent experiments for each cell type, each performed in triplicate. Error bars indicate SD.

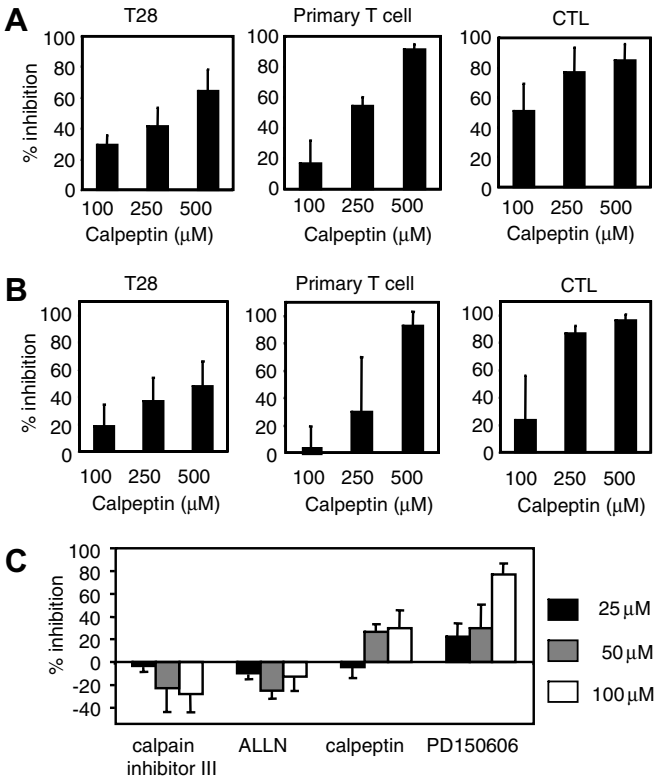

Fig. 2. Effects of calpain inhibitors on LFA-1 mediated adhesion. T28 cells, primary resting T cells or CTLs were incubated with the indicated concentrations of calpeptin, then stimulated with 500 (T28 cells), 250 (resting T cells), or 750 (CTLs) ng/ml ionomycin for 10 min at 37 °C in (A) or 50 ng/ml PMA for 10 min in (B). LFA-1-mediated adhesion was analyzed as in Fig. 1. Percentage inhibition of adhesion was calculated as described in Materials and methods. Results shown are the average of three independent experiments for each cell type, each performed in triplicate. Error bars indicate SD. (C) T28 cells were incubated with 25 (filled bars), 50 (grey bars), or 100 (open bars) μM calpeptin, ALLN, calpain inhibitor III or PD150606 then stimulated with 500 ng/ml ionomycin as in (A). Cell adhesion was analyzed as in Fig 1. Results are the averages of three independent experiments, each performed in triplicate. Error bars indicate SD.

Effects of calpain inhibitors

Pre-incubation of cells with calpeptin inhibited ionomycin-induced adhesion of T28 cells, CTLs, and primary T cells to ICAM-1 (Fig. 2A). To test the specificity of calpeptin treatment, its effect on PMA-induced activation of LFA-1 was also tested. PMA is thought to activate LFA-1 through the PKC pathway [26], independent of Ca^{2+} flux and calpains. Unexpectedly, calpeptin also inhibited PMA-induced LFA-1 activation in all three types of T cells (Fig. 2B). These results suggest that the calpeptin inhibition seen in Fig. 2 may be due to a non-specific effect.

Calpeptin, ALLN, and calpain inhibitor III are modified peptides that compete for the active sites of calpains whereas PD150606 targets the Ca^{2+} binding site of the protease [27]. ALLN and calpain inhibitor III failed to inhibit ionomycin-induced activation of LFA-1 whereas calpeptin and PD150606 inhibited LFA-1 activation in T28 cells (Fig. 2C).

Talin is not cleaved by ionomycin stimulation

Activated calpain is thought to cleave talin into a ~47 kDa head and a ~200 kDa tail domain [17,28]. Western blotting showed that *in vitro* treatment of isolated talin with calpain indeed cleaves talin and generates the tail fragment (Fig. 3A, first lane). In contrast, ionomycin treatment did not decrease the amount of full length talin, nor increase the cleaved tail fragment in T28 cells (Fig. 3A) or CTLs (data not shown). Ionomycin treatment of the human prostate cancer cell line LNCaP has been reported to activate calpains and induce cleavage of spectrin [25]. However, talin cleavage was not detectable in ionomycin-treated LNCaP cells (Fig. 3A).

Spectrin cleavage into two fragments of 145 and 150 kDa has been used as an assay for calpain activity in intact cells [25,27]. Western blotting with anti-spectrin antibody that recognizes the full length spectrin (280 kDa) and

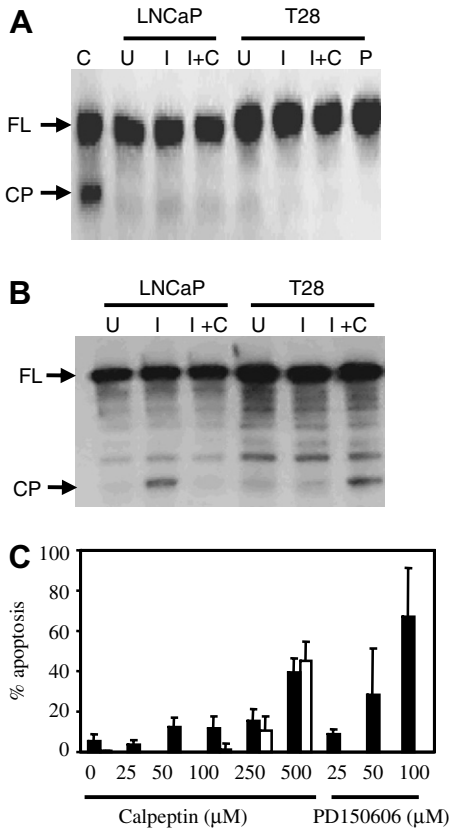

Fig. 3. Effects of ionomycin and calpeptin treatment. LNCaP or T28 cells were pre-treated with 20 (LNCaP) or 500 (T28 cells) μM calpeptin for 15 min. Cells were then treated with 7 μg/ml (LNCaP) or 500 ng/ml (T28 cells) ionomycin for 20 min. In (A), 15 μg of protein extracts were resolved on a 5% SDS–PAGE gel and immunoblotted with an anti-talin (Sigma) antibody. In (B), 30 μg of protein extracts were resolved on a 5% SDS–PAGE gel and immunoblotted with an anti-α-spectrin antibody. C, talin cleavage control; U, unstimulated (DMSO control); I, ionomycin stimulated; I + C, calpeptin pre-treated, ionomycin stimulated; P, PMA stimulated. Arrows marked by FL indicate the full length talin (240 kDa) in (A) and α-spectrin (280 kDa) in (B), and arrows marked by CP indicate cleavage products (~200 kDa in A) and ~150 kDa in (B). Talin cleavage product was generated by incubation of immunoprecipitated talin with calpain *in vitro*. In (C), T28 cells were pre-treated with the indicated concentrations of calpeptin then stimulated with 500 ng/ml ionomycin (black bars) or 50 ng/ml PMA (white bars) as in Fig. 2A. T28 cells were also pre-treated with PD150606 and stimulated with ionomycin as above. Cells were stained with Annexin V-PE and 7-AAD, and analyzed by FACS. Percentage apoptosis was calculated as described in Materials and methods. Results are the averages of three independent experiments. Error bars indicate SD.

the 150 kDa fragment showed spectrin cleavage in control LNCaP cells treated with ionomycin and inhibition of the cleavage with calpeptin. In contrast, spectrin cleavage was undetectable in T28 cells treated with ionomycin (Fig. 3B). Unexpectedly, treatment of T28 cells with ionomycin and 500 ng/ml calpeptin resulted in spectrin cleavage.

Since spectrin can also be cleaved by caspases [29], calpeptin may be inducing apoptosis in our experiments. Therefore, we stained the treated cells with Annexin V and 7-amino-actinomycin D (7-AAD) and analyzed by FACS. Both calpeptin and PD150606 in combination with ionomycin induced apoptosis in a dose-dependent manner (Fig. 3C). Calpeptinat 500 μM also induced apoptosis of PMA-stimulated cells. PD150606 is a more potent inhibitor of ionomycin-induced LFA-1 activation, and also induces apoptosis more strongly than calpeptin at the same concentration (100 μM). These results suggest that the inhibition of LFA-1 with these two calpain inhibitors may be due, at least in part, to the rapid induction of apoptosis.

Inhibitors of PI3K, but not PKC, inhibit activation of LFA-1 by ionomycin

The above results suggest that that calpain-mediated talin cleavage may not be responsible for ionomycin-induced LFA-1 activation. To look for alternative mediators of ionomycin-induced LFA-1 activation, we tested inhibitors of PKC and PI3K. Cells were pre-treated with the irreversible inhibitor Wortmannin and the specific but reversible inhibitor Ly294002 prior to stimulation with ionomycin. These agents inhibited LFA-1-mediated adhesion in a dose-dependent manner in both T28 cells (Fig. 4A). Neither inhibitor induced apoptosis above the level seen with ionomycin alone (data not shown), indicating that the inhibition of adhesion is not due to induction of apoptosis. The inhibitors also did not significantly inhibit PMA-

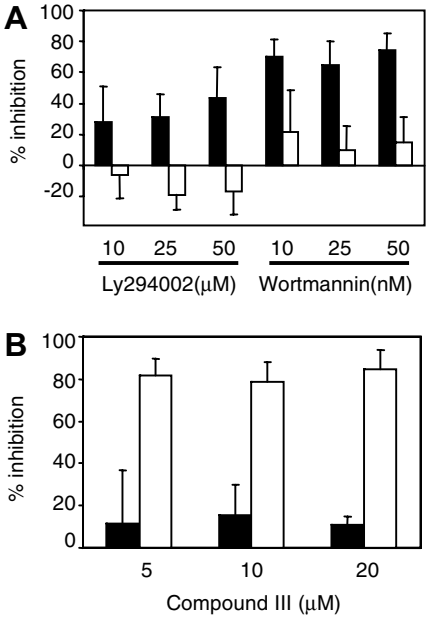

Fig. 4. Effects of PI3K and PKC inhibitors on LFA-1 mediated adhesion. (A) T28 cells were incubated with the indicated concentrations of Ly294002 or Wortmannin for 30 min. Cells were then stimulated with 500 ng/ml ionomycin (black bars) or 50 ng/ml PMA (white bars) for 10 min, and cell adhesion was analyzed as in (Fig. 1, top). Results shown are the averages of three independent experiments, each performed in triplicate. Error bars indicate SD. (B) T28 cells were incubated with the indicated concentrations of Compound 3 and stimulated as in (A). Results shown are the averages of three independent experiments, each performed in triplicate. Error bars indicate SD.

induced LFA-1 activation in T28 cells (Fig. 4A). The PKC inhibitor Compound 3 almost completely abrogated PMA-induced adhesion of T28 cells, but it did not affect ionomycin-induced LFA-1 activation (Fig. 4B). These results suggest that ionomycin-induced LFA-1 activation may involve activation of PI3K but not PKC.

Discussion

Our results dispute the view that calpain-mediated talin cleavage is an important step in LFA-1 activation. This view is based on three lines of studies. First, calpains cleave talin into the head and the tail domains in activated platelets [30], endothelial cells [31], and fibroblasts [32]. Second, calcium immobilizers activate LFA-1 on human T cell blasts and the calpain inhibitor calpeptin inhibits the activation of LFA-1 [19]. Third, overexpression of recombinant talin head domain activates LFA-1 [12] and β3 integrin [10,11]. Taken together, these studies imply that an increase in intracellular calcium activates calpains and induces talin cleavage to generate free talin head, which binds to the cytoplasmic tail of LFA-1, induces its conformational changes and activates its ligand-binding function [33]. However, our current results shown above proves that talin cleavage is undetectable in T cells treated with ionomycin or PMA, both of which activate LFA-1-mediated T cell adhesion to ICAM-1. Although ionomycin activates LFA-1-mediated adhesion of T cells and the calpain inhibitors inhibit the ionomycin-induced LFA-1 activation, the effects of the calpain inhibitors are most probably non-specific. First, calpeptin also inhibits PMA-induced activation of LFA-1, which does not involve increase in intracellular calcium in T cells and is unlikely to activate calpains. Second, combined with ionomycin treatment, calpain inhibitors induce apoptosis of T cells. Third, no cleavage of talin or spectrin is detectable in ionomycin-treated T cells. Therefore, it is highly unlikely that ionomycin-induced activation of LFA-1 is mediated by cleavage of talin by calcium-activated calpains.

It is still unclear whether calpains are expressed at significant level and effectively activated in ionomycin-treated T cells in our study. Interestingly, integrin-mediated signalling has been shown to induce calpain activation and talin cleavage in platelets [34] and fibroblasts [28]. In fibroblasts, calpains are recruited to focal adhesion complexes and cleave multiple proteins including talin and are thought to play an important role in integrin-induced signalling leading to cell spreading and motility [35]. Franco et al. have recently shown that talin cleavage by calpains regulates adhesion complex disassembly in fibroblasts [28]. In T cells, ionomycin treatment or β1 integrin-mediated adhesion induces translocation of calpains to Triton X-100-insoluble membrane/cytoskeleton fractions and cleavage of protein tyrosine phosphatase 1B, but not talin [36]. Stimulation of T cells with anti-CD3 antibody also induces calpain-mediated cleavage of α-actinin, but not talin [37]. Thus, activation of calpains and talin cleavage may be an important effect of integrin-mediated cell adhesion rather than a cause of integrin activation.

How ionomycin activates LFA-1-mediated T cell adhesion to ICAM-1 is still unclear. Our results with pharmacological inhibitors suggest that PI3K may mediate the effect of ionomycin. However, we have not been able to detect Akt activation in ionomycin-treated T cells (data not shown). It is possible that ionomycin activates the serine/threonine kinase $p70^{S6k}$, which is independent of Akt activation but is inhibited by PI3K inhibitors [38]. The possible role of $p70^{S6k}$ activation by calcium fluxing in LFA-1 activation remains to be determined.

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