An essential role for calmodulin in regulating human T cell aggregation

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Abstract After activation of T cells with either CD3 antibodies or phorbol esters, we have found that T cell–cell aggregation, integrin-dependent actin reorganisation and cell spreading are strongly suppressed by any of three structurally different calmodulin antagonists, without any effect on the amount of CD11/CD18 integrin binding to the actin cytoskeleton. However, only T cell receptor-induced, and not phorbol ester-induced, aggregation and cell spreading are prevented by inhibitors of phosphatidylinositide (PI) 3-kinase. These results suggest that PI 3-kinase lies upstream of calmodulin in the signalling pathway leading to T cell aggregation, cell spreading and actin reorganisation and that cell spreading and actin reorganisation are essential for T cell adhesion.

Key words: T lymphocyte; Adhesion; Calmodulin; Integrin; Cytoskeleton

1. Introduction

T cell adhesion is essential for the function of the immune system, and is involved in events like T cell cytotoxicity, T cell extravasation into sites of inflammation and the lymphatic system, and T cell stimulation of B cells to produce antibodies [1]. T cell adhesion to an antigen-presenting cell (APC) is a regulated multistep process, which begins when a migrating T cell detects an antigen–MHC (major histocompatibility complex) complex on the surface of another cell [2]. If the recognition is successful, the cell stops migrating and a part of its membrane surrounds the APC. This is followed by a series of events in which the CD11/CD18 integrins become capable of binding their ligands, the ICAMs (intercellular adhesion molecules) [3], to support cell–cell adhesion. The F-actin cortical cytoskeleton becomes polarised towards the APC [4] and there is a cytoskeleton-dependent rise in intracellular Ca^{2+} levels [5]. Integrin-mediated T cell adhesion has to be strictly regulated for the immune system to function properly [6], and thus cell adhesion is regulated by intracellular signalling pathways. Adhesion has been shown to be regulated at different levels [7], not only by regulating the affinity or avidity (surface clustering) of integrin receptors, but also by regulating so called post-receptor events [8], involving cytoskeletal changes and cell spreading.

Different signalling pathways are involved in the regulation of T cell binding to ICAM-1 through the CD11/CD18 integrins. There is evidence that the lipid kinase phosphatidylinositide 3-OH (PI 3)-kinase, as well as the mitogen-activated protein kinase pathway are involved in regulating T cell binding to immobilised ICAM-1 [9,10]. Also, the calcium-dependent protease calpain has been implicated in this process [11], as well as other signalling components, like the small GTP binding protein Rho [12] and protein phosphatases [13,14]. Importantly, however, the actin cytoskeleton also seems to play a major role in the regulation of integrin-mediated cell adhesion [8,15–17], but how signalling pathways and the cytoskeleton interact has remained elusive.

Here we have investigated the regulation of CD11/CD18 integrin-dependent primary T cell–cell adhesion using inhibitors of different signalling components. We show that activation of T cell–cell adhesion (homotypic aggregation) with antibodies against CD3, or with phorbol ester can be strongly suppressed by calmodulin antagonists. We also present further evidence which supports the notion that cell spreading and actin reorganisation after T cell activation are essential for adhesion, and that calmodulin is required for all these processes.

2. Materials and methods

2.1. Reagents

Ficoll-Hypaque was purchased from Amersham-Pharmacia. RPMI 1640 was from Gibco. Phorbol 12,13-dibutyrate (PDBu), FITC-conjugated phalloidin and LY 294002 from Sigma (St. Louis, MO, USA) and rapamycin, KN-62, W-7, cyclosporin (CsA), trifluoperazine and calmidazolium from Calbiochem (Nottingham, UK). Antibodies: pan-myosin antibody was from Berkeley antibody company (Babco, Richmond, CA, USA), myosin light chain (MLC) antibodies from Sigma (St. Louis, MO, USA), antibody against nuclear factor of activated T cells (NFAT) from Affiniti Research Prod., Exeter, UK, monoclonal antibody OKT3, which reacts with CD3, was used in the form of ascites fluid produced by hybridoma cells (clone CRL 8001: American Type Culture Collection, Rockville, MD, USA). The monoclonal anti-CD18 integrin antibodies R7E4 and R2E7B have been described previously [18]. Hydromount was from National Diagnostics, Atlanta, USA. Complete protease inhibitor cocktail tablets were from Roche Molecular Products (Mannheim, Germany). Microcystin-LR was provided by Dr L. Lawton, Robert Gordons University, Aberdeen, UK.

2.2. T cell isolation

The buffy coats used for isolation of cells were prepared from

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Abbreviations: ICAM, intercellular adhesion molecule; PI 3-kinase, phosphatidylinositide 3-OH kinase; PDBu, phorbol 12,13-dibutyrate; MLC, myosin light chain; mTOR, mammalian target of rapamycin; CsA, cyclosporin; CaMKII, calcium-calmodulin-dependent kinase II; NFAT, nuclear factor for activated T cells

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freshly drawn blood from healthy donors and were obtained from the Edinburgh and Helsinki blood transfusion services. Mononuclear leukocytes were isolated by Ficoll-Paque gradient centrifugation. T cells were enriched using nylon wool columns. T cells were suspended in RPMI 1640 with 10% foetal bovine serum (FBS), left in culture overnight at 37°C and used within 2 days.

2.3. Aggregation assay

These were performed essentially as described [19]. Briefly, cells were suspended in medium with 10% FBS and 40 mM HEPES at a cell density of 15 million cells/ml and aliquoted onto 96 well plates, 200 μl/well. Inhibitors were added for 30 min, after which the cells were activated for the indicated times. Samples were taken at the indicated timepoints and free cells were counted in triplicate. Aggregation percentage was counted as number of free cells after agonist stimulation subtracted from the number of free cells in control experiments, divided by the number of free cells in the control experiments.

2.4. NFATc bandshift assay

Cells were stimulated (1–2 million cells per sample) in RPMI 1640 medium+10% FBS, washed in phosphate-buffered saline (PBS) and suspended in 20 μl 40 mM Tris–HCl pH 8.0, 60 mM sodium pyrophosphate, 10 mM EDTA. 20 μl 10% sodium dodecyl sulfate (SDS) was added to lyse the cells, and the lysate heated for 20 min at 100°C in 1% SDS. Samples were subjected to SDS–polyacrylamide gel electrophoresis on 6% gels and NFAT was detected with a monoclonal antibody at 1 μg/ml.

2.5. Cellular fractionation

Cells were lysed for 15 min on ice in 10 mM sodium phosphate pH 7.4, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, protease inhibitor cocktail and 2 μM microcystin. After centrifugation for 5 min at 4000 rpm to remove nuclei, the supernatant was subjected to ultracentrifugation for 2 h at 100 000×*g* at 4°C. The pellet was designated the cytoskeletal fraction and the supernatant the soluble fraction. Cytoskeletal proteins were released with 300 mM NaCl and 10 mM ATP and 2 U DNase I, denatured with SDS, and subjected to electrophoresis. Proteins were detected by immunoblotting with anti-myosin (1:5000 dilution) or anti-CD18 (R2E7B) antibodies (1:2500 dilution).

2.6. Confocal microscopy

Confocal microscopy was performed using cells labelled with FITC-conjugated phalloidin to stain F-actin. Coverslips were coated with OKT3 in PBS overnight at 4°C or with poly-L-lysine for 20 min at room temperature and washed twice with PBS. Cells (500 000/coverslip) were preincubated with the inhibitors for 30 min at 37°C, pipetted onto the coverslips and left for 30 min at 37°C. Unbound cells were washed away and adhering cells were fixed for 10 min at 22°C with 1% formaldehyde/PBS. Cells were labelled with 1 μg/ml FITC–phalloidin in PBS/0.1% saponin/1% FBS for 20 min at 22°C. After washing with PBS, coverslips were mounted with Hydromount for confocal microscopy.

3. Results

3.1. Calmodulin is involved in regulating both OKT3 and PDBu-induced T cell aggregation

T cell aggregation is known to be largely dependent on CD11/CD18 integrins, as it can be blocked with the R7E4 antibody against the CD18 extracellular domain [14]. In order to study the mechanism by which CD11/CD18 integrins induced T cell–cell adhesion, we employed an aggregation assay in which human primary T cells were stimulated with soluble anti-CD3 antibody (OKT3). T cells from different buffy coats vary in the extent to which they aggregate in response to OKT3, and thus each aggregation assay was repeated several times. Since calcium ions have been implicated in T cell adhesion, we then examined the potential role of Ca-calmodulin in regulating T cell aggregation induced by OKT3.

Three structurally distinct antagonists of calmodulin, W-7, trifluoperazine and calmidazolium, all blocked T cell aggregation (Fig. 1A). All three inhibitors also blocked PDBu-induced cell aggregation (Fig. 1B).

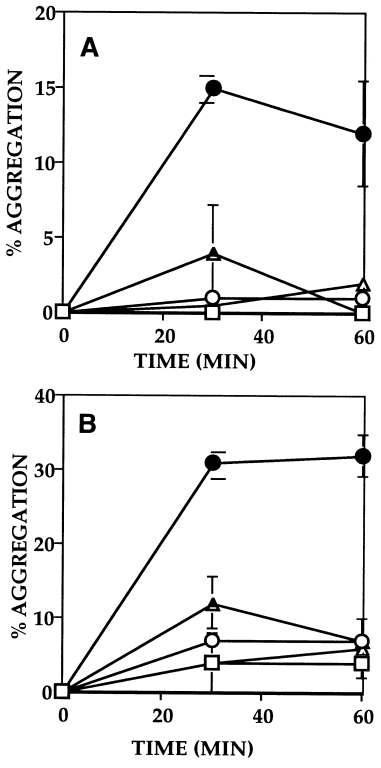

Fig. 1. OKT3- and PDBu-induced T cell aggregation is abolished by three different calmodulin antagonists. (A) T cells were incubated with vehicle alone (□), pretreated with vehicle and activated with OKT3 (●) or pretreated for 30 min with 50 μM W-7 (○), 15 μM trifluoperazine (▲) or 5 μM calmidazolium (△) and activated with OKT3 and assayed for aggregation (Section 2.3). (B) Same as (A) except that 200 nM PDBu replaced OKT3.

3.2. The effect of calmodulin antagonists on T cell–cell adhesion is not due to inhibition of calcineurin, calcium-calmodulin-dependent kinase II (CaMKII) or MLC kinase (MLCK)

It has been reported that calcineurin, the Ca-calmodulin-dependent phosphatase, and CaMKII, are involved in β3-integrin-mediated migration and adhesion of neutrophils, and in the regulation of β1-integrins, respectively [20,21]. We therefore investigated whether these enzymes are involved in regulating T cell–cell adhesion through the CD11/CD18 integrins. Although the three different calmodulin antagonists strongly suppressed T cell–cell adhesion, we found that they did not block the calcineurin-induced dephosphorylation of NFAT under the same conditions (Fig. 2A). In contrast CsA, which inhibits calcineurin and NFAT dephosphorylation, did not affect T cell–cell adhesion (Fig. 2B). Similarly KN-62, a relatively specific inhibitor of CaMKII, had no effect on adhesion (Fig. 2B). We also found that the calmodulin antagonists did not inhibit the calcium-calmodulin-dependent MLCK, because phosphorylation of the myosin P-light chain was unaffected (Fig. 2C). Myosin P-light chain phosphorylation was high in basal or stimulated cells, in the presence or absence of calmodulin antagonists (Fig. 2C). These results are considered further in Section 4.

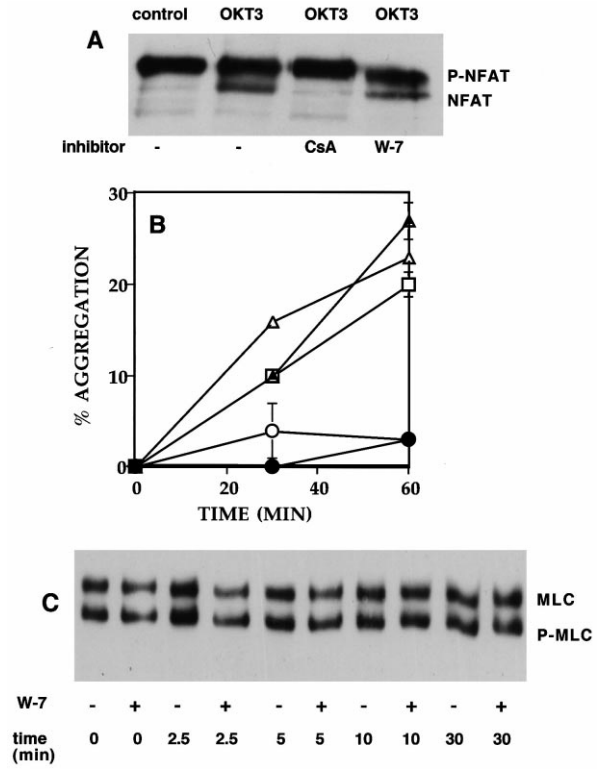

Fig. 2. Calcineurin, CaMKII and MLCK are not involved in regulating T cell–cell adhesion. (A) Cells were pretreated with 0.5 μg/ml CsA or 100 μM W-7 before activation with OKT3 for 30 min and analysis for dephosphorylated NFAT (NFAT) or phosphorylated NFAT (P-NFAT) (Section 2.4). (B) Cells were pretreated for 30 min with 0.1 mM W-7 (○) or 0.5 μg/ml CsA (△) or 10 μM KN-62 (□) or no additions (▲), then exposed to OKT3 for the times indicated followed by assay for aggregation. The closed circles show control experiments in which cells were incubated with no additions in the absence of OKT3. (C) Cells were pretreated for 30 min with W-7 (0.1 mM), before activation with OKT3. Proteins were precipitated with perchloric acid and analysed for myosin P-light chain (MLC) phosphorylation [28] by immunoblotting with an MLC antibody to detect phosphorylated MLC (P-MLC) and dephosphorylated MLC (MLC).

3.3. None of the inhibitors used affects integrin binding to the cell cytoskeleton

Many reports have emphasised the importance of integrin–cytoskeleton interactions in regulating adhesion. We therefore studied the binding of the CD18 chain to the filamentous actin-containing cytoskeleton using a cell fractionation assay. This assay makes use of the insolubility of filamentous actin and associated proteins in Triton X-100 and hence their sedimentation at high *g* forces [22,23]. In resting T cells, or in OKT3-activated T cells, only a small proportion of the CD11/CD18 integrins were attached to the cell cytoskeleton (Fig. 3A), and the level of attachment of total integrin did not change upon activation. This result is in agreement with earlier results in phorbol ester-stimulated T cells [22].

The integrins could be released from the cytoskeleton using DNase I, which breaks up filamentous actin, indicating that they are indeed bound to the actin-based cytoskeleton, and not just insoluble in Triton X-100 (data not shown). Myosin II, a cytoskeletal motor protein, was present mainly in the cell cytoskeleton, and the level of attachment did not change significantly upon cell activation (Fig. 3B). None of the compounds used to inhibit adhesion affected the distribution of integrins between the soluble and cytoskeletal fractions (Fig. 3C), indicating that a quantitative alteration of integrin cytoskeletal attachment is not involved in the change of integrin function.

3.4. Actin reorganisation in T cells in response to OKT3 and PDBu stimulation is profoundly inhibited by W-7

Since none of the inhibitors used changed the total attachment of integrins to the actin cytoskeleton, we wanted to examine whether there was a change in cytoskeletal organisation itself in response to stimuli. We therefore examined the effects of adding cells to coverslips, either coated with OKT3 or poly-L-lysine. We confirmed previous reports [15], which indicated that the T cell cytoskeleton underwent a profound change in response to OKT3 or phorbol ester stimulation. Firstly, cell spreading occurred both in OKT3- and phorbol ester-stimulated cells. Secondly, the actin-rich ring, which is present underneath the plasma membrane in resting cells, was broken down and actin-rich protrusions or pseudopods were seen extending from the cells (Fig. 4A–C). Strikingly, the calmodulin antagonist W-7 (Fig. 4D) affected actin reorganisation, indicating that this might be the mechanism by which it affects T cell aggregation. W-7 completely blocked actin reorganisation and cell spreading induced by OKT3 (Fig. 4D) or phorbol esters (data not shown), leaving the actin cytoskeleton in a dense ring beneath the plasma membrane.

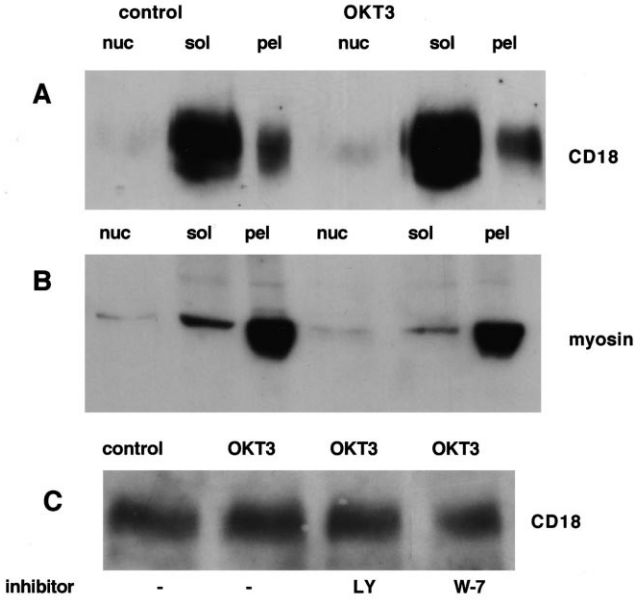

Fig. 3. Integrins are linked to the cytoskeleton in resting, activated and inhibitor-treated cells. Cells (25 million per sample) were stimulated for 30 min with OKT3 or left unstimulated (control) and lysed (Section 2.5). Nuclei were spun down (nuc) and the resulting supernatant was ultracentrifuged. The soluble fraction (sol), and the cytoskeletal pellet (pel) were denatured in SDS, subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose and analysed by immunoblotting with antibodies against CD18 (R2E7B) (A) and myosin (B). (C) Cells were pretreated for 30 min with 50 μM LY 294002 or 0.1 mM W-7 or in the absence of either inhibitor before activation for 30 min with OKT3 as indicated. Control samples were left untreated. The cytoskeletal fraction was isolated as above and CD18 was detected by immunoblotting.

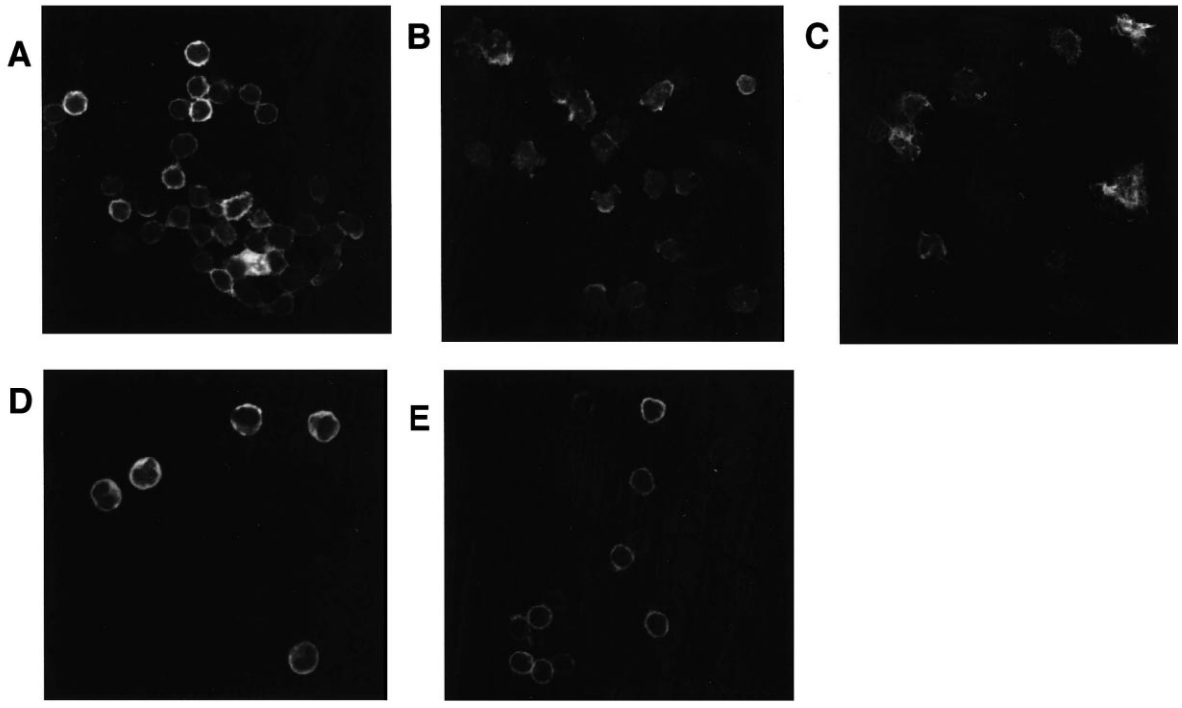

Fig. 4. The signalling pathways shown to be important for integrin-mediated cell–cell adhesion profoundly affect the actin cytoskeleton in T cells. Cells were coated on either poly-L-lysine (control and PDBu) or on OKT3-treated coverslips after preincubation for 30 min with inhibitors or vehicle alone. For PDBu activation, cells were mixed with the phorbol ester and then added to the coverslip. After 30 min activation, unbound cells were washed off gently and adhering cells were fixed. Following permeabilisation and staining in a single step, they were analysed by confocal microscopy. (A) Cells were left untreated; (B) cells were added to an OKT3-coated coverslip; (C) cells activated with 200 nM PDBu; (D) cells pretreated with 100 μM W-7 and activated with OKT3; (E) cells pretreated with 50 μM LY 294002 and activated with OKT3.

3.5. Effect of PI 3-kinase inhibitors on T cell aggregation

We confirmed earlier reports that OKT-induced T cell aggregation was prevented by the PI 3-kinase inhibitors LY 294002 and wortmannin (data not shown). In contrast, mammalian target of rapamycin (mTOR), a downstream effector of PI 3-kinase, is not involved in the process since rapamycin, a specific inhibitor of this protein kinase, had no effect on aggregation (data not shown). LY 294002 prevented OKT-induced cell spreading (Fig. 4E), but had no effect on phorbol ester-induced T cell aggregation and cell spreading (data not shown).

4. Discussion

T cell–cell adhesion is a complex process dependent on T cell activation, integrin activation and post-receptor events, like cell spreading and actin reorganisation. In this study we have examined the role of calmodulin and PI 3-kinase in regulating these processes using a number of relatively specific inhibitors (Fig. 5).

Calcium signals are reported to be important for integrin-mediated T cell adhesion [24], based on the use of calcium ionophores and chelators [11]. The protease calpain has been proposed to mediate calcium-induced cell adhesion, since calpeptin, an inhibitor of calpain, inhibits both integrin-mediated adhesion to coated ICAM-1 and clustering of the integrins on the T cell surface [11]. However, we have now shown that calcium ions have a further role, because three different calmodulin antagonists are very efficient blockers of T cell aggregation induced through either the T cell receptor or by phorbol ester. Nevertheless, despite the reported involvement of calcineurin and CaMKII in the regulation of other integrins [20,21], we excluded the involvement of these calmodulin-dependent enzymes, as well as MLCK from playing an essential role in calmodulin-regulated T cell aggregation (Fig. 2).

It could be argued that the apparent discrepancy between the inhibitory effects of the calmodulin antagonists on adhesion and their lack of effect on known calmodulin-dependent enzymes is the result of non-specific effects of these drugs. However, this seems improbable, since the three structurally different calmodulin antagonists affected T cell adhesion in a similar way. It is well known that calmodulin antagonists affect calmodulin-dependent proteins at different concentrations, depending on the strength of the interaction between calmodulin and the effector. Our results suggest that the calmodulin effector protein(s) that influences T cell adhesion interacts with calmodulin more weakly than with calcineurin, CaMKII or MLCK, such that the interaction is prevented by lower concentrations of inhibitors.

The cytoplasmic tail of the integrin CD18 chain is important for both cell adhesion and changes in the actin cytoskeleton [8]. Regulation of integrin avidity by clustering of receptors on the cell surface has been postulated to involve changes in integrin–cytoskeleton interactions [11,15,25,26]. Here we show that the CD11/CD18 integrins are partly attached to the F-actin cytoskeleton in control T cells and OKT3-activated cells, and the proportion attached does not change significantly after cell activation. This result is in agreement with our previous findings in phorbol ester-treated cells [22]. Moreover, treatment of the cells with inhibitors of aggregation did

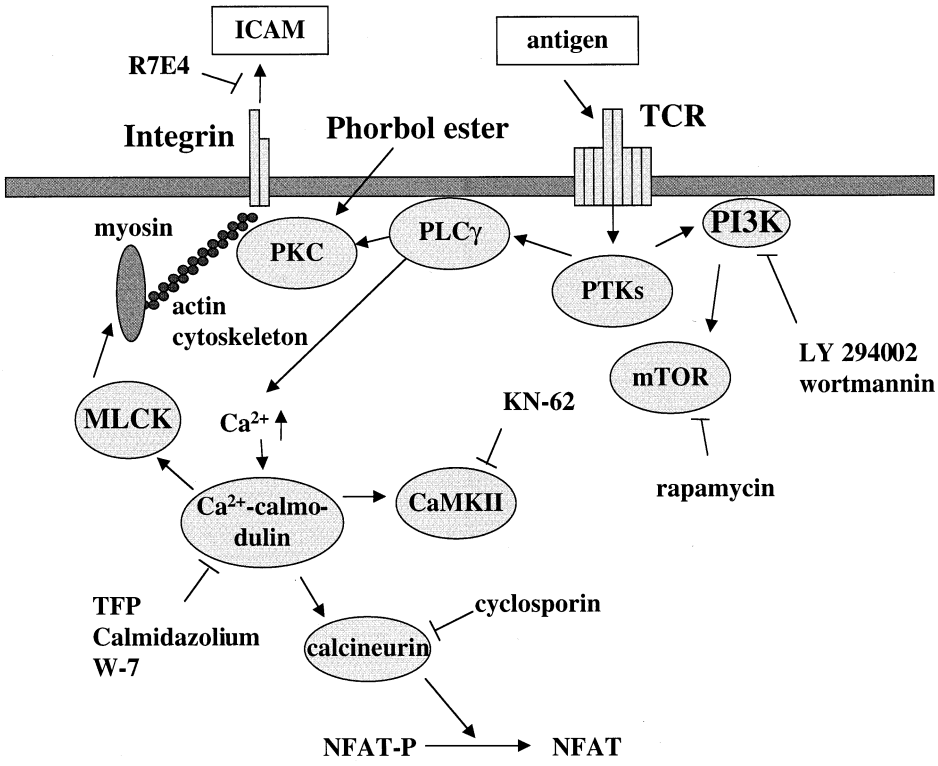

Fig. 5. Summary of the signalling pathways studied in this paper. Abbreviations: ICAM, intercellular adhesion molecule; R7E4, an antibody that blocks the binding of CD11/CD18 integrins to ICAMs; TCR, T cell receptor; MLCK, myosin light chain kinase; TFP, trifluoperazine; W-7, an inhibitor of calcium-calmodulin-dependent processes; CaMKII, calcium-calmodulin-dependent protein kinase II; KN-62, a cell-permeant inhibitor of CaMKII; NFAT, nuclear factor for activated T cells; PTKs, protein tyrosine kinases; PI3K, phosphatidylinositide 3-kinase; LY 294002 and wortmannin, cell-permeant inhibitors of PI3K; mTOR, mammalian target of rapamycin; PKC, protein kinase C; PLCγ, phospholipase Cγ.

not induce any significant changes in integrin distribution, indicating that an alteration in the amount of integrin bound to the cytoskeleton does not underlie the change in avidity of the cell–cell contact. However, it is possible that integrin phosphorylation, or some other signalling event, does lead to a qualitative change in integrin–cytoskeleton interactions after cell activation.

After T cell activation with either phorbol ester or through the T cell receptor complex [15], the actin cytoskeleton undergoes substantial reorganisation mediated by the interplay between integrins and actin. We found that actin reorganisation occurs in response to cellular stimuli, and that pretreatment of the cells with inhibitors of T cell–cell adhesion prior to activation altered actin reorganisation profoundly. The calmodulin antagonist W-7, as well as the PI 3-kinase inhibitor LY 294002 prevented OKT3-induced actin reorganisation. However, phorbol ester-induced cell spreading was only inhibited by the calmodulin antagonist, and not by the PI 3-kinase inhibitor, indicating that PI 3-kinase acts upstream of calmodulin in this system.

Only 15–20% of CD18 integrin-deficient (leukocyte adhesion deficiency type I or LADI) T cells succeed in reorganising their cytoskeleton and spreading on CD3 antibody-coated surfaces [15], indicating that the process is largely integrin-dependent. However, actin reorganisation and cell spreading are not necessarily ligand-dependent, since actin polarisation towards the T cell–APC contact area does not seem to involve integrin–ligand interaction [27]. Also, no integrin binding to ligand takes place in the assay we use for cell spreading and actin reorganisation. Thus, we think that the changes occurring in T cells that are responsible for integrin activation simultaneously induce integrin-, but not ligand-dependent changes in the cytoskeleton. Since the integrin cytoplasmic tail, and especially the cluster of three threonine residues at the C-terminus, is important both for cell adhesion and actin cytoskeletal changes [8], it is possible that threonine phosphorylation and/or altered binding of some other component to the CD18 chain after T cell activation induces subsequent actin reorganisation. The reorganisation of actin presumably also results in increased cell–cell adhesion through the integrins, which has been speculated to be dependent on cell spreading [16].

In summary T cell–cell adhesion is a complex, integrin-dependent process requiring multiple signalling pathways to be activated (Fig. 5). In this study we show that calmodulin plays an essential role in regulating T cell aggregation. We also show that there is a close correlation between actin reorganisation, cell spreading and cell adhesion through CD11/CD18 integrins, since inhibition of signalling pathways involved in regulating T cell–cell adhesion also inhibits the described actin reorganisation events.

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