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ICAM-1-Coupled Cytoskeletal Rearrangements and Transendothelial Lymphocyte Migration Involve Intracellular Calcium Signaling in Brain Endothelial Cell Lines[1]

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Endothelium of the cerebral blood vessels, which constitutes the blood-brain barrier, controls adhesion and trafficking of leukocytes into the brain. Investigating signaling pathways triggered by the engagement of adhesion molecules expressed on brain endothelial cells using two rat brain endothelial cell lines (RBE4 and GP8), we report in this paper that ICAM-1 cross-linking induces a sustained tyrosine phosphorylation of the phosphatidylinositol-phospholipase C (PLC)γ_1, with a concomitant increase in both inositol phosphate production and intracellular calcium concentration. Our results suggest that PLC are responsible, via a calcium- and protein kinase C (PKC)-dependent pathway, for p60^{Src} activation and tyrosine phosphorylation of the p60^{Src} substrate, cortactin. PKCs are also required for tyrosine phosphorylation of the cytoskeleton-associated proteins, focal adhesion kinase and paxillin, but not for ICAM-1-coupled p130^{Cas} phosphorylation. PKC's activation is also necessary for stress fiber formation induced by ICAM-1 cross-linking. Finally, cell pretreatment with intracellular calcium chelator or PKC inhibitors significantly diminishes transmonolayer migration of activated T lymphocytes, without affecting their adhesion to brain endothelial cells. In summary, our data demonstrate that ICAM-1 cross-linking induces calcium signaling which, via PKCs, mediates phosphorylation of actin-associated proteins and cytoskeletal rearrangement in brain endothelial cell lines. Our results also indicate that these calcium-mediated intracellular events are essential for lymphocyte migration through the blood-brain barrier. ***The Journal of Immunology,*** **2000, 165: 3375–3383.**

Lymphoid cells continuously patrol the body, infiltrating the tissues and re-entering the blood through the lymph. The former process, known as leukocyte extravasation, proceeds through multiple steps that involve a number of pairs of adhesion molecules (1). Initial interaction between selectins and their carbohydrate ligands precedes firm adhesion resulting from binding of activated leukocyte integrins with an Ig-like superfamily of adhesion molecules on endothelial cells. Morphological changes of leukocytes then accompany transmigration of leukocytes through the endothelial cell barrier. In the central nervous system, brain endothelial cells are joined by continuous tight junctions, constituting the blood-brain barrier, which strictly limits leukocyte infiltration. Nevertheless, in pathological situations such as multiple sclerosis or viral or bacterial infections, numerous activated lymphocytes, monocytes, or neutrophils can cross the blood-brain barrier (2).

It has become clear over the last few years that in addition to enabling leukocytes to adhere to endothelium, adhesion molecules are also involved in intracellular signal transduction. Leukocyte responses to integrin engagement have been extensively studied, while responses of endothelial cells have received much less attention. Nevertheless, leukocyte adhesion is known to be associated with alterations in the functional state of endothelium, affecting surface protein expression, secretory function, permeability to macromolecules, and leukocyte transmigration. These responses are associated with intracellular signals, including cytoskeletal modification, protein phosphorylation, and calcium influx (3).

Studies on adhesion molecules contributing to the different steps of leukocyte infiltration into brain tissue have pointed to the ICAM-1/LFA-1 interaction as one of the major pairs of adhesion molecules required for lymphocyte firm adhesion and infiltration (4, 5). ICAM-1 is barely detectable in normal brain cells, but its expression is enhanced on endothelial and glial cells during inflammatory situations such as multiple sclerosis and experimental allergic encephalomyelitis (6–8). In vitro, ICAM-1 expression can be up-regulated in response to proinflammatory cytokines, such as TNF-α, IL-1β, or IFN-γ (9). We have previously reported that ICAM-1 cross-linking on rat brain endothelial cell lines (RBE4 and GP8 cells) induces activation of the tyrosine kinase p60^{Src} (Src)[3] and an associated phosphorylation of the cytoskeletal-associated proteins cortactin (10), focal adhesion kinase (FAK), paxillin, and p130^{Cas} (11), along with activation of Rho and rearrangement of the actin-cytoskeleton (12).

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[3] Abbreviations used in this paper: Src, p60^{Src}; FAK, focal adhesion kinase; PKC, protein kinase C; PLC, phosphatidylinositol-phospholipase C; PLNC, peripheral lymph node-derived lymphocytes; RAM, rabbit anti-mouse Ab; bFGF, basic fibroblast factor; BAPTA-AM, 1,2,-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester; MAPTBM, (1,2,bis(*o*-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester).

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Intracellular calcium may be a key second messenger in the opening of the blood-brain barrier. Indeed, in brain endothelial cells, exposure to calcium ionophore A23187, which elevates intracellular calcium concentration, increases monolayer permeability (13). However, the mechanisms by which intracellular calcium increases are induced in response to leukocyte adhesion and the pathways which may mediate such a cellular response remains to be explored. Following leukocyte adhesion, production of inositol 1,4,5-trisphosphate and the binding of this molecule to its receptor calcium channel may be responsible for the initial increase in intracellular calcium concentration in endothelial cells. Indeed, this phenomenon has been shown to be associated with a number of cellular responses including stimulation of cell-cell adhesion molecules such as $\alpha_L\beta_2$ and VCAM-1 (14, 15). Because we have previously shown that ICAM-1-mediated intracellular signaling induced tyrosine phosphorylation of several proteins (10), we focused the present study on the phosphatidylinositol-phospholipase C (PLC)-γ (3), which is known to be regulated by tyrosine phosphorylation (16) and is a key enzyme responsible for inositol 1,4,5-trisphosphate generation. We have subsequently investigated the consequences of calcium signaling on cytoskeleton organization within brain endothelial cell lines in response to ICAM-1 engagement and the role of intracellular calcium and PKCs in controlling transendothelial migration of lymphocytes.

Materials and Methods

Reagents

Mouse mAbs to rat ICAM-1 (1A29) and to rat MHC class II (OX6) were purchased from Serotec (Wiesbaden, Germany). 3H8 was kindly provided by Dr. W. Hickey (Dartmouth Medical School, Hanover, NH). Isotype-matched mouse IgG, MOPC 21, was obtained from Sigma (St. Louis, MO). Rabbit anti-mouse (RAM) Abs were obtained from Dako (Trappes, France). Mouse mAbs specific for phosphotyrosine, Src, and cortactin were purchased from Upstate Biotechnology (Lake Placid, NY). Mouse mAbs specific for paxillin and $p130^{Cas}$ were obtained from Transduction Laboratories (Lexington, KY) and rabbit polyclonal Abs, anti-$PLC\gamma_1$, and anti-FAK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PMA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), (1,2,-bis(*o*-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester) (MAPTAM), A23187, thapsigargin, and cytochalasin D were obtained from Sigma. The PKC inhibitors bisindolylmaleimide, GF109203X (17), and Ro31-8220 and the PLC inhibitor U73122 were purchased from Calbiochem (San Diego, CA). [γ-^{32}P]ATP, myo-[2-^{3}H(N)]inositol were obtained from Dupont de Nemours (Les Ulis, France) and $^{51}NaCr_2O_7$ was obtained from Amersham (Bucks, U.K.)

Brain endothelial cell lines

RBE4 cells were isolated from rat brain cortex and immortalized with the plasmid pE1A-neo, containing the adenovirus E1A-encoding sequence followed by a neomycin-resistance gene (18). GP8 cells were isolated from rat brain cortex and immortalized with SV40 large T-Ag (19). RBE4 and GP8 cells have been extensively characterized, and maintain in culture the differentiated phenotype of cerebral endothelium (20–22). RBE4 and GP8 cells were grown, as previously described (11).

ICAM-1 cross-linking

RBE4 or GP8 cells were seeded at a density of 10^4 cells/cm^2, and after 3–4 days in culture, were incubated with serum- and basic fibroblast factor (bFGF)-free culture medium containing 100 U/ml IFN-γ, for 48 h. In the same conditions of IFN-γ pretreatment, RBE4 cells express MHC class II molecules (23). Cells were washed in PBS before treatments. Cross-linking of adhesion molecules was performed by treatment with specific mAbs for 10 min, and subsequent addition of RAM Abs for the indicated periods of time.

Calcium measurements

Endothelial cells were plated onto 35-mm dishes. Confluent cells were changed to serum- and bFGF-free medium containing 100 U/ml IFN-γ. The following day, cells were loaded with 10 μg/ml Fluo-3-AM (Molecular Probes, Eugene, Oregon) for 45–60 min at room temperature. Fluo-3-AM is a fluorescein-based dye, with an absorption maximum around 464 nm and a peak emission of 530 nm. The fluorescence intensity of Fluo-3-AM increases linearly with internal calcium concentrations. After extensive washing, cells were treated as described above in serum- and bFGF-free medium. Cells were observed by phase contrast microscopy and by fluorescence microscopy using the transillumination and epi-illumination pathways, respectively, of an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany). Fluorescence was excited using an Argon laser (Spectra Physics, Les Ulis, France) and images were recorded with a Silicium Intensified Target camera (LH4036; Lhesa, France) and digitized in real-time using a personal computer card frame grabber (Meteor, Matrox, Rungis, France). A customized program permitted microfluorometry quantification using the fluorescence intensity of individual cells (10×10 pixels square box, approximately corresponding to a 5×5 μm field). Photobleaching was found to be negligible on short time scales (typically < 2 min). However, when fluorescence was recorded over longer time scales, a time-lapse recording technique was used to limit photobleaching; fluorescence intensity data were acquired for 3 s every minute, while the laser was shut down between two successive recordings. Data reported correspond to the average fluorescence intensity over the 3-s recordings ($\pm$ SD).

Inositol phosphate analysis

Cells were grown to confluence in six-well plates and labeled with [^{3}H]inositol by adding serum- and bFGF-free medium containing 100 U/ml IFN-γ and 5 μCi/ml of [^{3}H]inositol for 24 h. Cells were washed twice with PBS and incubated for 30 min in incubation buffer (HEPES (pH 7.4), NaCl, $MgCl_2$, KCl, EDTA, glucose, and BSA) containing 10 mM LiCl. Cell treatments were then performed as described above in fresh incubation buffer, containing 10 mM LiCl. After 30 min, reactions were stopped by aspiration of the buffer and immediate extraction with 500 μl of chloroform/methanol (1:2 v/v). After the addition of 200 ml H_2O, and vortexing, phases were separated by centrifugation (15,000 rpm, 1 min). Upper aqueous phases containing [^{3}H]inositol phosphates were applied to Bio-Ras AG1 (formate form) columns (Isolab, Akron, OH). After washes with H_2O, then with 60 mM sodium-formate, and with 5 mM sodium-tetraborate, samples were eluted from the columns with 3 ml of 1 M ammonium formate and 0.1 M formic acid. Radioactivity in samples was counted by scintillation spectrometry with 10 ml Atomlight liquid scintillation mixture (Dupont de Nemours).

Immunoprecipitations and Western blotting

Following treatments, cells were washed with ice-cold PBS containing 1 mM orthovanadate and lysed for 30 min at 4°C in Nonidet P-40 buffer (10 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM orthovanadate, and 1% Nonidet P-40 with protease inhibitors 2 mM PMSF, 5 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin). Nuclei were discarded following centrifugation at $10{,}000 \times g$ for 10 min. Lysates were incubated overnight at 4°C with specific Abs, and subsequently for 2 h with agarose or RAM-coated agarose, respectively. Immunoprecipitates were collected by centrifugation and extensively washed in Nonidet P-40 buffer. Immunoprecipitated proteins were eluted with SDS-sample buffer, and resolved on 10% SDS-PAGE followed by immunoblotting as previously described (11).

In vitro Src kinase activity

After cell treatments, Src immunoprecipitations were performed as described above, samples were washed twice in kinase buffer (20 mM PIPES (pH 7), 10 mM $MnCl_2$, 1 mM PMSF, 0.1 μM orthovanadate, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin). Kinase activity of each sample was determined by autophosphorylation assay in 30 μl kinase buffer containing 1 mM ATP and 2 μCi [γ-^{32}P]ATP for 5 min. Reactions were terminated by the addition 15 μl 4$\times$ Laemmli buffer, and proteins were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membrane. The presence of phosphorylated proteins was revealed by autoradiography using an intensifying screen. Equal loading of Src protein in each lane was confirmed by Western blotting using anti-Src mAb.

Immunofluorescence

Cells were plated on glass coverslips. After 3 days of culture, cells were starved in serum- and bFGF-free medium for 24 h and then treated as described above. After washes with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, protected with glycine 0.1 M for 15 min, and blocked with 2% BSA and 0.05% saponin in PBS for 1 h. Cells were incubated for 1 h with tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma) for F-actin labeling. Immunofluorescence images

were collected using a scanning confocal microscope (MCR.1000; Bio-Rad, Hercules, CA).

Adhesion of peripheral lymph node cells to endothelial cells

Adhesion assays were conducted as previously described (24, 25) using cells harvested from Lewis rat peripheral lymph nodes. Briefly, peripheral lymph node-derived lymphocytes (PLNC; Ref. 3) were isolated and T lymphocytes were obtained after purification on nylon wool columns. These cells, which represent non-Ag activated T lymphocytes, are therefore non-migratory but highly adhesive when activated with the mitogen Con A (4, 26, 27). PLNC were activated for 24 h with 5 μg/ml type V Con A and washed twice in HBSS, and cells were labeled with 3 μCi ^{51}Cr per 10^6 cells in HBSS for 90 min at 37°C. After three washes with HBSS, cells were resuspended in RPMI 1640 medium containing 10% FCS. Endothelial monolayers grown on 96-well plates were prepared by removing the culture medium and washing the cells four times with HBSS. [^{51}Cr]-labeled PLNC (200 μl) at a concentration of 1×10^6 cells/ml were then added to each well and incubated at 37°C for 1.5 h. In each assay, γ-emissions from each of 12 replicate blank wells were determined to provide a value for the total amount of radioactivity added per well and to allow calculation of the specific activity of the cells. After incubation, nonadherent cells were removed with four separate washes from the four poles of the well with 37°C HBSS as previously described (24). Adherent PLNC were lysed with 2% SDS, the lysate was removed and γ-emissions were quantitated by spectrometry. Results are expressed as the fractional adhesion of PLNC to untreated endothelial cells. Results were obtained from a minimum of 6–12 separate wells per treatment. The results are expressed as the means ± SEM, and significant differences between groups were determined by Student's *t* test.

T lymphocyte transendothelial migration

The ability of the immortalized cells to support the transendothelial migration of Ag-specific T lymphocytes was determined using a well-characterized assay as described extensively elsewhere (4, 26, 27). Briefly, T lymphocytes were added (2×10^4 cells/well) to 96-well plates containing monolayers of brain endothelial cell lines. Lymphocytes were allowed to settle and migrate over a 4-h period. To evaluate the level of migration, cocultures were placed on the stage of a phase-contrast inverted microscope housed in a temperature-controlled (37°C), 5% CO_2-gassed chamber (Zeiss, Herts, U.K.). A 200×200–μm field was randomly chosen and recorded for 10 min spanning the 4-h time point using a camera linked to a time-lapse video recorder. Recordings were replayed at 160 times normal speed and lymphocytes that had either adhered to the surface of the monolayer or that had migrated through the monolayer were identified and counted. Lymphocytes on the surface of the monolayer were identified by their highly refractive morphology (phase-bright) and rounded or partially spread appearance. In contrast, cells that had migrated through the monolayer were phase-dark, highly attenuated, and were seen to probe under the endothelial cells in a distinctive manner (4, 26, 27). Treatment of endothelial cells with intracellular calcium chelators or PKC inhibitors was conducted before the addition of lymphocytes, following extensive washing and replacement into new media. Control data were expressed as the percentage of total lymphocytes within a field that had migrated through the monolayer. All other data were expressed as a percentage of the control migrations. A minimum of six wells per assay were performed. The results are expressed as the means ± SEM, and significant differences between the groups were determined by Student's *t* test.

Results

ICAM-1 cross-linking induces PLCγ_1 tyrosine phosphorylation

After specific immunoprecipitation of PLCγ_1, Western blot analysis revealed that ICAM-1 cross-linking strongly induced the tyrosine-phosphorylation of PLCγ_1 (Fig. 1*A*, *upper panel*). By contrast, treatment with an isotype-matched mAb (MOPC 21, mouse IgG1) and RAM or RAM alone had no effect (Fig. 1*A*, *upper panels*). Cross-linking of MHC class II molecules using the isotype-matched OX6 Ab did not induce any PLCγ_1 phosphorylation (not shown). This result was confirmed using the GP8 brain endothelial cell line (Fig. 1*A*, *right panels*). As shown in Fig. 1*A* (*lower panels*), similar amounts of PLCγ_1 protein were immunoprecipitated in all lanes. Phosphorylation of PLCγ_1, observed in response to ICAM-1 cross-linking, appeared within the first minute, increased until 20–30 min, then declined slowly over the next hour (Fig. 1*B*).

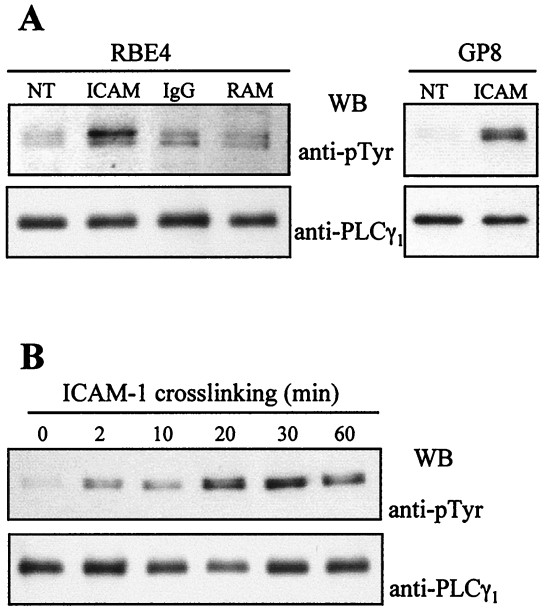

FIGURE 1. ICAM-1 cross-linking induces activation of a phosphoinositide pathway in brain endothelial cell lines. *A*, RBE4 or GP8 cells were either nontreated (*NT*) or stimulated (*ICAM*) with 10 μg/ml anti-ICAM-1 mAb (1A29) for 10 min and the subsequent addition of 10 μg/ml RAM for 30 min. As control, RBE4 cells were either submitted to cross-linking with isotype-matched mAb (*IgG*) or treated with RAM alone (*RAM*) for 30 min. Cell lysates were subjected to immunoprecipitations with anti-PLCγ_1 Ab. Immunoprecipitated proteins were analyzed by phosphotyrosine Western blotting (*upper panels*). After stripping of bound Abs, the same membranes were reincubated with anti-PLCγ_1 (*lower panel*). *B*, RBE4 cells were incubated with anti-ICAM-1 mAb for 10 min, then RAM was added for the indicated periods of time (cross-linking time). Cell lysates were subjected to immunoprecipitations with anti-PLCγ_1 Abs. Immunoprecipitated proteins were analyzed by anti-phosphotyrosine Western blotting (*upper panels*). After stripping of bound Abs, the same membrane was reincubated with anti-PLCγ_1 (*lower panel*). Results are representative of three to five experiments.

ICAM-1 cross-linking induced inositol phosphate production and increase of intracellular calcium concentration

Inositol phosphates appeared to be associated with enzymatic activation of PLCγ_1. Clustering of ICAM-1 molecules resulted in approximately a 2-fold increase of inositol phosphate production (Fig. 2*A*). Similar responses were observed when ICAM-1 cross-linking was performed with a different anti-ICAM-1 mAb (3H8; not shown). Isotype-matched mAb (MOPC 21) or RAM alone did not induce any significant increase in inositol phosphate production (Fig. 2*A*).

Because inositol 1,4,5-trisphosphate production is known to lead to mobilization of intracellular calcium, we investigated calcium signaling in RBE4 cells. Cells were loaded with the fluorochrome agent Fluo-3 and the change of fluorescence was recorded as a function of time in individual cells (Fig. 2*B*). Addition of anti-ICAM-1 mAb alone did not generate increases in intracellular calcium concentration. However, in parallel with the tyrosine phosphorylation of the PLCγ_1 (Fig. 1*B*), we observed a rapid elevation of intracellular calcium concentration 1 min after ICAM-1 cross-linking with RAM in a majority of cells (72% responding cells). This rapid increase was followed by sustained oscillations

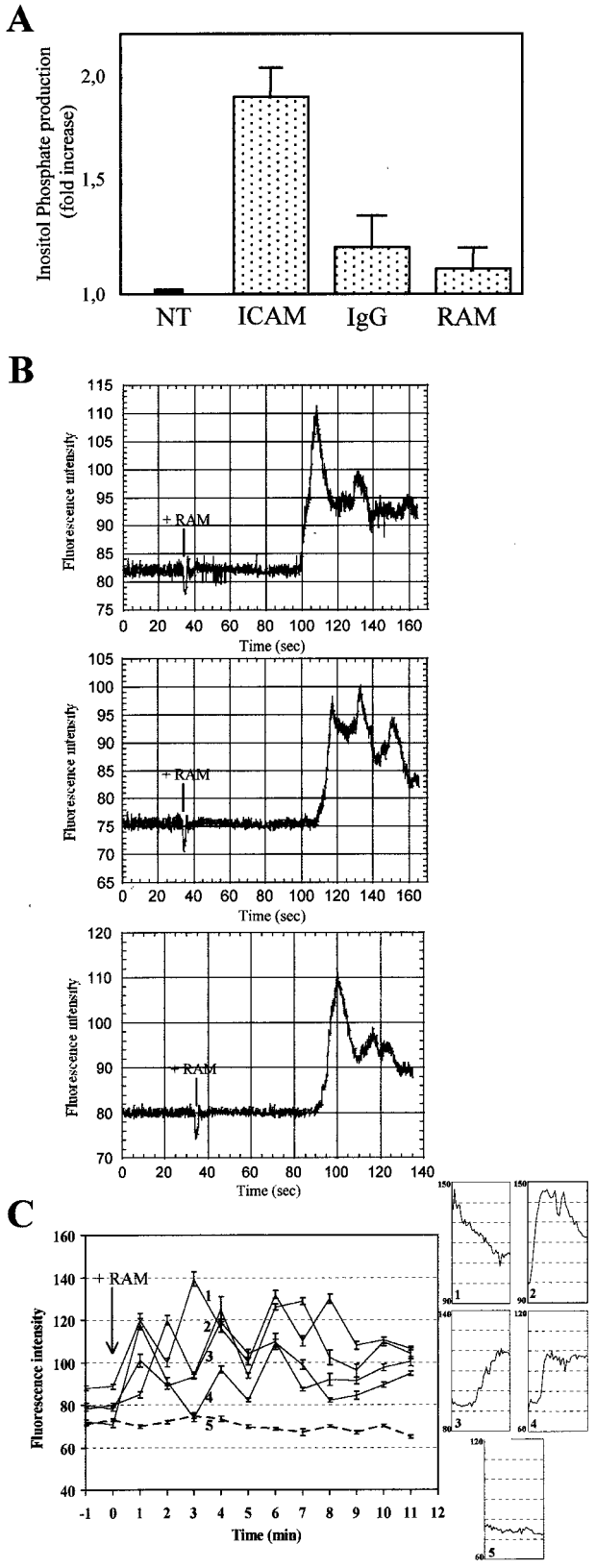

FIGURE 2. PLC activation and intracellular calcium increase in response to ICAM-1 cross-linking in RBE4 brain endothelial cell lines. *A*, RBE4 cells were either nontreated (*NT*) or stimulated (*ICAM*) with 10 μg/ml anti-ICAM-1 mAb (1A29) for 10 min and a subsequent addition of 10 μg/ml RAM for 30 min. As control, cells were either submitted to cross-linking with isotype-matched mAb (*IgG*) or treated with RAM alone (*RAM*) for 30 min. Phosphoinositide production was determined as described in *Materials and Methods*. Results are the mean ± SD of five independent determinations. *B*, Cells were loaded with Fluo-3-AM in serum- and bFGF-free medium and treated with anti-ICAM-1 mAb for 5 min and then RAM for the indicated period of time. Time-dependent recording of fluorescence levels in representative responding cells (72% of cells). Arrows indicate the addition of Abs. Results are representative of five independent experiments. *C*, Time-lapse recording of the fluorescence level in five different cells. Data points correspond to 3-s fluorescence measurement mean ± SD (see *Materials and Methods*). The panels on the right represent 10-s real-time recording of fluorescence intensity observed 4 min following ICAM-1 cross-linking in the same cells. A nonresponsive cell is shown as a control (*cell 5*).

over a period of 10 min or more (Fig. 2*C*). This result was confirmed using the anti-ICAM-1 mAb (3H8), whereas an irrelevant isotype-matched mAb (MOPC 21) cross-linked with RAM Abs or RAM Abs alone had no effect on intracellular calcium ion concentration (data not shown).

These results indicate that ICAM-1 cross-linking induces PLCγ_1 tyrosine phosphorylation with concomitant inositol phosphate production and an increase in calcium ion concentration within brain endothelial cell lines.

Calcium and PKCs mediate ICAM-1-mediated Src activation and cortactin phosphorylation

To determine whether intracellular calcium was required for Src activation, Src was immunoprecipitated and submitted to an autophosphorylation assay. Src activity was clearly enhanced in cells treated with the calcium ionophore, A23187, compared with nontreated cells (Fig. 3*A*). BAPTA-AM, a cell-permeable calcium chelator, not only abolished Src activation observed in response to A23187 but, more interestingly, also blocked Src activation in response to ICAM-1 cross-linking (Fig. 3*A*). Western blotting with anti-Src mAb confirmed that similar amounts of proteins were immunoprecipitated in all lanes (not shown). Calcium-dependent PKCs are known to mediate a number of intracellular responses. In this paper, we show that stimulation of cells by the phorbol ester PMA for 10 min induced a strong activation of Src (Fig. 3*B*). As expected, overnight pretreatment with PMA, which has been shown to down-regulate most PKC isoforms, including calcium-dependent PKCs, totally abolished this response. Under the same conditions, Src activation induced by ICAM-1 cross-linking or A23187 treatment was also largely reduced (Fig. 3*B*). The role of PKCs in mediating Src activation was further analyzed by using a specific PKC inhibitor, GF109203X. Pretreatment of RBE4 cells with this inhibitor prevented Src activation induced following ICAM-1 cross-linking, confirming that PKC activity was required for ICAM-1-coupled Src activation (Fig. 3*C*)

We have previously reported that ICAM-1 cross-linking induced tyrosine phosphorylation of the Src substrate, cortactin (10). This effect was mimicked by treatment of RBE4 cells with PMA or A23187 (Fig. 3*D*). In addition, inhibition of the calcium signal following treatments of cells with BAPTA-AM, or PKC inhibition by overnight PMA pretreatment, resulted in a drastic limitation of cortactin phosphorylation following ICAM-1 cross-linking (Fig. 3*D*). The role of PLC signaling was further analyzed by pretreating RBE4 cells with the PLC inhibitor U73122 or with thapsigargin, which induces calcium depletion from intracellular stores. In these conditions, ICAM-1-coupled cortactin phosphorylation was abolished, while PMA still induced phosphorylation of cortactin (Fig. 3*E*). In contrast, addition of the extracellular calcium chelator EGTA in the medium concomitantly with RAM Abs failed to inhibit cortactin phosphorylation induced by either PMA or ICAM-1 cross-linking (Fig. 3*E*).

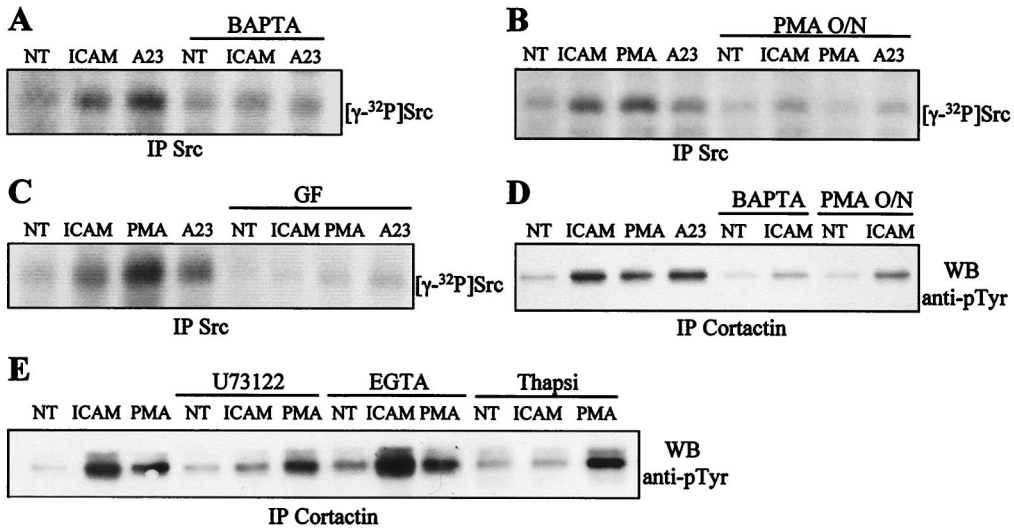

FIGURE 3. Calcium and PKCs mediate ICAM-1-coupled Src activation and cortactin phosphorylation. RBE4 cells were either nontreated (*NT*), submitted to ICAM-1 cross-linking for 30 min (*ICAM*), or treated with 10^{-5} M A23187 for 20 min (*A23*) or 160 nM PMA for 10 min (*PMA*). Where indicated (*BAPTA, PMA O/N, GF, U73122, Thapsi*), cells have been pretreated with 20 mM BAPTA-AM for 1 h, 160 nM PMA overnight (14 h), 20 μM GF109203X for 1 h, 2.5 μM U73122 for 30 min, and 10 μM thapsigargin for 30 min, respectively. When EGTA is indicated, 5 mM EGTA was added with RAM. *A–C*, Src was immunoprecipitated and assayed for Src kinase activity by autophosphorylation. Samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes submitted to autoradiography. *D* and *E*, Cortactin was immunoprecipitated and analyzed by anti-phosphotyrosine Western blotting. Results are representative of three independent experiments.

Taken together, these results indicate that activation of PLC, followed by increase of intracellular calcium concentration and activation of PMA-sensitive PKC isoforms, is the major pathway coupled to ICAM-1 cross-linking toward Src activation and cortactin phosphorylation

Intracellular calcium and PKCs are required for ICAM-1-coupled tyrosine phosphorylation of actin-associated proteins and actin cytoskeleton rearrangements

We have previously reported that ICAM-1 cross-linking induces tyrosine phosphorylation of the focal adhesion-associated proteins FAK, paxillin, and $p130^{Cas}$ (11). Because these three proteins have been described as potential Src substrates, we assessed the role of intracellular calcium and PKCs in ICAM-1-coupled tyrosine phosphorylation of these proteins. Western blot analysis of immunoprecipitated FAK or paxillin revealed that pretreatment with BAPTA-AM or overnight pretreatment with PMA totally abolished tyrosine phosphorylation of FAK (Fig. 4*A*) and paxillin (Fig. 4*B*). In contrast, $p130^{Cas}$ tyrosine phosphorylation was not affected (Fig. 4*C*). Accordingly, $p130^{Cas}$ association with GST-Crk still occurred, whereas paxillin did not bind GST-Crk under these conditions (Fig. 4*D*).

We have shown that ICAM-1 cross-linking induces actin rearrangement in RBE4 and GP8 cells (12). Because phosphorylation of FAK and paxillin has been reported to occur at focal adhesions, where stress fibers are connected with transmembranous integrins, we determined whether calcium signaling and PKCs were required for stress fiber formation observed upon ICAM-1 cross-linking. ICAM-1 was cross-linked on RBE4 cells following pretreatment of cells with BAPTA-AM (Fig. 5*d*), the PKC inhibitor GF109203X (Fig. 5*f*), or overnight treatment with PMA (Fig. 5*h*). Under these conditions, stress fiber formation was totally abolished compared with nonpretreated cells in which stress fibers were apparent following ICAM-1 cross-linking (Fig. 5*b*). These results indicate that intracellular calcium is required for ICAM-1-coupled cytoskeletal modifications and that PKCs play a key role in mediating these effects.

PLC, intracellular calcium, and PKC activation are required for efficient lymphocyte transmonolayer migration

As previously reported, rat brain endothelial cell monolayers can support the ICAM-1-dependent transendothelial migration of Ag-specific lymphocytes. Pretreatment of RBE4 monolayers with the PLC inhibitor U73122, followed by removal, exhaustive washing, and replacement with fresh media before coculture with lymphocytes, reduced transendothelial migration of Ag-specific lymphocytes to 53.1 ± 3.9% ($n = 24$, $p < 0.0001$; Fig. 6*A*, *left panel*). Pretreatment of RBE4 monolayers with intracellular calcium chelator (BAPTA-AM or MAPTAM) for 30 min dramatically reduced transendothelial migration of Ag-specific lymphocytes to 49.7 ± 3.4% and 58.3 ± 3.5% respectively ($n = 18$, $p < 0.0001$; Fig. 6*B*, *left panel*). To assess the potential role of endothelial PKCs during lymphocyte transmigration, both RBE4 and GP8 cells were pretreated with the PKC inhibitor GF109203X. This pretreatment resulted in a lower but still significant inhibition of lymphocyte migration (Fig. 6*C*, *left panel*). Similar results were obtained when using an alternative PKC inhibitor, Ro31-8220 (Fig. 6*C*, *left panel*). These results were confirmed from experiments conducted using GP8 cells (Fig. 6, *left panels*).

The viability of endothelial cell monolayers was not affected by the treatments, as shown by trypan blue exclusion following pretreatment and coculture with lymphocytes (data not shown). Adhesion of PLNC, which adhere as Ag-specific T-cells, but are not capable of transmigration through brain endothelial monolayers (12), was not affected by endothelial cell pretreatments (Fig. 6, *right panels*). In addition, it was also observed that endothelial cell pretreatment did not affect the adhesion of Ag-specific lymphocytes during time-lapse videomicroscopy because lymphocytes displayed normal spreading and motile behavior on the endothelial cell surface. These findings clearly indicate that the effects of the PLC and PKC inhibitors, as well as calcium chelators, in inhibiting lymphocyte migration are due to an effect on the endothelial support of lymphocyte migration and not to prevention of lymphocyte binding to endothelia.

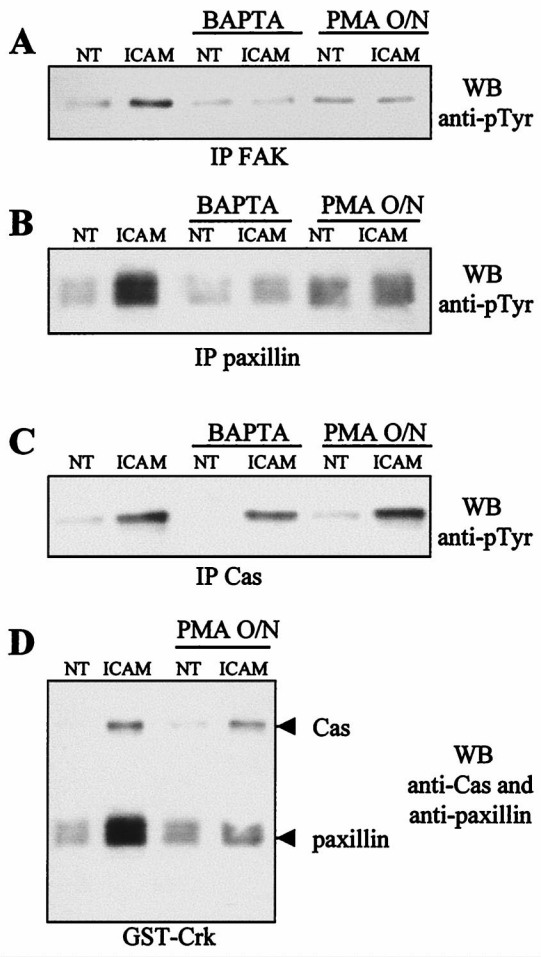

FIGURE 4. Intracellular calcium and PKCs are involved in ICAM-1-coupled cytoskeletal rearrangement. RBE4 cells were either nontreated (*NT*), or subjected to ICAM-1 cross-linking for 30 min (*ICAM*). Where indicated (*BAPTA* or *PMA O/N*), cells have been pretreated with 20 μM BAPTA-AM for 1 h or 160 nM PMA overnight (14 h), respectively. *A*, FAK was immunoprecipitated with specific Ab. Immunoprecipitated proteins were analyzed by anti-phosphotyrosine Western blotting. *B*, Paxillin was immunoprecipitated with specific mAb. Immunoprecipitated proteins were analyzed by anti-phosphotyrosine Western blotting. *C*, $p130^{Cas}$ was immunoprecipitated with specific mAb. Immunoprecipitated proteins were analyzed by anti-phosphotyrosine Western blotting. *D*, GST-Crk immobilized on glutathione-agarose beads were incubated with total cell lysates. Bound proteins were analyzed by SDS-PAGE and Western blotting with anti-paxillin and anti-$p130^{Cas}$ Abs.

Discussion

In the present study, we show that ICAM-1 cross-linking in brain endothelial cell lines induces sustained $PLC\gamma_1$ tyrosine phosphorylation, inositol phosphate production and increase in intracellular calcium ion concentration. Our data strongly suggest that this ICAM-1-induced pathway is responsible for Src activation and subsequent phosphorylation of the Src-substrates, cortactin, and FAK as for actin stress fiber formation, but not for $p130^{Cas}$ phosphorylation.

Calcium signals triggered by ICAM-1 cross-linking have been observed in fibroblasts (28), and inositol phosphate production has also been observed in astrocytes upon ICAM-1 stimulation (S. E.-M., unpublished results). In contrast, phosphorylation of cortactin, which has been reported in response to ICAM-1 cross-linking as well as to lymphocyte adhesion in endothelial cell lines (10, 12), is not observed in astrocytes following ICAM-1 cross-linking (S. E.-M., unpublished results). These observations suggest that the calcium-mediated signals observed in this paper may be specific to endothelial cells.

Tyrosine phosphorylation of $PLC\gamma$ has been reported in the case of other Ig superfamily receptors, like CD2 and CD3, that lack intrinsic kinase activity, but can activate protein tyrosine kinases of the Src family (29, 30). Although the biochemical link between ICAM-1 and $PLC\gamma$ phosphorylation is not known, ICAM-1-mediated $PLC\gamma_1$ phosphorylation was abolished by pretreatment with herbimycin, a well known inhibitor of Src family kinases (not shown). Among these kinases, $p53/p56^{lyn}$, which is highly expressed in brain endothelial cells, has been shown to be strongly activated 1 min after ICAM-1 cross-linking in B cells (31). Because our results strongly suggest that Src is not the kinase responsible for $PLC\gamma_1$ phosphorylation, the putative role of $p53/p56^{lyn}$ will need to be investigated in further studies.

Our results indicate that ICAM-1 cross-linking-induced Src activation is mediated by PKCs. Among the different PKC isoforms, conventional PKCs are under the combined control of diacyl glycerol and intracellular calcium, both being products of PLC activity. However, intracellular calcium increases can mediate PKC activation in some cell types (32). This phenomenon may therefore be responsible for the calcium-induced Src activation observed in the present study. To date, a direct effect of PKCs on Src activity has not been conclusively demonstrated, although PKC is capable of phosphorylating Src on serine 12 (33, 34) and Src-related kinases mediate PKC-dependent activation of the Ras/Raf pathway in T cells (35). It has been suggested that PKC-mediated Src activation in platelets may be a consequence of a PKC-mediated cellular relocalization of Src to a phosphatase responsible for its activation (36).

In addition to cortactin, FAK has also been described as a potential Src substrate (37). Activation of PKC is required for enhanced tyrosine phosphorylation of FAK and formation of focal adhesions. Our data has confirmed these observations and extended them to paxillin, which is usually associated with FAK at focal adhesions, and which is also phosphorylated in response to intracellular calcium increases or PKC activation. It is interesting to note that we have previously demonstrated that FAK and paxillin phosphorylation, in contrast to cortactin, required Rho activation (11). If Src is the kinase responsible for FAK and paxillin phosphorylation as well as for cortactin phosphorylation, our results suggest the existence of different intracellular pools of Src, which are or are not regulated by Rho.

In contrast, our observation that $p130^{Cas}$ phosphorylation and its association with Crk, which are both Rho dependent (11), but which do not depend on PKCs, strongly indicates that the calcium and Rho pathways are distinct. Because $p130^{Cas}$ phosphorylation is not inhibited by PKC depletion, Src is unlikely to be the kinase responsible for $p130^{Cas}$ phosphorylation. The cytosolic tyrosine kinase Abl has been shown to phosphorylate $p130^{Cas}$ in vitro; this phosphorylation is enhanced by binding of Crk to Abl (38). Indeed, we have observed a constitutive association of Abl with Crk in RBE4 cells (data not shown) and have previously described that following ICAM-1 cross-linking, phosphorylated $p130^{Cas}$ binds both Crk and C3G (11). We have previously suggested that this Rho-dependent pathway is responsible for JNK activation; in agreement with this hypothesis, we observed in this paper that JNK activity was, like $p130^{Cas}$ phosphorylation, insensitive to PKC depletion (data not shown).

In summary, ICAM-1 appears to be coupled to two independent pathways, mediated either by Rho or by intracellular calcium and PKCs in brain endothelial cells. The Rho-dependent pathway is responsible for $p130^{Cas}$ phosphorylation and JNK activation, while

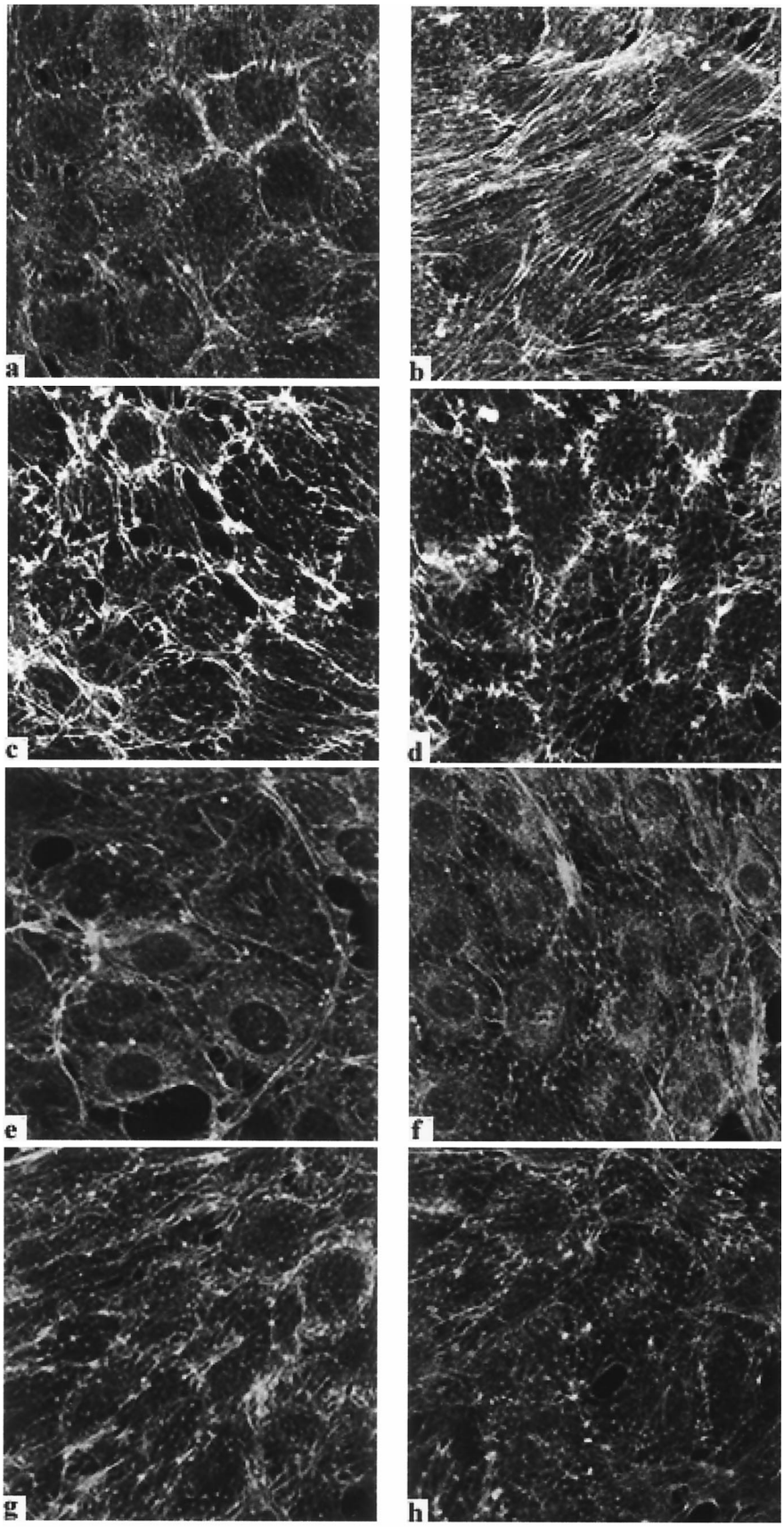

FIGURE 5. Intracellular calcium and PKCs are required for ICAM-1-coupled cytoskeletal rearrangement in RBE4 cells. RBE4 cells were either nonpretreated (*a* and *b*), or pretreated with 20 μM BAPTA-AM for 1 h (*c* and *d*), 20 μM GF109203X for 1 h (*e* and *f*), or 160 nM PMA overnight (14 h) (*g* and *h*). Cells were subsequently either treated with 10 μg/ml RAM for 30 min(*a, c, e,* and *g*) or subjected to ICAM-1 cross-linking (*b, d, f,* and *h*) for 30 min. Cells were fixed and stained for actin using 0.1 μg/ml tetramethylrhodamine isothiocyanate-labeled phalloidin. Results are representative of three independent experiments.

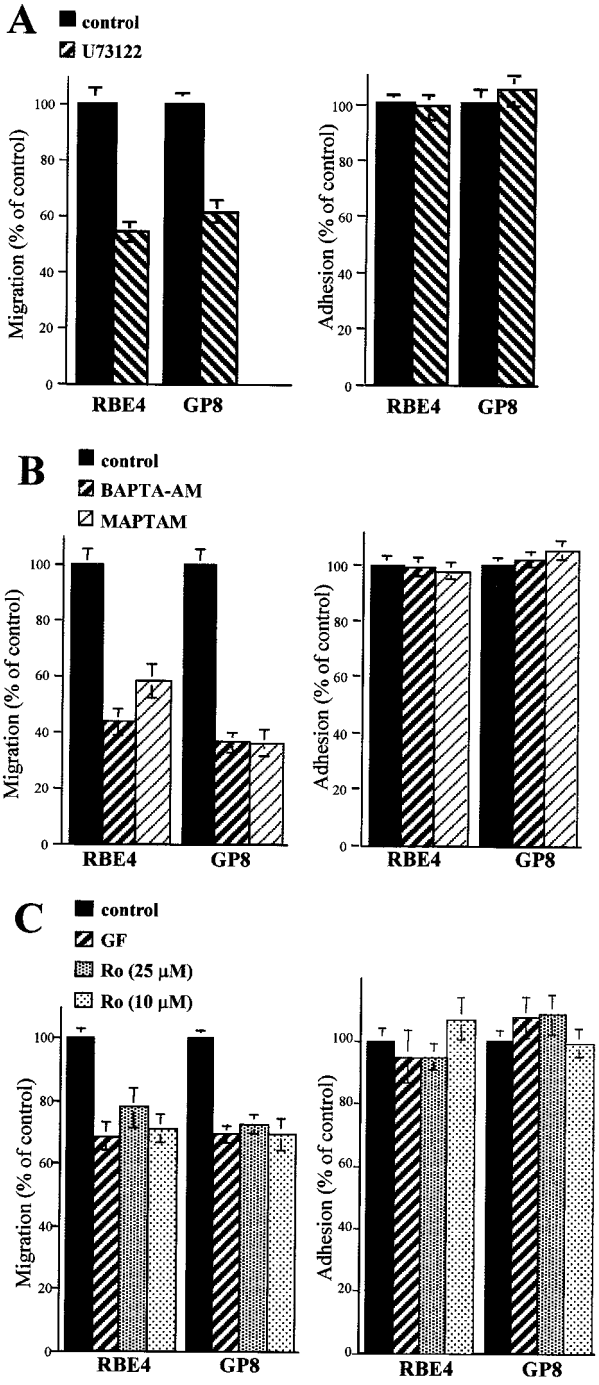

FIGURE 6. Effects of calcium chelators and PKC inhibitors on lymphocyte adhesion to and migration through brain endothelial monolayers. RBE4 cells or GP8 cells were either nonpretreated or treated with the following: *A*, PLC inhibitor (*U73122*, 25 μM); *B*, calcium chelators (*BAPTA-AM* or *MAPTAM*, 20 μM); or *C*, PKC inhibitors 20 μM GF109203X (*GF*), or 10 or 25 μM Ro31-8220 (*Ro*) for 1 h. After removal and vigorous washing, Ag-specific T cells (migration, *left panels*) or 5 μg/ml ^{51}Cr-labeled Con A-activated rat PLNC (adhesion, *right panels*) were added and allow to migrate over a 4-h period or adhere for 90 min, respectively. Results are the mean ± SEM of at least six replicates from at least three independent experiments.

the PKC-dependent pathway mediates Src activation and cortactin phosphorylation. These two pathways seem to converge to allow cytoskeletal rearrangements and phosphorylation of FAK and paxillin induced upon ICAM-1 cross-linking in brain endothelial cells.

Together with VCAM-1-mediated calcium signaling (15), ICAM-1-coupled calcium signaling may directly contribute to the intracellular calcium increase observed in endothelial cells following leukocyte adhesion (5, 28). It has been previously shown by us that leukocyte infiltration is blocked by anti-ICAM-1 Abs (26), as well as by endothelial cell pretreatment with cytochalasin D or the Rho-inhibitor C3 exoenzyme (12). Our findings confirm the active role of brain endothelial cells in promoting transendothelial migration of lymphocytes, and moreover they highlight the involvement of intracellular calcium signaling in this phenomenon. Intracellular calcium concentration controls several downstream effectors, including calcium-dependent PKCs. We observed in this paper that endothelial PKC activation is clearly involved, although to a lesser extent than calcium, in lymphocyte transmigration. PKC activation is correlated with tight junction regulation, via a mechanism involving actin reorganization (39). The role of PKCs during transendothelial migration may be due to their ability to mediate ICAM-1-coupled cytoskeletal rearrangements. Furthermore, PKCs may directly lead to the regulation of junctional complexes. Indeed, in epithelial cells, PKCs appear to be involved in the regulation of adherens junctions via phosphorylation of vinculin (40), and of tight junctions (41), possibly due to translocation of ZO-1 and phosphorylation of occludin (42, 43). In addition, PKCs have also been shown to regulate desmosomal junctions in HeLa cells (44). Thus, it is tempting to speculate that ICAM-1-coupled PKC activation may lead to interendothelial junction opening and, therefore, may contribute to leukocyte diapedesis.

Moreover, our results strongly suggest that calcium-dependent signals, other than PKC activation, are required for lymphocyte transmigration. Investigating other calcium-dependent pathways, we observed that endothelial cell treatment with FK-506, an inhibitor of calcium-dependent phosphatases failed to significantly inhibit lymphocyte migration (data not shown). This observation suggests that the inhibitory activity of FK506 on lymphocyte adhesion to and migration through the endothelium may reflect a regulation of T lymphocyte activation without affecting endothelial activation (45, 46). In contrast, we have observed that ICAM-1 cross-linking leads to phospholipase A_2 activation and concomitant arachidonic acid release (unpublished observations). Alternatively, intracellular calcium increases might lead to a local production of NO via activation of calcium-dependent endothelial NO synthase, which might in turn contribute to a rapid regulation of blood-brain barrier permeability (47), possibly via a direct regulation of cell-cell contacts (48).

Taken together, the findings presented in this study indicate that activation of endothelial ICAM-1 by cross-linking evokes signaling through 1) PLCγ activation, 2) elevation of intracellular calcium concentration, and 3) PKC activation in addition to the previously documented Rho-mediated signaling pathways (11). The integration by the endothelial cells of this complex set of responses to leukocyte adhesion likely reflects the active role played in vivo by vascular endothelium in leukocyte diapedesis.

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