# Lymphocyte Adhesion-dependent Calcium Signaling in Human Endothelial Cells

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***Abstract.*** Vascular endothelial cells (ECs) can undergo dramatic phenotypic and functional alterations in response to humoral and cellular stimuli. These changes promote endothelial participation in the inflammatory response through active recruitment of immune effector cells, increased vascular permeability, and alteration in vascular tone. In an attempt to define early events in lymphocyte-mediated EC signaling, we investigated cytosolic-free calcium ($Ca^{2+}$) changes in single, Fluo-3–labeled human umbilical vein ECs (HUVECs), using an ACAS interactive laser cytometer. Of all lymphocyte subsets tested, allogeneic CD3−, CD56+ natural killer (NK) cells uniquely elicited oscillatory EC $Ca^{2+}$ signals in cytokine (interleukin [IL]-1– or tumor necrosis factor [TNF])–treated ECs. The induction of these signals required avid intercellular adhesion, consisted of both $Ca^{2+}$ mobilization and extracellular influx, and was associated with EC inositol phosphate (IP) generation. Simultaneous recording of NK and EC $Ca^{2+}$ signals using two-color fluorescence detection revealed that, upon adhesion, NK cells flux prior to EC. Lymphocyte $Ca^{2+}$ buffering with 1,2-*bis*-5- methyl-amino-phenoxylethane-*N,N,N'*-tetra-acetoxymethyl acetate (MAPTAM) demonstrated that lymphocyte fluxes are, in fact, prerequisites for the adhesion-dependent EC signals. mAb studies indicate that the $\beta_2$ integrin–intercellular adhesion molecule (ICAM)-1 adhesion pathway is critically involved. However, ICAM-1 antisense oligonucleotide inhibition of IL-1–mediated ICAM-1 hyperinduction had no effect on EC $Ca^{2+}$ signaling in lymphocyte–EC conjugates, indicating that additional cytokine-induced EC alteration is required. These experiments combine features of lymphocyte–endothelial interactions, intercellular adhesion, EC cytokine activation and transmembrane signaling. The results implicate the IP/$Ca^{2+}$ second messenger pathway in EC outside-in signaling induced by cytotoxic lymphocytes, and suggest that these signals may play a role in EC alteration by lymphocyte adhesion.

LEUKOCYTE adhesion to the vascular endothelium has been implicated as an early event in pathologic processes ranging from allograft rejection to coronary atherosclerosis (31, 36). Leukocyte–endothelial cell (EC)[1] interactions are dynamic, involve both cell adhesion through specific receptor–ligand pairs and bidirectional cell signaling, and are dramatically affected by inflammatory mediators which can modulate adhesive membrane molecules in both cell types (30). Leukocyte adhesion is associated with alterations in the functional state of the endothelium (2), affecting permeability to macromolecules, surface protein expression, secretory function, and leukocyte transmigration. Although adhesion pathways have been studied extensively, mechanisms by which cell conjugate formation effect morphologic and functional alterations in EC are less clear.

Cytokines are known to activate ECs in a time course of hours to days (33). Some of these activation events also occur as a result of lymphocyte adhesion and, in this setting, undoubtedly involve the elaboration of some of the same activating mediators. Signal transduction in EC following lymphocyte contact is relatively unexplored. One mechanism of rapid signaling is elevation in cytosolic free $Ca^{2+}$, a path-

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1. *Abbreviations used in this paper*: $Ca^{2+}$, calcium; $[Ca^{2+}]_i$, intracellular calcium concentration; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; ICAM, intracellular adhesion molecule; IFN, interferon; IL, interleukin; IP, inositol phosphate; IP3, inositol (1,4,5)-trisphosphate; LFA, lymphocyte function-associated antigen; MAPTAM, 1,2-*bis*-5-methyl-amino-phenoxylethane-*N,N,N'*-tetra-acetoxymethyl acetate; MTOC, microtubule organizing center; NK, natural killer; PBMC, peripheral blood mononuclear cell; PLC phospholipase C; VCAM; vascular cell adhesion molecule.

way studied most extensively in electrically excitable cells, but more recently established in nonexcitable cells (17). In ECs, elevated cytosolic-free calcium concentrations ($[Ca^{2+}]_i$) have been recorded in response to a variety of soluble mediators including thrombin, histamine, and bradykinin. These elevations have been temporally, and in some settings causally, associated with prostacyclin production (23), von Willebrand factor secretion (5), increased permeability to macromolecules (13), and enhanced neutrophil transendothelial migration (22). Each of these cellular events can be a component of a physiologic or pathologic biological response.

ECs can be stimulators and targets of T cells and NK lymphocytes. We have previously demonstrated that, of all lymphocyte subsets tested, CD3−, CD56+ NK cells adhere most avidly to ECs (1), are the most efficient inducers of endothelial class II HLA molecules (32) and have the ability to recognize allogeneic ECs in an antigen-specific fashion (3). These recognition and activation events are all contact dependent. As a potential early event in these contact-related immune responses, we explored the possibility that lymphocyte adhesion could result in changes in EC $[Ca^{2+}]_i$. Surprisingly, of all subsets tested, NK cells uniquely promote striking EC $Ca^{2+}$ oscillations in an adhesion-dependent fashion. In addition, we demonstrate the co-requirement for NK cell signaling and an association with EC IP generation, evaluate the role of EC membrane molecules known to be involved with leukocyte adhesion, and dissect the sources of increased cytosolic $Ca^{2+}$ in this phenomenon.

## Materials and Methods

### Materials

Fluo-3/AM(acetoxymethylester), Fura red/AM, and pluronic acid were obtained from Molecular Probes (Eugene, Oregon). 1,2-*bis*-5-methyl-aminophenoxylethane-*N,N,N'*-tetra-acetoxymethyl acetate (MAPTAM) was obtained from Calbiochem-Behring Corp. (San Diego, CA). rInterleukin-1 (rIL-1) was a kind gift from S. Gillis (Immunex Corp., Seattle, WA). Tumor necrosis factor (TNF)-$\alpha$ and interferon (IFN)$\gamma$ were purchased from Collaborative Research, Inc. (Bedford, MA). Anti-ICAM-1 mAb (clone 84H10) was purchased from AMAC (Westbrook, ME). Anti- VCAM-1 (clone 2G7) and anti-E-selectin (clone 3B7) were kindly provided by W. Newman (Otsuka Pharmaceutical, Rockville, MD). mAbs leu 4-Fitc (anti-CD3), leu 3-Fitc (anti-CD4), leu 2-Fitc (anti-CD8), leu 11a-Fitc (anti-CD16), and leu 19-PE (anti- CD56) were purchased from Becton Dickinson Co. (San Jose, CA). Nickel chloride, heparin, nocodazole, and ionomycin were purchased from Sigma Chem. Co. (St. Louis, MO).

### Cell Isolation and Culture

Human umbilical vein ECs (HUVECs) were obtained by collagenase digestion of anonymous donor umbilical veins (as described in reference 18). After isolation, HUVECs were plated onto gelatin (0.5%)-coated tissue culture flasks and grown in HUVEC medium (medium 199, 15% [vol/vol] heat inactivated FBS, penicillin/streptomycin, heparin 50 $\mu$g/ml, and endothelial cell growth supplement 25 $\mu$g/ml) (Collaborative Research Inc., Bedford, MA). All EC cultures were single donor and were used between 3rd and 8th passage.

Peripheral blood mononuclear cells (PBMCs) were obtained through Ficoll-Hypaque gradient centrifugation of peripheral blood from volunteer donors. PBMCs were depleted of monocytes, B cells, and platelets by serial plastic adherence and passage over nylon wool columns. Lymphocyte subpopulations were isolated through negative immunoselection using a panning technique, as previously described (41). Subset purity was analyzed by flow cytometry on a FACSort (Becton Dickinson & Co., Mountain View, CA), as described (27). CD4+ (CD8−, CD16−), CD8+ (CD4−, CD16−), and CD56+, CD16+ (CD3−, CD5−) subsets were all greater than 90% pure, less than 2% contaminated with the depleted phenotype. The CD3−, CD56+ subset will hereafter simply be referred to as natural killer (NK) cells. To obtain alloactivated T cell subsets, PBMCs were cocultured for 7 d with the stimulating EC line at a 10:1 PBMC/EC ratio, after which the leukocytes were harvested and separated into purified T cell subsets if cell counts indicated proliferation had occurred.

### Calcium Measurements

All $Ca^{2+}$ measurements were performed on an ACAS 570 Interactive Laser Cytometer using a 488nm argon source (Meridian, Inc., East Lansing, MI). $[Ca^{2+}]_i$ was followed primarily by using the fluorescent calcium probe Fluo-3; this fluorescein-based dye has a peak emission wavelength of 530 nm when excited at 488 nm, and gains fluorescence intensity proportionally with increasing $[Ca^{2+}]_i$ (24). For simultaneous $Ca^{2+}$ measurements in two cell populations, Fura red was used as the second $Ca^{2+}$-sensitive probe. Fura red has a peak emission wavelength of 660 nm when exicted at 488 nm, and loses fluorescence intensity with increasing $[Ca^{2+}]_i$ (29). A 575 nm short pass dichroic filter followed by 530 nm band pass and 605 long pass filters were used to separate green (Fluo-3) and red (Fura red) fluorescence.

EC were plated onto 35 mm dishes or microtiter wells (Becton Dickinson Labware, Lincoln Park, NJ) and grown overnight in HUVEC medium with or without 20 U/ml of IL-1. The following day, EC were loaded with Fluo-3/AM (final concentration 2 $\mu$M) for 45–60 min using 0.5% pluronic acid at 22°C, washed twice, and placed in KRH (125 mM NaCl, 5 mM KCl, 1 mM $KH_2PO_4$, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes, 6 mM glucose) containing 5% FBS. For mab blocking experiments, antibodies were added during Fluo-3 loading and excess washed out with excess Fluo-3 according to protocol (above). Tissue culture dishes were placed onto the stage of the ACAS 570 and were visualized initially with an integrated, inverted microscope under phase contrast to select an appropriate field. Repeated laser excitation of a field (which generally contained 3–5 ECs) was performed with continuous recording of fluorescence data. Lymphocytes were resuspended in KRH 5% FBS and added vol/vol to ECs, and allowed to settle onto the EC monolayer during fluorescence measurement. For experiments involving protease inhibitors, a cocktail of 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml chymostatin, 2.5 $\mu$g/ml trypsin inhibitor, 4 KIU/ml aprotinin, and 1 mM AEBSF HCl was added to EC medium both during Fluo-3 loading and during NK-EC binding events. For experiments involving MAPTAM buffering of lymphocyte $Ca^{2+}$, lymphocytes were incubated at 22°C in 250 $\mu$M MAPTAM for 20 min followed by 1:10 dilution with medium, and then incubation at 37°C for 20 min. The lymphocytes were washed once and then resuspended in KRH for use in fluorescence assays. For experiments using nocodazole, lymphocytes were pretreated with 100 nM nocodazole for 1 h at 37°C, washed, and resuspended in KRH. Alternatively, NK cells were prestimulated with 20 ng/ml PMA for 15 min to release preformed granules, followed by 100 nM nocodazole for 8 h, and then washed and resuspended in KRH. Experiments were performed both after washing the nocodazole-treated cells prior to the assay and, because nocodazole effects can be rapidly reversible, with the inhibitor maintained in the medium during assays. In general, lymphocytes were added after several minutes of scanning had established a baseline level of EC fluorescence. Periodic simultaneous visualization with the inverted microscope permitted correlation of EC $Ca^{2+}$ changes with lymphocyte-EC appearance. ECs which did not demonstrate $Ca^{2+}$ increases were tested for adequate Fluo-3 loading by the addition of ionomycin (final concentration 1 $\mu$M). EC viability was evaluated at the end of scanning, typically at 15–20 min, by replacing the experimental medium with 0.1% (in PBS) trypan blue. Trypan blue exclusion in experimental ECs was compared by phase contrast microscopy with 70% methanol-treated or hypotonically lysed cells. Fluorescence intensity curves for individual EC were generated with the Kinetics software data analysis program available with the ACAS.

### Inositol Phosphate Analysis

Analysis of total IPs was performed as previously described (12). Briefly, ECs were grown to confluence in 12-well plates and labeled with [$^3$H]inositol (DuPont NEN Research Products, Boston, MA) by adding fresh growth media and 15 $\mu$Ci/ml of isotope for 36 h. At the time of assay, cells were washed once in KRH (5% FBS containing 5 mM LiCl) for 10 min. This was aspirated and stimulus was added in the presence of 5 mM LiCl. The reaction was stopped and IPs extracted by the addition of cold 20 mM formic acid and incubation for 30 min at 4°C. Extracts were run over an ion exchange column consisting of 2 ml Dowex AG1-X8 resin (formate form) (BioRad Laboratories, Hercules, CA) and unbound inositols washed off the

column with 3 ml 5 mM ammonium hydroxide. Inositol phosphates (IPs) were eluted with 4 M ammonium phosphate/0.2 mM formic acid. Inositols were counted using Ultima Gold scintillation fluid (Packard, Meriden, CT), and IP fractions were counted using UltimaFlow AF (Packard). IP counts are reported as the ratio of IP to total inositols (inositol counts + IP counts) to correct for cell number and loading within wells.

### *ICAM-1 Antisense Treatments*

ECs were treated with ICAM-1 message antisense and nonsense oligonucleotides as previously described (7). Briefly, ECs were grown to confluence in six-well tissue cultures plates. The cells were washed in Opti-MEM (GIBCO BRL, Gaithersburg, MD) and then incubated for 4 h at 37°C in Opti-MEM containing 10 μg/ml lipofectin (GIBCO BRL) and 50 ng/ml antisense or nonsense oligonucleotide. The cells were washed three times in fresh Opti-MEM to remove the lipofectin, followed by the readdition of oligonucleotide (50 ng/ml) in IL-1 containing medium and cultured overnight. The following day, the cells were washed in fresh grown medium and used immediately for flow cytometry and $Ca^{2+}$ measurement experiments. Oligonucleotides used were: antisense, 5′ to 3′, CCCCCACCACTTCCCCTCTC (designated 1939 in reference 7), and nonsense, 5′ to 3′ CCATCCCCACCCTCCTCCTC.

## *Results*

### *EC $Ca^{2+}$ Oscillations Induced by Lymphocyte Adhesion*

Fig. 1, cells 2 and 3, illustrate typical repetitive increases in intracellular $Ca^{2+}$ in individual IL-1 pretreated, lymphocyte-adherent EC. Similar results were obtained by pretreating the EC with TNF (40 U/ml) (data not shown). Cell 1 shows the stability of $[Ca^{2+}]_i$ in cytokine-activated EC in the absence of lymphocyte adhesion. Cell 4 is representative of lymphocyte-adherent EC without, or with very minimal changes in $[Ca^{2+}]_i$ which were occasionally observed as well. In resting (non-cytokine-treated) ECs, the magnitude and frequency of $[Ca^{2+}]_i$ increases were markedly reduced (data not shown, but curves are similar to that of cell 4, above), suggesting the induction of a required adhesive event (see below), membrane receptor, signaling component, or some combination of all three. IL-1 pretreatment did not affect the EC responsiveness to thrombin or histamine (not shown). If scanning is interrupted immediately after the first $[Ca^{2+}]_i$ increase in a field of EC and nonadherent lymphocytes removed, it is apparent that only lymphocyte-adherent EC are able to signal. Thus, the relationship between lymphocyte adhesion and EC $Ca^{2+}$ events occurs early and persists throughout the duration of the experiment, typically at least 20 min. Observations from a large number of these experiments indicate that there was no relationship between the strength of the $Ca^{2+}$ signal and the number of lymphocytes adherent to a specific EC.

In attempts to define whether a specific lymphocyte subset was effecting the EC $Ca^{2+}$ responses, assays were per-

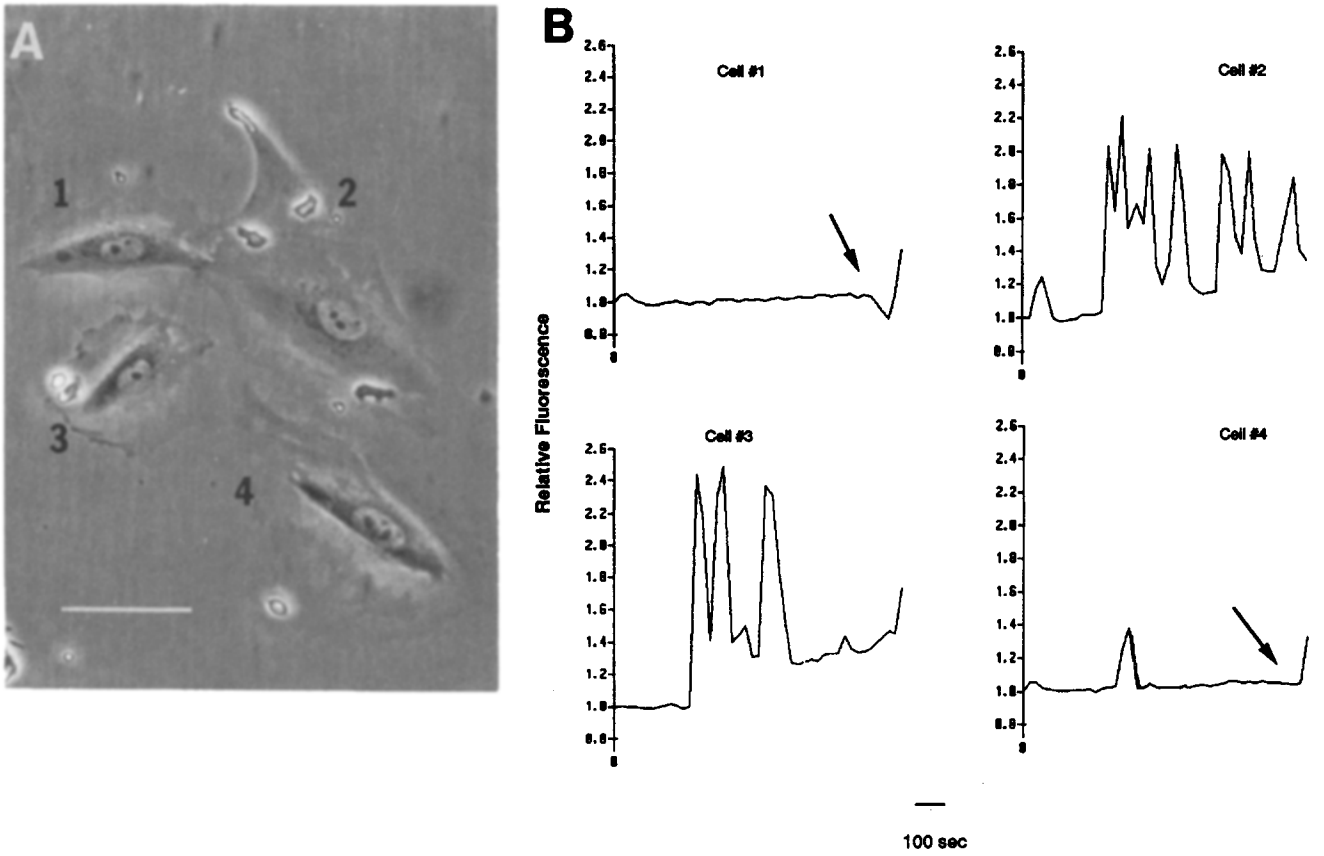


*Figure 1.* $Ca^{2+}$ signals in EC. Real time recordings of fluorescent signal, corresponding to cytosolic free $Ca^{2+}$ concentration, from individual EC in contact with lymphocytes. Photo (*A*) and fluorescence curves (*B*) of four EC and their adherent lymphocytes after washing to remove unbound cells. Cell *1* has no adherent lymphocytes and shows an expected flat $Ca^{2+}$ curve. Cell *2* shows a typical oscillatory pattern over the duration of the experiment, and has four adherent lymphocytes. Cell *3* has two bound lymphocytes and typical $Ca^{2+}$ oscillations. Cell *4* has a single bound lymphocyte and shows a brief $Ca^{2+}$ signal. Lymphocytes were added 60 s after the initiation of scanning. Ionomycin confirmed $Ca^{2+}$ responsiveness in cells *1* and *4* (*arrow*). Bar, 35 μM.

*Table I. EC $Ca^{2+}$ Signals in Response to Lymphocyte Subsets*

| Lymphocyte subset | Lymphocyte treatment | EC treatment | Adhesion* | EC $Ca^{2+}$ signal‡ |
|---|---|---|---|---|
| Histamine | – | IL-1 | – | + |
| Thrombin | – | IL-1 | – | + |
| CD56+(NK)§ | – | – | ++ | ± |
| CD56+(NK) | – | IL-1 or TNF | ++++ | + |
| CD4+ | – | IL-1 | + | – |
| CD4+ | alloactivation‖ | IL-1 | ++ | – |
| CD4+ | alloactivation | IFN + IL-1 | +++ | – |
| CD8+ | – | IL-1 | + | – |
| CD8+ | alloactivation | IL-1 | +++ | – |

* Grading system for adhesion as follows: + = 0–10% of labeled lymphocytes added; ++ = 11–20%; +++ = 21–30%; ++++ = >30%.
‡ Relative comparison for EC $Ca^{2+}$ signals as follows: + = typical $Ca^{2+}$ oscillations in a majority (>50%) of cells; ± = minimal $Ca^{2+}$ fluxes in a minority (<10%) of cells; – = no $Ca^{2+}$ fluxes.
§ All assays represented in this table were performed at a 20:1 lymphocyte/EC ratio.
‖ Cells were considered alloactivated if proliferation occurred in the 7-d co-culture prior to subset purification. ECs used in the $Ca^{2+}$ flux assays were the "relevant," stimulating EC line.

formed with purified subsets of T (CD4+ or CD8+) or NK (CD3−, CD56+) cells. Table I demonstrates that even when T cell–EC binding is dramatically augmented via alloactivation and specific allorecognition, as has been previously shown (11, 37), EC $Ca^{2+}$ responses were never seen. However, freshly isolated, resting NK cells, which do adhere to EC, reproducibly elicited EC $Ca^{2+}$ oscillations in IL-1–pretreated EC. As noted with unselected lymphocytes above, NK-adherent EC which failed to respond were also observed, especially at lower NK/EC ratios. However, only those EC with at least one bound NK cell demonstrated a $Ca^{2+}$ signal. At higher NK/EC ratios, virtually all EC demonstrated $Ca^{2+}$ signals (Table II), and it was with these higher ratios that all the following experiments were performed. These findings suggest that, of all lymphocyte subsets tested, the generation of contact-dependent EC $Ca^{2+}$ signals is a unique function of NK cells and that even within NK cells, there exists a subset responsible for this phenomenon, much more likely to be represented at higher NK/EC ratios. Despite NK lymphocytes bearing a substantial cytotoxic potential, EC viability in the signaling cells was ascertained by phase contrast microscopy and trypan blue exclusion at the end of the assays (not shown).

The subset data also demonstrate that, although intercellular adhesion is required, it is insufficient to simply trigger the noted response. However, we observed a qualitative nature of adhesion which correlated with the generation of EC $Ca^{2+}$ signals. Phenotypic alterations consisting of cell spreading, pseudopod formation and loss of refractility were consistently present in bound NK cells which promoted EC $Ca^{2+}$ fluxes (Fig. 2). Vigorous washing consistently failed to detach these avidly adherent NK cells. The correlation between this qualitative level of binding and EC $Ca^{2+}$ mobilization was strengthened by the observations that EC pretreatment with either IL-1 or TNF was associated with NK morphologic changes, promoted "high avidity" adhesion, and reproducibly induced EC $Ca^{2+}$ oscillations.

*Table II. Relationship of NK/EC Ratio to Signaling*

| NK/EC Ratio | NK bound per EC | Avidly bound NK per EC‡ | Percent EC with $Ca^{2+}$ fluxes |
|---|---|---|---|
| 2:1 | 0–1* | 0–1 | <10% |
| 10:1 | 1–3 | 0–2 | 40% |
| 25:1 | 2–14 | 1–8 | >90% |

* Binding numbers represent a range summarily determined in 40 independent experiments and defined by counting adherent lymphocytes per EC in 10 or more fields per experimental sample.
‡ "Avidly" is defined by morphological alterations in bound lymphocytes which resist detachment by vigorous washing.

### *The Role of Cell Adhesion Pathways*

The requirement for EC preactivation suggests that cytokine-dependent adhesion pathways may be important in generating EC $Ca^{2+}$-signals. Fig. 3 *A* demonstrates that a saturating concentration of anti-ICAM-1 mAb markedly reduces

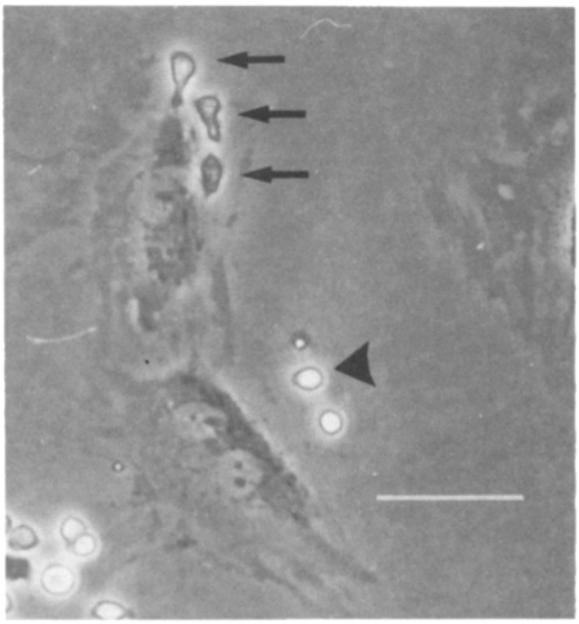

*Figure 2.* Phenotypic changes in tightly adherent NK cells. NK cells associated with $Ca^{2+}$ signals in EC displayed phenotypic alterations consisting of loss of circular shape and irregular projections along the periphery of the cell, as they appear to spread (cells indicated with *black arrows*). These NK cells also show darkening of the cell body in comparison to the brighter, more refractile, circular NK (*arrowhead*) suggesting that the darker cells have become flattened. This phenotype correlates with resistance to removal by washing, while the more refractile cells are easily washed off. Bar, 50 μM.

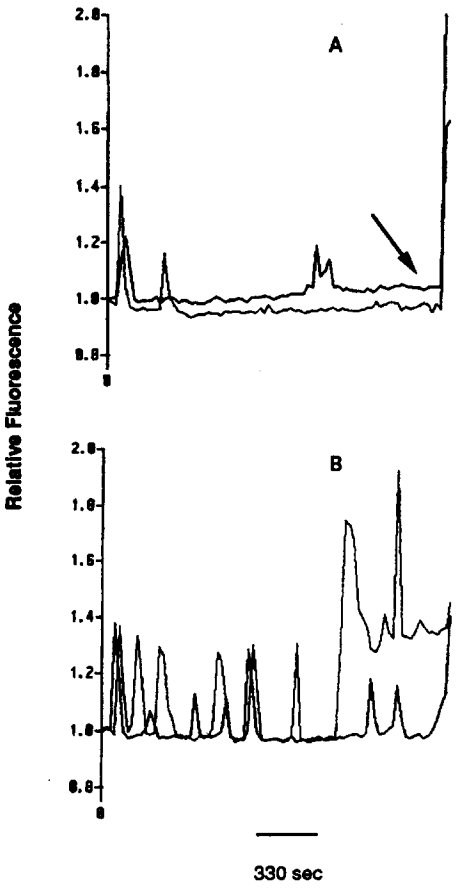


*Figure 3.* Effect of anti-ICAM-1 mAb on EC $Ca^{2+}$ signals. Representative fluorescence curves in two ECs pretreated with 10 $\mu$g/ml anti-ICAM-1 mAb (*A*), and two cells pretreated with isotype-matched control mAb (*B*), to which NK have bound. Although not completely eliminated, anti-ICAM-1 markedly reduced the $Ca^{2+}$ response. Anti-ICAM-1 had no effect on EC $Ca^{2+}$ responsiveness to histamine or thrombin (not shown). Lymphocytes were added 120 s prior to the initiation of scanning. Ionomycin was added in *A* (*arrow*) as a positive control for unresponsive cells. Cells in *A* are representative of observations from three experiments, 15 of 18 total EC.

$Ca^{2+}$ oscillations in IL-1–activated, NK-adherent ECs compared to irrelevant control antibody (Fig. 3 *B*). NK–EC conjugate formation still occurs, although it is quantitatively and qualitatively less. Specifically, the aforementioned characteristic NK phenotypic alterations were rarely seen. The strict correlation between NK–EC binding and EC $[Ca^{2+}]_i$ signals argues against the role for a soluble mediator, unless it acts in a contact-dependent fashion. mAbs directed against E-selectin and vascular cell adhesion molecule (VCAM)-1, other cytokine-induced molecules involved in lymphocyte–EC adhesion, failed to inhibit EC $Ca^{2+}$ oscillations. This information implicates a predominant role for $\beta_2$ integrin-ICAM pathway(s) in this phenomenon.

In an attempt to separate ICAM-1 hyperinduction from other potential consequences of cytokine activation, an ICAM-1 antisense oligonucleotide was added during IL-1 pretreatment of ECs. Fig. 4 demonstrates that despite the complete abrogation of the hyperinduced ICAM-1 component (*C*), $[Ca^{2+}]_i$ transients were equal to those observed in cytokine-treated, nonsense oligonucleotide controls (*B*) (equal to no oligonucleotide) and markedly greater than those seen in unstimulated EC with a similar (basal) level of ICAM-1 surface expression (*A*). Taken together with the mAb blocking data, these results suggest that some functional membrane ICAM-1 is required, perhaps to facilitate

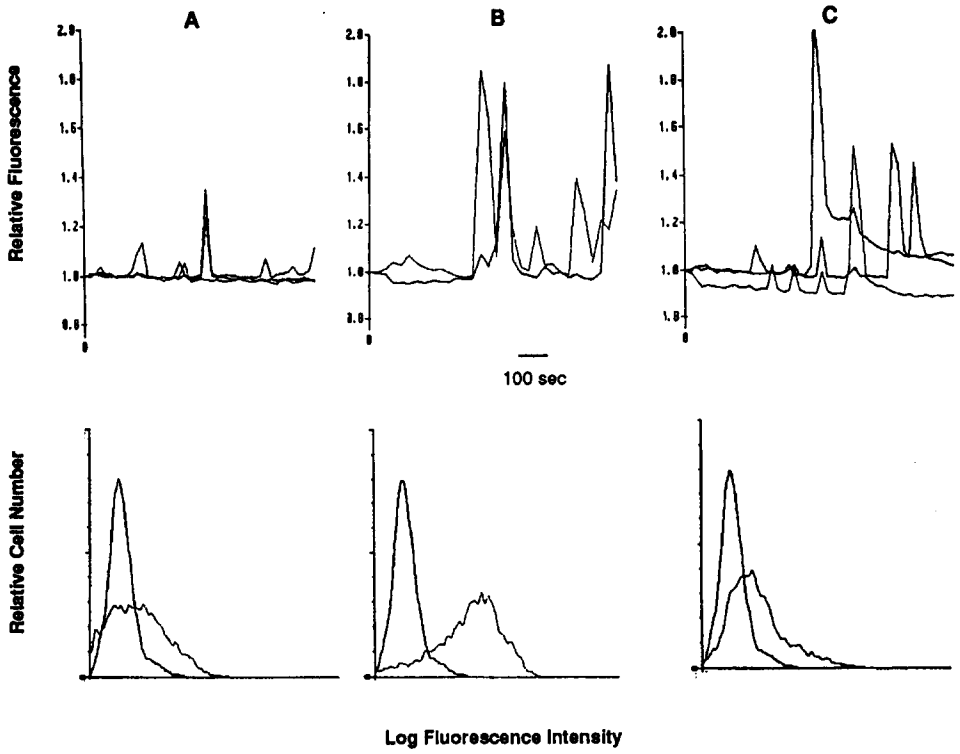


*Figure 4.* Effect of ICAM-1 antisense oligonucleotide on EC $Ca^{2+}$ signal. $Ca^{2+}$ curves (*top*) from NK-bound, resting ECs (*A*) (representative of 5 of 5 cells observed), IL-1 (20 U/ml)–stimulated, nonsense oligo-treated ECs (*B*) (representative of 7 of 9 cells observed), and NK-bound, IL-1 (20 U/ml)-stimulated, antisense oligo-treated ECs (*C*) (representative of 10 of 13 cells observed). The *bottom* shows FACS® profiles of ICAM-1 surface expression from each corresponding condition. ICAM-1 antisense treatment completely abrogated IL-1 hyperinduction of ICAM-1 (*C*, *bottom*). Lymphocytes were added 60 s after the initiation of scanning. FACS® profiles represent 5,000 EC.

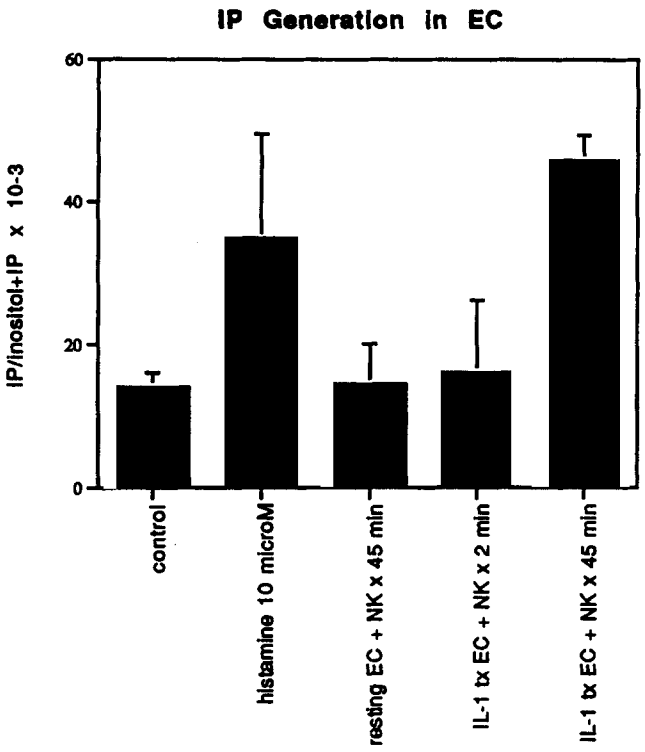


*Figure 5.* IP generation in EC upon NK binding. Increases in EC total IPs in resting and IL-1-treated ECs, both incubated with NK cells (3:1 NK/EC) for 45 min. 2 min was insufficient time for IP generation in both conditions. Histamine 10 $\mu$M was a positive control. Data are displayed as the CPM ($\times 10^{-3}$) ratio of IP to total inositols, and are representative of four separate experiments. *tx*, treated.

a specific mode of adhesion. However, constitutive ICAM-1 is not sufficient, and EC cytokine treatment must induce some new structure and/or function in EC required for cell contact-dependent $Ca^{2+}$ signaling.

### *EC Inositol Phosphates Generated by NK Binding*

In many physiological systems where $Ca^{2+}$ transients are intracellular messengers, the $[Ca^{2+}]_i$ increase is mediated through the activity of IP species liberated via phospholipase C activity at the plasma membrane (4). Fig. 5 demonstrates IP liberation in IL-1-preactivated, NK-adherent ECs. At early timepoints, insignificant IP generation correlated with a minimum of lymphocyte-EC contact. With sufficient time for EC-NK conjugate formation, however, there was dramatic IP increase. 45 min was chosen to allow sufficient signal to accumulate from a large population of ECs, and is not meant to coincide with the timing of $Ca^{2+}$ events as seen in individual cells. In fact, time course experiments demonstrated an IP3 rise at 4 min, consistent with the adhesion-dependent $Ca^{2+}$ kinetics, and accumulation throughout the 45-min period of analysis (data not shown). Resting EC, although NK bound, have insignificant IP increases, consistent with the fluorescence data showing minimal or absent $Ca^{2+}$ signals in the absence of cytokine preactivation. These data are in keeping with a model where the observed $[Ca^{2+}]_i$ increase is a physiologic signaling mechanism in ECs which involves IP second messengers, similar to that seen as a consequence of histamine or thrombin treatment.

### *The Source of Elevation in Cytosolic $Ca^{2+}$*

In other cell systems, $Ca^{2+}$ signals result from liberation of IP-sensitive intracellular stores, or inward movement of ex-

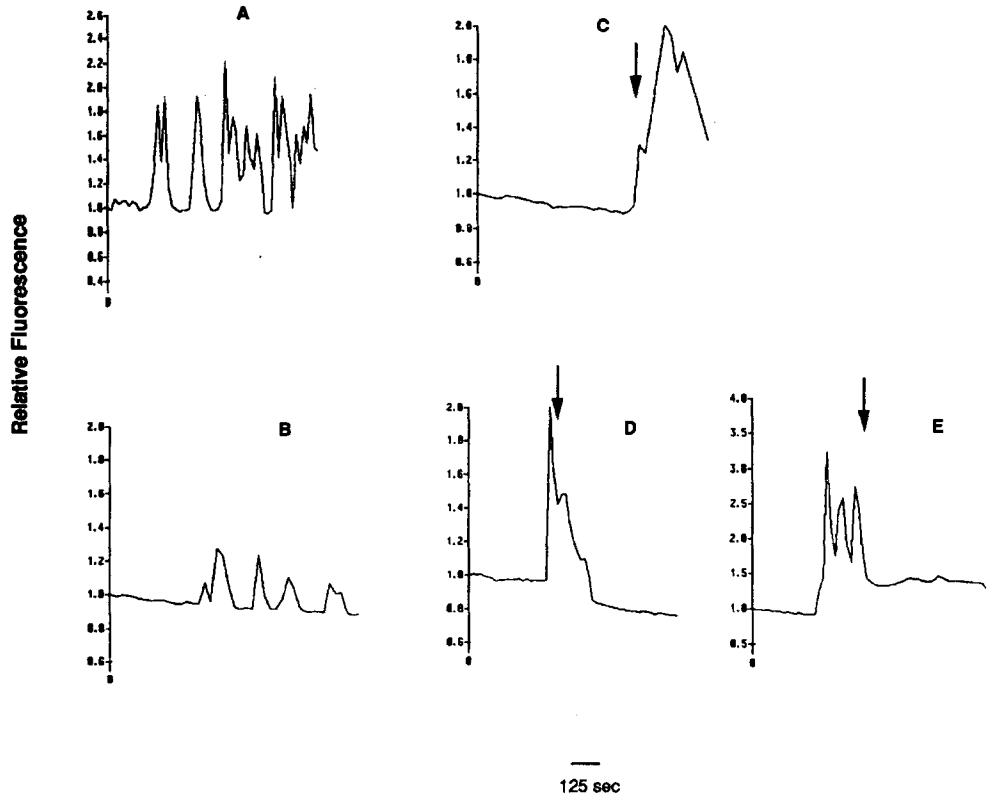


*Figure 6.* NK-mediated EC $Ca^{2+}$ mobilization and influx. Representative curves of $Ca^{2+}$ transients in single HUVECs bound by NK cells in complete, $Ca^{2+}$-containing medium (*A*), $Ca^{2+}$-free (dialyzed FBS, no additional $CaCl_2$) medium (*B*), $Ca^{2+}$ free then $Ca^{2+}$ repleted (1 mM, *arrow*) (*C*), $Ca^{2+}$-containing but NiCl-added (500 $\mu$M, *arrow*) medium (*D*), or low $Ca^{2+}$-containing (5% FBS, no additional $CaCl_2$) but EGTA added (250 $\mu$M, *arrow*) medium (*E*). Each curve representative of at least 12 EC observed over three experiments. Lymphocytes added 120 s after the initiation of scanning.

tracellular $Ca^{2+}$, or both. NK-EC binding is quantitatively identical in $Ca^{2+}$-containing (2 mM) and $Ca^{2+}$-free medium (data not shown). However, NK-induced EC $Ca^{2+}$ signals are markedly decreased in magnitude in $Ca^{2+}$-free conditions (Fig. 6 *B*). $Ca^{2+}$ repletion (final concentration 1 mM) after NK-EC conjugates were established resulted in a dramatic increase in intracellular $Ca^{2+}$ signal (Fig. 6 *C*), indicating that inward movement of extracellular $Ca^{2+}$ plays a major role in the noted responses. Calcium addition did not result in an increased signal in EC without associated NK. In $Ca^{2+}$-containing medium, typical contact-dependent oscillations were abruptly terminated by the addition of the nonselective ion channel blocker NiCl (Fig. 6 *D*), supporting the involvement of extracellular $Ca^{2+}$. This was further confirmed by performing experiments in low $Ca^{2+}$ medium (5% FBS, no additional $Ca^{2+}$), in which adhesion-dependent fluxes were abrogated by the addition if EGTA (Fig. 6 *E*). Finally, low level oscillations seen in $Ca^{2+}$-free medium suggest that intracellular $Ca^{2+}$ stores are mobilized upon NK adhesion. This observation, along with the IP data (above), are consistent with models of $Ca^{2+}$ signaling in EC and other nonexcitable cells in which signals are initiated by IP3-mediated release of intracellular stores, followed by extracellular $Ca^{2+}$ influx.

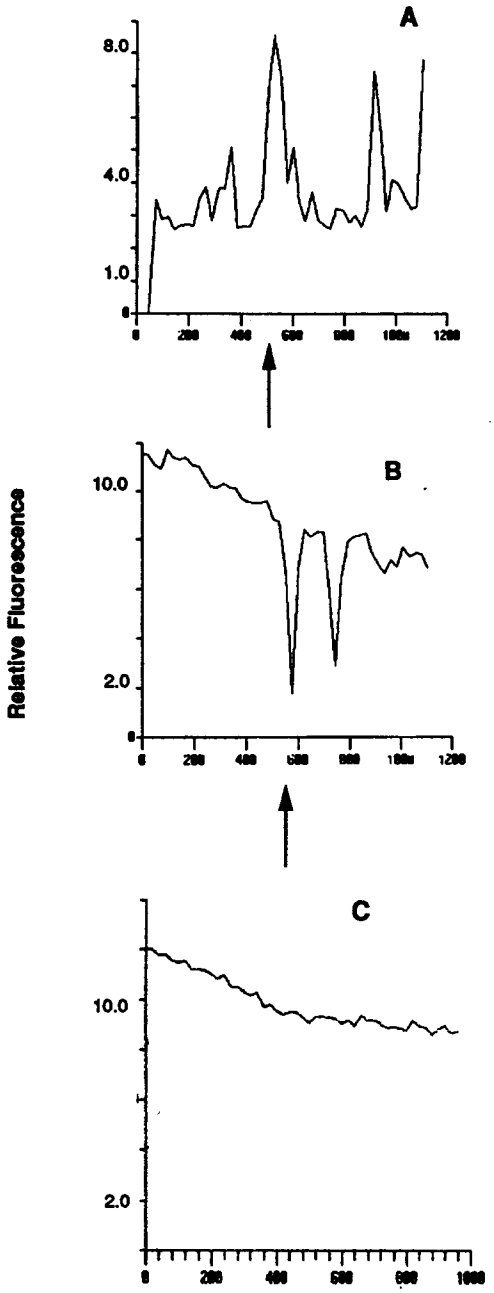


*Figure 7.* Bilateral signaling in EC and NK. $Ca^{2+}$ curves from a single NK cell (*A*) which has bound to an EC, whose simultaneous $Ca^{2+}$ curve is also displayed (*B*). Note that the $Ca^{2+}$ probe used in the EC in this experiment (Fura red) loses fluorescence intensity with increasing $Ca^{2+}$. NK $Ca^{2+}$ flux peaks at 520 s (*arrow*), the EC $Ca^{2+}$ increase peaks about 50 s later (*arrow*). *C* demonstrates the absence of EC $Ca^{2+}$ fluxes when bound by MAPTAM (250 $\mu$M)-pretreated NK cells. *B* is the medium control (non-MAPTAM) for *C*. Curves representative of three NK/EC pairs observed. Lymphocytes were added at time 0.

### *Requirement for NK Activation*

NK $[Ca^{2+}]_i$ elevations have been demonstrated in the setting of target recognition (15). Fig. 7 displays the $Ca^{2+}$ transients recorded from an individual NK cell as it binds to an IL-1 activated EC, and the relationship to subsequent $Ca^{2+}$ signals in the EC to which it is bound (*B*). These observations were made using simultaneous red (EC) and green (NK) fluorescence recording, as described in Materials and Methods. EC $[Ca^{2+}]_i$ signals began shortly after the NK transients in all cases, 50 s in the example shown.

To determine whether NK cell $Ca^{2+}$ fluxes were requisite events for EC signaling, NK cells were pretreated with the intracellular $Ca^{2+}$ buffer MAPTAM. 250 $\mu$M MAPTAM prevents thrombin (2 U/ml)-induced $Ca^{2+}$ increases in NK cells, but does not affect NK binding to IL-1–activated EC in quantitative binding assays (data not shown). Adherent MAPTAM-buffered NK cells did not elicit $[Ca^{2+}]_i$ elevations in EC (Fig. 7 *C*), suggesting that adhesion-mediated NK $Ca^{2+}$ mobilization and NK activation are, in fact, prerequisites for contact-dependent EC $Ca^{2+}$ signals.

Because many cell secretory pathways are $Ca^{2+}$ dependent, and NK lymphocytes are competent secretors of a variety of soluble mediators and lytic proteins, NK cells were pretreated for up to 8 h with the microtubule-disrupting agent nocodazole which, at 100 nM, has been shown to inhibit a variety of secretory processes, and to inhibit target cell lysis by NK cells (25). Nocodazole-treated NK cells were equally effective EC binders and inducers of $Ca^{2+}$ signals (Fig. 8 *B*), when compared with vehicle controls (Fig. 8 *A*). Furthermore, addition of a protease inhibitor mixture with a wide range of specificity to the experimental media failed to inhibit EC $[Ca^{2+}]_i$ increases whereas thrombin-induced EC $Ca^{2+}$ signals were abrogated (data not shown). These results argue against a role for secreted products and proteases in the genesis of EC signals.

## *Discussion*

In this study, we demonstrate that lymphocyte–EC adhesion results in characteristic EC cytosolic $Ca^{2+}$ signals. This process requires cytokine preactivation of EC, occurs in the setting of lymphocyte phenotypic changes and high-avidity adhesion, is associated with the production of EC IPs, and

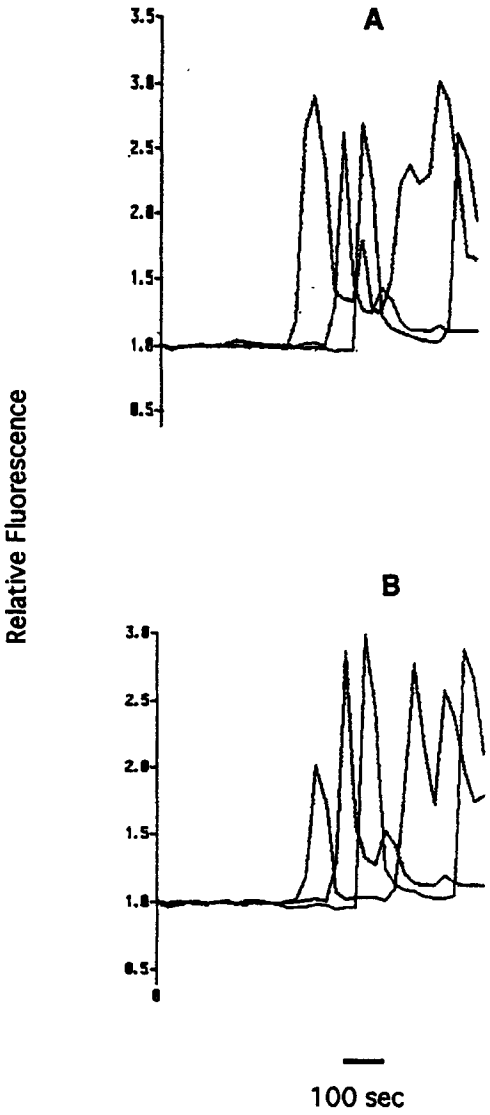


*Figure 8.* EC signaling in response to nocodazole treated NK. $Ca^{2+}$ curves from three EC bound by NK pretreated for 1 h with medium (*A*), or 100 nM nocodazole (*B*), which was maintained in the assay medium. Lymphocytes added 120 s after the initiation of scanning. Curves are representative of a total of nine EC observed in two experiments.

results in an oscillatory pattern of EC $[Ca^{2+}]_i$ similar to those reported for soluble mediators such as histamine. We believe this represents a novel pathway for contact-mediated outside-in signaling in vascular ECs.

The unique capacity of NK cells to trigger this form of activation was unexpected. NK cells are thought to be primarily involved with viral and tumor surveillance, both processes which require target cell lysis. Although human EC are NK-sensitive targets in vitro, we have demonstrated a variety of NK-mediated EC activation events which are, in and of themselves, potentially important and do not necessarily lead to cell death (2, 32). Although we did not evaluate whether those EC with NK-induced $Ca^{2+}$ fluxes would have eventually lysed, it is highly unlikely that the observed signals were a consequence of cell death. Besides EC trypan blue exclusion at the end of the assays, the dramatic oscillations observed are not characteristic of cell death, but implicate dynamically regulated $Ca^{2+}$ movements (17). In general, nonspecific membrane "leakiness" from cytotoxicity results in continuous accumulation of $Ca^{2+}$-fluorescent signals, and no return to baseline. Rare ECs which died during the assay demonstrated this fluorescent pattern and were excluded from analyses.

Despite the requirement for NK–EC adhesion, apparently identical conjugate formation can occur without consequent EC $Ca^{2+}$ signals. The features which promote an active response are unclear, but the phenomenon suggests a subset specificity at the level of NK or ECs, or both. This is underscored by our observation that higher NK/EC ratios augmented the likelihood of inducing EC $Ca^{2+}$ signals (Table II). With regard to allorecognition, which may well play a role in this phenomenon, NK clonal heteogeneity has been recently described (28). In addition, both mAbs (9) and cDNA probes (21) have been derived which define nonoverlapping members of potential NK receptor/activation families. Studies to define the subset specificity of this phenomenon and the potential relationship to these newly described NK markers are ongoing.

The similarity of these EC $Ca^{2+}$ signals to those induced by soluble mediators (histamine, bradykinin), together with the fact that activated NK lymphocytes secrete numerous factors, raised the possibility that the noted events involve elaborated mediators. Our data indicate that avid NK–EC binding is required (proximity is not sufficient) and that EC $Ca^{2+}$ transients are induced only in tightly NK-adherent EC. However, this does not exclude local and directed secretion from an adherent NK cell only to its bound congener cell. In fact, this is a well-recognized form of binary intercellular communication in which, as a result of mutual membrane molecular capping, the perinuclear golgi apparatus in the secretory cell receives a polarizing signal and reorients toward the area of cell–cell contact (39). The spectrum of NK-produced factors includes cytokines, growth factors, proteolytic enzymes such as granzymes, and the lytic, pore-forming protein perforin (14, 20, 26). Given that transport vesicles associate with microtubules as they travel to the cell surface, microtubule disruption with nocodazole would be expected to interfere with secretion. Although not specifically studied for all of these NK factors, this class of inhibitors has been demonstrated to abrogate secretion in a remarkably wide range of constitutive and regulated secretory events (6, 16), but had no effect on NK cell adhesion-mediated EC $Ca^{2+}$ signaling. Thus, it is highly unlikely that NK cell secretion of a soluble mediator, including the cytolytic pore-forming protein perforin, is involved in this intercellular communication.

Although the exact nature of activation is unknown, it is clear that lymphocyte–EC adhesion is absolutely required in this phenomenon. Furthermore, our data demonstrate that EC ICAM-1 is involved and, by association, $\beta 2$ integrin is as well. Both $\alpha_m\beta_2$ (CD11b/CD18, Mac-1) and $\alpha_L\beta_2$ (CD11a/CD18, LFA-1) are expressed on NK cells and are lymphocyte receptors for cell-bound ICAM-1. A logical analogy is the importance of LFA-1 in T cell adhesion-dependent signaling. It is generally believed that the mutual capping within LFA-1–ICAM-1 contact sites is vital for T cell signal transmission.

There is considerably less known regarding cell–cell inter-

actions leading to NK cell activation. We (3, 40) and others (8, 10) have recently demonstrated that NK cells do have specific antigen recognition capabilities, although the receptor and target structures are incompletely understood. As mentioned above, NK cell adhesion to NK-sensitive targets has been associated with NK $Ca^{2+}$ fluxes. In our system, NK cytosolic $Ca^{2+}$ increase was a prerequisite for EC $Ca^{2+}$ signaling. The significance of NK $Ca^{2+}$ rise is uncertain, but is consistent with cytoskeletal activation, postulated redistribution of membrane components, and the observed NK morphologic alterations. The molecular events which promote transition from early contact-triggered effector cell activation to cytoskeletal reorganization are unknown. The importance of the actin-based cytoskeleton was underscored by NK cell pretreatments with cytochalasin D, an inhibitor of actin polymerization which was initially used in attempts to delineate a role for cell secretion. Cytochalasin acts on a wide range of cytoskeletal elements, and its effect on secretory processes can be inhibitory or stimulatory depending upon the specific system (6), thus, the effect of cytochalasin on cell adhesion–dependent secretion is not predictable. Neither was it quantifiable in this NK-EC system because the "high avidity" adhesion, clearly a consequence of cytoskeletal rearrangments, was largely prevented, as were EC $Ca^{2+}$ fluxes (data not shown). This demonstrates the requirement for a dynamic actin cytoskeleton in this system.

Cytokine preactivtion of ECs, although not absolutely required, dramatically augments the noted EC $Ca^{2+}$ responses. It is possible that IL-1 simply enhances NK–EC adhesion. The effect of cytokine on the observed qualitative mode of adhesion would support this. However, the mechanism which links IL-1–induced adhesion and EC $Ca^{2+}$ signals is unclear. Although ICAM-1 is critically involved, the level of EC membrane expression, which is that feature of ICAM-1 known to be affected by IL-1, is not crucial. Antisense inhibition of IL-1–mediated increase in ICAM-1 surface expression results in basal ICAM-1 levels, yet $Ca^{2+}$ signaling is indistinguishable from controls with hyperinduced ICAM-1 expression. There are no reports of IL-1–mediated posttranslational modification of ICAM-1, direct transmembrane signaling via ICAM-1 has not been demonstrated, and we could not elicit $Ca^{2+}$ signals by ICAM-1 cross-linking (data not shown). Finally, at least 4 h of IL-1 treatment are required to generate IPs in response to NK binding (data not shown). ICAM-1 is required, as determined by mAb blocking experiments, but is not sufficient to trigger the noted responses. The kinetics of cytokine effect implicate a transcriptional event, yet the specific cytokine-induced activity is unknown. IL-1 and TNF activate multiple protein kinases (19), any of which may be involved in the required activation events, and were not investigated in this study.

Although EC $[Ca^{2+}]_i$ increases have been demonstrated in the setting of tumor cell (34) and neutrophil (22) contact, this is the first documentation of $Ca^{2+}$ signals recorded in a single, suspension cell-adherent EC. This is also the first report of EC IP generation triggered by leukocyte adhesion. Although our results do not define the exact relationship between these $Ca^{2+}$ fluxes and endothelial IP production, they implicate a role for inositol (1,4,5)–trisphosphate (IP3) as a second messenger at an early signal transduction step. IP3 liberated by phospholipase C activity acts on IP3 receptors to mobilize intracellular stores of $Ca^{2+}$, generating a rise in free cytosolic $Ca^{2+}$. In EC, other IP species, specifically inositol (1,3,4,5)-tetrakisphosphate, may act directly on the plasma membrane to facilitate extracellular $Ca^{2+}$ entry. Other signals which may direct extracellular $Ca^{2+}$ entry include $Ca^{2+}$ (via ryanodine receptors, recently described in EC), or CIF, a recently reported factor which is generated in response to depletion of intracellular stores and acts on the plasma membrane to promote extracellular $Ca^{2+}$ entry (35). Our data are consistent with mobilization of intracellular stores, since we detect signal in $Ca^{2+}$-free conditions. We also implicate a contribution of extracellular $Ca^{2+}$ to the strength of the signal (Fig. 6 *B*), and oscillations were frequently superimposed on an elevated baseline (Fig. 3 *B* and Fig. 1, cell *3*), suggesting a plateau from continuous extracellular $Ca^{2+}$ influx. Finally, refilling of intracellular stores is critical for the observed signals, since maneuvers which block extracellular entry of $CA^{2+}$ (EGTA and NiCl) abrogate further signaling (Fig. 6, *E* and *D*). The specific pathways involved in this phenomenon remain to be identified.

If cytokine activation is largely a prerequisite for these NK-induced $Ca^{2+}$ signals and resting NK cells can interact with ECs, what could trigger the cascade? A recent report demonstrated that EC hypoxia, in vitro and in vivo, produced a strong induction of EC IL-1 transcription. Functionally upregulated IL-1 had an autocrine effect, with augmented leukocyte adherence partially blockable by anti–ICAM-1 and –E-selectin antibodies (38). As such, conditions which compromise flow and/or promote vessel hypoxia could initiate autocrine cytokine effects, NK cell adhesion, EC $Ca^{2+}$ signals and further consequences. Hypoxia could be an initiating component in the setting of vascularized allograft pathology or native vasculopathy. We do not know at this time whether the noted $Ca^{2+}$ responses occur only in allogeneic combinations. Finally, there are well-recognized consequences of elevating EC cytoslic $Ca^{2+}$. Naturally anticoagulant, the endothelium becomes prothrombotic by expressing (and secreting) von Willebrand factor, platelet activating factor, and P-selectin, all in $Ca^{2+}$-dependent fashion. The production of $PGI_2$ and nitric oxide, both endothelial-derived vasodilatory substances, is also largely $Ca^{2+}$ dependent.

In summary, we have demonstrated contact-dependent EC $Ca^{2+}$ signals induced by NK lymphocyte adhesion. These are typical oscillating $Ca^{2+}$ signals, similar to those seen in histamine- activated ECs, and involve EC IP generation. Although ICAM-1 is required, an as yet unidentified cytokine-mediated alteration in EC structure and/or function is involved. We believe this represents a novel pathway of outside-in signaling in ECs, and that these observations have important implications for leukocyte-directed alteration in EC function.

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