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SHORT NOTE

Multiple Integrins Share the Ability to Induce Elevation of Intracellular pH

MARTIN ALEXANDER SCHWARTZ,*,[1] DONALD E. INGBER,† MICHAEL LAWRENCE,‡
TIMOTHY A. SPRINGER,‡ AND CLAUDE LECHENE*,§

*Department of Cellular and Molecular Physiology, Harvard Medical School; †Department of Surgery, Childrens Hospital; ‡Department of Pathology, Harvard Medical School and Center for Blood Research; and §Department of Medicine, Brigham and Womens Hospital, Boston, Massachusetts 02115

Previous work has shown that adhesion of anchorage-dependent cells to fibronectin via integrin α5β1 leads to activation of the Na-H antiporter and a rise in intracellular pH (pHi). We now show that adhesion of bovine capillary endothelial cells (BCE) to fibrinogen; collagens type III, IV, and V; laminin; and vitronectin; ligands that bind other members of the integrin family, resulted in significant elevations in pHi. Other ligands (basic fibroblast growth factor, concanavalin A, and thrombin), which bind cells when immobilized on plastic, but that do not bind integrins and do not support cell growth, do not elevate pHi. Adhesion to an antibody against integrin αvβ3 also elevates pHi. Adhesion of peripheral human T lymphocytes to an antibody against the integrin LFA-1 induced a rise in pHi. Antibodies to CD2 or ICAM-2 had only slight effects on pHi, whereas an antibody to the T cell receptor complex that strongly activates T cells induced a large increase in pHi. We conclude that elevation of pHi by integrins is specific and is a property shared by many members of the integrin family. © 1991 Academic Press, Inc.

INTRODUCTION

The integrins comprise a family of cell surface receptors that mediate cell–cell and cell–extracellular matrix (ECM) interactions [1–3]. These receptors are heterodimers, composed of an α and β subunit. The β1 subunit can form dimers with α subunits 1 through 6 and αv, to form a subfamily of ECM receptors that is expressed in a wide range of cell types. The β2 subunit dimerizes with CD11a, CD11b, and CD11c to form LFA-1, Mac-1, and p150,95, a subfamily that is expressed on lymphoid and myeloid cells, and that is involved in cell–cell interactions. The β3 subunit dimerizes with αv and platelet GPIIb to form another widely expressed subfamily of ECM receptors.

We have demonstrated that adhesion of anchorage-dependent cells to fibronectin (FN), or to their endogenous ECM, of which FN is a major component [4], induces elevation of intracellular pH (pHi), due to activation of the Na-H antiporter [5–7]. Activation of the antiporter by adhesion to fibronectin is mediated by local clustering of the fibronectin receptor, integrin α5β1 [8]. This activation of the antiporter and elevation of pHi is closely linked to mitogenesis [7, 9, 10]. Other ECM proteins are also able to support growth of anchorage-dependent cells [11], and in T lymphocytes, the integrin LFA-1 has been shown to mediate mitogenic effects under some conditions [12, 13]. Thus, integrins other than α5β1 are probably able to generate transmembrane signals that regulate cell growth. The experiments reported here were aimed at determining whether integrins other than α5β1 also share the ability to elevate pHi.

MATERIALS AND METHODS

Proteins. Fibronectin was prepared from human plasma as described [14]. Vitronectin, laminin, and collagens were purchased from CalBiochem (San Diego, CA). Fibrinogen (CalBiochem) was used either without further treatment or after passage through gelatin-Sepharose to remove any contaminating fibronectin (a generous gift from Dr. Tailan Tuan, Orthopedic Hospital, Los Angeles), which gave identical results. Concanavalin A and thrombin were purchased from Sigma Chemicals (St. Louis, MO). Recombinant basic FGF was a gift from Takeda Chemical Industries, Japan. Antibody 609 to the vitronectin receptor was a gift from Dr. David Cheresh. Monoclonal antibody OKT3 was obtained from the American Type Culture Collection (Rockville, MD). Monoclonal antibody CD2.1 to CD2 [15] was obtained from Dr. D. Olive. Monoclonal antibody IC2.2 to ICAM2 was prepared in one of our laboratories and will be described in a future publication (De Fougerolles and Springer, in preparation). Monoclonal antibody TS122 to LFA-1 was prepared in one of our laboratories [16].

Cells. Bovine capillary endothelial (BCE) cells were isolated and cultured as described [7, 17]. For pH experiments, cells were plated in 35-mm bacterial plastic dishes (Falcon, Oxnard, CA) in defined serum-free medium containing saturating basic FGF [7]. The plastic was coated with FN or other proteins, then blocked with 10 mg/ml BSA, as described [7]. For some experiments, three to four small areas in each dish were coated with 50 μl droplets containing saturating concentrations of various adhesive proteins, then each droplet was rinsed and the entire dish was coated with 10 mg/ml BSA. No adhesion of cells was observed to dishes without FN or to areas outside the droplets of adhesive proteins. For experiments with 4.5 μm beads, dishes were coated with 0.5 μg FN/dish; this coating promotes

[1] To whom reprint requests should be addressed.

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attachment, but cells spread minimally and have a low pHi and low levels of DNA synthesis [7]. Approximately 50,000 cells were plated in each dish, and cells were used for pHi measurements 24 h after plating. Experiments with hexamethylene amiloride (HMA) were carried out in medium with 50 m*M* sodium, where choline chloride was added to keep osmotic strength constant. This medium does not perturb pHi and supports cell growth [7], but because HMA competitively inhibits sodium binding to the antiporter, lower concentrations of HMA can be used [18].

Human peripheral T cells were isolated from whole blood by plastic adherence and nylon wool filtration and were 95% CD3+, and 97% CD2+. Cells were stored at 4°C in RPMI medium supplemented with 5% fetal bovine serum and were used 16–24 h after isolation. T cells ($1-2 \times 10^6$) were transferred to 35-mm dishes in RPMI/5% serum and warmed to 37°C for at least 1 h before pHi measurements.

Beads. Beads (4.5 μm) covalently derivatized with affinity-purified goat anti-mouse IgG (Dynal M450 beads, Robbins Scientific, Mountain View, CA) were incubated with a 5- to 20-fold molar excess of monoclonal IgG for 2–18 h, according to the manufacturers directions. The beads were collected by centrifugation at 4000 rpm for 4 min in a Beckman microfuge 11 (Fullerton, CA), rinsed 2×, and resuspended at a density of 2×10^7 beads/ml in DMEM containing 1 mg/ml BSA. Beads (40 μl) were added to each dish without mixing or swirling, to obtain gradients of bead density across the dish. Cells were incubated 1–2 h as described in the text, then loaded with BCECF and transferred to the microscope stage. pHi was measured in cells with different numbers of beads bound by examining cells from areas of the dish with different bead densities.

pHi measurements. Measurements of pHi in single cells were carried out as described [5, 6]. Briefly, cells were loaded with the pH-sensitive fluorescent dye BCECF (2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein) by incubation with the membrane-permeant acetoxy methyl ester at 1–3 μ*M*. pHi was determined by exposing each cell to excitation light of 440 and 495 nm and recording the intensity of emitted light at 530 nm with a photometer. Fifteen to twenty cells were measured, and absolute pHi was determined by comparing the 495/450 ratio to a calibration curve prepared by using the ionophore nigericin to equilibrate pHi with medium of known pH. pHi measurements were made at 37°C in standard DMEM or RPMI with bicarbonate, under 5% CO_2.

RESULTS

The first experiment was aimed at determining whether different ECM proteins, that bind different integrins, can elevate pHi in BCE cells. Cells were plated on plastic coated with saturating concentrations of a variety of ECM proteins. As negative controls, plastic was coated with bFGF, thrombin, or conconavalin A. These proteins induce attachment of cells to the plastic via receptors other than integrins [19–21], and induce minimal cell spreading or DNA synthesis (not shown). Cells were plated in dishes with 3–4 areas coated with different proteins, so that pHi could be measured in cells on different proteins but in the same dish at the same time.

The increases in pHi relative to basic fibroblast growth factor (bFGF) for cells on different adhesive proteins are shown in Fig. 1. These data clearly demonstrate that adhesion of cells to all of the ECM proteins induced significant elevations of pHi. Addition of 40 μ*M* HMA, a specific inhibitor of the Na-H antiporter [18] abolished the increases in pHi (not shown), demonstrating that the antiporter was necessary for the change in pHi. Con A, bFGF, and thrombin did not induce significant increases in pHi. Since the ECM proteins all bind to cells via integrins, these results imply that a number of integrin receptors must be capable of elevating pHi similar to integrin $\alpha 5\beta 1$.

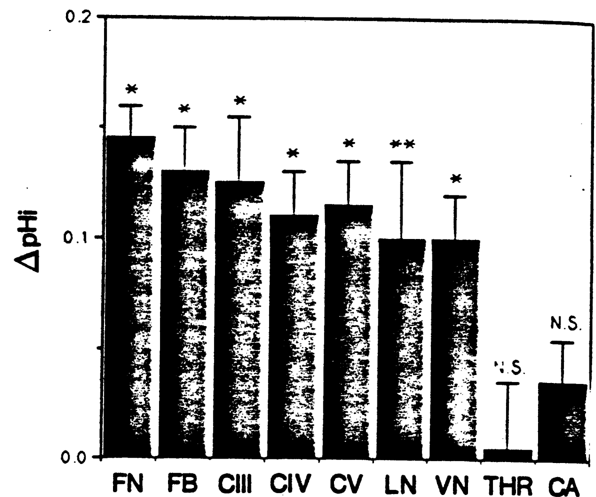

FIG. 1. Effect of different adhesive proteins on pHi in BCE cells. BCE cells were plated on plastic coated with saturating levels of fibronectin (FN); fibrinogen (FB); collagens type III (CIII), IV (CIV) and V (CV); laminin (LN); vitronectin (VN); thrombin (THR); and concanavalin A (CA). Cells were also plated on bFGF, and the increase in pHi was calculated relative to cells on bFGF for each experiment. Values are means ± standard deviations from five experiments, except for thrombin, which was from three experiments. Statistical significance was determined by the unpaired Student *t* test. $^*P < 0.0005$; $^{**}P < 0.01$; N.S., not significant. pHi of cells on bFGF was 7.07 ± 0.08.

To test this idea more directly, we stimulated cells with anti-integrin antibodies immobilized on 4.5 μm beads. Previous work in our laboratories has shown that binding of these beads coated with FN or with antibodies to the integrin $\beta 1$ or $\alpha 5$ subunits was highly specific and was sufficient to trigger a dose-, time-, and antiporter-dependent increase in pHi [8].

BCE cells were incubated for 2 h with beads coated with antibodies to the integrin $\beta 1$ subunit, or to integrin $\alpha v\beta 3$. pHi measurements showed that binding of 10 or more anti-$\alpha v\beta 3$ beads induced an increase in pHi of 0.09 ± 0.015 units, similar to the $\beta 1$ antibody (pHi increase of 0.11 pH units; 8). This result demonstrates directly that a member of a distinct integrin family can trigger an increase in pHi.

The effects of stimulating LFA-1 on peripheral human T lymphocytes was also examined. T cells were incubated for 1 h with beads coated with anti-LFA-1 antibody [16], and pHi was determined. As a positive control, cells were incubated with beads coated with the monoclonal antibody OKT3, which binds a component of the T cell receptor complex and strongly activates T cells [22]. As negative controls, beads coated with antibodies to ICAM-2 and CD2 were used. CD2 is present at levels equal to or slightly higher than LFA-1 [23, 24]; ICAM2 is present at about one-half the level of LFA.1 (de Fougerolles and Springer, in preparation).

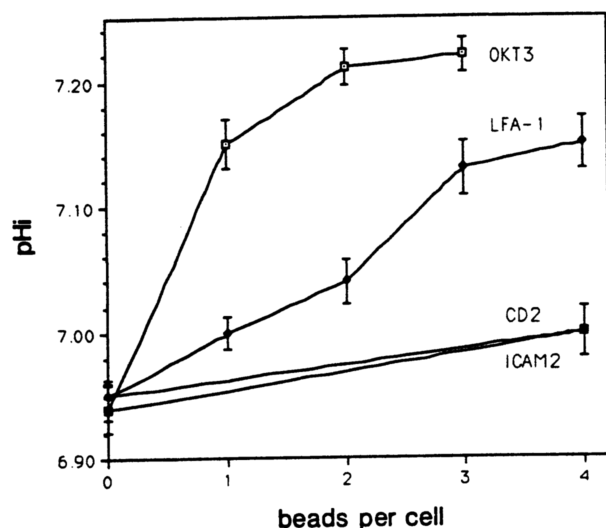

FIG. 2. Effect of antibody-coated 4.5 μm beads on pHi in T lymphocytes. T lymphocytes were incubated with antibody-coated beads for 1 h, then pHi (±SE) was measured as a function of beads bound per cell.

T cells bind beads coated with these four antibodies equally well. OKT3 was highly effective at elevating pHi; binding increased pHi by 0.2–0.25 pH units, and one bead gave a nearly maximal response (Fig. 2). Anti-LFA-1 beads elevated pHi by 0.15–0.2 pH units, though binding of three beads was required for a near-maximal effect. Binding of anti-CD2 or anti-ICAM2 beads had a much smaller effect on pHi (four beads bound per cell increased pH by 0.06 ± 0.01 units, for both antibodies), indicating that the increases in pHi were specific. These increases in pHi were completely abolished by addition of 40 μm hexamethylene amiloride (not shown). Thus, a member of the β2 integrin family appears to be capable of elevating pHi by activating the Na-H antiporter.

DISCUSSION

The experiments demonstrating that multiple ECM proteins induce alkalinization in BCE cells suggest strongly that multiple integrins function in a similar manner to activate the intracellular pathways that modulate pHi. Exact assignment of which integrins are responsible for the effects of each ECM protein is, however, probably not possible at this time, since most of the ECM proteins bind to several integrins [1–3]. Experiments with antibodies clearly show that members of the β1, β2, and β3 subfamilies activate the antiporter and elevate pHi. It therefore seems highly likely that signaling is a general property of integrins.

Integrins have been implicated in regulation of cell growth and differentiation in a wide range of systems. Since pHi is not only a potential regulator of cell growth and differentiation, but also a marker for activation of other regulatory pathways, these results suggest that signaling by integrins is likely to be of widespread importance.

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