Membrane Targeting and Coupling of NHE1-Integrin $\alpha_{IIb}\beta_3$-NCX1 by Lipid Rafts following Integrin-Ligand Interactions Trigger Ca$^{2+}$ Oscillations*\textsuperscript{[S]}

Received for publication, June 5, 2008, and in revised form, October 21, 2008. Published, JBC Papers in Press, November 7, 2008, DOI 10.1074/jbc.M804334200

Yung-Hsiang Yi,\textsuperscript{a} Pei-Yun Ho,\textsuperscript{b} Tung-Wei Chen,\textsuperscript{c} Wen-Jie Lin,\textsuperscript{a} Vladimir Gukassyan,\textsuperscript{c} Tsung-Heng Tsai,\textsuperscript{c} Da-Wei Wang,\textsuperscript{d} Tien-Shen Lew,\textsuperscript{e} Chih-Yung Tang,\textsuperscript{e} Szecheng J. Lo,\textsuperscript{f} Tsung-Yu Chen,\textsuperscript{f} Fu-Jen Kao,\textsuperscript{g} and Chi-Hung Lin\textsuperscript{a}\textsuperscript{h}\textsuperscript{i}\textsuperscript{j}

From the \textsuperscript{a}Institute of Microbiology and Immunology, National Yang-Ming University, Taipei 112, Taiwan, the \textsuperscript{b}Institute of Biophotonics, National Yang-Ming University, Taipei 112, Taiwan, the \textsuperscript{c}Institute of Information Science, Academia Sinica, Taipei 11529, Taiwan, the \textsuperscript{d}Department and Institute of Physiology, National Yang-Ming University, Taipei 112, Taiwan, the \textsuperscript{e}Department of Physiology, National Taiwan University, Taipei 100, Taiwan, the \textsuperscript{f}Department of Life Science, Chang Gung University, Taipei 333, Taiwan, the \textsuperscript{g}Center for Neuroscience and Department of Neurology, University of California, Davis, California 95618, the \textsuperscript{h}Department of Surgery, Taipei Veteran General Hospital, Taipei 112, Taiwan, and the \textsuperscript{i}National Nano Device Laboratory, Hsinchu 30078, Taiwan.

The cyclic calcium release and uptake during calcium oscillation are thought to result from calcium-induced calcium release (CICR); however, it is unclear, especially in nonexcitable cells, how the initial calcium mobilization that triggers CICR occurs. We report here a novel mechanism, other than conventional calcium channels or the phospholipase C-inositol trisphosphate system, for initiating calcium oscillation downstream of integrin signaling. Upon integrin $\alpha_{IIb}\beta_3$ binding to fibrinogen ligand or the disintegrin rhodostomin, sodium-proton exchanger NHE1 and sodium-calcium exchanger NCX1 are actively transported to the plasma membrane, and they become physically coupled to integrin $\alpha_{IIb}\beta_3$. Lipid raft-dependent mechanisms modulate the membrane targeting and formation of the NHE1-integrin $\alpha_{IIb}\beta_3$-NCX1 protein complex. NHE1 and NCX1 within such protein complex are functionally coupled, such that a local increase of sodium concentration caused by NHE1 can drive NCX1 to generate sodium efflux in exchange for calcium influx. The resulting calcium increase inside the cell can then trigger CICR as a prelude to calcium oscillation downstream of integrin $\alpha_{IIb}\beta_3$ signaling. Fluorescence resonance energy transfer based on fluorescence lifetime measurements is employed here to monitor the intermolecular interactions among NHE1-integrin $\alpha_{IIb}\beta_3$-NCX1, which could not be properly detected using conventional biochemical assays.

In many excitable or nonexcitable cells, the concentration of free intracellular calcium oscillates with a period ranging from a few seconds to a few minutes. Such calcium oscillations are involved in a wide variety of cellular functions (1, 2). It is generally believed that, except for minor variations, the cyclic increase and decrease of calcium results from an autocatalytic release of calcium in a process called calcium-induced calcium release (CICR),\textsuperscript{2} followed by a slow negative feedback that terminates calcium release. The cytoplasmic free calcium is then taken up into the organelles to reset the cycle. Despite a general agreement on how calcium oscillation proceeds once the system has been turned on, various different mechanisms have been proposed to explain how the initial calcium mobilization is generated that triggers CICR.

As a general rule, calcium entry through voltage-gated channels in electrically excitable cells (3) or through agonist-receptor interactions in nonexcitable cells, such as epithelial cells, hepatocytes, or oocytes (4), is thought to initiate the CICR process (1, 2). Typically, in nonexcitable cells, the binding of an agonist, such as a hormone, a growth factor, or an extracellular matrix, to the corresponding cell surface receptor initiates a series of reactions that end in the activation of phospholipase C (PLC) and the production of the secondary messenger inositol trisphosphate (IP$_3$) (1, 2, 4). IP$_3$ is thought to induce calcium release from the internal endoplasmic reticulum or mitochondria store, and governs the CICR mechanisms that modulate calcium oscillations (1, 2, 4). However, not all calcium oscillations found in nonexcitable cells are initiated by the PLC-IP$_3$ pathway (5). We report here a novel pathway whereby ion exchangers NHE1 and NCX1, by interacting with integrin $\alpha_{IIb}\beta_3$, can play an active role in mobilizing intracellular calcium that triggers calcium oscillation. Integrin $\alpha_{IIb}\beta_3$ is the most abundant membrane protein found in platelets (6). Many functions of platelet are mediated.

\textsuperscript{[S]} Microscopy for this study was supported by the Biophotonics Interdisciplinary Research Center and the General Research Center of National Yang-Ming University. This work was supported by Ministry of Education, Aim for the Top University Plan and National Science Council, Taiwan Grants NSC 91-2320-B-010-051, NSC (94-96)-2627-B-010-017, and NSC 95-2321-B-010-005. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{[2]} The abbreviations used are: CICR, calcium-induced calcium release; CHO $\alpha_{IIb}\beta_3$, Chinese hamster ovary cells expressing exogenous human integrin $\alpha_{IIb}$ and integrin $\beta_3$; Fg, fibrinogen; rho, rhodostomin; PLL, poly-L-lysine; Tg, thyssagargin; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; M6PC, methyl-$\beta$-cyclodextrin; PLC, phospholipase C; IP$_3$, inositol trisphosphate; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; TIRFM, total internal reflection fluorescence microscopy; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester; TRITC, tetramethylrhodamine isothiocyanate; DRM, detergent-resistant membrane.
Coupling of NHE1-Integrin $\alpha_{\text{IIb}}\beta_3$-NCX1 and Calcium Oscillation

by integrin $\alpha_{\text{IIb}}\beta_3$ (7, 8), and these are tightly associated with the control of intracellular calcium concentrations (9). Binding of integrin $\alpha_{\text{IIb}}\beta_3$ with plasma fibrinogen (Fg) or von Willebrand factor, for example, elicits a complicated series of calcium events, including calcium oscillations, which modulate both the inside-out and outside-in signaling pathways and regulate platelet thrombus formation (9). Calcium oscillations could also be induced in human platelets by plating the cells on a substrate coated with Fg or rhodostomin (rho), a disintegrin protein isolated from snake venom (10, 11). Like platelets, Chinese hamster ovary cells expressing exogenous integrin $\alpha_{\text{IIb}}\beta_3$ on their plasma membrane (CHO $\alpha_{\text{IIb}}\beta_3$) also exhibit active calcium oscillations when plated on substrates coated with Fg or rho (12). Using CHO $\alpha_{\text{IIb}}\beta_3$ cells as an experimental model, we report here that calcium oscillations downstream of integrin $\alpha_{\text{IIb}}\beta_3$ signaling could be readily triggered by the combined function of two ionic exchangers, the sodium-proton exchanger NHE1 and the sodium-calcium exchanger NCX1, which are actively recruited from intracellular vesicles to plasma membranes and form molecular complexes with integrin $\alpha_{\text{IIb}}\beta_3$ by a lipid microdomain- or lipid raft-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Models, Preparation of Substrates, and Pharmacological Treatments**—The CHO $\alpha_{\text{IIb}}\beta_3$ cell line and human integrin $\beta_3$ cDNA was a gift from Dr. M. H. Ginsberg (The Scripps Research Institute, La Jolla, CA). Purifications of recombinant rho and isolations of human platelets from volunteers were as described previously (11). The fluorescently labeled proteins were made by using pDNR-Dual donor vector that contained gene constructs of interest and acceptor vector (pLP-AcGF1P-C or pLP-AcmRFP1-C) that contained fluorescence protein tags (BD Biosciences Clontech). A donor vector encoding an mRFP gene (13) (pLP-AcmRFP1-C) was constructed in the laboratory, as described previously (18). Briefly, time-correlated single photon counting was built on a modified two-photon laser-scanning microscope (Olympus FV300). Two-photon fluorescence was excited using an 800-nm mode-locked femtosecond laser (Mira F-900; Coherent Inc., Palo Alto, CA) that operated at 76 MHz. Fluorescence emission was detected using a photon-counting photomultiplier (H7422P-40; Hamamatsu Photonics KK) and SPC-830 PC board (Becker & Hickl GmbH, Berlin, Germany). Images were taken at 256 x 256 pixel resolution. Data analysis via model function fitting, along with the instrument response function deconvolution and color-coding was conducted by SPCImage version 2.8 software package (Becker & Hickl). In Fig. 3e, the histogram of all image pixels was plotted as a function of fluorescence lifetime value and smoothed by the Sabitzky-Golay algorithm to approximate a normal distribution (upper panel), from which mean and S.D. were quantified (Fig. 3e, lower panel, and Fig. 4c).

**Isolation and Characterization of Cholesterol-enriched Cell Fractions**—Protocols for isolating lipid rafts were as previously described (19) with some modifications. Briefly, CHO $\alpha_{\text{IIb}}\beta_3$ cells were plated on different substrates as indicated at 37 °C for 20 min, lysed with lysis buffer (20 mM Tris-HCl at pH 7.5, 0.5 mM EDTA, 0.1% protease inhibitor cocktail, 2 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) at 4 °C for 4 h. A 10–60% continuous sucrose gradient was used to fractionate total cell lysate into 13 fractions, collected from the top. The cholesterol content within each fraction was analyzed by the
Amplex Red cholesterol assay kit (Molecular Probes). The protein content within each fraction was first precipitated with 10% tricholoroacetic acid and then analyzed by 6.5% SDS-PAGE and Western blotting, using 1:1000 anti-integrin α₂β₃, 1:1000 anti-NHE-1 (Chemicon), and 1:500 anti-NCX-1 (Swant, Bellinzona, Switzerland) as primary antibodies, followed by peroxidase-conjugated secondary antibody, and detected by an enhanced chemiluminescence system (Pierce).

RESULTS

**Net Calcium Influxes Generated by Combined NHE1-NCX1 Effects Induced Calcium Oscillations in CHO α₂β₃ Cells**

![Image](image_url)

**FIGURE 1.** NHE1 and NCX1 were involved in calcium oscillations of CHO α₂β₃ cells or human platelets when grown on Fg or rho substrates. a, calcium imaging was performed using Fluo-4 in single live CHO α₂β₃ cell grown on control PLL, Fg, or rho substrates. Changes of intracellular calcium concentration were quantified over time using fluorescence intensity taken at 10 min after cell plating as the reference. b, the percentage of CHO α₂β₃ cells or human platelets exhibiting active calcium oscillations was calculated. Cells grown on the substrates as indicated were pretreated with calcium-free medium (5 mM EGTA), 3 μM Tg, 20 μM BAPTA-AM, 2 μM U73343 or U73122, 5 μM xestospongin C, 25 μM EIPA, 80 μM bepridil, 40 μM KBR7943, or 1, 5, or 10 μM MiCD before calcium imaging. Data shown are mean ± S.D. from more than 100 cells in three separate experiments. c, sodium imaging was done using Sodium Green. CHO α₂β₃ cells grown on rho substrate were pretreated with mock solution (black trace), 25 μM EIPA alone (blue trace), 80 μM bepridil alone (red trace), or both (purple trace) at 37 °C for 10 min before sodium imaging. Changes of intracellular sodium concentration over time were quantified in single live cell. d, -fold changes of intracellular sodium concentration were calculated by dividing the fluorescence intensity of Sodium Green measured at 40 min by that taken at 10 min after cell plating (F₄₀/F₉₀). CHO α₂β₃ cells grown on rho substrate were pretreated with drugs as indicated. Data shown are mean ± S.D. from at least 85 cells in three separate experiments. e, differential interference contrast images of CHO α₂β₃ cells grown on rho substrate, pretreated or not with the drugs as indicated for 20 min. Bar, 5 μm. *p ≤ 0.0001.
**Coupling of NHE1-Integrin α<sub>IIb</sub>β<sub>3</sub>-NCX1 and Calcium Oscillation**

Grown on Fibrinogen/Rhodostomin Substrates—CHO α<sub>IIb</sub>β<sub>3</sub> cells adhered strongly to the surface coated with Fg (12) or the disintegrin rho (supplemental Fig. 1a). The interactions between integrin α<sub>IIb</sub>β<sub>3</sub> and such ligands could further induce cell spreading (supplemental Fig. 1, b and c, and supplemental Movie 1), which coincided with the occurrence of intracellular calcium oscillations that started at ~10 min after plating CHO α<sub>IIb</sub>β<sub>3</sub> cells on a substrate coated with either Fg or rho (Fig. 1a and supplemental Movie 2); growing CHO α<sub>IIb</sub>β<sub>3</sub> cells on the control PLL-coated substrate, on the other hand, did not result in any noticeable calcium transient.

More than 60% of the adherent CHO α<sub>IIb</sub>β<sub>3</sub> and human platelet cells on Fg/rho substrates exhibited calcium oscillations (Fig. 1b, open bars). Treating these cells with calcium-free medium, Tg, or BAPTA-AM effectively inhibited calcium oscillation (Fig. 1b, gray and black bars), suggesting that both extracellular and intracellular calcium pool participated in the calcium oscillation triggered by Fg/rho. Interestingly, inhibition of calcium mobilization through the conventional PLC-IP<sub>3</sub> pathway by U73122 or xestospongin C only partially inhibited the calcium oscillations triggered by Fg/rho (Fig. 1b, dark blue and purple bars), whereas control homolog U73343 had no obvious effect (Fig. 1b, light blue bars). Also, Goncalves et al. (20) had previously reported that platelets isolated from PLC knock-out mice still exhibited calcium oscillation when triggered by Fg, despite having a delayed onset. Taken together, these findings suggested the presence of additional pathway(s), other than PLC, that might be involved in triggering the calcium oscillations downstream of integrin α<sub>IIb</sub>β<sub>3</sub>.

To delineate the putative alternative pathway, we noticed that NHE1 and NCX1 have been reported to be involved in calcium mobilization of platelet (21, 22) and activation of platelets (or inside-out signaling) (23–25). In our system, treating CHO α<sub>IIb</sub>β<sub>3</sub> cells with bepridil and KB R7943, which inhibited the function of sodium-calcium exchanger NCX1, could effectively inhibit the calcium oscillation induced by Fg or rho substrates (Fig. 1b, red bars). NCX1 could potentially generate a local increase of calcium (to trigger CICR) by operating in a “reverse” mode, using sodium efflux in exchange for calcium influx (26).

The question then was how the high local sodium concentration was generated that drove NCX1 in the reverse mode. Indeed, treating CHO α<sub>IIb</sub>β<sub>3</sub> cells grown on Fg or rho substrates with bepridil did cause a progressive increase of sodium (Fig. 1, c and d, and supplemental Movie 3) and cell swelling as more water flowed inside the cell (Fig. 1e). We noticed that the sodium increase and cell swelling caused by bepridil could be reversed by co-treating the cells with EIPA that perturbed the function of sodium-proton exchanger NHE1 (Fig. 1, c–e). Furthermore, EIPA treatment alone also prevented calcium oscillation (Fig. 1b, pink bars). These results suggested that NHE1 could use proton efflux in exchange for sodium influx; the neighboring NCX1 could then take the local high sodium to drive calcium influx (27–31). Such combined NHE1-NCX1 activity appeared to also function in human platelets, since either EIPA or bepridil could effectively inhibit calcium oscillation induced by the cells’ binding to Fg (Fig. 1b).
Targeting of NHE1-NCX1 to the Plasma Membrane When Plating CHO α1β3 Cells on Fibrinogen/Rhodostomin Substrates—Endogenous NHE1 and NCX1 proteins in CHO α1β3 cells were revealed by Western blotting analysis (Fig. 2a). Although the amounts of ion exchanger did not change significantly when the cells were plated on different substrates, their subcellular distributions varied. In Fig. 2, b and c, immunofluorescence staining showed that the ion exchangers were present mainly in the cytoplasm as punctate vesicles but were absent from the cell membrane of CHO α1β3 cells when plated on PLL control substrate for 20 min. In contrast, CHO α1β3 cells grown on Fg or rho substrates for 20 min resulted in recruitment of NHE1-NCX1 from the intracellular vesicular compartments to the plasma membranes (arrowheads), where they co-localized with integrin α1β1 (asterisks). As shown in the zoomed-in image sequence, vesicular or tubular structures that contained NHE1-mRFP were noticed to appose the plasma membrane to where the bead bound (arrowheads). We have also employed total internal reflection fluorescence microscopy (TIRFM) to monitor fusion by an intracellular vesicle to the plasma membrane (supplemental Movie 4). A typical example of such vesicle fusion event is shown in Fig. 2e. A vesicle containing GFP-labeled NHE1 was seen entering the evanescent field of TIRFM (Fig. 2e, red arrowheads); the vesicle’s fluorescence then locally dispersed in a pattern that was characteristic of vesicle fusion (yellow arrowheads). In contrast, we seldom detected vesicle in the evanescent field; nor did we see any discernible occurrence of vesicle fusion if plating CHO α1β3 cells on PLL substrate (supplemental Movie 5).

**FLIM-FRET Measurements Revealed Direct Molecular Interactions between Integrin α1β1 and Ion Exchanger NHE1 or NCX1—**Despite the strong functional link between integrin α1β3 and the ion exchangers, their protein-protein interactions in a convincing way. Does integrin α1β3 bind to the ion exchangers? To further explore this question, we employed the FRET technique to explore this question, we employed the FRET technique to

![FIGURE 2.](image-url)

**FIGURE 3.** FLIM-FRET experiments revealed intermolecular binding between integrin α1β1, and NHE1 or NCX1. a, CHO α1β3 cells were plated on different substrates as indicated at 37 °C for 20 min and then subjected to immunofluorescence staining. NHE1 labeled with fluorescein isothiocyanate was used as FRET donor, whereas integrin α1β1 labeled with TRITC was used as a FRET acceptor. In donor-only control, the FRET acceptor was omitted. Both the FRET donor’s fluorescence intensity (Int) and lifetime (Lt) were recorded and displayed in gray scale or pseudocolor (ranging from 1200 to 2500 ps), respectively. In zoom-in insets, strong FRET focal areas (red spots as indicated by arrowheads) were noted along the plasma membrane when CHO α1β3 cells were plated on Fg or rho substrates but not on the control PLL substrate. b and c, NCX1-integrin α1β1 and NCX1-NHE1 pair was used as the FRET pair, respectively. d, the same setting as in a, but the FRET donor’s fluorescence intensity and lifetime were recorded before and after photobleaching the FRET acceptor. e, FRET donor’s fluorescence lifetime values of all image pixels from the FLIM images as shown in a–c were plotted as histograms (thin traces), to which the Sabitzky-Golay algorithm was applied to approximate normal distributions (thick traces). From individual histograms’ normal distributions, Means ± S.D. were calculated from at least seven sets of cells in three separate experiments (lower panel), * p < 0.0001; ** p = 0.005; bar, 5 μm.
**Coupling of NHE1-Integrin $\alpha_{\text{IIIb}}\beta_3$-NCX1 and Calcium Oscillation**

**a**

![Image of FLIM-FRET results between integrin $\alpha_{\text{IIIb}}$ and individual ion exchangers.](image)

**b**

|      | Fluorescence lifetime (ps) |
|------|---------------------------|
| Donor only | 2250 ± 40              |
| Mock     | 2150 ± 100              |
| Gns     | 2050 ± 300              |
| LY294002 | 2000 ± 200              |
| Cal C   | 1950 ± 150              |
| Tg      | 1900 ± 100              |
| Noc     | 1850 ± 50               |
| MgCD    | 1800 ± 25               |

**FIGURE 4.** Binding to rho by integrin $\alpha_{\text{IIIb}}$ on the cell membrane triggered integrin-NHE1 coupling in the intracellular vesicles. *a* FRET was recorded when a CHO $\alpha_{\text{IIIb}}\beta_3$ cell expressing NHE1-GFP and integrin $\beta_3$-mRFP as FRET pair was held in contact with a rho bead (asterisk). In donor-only control, the FRET acceptor was omitted. FLIM images were captured at the time points indicated. Note the progressive increase of punctate areas inside the cell whose lifetime values were significantly reduced and color-coded in yellow/red (red symbols), the same setting as in *a*, but the cells were pretreated with 200 μM genistein (Gns), 200 μM LY294002, 250 nm calphostin C (Cal C), 3 μM Tg, 500 nm nocodazole (Noc), or 20 μM MgCD for 10 min before interacting with the rho bead. Means ± S.D. of the fluorescence lifetime was measured as in Fig. 3e. ***, p < 0.005; bar, 5 μm.**

The CHO $\alpha_{\text{IIIb}}\beta_3$ cells grown on Fg or rho substrates significantly shifted to yellow/red across the entire cell, as compared with the cells grown on the control PLL substrate or the cells containing FRET donor only. In the latter two cases, long lifetime values were obtained, and the FLIM images were pseudocolor-coded in blue/green. Note also that in CHO $\alpha_{\text{IIIb}}\beta_3$ cells grown on Fg or rho substrates, certain focal spots along the plasma membranes exhibited particularly strong FRET (red spots indicated by arrowheads). These were the regions where strong intermolecular interactions between NHE1 and integrin $\alpha_{\text{IIIb}}$ took place. Similar findings were obtained using NCX1 as FRET donor and integrin $\alpha_{\text{IIIb}}$ as FRET acceptor (Fig. 3b). Interestingly, FRET between NCX1 and NHE1 (Fig. 3c) was less evident than FRET between individual ion exchangers and integrin $\alpha_{\text{IIIb}}$.

To confirm that the reduction of NHE1 donor’s FLIM was actually due to the energy transfer (or FRET) to the integrin $\alpha_{\text{IIIb}}$ acceptor, we photobleached the acceptor fluorochrome and repeated the donor’s lifetime measurement (Fig. 3d). Indeed, photobleaching of the FRET acceptor effectively prevented the reduction of donor fluorochrome’s lifetime, suggesting that detecting decrease of the donor’s lifetime was an accurate way to reveal the occurrence of FRET. To quantify the degrees of FRET, lifetime values of individual image pixels were calculated and displayed in histograms or as mean ± S.D. (Fig. 3e). Using NHE1-integrin $\alpha_{\text{IIIb}}$ and NCX1-integrin $\alpha_{\text{IIIb}}$ as FRET pairs, we noticed that CHO $\alpha_{\text{IIIb}}\beta_3$ cells plated on the Fg (red symbols) or rho (blue symbols) substrates exhibited profound donor lifetime reductions, compared with the cells grown on the PLL substrate (black symbols), or the cells containing donor fluorochrome only (gray symbols). Note also from these results that FRET between the NCX1 and NHE1 pair was less evident than FRET pairs between integrin $\alpha_{\text{IIIb}}$ and individual ion exchangers.

**Molecular Interactions between Integrin $\alpha_{\text{IIIb}}\beta_3$ and Ion Exchangers Also Occurred in Intracellular Vesicular Compartments**—As shown in Fig. 3a, FRET between integrin $\alpha_{\text{IIIb}}\beta_3$ and ion exchangers was found not only on the cell membrane but also in the intracellular vesicular compartments. To further explore this phenomenon, time lapse FLIM imaging (Fig. 4) using NHE1-GFP and integrin $\beta_3$-mRFP as the donor and acceptor of FRET pair, respectively, was performed on a
live CHO α<sub>IIbβ<sub>3</sub> cell, which was held in contact with a 5-μm rho-coated bead (asterisks). The resulting donor’s intensity and FLIM images were shown in gray scale and pseudocolor (ranging from 1600 to 2600 ps), respectively. We noticed that FRET-positive areas (yellow/red) did appear in the cytoplasm as punctate intracellular vesicles, and these progressively increased over time (Fig. 4a), whereas the donor-only control remained free of FRET. Interestingly, such FRET between integrin β<sub>3</sub> and NHE1 in the intracellular vesicular compartments could be effectively reduced by treating the cell with Tg that depleted intracellular calcium store or partially with MβCD that disrupted lipid raft integrity (Fig. 4b). On the other hand, genistein, a tyrosine kinase inhibitor; LY294002, a phosphatidylinositol 3-kinase inhibitor; calphostin C, a protein kinase C inhibitor; or nocodazole, a microtubule toxin, had no obvious effect on FRET between integrin β<sub>3</sub>-NHE1 in the intracellular vesicular compartments. These results indicated that following the CHO α<sub>IIbβ<sub>3</sub></sub> cell’s binding to a Fg/rho substrates intermolecular interactions between integrin α<sub>IIbβ<sub>3</sub></sub> and ion exchangers took place not only on the plasma membrane, where the ligands bound to the integrin receptors, but also in vesicles many μm away from the ligand-receptor binding sites.

**Lipid Microdomain-mediated Targeting of NHE1 and NCX1 to the Plasma Membrane and Their Interactions with Integrin α<sub>IIbβ<sub>3</sub></sub>**—Previous reports indicated that lipid rafts were involved in integrin downstream signaling (33, 34) and platelet activation (35). We have shown in Fig. 4b that disruption of lipid rafts by MβCD affected intermolecular interactions between integrin α<sub>IIbβ<sub>3</sub></sub> and ion exchanger NHE1 or NCX1. The same drug treatment also reduced calcium oscillations in CHO α<sub>IIbβ<sub>3</sub></sub> cells grown on Fg/rho substrates in a dose-dependent manner (Fig. 1b, green bars). These results suggested a role played by lipid rafts, or lipid microdomains, in modulating the observed calcium oscillations in CHO α<sub>IIbβ<sub>3</sub></sub> cells grown on Fg/rho substrates. The presence of lipid rafts was revealed by staining CHO α<sub>IIbβ<sub>3</sub></sub> cells’ GM1 with fluorescently labeled cholera toxin B. We noticed that in CHO α<sub>IIbβ<sub>3</sub></sub> cells grown on control PLL substrate, GM1, like NCX1 or NHE1, was present as punctate intracellular vesicles but absent from the plasma membranes (Fig. 5a). In CHO α<sub>IIbβ<sub>3</sub></sub> cells plated on Fg surfaces, GM1 appeared to transport to the plasma membrane (arrowheads) together with NHE1 (Fig. 5a) or NCX1 (supplemental Fig. 2a). Furthermore, disruption of lipid rafts by MβCD profoundly inhibited targeting of GM1 to the plasma membrane, and as a result, NHE1 (Fig. 5b) and NCX1 (supplemental Fig. 2b) were retained in the cytoplasmic vesicles (green arrowheads). Plating CHO α<sub>IIbβ<sub>3</sub></sub> cells on rho substrate also resulted in membrane targeting of GM1, and such recruitment to the plasma membrane was lipid raft-dependent (data not shown).

We also conducted biochemical assays to assess the recruitments of integrin α<sub>IIbβ<sub>3</sub></sub> and ion exchangers to lipid rafts when plating CHO <sub>IIbβ<sub>3</sub></sub> cells on Fg or rho substrates, as compared with the control PLL substrate. As shown in Fig. 5c, CHO α<sub>IIbβ<sub>3</sub></sub>
Coupling of NHE1-Integrin $\alpha_{IIb}\beta_3$-NCX1 and Calcium Oscillation

**FIGURE 6.** A model summarizes the findings of this study. Most of NHE1 and NCX1 (blue ovals) were present in intracellular vesicles in control CHO $\alpha_{IIb}\beta_3$ cells (1'). Upon binding to ligands, such as Fg or rho disintegrin (purple circles) (2), integrin $\alpha_{IIb}\beta_3$ (green symbols) on the cell membrane elicits signaling events that trigger intermolecular binding between the integrin $\alpha_{IIb}\beta_3$ and the ion exchangers that can occur on the intracellular vesicles (3) or on the plasma membranes (4). The transports and/or interactions of the integrins and ion exchangers are mediated by a lipid raft-dependent mechanism (red-shaded membrane). Functional coupling between NHE1 and NCX1 is able to generate net calcium influx (5) that triggers CICR as a prelude to calcium oscillation downstream of integrin $\alpha_{IIb}\beta_3$ signaling.

cells plated on PLL or Fg substrates for 20 min were subjected to detergent treatments; the detergent-resistant membrane (CRM) that was lipid raft-enriched could be isolated using sucrose gradient fractionation protocols (19, 33). The content of cholesterol, as well as integrin $\alpha_{IIb}$, NHE1, and NCX1, within each fraction was determined. We found that high cholesterol contents, indicative of DRM, were enriched in the first three fractions from the top. From the Western blotting analyses, we noticed that when CHO $\alpha_{IIb}\beta_3$ cells were grown on Fg substrate, there were significantly higher contents of integrin $\alpha_{IIb}$, NHE1, and NCX1 in the DRM fractions (or these proteins’ distributions in fractions shifted to the DRM fractions) than when growing CHO $\alpha_{IIb}\beta_3$ cells on the control PLL substrate.

Caveolin-1 is another protein marker for lipid rafts. Using NHE1-caveolin 1 or NCX1-caveolin 1 as FRET pairs, we concluded from the FLIM-FRET experiments shown in Fig. 5d and from mean FLIM values shown in Fig. 5e that close intermolecular apposition between NHE1/NCX1 and caveolin-1 did occur (Fig. 5e, red bars), not only along the cell membrane (Fig. 5d, arrowheads) but also over the entire cell. As controls, there was no significant FRET taking place when the FRET acceptor was omitted in the experiments (Fig. 5e, black bar) or when CD45, a membrane protein excluded from the lipid raft, was used as the FRET acceptor (blue bars).

**DISCUSSION**

We report here a novel mechanism downstream of integrin $\alpha_{IIb}\beta_3$ signaling whereby a net calcium influx generated by the combined effect of NHE1 and NCX1 ion exchangers is capable of inducing calcium oscillation in a CHO $\alpha_{IIb}\beta_3$ cell model, as well as in native human platelets. The current working model is depicted in Fig. 6. In the control condition, integrin $\alpha_{IIb}\beta_3$ is present mainly on the plasma membrane (Fig. 6, 1) and in some intracellular vesicles. NHE1 and NCX1, on the other hand, are found mostly in intracellular vesicular compartments (Fig. 6, 1') (see Fig. 2a). Upon binding to ligands, such as Fg or rho disintegrin (Fig. 6, 2), integrin $\alpha_{IIb}\beta_3$ on the cell membrane elicits signaling events that trigger intermolecular binding between the integrin $\alpha_{IIb}\beta_3$ and the ion exchangers that can occur on the intracellular vesicles (Fig. 6, 3) or on the plasma membranes (Fig. 6, 4). The transports and/or interactions of the integrins and ion exchangers are mediated by a lipid raft-dependent mechanism. Close molecular apposition between NHE1 and NCX1 render both ion exchangers functionally coupled. NHE1 can generate local high intracellular sodium concentration, which facilitates NCX1 to operate in reverse mode using sodium efflux to drive calcium influx (Fig. 6, 5) (see Fig. 1). The resulting increase of intracellular calcium represents an alternative pathway to trigger CICR as a prelude to calcium oscillation downstream of integrin $\alpha_{IIb}\beta_3$ signaling. Our experimental results strongly suggest that plasma membrane targeting and intermolecular coupling among integrin $\alpha_{IIb}\beta_3$ and ion exchangers NHE1 or NCX1 are dependent on the integrity of lipid rafts (see Figs. 4 and 5).

Compared with the conventional PLC-IP$_3$ pathway that mostly triggers calcium oscillations following receptor binding to soluble agonists, the NHE1-NCX1 system described here may represent an alternative pathway for the integrins to induce calcium oscillation during cell-substrate interactions that govern cell adherence, spreading, and migration (1, 2, 36–39), cancer metastasis (40), platelet activation (23–25), and aggregation (9, 22).

Note that although Fg and the disintegrin rho could both trigger calcium oscillations downstream of integrin $\alpha_{IIb}\beta_3$, there are differences between these two ligands. As shown in Fig. 1b, calcium oscillation induced by rho could be totally abolished by inhibiting NCX1 function using bepridil, whereas calcium oscillation triggered by Fg was only partially inhibited by the drug. The difference may be due to the fact that the much larger Fg could bind to membrane receptors other than integrin $\alpha_{IIb}\beta_3$ heterodimer (41), whereas the much smaller rho protein can only interact with integrin $\alpha_{IIb}\beta_3$ (11).

Despite strong functional links and close intermolecular apposition between integrin $\alpha_{IIb}\beta_3$ and NHE1 or NCX1 as detected by FRET experiments, stable physical binding among these molecules could not be unequivocally demonstrated using conventional biochemical assays, such as co-immunoprecipitation experiments. These results suggest that the intermolecular interactions among components of the proposed
NHE1-integrin α_{IIIb}β_{3}-NCX1 molecular complex are either too transient or too weak to sustain the preparation procedures associated with the biochemical assays or too sensitive to the detergent treatment typically applied during co-immunoprecipitation experiments. We demonstrate here that biophotonic approaches, such as FLIM-FRET, could overcome this difficulty and disclose intermolecular interactions that cannot be otherwise revealed using conventional biochemical methodologies. FRET can also be applied to monitor dynamic intermolecular interactions in live cells or even in situ of a subcellular focal area where cellular events of interest may be only short lived. Note that in the current study, FRET is determined by recording the reduction of the FRET donor’s lifetime as the FRET acceptor approaches. Such a FLIM-FRET imaging platform should be complementary to the conventional ratio-imaging FRET that is based on fluorescence intensity measurements. FLIM-FRET is less sensitive to the noise caused by fluorescence photobleaching or local fluctuations of fluorochrome concentrations, but its current temporal resolution, at less than a frame/s, is less advantageous than the ratio imaging that can easily reach at least 10 to even hundreds of frames/s. FRET occurs if the intermolecular proximity is less than 10 nm, which is generally accepted as the distance to define intermolecular binding (18, 32). It is interesting to know that FRET does occur between integrin α_{IIIb}β_{3} and NHE1 or NCX1 but not much between NHE1 and NCX1, despite the fact that both ion exchangers are co-localized and functionally coupled to each other. We believe that NHE1 and NCX1 may be brought into proximity by individually binding to integrin α_{IIIb}β_{3} heterodimer or by restricting their distributions within a membrane microdomain confined by the lipid rafts.

We have reported previously that plating CHO α_{IIIb}β_{3} cells on Fg or rho substrates could elicit transport of integrin α_{IIIb}β_{3} proteins from intracellular vesicular pool to the cell membrane (14); it appears that NHE1 and NCX1 can also undergo such targeting from intracellular vesicular compartments to the plasma membrane. A major presence of ion exchanger in the intracellular vesicles found in this study is rather new. Previously, NCX had been located to mitochondria (42) or the nuclear envelope (43); however, it is unlikely that NCX located in intracellular vesicles found in this study is rather new. Previous studies have shown to play important roles in various pathophysiological processes, including ischemia-induced reperfusion injuries in myocardium (48) or myocardial stretching (49), salt-dependent hypertension (50), and primary hypertension (51). Our results link NHE and NCX to integrin signaling and may help delineate some of the currently unidentified mechanisms underlying these ion exchanger-related disease processes.

REFERENCES

1. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 11–21
2. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Nat. Rev. Mol. Cell. Biol. 4, 517–529
3. Wang, S. Q., Song, L. S., Lakatta, E. G., and Cheng, H. (2001) Nature 410, 592–596
4. Boitano, S., Dirksen, E. R., and Sanderson, M. J. (1992) Science 258, 292–295
5. Elliott, A. C. (2001) Cell Calcium 30, 73–93
6. Kieffer, N., and Phillips, D. R. (1990) Annu. Rev. Cell Biol. 6, 329–357
7. Phillips, D. R., Charo, I. F., and Scarborough, R. M. (1991) Cell 65, 359–362
8. Jackson, S. P. (2007) Blood 109, 5087–5095
9. Jackson, S. P., Nesbitt, W. S., and Kulkarni, S. (2003) J. Thromb. Haemost 1, 1602–1612
10. Jen, C. J., Chen, H. L., Lai, K. C., and Usami, S. (1996) Blood 87, 3775–3782
11. Chang, H. H., Lin, C. H., and Lo, S. J. (1999) Exp. Cell Res. 250, 387–400
12. Ylanne, J., Chen, Y., O’Toole, T. E., Loftus, J. C., Takada, Y., and Ginsberg, M. H. (1993) J. Cell Biol. 122, 223–233
13. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7877–7882
14. Hsieh, C. F., Chang, B. I., Pai, C. H., Chen, H. Y., Tsai, J. W., Yi, Y. H., Chiang, Y. T., Wang, D. W., Chi, S., Hsu, L., and Lin, C. H. (2006) J. Biol. Chem. 281, 25466–25474
15. Liang, W. N., Wu, T. W., Yao, R. L., Chen, J. Y., and Lin, C. H. (2006) J. Cell Sci. 119, 11–22
16. Lin, C. H., Thompson, C. A., and Fischer, P. (1994) Curr. Opin. Neurobiol. 4, 640–647
17. Liang, W. N., Tsai, J. W., Yu, P. M., Wu, T. W., Yang, S. C., Chau, Y. P., and Lin, C. H. (1999) Hepatology 30, 748–760
18. Ghukasyan, V., Hsu, Y. S., Kung, S. H., and Kao, F. J. (2007) J. Biomed. Opt. 12, 024016
19. Dorathy, D. J., Lincz, L. F., Meldrum, C. J., and Burns, G. F. (1996) Biochem. J. 319, 67–72
20. Goncalves, I., Hughan, S. C., Schoenwaelder, S. M., Yap, C. L., Yuan, Y., and Jackson, S. P. (2003) J. Biol. Chem. 278, 34812–34822
21. Siffert, W., and Akkerman, J. W. (1987) Nature 325, 456–458
22. Roberts, D. E., McNicol, A., and Bose, R. (2004) J. Biol. Chem. 279, 19421–19430
23. Siffert, W., and Akkerman, J. W. (1988) Trends Biochem. Sci. 13, 148–151
24. Shiraga, M., Tomiyama, Y., Honda, S., Suzuki, H., Kosugi, S., Tadokoro, S., Kanakura, Y., Tanoue, K., Kurata, Y., and Matsuzawa, Y. (1998) Blood 92, 3710–3720
25. Rosskopf, D. (1999) J. Thromb. Thrombolysis 8, 15–24
26. Liu, P. S., and Kao, L. S. (1990) Cell Calcium 11, 573–579
27. Borle, A. B., and Bender, C. (1991) Am. J. Physiol. 261, C482–C489
28. Gillespie, J. I., Otun, H., Greenwell, I. R., and Dunlop, W. (1992) Exp. Physiol. 77, 141–152
29. Thomas, M. J., Sjaastad, I., Andersen, K., Helm, P. J., Wasserstrom, J. A., Sejersted, O. M., and Ottersen, O. P. (2003) J. Mol. Cell Cardiol. 35, 1325–1337
30. Viachenko-Karpinski, S., Terentyev, D., Jenkins, L. A., Lutherer, L. O.,
Coupling of NHE1-Integrin α₅β₃-NCX1 and Calcium Oscillation

31. Huang, J., Hove-Madsen, L., and Tibbits, G. F. (2008) Am. J. Physiol. 294, C516–C525
32. Wang, Y., and Chien, S. (2007) Methods Enzymol. 426, 177–201
33. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–39
34. Wang, X., Huang, D. Y., Huang, S. M., and Huang, E. S. (2005) Nat. Med. 11, 515–521
35. Bodin, S., Tronchere, H., and Payrastre, B. (2003) Biochim. Biophys. Acta 1610, 247–257
36. Demaurex, N., Downey, G. P., Waddell, T. K., and Grinstein, S. (1996) J. Cell Biol. 133, 1391–1402
37. Tominaga, T., and Barber, D. L. (1998) Mol. Biol. Cell 9, 2287–2303
38. Dreval, V., Dieterich, P., Stock, C., and Schwab, A. (2005) Cell Physiol. Biochem. 16, 119–126
39. Hynes, R. O. (2002) Cell 110, 673–687
40. Cardone, R. A., Casavola, V., and Reshkin, S. J. (2005) Nat. Rev. Cancer 5, 786–795
41. Altieri, D. C., Agbanyo, F. R., Plescia, J., Ginsberg, M. H., Edgington, T. S., and Plow, E. F. (1990) J. Biol. Chem. 265, 12119–12122
42. Crompton, M., Moser, R., Ludi, H., and Carafoli, E. (1978) Eur. J. Biochem. 82, 25–31
43. Xie, X., Wu, G., Lu, Z. H., Rohowsky-Kochan, C., and Ledeon, R. W. (2004) Neurochem. Res. 29, 2135–2146
44. Nass, R., and Rao, R. (1998) J. Biol. Chem. 273, 21054–21060
45. Bossuyt, J., Taylor, B. E., James-Kracke, M., and Hale, C. C. (2002) FEBS Lett. 511, 113–117
46. Bullis, B. L., Li, X., Singh, D. N., Berthiaume, L. G., and Fliegel, L. (2002) Eur. J. Biochem. 269, 4887–4895
47. Petrie, R. J., Schnetkamp, P. P., Patel, K. D., Awasthi-Kalia, M., and Deans, J. P. (2000) J. Immunol. 165, 1220–1227
48. Fliegel, L. (2005) Int. J. Biochem. Cell Biol. 37, 33–37
49. Cingolani, H. E., Perez, N. G., and Camilion de Hurtado, M. C. (2001) News Physiol. Sci. 16, 88–91
50. Iwamoto, T., and Kita, S. (2006) Kidney Int. 69, 2148–2154
51. Orlov, S. N., Adragna, N. C., Adarichev, V. A., and Hamet, P. (1999) Am. J. Physiol. 276, C511–C536