Integrin-mediated membrane blebbing is dependent on the NHE1 and NCX1 activities

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Integrin-mediated signal transduction and membrane blebbing have been well studied to modulate cell adhesion, spreading and migration[1-6]. However, the relationship between membrane blebbing and integrin signaling has not been explored. Here we show that integrin-ligand interaction induces membrane blebbing and membrane permeability change. We found that sodium-proton exchanger 1 (NHE1) and sodium-calcium exchanger 1 (NCX1) are located in the membrane blebbing sites and inhibition of NHE1 disrupts membrane blebbing and decreases membrane permeability change. However, inhibition of NCX1 enhances cell blebbing to cause cell swelling which is correlated with an intracellular sodium accumulation induced by NHE1[7]. These data suggest that sodium influx induced by NHE1 is a driving force for membrane blebbing growth, while sodium efflux induced by NCX1 in a reverse mode causes membrane blebbing retraction. Together, these data reveal a novel function of NHE1 and NCX1 in membrane permeability change and blebbing and provide the linkage of integrin signal and membrane blebbing.

Plasma membranes often undergo dynamic, local expansion and retraction, forming transient, small spherical blebs which initiate from the detachment of plasma membrane from the actin cortex followed by lipid flow into the blebbing site. These blebs expand by a transient increase of intracellular pressure and retract when myosin motor track along actin filament located beneath the bleb. Membrane blebbing has been well-documented to participate in many cellular activities such as cell spreading, migration, virus entry, cytokinesis and apoptosis[4-6]. Although integrin receptors, constituting 24 αβ heterodimers that result from combinations between 18 α and 8 β subunits, and their extracellular matrix (ECM) ligands i.e., collagen, laminin and fibronectin etc., also regulate many of these cell activities[1-3], it is unclear whether integrin-mediated signals are involved in membrane blebbing that can cause the cells to exhibit cell adhesion and spreading.

Based on the ligand specificity and restriction expression on leukocytes, the 24 integrins are divided into four subfamilies, RGD (Arginine-Glycine-Aspartic Acid), collagen, laminin receptors and leukocyte receptors[1, 2]. Integrin$_{\alpha IIb\beta 3}$ is one of RGD receptors which is only expressed on platelets and modulates platelet adhesion and spreading that is critical for thrombosis[3, 8-11]. Previously, we demonstrated that the activation of platelets and CHO$_{\alpha IIb\beta 3}$ cells by fibrinogen (Fg) and RGD-containing rhodostomin [RHO(RGD)], a snake venom from *Agkistrodon rhodostoma*, can induce not only cell spreading but also intracellular calcium oscillation[7-12]. We also found that integrin$_{\alpha IIb\beta 3}$ down-stream signals induced intracellular NHE1 and NCX1-integrin$_{\alpha IIb\beta 3}$ interaction leading to the formation of functional complexes in lipid raft microdomain[7]. This finding is interesting because NHE1 and NCX1 are functionally coupled in which NHE1 drives sodium ion influx in turn to activate NCX1 in a reverse mode to generate a calcium influx and to modulate intercellular calcium oscillation[7]. Whether other ion transportation can be triggered by integrin signaling has less explored.

In this report, we measured ionic flow across the plasma membrane by using whole cell voltage-clamp recording to show that plating the $CHO_{\alpha IIb\beta 3}$ cells onto Fg and RHO(RGD) substrates induced a rapid, dramatic ion current change (around -2 nA to -3 nA) (Fig. 1a, b), suggesting a significant change in membrane permeability. Control experiments showed that no such permeability change occurred when $CHO_{\alpha IIb\beta 3}$ cells were plated onto poly-L-lysine (PLL)-, bovine serum albumin (BSA)- or RHO(RGE) (a glutamic acid substituted rhodostomin)[9-11]-coated substrates (Figure 1a and 1b). Similarly, no such permeability change occurred when the parental CHO cells (data not show) and CHO cells expressing $integrin_{\alpha v\beta 3}$ were plated onto the RHO(RGD)-coated substrate (Fig. 1c). These data indicated that membrane permeability change was $integrin_{\alpha IIb\beta 3}$–ligand interaction specific, and revealed a novel cell response that the activation of $integrin_{\alpha IIb\beta 3}$ could induce cell membrane permeability change.

In addition, these experiments revealed that membrane permeability change were accompanied by striking membrane blebbing, which could be monitored by time-lapse phase microscopy. Membrane blebbing after $CHO_{\alpha IIb\beta 3}$ cells attached onto the Fg-coated substrate was observed before cell spreading (Fig. 2a upper panels and supplemental Movie 1). In contrast, $CHO_{\alpha IIb\beta 3}$ cells plated onto the control bovine fetal albumin (BSA)-coated substrate did not show noticeable blebbing (Fig. 2a, lower panels and supplemental Movie 2). To test whether this integrin-induced membrane blebbing also occurs in other types of cells when they attach onto a specific substrate, we tested human platelets, which naturally contain abundant $integrin_{\alpha IIb\beta 3}$. We observed membrane blebbing in platelets when they were seeded onto Fg-coated substrate before spreading (indicated by *arrows* in Fig. 2b and supplemental Movie 3). We also found a similar result of blebbing when NIH3T3 cells, a mouse fibroblast cell containing two members of the RGD receptor ($integrin_{\alpha v\beta 3}$ and $integrin_{\alpha 5\beta 1}$)[13,], which is commonly used for cell adhesion and migration, plated onto the fibronectin (Fn)-coated substrate (indicated by *arrowheads* in upper panels of Fig. 2c and supplemental Movie 4). Occasionally, elongation of filopodium (*yellow arrowheads* in Fig. 2c) in the membrane blebbing site of NIH3T3 cells was detected. In contrast, NIH3T3 cells attached onto BSA-coated plates did not show noticeable blebbing (Fig. 2c, lower panels and supplemental Movie 5). Taken together, these data demonstrate for the first time that integrin-ligand interaction could induce membrane blebbing prior to cell spreading.

Since $integrin_{\alpha IIb\beta 3}$ down-stream signals can target NHE1 (a sodium-proton exchanger) and NCX1 (a sodium-calcium exchanger) onto the plasma membrane[7] with many other studies documented that NHE1 and NCX1 associated proteins are present in the membrane blebs site [5, 6, 14-16], we wanted to know whether the two ion exchangers are located on bleb sites and involved in inducing blebbing and membrane permeability change. By immunofluorescence staining, we detected that NHE1 was located on membrane blebbing sites when $CHO_{\alpha IIb\beta 3}$ cells attached onto the Fg substrate (Fig. 3a). This is the first finding showing NHE1 can be located

on blebs and suggesting that NHE1 and its associated proteins play a role in blebbing. While $CHO_{\alpha IIb\beta 3}$ or NIH3T3 cells were pretreated with an NHE1 inhibitor [EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride], no membrane blebs were observed when cells attached onto Fg or Fn substrates (Fig. 3b, c, lower panels). Moreover, this inhibitor also completely blocked cell permeability change (Fig. 3d, e). These data indicate that NHE1, which can pump sodium ions into cells, is required for integrin-mediated cell membrane blebbing and permeability change.

Since NHE1 and NCX1 are known to be functionally coupled, we then tested the role of NCX1 in the membrane blebbing by the treatment of $CHO_{\alpha IIb\beta 3}$ cells with an NCX1 inhibitor (bepridil). Immunofluorescence staining revealed that NCX1 of untreated $CHO_{\alpha IIb\beta 3}$ cells was indeed present in membrane blebbing sites when cells attached onto Fg substrate (indicated by *arrows* in Fig. 4a). Moreover, we found that $CHO_{\alpha IIb\beta 3}$ cells pretreated with an NCX1 inhibitor (bepridil), failed to bleb (control; indicated by *arrowheads* in Fig. 4b); rather the cells became swollen (*double arrowheads* in Fig. 4b). We also showed that an intracellular sodium accumulation accompany with cell blebbing or swelling in the bepridil-pretreated $CHO_{\alpha IIb\beta 3}$ cells when cells attached onto Fg substrate (Fig. 4c and supplemental Movie 6)[7]. Together with the results demonstrating that NCX1 is located on the plasma membrane blebbing site (Fig. 4a), we suggest that NCX1 plays a compensation role with NHE1 by refluxing sodium ions outside of cells and simultaneously influx calcium ions to modulate the membrane blebbing retraction.

Although membrane blebbing has been well documented in many cell types, little is known about its detailed mechanism[4-6]. Our study showed for the first time that membrane blebbing occurring in the $CHO_{\alpha IIb\beta 3}$ cells can be induced by $integrin_{\alpha IIb\beta 3}$ signals (Fig. 2a). Our study also showed that membrane blebbing occurs in human platelets when they adhere onto Fg substrate before full spreading (Fig. 2b) indicating that membrane blebbing can be induced by down stream signals of $integrin_{\alpha IIb\beta 3}$. Membrane blebbing in platelets has not been observed earlier probably because its small size and blebs occurring time is short and quick which did not notice by previous studies[8-11, 17]. Combined studies of $CHO_{\alpha IIb\beta 3}$ cells and platelets with the results that the membrane blebbing in NIH3T3 cells when they adhere onto Fn substrate (Fig. 2c). Taken together, these data suggest that integrin-mediated membrane blebbing is a common phenomenon for cell adhesion and spreading[18].

Although cell spreading accompany with cell volume change which is modulated by the NHE1 activity has been well documented[14, 15, 19, 20]. It's not known whether NHE1 is required for the $integrin_{\alpha IIb\beta 3}$-mediated cell permeability change and membrane blebbing during cell spreading. In many cell models, NHE1 is known to operate in parallel with Cl^-/HCO_3^- exchanger causing cell uptake of sodium and chloride ions[21]. This uptake can induce the influx of osmotically obliged water which results in an increase of intracellular volume and pressure to cause cell blebbing or swelling[21]. Our study links the $integrin_{\alpha IIb\beta 3}$-mediated cell permeability change and membrane blebbing which are dependent on intracellular sodium and

chloride influx and efflux. We thus suggest that NHE1, Cl^-/HCO_3^- exchanger and water channel cooperatively participate in the integrin-mediated cell permeability change and membrane blebbing.

Many actin associated proteins, such as ERM protein, Rho A, ROCK and ankyrin, have been found to interact with NHE1 and NCX1 at the membrane[5, 6, 14-16]. This study is the first to demonstrate that NHE1 and NCX1 are located on the membrane blebbing sites (Fig. 3a, 4a). Together with our and others' previous studies showing that sodium and calcium ions are enriched in the blebbing sites[7, 22, 23](Fig. 4c), we propose a model to explain how membrane blebs are involved in the expanding and retraction steps. In this model, NHE1 and NCX1 are key players in forming membrane blebbing, accompanying with the influx calcium and actin-associated proteins to control the dynamic change of membrane blebbing (Fig. 5). When integrins are activated by binding to their substrates, NHE1 and NCX1 in vesicles are targeted to the plasma membranes[7] (Fig. 5, panel 1), followed by the elevation of superoxide induced by NADPH oxidase activates NHE1 thus causing cytosolic alkalization and sodium ion influx[24] (Fig. 5, panels 2 and 3). The sodium ion influx generated by NHE1 can increase the intracellular pressure which is an initial driving force triggering membrane bleb growth thus forming a nucleation site of membrane blebbing (Fig. 5, panel 3). While the sodium influx continues, membrane blebbing grows. NCX1 located on membrane blebbing sites then plays a reverse mode to decrease the intracellular pressure by refluxing sodium ions outside of cells and influxing calcium ions into cells[7, 25] (Fig. 5, panel 4). Subsequently, a higher concentration of calcium accumulated locally in the membrane bleb induces myosin-mediated contraction, accompanied by the activation of actin-associated proteins located underneath the membrane bleb, causing membrane blebbing retraction (Fig. 5, panel 5)[6, 26].

Methods

Cell models, preparation of substrates, and pharmacological treatments. The $CHO_{\alpha IIb\beta 3}$ and $CHO_{\alpha v\beta 3}$ cell lines were a gift from Dr. M. H. Ginsberg (The Scripps Research Institute, La Jolla, CA). Purifications of recombinant rhodostomin [both wild-type RHO(RGD) and mutant RHO(RGE)] and isolations of human platelets from volunteers were as described previously[8]. Human Fg, poly-L-lysine (PLL), bovine serum albumin (BSA), EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride), and bepridil were purchased from Sigma (Sigma, St Louis, MO). Sodium Green dye were obtained from Molecular Probes, Inc. (Eugene, OR). Preparation of substrates was done according to the methods described previously[7].

Whole cell voltage-clamp recording. For whole cell voltage-clamp recording, we used a commercially available amplifier and data acquisition software (Axopatch 200B, Axon Instruments, Union City, CA) to acquire data. For the preparation of cell suspensions, cells were harvested by using 1X trypsin solution and dissociated using a standard medium and kept in a suspension for 10 min before use. Suspended cells in Dulbecco's modified Eagles' medium (supplemented with 10% fetal bovine serum) (Life Technologies/Gibco-BRL Cleveland, OH) were plated onto different substrates for 10 min and then transfer to the recording chamber containing extracellular electrolyte solutions to perform whole cell voltage-clamp recording under a phase contrast microscope (DM IRE, Leica Microsystems, Wetzlar, Germany). The ionic compositions of electrolyte solutions had extra 1 mM $CaCl_2$ in the extracellular electrolyte solution and the same of other ions, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, pH at 7.35, in both extracellular and intracellular electrolyte solutions.

Living cell image. Living cell and sodium image was recorded according to the methods described previously with some modifications[7]. For phase, DIC (differential interference contrast) or sodium image, suspended cells were plated for 5 min and observed under a phase, DIC time-lapse or fluorescence microscope in a climate chamber to maintain cell viability and activity.

Immunofluorescence staining. Suspended cells were plated for 5 min and subjected to immunofluorescence staining according to the methods described previously[7]. All stained samples were observed under a BD *CARV II*™ confocal microscope (BD Bioscience, San Jose, CA).

Supplemental Information

Supplemental Movie 1

$CHO_{\alpha IIb\beta 3}$ cells were plated onto Fg substrate at 37^{o}C and cell images were recorded using time-lapsed (1:30 time condensation) phase video microscopy.

Supplemental Movie 2

$CHO_{\alpha IIb\beta 3}$ cells were plated onto BSA substrate at 37^{o}C and cell images were recorded using time-lapsed (1:30 time condensation) phase video microscopy.

Supplemental Movie 3

Platelet cells were plated onto Fg substrate at 37^{o}C and cell images were recorded using time-lapsed (1:30 time condensation) DIC video microscopy.

Supplemental Movie 4

NIH3T3 cells were plated onto Fn substrate at 37^{o}C and cell images were taken using time-lapsed (1:30 time condensation) phase video microscopy.

Supplemental Movie 5

NIH3T3 cells were plated onto BSA substrate at 37^{o}C and cell images were recorded using time-lapsed (1:30 time condensation) phase video microscopy.

Supplemental Movie 6

NIH3T3 cells were plated onto Fn substrate at 37^{o}C and cell sodium images were taken using time-lapsed (1:30 time condensation) fluorescence video microscopy and displayed by pseudo-color.

ACKNOWLEDGEMENTS

We would like to express our thanks to Drs Simon Silver (CGU visiting professor) and Tung-Tien Sun (New York University) for reviewing the manuscript. Microscopy and electrophysiology equipment for this study were supported by the Department of Biomedical Sciences and Molecular Medicine Research Center of Chang Gung University and Biophotonics Interdisciplinary Research Center and the Genome Research Center of National Yang-Ming University. This work was supported by grants from the Chang Gung Memorial Hospital, the Ministry of Education (Aim for the Top University Plan) and the National Science Council, R.O.C.

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Figure legends

Figure 1. Detection of integrin$_{\alpha IIb\beta 3}$-mediated cell permeability change by the whole cell voltage-clamp technique. (**a**) CHO$_{\alpha IIb\beta 3}$ cells attached on different substrates as indicated and the plasma membrane permeability change was monitored by whole cell voltage-clamp recording. The current change of cells attached on various substrates is shown by different color traces: CHO$_{\alpha IIb\beta 3}$ cells on RHO(RGD), *red trace*; fibrinogen (Fg), *orange trace*; mutant rhodostomin RHO(RGE), *blue trace;* poly-L-lysine (PLL), *black trace*, and BSA, *green trace*. (**b**) The quantitative summary of membrane permeability change data from three independent experiments as shown in panel (**a**). Noted that integrin$_{\alpha IIb\beta 3}$ specific substrates, Fg and RHO(RGD), can induce cell permeability change. (**c**) CHO$_{\alpha v\beta 3}$ and CHO$_{\alpha IIb\beta 3}$ cells were attached onto RHO(RGD) substrate and monitored the plasma membrane permeability change by using whole cell voltage-clamp recording. Noted that RHO(RGD) induced cell permeability change was observed in CHO cells expressing integrin$_{\alpha IIb\beta 3}$ but not integrin$_{\alpha v\beta 3}$. Error bars indicate SEM.

Figure 2. Recording of cell membrane blebbing when cells attached on various substrates. (**a**) CHO$_{\alpha IIb\beta 3}$ cells attached onto Fg or control BSA substrates. Time-lapse phase imaging was done and the time interval for the image sequence shown is 5 sec. Noted the cell membrane blebbing occurred before cell fully spreading induced by Fg substrate (upper panels, *arrowheads*) but not by BSA substrate (lower panels). (**b**) Platelets attached onto Fg substrate. Time-lapse differential interference contrast (DIC) imaging was performed to monitor membrane blebbing and spreading. The time interval for the image sequence is 5 sec. Noted the cell membrane blebbing occurred before the platelet was fully spreading induced by Fg substrate (*arrows*)**.** (**c**) NIH3T3 cells attached onto Fn or control BSA substrates. The time-lapse phase imaging was performed in the time interval for 5 sec. Noted the cell membrane blebbing occurred before cell fully spreading induced by Fn substrate (upper panels, *arrowheads*) but not by BSA substrate (lower panels). Single filopodium elongation occurred in the blebbing site is shown in *yellow arrowheads* Numbers on panels **a-c** indicate time in min. Scale bar represents 5 μm.

Figure 3. NHE1 located on the membrane blebbing site and modulates membrane blebbing and permeability change. (**a**) CHO $_{\alpha IIb\beta 3}$ cells plated onto Fg substrate were subjected to immunofluorescence staining to reveal the distributions of integrin$_{\alpha IIb}$ and NHE1. Cell images were taken under a DIC (left panel) and fluorescence (middle two panels) microscope. Co-localization of integrin$_{\alpha IIb}$ and NHE1 on plasma membrane is shown by the merged picture (right panel). Noted that NHE1 is located to the membrane blebbing sites when plated onto Fg

substrate (*arrows*). The inset is enlarged from the portion of cell indicated by a square box. (**b**) $CHO_{\alpha IIb\beta 3}$ or (**c**) NIH3T3 cells pretreated with or without NHE1 inhibitor, EIPA, were seeded onto the Fg or Fn substrates respectively. Time-lapse phase images were taken and the time interval for the image sequence is 5 sec. Noted the blebbing growth (*arrowheads*) is induced by Fg or Fn substrates (upper panels) but is inhibited by the EIPA pretreatment (lower panels). (**d**) $CHO_{\alpha IIb\beta 3}$ cells attached onto RHO(RGD) substrate in the presence (*red trace*) or absence (*black trace*) of EIPA and were monitored the permeability change of plasma membrane by using whole cell voltage-clamp recording. (**e**) The quantitative data of membrane permeability change from three independent experiments shown as in panel (**d**). Noted that the cell permeability change is significantly reduced when the $CHO_{\alpha IIb\beta 3}$ cells were pretreated with EIPA. Error bars indicate SEM. Numbers on panels **b-c** indicate time in min. Scale bar represents 5 μm.

Figure 4. NCX1 locates on the membrane blebbing site and modulates membrane blebbing. (**a**) $CHO_{\alpha IIb\beta 3}$ cells plated onto Fg substrate were subjected to immunofluorescence staining to reveal the distributions of $integrin_{\alpha IIb}$ and NCX1. Cell images were taken under a DIC (left panel) and fluorescence (middle two panels) microscope. Co-localization of $integrin_{\alpha IIb}$ and NCX1 on the plasma membrane is shown on the merged picture (right panel). The inset is enlarged from the portion of cell indicated by a square box. Noted that NCX1 is located to the membrane blebbing sites when cells were attached onto Fg substrate (*arrows*). (**b**) $CHO_{\alpha IIb\beta 3}$ cells were attached onto RHO(RGD) substrate in the presence of NCX1 inhibitor, bepridil. The time-lapse DIC imaging was performed to monitor membrane blebbing and cell swelling. The time interval for the image sequence is 5 sec. Noted that the cell membrane blebbing occurred (*arrowheads*) before the cell swelling (*double arrowheads*) when the cell pretreated with bepridil, was attached onto RHO(RGD) substrate. (**c**) Sodium ion distribution in the bepridil-treated NIH3T3 cell which was attached onto Fn substrate. The time-lapse fluorescence imaging was performed to monitor the sodium ion concentration change which is indicated by pseudo-color. Arrowheads indicate the membrane bleb. Numbers on panels **b-c** indicate time in min. Scale bar represents 5 μm.

Figure 5. The proposed model that integrin-mediated membrane blebbing is dependent on the activities of NHE1 and NCX1. The integrin-ligand interaction induces NHE1 and NCX1-containing vesicles targeting onto the membrane (1'). After cell attachment onto substrate, the NADPH oxidase is activated through integrin signals to elevate the concentration of superoxide which in turn activates NHE1 to cause sodium influx (2'). The sodium influx can further increase the intracellular pressure as a driving force to cause membrane blebbing nucleation and growth (3'). A higher intracellular sodium concentration activates NCX1 in a reverse mode to cause the efflux of intracellular sodium and calcium influx (4'). The locally

increasing intracellular calcium driven by NCX1 induces the myosin-mediated contraction to cause membrane blebbing retraction (5’). This model is modified from Fackler and Grosse (2008) and the symbols of figure are indicated. Various sizes of ion symbols represent the relative concentration of ions in intracellular (i) or extracellular (o) compartments.