Molecular Proximity of Kv1.3 Voltage-gated Potassium Channels and β_1-Integrins on the Plasma Membrane of Melanoma Cells: Effects of Cell Adherence and Channel Blockers

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ABSTRACT Tumor cell membranes have multiple components that participate in the process of metastasis. The present study investigates the physical association of β1-integrins and Kv1.3 voltage-gated potassium channels in melanoma cell membranes using resonance energy transfer (RET) techniques. RET between donor-labeled anti–β1-integrin and acceptor-labeled anti-Kv1.3 channels was detected on LOX cells adherent to glass and fibronectin-coated coverslips. However, RET was not observed on LOX cells in suspension, indicating that molecular proximity of these membrane molecules is adherence-related. Several K^+ channel blockers, including tetraethylammonium, 4-aminopyridine, and verapamil, inhibited RET between β1-integrins and Kv1.3 channels. However, the irrelevant K^+ channel blocker apamin had no effect on RET between β1-integrins and Kv1.3 channels. Based on these findings, we speculate that the lateral association of Kv1.3 channels with β1-integrins contributes to the regulation of integrin function and that channel blockers might affect tumor cell behavior by influencing the assembly of supramolecular structures containing integrins.

KEY WORDS: Kv1.3 channel • integrin • resonance energy transfer • spectroscopy • receptors, signaling

INTRODUCTION

Integrins are a major family of cell surface heterodimeric transmembrane glycoproteins that broadly participate in the control of cell adhesion and motility, and, for tumor cells, their invasive and metastatic potential (Dedhar, 1990; Hynes, 1992; Albelda, 1993). Each heterodimer is composed of noncovalently associated α and β chains. Multiple α and β chains exist, which can be assembled in several fashions to yield many integrins (Hynes, 1992). For example, β1 integrins are assembled from multiple α chains (CD49a-f) and the β1 chain to yield receptors for extracellular molecules such as laminin, collagen, and fibronectin. Integrins provide a transmembrane signaling conduit for both chemical and mechanical signals (Hynes, 1992; Wang et al., 1993). Integrins also mediate transmembrane communication in the opposite direction from the cell interior to the extracellular environment, which is known as inside-out signaling (Shaw et al., 1993). Integrins have been shown to interact with several other membrane-associated proteins (Petty et al., 2002). For example, integrins can form noncovalent interactions with GPI-linked proteins (urokinase receptors, Fcγ receptor type III, and CD14), Fcγ receptor type IIA, tetraspans, CD98, IAP (integrin-associated protein), and others. Not surprisingly, integrins can participate in, or in some cases mediate, transmembrane signaling on behalf of a partner protein (for a recent review see Petty et al., 2002). Furthermore, lateral interactions can regulate the function of both the integrin and the partner protein. Thus, integrins demonstrate great flexibility in their assembly and interactions with other components on the outside, inside, and within the plasma membrane.

Recent studies have suggested that lines of communication exist between cell surface integrins and K^+ channels. When fibronectin binds to integrins on tumor cell membranes, K^+ channels are activated, thus leading to membrane hyperpolarization. Hyperpolarization precedes a marked cell spreading in erythroleukemia cells (Arcangeli et al., 1991) and serves as a commitment signal to neuritogenesis in neuroblastoma cells (Arcangeli et al., 1993). Fibronectin binding promotes activation of Ca^{2+}-dependent K^+ channels in murine erythroleukemia cells, HERG K^+ channel currents in human preosteoclastic leukemia cells, and inwardly rectified K^+ channels in neuroblastoma cells (Arcangeli et al., 1989, 1991; Becchetti et al., 1992; Hofmann et al., 2001). Laminin also promotes inwardly rectifying K^+ currents in neuroblastoma cells (Arcangeli et al., 1996). Certain antibodies against β1-integrins mimic the effects of laminin, further supporting the integrin-related activation of K^+ channels. Complementary evidence using K^+ channel blockers (e.g., tetraethylammonium [TEA],*

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4-aminopyridine [4-AP], quinine, verapamil, diltiazem, cetiedil) have demonstrated cross-talk between K^+ channels and integrin function. Migration of transformed renal epithelial cells and human melanoma cells requires K^+ channel activity (Schwab et al., 1994, 1999). More specifically, voltage-gated K^+ channels have been linked with integrin activation and integrin-mediated processes (Lewis and Cahalan, 1995). In contrast to the inhibitory effects of K^+ channel blockers, elevated extracellular K^+, in the absence of a "classical" immunological stimulatory signal, is a sufficient stimulus for activation of T cell β1 integrins and induction of integrin-mediated adhesion and migration (Levite et al., 2000). Thus, the cooperation of integrins and K^+ channels in eliciting cell functions has been established.

One potential mechanism to account for integrin–K^+ channel cross-talk is physical interactions between these two cell surface molecules. Indeed, recent studies have suggested that at least two types of K^+ channels can be immunoprecipitated with integrins (McPhee et al., 1998; Levite et al., 2000). Using resonance energy transfer (RET) techniques, we now extend these previous studies by demonstrating the molecular proximity of the Kv1.3 channel and β1 integrins on living cell membranes. We further show that this interaction is promoted by cell adherence and inhibited by channel blockers. This latter observation leads to the speculation that the disassembly of supramolecular complexes containing integrins and K^+ channels may be important in the action of certain antimetastatic drugs.

MATERIALS AND METHODS

Materials

Fibronectin was purchased from Life Technologies. TRITC and FITC were obtained from Molecular Probes, Inc. TEA, 4-AP, apamin, and verapamil were purchased from Sigma-Aldrich.

Antibodies

A FITC-conjugated monoclonal antibody (mAb) against CD29 (β_1 integrin subunit; K20 clone, IgG_{2a} isotype) was purchased from Immunotech (Beckman Coulter). Polyclonal rabbit anti-Kv1.3 antibody, monoclonal goat anti–rabbit antibody, and TRITC-conjugated monoclonal goat anti–rabbit antibody were purchased from Chemicon International Inc.

For TRITC antibody conjugation, the pH of antibody solutions was increased by overnight dialysis against carbonate buffer (pH 9.6; 0.2 M). TRITC was then added at a molar ratio 1:13 for 3 h with shaking followed by overnight dialysis against PBS (pH 7.4) and Sephadex G25 column chromatography.

Cell Culture

The human amelanotic malignant melanoma cell line LOX was provided by Dr. Oystein Fodstad (Institute for Cancer Research, Norwegian Radium Hospital, Oslo, Norway). LOX cells were cultured in Dulbecco's modified Eagle medium (Life Technologies) and RPMI-1640 (Life Technologies) medium at 1:1 ratio supplemented with 10% heat-inactivated fetal calf serum (Life Technologies), 5% Nu-serum IV (Becton Dickinson), and 0.01% antibiotic-antimicotic (Life Technologies) in an atmosphere of 5% CO_2 at 37°C.

Indirect Immunofluorescence Staining

To study Kv1.3 channel and β1-integrin localization on cell surfaces, LOX cells were incubated for 2 h on uncoated or fibronectin-coated glass coverslips. Coverslips were coated with fibronectin (10 μg/ml) in PBS (pH 7.2) by incubation for 2 h at 37°C or overnight at 4°C, followed by extensive washing. Cells attached to the coverslip were fixed with 3% paraformaldehyde for 15 min at room temperature. Coverslips with attached cells were washed several times with HBSS. Cells were then labeled with first step polyclonal rabbit antibody against Kv1.3 channel (10 μg/ml) for 1 h at room temperature followed by second-step TRITC-conjugated goat anti–rabbit IgG antibody for 1 h at room temperature. In other experiments a TRITC-conjugated anti-Kv1.3 antibody was employed. After several washes, cells were fixed again with 3% paraformaldehyde and blocked with 3% BSA in HBSS, followed by labeling with FITC-conjugated anti-β1 integrin/CD29 mAb for 1 h at room temperature. After extensive washing the coverslip was inverted and mounted on a slide. The stained cells were observed using fluorescence microscopy.

Fluorescence Microscopy

Cells were observed using an axiovert fluorescence microscope (ZEISS) with mercury illumination interfaced to a Scion image processing system. A narrow bandpass discriminating filter set (Omega Optical) was used with excitation at 485/22 nm and emission at 530/30 nm for FITC and an excitation of 540/20 nm and emission at 590/30 nm for TRITC. Long-pass dichroic mirrors at 510 and 560 nm were used for FITC and TRITC, respectively. For RET imaging, a 485/22 nm narrow bandpass discriminating filter was used for excitation and a 590/30 nm filter was used for emission. The fluorescence images were collected with an intensified charge-coupled device camera (Geniisys; Dage-MTI). DIC photomicrographs were taken using ZEISS polarizers and a charge-coupled device camera (Model 72; Dage-MTI).

Single Cell Emission Spectrophotometry

Energy transfer was also examined by means of a microscope spectrophotometer apparatus. Fluorescence emission spectra were collected from single cells by a Peltier-cooled IMAX camera and intensifier (Princeton Instruments, Inc.) attached to a ZEISS Axiovert fluorescence microscope (Petty et al., 2000). Winspec software (Princeton Instruments, Inc.) was used to analyze spectrophotometric data. In some cases spectral subtractions were performed to highlight changes. Intensity levels were obtained by calculating differences between the intensity levels of the cell RET peaks and the background intensity levels. Intensity levels are given as the mean ± SE. *P* values were calculated using Microsoft Excel 2000 software.

RESULTS

Physical Proximity of Kv1.3 Potassium Channels and β1 Integrins on Adherent but not Nonadherent LOX Cells

To assess the physical proximity of Kv1.3 channels and β1-integrins on LOX melanoma cells, RET experi-

**Abbreviations used in this paper:* 4-AP, 4-aminopyridine; RET, resonance energy transfer; TEA, tetraethylammonium.

ments were conducted on cells labeled with donor- and acceptor-conjugated antibodies directed against Kv1.3 and the common chain of β1-integrins. Experiments were first performed using cells in suspension. Cells were detached from tissue culture plates, fixed with paraformaldehyde, washed extensively, and then labeled with fluorescent antibodies directed against the Kv1.3 channel and β1-integrin molecules. Immunofluorescence microscopy showed uniform distributions of β1-integrins and Kv1.3 channels on the LOX cell surface (Fig. 1, A–D). RET imaging experiments did not demonstrate energy transfer (Fig. 1 D). Moreover, single cell emission spectrophotometry did not reveal energy transfer between these two labels on LOX cells Fig. 1, E–H. Thus, these two molecules are expressed on LOX cells, but are not in the physical proximity of one another on nonadherent cells.

LOX cells were next studied while adherent to glass or fibronectin-coated coverslips. Fluorescence microscopy shows that both anti–β1-integrin and anti-Kv1.3 label LOX cells adherent to glass (Fig. 2, A–D). Labeling is also observed after adherence to fibronectin-coated coverslips (Fig. 2, E–H), which results in a greater number of morphologically polarized cells. It also results in nonuniform distributions of β1-integrin and Kv1.3 channel labeling, which resemble one another (Fig. 2, E–H). RET between FITC-labeled anti–β1-integrin and a TRITC-labeled second-step antibody attached to anti-Kv1.3 was demonstrated by emission spectrophotometry and immunofluorescence imaging. Fig. 2 illustrates the sensitization of acceptor fluorescence (TRITC) as a result of RET between these labeled membrane proteins. RET was observed during adherence to both glass and fibronectin-coated surfaces (Fig. 2, I and K, respectively). Difference spectra (Fig. 2, J and L) underscore the appearance of acceptor emission at ~585 nm (compare with Fig. 1 H). Since RET is only possible when two molecules are separated by ~7 nm or less (Szollosi et al., 1987), we suggest that β1-integrins and Kv1.3 channels are in close physical proximity on adherent LOX cells. The average RET intensity level was indistinguishable between LOX cells adherent to glass or fibronectin-coated coverslips (Table I). We were concerned that the TRITC-labeled second-step antibody might bind to the first step anti-CD29 reagent thereby promoting RET. This nonspecific effect is unlikely to be true since RET was not observed on nonadherent cells using the same protocol. Nonetheless, we rigorously eliminated this remote possibility using several controls. First, adherent cells were fixed then labeled with FITC-conjugated anti-CD29 and the TRITC-conjugated second-step goat anti–rabbit antibody. No rhodamine fluorescence or RET was observed on adherent cells, suggesting that cross-reaction between these reagents cannot explain the RET signal (unpublished data). Second, binding of anti-CD29 could not be inhibited by blocking the second-step

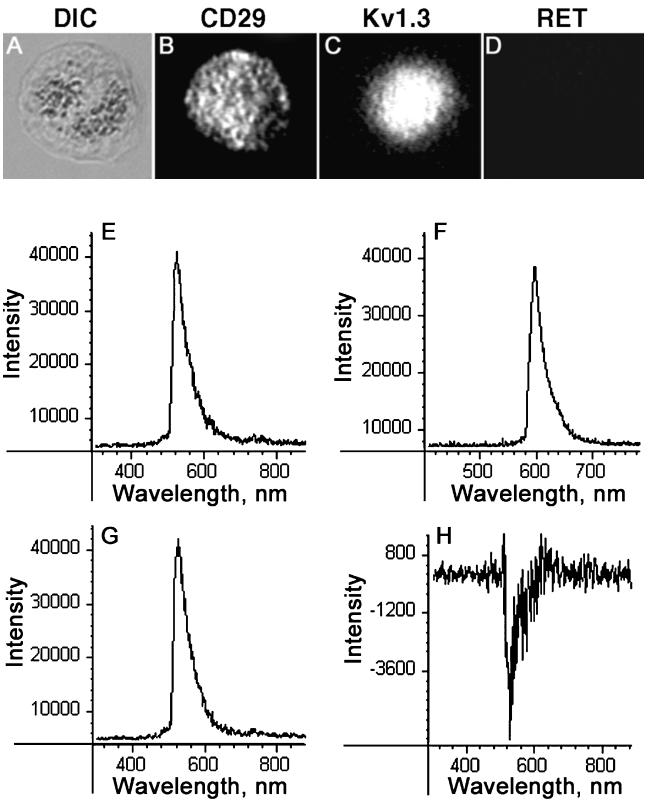


FIGURE 1. An absence of RET between β1 integrins (CD29) and Kv1.3 potassium channels on LOX cells in suspension as determined by RET imaging and microspectrophotometry. (A–D) Representative immunofluorescence microscopy experiments of nonadherent cells labeled with anti-CD29 (B) and anti-Kv1.3 (C) are shown. The corresponding DIC image is shown in A. Although cells are labeled with anti-CD29 and anti-Kv1.3 channel reagents, no RET is observed between these labels (D). (E–H) LOX cells in suspension were examined by fluorescence emission microspectrophotometry. The data shown here and elsewhere are plotted as intensity (photon counts) vs. wavelength (nm). LOX cells in suspension were labeled with FITC-conjugated anti-CD29 mAb only (E), first step rabbit anti-Kv1.3 Ab and second step goat anti–rabbit TRITC-conjugated mAb only (F), or both FITC-conjugated anti-CD29 mAb and rabbit anti-Kv1.3 Ab followed with TRITC-conjugated goat anti–rabbit Ab (G). The difference spectrum obtained by mathematical subtraction of anti–β1-integrin FITC (E) from RET spectrum (G) is shown in H. (See text for additional controls.) LOX cells in suspension revealed no RET between β1 integrins and Kv1.3 channels.

reagent by a nonspecific mouse IgG2a reagents. In the third type of experiment the anti-Kv1.3 reagent was directly conjugated to TRITC. When adherent LOX cells were directly labeled with FITC–anti-CD29 and TRITC–anti-Kv1.3 reagents, RET was observed. Thus, RET could not be explained by the second-step label. However, the total RET intensity is somewhat diminished using direct labeling due to fewer acceptors in the vicinity of the donor. Thus, cell adherence accompanies integrin-to-Kv1.3 channel proximity.

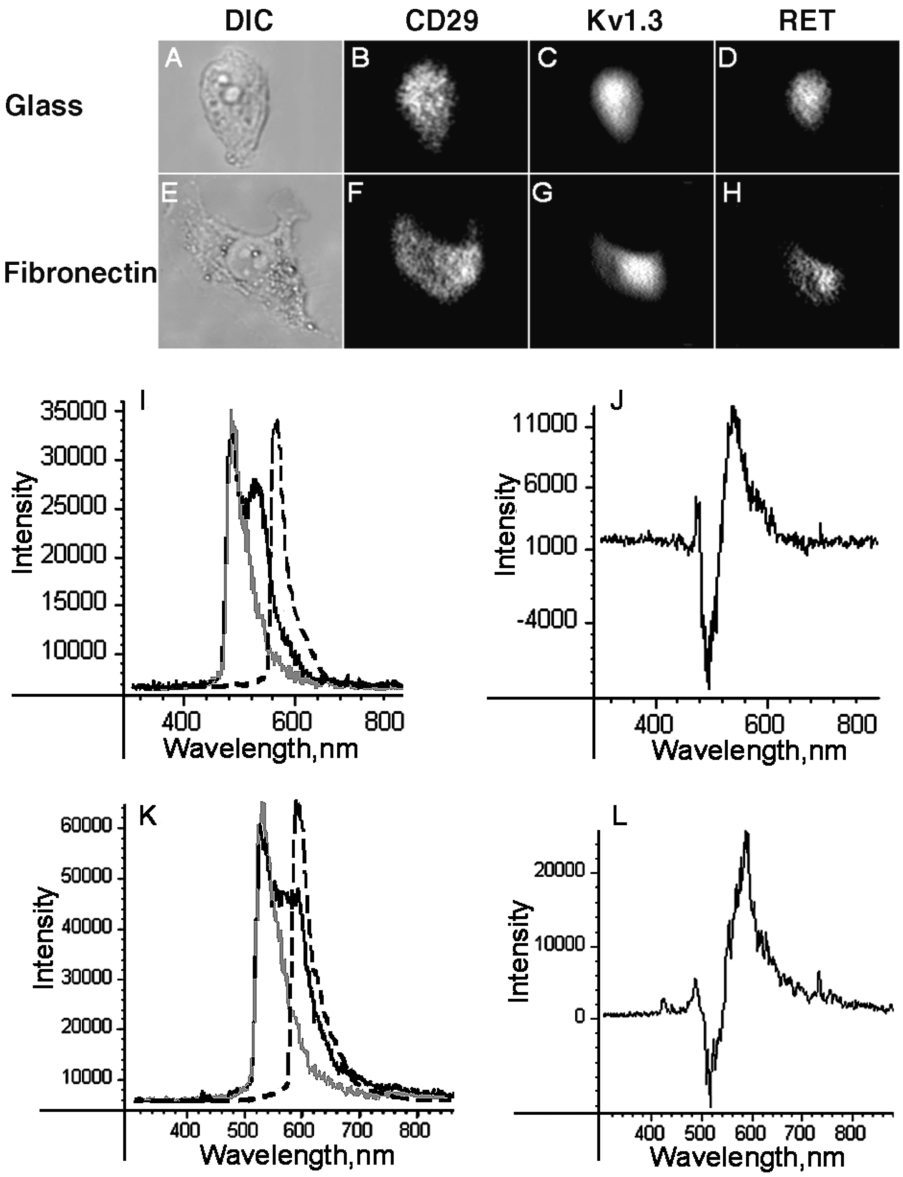


FIGURE 2. Resonance energy transfer between FITC-labeled β1-integrin and TRITC-labeled Kv1.3 potassium channel on LOX cells adherent to glass or fibronectin-coated coverslips. (A–H) Representative DIC (A and E) and immunofluorescence images of β1-integrin (B and F) and Kv1.3 channel (C and G) labeling on LOX cells adherent to the glass (A–D) and fibronectin-coated (E–H) coverslips. Note the morphological polarization and spreading of the cells in A and E. These cells displayed significant levels of RET (D and H). (I–L) Cells adherent to glass (I and J) or fibronectin (K and L) were examined by fluorescence spectroscopy. Cells were labeled with FITC-conjugated anti–β1-integrin Ab only (gray line in I and K), rabbit anti-Kv1.3 Ab/goat anti–rabbit TRITC-conjugated mAb only (dashed line in I and K), or both anti–β1-integrin and rabbit anti-Kv1.3 Ab/goat anti–rabbit TRITC-conjugated mAb (black line in I and K). Emission RET spectrophotometry detected RET between β1-integrin and Kv1.3 molecules on LOX cells adherent to glass and fibronectin-coated coverslips (appearance of second peak or shoulder in the FITC emission spectra; black lines in I and K). The difference spectra obtained by mathematical subtraction of anti–β1-integrin FITC spectrum (gray line in A and C) from RET spectrum (black line in I and K) represents the RET between integrin and channel. These spectra are shown in J and L for LOX cells adherent to glass or fibronectin-coated coverslips, respectively. (Compare with Fig. 1 H.)

Potassium Channel Blockers Inhibit RET between Kv1.3 Channels and β1 Integrins

We investigated the effect of K^+ channel blockers on RET between Kv1.3 channels and β1-integrins. The inhibitors TEA, which blocks voltage-gated K^+ channels and large conductance Ca^{2+}-dependent K^+ channels, and 4-AP, which blocks only voltage-gated K^+ channels, were tested. These "classical" K^+ channel blockers are known to inhibit Kv1.3 K^+ channel currents (Grissmer et al., 1994). Treatment of LOX cells with TEA or 4-AP blocked RET between β1-integrins and Kv1.3 channels as shown by both emission spectrophotometry and fluorescence imaging (Figs. 3 and 4). Emission spectrophotometry data show that treatment of LOX cells with 10^{-3} M TEA or 10^{-4} M 4-AP reduced the number of RET positive cells by 77% and 93%, respectively (Table II). Apamin, an irrelevant small conductance Ca^{2+}-dependent K^+ channel blocker, was used as a negative control. Adherence of cells to fibronectin-coated coverslips in the presence of 10^{-9} M apamin had no effect on RET between β1-integrins and Kv1.3 channels (Figs. 3 and 4). Thus, certain K^+ channel blockers inhibit β1-integrin-to-Kv1.3 channel proximity.

We next tested the effect of verapamil on RET between Kv1.3 channels and β1-integrins. Although a "classical" Ca^{2+} channel antagonist, verapamil has recently been shown to block Kv1.3 channels (Pancrazio et al., 1991; Rauer and Grissmer, 1996, 1999; Madeja et al., 2000; Robe and Grissmer, 2000). It has been shown that at low concentrations verapamil blocks Ca^{2+} channels, whereas higher concentrations block K^+ channels (Pancrazio et al., 1991; Rauer and Grissmer, 1996). LOX cells were incubated on fibronectin-coated cover-

TABLE I

Quantitative Summary of RET Intensity Levels between β1-integrin and Kv1.3 Potassium Channel

Substrate	Treatment	N	CN	RET	*P*
				arbitrary units	
Cell suspension		4	90	5,142 ± 412[a]	> 0.01[c]
Glass		3	107	18,177 ± 107	
Fibronectin		3	150	18,168 ± 603	> 0.001[d]
Fibronectin	10^{-9} M apamin	3	85	16,779 ± 712	> 0.01[d]
Fibronectin	10^{-3} M TEA	3	80	5,416 ± 395[a]	> 0.01[c]
Fibronectin	10^{-4} M 4-AP	3	92	5,538 ± 162[a]	> 0.01[c]
Fibronectin	10^{-7} M verapamil	3	88	11,381 ± 805	< 0.01[d]
Cell suspension	10^{-4} M verapamil	3	100	4,969 ± 124[a]	> 0.01[c]
Cell suspension control		4	80	5,267 ± 513[b]	
TEA control		3	80	5,110 ± 441[b]	
4-AP control		3	90	5,588 ± 538[b]	
M verapamil control		3	93	5,178 ± 541[b]	
Background control		3	60	5,062 ± 67[e]	
Baseline		3	60	5,015 ± 18[f]	

N, the number of independent trials; CN, the number of cells measured; RET, intensity level.

[a]The RET intensity level was obtained as a mathematical difference between intensity level at 590 nm of RET spectrum and intensity level at 590 nm of RET spectrum from cells labeled with FITC-conjugated mAb only.

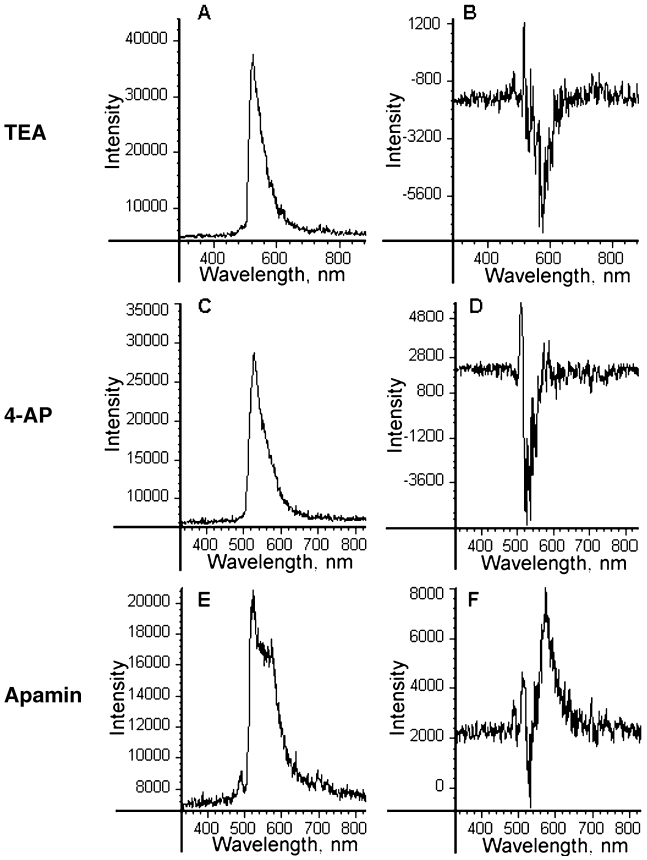
[b]The intensity level at 590 nm of RET spectrum from cells labeled with FITC-conjugated mAb only.

[c]The RET level for the given treatment was compared with the RET level for that treatment control.

[d]The RET level for the given substrate was compared with the RET level for the glass.

[e]The instrumentation base line in the presence of the slide with unlabeled cells.

[f]The instrumentation base line.



slips in the presence of various concentrations of verapamil. Verapamil at 100 μM inhibited spreading and polarization of LOX cells on fibronectin-coated coverslips. Less dramatic changes were observed at lower doses. LOX cells adherent to fibronectin-coated coverslips were incubated with 0.1, 1, and 10 μM verapamil for 30 min, then fixed and labeled with the Kv1.3 channel and β1-integrin reagents. Verapamil decreased the number of RET-positive cells in a dose-dependent fashion (Table II; Figs. 5 and 6). Representative RET spectra (Fig. 6) demonstrate RET between β1-integrins and Kv1.3 channels on cells treated with 0.1 μM verapamil, but not between these molecules at 100 μM verapamil. Thus, the verapamil studies are consistent with an effect of this compound on K^+ channels.

FIGURE 3. Inhibition of RET between β1-integrins and Kv1.3 channels by K^+ channel blockers. The effect of K^+ channel blockers on RET between β1-integrins and Kv1.3 channels was investigated using emission spectrophotometry. LOX cells were allowed to adhere to the fibronectin-coated coverslips at the presence of 10^{-3} M TEA (A and B), 10^{-4} M 4-AP (C and D), or 10^{-9} M apamin (E and F). The cells were fixed then labeled with anti–β1-integrin and anti-Kv1.3 channel antibodies as described above. Representative emission (A, C, and E) and difference (B, D, and F) spectra are shown. RET is absent in cells treated with TEA and 4-AP (A–D). However, RET was observed in the presence of apamin, a K^+ channel blocker that has no effect on Kv1.3 channels.

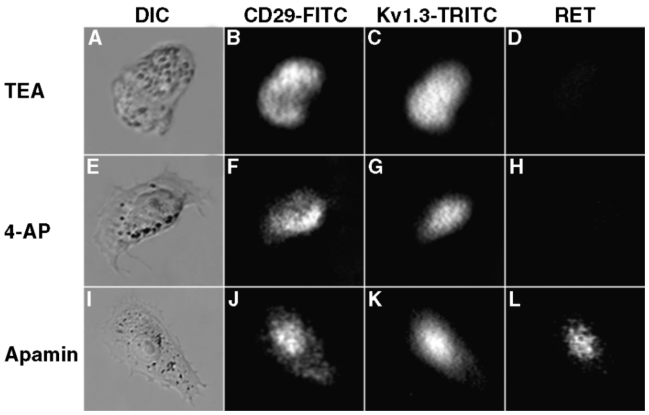


FIGURE 4. Inhibitory effect of potassium channel blockers on RET between β1-integrins and Kv1.3 channels. The effect of K^+ channel blockers on RET between β1-integrins and Kv1.3 channels was investigated using fluorescence microscopy. LOX cells adherent to the fibronectin-coated coverslips in the presence of 10^{-3} M TEA (A–D), 10^{-4} M 4-AP (E–H), or 10^{-9} M apamin (I–L) were labeled with anti–β1-integrin and anti-Kv1.3 channel reagents. Columns 1–4 show (a) DIC, (b) FITC fluorescence of anti-β1-integrin, (c) TRITC fluorescence of anti-Kv1.3 channel, and (d) RET between these two reagents. Note that although all of the cells were labeled with both reagents, RET was only observed in the presence of apamin.

TABLE II

Inhibitory Effect of Potassium Channel Blockers on the RET between β1-integrin and Kv1.3 Potassium Channel on LOX Cell Membranes

Substrate	Inhibitor	N	CN	% RET	*P*
Glass	No inhibitor	3	107	88.3 ± 5.2	
	No inhibitor	3	150	84.0 ± 9.9	> 0.001[a]
	10^{-3} M TEA	3	100	19.0 ± 3.8	< 0.001[b]
	10^{-4} M 4-AP	3	75	6.0 ± 2.0	< 0.001[b]
Fibronectin	10^{-9} M apamin	3	100	80.0 ± 3.5	> 0.001[b]
	10^{-7} M verapamil	3	150	52.0 ± 9.5	< 0.001[b]
	10^{-6} M verapamil	3	150	28.0 ± 8.1	< 0.001[b]
	10^{-5} M verapamil	3	150	13.3 ± 7.1	< 0.001[b]
Cell suspension	10^{-4} M verapamil	3	150	14.0 ± 5.0	< 0.001[b]

N, the number of independent trials; CN, the number of cells measured; % RET, percentage of cells with RET between integrin and Kv1.3 potassium channel.

[a]This value was compared with the % RET value for LOX cells adherent to the glass at the absence of inhibitors.

[b]This value was compared with the % RET value for LOX cells adherent to the fibronectin in the absence of inhibitors.

DISCUSSION

Our resonance energy transfer studies demonstrate molecular proximity between β1 integrins and Kv1.3 channels at tumor cell surfaces, thus suggesting that a direct line of communication exists between these two key cell surface regulatory systems. Recent studies have indicated that integrins are capable of forming supramolecular complexes with other cell surface molecules (Petty et al., 2002). For example, β1 integrins interact with the urokinase-type plasminogen activator receptors (Chapman, 1997; Xue et al., 1997), which may contribute to the coordination of adhesive/proteolytic events during in vivo cell motility. Moreover, additional molecules may also interact with integrins in cell membranes. Recently, Levite et al. (2000) have suggested that integrins interact with Kv1.3 channels on lymphocyte membranes. Our studies agree with those of Levite et al. (2000), although we have used a different technique and cell type. Furthermore, our studies of living cells indicate that the β1 integrin–Kv1.3 channel interaction is not static, but rather can be quite dynamic, depending upon the experimental circumstances. β1 integrin–Kv1.3 channel proximity is affected by cell adherence and the presence of K^+ channel blockers. Thus, a direct physical interaction between β1 integrins and Kv1.3 channels may contribute to cell signaling and functions.

Previous studies have demonstrated that K^+ channel blockers can inhibit integrin-mediated cell adhesion to the extracellular matrix, proliferation, migration, and metastasis (Lewis and Cahalan, 1995; Lepple-Wienhues et al., 1996; Xu et al., 1996; Schwab et al., 1999; Yao and Kwan, 1999). Moreover, the availability of functionally active Kv1.3 channels is a prerequisite for β1-integrin–mediated activation of T cells (Levite et al., 2000). In the present studies, we found that treatment of LOX cells with TEA or 4-AP dramatically reduced the number of RET-positive cells and led to an almost uniform distribution of Kv1.3. However, melanoma cell treatment with apamin, an irrelevant K^+ channel blocker, had no effect on the RET between Kv1.3 channels and β1-integrins. Thus, it would appear that a functional K^+ channel is required for both the molecular proximity of β1 integrins and Kv1.3 channels and certain integrin functions.

We investigated the effect of verapamil on RET between β1-integrins and Kv1.3 channels. Verapamil, a well-known blocker of L-type voltage-gated Ca^{2+} channels, has been shown to affect K^+ channels as well. Depressive effects on K^+ conductance have been described in lymphocytes (DeCoursey et al., 1985), alveolar epithelial cells (Jacobs and DeCoursey, 1990), small cell lung cancer cells (Pancrazio et al., 1991), kidney cells (Bleich et al., 1990), enterocytes (Tatsuta et al., 1994), rat prostatic cancer cells (Fraser et al., 2000), and human prostate cancer cells (Rybalchenko et al., 2001). It was shown that verapamil blocks voltage-gated Ca^{2+} channels at nanomolar concentrations and can block *Shaker* voltage-gated K^+ channels at micromolar concentrations (Chandy et al., 1993). When we investigated the effect of several verapamil concentrations, we found that RET was inhibited at higher doses of verapamil (~100 μM), thus suggesting an important role for K^+ channels. The formation of β1-integrin–Kv1.3 channel supramolecular proximity complexes may be

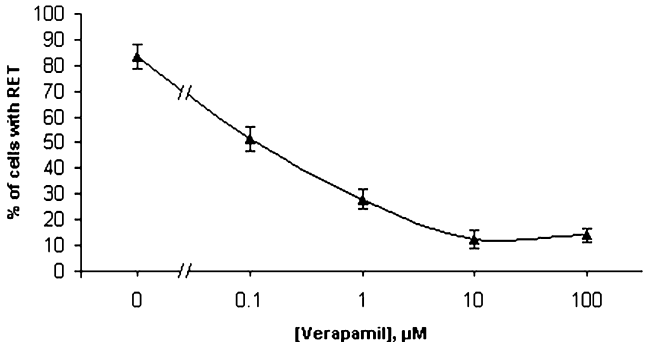


FIGURE 5. Dose-dependent inhibitory effect of verapamil on RET between β1-integrins and Kv1.3 channels. LOX cells were allowed to adhere to fibronectin-coated coverslips at the absence and presence of 0.1 μM to 10 μM verapamil for 2 h. Since cells exposed to 100 μM verapamil could not adhere, experiments at this dose were performed in suspension. The cells were fixed, labeled with anti-β1-integrin and anti-Kv1.3 channel reagents as described, then examined with emission spectrophotometry. The number of cells exhibiting RET were counted and are shown here as a percentage of the total number of cells.

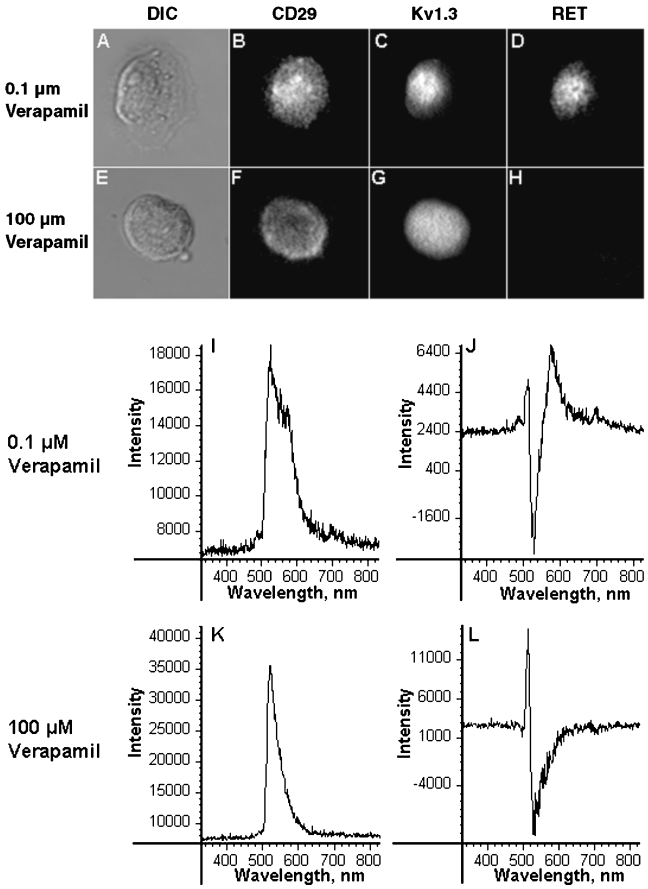


FIGURE 6. Effect of verapamil treatment on RET between β1-integrins and Kv1.3 channels. (A–H) Representative DIC (A and E) and immunofluorescence images of β1-integrin (B and F) and Kv1.3 channel (C and G) labeling on LOX cells on fibronectin-coated coverslips in the presence of 0.1 μM (A–D) and 100 μM (E–H) verapamil. These cells displayed significant levels of RET in the presence of 0.1 μM verapamil (D), but not in the presence of 100 μM verapamil (H). (I–L) Spectrophotometry experiments were conducted in the presence of 0.1 μM verapamil (I and J) or 100 μM verapamil (K, L). Cells were labeled as described above. Emission RET spectrophotometry detected RET between β1-integrins and Kv1.3 channels on LOX cells in the presence of 0.1 μM verapamil, but not in the presence of 100 μM verapamil. The difference spectra obtained by mathematical subtraction of anti-β1-integrin FITC spectrum (unpublished data) from the RET spectrum (I) represents the RET between integrin and channel. These spectra are shown in J and L. These data indicate that RET can be observed at doses consistent with binding to calcium channels, but not at doses more consistent with binding to K^+ channels.

particularly important in view of the reduced in vitro invasiveness and the small, but significant reduction in in vivo metastasis found using verapamil (Yohem et al., 1991; Taylor et al., 1997; Farias et al., 1998; Iishi et al., 2001). Although verapamil per se is not likely to be broadly used in managing disease, the identification of a molecular pathway contributing to its action, such as the control of β1-integrin-to-Kv1.3 channel interactions, may lead to more effective therapeutic approaches. One practical example of this would be screening for new drugs based on the inhibition of these interactions.

Several potential mechanisms of β1-integrin-to-Kv1.3 channel communication can be envisioned. First, a cis-partnership might lead to reciprocal changes in integrin and channel conformation, thus affecting their functions. Both integrins and Kv channels are known to undergo conformational changes (e.g., Yellen et al., 1994; Ma et al., 2002). Furthermore, channel blockers might result in channel conformational changes that are not favorable for channel–integrin interactions thereby leading to an inhibition of RET. Second, it has been shown that upon activation and gating the Kv1.3 channel becomes phosphorylated by a tyrosine kinase (Holmes et al., 1996). Thus, as a result of interacting with integrins, the channel may provide integrins with access to both its channel activity and additional signaling molecules. In other words, the channel (or integrin) could be viewed as a scaffolding protein for the integrin (or channel), thereby affecting its transmembrane signaling capacity. For example, kinases associated with integrins may mediate the phosphorylation of Kv1.3. Thus, integrin–channel communication in these supramolecular proximity complexes could take on several forms. However, we have not shown that integrins directly bind to K^+ channels. Similarly, immunoprecipitation demonstrates that integrins and Kv1.3 channels coimmunoprecipiate (Levite et al., 2000), but this does not prove that the molecules directly interact as some other component of the precipitate could link the two together. The positive energy transfer suggests that these membrane proteins are nearest neighbors; thus,

a linker protein is unlikely to come between the integrin and channel. One potential route to sorting out these possibilities and to identify relevant binding regions within these proteins is the use of chimeric integrins and channels coupled with evaluation of molecular proximity.

Integrins and K^+ channels collaborate to perform adherence-related cell functions. This reciprocal partnership is illustrated by the ability of integrin ligation to promote K^+ currents and the ability of K^+ channel blockers to inhibit integrin-mediated adherence. Although the functions of integrins are clear enough because they directly attach to basement membranes, other cells, and glass, what adherence-related functions might K^+ channels serve? Several possibilities might be considered. Voltage-gated channels respond to changes in the membrane potential (i.e., an influx of Na^+ or Ca^{2+}). Oscillating membrane potentials and Ca^{2+} levels have been found in migrating cells (e.g., Petty, 2001). Thus, K^+ channels might serve the crucial role of resetting the local signaling apparatus. This is likely important since cell migration requires that integrins undergo numerous cycles of attachment and release. Integrin-to-ligand binding causes K^+ channel activation (Arcangeli et al., 1989, 1991, 1996; Becchetti et al., 1992), which may enhance Ca^{2+} influx. For example, inhibition of K^+ channels by TEA, 4-AP, and verapamil block Ca^{2+} influx (Lepple-Wienhues et al., 1996; Yao and Kwan, 1999). Thus, repetitive changes in membrane potential, integrin avidity (adherence vs. detachment), and Ca^{2+} signals (which participate in resculpting the cytoskeleton) participate in the choreography of cell adherence. Emerging technologies, such as high-speed imaging (see below), should allow these events at adherence sites to be precisely mapped. Finally, activation of K^+ channels will, in general, cause a loss of K^+ and Cl^-, thereby reducing cell water and volume. Such a reduction in volume might be crucial in in vivo cell migration such as the migration of tumor cells across endothelial cell barriers (e.g., Soroceanu et al., 1999).

A quantitative understanding of the structure and function of membrane complexes of tumor cells is likely to dramatically increase our understanding of tumor cell motility, an integral feature of metastasis. Although it has been demonstrated that integrins interact with both urokinase-type plasminogen activator receptors and Kv1.3, whether or not all three molecules are simultaneously present in the same assembly is not known. One possible approach to address this question is the use of two-step RET, wherein energy is transferred among three molecules from donor to acceptor/donor to acceptor. Moreover, recent developments in high-speed imaging (Petty and Kindzelskii, 2000, 2001; Petty et al., 2000) make it possible to spatiotemporally map signals emanating from adherence sites. For example, it is possible to image the ignition and propagation of metabolic waves at adhesion sites (Petty and Kindzelskii, 2000). The direct observation of protein–protein complexes and their signals will greatly enhance our ability to link molecular interactions with cell behavior.

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