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HERG K^+ Channels Activation during β_1 Integrin-mediated Adhesion to Fibronectin Induces an Up-regulation of $\alpha_v\beta_3$ Integrin in the Preosteoclastic Leukemia Cell Line FLG 29.1*

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Integrin receptors have been demonstrated to mediate either "inside-to-out" and "outside-to-in" signals, and by this way are capable of regulating many cellular functions, such as cell growth and differentiation, cell migration, and activation. Among the various integrin-centered signaling pathways discovered so far, we demonstrated that the modulation of the electrical potential of the plasma membrane (V_{REST}) is an early integrin-mediated signal, which is related to neurite emission in neuroblastoma cells. This modulation is sustained by the activation of HERG K^+ channels, encoded by the *ether-à-go-go-related* gene (*herg*). The involvement of integrin-mediated signaling is being discovered in the hemopoietic system: in particular, osteoclasts are generated as well as induced to differentiate by interaction of osteoclast progenitors with the stromal cells, through the involvement of integrin receptors. We studied the effects of cell interaction with the extracellular matrix protein fibronectin (FN) in a human leukemic preosteoclastic cell line (FLG 29.1 cells), which has been demonstrated to express HERG currents. We report here that FLG 29.1 cells indeed adhere to purified FN through integrin receptors, and that this adhesion induces an osteoclast phenotype in these cells, as evidenced by the appearance of tartrate-resistant acid phosphatase, as well as by the increased expression of CD51/$\alpha_v\beta_3$ integrin and calcitonin receptor. An early activation of HERG current (I_{HERG}), without any increase in *herg* RNA or modifications of HERG protein was also observed in FN-adhering cells. This activation is apparently sustained by the β_1 integrin subunit activation, through the involvement of a pertussis-toxin sensitive G_i protein, and appears to be a determinant signal for the up-regulation of $\alpha_v\beta_3$ integrin, as well as for the increased expression of calcitonin receptor.

The integrin family of adhesion receptors functions not only as ligands for the extracellular matrix (ECM),[1] but can influence many aspects of cell behavior, including morphology, adhesion, migration, as well as cellular proliferation and differentiation. In fulfilling these functions, integrins are not simply adhesion receptors, but can affect many signaling pathways, and therefore impinge upon complex cellular activities. Therefore integrin function itself is highly regulated, largely through the formation of specific associations with both structural and regulatory components within the cell. Recently, much research has focused on elucidating the molecular mechanisms, which control integrin function and transduce integrin-mediated signaling events.

In particular, in the hemopoietic system, it is well known that adhesive interactions between hemopoietic precursors and the bone marrow microenvironment play a critical role in regulating hemopoiesis (1, 2), and that such interactions are often mediated by integrin receptors (3, 4). Among hemopoietic precursors, the progenitors of osteoclasts are formed through a contact-dependent interaction between bipotential osteoclast-macrophage precursors and stromal cells, which express osteoclast forming activity (5, 6). Furthermore, osteoclast differentiation and activation leading to bone remodeling is finely regulated by interaction of osteoclasts with stromal cells (reviewed in Ref. 7) through the involvement of integrin receptors, which are numerous on the osteoclast plasma membrane (8). Therefore, osteoclast precursors can be envisaged as a good model wherein studying the relationships between the ECM, integrin-mediated signals, and the induction of cell differentiation/activation.

Among the various integrin-centered signaling pathways discovered so far (9, 10), we demonstrated that the modulation of V_{REST} is an early integrin-mediated signal, which is related to neurite emission in neuroblastoma cells (11–13). This modulation is sustained by the activation of a peculiar type of K^+ channels, the HERG channels (14, 15), encoded by the *ether-à-*

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[1] The abbreviations used are: ECM, extracellular matrix; *herg,* human *eag*-related gene; HERG, *herg*-encoded protein; I_{HERG}, HERG current; FN, fibronectin; VN, vitronectin; V_{REST}, membrane resting potential; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRAP, tartrate-resistant acid phosphatase; RT-PCR, reverse transcriptase polymerase chain reaction; CtR, calcitonin receptor; mAb, monoclonal antibody; bp, base pair(s); PKC, protein kinase C; PTX, pertussis toxin; gapdh, glyceraldehyde-3-phosphate dehydrogenase.

go-go-related gene (*herg*). These are channels with limited hyperpolarizing potency, clamping V_{REST} to substantially depolarized values (around −30 mV), and are expressed in the heart (16–18), embryonic neuroblasts (19), as well as in tumor cells of various histogenesis (12). We found that a human leukemia cell line, FLG 29.1 cell, expressed *herg*, and an HERG current (I_{HERG}) with a very fast deactivation kinetics, which apparently justifies the low, depolarized value of their V_{REST} (12, 20). These cells derive from a patient with an M5a-type leukemia (21) and represent immature preosteoclastic precursors, as revealed by their treatment with phorbol esters (22). They are capable of adhering to bone endothelium, possibly through fibronectin (FN) molecules produced by the endothelial cells (23), and this interaction could influence the maturation of these cells through the osteoclastic pathway. We therefore used the preosteoclastic FLG 29.1 cells to study HERG K^+ channel-centered signals, elicited by integrin-mediated adhesion to the ECM molecule FN, as a model for hemopoietic precursors, and their relationships with the process of osteoclastic differentiation.

We report here that FLG 29.1 cells indeed adhere to purified FN through integrin receptors, and that this adhesion is accompanied by the appearance of osteoclast differentiation markers, namely tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CtR), as well as CD51/$\alpha_v\beta_3$ integrin. FN also induces the activation of I_{HERG}, without any increase in *herg* RNA or modifications of HERG protein. This activation is apparently sustained by the β_1 integrin subunit activation, through the involvement of a pertussis toxin-sensitive G_i protein, and appears to be a determinant signal for the up-regulation of $\alpha_v\beta_3$ integrin, as well as for the increased expression of CtR.

EXPERIMENTAL PROCEDURES

Cell Culture—FLG 29.1 were obtained in Dr. P. A. Bernabei's laboratory (Hematological Unit, Florence, Italy) as previously reported (22). Cells were routinely cultured in RPMI medium (Hyclone) supplemented with 10% fetal calf serum (Hyclone) (complete medium) and incubated at 37 °C in 10% CO_2. For experiments, cells were harvested from freshly seeded preparatory cultures and resuspended (2.5×10^5 cells/ml) in RPMI containing 250 μg/ml heat-inactivated bovine serum albumin (BSA) (RPMI + BSA) (for cell adhesion experiments) or in complete medium (for differentiation experiments). Cells were then plated onto either 35-mm Petri dishes (Costar) (for patch clamp experiments) or dishes in which glass slides had been accommodated (for immunocytochemistry experiments) or 10-cm Petri dishes (for RNA or protein extraction).

Preparation of Substrates and Coating of Culture Dishes—Heat-inactivated BSA was prepared as described previously (11). Coating of culture dishes with FN or vitronectin (VN) was performed by adding the two proteins, prepared as reported in Ref. 11, at 100 μg/ml in RPMI at 37 °C for 1 h. Thereafter, FN or VN solution was poured off and dishes were further incubated for 30 min with RPMI + BSA to saturate the free binding sites of the culture dish surface. Dishes were then immediately used for experiments or stored with PBS at 4 °C and used afterward (1 or 2 days).

Experiments with substrate-bound as well as soluble anti-integrin antibodies were performed by using the following antibodies: (*a*) anti-β_1 monoclonal antibodies: mAb BV7 (24) and mAb TS2/16; (*b*) anti-β_3 monoclonal antibody (mAb B212) (25); (*c*) anti-α_3 monoclonal antibody (F2) (26), and following the procedure essentially according to Arcangeli *et al.* (13). Briefly, the antibodies were diluted at 20 μg/ml in RPMI, and added to Petri dishes at 37 °C for 1 h. After coating the plastic surface, dishes were further incubated for 30 min with RPMI + BSA, as above, and thereafter immediately used for patch clamp experiments (see below). For experiments with soluble anti-β_1 antibody (mAb TS2/16), the antibody was diluted at 20 μg/ml in RPMI medium + BSA, and cells were incubated in this solution at 2.5×10^5/ml for 10 min at room temperature, then transferred to BSA-coated Petri dishes, and incubated for further 15 min at 37 °C.

Adhesion Assay—Adhesion assay was performed essentially according to Arcangeli *et al.* (11). Briefly, stock cultures were radiolabeled during 36 h of exponential growth in RPMI medium containing 1 μCi/ml [*methyl*-^{3}H]thymidine (specific activity 24 Ci/mmol). After this time, cells were harvested, pelleted, and resuspended in RPMI + BSA. Aliquots of cells (14×10^3) were inoculated into each well of 96-well clusters (Corning-Costar), previously coated with the adhesive proteins (see above). In the appropriate samples, blocking antibodies to FN-receptor (αFN-R; 1:50 dilution) (11), β_3 subunit (mAb B212, 9 μg/ml) (25), or α_v subunit (mAb L230) (10 μg/ml) were added at time 0. When the mAb B212 was used, cells were preincubated with the antibody for 20 min at 4 °C, according to Defilippi *et al.* (25). When needed, GRGDSP (Telios) or GRGESP (Telios) were added just before cell seeding at 0.5 μg/ml (final concentration). After 60 min of incubation, the medium was aspirated off and adherent cells gently rinsed twice with PBS containing divalent cations; the cells were then solubilized with 50 μl of 1% SDS in 0.1 N NaOH for 1 h. Radioactive solubilized cells were quantified by scintillation counting and compared on a percent basis with the radioactivities of the FN-seeded cells.

Induction of Cell Differentiation—For induction of cell differentiation, TPA (Sigma) was dissolved in dimethyl sulfoxide at 10^{-3} M and added to complete medium at a final concentration of 10^{-6} M.

Pertussis Toxin Treatment—Pertussis toxin (Calbiochem) dissolved in water was added to cell cultures at a final concentration of 100 ng/ml, and cells were incubated in its presence for 14–20 h. After this time, cells were harvested and resuspended in RPMI + BSA in the absence of the toxin, and seeded on BSA-, FN-, or anti-β_1 antibody (mAb TS2/16)-coated dishes for 1 h.

Patch Clamp Recordings—Cells plated on dishes were incubated at 37 °C for various times. Patch clamp experiments were performed at room temperature with an amplifier Axopatch 1-D (Axon Instruments, Foster City, CA), replacing the Petri dishes every 30 min. The whole cell configuration of the patch clamp technique (27) was employed using pipettes (borosilicate glass; Hilgenberg, Germany) whose resistance was in the range 3–5 MΩ. Extracellular solutions were delivered through a 9-hole (0.6 mm), remote-controlled linear positioner placed near the cell under study. The standard extracellular solution contained (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, Hepes-NaOH 10, glucose 5, pH 7.4. The standard pipette solution at $[Ca^{2+}] = 10^{-7}$ M contained (mM): K^+ aspartate 130, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 4, EGTA-KOH 10, Hepes KOH 10, pH 7.4. Gigaseal resistance were in the range 1–10 GΩ. Whole cell currents were filtered at 2 KHz. For precise measurement of the gating parameters of the inward rectifier channels, we carefully compensated pipette and cell capacitance and the series resistance before each voltage-clamp protocol run. The density of inward I_{HERG} was calculated at $[K^+]_o = 40$ mM as the peak current elicited at a voltage of −120 mV after 20 s preconditioning at 0 mV, normalized by the cell capacitance. The values of the peak current refer to the current obtained after subtraction of the traces obtained in the presence of 5 mM Cs^+ or 1 μM WAY 123,398, according to Ref. 19. The I_{HERG} activation curves were measured according to Refs. 14 and 15. Resting potential (V_{REST}) was measured at 5 mM $[K^+]_o$ in current-clamp mode (I = 0). The leakage conductance g_L was calculated at 5 mM $[K^+]_o$ by a ramp protocol ranging from −100 to +60 mV and lasting 1280 ms, from a holding potential of 0 mV, as the slope of the current trace obtained in the range −80 to −40 mV. The relatively slow rate of voltage change produced a negligible capacitive current. The cell capacitance was obtained directly by reading the position of the amplifier knob of the cell capacitance compensation. Input resistance of the cells was in the range 2–6 GΩ. For data acquisition and analysis, pClamp software (Axon Instruments) and Origin (Microcal Software, Northampton, MA) were routinely used.

RNA Extraction and Northern Blot—Total RNA was extracted from FLG 29.1 cells by the guanidinium/isothyocyanate method (28). Ten or 20 μg of total RNA were loaded on a formaldehyde-formamide reducing agarose gel run at 80 mA for 3–4 h. Six micrograms of an RNA ladder (Life Technologies, Inc.) were also loaded. After staining with ethidium bromide, the gel was photographed and the position of the standards marked. RNA was then transferred by Northern blot onto a nylon membrane (Hybond N^+; Amersham Pharmacia Biotech) and hybridized in Church Buffer (Na phosphate monobasic, 0.5 M, pH 7.2, 7% SDS, 1 mM EDTA) containing 10^8 cpm/mg DNA of the appropriate probe (see below), at 65 °C overnight. Filter were then washed twice in Na phosphate 50 mM, SDS 1% for 5 min at room temperature, and once at 65 °C for 30 min, and exposed to x-ray film (Hyperfilm; Amersham Pharmacia Biotech) overnight at −70 °C for HERG probe, 5 min at −70 °C for 18S probe.

Probes and Plasmids—A *Bam*HI-*Hin*dIII full-length (3.5 kilobase) fragment of the HERG gene cloned in SP64 vector (29) kindly gifted by Dr. M. Keating (University of Utah, Salt Lake City, UT) was random priming labeled using [^{32}P]dCTP (Amersham Pharmacia Biotech), and the probe purified on Sephadex columns as described (28). An 18S probe

(Ambion) was labeled as above.

Detection of Calcitonin Receptor by Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted as reported above; cDNA was then synthesized from 1 μg of RNA using 200 units of reverse transcriptase SuperScript II (Life Technologies), plus 200 μM of each dNTP and 2.5 μM random hexamers, in a 20-μl final reaction volume, for 50 min at 42 °C. 5 μl of such reaction were used to perform the specific amplification of the CtR, using 2.5 units of Platinum *Taq* polymerase (Life Technologies), 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 μM of the specific primers (see below). Amplification was performed in a Robocycler (Stratagene), after an activation step at 94 °C for 2 min, for 30 cycles with 30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C. The primers used were: 5′ sense oligonucleotide, GTATTGTCCTATCAGTTCTGCC; and 3′ antisense oligonucleotide, AGAGATAATACCACCGCAAGCG. These primers encompass a nucleotide region from 265 to 842 of the human CtR cDNA, and give rise to two bands, one 577 bp long, corresponding to the human CtR1 and one 529 bp long, corresponding to the human CtR2 (30). Preliminary experiments (not reported) demonstrated that under the conditions used in these experiments the amplification reaction was in the exponential phase, as reported by Beaudreuil *et al.* (30), thus allowing a semiquantitative analysis of the PCR products (see below). Moreover, the two bands obtained from such experiments were purified and sent off for sequencing, resulting in being 100% identical to the CtR sequences present in GeneBank™. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as internal control, from 2 μl of the same cDNA, under the above conditions, and using the following primers, which comprise a sequence between nucleotide 457 and 595 of the *gapdh* gene: 5′ primer sense, AACAGCCTCAAGATCATCAGCAA; 3′ primer antisense, CAGTCTGGGTGGCAGTGAT. Samples of the PCR products (35 μl) were separated by gel electrophoresis on 3% agarose, and the bands, acquired by an HP 4C scanner, were analyzed by Scion Image software. Signals of the CtR cDNA were normalized using the values of the corresponding products from the *gapdh* amplifications.

Immunoprecipitation of Integrins from Cell Surface Biotinylated Cells—Cells prepared as above were placed on ice and washed twice with Hank's balanced salts (1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5 mM KCl, 138 mM NaCl, 5.6 mM D-glucose, 25 mM HEPES, pH 7.4) and biotinylated by adding 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in Hank's buffer for 15 min. Cells were washed twice with Hank's solution, labeling was repeated and the reaction was stopped by washing three times with serum-free Dulbecco's modified Eagle's medium + 0.6% BSA. Labeled cells were lysed in ice for 20 min with lysis buffer (Tris-HCl 20 mM, pH 7.4, NaCl 150 mM, glycerol 10%, Triton X-100 1%, phenylmethanesulfonyl fluoride 1 mM, aprotinin 0.15 units/ml, leupeptin 10 μg/ml, NaF 100 mM, Na vanadate 2 mM). The supernatant was then cleared by centrifugation at 16,000 × *g*.

For immunoprecipitation of integrin subunits, 0.5–1 mg of total cell lysate was incubated overnight at 4 °C with polyclonal antibodies raised against the cytoplasmic tails of α_v or β_1 integrins (a gift from Prof. G. Tarone, University of Torino, Italy). Immunocomplexes were then bound to protein A-Sepharose beads and recovered by centrifugation. Bound material was eluted by boiling beads in nonreducing Laemmli buffer (Tris-HCl 62.5 mM, pH 6.8, glycerol 10%, SDS 0.2%, bromphenol blue 0.00125%), analyzed by 6% SDS-PAGE under nonreducing conditions, and transferred to a nitrocellulose sheet. The membrane was incubated 30 min at 42 °C in T-TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.3% Tween), containing 5% BSA, washed with T-TBS, and then biotinylated integrins were detected by incubating blots in TBS, 1% BSA, containing 0.2 μg/ml horseradish peroxidase-conjugated streptavidin (Pierce) for 30 min, washed again with T-TBS, and developed with a chemiluminescent substrate (ECL, Amersham Pharmacia Biotech). Images were then acquired by means of an HP4C scanner, and densitometric analysis performed using the QuantiScan software.

Immunoblot—Immunoblot with anti-PKC_α antibodies was performed using a total cell protein extract. Cells, plated on 60-mm Petri dishes covered with BSA or FN, respectively, were collected by centrifugation, washed three times with ice-cold PBS and lysed on ice with lysis buffer (Tris-HCl 20 mM, pH 7.4, NaCl 150 mM, glycerol 10%, Triton X-100 1%, phenylmethanesulfonyl fluoride 1 mM, aprotinin 0.15 units/ml, leupeptin 10 μg/ml, NaF 100 nM, Na vanadate 2 mM). The supernatant was then cleared by centrifugation at 16,000 × *g*, for 10 min at 4 °C.

Immunoblot with anti-HERG antibodies was performed using a crude membrane fraction obtained from cells seeded on 10-cm Petri dishes, covered with BSA or FN, respectively. For membrane extraction, all steps were performed at 4 °C. Cells were washed with PBS, scraped off, and homogenized into 2.5 ml of TE buffer (Tris 10 mM, pH 7.5, EDTA 1 mM) plus a protease inhibitor mixture (Pefabloc 0.5 mM, pepstatin 1 μM, benzamidine 1 mM, aprotinin 4 μg/ml, iodacetamide 1 mM, phenanthroline 1 mM, leupeptin 1 μg/ml). Debris and nuclei were removed by centrifugation at 3,600 × *g* for 10 min, and membrane fraction was pelleted by centrifugation at 110,000 × *g* for 40 min. The membrane-enriched pellet was then solubilized in 50 mM Tris-HCl, 15 mM β-mercaptoethanol, and 1% SDS.

In any case, membrane as well as total cell proteins were then heated in reducing Laemmli buffer at 95 °C for 3 min, separated by 7.5% (for experiments with anti-PKC_α antibodies) or 6% (for experiments with anti-HERG antibodies) SDS-PAGE and transferred to a nitrocellulose sheet. After transfer, membranes were blocked for 4 h at room temperature with PBS + Tween-20 0.1% (T-PBS), containing 5% BSA (T-PBS-BSA) and then incubated overnight at 4 °C with anti-PKCα antibodies (Santa Cruz Biotechnology), diluted 1:3000 in T-PBS-BSA or with purified rabbit polyclonal anti-HERG antibody (a kind gift from Dr. Nerbonne, University of Washington, St. Louis, MO) diluted 1:500 in T-PBS-BSA. Membranes were washed 3 times with T-PBS and incubated with anti-rabbit peroxidase-conjugated secondary antibodies (Sigma) diluted 1:10,000 in T-PBS-BSA for 1 h at room temperature. After 3 washes with T-PBS, the immunoreactivity was determined by a chemiluminescent reaction (ECL) (Amersham Pharmacia Biotech).

Immunocytochemistry—Cells were seeded on glass slides covered with BSA or FN, and incubated for 24 h as above. After this time, cells were air-dried, fixed, and then treated with a 1:50 dilution of the primary antibody (anti-vitronectin receptors, Celbio) in 0.05 M Tris-HCl, pH 7.6, solution in a humidified chamber for 30 min. Then the slides were treated with an alkaline phosphatase anti-alkaline phosphatase kit (TOP-Line Histology) according to manufacturer's procedure. Slides were then counterstained with Giemsa, and photographed through a Nikon microscope, then acquired through a Minolta scanner.

Tartrate-resistant Acid Phosphatase Staining—Cells were cultured in slide-containing Petri dishes (see above), in complete medium. In the appropriate samples TPA (10^{-6} M, final concentration) was added at time 0. At different times of incubation, cells in suspension were harvested and processed for cytospin slide preparations; cytospin preparations as well as cells remaining adherent on the glass slides were fixed in 60% acetone in citrate buffer, pH 5.4, for 30 s, washed twice in distilled water, air-dried, and then stained for TRAP using a commercially available kit (Sigma), and following the manufacturer's instructions. Stained cultures were examined under light microscopy at a magnification of × 250, photographed, and acquired as above.

RESULTS

To study the relationships between ECM and integrin-mediated signals in FLG 29.1 leukemic cells, these cells were seeded on a FN-enriched substratum and the mechanisms sustaining cell adhesion were evaluated. In fact it had been previously shown that these cells could adhere to endothelial cells through the FN molecules covering endothelial cell surfaces (23) (see Introduction). Since FLG 29.1 cells express various classes of integrin receptors, mainly belonging to the β_1 and β_3 classes, and associated with different α subunits (α_2, α_3, α_4, α_5 and α_v) (23)[2] the role of these two different integrin classes on cell adhesion was evaluated. As shown in Fig. 1, about 50% of the FLG 29.1 cells adhere to FN, while this adhesion is significantly lower (about 35%) on VN, another integrin-recognized substrate. Cell adhesion to FN is impaired by blocking antibodies to FN receptor ($\alpha_5\beta_1$), while unaffected by blocking antibodies to β_3 subunit (25), ruling out the involvement of β_3 subunit in leukemia cell adhesion to FN. As to cell adhesion to vitronectin, it is 40% inhibited by blocking antibodies to FN receptor, while only slightly affected by blocking antibodies to the β_3 subunit (see Fig. 1). However, FLG 29.1 adhesion to VN is impaired by RGD containing peptides, as well as anti-α_v antibodies, suggesting the involvement of an integrin receptor containing the α_v subunit in this adhesion. Since the polyclonal anti-FN-R antibodies contain a great amount of antibodies against epitopes belonging to the β_1 subunit, the most plausible explanation for this finding is that FLG 29.1 cells recognize VN

[2] G. Hofmann, P. Defilippi, and A. Arcangeli, unpublished results.

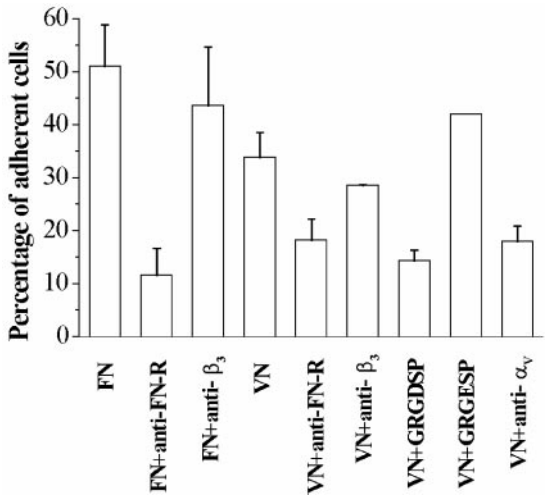

FIG. 1. **Effects of various treatments on FLG 29.1 cell adhesion.** Cells were seeded on FN- or VN-coated substrates for 60 min and the adhesion test performed as described under "Experimental Procedures." Anti-FN-R antibodies (dilution 1:50), anti-α_v antibodies (mAb L230, 10 μg/ml), GRGDSP (Telios) and GRGESP (Telios) peptides (0.5 mg/ml) were added at time 0 just before cell seeding. Anti-β_3 antibodies (mAb B212, 9 μg/ml) were added to the cell suspension and preincubated at 4 °C for 20 min before cell seeding. Values are expressed as the percentage of adherent cells in the various samples on the total number of cells inoculated. Values are mean ± S.E. of data carried out in quadruplicate samples and obtained in three separate experiments.

through their $\alpha_V\beta_1$ integrins, a heterodimer that is indeed expressed in these cells (see also Fig. 3). On the whole, data presented in Fig. 1 confirm that FLG 29.1 cells firmly adhere to FN, mainly through their $\alpha_5\beta_1$ integrin receptors.

The effect of FLG 29.1 cell adhesion to FN on cell differentiation was then evaluated: since FLG 29.1 cells show a preosteoclastic phenotype, as revealed by the appearance of osteoclastic markers upon TPA treatment (22), three of these markers, namely the CD 51/$\alpha_V\beta_3$, CtR (30), as well as TRAP (31) were studied. When FLG were left to adhere to FN for 24 h (in complete medium) the appearance of immunoreactivity to CD 51 was first detectable: in fact, this marker cannot be easily detected with this method in control cells (Fig. 2*A*), while it appears in FN-seeded cells (Fig. 2*B*). CD51 is also strongly reactive in cells treated with TPA (Fig. 2*C*), *i.e.* the classic inducer of osteoclastic differentiation of these cells (22), indicating that the increase in immunoreactivity to CD51 correlates with cell differentiation. The expression of TRAP could be also detected after longer times of incubation: as shown in the figure, while control cells barely (less than 10% of cells) express TRAP (*panel D*), about 68% of cells cultured on FN-coated dishes for at least 6 days display the marker (*panel E*), which is detectable either in resuspended (*E*) and spread (*E′*) cells. Here again the expression of TRAP in TPA-treated cells is reported as an internal control (*panel F*). In *panel G* is reported the expression pattern of CtR as revealed by RT-PCR: two thin PCR bands of 577 and 529 bp, corresponding to hCtR1 and hCtR2, respectively (as assessed by sequencing the PCR products, see "Experimental Procedures") can be detected in control cells (*lane 1*). The intensity of either bands is significantly increased (see also *inset* to *panel G*) in cells cultured for 1 week on FN-coated dishes (*lane 2*), and, at a roughly identical extent, in TPA-treated cells (*lane 3*).

Since CD51 represents the vitronectin receptor $\alpha_v\beta_3$, the modulation of this integrin was studied in more detail. As shown in Fig. 3*A*, FLG 29.1 control cells express the α_v integrin subunit, which associates with the β_1 and, although at very low levels, with the β_3 subunit (see legend to the figure). These results were confirmed by cytofluorimetric experiments performed with antibodies against external epitopes of the three integrin subunits (not shown). Moreover, expression of $\alpha_V\beta_3$ integrin was 50% increased in FN-seeded cells (see *inset* to Fig. 3*A* and the legend to the figure), as compared with control BSA-seeded cells. This up-regulation is induced, although at a much higher extent, also by treatment with TPA (see *lane c* of Fig. 3*A*, and *inset* to the figure), indicating here again that the increase in $\alpha_V\beta_3$ integrin correlates with cell differentiation. As shown in *panel B*, another integrin expressed on FLG 29.1 plasma membrane, namely the β_1 subunit and its associated α(s), is not affected by adhesion of leukemic cells to FN. Moreover, a cytoplasmic protein, namely PKCα, was not affected at all by cell adhesion to FN (*panel C*), ruling out any aspecific potentiating effect on protein synthesis operated by this adhesion.

On the whole, FLG 29.1 cells appear to be a suitable model for studying the electric signals induced by integrin activation, since these cells undergo a strong $\alpha_5\beta_1$-mediated adhesion to FN and this adhesion is capable of inducing the differentiation of these cells through the osteoclastic pathway, and, in this process, of sustaining an up-regulation of another integrin expressed in these cells, namely the $\alpha_v\beta_3$. The effect of integrin activation on various biophysical parameters was then studied in FLG 29.1, using the whole cell configuration of the patch clamp technique, after seeding the cells for different times on a FN- or a BSA-coated substratum, the latter taken as a control.

In Fig. 4 the time course of V_{REST} is reported: this potential starts to be hyperpolarized in FN-seeded FLG 29.1 cells as soon as 15 min after seeding, reaching a value significantly different from that of the BSA-seeded control cells (-28.6 mV ± 2.6 *versus* -12.9 mV ± 2.3; $p < 0.03$) after 1 h of incubation. Thereafter, V_{REST} of FN-seeded FLG 29.1 cells returns to starting values, identical to that of control cells.

Since we previously demonstrated that only two currents contribute to regulate V_{REST} in FLG 29.1 cells (12, 20), namely I_{HERG} and the leakage, values relative to these parameters were evaluated and are reported in Table I: I_{HERG} density is significantly increased after 1 h of cell adhesion to FN, as compared with the controls. This activating effect of FN on I_{HERG} declines thereafter, so that the I_{HERG} density in FN-seeded cells displays values even lower than those of control cells, after 2 h of incubation. This increase in I_{HERG} density is not accompanied by variations in biophysical characteristics of the current, such as the activation or the deactivation properties (not shown). On the other hand, the leakage conductance is not significantly increased by cell adhesion to FN: in fact, this parameter is even higher in BSA-seeded cells after 1 h of incubation, while is not significantly different in the two experimental conditions after 2 h. This effect on the leakage conductance exerted by cell adhesion is different from that elicited by laminin in human neuroblastoma cells (13), where a concomitant activation of I_{HERG} and leakage conductance is observed.

The integrin involvement in the mechanism underlying the FN-induced HERG channel activation was further evaluated at 1 h of incubation and results are reported in Table II: as shown in the table, the effect of FN is not unique, since also the adhesion to VN induces the activation of I_{HERG} in FLG 29.1 cells, although at a slightly lower extent as compared with FN. This result suggests, even in this case as in neuroblastoma cells (13), the involvement of the β_1 subunit of integrin receptors in the increment of I_{HERG}: this is clearly proven by the fact that FLG 29.1 cell adhesion to dishes coated with mAb BV7 or TS2/16 to the β_1 subunit (24, 13) (bound anti-β_1-ab in Table II) induces a significant increase in I_{HERG} density, whose intensity is even greater than that induced by adhesion on the whole FN

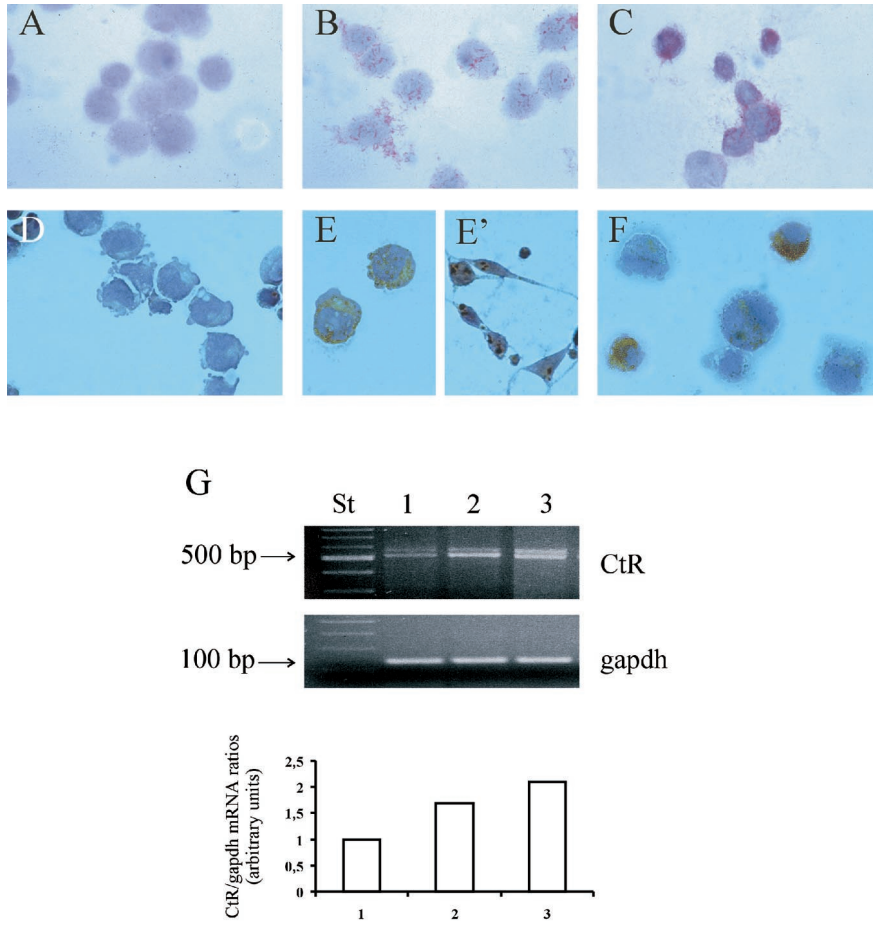

FIG. 2. **Effects of adhesion to FN on FLG 29.1 cell osteoclastic differentiation.** Cells were seeded on BSA- or FN-coated dishes or slides in RPMI complete medium and incubated for different times. As a control of osteoclastic differentiation, cells were incubated in RPMI complete medium containing 10^{-6} M TPA. At the end of the incubation, cells seeded on coated slides were used for immunocytochemistry experiments by treating with anti-CD51 antibodies, or revealing the TRAP (see "Experimental Procedures"), while cells seeded on coated dishes were used for RNA extraction and RT-PCR of the CtR. *A-C,* immunocytochemistry using a monoclonal antibody raised against the osteoclastic marker CD51; *A,* control, BSA-seeded cells; *B,* cells adherent to FN; *C,* TPA-treated cells. *D-F,* tartrate-resistant acid phosphatase after 6 days of culture. *D,* control; *E,* cells adherent to FN; *E′*, cells cultured on FN, collected for cytocentrifugation; *F,* TPA-treated cells. *G,* RT-PCR of CtR after 1 week of culture: *upper panel*, RT-PCR for CtR; *lower panel,* RT-PCR for gapdh. *St,* standard 100 bp (New England Biolabs); *lane 1,* control; *lane 2,* cells adherent to FN; *lane 3,* TPA-treated cells. *Inset*: densitometric analysis of CtR expression. Values are expressed as arbitrary units (see "Experimental Procedures").

molecule. Moreover, I_{HERG} density was also increased at the same extent by antibody-induced clustering of β_1 integrin subunits on FLG 29.1 cells kept in suspension (soluble anti-β_1-ab in Table II). These data indicate that antibody-induced β_1 clustering is sufficient to trigger HERG channel activation. The specificity of the involvement of the β_1 subunit in HERG channel activation is confirmed by data, also reported in Table II, showing that dishes coated with antibodies directed against either an α subunit (anti-α_3) or the β_3 subunit do not substantially increase I_{HERG} density. On the whole, data reported in Fig. 4 and Tables I and II demonstrate that FLG 29.1 adhesion on FN induces a cycle of V_{REST} hyperpolarization, which peaks after 1 h of incubation and is conceivably sustained by a concomitant increase in I_{HERG}, the latter being induced by the engagement of β_1 integrin subunit with its ligand.

We then analyzed whether the above reported increment in I_{HERG} density could be reconducted to variations in *herg* RNA level and/or modification of the HERG protein on the plasma membrane. In Fig. 5 the results of this analysis are reported. Fig. 5*A* shows a Northern blot performed with RNA extracted from cells seeded on BSA- or FN-coated dishes for 1 h, and probed with *herg* or a control gene (ribosomal *18S*). It is evident that FLG 29.1 cell adhesion to FN is not accompanied by any variations in *herg* RNA level as compared with the controls. The effect of cell adhesion on HERG protein expression on the plasma membrane is shown in Fig. 5*B*, where an immunoblot performed on membrane proteins extracted from FLG 29.1 cells seeded on BSA or FN for 1 h, and revealed with an anti-HERG antibody is reported. Two protein bands are evident in FLG 29.1 membrane extracts, one upper band of 155 kDa, and a lower band of 135 kDa. According to data reported so far (32),[3] the 155-kDa isoform represents the completely mature, fully glycosylated form of the protein, expressed on the plasma membrane, while the 135-kDa band is the immature, core-glycosylated protein. It is evident that FN adhesion does not alter this HERG protein profile, neither quantitatively, nor qualitatively.

Therefore, the FN-induced increment in I_{HERG} could be realized by the action of a short range modulator of channel activity: a possible candidate could be envisaged in a pertussis toxin-sensitive G_i protein interposed between β_1 integrins and channel protein, as in neuroblastoma cells (11). And in fact, as shown in Fig. 5*C*, when FLG 29.1 cells are seeded on FN- (*lane d*) or activating anti-β_1 antibody-coated dishes (*lane e*), in the presence of PTX, no increase in I_{HERG} density can be observed as compared with BSA-seeded control cells. It is worth noting that PTX-treated cells do adhere to FN, but do not spread on the substrate (*panels c* and *c′*), an effect already reported for PTX on melanoma cells (33). It is important to note that patch clamp experiments were performed either on adherent round or spread cells, and no difference in I_{HERG} density as well as biophysical characteristics of the current were detected in the two conditions (not shown). This rules out that the lack of effect of FN on I_{HERG} in PTX-treated cells can be due to the incapacity of the cells to undergo cell spreading.

The question now arises as to whether the FN-induced, β_1-mediated electric signal is a necessary step in the pathway leading to osteoclastic differentiation of leukemic cells. Since we showed that this pathway can be evidenced by at least three markers (CD 51/$\alpha_v\beta_3$ integrin, TRAP, and CtR receptor), ex-

[3] L. Guasti, D. Crociani, and A. Arcangeli, unpublished results.

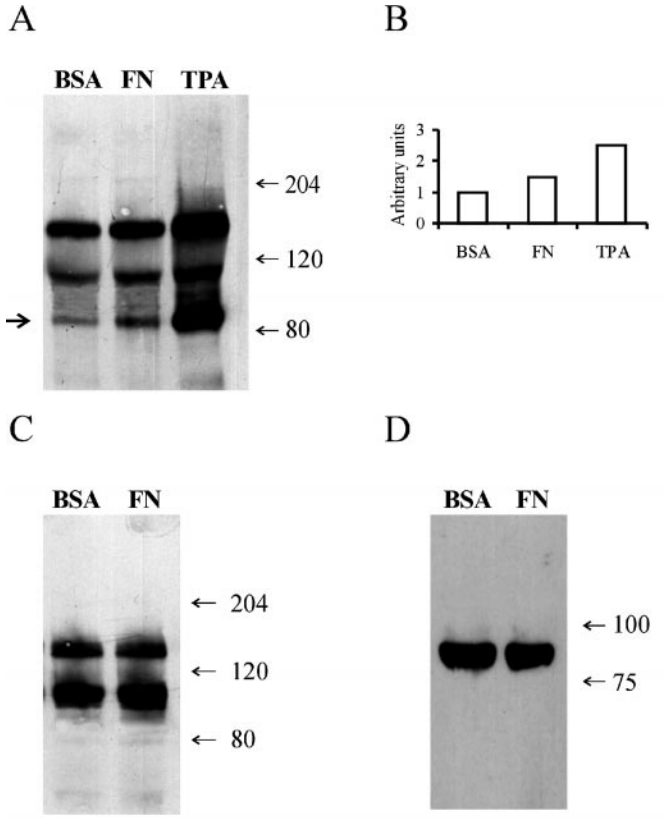

FIG. 3. **Effect of cell adhesion to FN on integrin expression on FLG 29.1 cells.** Cells were seeded on BSA- or FN-coated dishes in RPMI complete medium and incubated for different times. As a control of osteoclastic differentiation, cells were incubated in RPMI complete medium containing 10^{-6} M TPA. At the end of the incubation, membrane proteins were biotinylated, extracted, and immunoprecipitated with various anti-integrin antibodies. *A,* immunoprecipitation of $\alpha_v\beta_3$ integrin from BSA- or FN-seeded cells or TPA-treated cells. $\alpha_v\beta_3$ was immunoprecipitated using a polyclonal antibody raised against the integrin α_v. Molecular weight markers (Bio-Rad) are reported on the *right*. In nonreducing conditions, the β_3 integrin subunit migrates as an 80-kDa band (see *arrow*), while the associated α_v as a 150-kDa band. α_v also complexes with the β_1 subunit, which migrates as a 110-kDa band. *B,* densitometric analysis of the amount of β_3 band; the analysis gave the following results: control (BSA) = 1, FN = 1.5, TPA = 2.5 (indicated in arbitrary units). *C,* immunoprecipitation using a polyclonal antibody raised against the β_1 integrin subunit, performed on BSA- or FN-seeded cells; note that neither the β_1 (the *lower band*) nor the associated α(s) (the *upper band*) appear to be modulated by cell adhesion; *D,* immunoblot on a cell total protein extract revealed by anti-PCKα antibodies, performed on BSA-or FN-seeded cells. The molecular weight standard is from Bio-Rad.

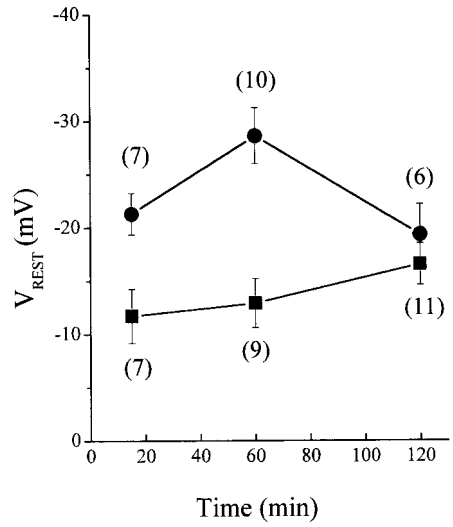

FIG. 4. **Modulation of V_{REST} of FLG 29.1 cells by cell adhesion.** Cells from preparatory cultures were resuspended in RPMI + BSA and seeded on Petri dishes previously coated with the indicated substrates. At various times of incubation V_{REST} was measured by the patch clamp technique (whole cell configuration; I = 0) (see "Experimental Procedures"). Values are mean ± S.E. of the number of cells reported in parentheses. *Closed circles,* BSA. *Closed squares,* FN.

TABLE I
Effects of cell adhesion on various biophysical parameters of FLG29.1 cells at different times of incubation

Cells were seeded on BSA- or FN-coated dishes, and various biophysical parameters were studied at different times of incubation by means of the patch-clamp technique (see "Experimental Procedures").

Treatment	I_{HERG}[a] density	*t* Test	g_L	*t* Test[b]
	pA/pF		*pS*	
BSA (1 h)	28.9 ± 4.2 (16)		0.82 ± 0.12 (14)	
		$p = 0.0053$		$P = 0.04$
FN (1 h)	45.4 ± 3.5 (16)		0.56 ± 0.09 (18)	
BSA (2 h)	26.5 ± 5.2 (6)		0.46 ± 0.19 (6)	
		NS		NS[c]
FN (2 h)	17.4 ± 3.7 (6)		0.6 ± 0.13 (6)	

[a] I_{HERG} density was calculated by dividing the HERG peak current by the cell capacitance. g_L, leakage conductance. Values are mean ± S.E. of recordings obtained from the number of cells reported in parentheses.
[b] *p* = Student's *t* test (two populations).
[c] NS, not significant.

TABLE II
Effects of various substrates or anti-integrin antibodies on HERG current density

Cells were seeded on BSA-, VN-, or anti-integrin antibodies-coated dishes, or incubated with soluble anti-β_1 antibodies, as explained under "Experimental Procedures".

Treatment	I_{HERG}[a] density	*t* Test[b]
	pA/pF	
BSA	28.8 ± 3.5 (22)	
VN	40.6 ± 3.8 (15)	$p = 0.034$
Bound anti-β_1-ab	53.1 ± 3.4 (14)	$p = 0.005$
Soluble anti-β_1-ab	51.8 ± 8.9 (14)	$p = 0.009$
Bound anti-β_3-ab	31.8 ± 4.1 (14)	NS[c]
Bound anti-α_3-ab	25.0 ± 4.5 (5)	NS

[a] I_{HERG} density, calculated as reported in the legend to Table I, was measured after 1 h of incubation. Values are mean ± S.E. of recordings obtained from the number of cells reported in parentheses.
[b] *p* = Student's *t* test (two populations).
[c] NS, not significant.

periments were performed evaluating the expression of such markers in cells incubated on a BSA- or FN-enriched substratum for different times, with or without a specific inhibitor of HERG channels, namely WAY 123,398. In the latter conditions, the I_{HERG} was completely and irreversibly blocked (not shown) (15). As shown in Fig. 6, *panel A*, incubation of FLG 29.1 cells with 40 μM WAY 123,398 for 24 h (*lane b*) impairs the increase in $\alpha_v\beta_3$ expression induced by cell adhesion to FN (which is reported for comparison in *lane c*). Moreover, WAY 123,398 addition can also impair the increased expression of CtR (*panel B, lanes 2* and *3*), which can be observed after a 1-week incubation of FLG 29.1 cells on FN-coated dishes.

DISCUSSION

Integrin receptors are known to mediate either "inside-to-out" and "outside-to-in" signals (9), and, in this way, can regulate many cellular functions, such as cell growth and differentiation, cell migration and/or activation. We present evidence that β_1 integrin receptors can elicit the activation of a hyperpolarizing, HERG-sustained, K^+ current in leukemic hemopoietic precursors (FLG 29.1 cells), and that this signal apparently

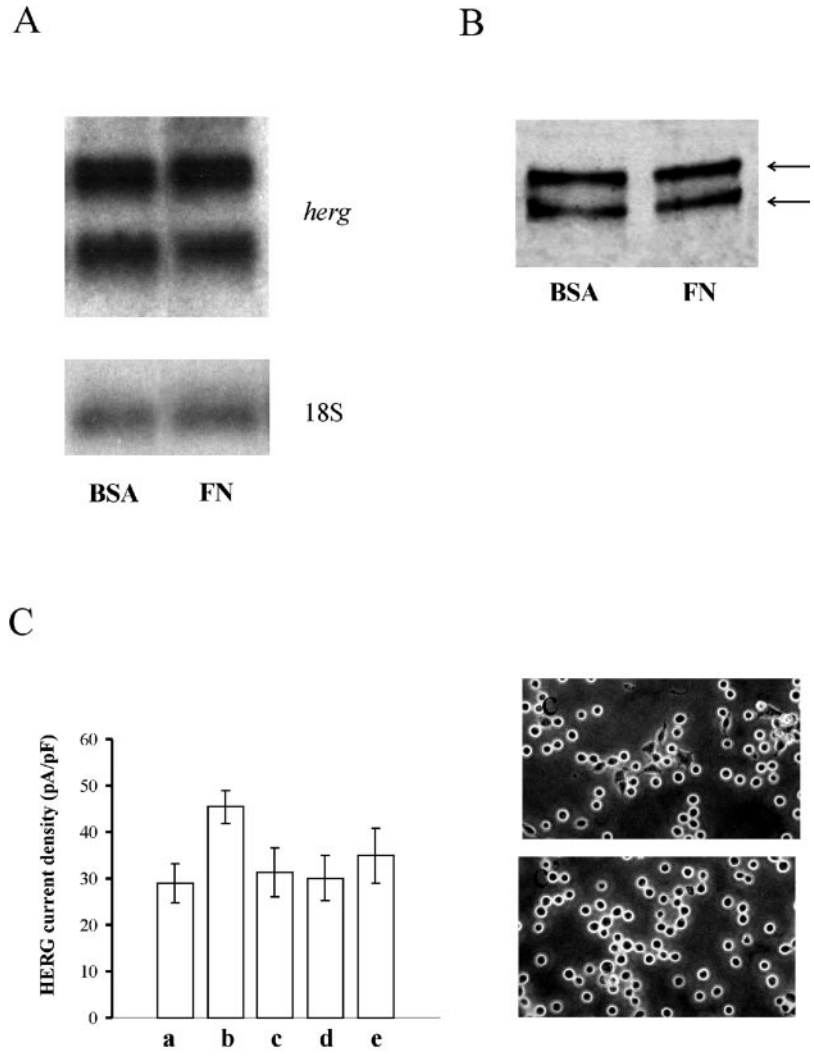

FIG. 5. **Analysis of the mechanisms sustaining HERG current activation by cell adhesion to FN.** FLG 29.1 cells were seeded for 1 h on BSA- or FN-coated dishes and then RNA or membrane proteins were extracted. Extracted RNA was analyzed by Northern blot using the *herg* and 18S probes. Membrane proteins extracts were electrophoresed by SDS-PAGE, transferred on nitrocellulose sheets, and immunoblotted with anti-HERG antibodies. *A, herg* gene expression; the *upper bands* (4.0 and 2.2 kDa) are the two *herg* bands; the *lower* represents the control gene *18S*. *B,* Western blot analysis with anti-HERG antibodies. Two bands relative to HERG protein (155 and 135 kDa, respectively) are reported. *C,* HERG current density of FLG 29.1 cells seeded on BSA (control) (*a*), FN (*b*), BSA after a 12–16 h preincubation with PTX 100 nM, FN after a 12–16 h preincubation with PTX, 100 nM (*d*); anti-β_1 antibodies after a 12–16 h preincubation with PTX, 100 nM (*e*). *c* and *c′*, effect of PTX treatment on cell spreading on FN-coated dishes. *c,* FN; *c,* FN after a 12–16-h preincubation with PTX, 100 nM.

modulates the increased expression of $\alpha_v\beta_3$ integrins on the plasma membrane, a phenotypic marker of osteoclastic differentiation in these cells.

An integrin-mediated electric signal had been previously reported to occur in murine erythroleukemic (34), as well as murine and human neuroblastoma cells (11, 13). While in murine leukemic cells, adhesion to FN leads to the activation of Ca^{2+}-dependent K^+ channels, the electric signal elicited by FN in FLG 29.1 leukemic cells is represented by a hyperpolarization of V_{REST}, mediated by the activation of HERG K^+ channels, as occurs in neuroblastoma cells (11, 13). Therefore, HERG channels appear to be frequently involved in the signaling pathway induced by integrins in cells of different lineages.

An association between integrins and K^+ channels has been reported to occur between β_1 subunit and GIRK channels in transfected cells (35), although integrins do not appear to be essential for a functional GIRK expression (36), despite the physical association of the two proteins. A functional association between integrins and a K^+ channel different from HERG involves the Kv 1.3 channels in human lymphocytes: in these cells, however, the modulation of the K^+ current is the trigger for β_1 integrin activation, which leads to an enhanced functioning of the adhesive receptor (37).

In our case, the integrin-induced HERG activation does not imply any modulation in *herg* RNA or HERG protein level, but is mediated by a short-range mechanism of activation. In particular, in human leukemic, as in neuroblastoma cells (11), a G_i protein is involved in the functional association between integrins and HERG channels. Integrins are known to modulate the activity of the Rho family of small GTPases (reviewed in Ref. 38), but also a physical and functional association between integrins, the integrin-associated protein and trimeric G_i proteins has been reported (39, 40). These proteins constitute a signaling complex, involved in many cellular functions, such as chemotaxis (41) and platelet aggregation (39), possibly through the activation of β_3 integrins. In such models, the stimulation of G_i induces an inhibition of adenyl cyclase, which ultimately leads to a decrease in intracellular cAMP. We have no evidence whether such a mechanism can be also invoked in our model, or whether a direct effect of G_i occurs on HERG channels as reported for other types of K^+ channels (42).

Another point to be stressed here is that HERG channels, and their mediated electric variation of V_{REST}, appear to be more frequently involved in processes of cell differentiation (11, 13, 43, 44). In fact, we show here that leukemic cells apparently start undergoing a process of osteoclastic differentiation, after adhesion to FN, as evidenced by the appearance of osteoclast markers, such as TRAP, CtR, as well as CD51/$\alpha_v\beta_3$ integrin. This is an important demonstration of a differentiating effect of the ECM on cells of the hemopoietic lineage, which couples with recent evidence showing a cooperative effect of FN with cytokines and growth factors on the maintenance and growth of CD34+ hemopoietic stem cells (45).

Moreover, data reported in this paper demonstrate that this differentiation process includes the appearance of immunoreactivity to CD51, *i.e.* by the increased expression of $\alpha_v\beta_3$ inte-

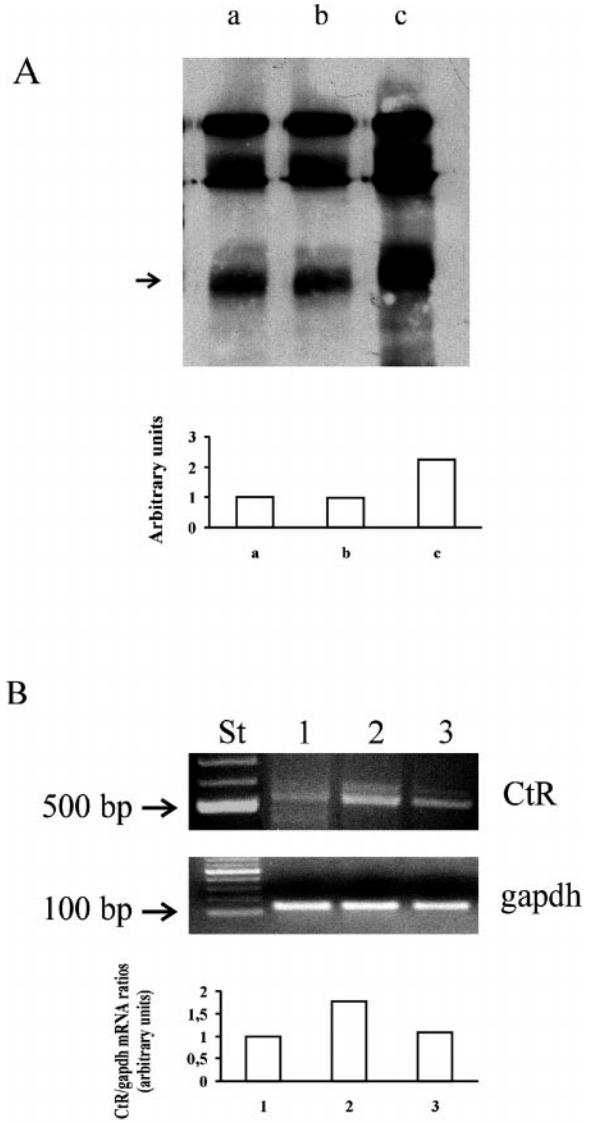

FIG. 6. **Effect of treatment with blockers of HERG currents on $\alpha_v\beta_3$ and CtR expression.** Cells were seeded on BSA- or FN-coated dishes in the absence or presence of 40 μM WAY 123.398 for different times. After 24 h of incubation, cells were used for protein extraction. Biotinylated proteins were then immunoprecipitated with anti-α_v polyclonal antibodies as reported in Fig. 2. After 1 week of incubation, cells were used for RNA extraction and RT-PCR of the CtR (see "Experimental Procedures"). *A,* immunoprecipitation of $\alpha_v\beta_3$ by means of anti-α_v antibodies. The β_3 coimmunoprecipitating with α_v is the band indicated by the *arrow*. *Lanes: a,* control, BSA-seeded cells + WAY 123,398; *b,* FN + WAY 123,398; *c,* FN. *B,* RT-PCR of CtR after 1 week of culture: *upper panel*, RT-PCR for CtR; *lower panel,* RT-PCR for gapdh. *St,* standard 100 bp (New England Biolabs), *lane 1*, control; *lane 2,* cells adherent to FN; *lane 3,* cells adherent to FN + WAY 123,398. *Inset*: densitometric analysis of CtR expression. Values are expressed as arbitrary units (see "Experimental Procedures").

grin on the plasma membrane, the integrin involved in osteoclast adhesion to the bone matrix, a necessary step for bone readsorption (8). The demonstration of an up-regulation of an integrin receptor, induced by the engagement of a different integrin subunit with the ECM deserves more attention. Moreover, we show here that drugs, which specifically block HERG channels, such as anti-arrhythmic drugs, impair either this up-regulation of $\alpha_v\beta_3$ integrin or the increased expression of CtR. Therefore, the activation of HERG channels is capable of mediating the up-regulation of membrane receptors, including integrins, an interesting finding unexplored so far. In fact, in T lymphocytes, the activation of Kv 1.3 channels induced an increased function of β_1 integrins, possibly through a conformational change of the integrin molecule; however, an increased expression of this integrin subunit on the plasma membrane, as in our case, is not reported. While the effect of HERG channels on CtR appears to happen at the RNA level, the mechanism leading, in our model, to the up-regulation of β_3 integrins remains to be explored, and a transcriptional activation could be invoked. However, a direct electrostatic effect on the protein could also occur, thus candidating some classes of integrins as voltage-dependent proteins (46), capable of being influenced themselves by the biophysical modifications of the plasma membrane. On the whole, a mechanism of cross-talk between integrin subunits could be hypothesized, centered on the activity of K^+ HERG channels, which can be influenced by integrins (β_1 subunits, mainly), and can activate diverse proteins, either membrane-endowed, such as integrins themselves (β_3 subunits mainly), and hormone receptors, or cytoplasmic, such as $pp125^{FAK}$ (47).

On the whole, these data support the hypothesis that HERG channels represent an important molecular device, involved both in the integrin-mediated outside-to-in and inside-to-out signaling and by this way in some signaling pathways controlling cell differentiation in the hemopoietic system.

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