

Integrin-mediated Neurite Outgrowth in Neuroblastoma Cells Depends on the Activation of Potassium Channels

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Abstract. Electrical signals elicited by integrin interaction with ECM components and their role in neurite outgrowth were studied in two clones (N1 and N7) isolated from 41A3 murine neuroblastoma cell line. Although the two clones similarly adhered to fibronectin (FN) and vitronectin (VN), this adhesion induced neurite outgrowth in N1 but not in N7 cells. Patch clamp recordings in whole cell configuration showed that, upon adhesion to FN or VN but not to platelet factor 4 (PF4), N1 cells undergo a marked (≈ 20 mV) hyperpolarization of the resting potential (V_{rest}) that occurred within the first 20 min after cell contact with ECM, and persisted for ~ 1 h before reverting to the time zero values. This hyperpolarization was totally absent in N7 cells. A detailed analysis of the molecular mechanisms involved in N1 and N7 cell adhesion to ECM substrata was performed by using antibodies raised against the FN receptor and synthetic peptides variously competing with the FN or VN binding to integrin receptors (GRGDSP and GRGESP). Antibodies, as well as GRGDSP, abolished adhesion of N1 and N7 clones to FN and VN, revealing a similar implication of integrins in the adhesion of these clones to the ECM proteins. However, these anti-adhesive treatments, while ineffective on V_{rest} of N7 cells, abolished in N1 cells the FN- or VN-induced hyperpolarization

and neurite outgrowth, that appeared therefore strictly associated and integrin-mediated phenomena. The nature of this association was deepened through a comparative analysis of the integrin profiles and the ion channels of N1 and N7 cells. The integrin immunoprecipitation profile resulted very similarly in the two clones, with only minor differences concerning the αV containing complexes. Both clones possessed Ca^{2+} and K^+ delayed rectifier (K_{DR}) channels, while only N1 cells were endowed with inward rectifier K^+ (K_{IR}) channels. The latter governed the V_{rest} , and, unlike K_{DR} channels, were blocked by Ba^{2+} and Cs^+ . By moving patched cells in contact with FN-coated beads, it was shown that K_{IR} channel activation was responsible for the FN-mediated hyperpolarization of V_{rest} . Treatment with Pertussis toxin (PTX) abolished this hyperpolarization and neurite outgrowth, indicating that a G protein is interposed between integrins and K_{IR} channels and that the activation of these channels is required for neuritogenesis. In fact, the block of K_{IR} channels by Cs^+ abolished both hyperpolarization and neurite outgrowth, provided that the cation was supplied during the first two hours after N1 cell contact with FN. This Cs^+ -sensitive commitment time for neuritogenesis coincided with the time length of the FN-induced hyperpolarization.

EXTRACELLULAR matrix (ECM)¹ is a complex network of interacting molecules which, besides acting as an inert scaffold that stabilizes the physical structure of tissues, regulates fundamental cell processes, such as migration, differentiation and growth (McDonald, 1988; McClay and Ettersohn, 1987). In addition, ECM is implicated in pathogenesis of important diseases characterized by

aberrant cell adhesion, including cancer (Liotta et al., 1986; Juliano, 1987). It is composed by various types of molecules, as collagens, proteoglycans (Ruoslahti, 1988a) and "adhesive proteins," among which the best characterized are fibronectin (FN) (Ruoslahti, 1988b; Yamada, 1989; Aota et al., 1991), vitronectin (VN) (Hayman et al., 1985) and laminin (LM) (Martin and Timpl, 1987). Different cellular receptors for ECM components have been identified: glycoproteins belonging to the integrin superfamily (Ruoslahti, 1991; Hynes, 1992), heparan sulfate (Culp et al., 1986), gangliosides (Yamada et al., 1981; Mugnai et al., 1988a), proteoglycans (Harper and Reisfeld, 1983; Ruoslahti,

1. *Abbreviations used in this paper:* ECM, extracellular matrix; FN, fibronectin; K_{DR} , K^+ delayed rectifier; K_{IR} , K^+ inward rectifier; MELC, murine erythroleukemia cells; NB, neuroblastoma; PTX, Pertussis toxin; VN, vitronectin; V_{rest} , resting potential.

1988a). Integrin receptors are $\alpha\beta$ heterodimers, with various types of α and β subunits variously combined to give rise to a variety of binding specificities (Hynes, 1992). Both subunits are glycoproteins, endowed with a transmembrane segment, a short cytoplasmic domain and an extended extracellular domain (reviewed in Hynes, 1992). The extracellular ligand binding domain in most cases specifically recognizes an RGD tripeptide sequence of the adhesive proteins (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984; Ruoslahti and Pierschbacher, 1987), while cytoplasmic domains interact with cytoskeletal proteins and perhaps with other cytoplasmic components (Hynes, 1992). Increasing evidence indicates that two types of signaling occur via integrins (Hynes, 1992): (a) inside-to-out, regulating the conformation and affinity of the receptor from inside the cell; and (b) outside-to-in, triggering intracellular events after ligand-receptor binding. Outside-to-in signals involve tyrosine phosphorylation (Kornberg et al., 1991; Guan et al., 1991), transient elevation of intracellular Ca^{2+} (Jaconi et al., 1991), an increase in intracellular pH (Ingber et al., 1990) and the activation of K^+ ion channels (Arcangeli et al., 1991; Becchetti et al., 1992a).

Knowledge about cell adhesion mechanisms to ECM components is being stimulated by studies aimed at revealing alterations of these mechanisms in tumor cell population relevant to neoplastic progression and metastatic spreading. In fact, a peculiar cell interaction with ECM components may influence clonal selection during tumor progression (Liotta et al., 1986; Juliano, 1987), as well as differentiation towards a normal phenotype (Patel and Lodish, 1987; Tomaselli et al., 1987); moreover specific interaction with target organs may account for the selectivity of metastatic colonization (Roos, 1984; Rusciano et al., 1991).

Since their establishment in culture, neuroblastoma (NB) cell lines offered leads to studying the role of cell adhesion in tumor cell progression and differentiation. These lines are originated from the malignant conversion of a presumptive precursor from the embryonic neural crest (reviewed in Abemayor and Sidell, 1989); they are tumorigenic and metastasizing in vivo, although their spontaneous differentiation may occur, with remission of the malignant disease (Evans et al., 1976). In vitro, NB cells can differentiate, expressing multiple phenotypes (Prasad, 1975; Abemayor and Sidell, 1989), upon addition of soluble factors, such as retinoic acid or NGF (Sidell, 1982; Sonnefeld and Ishii, 1982; Abemayor and Sidell, 1989), as well as in culture on appropriate substrata, including FN or LM (Denis-Donini and Augusti-Tocco, 1980; Culp and Barletta, 1990). The substrata-induced differentiation is frequently neuronal-like, and can be morphologically quantified by counting cellular processes displaying neurite-like characteristics (Bottenstein, 1981; Abemayor and Sidell, 1989). This induction of neurite outgrowth in differentiating NB cells has been intensively studied at the molecular level; interestingly, it is frequently accompanied by modulation of cellular oncogenes (Thiele and Israel, 1988; Abemayor and Sidell, 1989), as well as by the generation of ionic signals at the plasma membrane level (Koike, 1978; Reboulleau, 1986; Abemayor and Sidell, 1989; Doherty et al., 1991; Saffel et al., 1992; Williams et al., 1992).

In a previous study on murine erythroleukemia cells (MELC) we demonstrated for the first time that an electric

signal is elicited by FN-integrin interaction, consisting in a plasma membrane hyperpolarization, sustained by the activation of Ca^{2+} -dependent K^+ channels (Arcangeli et al., 1991; Becchetti et al., 1992a). In the work we present here, we investigated whether a similar signal could be evoked in NB cells by cell adhesion to ECM proteins and whether this signal was implicated in the induction of neuronal differentiation. We used two clones (N1 and N7) of the 41A3 neuroblastoma cell line derived from the mouse C1300 parental cell line (Augusti-Tocco and Sato, 1969; Denis-Donini and Augusti-Tocco, 1980), only one of which (N1) emitted neurites after adhesion to FN-enriched substrates. Only the neurite-sprouting clone underwent a marked hyperpolarization during adhesion to FN and both neurite elongation and hyperpolarization turned out to be integrin-mediated phenomena. The hyperpolarization occurred through a G-protein-mediated activation of a particular type of K^+ channel ("inward rectifier" K^+ channel) (Hille, 1992). Interestingly, while the integrin profile in the two clones was substantially similar, the above channels were not demonstrable in the nondifferentiating cells. The block of these channels drastically inhibited neurite outgrowth in the differentiating clone, suggesting that the integrin-mediated membrane hyperpolarization was a triggering signal of neuritogenesis.

Materials and Methods

Cell Culture

The N1 and N7 subclones of 41A3 murine neuroblastoma cells (Augusti-Tocco and Sato, 1969; Denis-Donini and Augusti-Tocco, 1980) were isolated by limiting dilution. Cells were routinely cultured in DME, containing 4.5 g/liter of glucose and 10% FCS, and incubated at 37°C in a humidified atmosphere, with 10% CO_2 in air. Cells from a subconfluent culture were detached with 0.25% trypsin (Sigma Immunochemicals, St. Louis, MO), centrifuged at 250 g for 10 min and resuspended in DME containing 250 $\mu\text{g}/\text{ml}$ of heat-inactivated BSA (DME + BSA) at a cell concentration of $3\text{--}5 \times 10^4$ cells/ml to be used for various types of experiments. Cells were used for no more than 20 passages in culture.

Preparation of Substrata and Coating of Culture Dishes and Latex Beads

Heat-inactivated BSA was prepared by heating a BSA (Miles Inc., Kankakee, IL) solution (25 mg/ml) in Dulbecco's modified PBS at 70°C for 1 h. FN was purified from human plasma by affinity chromatography on gelatin, according to Ruoslahti et al. (1982). VN was prepared from human plasma according to Yatohgo et al. (1988); platelet factor 4 (PF4) was prepared from human platelets according to Levine and Wohl (1976). Coating of culture dishes was performed by adding the adhesive proteins at 20 $\mu\text{g}/\text{ml}$ in DME at 37°C for 1 h. Thereafter dishes were rinsed with PBS and further incubated for 1 h with DME + BSA. Dishes were then rinsed again with PBS and immediately used for experiments. Latex microbeads (Sigma Immunochemicals; diameter 11.6 μm) were coated with FN by using Grinnell's method (Grinnell, 1980). A few microliters of a saline solution containing coated beads (0.4×10^8 beads/ml) were added to BSA-coated petri dishes containing NB cells immediately before the patch-clamp experiments. Care was taken to avoid contacts of the beads with NB cells before the patch clamp measurements.

Cell Treatment with Ant-FN Receptor Antibodies and Synthetic Peptides

Rabbit antiserum, raised against BHK cells (α -BHK) and containing anti-FN receptor antibodies (Tarone et al., 1982) was dissolved in DME + BSA and added to cell cultures at time zero at the final dilution of 1:25. The hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP, Telios) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP, Telios) were dissolved in PBS at 10 mg/ml, diluted

in DME + BSA (final concentration 0.5 mg/ml) and added to dishes just before cell seeding.

Patch-Clamp Recordings

Cells, prepared as described above were seeded into petri dishes (35 mm diameter; Costar Corp., Cambridge, MA) previously coated with various substrata, and incubated at 37°C for 15–20 min in the absence or in the presence of antibodies or peptides. Patch clamp experiments were performed at room temperature with an amplifier Axopatch 1-D (Axon Instruments, Inc., Foster City, CA) substituting the petri dishes every 30–45 min. The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used by using pipettes (borosilicate glass, Hilgenberg, Germany), whose resistances were in the range of 5–10 M Ω . Extracellular solutions were delivered with hypodermic needles inserted into a capillary with a small hole (inner diameter 0.4 mm), positioned near the cell under study. The standard extracellular solution contained (in mM): NaCl 130, KCl 5, CaCl₂ 2, MgCl₂ 2, Hepes-NaOH 10, glucose 5, pH 7.4. The extracellular solution with high K⁺ contained (in mM): NaCl 93 (or 0), KCl 40 (or 130), CaCl₂ 2, MgCl₂ 2, Hepes-NaOH 10, pH 7.4. The standard pipette solution at [Ca²⁺]_i = 10⁻⁷ M (pCa 7) contained (mM): K⁺-Aspartate 130, NaCl 10, MgCl₂ 2, CaCl₂ 4, EGTA-KOH 10, Hepes 10, pH 7.4. Gigaseals resistances were in the range of 5–20 G Ω . Whole-cell currents were filtered at 1 KHz. For a precise measurement of the gating parameters and tail currents of the inward rectifier channels, we carefully compensated pipette and cell capacity and the series resistance before each voltage clamp protocol run. When necessary, membrane potential was measured in current-clamp mode ($I = 0$). During data acquisition and analysis, the pClamp software (Axon Instruments, Inc.) was routinely used. I-V plots were obtained in voltage-clamp with ramp commands between -80 and +60 mV. The ramp lasted 1280 ms. The relatively slow rate of change of voltage produced a disregardable capacitive current. Resting resistance of the cells were in the range of 2–6 G Ω .

Adhesion Assays

To quantify the attachment of cells to various substrata, stock cultures were radiolabeled during 24 h of exponential growth in medium containing 0.5 μ Ci/ml of methyl-³H-thymidine (specific activity, 10–20 Ci/mmol). After this time, cells were detached as previously described, pelleted and resuspended in DME + BSA. Aliquots of cells (5×10^3) were inoculated into each well of 96-well clusters (Costar Corp.), previously coated with a solution of the selected adhesive protein in DME (20 μ g/ml), and then incubated for 30 min at 37°C. At the end of this time, medium was aspirated off and adherent cells gently rinsed twice with PBS containing divalent cations; the cells were then solubilized with 50 μ l of 1% SDS in 0.1 N NaOH for 1 h. Radioactive solubilized cells were quantified by scintillation counting (with correction for quenching), and compared on a percent basis with the radioactivities of aliquots of the original cell suspension.

Neurite Count

NB cells were plated on 35-mm petri dishes (Costar Corp.) coated with various proteins as described above and incubated for 5 h at 37°C. Cells were then fixed with 2.5% glutaraldehyde and observed by phase-contrast microscopy. Neurite outgrowth was quantified as previously described (Mugnai et al., 1988b) by scoring cell processes longer than one cell body. At least 250–300 cells per dish were counted.

Immunoprecipitation of Integrins

Mouse integrins were identified with polyclonal antisera directed to synthetic peptides reproducing amino acid sequences of the cytoplasmic domains of β 1, α 1, α 2, α 3, α 5, and α V integrin subunits. The following peptides, obtained from Multiple Peptide System (San Diego, CA) were used: β 1, CTTVVNPKYEGK; α 1, KIGFKRPLKMKMEK; α 2, KYEKMT-KNPDEIDETTELSS; α 3, CRIQPSETERLTDDY; α 4, KLQENRRD-SWSYINSKSNDD; α 5, KRSLPYGTAMEKAQLKPPATSDA; and α V, KKRVRPPQEEQERE. Peptides were coupled to a hemocyanin with glutaraldehyde (approximate peptide/carrier molar ratio of 50:1) and rabbits were injected with 500 μ g of the conjugate in complete Freund adjuvant. Antibodies reacted specifically with the peptide sequence used for immunization, as determined by ELISA assay on peptide-BSA conjugates. The antibodies were specific to the appropriate subunit and did not show cross reaction with other integrin subunits as demonstrated by immunoprecipita-

tion assay on different cell lines (Defilippi et al., 1991, 1992; Rossino et al., 1991). Due to the highly conserved sequence of the COOH-terminal region of integrin subunits, all these antibodies react with integrins of several animal species. Polyclonal antibodies directed to COOH-terminal peptides of the human β 3 integrin subunit was a kind gift of James Gailit (SUNY, Stony Brook, NY). The mAb GoH3 to integrin α 6 was a kind gift from Dr. Arnou Sonnenberg (The Netherlands Cancer Center, Amsterdam, The Netherlands). This antibody, although directed to the human α 6, reacts with the mouse protein as well.

For immunoprecipitation, cells, grown to semiconfluence in 10-cm petri dishes (Costar Corp.), were metabolically labeled with a Tran-³⁵S-label (ICN Biomedicals, Inc., Costa Mesa, CA) (containing L-Methionine [³⁵S] and L-Cysteine [³⁵S], s.a. 1242 Ci/mmol) by overnight incubation in methionine-free DME (ICN Biomedicals) with 10% FCS and 100 μ Ci of ³⁵S per dish. To analyze integrins from cell extracts, labeled cells were washed with ice cold PBS and extracted for 20 min at 4°C with 0.5% Triton X-100 in 20 mM Tris(hydroxymethyl)aminomethane (TrisHCl), pH 7.4, 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂ (TBS-Triton buffer) containing 10 μ g/ml leupeptin, 4 μ g/ml pepstatin and 0.1 TIU/ml aprotinin (all from Sigma Immunochemicals). After centrifugation at 16000 g at 4°C for 10 min, extracts were incubated with the specific antibodies for 1 h at 4°C with gentle agitation. Soluble immunocomplexes were bound to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). When primary monoclonal antibodies were used, the resin was preincubated with rabbit anti-mouse Ig. After washing, bound material was eluted by boiling beads in 1% SDS (Pierce, Rockford, IL) and analyzed by SDS-PAGE electrophoresis under non reducing conditions, as described previously (Defilippi et al., 1992).

Pertussis Toxin Treatment

Pertussis toxin (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) was dissolved in 100 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 at 100 μ g/ml. The toxin 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 detached, resuspended in DME + BSA in the absence of the toxin, and seeded on FN or BSA coated dishes for experiments.

Induction of Cell Differentiation by Retinoic Acid

Retinoic acid (Sigma Immunochemicals) was dissolved in ethanol at 1 mg/ml and stored at -20°C in dark bottles. Cells were seeded at $\sim 10^4$ cells/ml in DME + FCS 10% and after 24 h the medium was changed to DME + FCS 1% + Retinoic acid 10⁻⁷ M (final concentration). After further 48 h of incubation cells were fixed with 2.5% glutaraldehyde.

Results

Relationships between Neurite Emission and the Resting Potential in Different Clones of Neuroblastoma Cells

As a first approach to investigate the electrical signals elicited in NB cells during adhesion and the relationships between these signals and cell differentiation, the time course of the resting potential (V_{rest}) was followed in the cells of N1 and N7 clones plated on either BSA- or FN-coated dishes.

After 5 h of incubation on BSA-coated substrata, both N1 and N7 cells maintained their time zero shape, appearing round and loosely adhering to the substratum (not shown). When incubated for the same time on FN-coated substrata, N1 cells appeared strongly adherent, with long slender neurites sprouting from the round refractile cell body (Fig. 1 a). On the other hand, N7 cells incubated for 5 h on FN-coated substrata, although strongly adherent, displayed a spread morphology, with no neurite emission (Fig. 1 c). It is worth noting here that the defect of neuritogenesis in the N7 clone is apparently restricted to the integrin-mediated pathway. In fact these cells, as well as N1 cells, maintain the capability, characteristic of the parental cell line C1300 (Denis-Donini

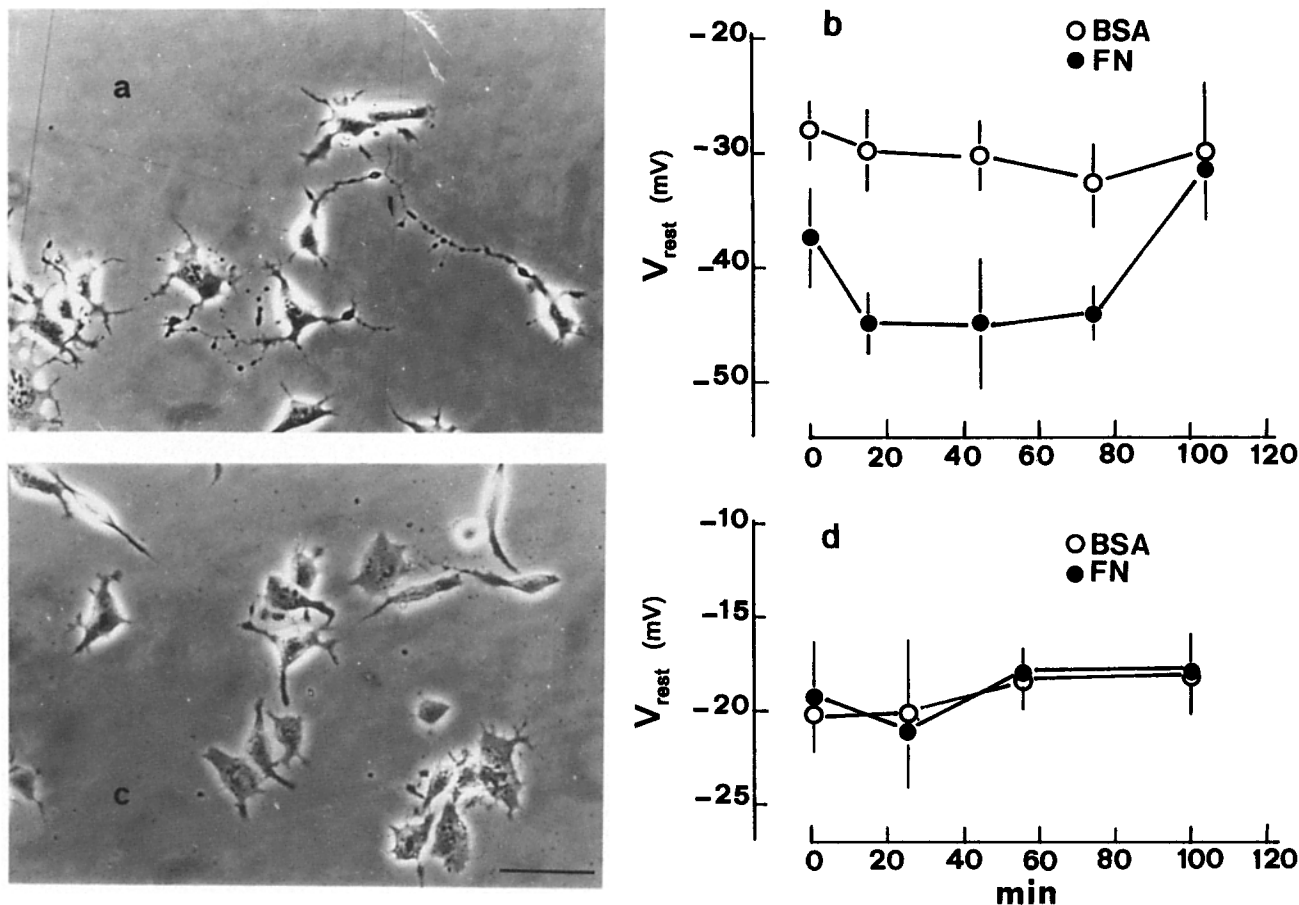


Figure 1. Neurite emission and V_{rest} response of N1 and N7 cells plated on BSA- or FN-coated dishes. (a and c). Morphology of N1 and N7 cells, respectively, after 5 h of incubation on FN-coated dishes. Cells, resuspended in DME + BSA medium were plated on FN-coated dishes and, at the end of incubation, fixed with 2.5% glutaraldehyde and photographed under phase-contrast microscopy. (b and d). Time course of V_{rest} of N1 or N7 cells, respectively, plated either on BSA- (○) or FN- (●) coated dishes in DME + BSA. At indicated times, dishes were transferred to the patch clamp apparatus and V_{rest} measured in whole cell configuration, in current clamp conditions ($I = 0$). Values are means \pm SEM of single cell measurements carried out in three separate experiments ($n = 10$). Bar, 50 μ m.

and Augusti-Tocco, 1980), to elongate neurites when cultured for 2–3 d in low serum in the presence of Retinoic acid (Fig. 2).

Interestingly, the above-mentioned different capacity to emit neurites on FN substrata displayed by the two clones was associated with remarkable differences in the V_{rest} time course during the first 2 h of adhesion. In fact, in N1 cells the potential remained fairly constant at least for 2 h after seeding on BSA, whereas in cells plated on FN it underwent a progressive hyperpolarization that attained its maximum (~ -45 mV) at 15 min and persisted for 80–90 min (Fig. 1 b). This FN-induced hyperpolarization was totally absent in N7 cells, whose V_{rest} remained constant at ~ -20 mV, either on BSA or FN (Fig. 1 d).

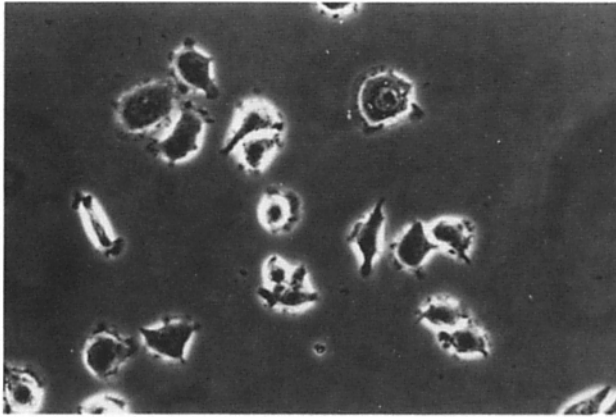
These data suggested a correlation between the ability of NB cells to differentiate upon FN-mediated adhesion and the hyperpolarizing response elicited by this adhesion.

Hyperpolarization and Neurite Emission Are Both Integrin-mediated Phenomena in N1 Cells

The links between the adhesion-promoted effects on V_{rest} and neurite emission were explored by studying the molecular mechanisms of adhesion and the consequences of induced

alterations of these mechanisms. This was pursued by seeding the cells on substrata recognized by different cellular receptors for ECM proteins and by using various inhibitors of integrin binding to these proteins. In Table I estimates of cell adhesiveness (percent of adherent cells at 30 min after seeding) under various conditions are compared to measurements of V_{rest} at 15 min and of neuritogenesis (percent neurite-emitting cells at 5 h). As expected, N1 cells neither adhered nor emitted neurites on BSA, displaying an average V_{rest} of -22 mV. On the contrary, these cells effectively adhered to FN ($\sim 70\%$), both hyperpolarizing and emitting neurites. N1 cell adhesion to FN was drastically reduced by the addition of GRGDSP, a hexapeptide that competes with the FN binding to receptors (Pierschbacher and Ruoslahti, 1984). Remarkably, this peptide abolished both V_{rest} hyperpolarization and neurite emission of N1 cells (Table I), implying that these effects are specifically elicited by FN-integrin interaction. This specificity was stressed by the fact that the non-inhibitory peptide GRGESP was ineffective on the above parameters. Thus, adhesion to FN, as well as its electrical and biological effects, are strictly dependent on the interaction between the cell binding domain of FN and the relative integrin receptor. The direct implication of integrins in

A



B

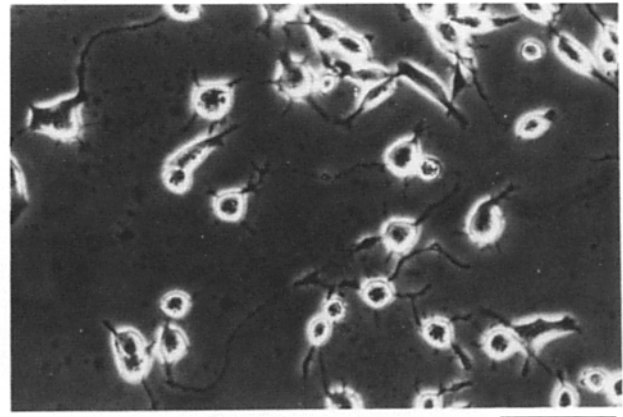


Figure 2. Neurite outgrowth induced by Retinoic acid in N7 cells. (A) Morphology of N7 cells in control culture incubated for 48 h in DME + FCS 10%. (B) Morphology of N7 cells after 2 d of incubation in DME + FCS 1% + Retinoic acid 10^{-7} M. Cells were cultured for 24 h in DME + FCS 10%, thereafter medium was changed and Retinoic acid was added at a final concentration of 10^{-7} M in DME + FCS 1%. After 2 d of incubation cells were fixed with 2.5% glutaraldehyde and photographed under phase-contrast microscopy. Bar, 50 μ m.

the adhesion and relative phenomena in N1 cells was definitely confirmed by the abolition of these phenomena by antibodies raised against the FN receptor (Table I).

Similar conclusions could be drawn from experiments where N1 cells were seeded on VN, another integrin-dependent substratum, to which these cells effectively adhered, hyperpolarizing and emitting neurites at a similar extent as on FN. Here again, both cell adhesion and V_{rest}

hyperpolarization were abolished by GRGDSP, without being affected by the control peptide GRGESP.

On the whole these data, while demonstrating that N1 cells do in fact recognize insoluble FN or VN on the substratum by means of their plasma membrane integrins, opened the question of whether V_{rest} hyperpolarization and neurite emission were consequences of the adhesion per se, or were specifically operated by an integrin-mediated signal elicited

Table I. Effects of Various Treatments on Cell Adhesion V_{rest} and Neurite Outgrowth of N1 and N7 Cells

Substratum	Percent adherent cells	V_{rest} (mV)	Percent cells with neurites
N1 cells			
BSA	9.4 ± 6.4 (2)	-22.6 ± 1.5 (24)	0
FN	66.0 ± 11.7 (4)	-41.3 ± 1.8 (18)	52.4 ± 3.6 (3)
FN+GRGDSP	29.6 ± 12.8 (2)	-23.6 ± 3.2 (10)	0
FN+GRGESP	73.6 ± 8.5 (3)	-38.0 ± 2.9 (6)	49.6 (1)
FN+ α BHK	12.4 ± 1.8 (2)	-19.7 ± 4.6 (6)	0
VN	77.6 ± 12.8 (3)	-38.6 ± 1.3 (13)	34.8 ± 2.6 (3)
VN+GRGDSP	4.2 ± 3.4 (4)	-17.1 ± 2.2 (11)	2.3 ± 1.9 (3)
VN+GRGESP	74.6 ± 24.3 (2)	-35.0 ± 5.4 (4)	32.2 (1)
PF4	69.6 ± 3.1 (3)	-18.7 ± 2.0 (17)	2.6 ± 1.0 (2)
N7 cells			
BSA	10.9 ± 5.7 (2)	-18.4 ± 1.8 (18)	0
FN	71.6 ± 7.8 (3)	-16.5 ± 2.4 (14)	4.1 ± 0.5 (2)
FN+GRGDSP	21.3 (1)	—	0
FN+GRGESP	66.5 ± 13.3 (3)	—	4.2 (1)
FN+ α BHK	28.6 (1)	—	—
VN	70.3 ± 3.8 (2)	-17.0 ± 2.2 (14)	4.6 ± 2.2 (2)
VN+GRGDSP	12.3 ± 0.7 (2)	—	0
VN+GRGESP	62.7 ± 3.4 (2)	—	3.7 (1)

Cells were plated on petri dishes coated with FN, VN, or PF4 as described in Materials and Methods. Before cell plating GRGDSP or GRGESP peptides (500 μ g/ml) or α -BHK antibodies (1:25 dilution) were added to the dishes. For cell adhesiveness assay, [3 H]thymidine labeled cells were detached after 24 h of labeling and plated on 96-well clusters previously coated with various proteins, in the absence or in the presence of peptides, or anti-integrin (BHK) antibodies. After 30 min of incubation, the cell radioactivity was counted by liquid scintillation. Values are means \pm SEM of the number of experiments listed in parentheses, each carried out in quadruplicate. For V_{rest} measurements cells were used 15 min after seeding, and the potential measured in single cells by means of the patch clamp technique, in whole cell configuration and current clamp conditions ($I = 0$). Values are means \pm SEM of measures on single cells. For determination of neurite outgrowth cells were fixed with 2.5% glutaraldehyde after 5 h of incubation. The percentage of cells with neurites was determined under phase contrast microscopy by counting at least 300 cells per experiment. Values are means \pm SEM of the number of experiments listed in parentheses.

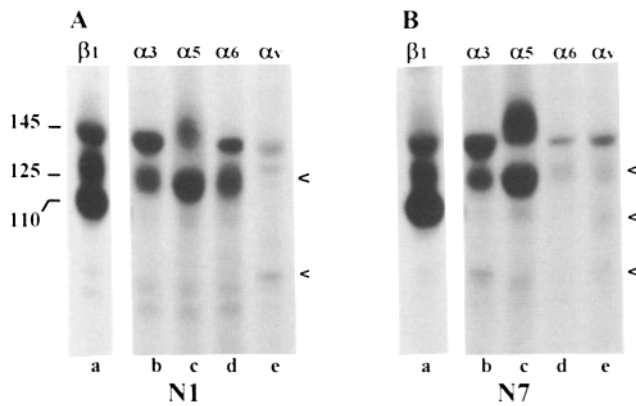


Figure 3. Integrin profile of N1 and N7 clones. Cells were metabolically labeled with [³⁵S]methionine and cysteine and extracted with detergents. Integrins were immunoprecipitated with antibodies specific for $\beta 1$ (lane a), $\alpha 3$ (lane b), $\alpha 5$ (lane c), $\alpha 6$ (lane d), and αV (lane e). (A) clone N1; (B) clone N7. Numbers on the left indicate the estimated molecular mass of the α (145 kD), $\beta 1$ (125 kD) and of the immature form of $\beta 1$ (110 kD). The putative β subunits associated with αV in N1 and N7 clones are indicated by arrowheads on the right of each panel.

by this adhesion. The answer to this question was provided by experiments in which N1 cells were plated on substrata enriched with platelet factor 4 (PF4), an integrin-independent adhesion molecule that recognizes the cell proteoglycans (Culp et al., 1986). As reported in Table I, these experiments showed that N1 cells effectively adhere to PF4, without hyperpolarizing nor emitting neurites. Thus, hyperpolarization and neuritogenesis are strictly correlated and integrin-mediated phenomena, that cannot be induced by adhesion per se.

The Dissociation of Integrin-mediated Adhesion Process from V_{rest} Hyperpolarization in N7 Cells

Recalling that, in N7 cells, adhesion to FN was dissociated from V_{rest} hyperpolarization and neuritogenesis (Fig. 1, c and d), comparison of N7 with N1 clone offered at this point the possibility to test whether such a dissociation was attributable to differences between the two clones in the adhesion process to ECM proteins.

As shown in Table I, the adhesion pattern to FN and VN displayed by N7 cells turned out to be substantially the same as that of N1 cells, namely strictly dependent on integrin receptors. Note that, as in the case of FN, the N7 adhesion to VN did not produce either hyperpolarization or neurite emission.

Thus, in N7 cells the integrin involvement in cell adhesion did not elicit electrical and differentiative signals as it occurred in N1 clone. This could be due to one of the following possibilities: (a) a difference in integrin profile between the two clones; or (b) the lack of a purportive functional link between integrins and the V_{rest} hyperpolarizing mechanism in N7 cells. The first possibility was experimentally tested by comparing the integrin profile of the two clones.

Integrins expressed on N1 and N7 clones were analyzed by immunoprecipitation from metabolically labeled cells and sodium dodecylsulphate polyacrylamide gel electrophoresis. Polyclonal antibodies to COOH-terminal sequences of integrin molecules, showing wide cross-reactivity among

integrins of different animal species, were used. The $\alpha 6$ subunit was identified by the mAb GoH3 (Sonnenberg et al., 1988), known to react with both human and mouse proteins. As shown in Fig. 3, both N1 and N7 clone expressed several integrin complexes. Antibodies to $\beta 1$ immunoprecipitated two major bands corresponding to β (125 kD) and its associated α subunits (145 kD) (Fig. 3, lanes a). A third band of lower molecular mass (110 kD) was also present and corresponded to an immature form of the $\beta 1$ subunit (Defilippi et al., 1991). The α subunits were identified by specific antibodies as the $\alpha 3$, $\alpha 5$, $\alpha 6$, and αV (Fig. 3, lanes b-e). $\alpha 1$, $\alpha 2$, and $\alpha 4$ were not expressed on these cells.

The integrin profile of N1 and N7 clones were similar (Fig. 3, A and B). Minor differences were observed in the amount of the laminin receptor $\alpha 6/\beta 1$ and in the labeling intensity of the $\alpha 5$ subunit (fibronectin receptor). In addition, the β subunits associated with the αV appear to differ slightly between the two clones. Both clones express an αV -associated $\beta 1$ integrin, but the expression is higher in N7 as compared to N1 cells (Fig. 3, upper arrowheads in lanes e: the N1 cell $\beta 1$ is scarcely visible in the photo). In addition, both N1 and N7 cells express an αV -associated β_x (90 kD) (lowest arrows in lanes e). N7 cells express a third αV -associated β subunit (middle arrow), that is missing in N1 cells. αV is known to associate with several different β subunits including $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 8$ (Vogel et al., 1990; Hynes, 1992). Both N1 and N7 clones were negative for $\beta 3$, as determined by immunoprecipitation with antibodies to human $\beta 3$ COOH-terminal peptide (not shown). The exact nature of these β subunits remains to be determined due to the scarcity of reagents for these molecules. On the whole, N7 cells displayed a wider spectrum of αV -associated β subunits than N1 cells, while quantitative differences in this spectrum, if significant, are in favor of N7 cells.

Thus, recalling that among the integrins expressed on the two neuroblastoma clones the possible receptors for fibronectin and vitronectin are $\alpha 5/\beta 1$ and the αV -containing complexes (Vogel et al., 1990; Hynes, 1992), the differences observed in the expression of these complexes are not likely to account for the clear cut difference in the neurite extension response between the two clones.

At this point, the lack of electrical response to the integrin-mediated adhesion in N7 cells seems to rely on the absence of a functional link between these integrins and the hyperpolarizing mechanism.

This in turn implied the existence of such a link in N1 cells, to be sought through a detailed electrophysiological analysis of ion channels regulating V_{rest} in the two clones.

The Identification of Ion Channels of the N1 Cell Plasma Membrane

The identification of ion channels of N1 cells was carried out by electrophysiological procedures apt to characterize them at their maximum level of activation, irrespective of the substratum onto which the cells were seeded.

Fig. 4 A illustrates the I-V plot obtained in an N1 cell in standard extracellular saline, showing a relatively steep activation of an outward current at about -10 mV. This current was 80% blocked by 30 mM tetraethylammonium (TEA) (not shown), while was insensitive to 2 mM Ba^{2+} and 5 mM Cs^+ (see below). These features are typical for the K^+ cur-

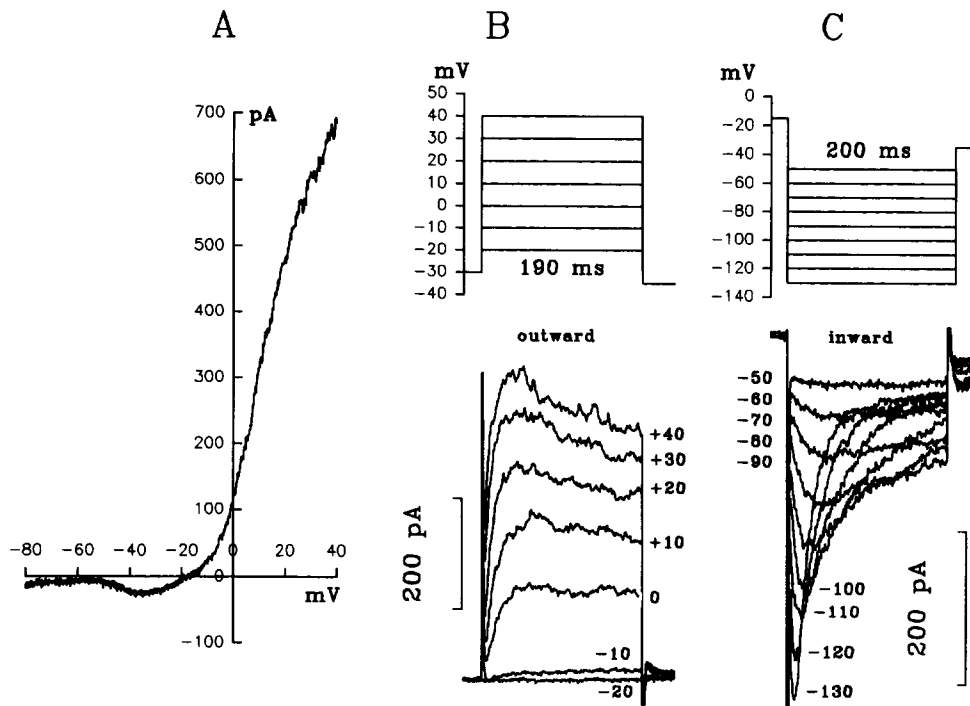


Figure 4. Potassium currents registered in whole cell configuration in single N1 cells. (A) I-V plot in standard extracellular saline ($[K^+]_o = 5$ mM). (B) The depolarizing voltage steps (upper panel) imposed to the holding potential, and the corresponding current traces (lower panel) characterizing the K_{DR} channel. Extracellular saline containing low K^+ concentration ($[K^+]_o = 5$ mM). (C) The hyperpolarizing voltage steps (upper panel) imposed to the holding potential and the corresponding current traces (lower panel) characterizing the K_{IR} channels. Extracellular saline containing high K^+ concentration ($[K^+]_o = 40$ mM).

rent operated by the so called “delayed rectifier” K^+ (K_{DR}) channels (Hodgkin et al., 1949). This type of channel is present and active in the excitable cells, accounting for their strong membrane polarization at rest (Hille, 1992). In Fig. 4 B (lower panel) are shown the superimposed traces of this current obtained by imposing the series of depolarizing voltage pulses indicated in the upper panel. The currents elicited by these pulses persisted unmodified for more than 200 ms, revealing a low inactivation kinetics, typical of K_{DR} channels.

No other current was revealed by the I-V plot of Fig. 4 A, except the small inward inflection around -35 mV, that was attributable to the transient, tiny, Ca^{2+} current mediated by Ca^{2+} channels of type “T,” almost ubiquitous in excitable cells (Tsien et al., 1988) and already described in these cells (Becchetti et al., 1992b).

However, after changing $[K^+]_o$ from 2 to 40 mM, and by imposing the hyperpolarizing voltage commands shown in Fig. 4 C (upper panel), we obtained the superimposed set of current traces reported in the lower panel. This current is very similar to that described by Bauer et al. (1990) in GH3 cells and identified, in the same potential range, as due to the K^+ current mediated by the “inward rectifier” channels (K_{IR}). The latter are typically present in heart muscle cells (Sakmann and Trube, 1984), although described in non-excitable cells, including eggs (Hagiwara and Takahashi, 1974; Hille, 1992). The peculiarity of these channels is their exclusive activation at membrane potentials more negative than E_K . This makes it that K_{IR} channels are barely visible under experimental conditions as those of Fig. 4 A, whereas they can be easily detected by clamping E_K to strongly depolarized values. Typically, in the physiological range of membrane potentials, the K_{IR} channels are scarcely conducting (Fukushima, 1982; Mazzanti and DiFrancesco, 1989). However, their currents are not disregardable, because they counteract inward currents, thus producing the

balance which sets the resting potential. The physiological role usually invoked for K_{IR} channels is simply that of sustaining depolarized membrane potentials (where normally high K^+ currents should flow), without dissipating ionic gradients and keeping to a minimum the metabolic energy consumption of the Na-K-ATPases (Hille, 1992).

The Comparative Analysis of K^+ Channels in N1 and N7 Cells, and the Lack of K_{IR} in N7 Cells

The pharmacological properties of K^+ channels in both clones of neuroblastoma cells were analyzed in experiments where cell currents were studied either in the absence or in the presence of classical inhibitors of these channels, such as Cs^+ and Ba^{2+} . As exemplified in Fig. 5, A and B (upper panels) neither 5 mM Cs^+ , nor 2 mM Ba^{2+} significantly affected the I-V plot recorded from a single N1 cell, indicating that the K_{DR} currents, which are suitably revealed by this plot, were left unaltered by these inhibitors. When performed on N7 cells this type of analysis demonstrated an identical I-V plot, equally unaffected by Ba^{2+} (Fig. 5 C, upper panel) and Cs^+ (not shown). This leads us to conclude that N7 cells possess K_{DR} channels with the same functional and pharmacological properties as those previously pointed out for N1 cells. However, a striking difference between the two clones emerged when the comparative analysis was extended to the K_{IR} channels by the step hyperpolarization protocol shown in Fig. 5, A-C (lower panels). In fact, this protocol failed to reveal any K_{IR} current in N7 (Compare C with A and B, open symbols), supporting the conclusion that K_{IR} channels are either lacking or totally ineffective in these cells. The same conclusion was attained in experiments where $[K^+]_o$ was raised up to 40 mM, a procedure that, by lowering E_K close to V_{rest} , increases the accuracy of revealing K_{IR} currents (Hagiwara and Yoshii, 1979).

On the whole, the comparison between N1 and N7 clones

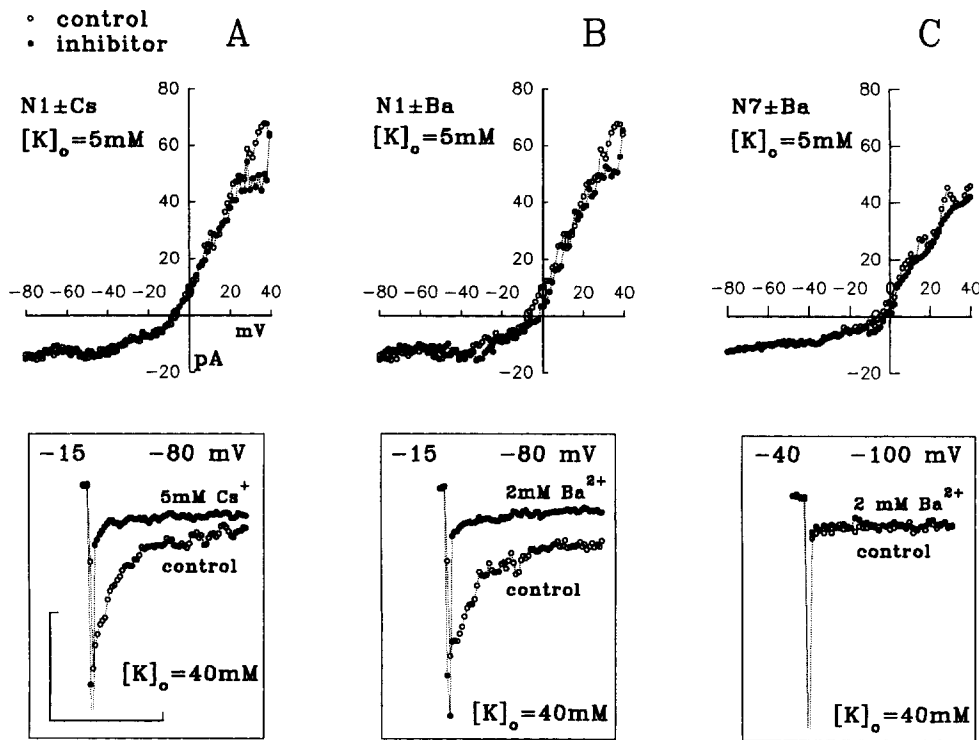


Figure 5. Effects of Cs^+ and Ba^{2+} on the K^+ currents in two N1 cells (A and B) and one N7 cell (C). In the upper panels I-V plots in the range $-80/+40$ mV were obtained either in the absence (\circ) or the presence (\bullet) of 5 mM Cs^+ (A) or 2 mM Ba^{2+} (B and C) with the standard extracellular $[\text{K}^+]_o = 5$ mM. In the lower insets (same cells of the upper panels) are shown the superimposed traces of K_{IR} currents elicited at the indicated membrane potentials, either without, or with the indicated inhibitors (same symbols of the upper panels). Measurements were carried out in whole-cell configuration, with $[\text{K}^+]_o = 40$ mM to increase the current amplitude. Calibration bars indicate 50 pA (vertical) and 400 ms (horizontal).

revealed that, while the integrin-mediated adhesion to FN or VN is sustained by an identical process, the electrical unresponsiveness of N7 to this adhesion is associated with the lack of K_{IR} channels; this suggested that K_{IR} channels were in fact responsible for the adhesion-promoted hyperpolarization of V_{rest} in N1 cells. In this light the comparison between the two clones proposed a valuable model to investigate the correlation between the electrical events and the integrin-mediated neuritegenesis. The suitability of this model was reinforced by the fact that, at difference from the K_{DR} current, the K_{IR} currents of N1 cells were severely blocked by Cs^+ as well as by Ba^{2+} . (Fig. 5, A and B, lower panels), so that, from an electrical point of view, the addition of these inhibitors should revert the N1 clone to the N7 behavior.

The V_{rest} Regulation by K_{IR} in N1 Cells

The depolarized (-22.6 mV) average value of V_{rest} registered in N1 cells plated on BSA (see Table I) suggested a low basal activation of hyperpolarizing conductances in these conditions. This was confirmed by the fact that, after decreasing $[\text{K}^+]_o$, the average V_{rest} remained practically unchanged (-22 ± 1.56 mV, $n = 32$). However, analyzing data obtained on single cells, it emerged that, while V_{rest} ranged between -10 and -43 mV, the addition of 2 mM Ba^{2+} or the increase in $[\text{K}^+]_o$ always determined a cell depolarization directly proportional to the initial value of this potential. Furthermore, in the few cells ($\sim 7\%$) with initial V_{rest} around -40 mV, Ba^{2+} addition reverted the potential to -22 mV. On the contrary, the addition of TEA at concentrations blocking the K_{DR} channels never depolarized the cells more than 1–2 mV.

This leads us to conclude that, beyond a basal level of ~ -20 mV, V_{rest} is modulated in N1 cells by the state of activation or channel density of K_{IR} channels.

The Integrin-mediated Hyperpolarization Is Sustained by the Activation of K_{IR} Channels

The elucidation of N1 cells electric response to the integrin-mediated adhesion required the follow up of the very early events of this response at the single cell level. This was obtained by adopting the following experimental protocol, previously described for MELC (Becchetti et al., 1992a). Briefly, a single cell, sedimented onto BSA-coated dishes, was patch-clamped and immediately moved in gentle contact with an FN-coated bead. Continuous switching between voltage- and current-clamp allowed measurements of K_{IR} and K_{DR} currents, or V_{rest} . A typical experiment obtained with this procedure is reported in Fig. 6, showing that contact with the FN-coated bead led to a direct activation of K_{IR} channels.

In A, the maximum K_{IR} currents (peak I_{IR} current) and V_{rest} at the corresponding times, were plotted versus the time elapsed from the contact. At ~ 650 s, 2 mM Ba^{2+} was added by perfusion for 25 s and then washed out. As shown, the K_{IR} current (\bullet) and V_{rest} (\circ) displayed very similar time courses. Note that, within 810 s from the contact, the K_{IR} current is threefold increased (insets). Ba^{2+} addition produced an almost complete block of the current, while reverting the V_{rest} to the time zero values. Both the current block and the potential reversal were totally removed after Ba^{2+} withdrawal.

Contrary to the K_{IR} current, the K_{DR} current was only slightly influenced by the cell contact with the FN-coated beads. This is evident from Fig. 6 B, showing the I-V plot recorded at times 0, 120, and 810 s, in the same cell as in Fig. 6 A. Between 0 and 810 s only a minor increase in K_{DR} current occurred, while, as expected, this current was totally insensitive to Ba^{2+} .

On the whole, these electrophysiological data indicated that K_{IR} are the only channels involved in the hyperpolarization.

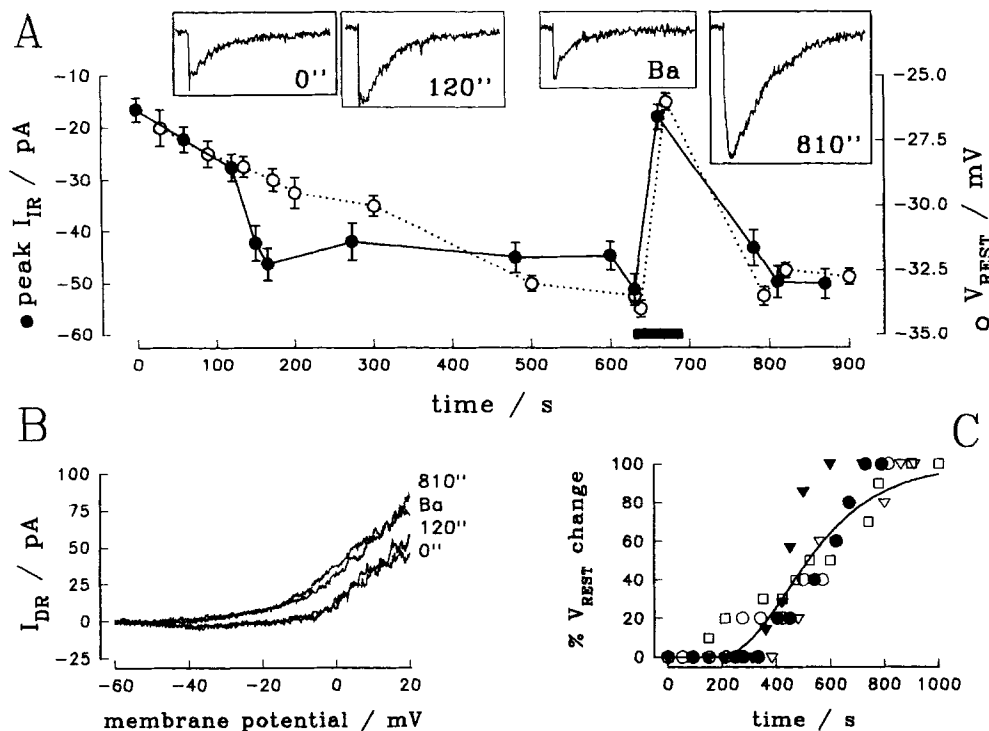


Figure 6. (A) Time course of the peak K_{IR} currents (peak I_{IR} , left axis, ●) and V_{rest} (right axis, ○) after an NI cell contact to an FN-coated bead. Cells were plated on BSA-coated dish containing DME + BSA medium, to which FN-coated beads (diameter 11.6 μ m) were added and left to sediment. A single cell was then patch-clamped (whole cell) and moved in contact with a bead. V_{rest} and K_{IR} measurements were taken in current- or voltage-clamp conditions, respectively. (Insets) K_{IR} current recordings elicited at -100 mV (for 250 ms) from a holding potential of -10 mV at the indicated times. After ~ 650 s from the initial contact and for the indicated time (black horizontal bar) 2 mM Ba^{2+} was applied and then washed out. Note the blocking effect of Ba^{2+} on the K_{IR} current and the concomitant depolarization. (B) I-V plot of the K_{DR} current taken at the indicated times after contact in the same cell used in A, by applying a ramp protocol between -80 and $+20$ mV. Note the lack of significant activation of this current after contact and its insensitivity to Ba^{2+} . Recordings were corrected for a linear leakage (leakage conductance 1.9/4.5 G Ω). (C) Time course of the percent (%) V_{rest} change (hyperpolarization) recorded in five experiments similar to that shown in A. Data were normalized to the maximal hyperpolarization obtained in each experiment. Different symbols refer to different cells. The best fitting line corresponds to the function $(1 - e^{-(t-91)/192})^4$.

tant depolarization. (B) I-V plot of the K_{DR} current taken at the indicated times after contact in the same cell used in A, by applying a ramp protocol between -80 and $+20$ mV. Note the lack of significant activation of this current after contact and its insensitivity to Ba^{2+} . Recordings were corrected for a linear leakage (leakage conductance 1.9/4.5 G Ω). (C) Time course of the percent (%) V_{rest} change (hyperpolarization) recorded in five experiments similar to that shown in A. Data were normalized to the maximal hyperpolarization obtained in each experiment. Different symbols refer to different cells. The best fitting line corresponds to the function $(1 - e^{-(t-91)/192})^4$.

zation elicited by the NI cell contact with FN-coated beads, the other channels being neither activated nor inactivated. An average time course of V_{rest} after contact is reported in Fig. 6 C, where data obtained from five separate cells were normalized as a fraction of maximum potential registered within the corresponding single cell recording, and plotted versus the time after contact with the FN-coated bead. It is evident from this figure that hyperpolarization, that is the K_{IR} activation, starts after a 50–150 s delay after contact, manifesting as an early event triggered by the FN-integrin interaction.

The Dependence of FN-induced Hyperpolarization on G Protein Activation

Since many K^+ channels are regulated by G proteins (Birbaumer et al., 1990), these proteins appeared reasonable candidates for the molecular link between integrins and K_{IR} channels in NI cells. This possibility was verified by testing the electric response to the adhesion of these cells to FN after treatment with Pertussis toxin (PTX), a potent inhibitor of G activating proteins (Kaslow and Burns, 1992). In these experiments cells were incubated overnight with PTX and plated either on BSA or FN for 15 min in the absence of the toxin. As shown in Table II, treatment of NI cells with PTX, while not affecting the V_{rest} of cells plated on BSA, abolished the FN-induced hyperpolarization. Recalling that V_{rest} is regulated by K_{IR} channels (see above), we draw the following suggestions from these data: (a) the lack of PTX effects indicates a minimum level of G protein dependent activation of these channels in BSA-plated cells, in keeping with their depolarized V_{rest} ; and (b) the FN-induced activa-

tion of K_{IR} channels strictly depends on the channel interaction with the activating G protein. Remarkably, the effects of PTX on the electric response were once again indissociable from those on neurite emission, that was in fact drastically reduced by the toxin. In this light, a G protein dependent signaling pathway seems involved in both the electrical and differentiative responses elicited by integrin-mediated adhesion of NI cells.

The Block of K_{IR} Channels Inhibits Neurite Emission

Data so far provided evidence that, at any level studied, K_{IR} channel activation and neuritogenesis are correlated phenomena. The sensitivity of K_{IR} to Cs^+ and Ba^{2+} could at this

Table II. Effect of PTX Pretreatment on V_{rest} and Neurite Outgrowth of NI Cells

Treatment	V_{rest} (mV)	Percent cells with neurites
BSA \pm PTX	-18.0 ± 6.0 (3)	0
FN	-36.4 ± 2.1 (21)	29.6 ± 1.6 (5)
FN+PTX	-19.6 ± 3.1 (13)*	11.4 ± 3.1 (2)†

Control cells or cells treated overnight with PTX (100 ng/ml) were resuspended in DME+BSA and plated on BSA- or FN-coated dishes. V_{rest} was measured 15 min after cell seeding in whole cell configuration and current clamp conditions ($I = 0$). Values are means \pm SEM of the number of measurements listed in parentheses and carried out in four separate experiments. Neurite count was performed at the end of a 5 h incubation after fixing the cells with 2.5% glutaraldehyde under phase-contrast microscopy. Values are means \pm SEM of ~ 8 –10 fields containing 25–30 cells (~ 250 cells counted) obtained from the number of experiments listed in parentheses.

* $p < 0.001$.

† $p < 0.005$.

(t test for independent samples, compared with FN.)

Table III. Effects of Cs⁺ on V_{rest} and Neurite Outgrowth in N1 Cells

Treatment	V _{rest} (mV)	Cells per field	Percent cells with neurites
FN	-35.0 ± 1.2 (6)	27.2 ± 2.4 (7)	33.2 ± 2.8 (7)
FN+CsCl	-20.6 ± 2.1 (10)*	26.5 ± 1.0 (4) NS	10.1 ± 1.3 (4)*
FN+NaCl	ND	32.3 (1)	27.5 (1)

Cells were resuspended in standard DME + BSA containing 5 mM CsCl or NaCl, and plated on FN-coated dishes. V_{rest} was measured 15 min after seeding in whole cell configuration and current clamp conditions (I = 0). Values are means of the number of determinations listed in parentheses, obtained in three separate experiments. Neurite count was performed at the end of a further 5-h incubation, after cell fixing with 2.5% glutaraldehyde. The number of cells per field and the percentage of cells emitting neurites was determined under phase-contrast microscopy 5 h after cell plating. Values are means ± SEM of ~8-10 fields containing 25-30 cells (~250 cells counted), obtained in the number of experiments listed in parentheses.

* *p* < 0.001 (*t* test for independent samples, compared with FN).
NS, not significantly different as compared with FN.

point be exploited to discriminate whether these phenomena were linked in a causal sequence. To this purpose, Ba²⁺ resulted scarcely suitable since, at the required concentrations, it tended to precipitate in the DME medium to be used for this type of experiments. This inconvenience was not presented by Cs⁺, that equally blocked the K_{IR} channels (see Fig. 5 A, lower panel). This Cs⁺ block, like the Ba²⁺ block (see Fig. 6 A), resulted totally reversible after washing out of the cation (not shown). As shown in Table III, Cs⁺ prevented the FN induced V_{rest} hyperpolarization and this block was associated with the inhibition of neuritogenesis. It is worth stressing that Cs⁺ had no apparent aspecific toxic effects under these conditions. In fact, the cation did not alter the percentage of adherent cells per field, a reliable viability test for these cells, that detach from substrata any time they are seriously damaged; this, however, did not exclude that Cs⁺ might aspecifically impair neuritogenesis. That this was not the case and that abolition of neuritogenesis was a consequence of the impairment of the electrical response to integrin activation was confirmed by the experiments reported below.

In Fig. 7 A is reported an experiment in which the cells were plated on FN-coated dishes in DME + BSA containing 5 mM Cs⁺. This cation was removed after different times (0-140 min) and the cells further incubated in DME + BSA

for the time required for neuritogenesis (5 h). When Cs⁺ was removed within the first 20-30 min after cell plating, neuritogenesis normally occurred thereafter, while it was progressively inhibited as the time of exposure to Cs⁺ was incremented. This meant that exposures to Cs⁺ longer than 20-30 min inhibited the capability of NB cells to emit neurites in response to FN substrata, despite the reversibility of K_{IR} block after Cs⁺ removal. The inhibition of neuritogenesis could be accounted for by one of the following alternatives: (a) longer incubations than 20-30 min in Cs⁺ irreversibly compromised the cell viability (or at least the capability to elongate neurites); or (b) the very first time of contact with FN represented a crucial time for commitment to neuritogenesis, during which Cs⁺ must be absent. That the second possibility was true was demonstrated by the experiments reported in Fig. 7 B, where the cells were plated on FN-coated dishes in DME + BSA without Cs⁺, that was added after various times (0-140 min) of incubation. After addition, Cs⁺ was always maintained in the culture medium for the successive 5 h. When Cs⁺ was added after the first 2 h after plating, it was totally ineffective on neuritogenesis, indicating that this process was not aspecifically compromised by this cation. On the contrary, the inhibitory effect of Cs⁺ on neuritogenesis fully manifested when addition was performed within the first 20-30 min of contact with the

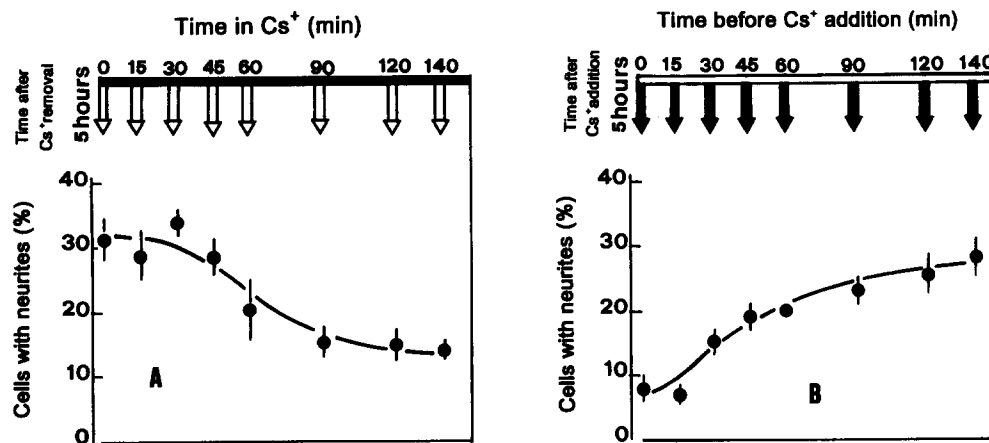


Figure 7. Effects of Cs⁺ on FN-induced neurite emission by N1 cells in relation to the time elapsed from the cell contact with the substratum. (A) Cells were resuspended in DME + BSA medium containing 5 mM CsCl and plated on FN-coated dishes. At the indicated times, the supernatant medium was gently removed and substituted with DME + BSA without CsCl; the cells were further incubated for the time required for neurite outgrowth (5 h) in the absence of the cation

(open arrows). (B) Cells were plated on FN-coated dishes in DME + BSA medium without CsCl. At the indicated times, the supernatant was substituted with medium containing 5 mM CsCl and the cells were further incubated for 5 h in the presence of the cation (closed arrows). Neurite outgrowth was measured at the end of the total time of incubation (time without+time with Cs⁺). Experimental values are means ± SEM of 10 measurements.

FN-coated dishes. It is concluded that Cs^+ , while devoid of any aspecific toxic effect on neuritogenesis, inhibited this process within the first 90 min of contact, that is the time length of the FN-induced V_{rest} hyperpolarization (see Fig. 1 *b*).

Discussion

In the present study we provided evidence that, upon interaction with ECM proteins, integrins activate in NB cells an outside-to-in pathway that converges into an electric signal directly implicated in the commitment of neurite outgrowth.

On the basis of data obtained so far, this pathway can be confidently outlined as a transducing sequence from integrin receptors to K_{IR} channels, via a PTX-sensitive G protein.

Integrin implication at the origin of this sequence was supported by the fact that antibodies directed against these receptors, as well as inhibition of their binding to FN or VN by competitive peptides, abolished the whole triad "cell adhesion, V_{rest} hyperpolarization, neurite outgrowth" (Table I). This implication however, cannot be ascribed to the adhesive process per se, that occurred at an even stronger extent for N7 cells on FN or VN and for N1 cells themselves on PF4, without producing V_{rest} hyperpolarization or neurite outgrowth. Therefore, these N1 cell responses appear to rely on integrin ability to act as true signaling receptors (Hynes, 1992). Moreover, the implication of the RGD-recognizing sequence of the integrin receptors in the elicitation of these responses, suggests that transition of integrins to the "activated" state is produced by their specific interaction with the cell binding domain of ECM proteins.

Although indirect, the evidence for the G protein involvement in the integrin-dependent pathway is rather convincing, since PTX abolished hyperpolarization and neurite outgrowth in N1 cells plated on FN, without altering their viability and V_{rest} on BSA.

On the other hand, patch clamp experiments unequivocally demonstrated that the integrin-elicited signal has its hyperpolarizing target in the K_{IR} channels, that govern V_{rest} in N1 cells. Thus, the most simple explanation of our data is that a PTX-sensitive G protein functionally links integrins to K_{IR} channels. A similar pathway was proposed by Williams et al. (1992) and Saffel et al. (1992) to account for the CAM-dependent activation of Ca^{2+} channels, a crucial step to neurite outgrowth in PC12 cells. In the above sequence, the integrin-mediated activation of the G protein could be attributed to a hypothesized capability of integrins to act as coreceptors with the G protein-coupled receptors (Hynes, 1992).

Increasing evidence is being gathered that K_{IR} channels are indeed modulated by G proteins (Inoue and Yoshii, 1992). In fact, receptors for substance P and TRH were found to control K_{IR} channels in brain neurons (Stanfield et al., 1985; Nakajima et al., 1988) and in GH3 cells (Bauer et al., 1990; Barros et al., 1992), respectively. The same was found for Angiotensin II receptors in mouse renal juxtaglomerular cells (Kurtz and Penner, 1989). In all these cases however, the G protein control was in fact inhibitory, and the final effect was a depolarization, putatively due to a voltage shift of the steady state inactivation curve (Bauer et al., 1990).

The activation of K^+ channels downstream of integrin

binding to FN was demonstrated for the first time by our study on MELC (Arcangeli et al., 1991; Becchetti et al., 1992*a*). This activation concerned the Ca^{2+} -dependent K^+ channels, whose functional linkage with the integrins was probably represented, rather than by a G protein, by a cytoplasmic kinase, activated at the adhesion site and diffusing within the cell. Thus, hyperpolarization is a recurrent effect of integrin activation in various cell types, that involves the V_{rest} -regulating K^+ channels, although the category and functional link to integrins of these channels differ from one cell type to another.

In the case of N1 cells, activation of K_{IR} channels is an essential condition for the promotion of neurite outgrowth through the integrin-mediated pathway. This conclusion is supported by the following considerations: (*a*) K_{IR} channels are not demonstrable in the N7 clone, that fails to emit neurites upon the integrin-mediated adhesion to ECM proteins. Most probably, this undetectability reflects the absence of the channels, as no K_{IR} current was elicited by voltage step protocols apt to produce their maximal activation. This seems to rule out that in N7 cells K_{IR} channels, although present, are not activated via the integrin-dependent pathway because of some upstream block of this pathway. Such conclusion is consistent with the analysis of the integrin profile of N7 as compared to N1 cells. In fact, among the integrins expressed on the two clones, the minor differences in the possible receptors for FN and VN ($\alpha 5\beta 1$, αV containing complexes) (Fig. 3), cannot be easily taken as responsible for the all or null response of V_{rest} in the two clones. The possibility remains to be explored, however, that a minor $\alpha 5/\beta 1$, αV containing, component is the only way to bring the signal onto the G protein(s) controlling K_{IR} channels. (*b*) The block of K_{IR} channels with Cs^+ reverted the outside-to-in response of N1 to that of N7 cells, dissociating adhesion from either hyperpolarization and neurite outgrowth. This dissociation was produced only when Cs^+ was present for the first 2 h after N1 cells contacted the FN-enriched substrata and, although occurring with no apparent side effects on cell viability and adhesiveness, persisted after Cs^+ was removed from the culture. On the contrary, when the cation was added after the first 2 h, its presence in the culture throughout the successive time was fully compatible with a normal neuritogenesis (Table III and Fig. 7).

On the basis of these data we conclude that (*a*) although neuritogenesis requires a relatively long time (~ 5 h) to be accomplished, its commitment (meant as the irreversible switch on of the underlying genomic and/or biochemical programs) is performed within the first two hours of cell adhesion to ECM proteins; (*b*) K_{IR} channels must be operative during this commitment; and (*c*) when produced after commitment, inhibition of K_{IR} channels is without consequences on neuritogenesis. At this point, it should be stressed that the hyperpolarization sustained by K_{IR} channels lasts at its maximum between 30 and 90 min after cell plating on FN- or VN-enriched substrata, and then spontaneously reverts to the time zero value at 120 min (Fig. 1 *b*). Thus, whatever the integrin-mediated mechanism responsible for the K_{IR} channel activation, this mechanism is only operative during the commitment time of neuritogenesis, resulting down regulated at the end of this time.

Altogether, these data support the view that a definite period of K_{IR} channel-mediated hyperpolarization is the

commitment signal to neuritogenesis in N1 cells. This view would in fact imply that the Cs^+ inhibition of the electrical signal is a sufficient condition to prevent neurite outgrowth, and account for the ineffectiveness of the cation after commitment has been accomplished and K_{IR} channels are spontaneously closed.

The above interpretation of our data opens intriguing questions on the way of triggering neuritogenesis by the electrical signals. Neuritogenesis is a poorly known process, whose transduction elements are largely undefined not only for the adhesion-mediated way, but also for the neurotrophic agents, like NGF (Bixby and Harris, 1991; de Curtis, 1991). However, the numerous studies on this topic point out a remarkable redundancy in molecular mechanisms responsible for this process, possibly to avoid irreparable impairment caused by single mutations (Bixby and Harris, 1991). This redundancy should favor combinational pathways, such that neuritogenesis can be achieved by the concerted function of several molecule-receptor systems, including different classes of adhesion molecules (integrins, CAM) and receptors for classical neurotrophic factors, like NGF (Bixby and Harris, 1991). In our model, a reminiscence of this redundancy is possibly the fact that the N7 cells maintain the capability of neurite outgrowth in response to a soluble inducer of neuronal differentiation, such as Retinoic acid, a feature shared with N1 cells and the parental cell line (Fig. 2). The different time course between the adhesion-mediated pathway (5 h) and the Retinoic acid-mediated one (2 d), argues in favor of different induction mechanisms involved in these pathways.

The involvement of electrical events in this complex picture was suggested by Doherty et al. (1991) in PC12 cells: they provided indirect evidence that neurite outgrowth can be produced in these cells by opening voltage-dependent calcium channels, via the plasma membrane depolarization in high external potassium concentrations, or by calcium agonists; both these treatments mimicked the CAM- or N-cadherins-induced neuritogenesis (Saffel et al., 1992; Williams et al., 1992). In N1 cells, the hyperpolarizing nature of the electrical integrin-mediated signal rules out a similar mechanism. Consistently, the integrin-dependent neurite outgrowth turned out to be insensitive to calcium antagonists in PC12 cells (Doherty et al., 1991).

On the whole, the explanation we favor of our data is that in neuroblastoma cells hyperpolarization activates voltage-dependent membrane protein(s) capable of promoting the biochemical cascade that leads to neurite outgrowth. Although the existence, not only the species, of this protein is so far merely speculative, evidence is being gathered that activity of most integral and peripheral proteins of the plasma membrane are influenced by trans- and intramembrane electric fields (Brown, 1990; Tsong and Astumian, 1988). In fact, due to the minimal width and the low dielectric constant of the plasma membrane, even a few millivolt variation of V_{rest} can produce intense changes of the electrostatic forces governing the protein interaction within, or at the periphery of, the membrane, with decisive effects on cell programs and behavior (Arcangeli et al., 1993). We propose here that signal protein(s) (for instance tyrosinkinase receptors, G coupled receptors, G protein), responsible for one of the mechanisms promoting neurite outgrowth, "sense" the variations of V_{rest} produced by the integrin-dependent activation of K_{IR} channels. Whatever the merit of these speculations, the elec-

trical aspects deserve attention in any further analysis of the adhesion-promoted response in neuronal differentiation and, more specifically, in neurite outgrowth. Moreover, the implication of electric fields in the membrane signal transduction systems can contribute to unravel so far elusive aspects of cell-cell, or cell-matrix interactions during development or cell differentiation, as well as of cancer invasion and metastatic spread.

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