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_m) 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_m 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 Ca²⁺ and K⁺ delayed rectifier (KDR) channels, while only N1 cells were endowed with inward rectifier K⁺ (Kir) channels. The latter governed the V_m, and, unlike KDR channels, were blocked by Ba²⁺ and Cs⁺. By moving patched cells in contact with FN-coated beads, it was shown that Kir channel activation was responsible for the FN-mediated hyperpolarization of V_m.

Treatment with Pertussis toxin (PTX) abolished this hyperpolarization and neurite outgrowth, indicating that a G protein is interposed between integrins and Kir channels and that the activation of these channels is required for neuritogenesis. In fact, the block of Kir 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.

Xtracellular matrix (ECM) 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, 1988).
Integrin receptors are αβ 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 Ca²⁺ (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-Domini 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; Rebolleau, 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 Ca²⁺-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 (NI and N7) of the 41A3 neuroblastoma cell line derived from the mouse Cl300 parental cell line (Augusti-Tocco and Sato, 1969; Denis-Domini and Augusti-Tocco, 1980), only one of which (NI) 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 NI and N7 subclones of 41A3 murine neuroblastoma cells (Augusti-Tocco and Sato, 1969; Denis-Domini and Augusti-Tocco, 1980) were isolated by limiting dilution. Cells were routinely cultured in DMEM containing 4.5 g/liter of glucose and 10% FCS, and incubated at 37°C in a humidified atmosphere, with 10% CO₂ in air. Cells from a subconfluent culture were detached with 0.25% trypsin (Sigma Immunchemicals; St. Louis, MO), centrifuged at 250 g for 10 min and resuspended in DMEM containing 250 μg/ml of heat-inactivated BSA (DMEM + BSA) at a cell concentration of 3–5 × 10⁵ 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 Yamada 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 μg/ml in DMEM at 37°C for 1 h. Thereafter dishes were rinsed with PBS and further incubated for 1 h with DMEM + BSA. Dishes were then rinsed again with PBS and immediately used for experiments. Latex microbeads (Sigma Immunchemicals; diameter 11.6 μm) were coated with FN by using Grinnell's method (Grinnell, 1981). A few microliters of a saline solution containing coated beads (0.4 × 10⁹ 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 antiseraum, raised against BHK cells (α-BHK) and containing anti-FN receptor antibodies (Tarone et al., 1982) was dissolved in DMEM + BSA and added to cell cultures at time zero at the final dilution of 1:25. The hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GGRDSP, 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 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 rain. 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 routines 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 disregarable capacitive current. Resting resistance of the cells were in the range of 2-6 GΩ.

**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 μCi/ml of methyl-3H-thymidine (specific activity, 10-20 Ci/mmol). 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. Pertussis toxin (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) was dissolved in 100 mM sodium phosphate, 500 mM sodium chloride, pH 7.0 at 100 μg/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 ~10⁴ cells/ml in DME + BSA. Aliquots of cells (5 × 10⁴) were inoculated into each well of 96-well clusters (Costar Corp.), previously coated with a solution of the selected adhesive protein in DME (20 μg/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 μl 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 antiserum directed to synthetic peptides reproducing amino acid sequences of the cytoplasmic domains of β1, α1, α2, α3, α6, α5, and αV integrin subunits. The following peptides, obtained from Multiple Peptide System (San Diego, CA) were used: β1, CTVTVNNPKYEGK; α1, KIGFKRPLKMKKE; α2, KYEKMTIKNPQDDETTLSS; α3, CRIQAPETERLTDY; α4, KLQENRRDKSWYVMKSN; α5, KSLYFGATMEKQALPKIPATDA; and αV, KKRVRPQPQEERE. Peptides were coupled to a hemocyanin with glutaraldehyde (approximate peptide/capristic molar ratio of 50:1) and rabbits were injected with 500 μg 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 immunoprecipitation assay on different cell lines (Dellipoti et al., 1991, 1992; Rossini 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 β3 integrin subunit was a kind gift of James Galili (SUNY, Stony Brook, NY). The mAb GoH3 to integrin α6 was a kind gift from Dr. Arnold Sonnenberg (The Netherlands Cancer Center, Amsterdam, The Netherlands). This antibody, although directed to the human α6, 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-3S label (ICN Biomedical, Inc., Costa Mesa, CA) containing 1-2-Methionine (35S) and l-Cysteine (35S), s.a. 1242 Ci/mmol) by overnight incubation in methionine-free DME (ICN Biomedical) with 10% FCS and 100 μCi 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 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 TITU/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 (Dellipoti 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 70 at 100 μg/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.

**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 (Vrest) was followed in the cells of N1 and N7 clones plated on either BSA- or FN-coated dishes. After 5 h of incubation cells were fixed with 2.5% glutaraldehyde.
Figure 1. Neurite emission and $V_{\text{rest}}$ 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 $V_{\text{rest}}$ 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 $V_{\text{rest}}$ measured in whole cell configuration, in current clamp conditions ($I = 0$). Values are means ± SEM of single cell measurements carried out in three separate experiments ($n = 10$). Bar, 50 μm.

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 $V_{\text{rest}}$ 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. 1b). This FN-induced hyperpolarization was totally absent in N7 cells, whose $V_{\text{rest}}$ remained constant at ~20 mV, either on BSA or FN (Fig. 1d).

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 $V_{\text{rest}}$ 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 $V_{\text{rest}}$ 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 $V_{\text{rest}}$ of ~22 mV. On the contrary, these cells effectively adhered to FN (~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 $V_{\text{rest}}$ 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
the adhesion and relative phenomena in N1 cells was definitively 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 \( V_{\text{m}} \) 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 \( V_{\text{m}} \) hyperpolarization and neurite emission were consequences of the adhesion per se, or were specifically operated by an integrin-mediated signal elicited from the substratum.

### Table I. Effects of Various Treatments on Cell Adhesion \( V_{\text{m}} \) and Neurite Outgrowth of N1 and N7 Cells

| Substratum   | Percent adherent cells | \( V_{\text{m}} \) (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+\( \alpha \)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+\( \alpha \)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 (3)         | -                        | 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 \( \mu \)g/ml) or \( \alpha \)-BHK antibodies (1:25 dilution) were added to the dishes. For cell adhesiveness assay, \([3H]\)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 ± SEM of the number of experiments listed in parentheses, each carried out in triplicate. For \( V_{\text{m}} \) 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 ± 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 ± SEM of the number of experiments listed in parentheses.
by immunoprecipitation from metabolically labeled cells and sodium dodecylsulphate polyacrylamide gel electrophoresis, showing wide cross-reactivity among resis. Polyclonal antibodies to COOH-terminal sequences of comparing the integrin profile of the two clones.

Possibilities: (a) a difference in integrin profile between the integrins and the V~t hyperpolarizing mechanism in two clones; or (b) the lack of a purportive functional link between integrins and the V~t hyperpolarizing mechanism in N7 cells. The first possibility was experimentally tested 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~t Hyperpolarization in N7 Cells

Recalling that, in N7 cells, adhesion to FN was dissociated from V~t 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 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~t 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 α6 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 β1 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 β1 subunit (Defilippi et al., 1991). The α subunits were identified by specific antibodies as the α3, α5, αv, and αV (Fig. 3, lanes b–e). α1, α2, and αv 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 α6/β1 and in the labeling intensity of the α5 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 β, integrin, but the expression is higher in N7 as compared to N1 cells (Fig. 3, upper arrowheads in lanes a: the N1 cell β, is scarcely visible in the photo). In addition, both N1 and N7 cells express an αv-associated β, (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 β1, β3, β5, β6, and β8 (Vogel et al., 1990; Hynes, 1992). Both N1 and N7 clones were negative for β3, as determined by immunoprecipitation with antibodies to human β3 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 α5/β1 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~t 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 Ba²⁺ and 5 mM Cs⁺ (see below). These features are typical for the K⁺ cur-
rent operated by the so called "delayed rectifier" K⁺ (K_Dm) 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_Dm 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, Ca²⁺ current mediated by Ca²⁺ 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-excitible cells, including eggs (Hagiwara and Takeda, 1974; Hille, 1992). The peculiarity of these channels is their exclusive activation at membrane potentials more negative than E_L. 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_L 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 disregarded, 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 KIR 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 Ba²⁺. As exemplified in Fig. 5, A and B (upper panels) neither 5 mM Cs⁺, nor 2 mM Ba²⁺ 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 Ba²⁺ (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_L close to V_M, increases the accuracy of revealing K_IR currents (Hagiwara and Yoshii, 1979).

On the whole, the comparison between N1 and N7 clones
revealed that, while the integrin-mediated adhesion to FN or VN is sustained by an integrin process, the electrical unresponsiveness of N7 to this adhesion is associated with the lack of Kᵢᵣ channels; this suggested that Kᵢᵣ channels were in fact responsible for the adhesion-promoted hyperpolarization of Vᵢᵣ 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 neuritogenesis. The suitability of this model was reinforced by the fact that, at difference from the Kᵢᵣ current, the Kᵢᵣ currents of N1 cells were severely blocked by Cs⁺ as well as by Ba²⁺. (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 Vᵢᵣ Regulation by Kᵢᵣ in N1 Cells

The depolarized (−22.6 mV) average value of Vᵢᵣ 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 [K⁺]₀, the average Vᵢᵣ remained practically unchanged (−22 ± 1.56 mV, n = 32). However, analyzing data obtained on single cells, it emerged that, while Vᵢᵣ ranged between −10 and −43 mV, the addition of 2 mM Ba²⁺ or the increase in [K⁺]₀, always determined a cell depolarization directly proportional to the initial value of this potential. Furthermore, in the few cells (∼7%) with initial Vᵢᵣ around −40 mV, Ba²⁺ addition reverted the potential to −22 mV. On the contrary, the addition of TEA at concentrations blocking the Kᵢᵣ channels never depolarized the cells more than 1–2 mV.

This leads us to conclude that, beyond a basal level of ∼−20 mV, Vᵢᵣ is modulated in N1 cells by the state of activation or channel density of Kᵢᵣ channels.

The Integrin-mediated Hyperpolarization Is Sustained by the Activation of Kᵢᵣ 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 Kᵢᵣ and Kᵢᵣ currents, or Vᵢᵣ. 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 Kᵢᵣ channels.

In A, the maximum Kᵢᵣ currents (peak Kᵢᵣ current) and Vᵢᵣ at the corresponding times, were plotted versus the time elapsed from the contact. At ∼650 s, 2 mM Ba²⁺ was added by perfusion for 25 s and then washed out. As shown, the Kᵢᵣ current (●) and Vᵢᵣ (○) displayed very similar time courses. Note that, within 810 s from the contact, the Kᵢᵣ current is threefold increased (insets). Ba²⁺ addition produced an almost complete block of the current, while reverting the Vᵢᵣ to the time zero values. Both the current block and the potential reversal were totally removed after Ba²⁺ withdrawal.

Contrary to the Kᵢᵣ current, the Kᵢᵣ 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 Kᵢᵣ current occurred, while, as expected, this current was totally insensitive to Ba²⁺.

On the whole, these electrophysiological data indicated that Kᵢᵣ are the only channels involved in the hyperpolarization.
Figure 6. (A) Time course of the peak $K_{\text{IR}}$ currents (peak $I_{\text{IR}}$, left axis, $\bullet$) and $V_{\text{rest}}$ (right axis, $\circ$) after an N1 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 $\mu$m) were added and left to sediment. A single cell was then patch-clamped (whole cell) and moved in contact with a bead. $V_{\text{rest}}$ and $K_{\text{IR}}$ measurements were taken in current- or voltage-clamp conditions, respectively. (Insets) $K_{\text{IR}}$ 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 $K_{\text{IR}}$ current and the concomitant depolarization. (B) I-V plot of the $K_{\text{IR}}$ 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 GΩ). (C) Time course of the percent (%) $V_{\text{rest}}$ 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 $(1 - e^{-0.99t/192})$.

**zation elicited by the N1 cell contact with FN-coated beads, the other channels being neither activated nor inactivated.**

An average time course of $V_{\text{rest}}$ 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 $K_{\text{IR}}$ 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 (Birnbaumer et al., 1990), these proteins appeared reasonable candidates for the molecular link between integrins and $K_{\text{IR}}$ channels in N1 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 N1 cells with PTX, while not affecting the $V_{\text{rest}}$ of cells plated on BSA, abolished the FN-induced hyperpolarization. Recalling that $V_{\text{rest}}$ is regulated by $K_{\text{IR}}$ 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 $V_{\text{rest}}$; and (b) the FN-induced activation of $K_{\text{IR}}$ 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 N1 cells.

**The Block of $K_{\text{IR}}$ Channels Inhibits Neurite Emission**

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

**Table II. Effect of PTX Pretreatment on $V_{\text{rest}}$ and Neurite Outgrowth of N1 Cells**

| Treatment | $V_{\text{rest}}$ (mV) | Percent cells with neurites |
|-----------|------------------------|---------------------------|
| BSA ± PTX | $-18.0 \pm 6.0$ (3)    | 0                         |
| FN       | $-36.4 \pm 2.1$ (21)   | 29.6 ± 1.6 (5)            |
| FN + PTX | $-19.6 \pm 3.1$ (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. $V_{\text{rest}}$ was measured 15 min after cell seeding in whole cell configuration and current clamp conditions ($I_0 = 0$). Values are means ± 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 ± 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.)
The percentage of cells emitting neurites was determined under phase-contrast microscopy 5 h after cell plating. Values are means \( \pm \) SEM of ~8-10 fields containing experiments. Neurite count was performed at the end of a further 5-h incubation, after 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 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 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 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 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 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 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 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 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 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 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 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.

| Treatment        | \( V_{\text{rest}} \) (mV) | Cells per field | Percent cells with neurites |
|------------------|-----------------------------|----------------|----------------------------|
| FN               | \(-35.0 \pm 1.2\) (6)       | 27.2 \( \pm \) 2.4 (7) | 33.2 \( \pm \) 2.8 (7) |
| FN+CsCl          | \(-20.6 \pm 2.1\) (10)*     | 26.5 \( \pm \) 1.0 (4)  NS 10.1 \( \pm \) 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. \( V_{\text{rest}} \) 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 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 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 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 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 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 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 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 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.

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

It is worth stressing that Cs\(^+\) had no apparent aspecific block was associated with the inhibition of neuritogenesis. 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 uneffective 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 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 \( \pm \) SEM of 10 measurements.](Image)
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 Vrest hyperpolarization (see Fig. 1b).

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 Kᵦ 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, Vrest 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 Vrest 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 Vrest on BSA.

On the other hand, patch clamp experiments unequivocally demonstrated that the integrin-elicted signal has its hyperpolarizing target in the Kᵦ channels, that govern Vrest in N1 cells. Thus, the most simple explanation of our data is that a PTX-sensitive G protein functionally links integrins to Kᵦ channels. A similar pathway was proposed by Williams et al. (1992) and Saffel et al. (1992) to account for the CAM-dependent activation of Ca²⁺ 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 Kᵦ channels are indeed modulated by G proteins (Inoue and Yoshii, 1992). In fact, receptors for substance P and TRH were found to control Kᵦ 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., 1992a). This activation concerned the Ca²⁺-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 Vrest-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 Kᵦ 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) Kᵦ 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 Kᵦ current was elicited by voltage step protocols apt to produce their maximal activation. This seems to rule out that in N7 cells Kᵦ 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 (α5β1, αV containing complexes) (Fig. 3), cannot be easily taken as responsible for the all or null response of Vrest in the two clones. The possibility remains to be explored, however, that a minor α5/β1, αV containing, component is the only way to bring the signal onto the G protein(s) controlling Kᵦ channels.

(b) The block of Kᵦ 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) Kᵦ channels must be operative during this commitment; and (c) when produced after commitment, inhibition of Kᵦ channels is without consequences on neuritogenesis. At this point, it should be stressed that the hyperpolarization sustained by Kᵦ 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. 1b).

Thus, whatever the integrin-mediated mechanism responsible for the Kᵦ 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 Kᵦ 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 Kₐ 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 $V_{\text{rest}}$ 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 $V_{\text{rest}}$ produced by the integrin-dependent activation of Kₐ channels. Whatever the merit of these speculations, the electrical 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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