Elevated Glucocorticoid Receptor Transactivation and Down-regulation of α1 Integrin Are Associated with Loss of Plasma Membrane Ca\textsuperscript{2+}-ATPase Isoform 1*

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We have previously shown that inhibition of expression of the plasma membrane Ca\textsuperscript{2+}-ATPase isoform 1 in PC6 cells leads to loss of nerve growth factor-mediated neurite extension (Brandt, P. C., Sisken, J. E., Neve, R. L., and Vanaman, T. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13843–13848). Cells lacking plasma membrane Ca\textsuperscript{2+}-ATPase 1 do not attach to collagen-coated plates as tightly as controls, suggesting that a defect in adhesion might be underlying the inability to extend neurites. We report here that cell lines lacking plasma membrane Ca\textsuperscript{2+}-ATPase 1 do not produce α1 integrin, which is required for both collagen adherence and neurite extension. Because α1 integrin gene transcription can be down-regulated by glucocorticoids, the response of cells to glucocorticoids was investigated. Cortisol-dependent transactivation from the mouse mammary tumor virus promoter in cells lacking plasma membrane Ca\textsuperscript{2+}-ATPase 1 was stimulated 145–216-fold over untreated cells compared with 15–26-fold for controls. This increase was not due to increased binding affinity of the receptor for cortisol, an increased number of cortisol-binding sites, or increased translocation of the receptor to the nucleus. Expression of additional glucocorticoid receptor-dependent genes required for neurite extension must also be altered in cells missing the plasma membrane Ca\textsuperscript{2+}-ATPase 1 because constitutive expression of α1 integrin did not restore their nerve growth factor-mediated neurite extension capability. The impact of plasma membrane Ca\textsuperscript{2+}-ATPase isoform 1 on other signaling systems and the resultant profound yet subtle effects on PC6 cells strongly suggests that it plays an important role in modulating signal transduction pathways downstream of Ca\textsuperscript{2+}-mediated signals.

The plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA)\textsuperscript{1} has generally been thought of as a housekeeping enzyme with the responsibility of reducing cytosolic calcium levels to below an activation threshold. The discovery of multiple PMCA genes and multiple alternatively spliced mRNAs deriving from the primary transcripts of these genes, yielding 25 or more possible isoforms (for review see Ref. 1), suggests that perhaps there is more to the function of the PMCA than simple calcium homeostasis. This idea was further bolstered by the observation that all of the PMCA isoforms have unique temporal, developmental, and cell-specific expression patterns, implying that they may play unique roles that depend on the calcium handling requirements of particular cell types at various stages of differentiation and development (1–4).

One observation that has consistently been made for PMCA mRNAs is that PMCA1b is seen in all mammalian tissues and cells examined (1). This would suggest that if there is a housekeeping function for PMCAs, PMCA1b might be the isoform carrying out that role. Other isoforms would then be expressed as needed by cells to meet their special calcium handling requirements. However, when expression of all PMCA1 proteins was blocked with an antisense RNA that prevented translation of PMCA1 mRNAs in the pheochromocytoma cell line, PC6 (a PC12 derivative (5)), there were no major discernible effects on the undifferentiated cells (6). If PMCA1b had been solely responsible for maintenance of resting calcium levels, then these levels should have been elevated in PMCA1(−) cells. However, a major increase in resting cytosolic calcium was not observed within the limits of the detection using aequorin. In fact, the only change in calcium homeostasis seen was a modest decrease in the rate of removal of cytosolic calcium following stimulation of inositol 1,4,5-trisphosphate-dependent release with bradykinin. Clearly, other calcium handling mechanisms in the cell compensated for the loss of PMCA1.

The fact that loss of all PMCA1 isoforms had minimal effects on undifferentiated cells suggests that PMCA1 isoforms play some other role in the cell. PC6 cells lacking PMCA1 can no longer extend neurites in response to NGF (6) or other differentiating agents,\textsuperscript{2} despite the fact that NGF signaling pathways are intact. Also, PC12 cells treated with NGF (7) or myocytes differentiated by contact (4) up-regulate splicing of PMCA1 a and c, which are isoforms found exclusively in excitatory tissues. These data, taken together, provide a basis for hypothesizing that PMCA1 isoforms may play a role in the signaling pathways involved in controlling steps in excitatory cell differentiation. To test this hypothesis, studies were undertaken to identify alterations in regulatory pathways in PMCA1(−) cells that prevent them from extending neurites in

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† The abbreviations used are: PMCA, plasma membrane Ca\textsuperscript{2+}-ATPase; PMCA1(−), PC6 cells lacking PMCA1; GR, glucocorticoid receptor; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; DPBS, Dulbecco’s modified PBS; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing Tween-20; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; CAT, chloroamphenicol acetyltransferase; MMTV, murine mammary tumor virus; CMV, cytomegalovirus.

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2 P. C. Brandt, unpublished observation.

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response to NGF. As detailed here, it was found that cells lacking PCMA1 have an elevated glucocorticoid receptor (GR) transactivation response that may be repressing expression of genes necessary for neurite extension.

**EXPERIMENTAL PROCEDURES**

**Cells Lines**—The cell lines used in these experiments were described previously (6). The wild type cell line is PC6, a derivative of PC12 cells developed by Pittman et al. (5). RSVV-2 and RSVV-9 are two cell lines expressing PCMA1 antisense RNA that inhibit expression of PCMA1 protein to immunologically undetectable levels (6). These cell lines are referred to in the text as “PMCA1−/−” cell lines RSVV-2 and RSVV-4. Two control cell lines expressing the same PCMA1 cDNA sequence in the sense orientation. If translated, this cDNA would encode only the first 89 amino acids of PCMA1. However, this fragment has never been detected.

Unless otherwise specified, cells were maintained in PC6 medium (Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose (Sigma), 5% fetal bovine serum (Life Technologies, Inc.), 10% horse serum (Life Technologies, Inc. or Hyclone, Logan, UT), penicillin (Sigma), and streptomycin (Sigma). Cells were grown in a humidified 5% CO2/95% air environment at 37 °C. All experiments and routine passaging of cells were carried out in CellTissue culture dishes from Sarstedt (Newton, NC) except for collagen binding assays. Where noted, fetal bovine serum had been stripped of steroids by treatment with dextran-coated charcoal (Hyclone) was substituted for normal fetal bovine serum and horse serum in the medium.

**Collagen Binding Assays**—The procedure for preparing collagen-coated plates was essentially as described by Turner et al. (8). 35-mm plastic bacterial culture dishes (Falcon 1008) were coated with 0, 10, or 20 μg/ml collagen. Where noted, 0.5 mg/ml freshly prepared NHS-sulfo-biotin (Pierce) and incubated for 30 min at 4 °C with constant mixing by inversion. The cells were then moved by carefully adding 2 ml of DPBS and applying a sheer force for 1 min followed by 5 min on ice. Nonidet P-40 was added to 1% final concentration never exceeded 0.1% (v/v). The cells were washed twice with PBS, scraped from the plate in a minimal volume of PBS, and collected by centrifugation at 5000 × g for 5 min. The cell pellets were resuspended in 200 μl of PBS containing 1 mg/ml phenylmethylsulfonyl fluoride and lysed by three rounds of freeze-thaw from −70 °C to 37 °C.

**Immunochemistry**—Cells were allowed to attach for 1 h under standard incubation conditions after which a shear force was applied by placing the cells on an orbital platform rotating at 90 rpm for 30 s. The detached cells were immediately removed by aspiration. Loosely attached cells were removed by carefully adding 2 ml of DPBS and applying a shear force for an additional 30 s. The cells were washed again, and those still attached were treated with 1% Triton X-100 (PBS) in PBS and 1% biotinylated Antibody (Qiagen) was added and incubated for 1 h at room temperature. Membrane proteins were released by incubation with 1% NP-40 for 2 min at 4 °C. The cell pellets were resuspended in 1 ml of PBS containing 0.5 mg/ml freshly prepared NHS-sulfo-biotin (Pierce) and incubated for 30 min at 4 °C with constant mixing by inversion. The cells were then pelleted and washed twice with 1 ml of Tris-buffered saline (TBS).

**Glucocorticoid Receptor Regulation by PMCA1**

Glucocorticoid Receptor Transactivation Assays—Cells plated the previous evening at 60–70% confluency in 35- or 100-mm dishes were transiently transfected with the plasmids pMMTV-CAT and pcMV-gal. The transfection efficiency was determined by liquid scintillation counting of the lysates. A control of 10⁵ cells was added from a 1 mM stock made in ethanol, so the final ethanol concentration never exceeded 0.1% (v/v). The cells were washed twice with PBS, scraped from the plate in a minimal volume of PBS, and collected by centrifugation at 5000 × g for 5 min. The cell pellets were resuspended in 200 μl of PBS containing 1 mg/ml phenylmethylsulfonyl fluoride and lysed by three rounds of freeze-thaw from −70 °C to 37 °C. The freeze-thaw lysis buffer, which was pelleted by centrifugation at 12,000 × g for 4 min at 10 °C. Chloramphenicol acetyltransferase (CAT) activity was measured according to the method of Seed and Sheen (10) using [³⁵S]chloramphenicol from NEN Life Science Products and butyryl coenzyme A from Sigma. β-Galactosidase activity was measured by hydrolysis of o-nitrophenylgalactopyranoside (Stratagene, San Diego, CA). Protein content of extracts was measured by bicinchoninic acid complex formation with reduced copper ion (Pierce) with TCA precipitated proteins.

Control for variations in transfection efficiencies among different cell lines CAT activity from the MMTV promoter was normalized to β-galactosidase activity constitutively expressed under control of the CMV promoter. All experiments were done in triplicate or greater.

**Determination of Glucocorticoid-binding Site Number and Affinity**—The number of glucocorticoid-binding sites and the affinity of those binding sites in PCMA1(−) cells and controls was determined by whole cell extraction of [³¹H]Htriamcinolone acetonide (NEN Life Science Products) as described previously (11).

**Glucocorticoid Nuclear Translocation Assays**—The procedure followed was a slightly modified version of that previously used by Zhou et al. (12). Cells at 60–70% confluence in 10-cm dishes were treated with 1 μM dexamethasone, or an equal amount of ethanol, in DMEM containing 5% steroid-free fetal bovine serum and 0.1 trypsin inhibitory unit/ml aprotinin (Sigma) at room temperature for 24 h. After treatment, the cells were washed one time in DPBS, collected, and resuspended in 2 ml of DPBS by scraping, and centrifuged at 1000 × g for 2 min at 4 °C. The cell pellets were then resuspended in 1 ml of PKC sonication buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM MgCl₂, 1 μM pepstatin A, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1 trypsin inhibitory units/ml aprotinin) on ice for 1 h, and debris was removed by centrifugation at 13,000 × g for 4 °C for 30 min (9). α1 integrin was initially immunoprecipitated with the 3A1 monoclonal antibody (9) (a generous gift of Dr. David Turner, SUNY-Syracuse Health Science Center). In later experiments, a commercially available hamster antibody against rat α1 integrin was used (Pharmingen, San Diego). β1 integrin was immunoprecipitated with a rabbit polyclonal antibody (generous gift of Dr. Louis Reichardt, University of California, San Francisco). Approximately 20 μg of IgG was added to 0.5 mg of cell extract and incubated 1 h at 4 °C. In the case of the hamster antibody, 25 μg of rabbit anti-hamster IgG was added after 1 h of incubation with the anti-α1 integrin 3A1 monoclonal antibody. The immunoprecipitated material was continued for an additional hour. Immune complexes were then recovered by incubating reactions with 20 μl of protein A-Sepharose (50 μl slurry) for rabbit antibodies or protein G-Sepharose (50 μl slurry) for mouse antibodies at 4 °C with constant mixing by inversion for 1 h. The complexes were collected by centrifugation, washed three times in lysis buffer, and prepared for SDS-PAGE as follows.
avidin-alkaline phosphatase conjugate.

to determine whether changes in
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identical to the one used for fluorography in
immunoprecipitated proteins were resolved on a 5% SDS-PAGE gel, impregnated with fluor, and placed on x-ray film.

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PMCA1(sion might explain the altered adherence and inability of

ab[35S]methionine and cysteine and cells surface labeled with NHS-sulfo-biotin were treated with a

conjugate or impregnating the gel with a fluor and exposing it to x-ray film overnight.

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by blotting the proteins to Immobilon-P membranes and detecting the biotin-labeled cell surface proteins with avidin-alkaline phosphatase

for two weeks in PC6 medium.

positive clones were plated and exposed to 100 ng/ml 2.5S murine NGF

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facturer's recommended protocol. pWE3 encodes a puromycin-resis-

resultant plasmid was designated p

2 integrin—

expression of the coating collagen was raised to 10

ng/ml, the attach-

ment of the control cell lines increased to between 25 and 50%, whereas the PMCA1(−) cell lines remained at ~12%. This would suggest that the PMCA1(−) cells do not have a functional collagen receptor on their surface. This was further confirmed by the demonstration that increasing the collagen concentration to 50 µg/ml decreased the attachment of PMCA1(−) cells to less than 5%. This is likely due to the loss of the nonspecific binding sites on the plate at higher concentrations of collagen, which are therefore unavailable for PMCA1(−) cell attachment.

Having determined that there was decreased α3β1-dependent attachment in PMCA1(−) cells, the expression of these two integrins was examined. First, a study was conducted to deter-

mine whether a functional α3β1 pair was being expressed, the ability PMCA1(−) cells to bind to collagen was assayed because collagen is the preferred substrate for α3β1 integrins. Equal numbers of labeled cells were applied to untreated, nontissue culture plastic dishes that had been precoated with 0, 10, or 50 µg/ml calf skin collagen, type I, and a sheer force was applied. The cells remaining on the plate were quantified and expressed as a percentage of those initially plated (Fig. 1A). When no collagen was applied to the plates, 12–15% of all cells attached. This probably represents nonspecific interactions with the plastic that allow weak binding. When the concentra-
tion of the coating collagen was raised to 10 µg/ml, the attach-
ment of the control cell lines increased to between 25 and 50%, whereas the PMCA1(−) cell lines remained at ~12%. This would suggest that the PMCA1(−) cells do not have a functional collagen receptor on their surface. This was further confirmed by the demonstration that increasing the collagen concentration to 50 µg/ml decreased the attachment of PMCA1(−) cells to less than 5%. This is likely due to the loss of the nonspecific binding sites on the plate at higher concentrations of collagen, which are therefore unavailable for PMCA1(−) cell attachment.

Previously, we had reported that blockade of PMCA1 protein synthesis with antisense RNA led to the inability of PC6 cells to extend neuritic processes when treated with NGF (6). While trying to understand the mechanisms underlying this result, it was observed that RSV9-2 and RSV9-9, the two PMCA1(−) cell lines, did not attach to standard tissue culture plastic as well as the controls. Examination of the literature showed that for

PC12 cells, the parental line of PC6, neutralizing antibodies to α1 integrin blocked neurite extension (9). We therefore elected to determine whether changes in α1 integrin binding or expres-

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α1 Integrin Is Absent from PMCA1(−) Cells—PC12 cells con-
tain two αβ integrin pairs, α1β1 and α6β1, of which the α6β1 integrin pair is required for neurite extension (14). To deter-

Results

Establishment of PMCA1(−) Cell Lines Stably Expressing α1 Integrin—The rat α1 integrin cDNA (13) (a generous gift of Dr. Louis Reichardt, University of California, San Francisco) was cloned behind the constitutively expressed CMV promoter of the plasmid pCB6+. The resultant plasmid was designated pα1-I. RSV9-2 and RSV9-9 cells were co-transfected with pα1-I and pWE3 (ATCC) at a 10 to 1 molar ratio

using Effectene transfection reagent (Qiagen) according to the manu-

facturer's recommended protocol. pWE3 encodes a puromycin-resis-
tance gene and recombinants were selected with 0.5 µg/ml puromycin (Sigma). Puromycin-resistant colonies were isolated and expanded. Ex-

pression of α1 integrin in individual isolates was determined by cell

surface labeling with NHS-sulfo-biotin as described above. α1 integrin

positive clones were plated and exposed to 100 ng/ml 2.5S murine NGF for two weeks in PC6 medium.

RESULTS

Previously, we had reported that blockade of PMCA1 protein synthesis with antisense RNA led to the inability of PC6 cells to extend neuritic processes when treated with NGF (6). While trying to understand the mechanisms underlying this result, it was observed that RSV9-2 and RSV9-9, the two PMCA1(−) cell lines, did not attach to standard tissue culture plastic as well as the controls. Examination of the literature showed that for

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Fig. 1B shows that α₁ integrin was not present on the cell surface of PMCA1(−) cells but was in the wild type and control lines. To determine whether α₁ integrin was synthesized but possibly not successfully transported to the cell surface, cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine, and α₁ integrin was immunoprecipitated, resolved by SDS-PAGE, and fluorographed. As seen in Fig. 1B, no α₁ integrin was detected in PMCA1(−) cell lines.

To determine whether the expression of all integrins was affected in PMCA1(−) cells, β₁ integrin was also immunoprecipitated from extracts with β₁ integrin-specific antibodies. Cells again were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine and then surface labeled with NHS-sulfobiotin. The β₁ integrin antibody used for immunoprecipitation was able to co-precipitate any associated α integrins. In this way, it could be determined whether there was an up-regulation of α₁ integrin or induction of another α integrin to compensate for the loss of α₁. Half of the precipitated samples were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and detected with avidin-alkaline phosphatase conjugate, whereas the other half were resolved and fluorographed directly. The resulting fluorograms shown in Fig. 1 (C and D) confirmed the previous finding that α₁ integrin was not produced in PMCA1(−) cell lines. The cell surface labeling protocol employed in these studies does not label β₁ integrin with high efficiency. Therefore, it is not clearly seen in the blot shown in Fig. 1D. However, the β₁ integrin is easily seen in the [³⁵S]-labeled samples shown to the right in Fig. 1C.

These results show that synthesis of β₁ integrin production is not concomitantly decreased with the loss of α₁ integrin in PMCA1(−) cells. Also, α₁ integrin did not appear to be up-regulated in PMCA1(−) cells to compensate for the loss of α₁ integrin. The identity of α₁ integrin was independently determined by separate immunoprecipitation experiments with α₁ integrin-specific antibodies (data not shown). The band migrating between α₁ and β₁ integrins in Fig. 1C is still observed and may be a previously unidentified α integrin in PC6 cells. However, even if it is an α integrin, it also does not appear to be consistently up- or down-regulated by the loss of α₁ integrin in PMCA1(−) cells.

PMCA1(−) Cells Have Elevated Glucocorticoid Receptor Transactivation Activity—Zhang et al. (15) have shown that in some PC12 cell lines (the parental line of PC6) transcription of the α₁ integrin gene can be down-regulated by glucocorticoids. To determine whether a more responsive GR might be responsible for down-regulation of α₁ integrin in PMCA1(−) cells, cortisol-dependent transcription of the CAT reporter gene under control of the MMTV promoter was assayed. The MMTV promoter contains several tandem glucocorticoid response elements and is responsive to GR-mediated transcriptional activation. The cells were co-transfected with pMMTV-CAT and pCMVβgal and analyzed for induction of cortisol-stimulated transcription of the CAT gene in steroid-free medium that was normalized to constitutive β-galactosidase activity to control for transfection efficiency. As seen in Fig. 2, the PMCA1(−) cells are much more responsive to cortisol than controls. The cortisol-induced GR transactivation of controls was 15.3–25.8-fold, whereas the PMCA1(−) cell lines showed 145- and 216-fold induction for RSV9-2 and RSV9-9, respectively.

The nearly 10-fold elevation in glucocorticoid responsiveness could be explained by variety of mechanisms. One possibility is that there is an increase in the total number of glucocorticoid-binding sites, either because of more total receptor molecules or because of increased formation of the corticosteroid-binding competent “activated” 9S receptor complex (16) in the cytosol. As a first step in determining whether either of these possibilities may have contributed to elevated glucocorticoid response seen in PMCA1(−) cells, the number of binding sites and their affinity were determined by whole cell binding assays of [³H]triamcinolone acetonide. As seen in Fig. 3A, these analyses showed that there was no increase in the number of glucocorticoid-binding sites (~4300 sites/cell) or the affinity of those binding sites for the steroid (~1.8 × 10⁻⁹ M).

Because changes in binding of glucocorticoids by the glucocorticoid receptor did not appear to be responsible for enhanced glucocorticoid receptor response, studies were performed to determine whether there was an alteration in the amount of steroid-bound receptor translocated into the nuclei of PMCA1(−) cell lines. Cells were stimulated with dexamethasone and fractionated into nuclear and cytosolic components. The fractions were resolved by SDS-PAGE, and the GR was detected by immunoblotting with a GR-specific polyclonal antibody as shown in Fig. 3B. Although the bulk of the receptor remained in the cytosol after treatment with dexamethasone, there was a clear increase in the amount of GR detected in the nucleus after treatment with cortisol in all cell lines. There did not appear to be an appreciable difference in the amount of total GR translocated in the PMCA1(−) cells compared with the controls. Nor was there an increase in the total amount of GR in PMCA1(−) cell lines. However, one noticeable difference was the presence of a pair of bands in the control cell extracts, designated GR', that were greatly diminished or missing in samples from the PMCA1(−) cell lines. The identity of these bands has not yet been established. However, they may represent forms of the GR altered by phosphorylation or some other regulatory posttranslational modification that is lacking in PMCA1(−) cells.

Constitutive Expression of α₁ Integrin Does Not Restore Neurite Extension—To determine whether loss of α₁ integrin was the sole lesion preventing neurite extension, a rat α₁ integrin cDNA was constitutively expressed in PMCA1(−) cells. Purinoicin-resistant clones were selected and screened for cell surface expression of α₁ integrin. Positive clones were tested for the ability to extend neurites in response to NGF. The data in Fig. 4 show the expression of α₁ integrin in selected clones.
Because the number of cells used for labeling with NHS-sulfo-biotin varied between $10^6$ and $10^7$, the relative intensity of the $\alpha_1$ integrin bands was not absolutely quantitative, but in most cases the expression was on the order of that seen in wild type cells. For unknown reasons, of the 12 puromycin-resistant RSV9-2 clones examined only two were found to express de-

![Figure 3](image3.png)

**FIG. 3.** Determination of glucocorticoid receptor steroid-binding and nuclear localization properties. A, whole cells were incubated with $[^3H]$triamcinolone acetonide, a synthetic glucocorticoid analog, for 60 min. The cells were washed with DPBS and lysed in SDS, and the specifically bound triamcinolone in the extract was determined by liquid scintillation counting. Error bars represent standard error for triplicate measurements. B, cells were treated with 1 $\mu$M dexamethasone for 1 h and then fractionated into nuclear and cytosolic components. 100 $\mu$g of nuclear and cytosolic extracts from each cell line were subjected to Western blot analysis using a polyclonal GR antibody.

![Figure 4](image4.png)

**FIG. 4.** Constitutive expression of $\alpha_1$ integrin does not rescue NGF-mediated neurite extension. Stably transfected cell lines constitutively expressing $\alpha_1$ integrin were established from each PMCA1(-) cell line and the wild type PC6 cells. Puromycin-resistant cells were screened for the ability to express $\alpha_1$ integrin by cell surface labeling with NHS-sulfo-biotin and immunoprecipitating with an $\alpha_1$ integrin-specific antibody. Those clones expressing $\alpha_1$ integrin were plated, grown in the presence of 2.5 $\mu$g murine NGF for 2 weeks and plated.
tectable levels of α1 integrin, and those were at low levels compared with the expression seen in the α1 integrin-positive RSV9-9 clones. Clones expressing α1 integrin were exposed to 100 ng/ml 2.5 S murine NGF for 2 weeks. The constitutive expression of α1 integrin did not compensate for its loss in PMCA1(−) cells because none of the clones were able to extend neurites longer than the parental PMCA1(−) cell lines (Fig. 4). This suggests that multiple components necessary for NGF-dependent neuronal differentiation are affected by loss of PMCA1.

DISCUSSION

In the last ten years, the number of PMCA isoform mRNAs that have been cloned has reached at least 25 (1). These isoforms have shown unique temporal and spatial expression in tissues (2, 4, 17–26) and inducibility as cells differentiate (4, 7). Although it has not yet been shown that each of these mRNAs is translated into a functional protein, the complexity of their expression would suggest that they are used to address specific calcium handling requirements of the multitude of different cell types in animals.

In an attempt to understand the need for so many different PMCA isoforms, all members of the PMCA family of proteins were blocked with antisense RNA in PC6 cells (6). It was found that although PMCA1(−) cells were essentially indistinguishable from sense RNA controls and the wild type cell lines in the undifferentiated state, they were unable to extend neuritic processes when treated with NGF.

The data presented here showed that a cell surface protein essential for neurite extension, α1 integrin (9), was not present in PMCA1(−) cells. This was not the sole cause of loss of the ability to extend neurites, however, because stable expression of α1 integrin in PMCA1(−) cells did not restore this capability. This is not entirely surprising. Because the PC12 and PC6 cell lines are similar to the adreno-neural precursor cells of the neural crest, they can be differentiated into a sympathetic-like, dopaminergic neurons on treatment with NGF or a chromaffin-like cell (28, 29). In the former case, NGF induces a variety of neural crest cells, they can be differentiated into a sympathetic-like, dopaminergic neurons on treatment with NGF or a chromaffin-like cell (28, 29).

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