Regulation of Ionic Currents in Pheochromocytoma Cells by Nerve Growth Factor and Dexamethasone

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Growth factors and hormones induce differentiation of clonal pheochromocytoma (PC12) cells, which are derived from rat adrenal medulla chromaffin cells. On application of nerve growth factor (NGF), PC12 cells extend neurites and express properties characteristic of autonomic ganglion cells. In contrast, incubation of PC12 cells with a corticosteroid, dexamethasone (DEX), does not induce neurite formation but causes an increase in tyrosine hydroxylase activity, suggesting that the cells become chromaffin cell-like. The ability of NGF and DEX to regulate ionic currents has been less well studied. Therefore, we examined how long-term NGF and DEX treatments affected voltage-dependent Na, Ca, and K currents in PC12 cells. Voltage-dependent Na currents were observed only in a small fraction of the PC12 cells in the absence of NGF or DEX. Virtually all NGF-treated cells expressed Na currents within 7 d. DEX increased the number of cells expressing voltage-dependent Na current slowly over 3 weeks, but, unlike NGF, DEX did not change Na current density. Both NGF and DEX also affected the expression of voltage-dependent Ca currents. Most of the untreated cells had only sustained, high-threshold voltage-dependent Ca currents. Chronic application of NGF or DEX increased the fractions of the cells that showed transient, low-threshold T-type Ca currents in addition to the high-threshold currents. The T-type Ca current density, however, increased significantly only in NGF-treated cells. Neither DEX nor NGF affected the voltage-dependent K currents. These results suggest that the expression of voltage-dependent Na and Ca currents are differentially regulated by NGF and DEX. The distinction between treated and untreated cells decreased after 3 weeks in culture as older untreated cells showed increases in the fraction of cells expressing both Na and low-threshold Ca currents. A PC12 subline selected for adherence to uncoated plastic also showed increased fraction of cells expressing these currents, suggesting that interactions with substrate may also influence ionic current expression.

Peptide growth factors and steroid hormones play important roles in the differentiation of excitable cells such as neurons and endocrine cells. Differentiation of excitable cells is often accom-

panied by changes in electrical properties (see Spitzer, 1985, for review). Many lines of evidence suggest that the presence of nerve growth factor (NGF) and glucocorticoids are important to the differentiation of postnatal adrenal medullary cells. Clonal PC12 cells, derived from rat adrenal chromaffin cells, have been used as a model system for the study of neuronal differentiation. Rat adrenal chromaffin cells and rat pheochromocytomas show similar changes in process outgrowth, catecholamine content, and tyrosine hydroxylase activity when treated with NGF or glucocorticoids (Tishler et al., 1982b, 1983).

PC12 cells are normally spherical in shape and do not generate action potentials. In the presence of NGF, however, the cells develop long, branching processes. This morphological differentiation is accompanied by increased synthesis and storage of ACh (Greene and Rein, 1977) and development of electrical excitability (Dichter et al., 1977), which is, in part, a result of the enhanced expression of Na^+ channels (Rudy et al., 1982; Mandel et al., 1988). PC12 cells have also been shown to respond to cortical steroids such as dexamethasone (DEX) by increasing the activity of tyrosine hydroxylase, an enzyme involved in the synthesis of catecholamines. Glucocorticoids, such as DEX, also inhibit neurite outgrowth (Unsicker et al., 1978; Tishler et al., 1982b). It has been suggested that NGF causes PC12 cells to develop toward autonomic neurons, whereas glucocorticoids cause them to develop toward a chromaffin cell-like phenotype (Tishler et al., 1982b, 1983). Little is known, however, about the effect of DEX on the electrophysiological properties of PC12 cells.

Several different types of Ca^{2+} channels, such as L-, N-, and T-type Ca^{2+} channels, have been characterized (see Tsien et al., 1987 for review). High-threshold (L and N) Ca^{2+} channels activate at relatively depolarized voltages (> -10 mV) and can be distinguished by their slow inactivation and pharmacological properties. Low-threshold, T-type Ca^{2+} channels begin to activate at more hyperpolarized voltages (< -70 mV) and inactivate rapidly with maintained depolarization. Each type of Ca^{2+} channel may serve a different physiological function. The distribution of the types of voltage-dependent Ca^{2+} channels is different among different cell types. Adrenal chromaffin cells, for example, do not express T-type Ca^{2+} channels, while cells from many other tissues express both T-type and L-type Ca^{2+} channels (Tsien et al., 1987). Both L- and N-type channels have been implicated in the control of neurotransmitter release (Rane et al., 1987; Hirning et al., 1988).

Pharmacological evidence has suggested that undifferentiated PC12 cells, like chromaffin cells, express primarily a dihydropyridine-sensitive, high-threshold voltage-dependent Ca current (Kongsamut and Miller, 1986). A minor dihydropyridine-insensitive Ca^{2+} component which increases after the ap-

plication of NGF has also been observed (Takahashi et al., 1985; Kongsamut and Miller, 1986; Streit and Lux, 1987). These observations are consistent with the development of a neuronal phenotype in the presence of NGF.

The amount and temporal pattern of Ca influx can be regulated by voltage-dependent Na, Ca, and K channels. Because the regulation of intracellular levels of Ca^{2+} is important to many cellular processes, including the differentiation of excitable cells (see Kater et al., 1988, and Miller, 1988), we hypothesized that DEX or NGF would differentially affect the expression of these ion channels. Although the effects of NGF on voltage-dependent Na currents of PC12 cells have been studied (e.g., Mandel et al., 1988), neither the effects of NGF on other ion currents nor the effects of DEX on the electrical properties of PC12 cells have been systematically examined. We show here that chronic incubation of PC12 cells with DEX and/or NGF induces distinct changes in cellular morphologies and changes in voltage-dependent Na and Ca currents, but not in voltage-dependent K currents.

Materials and Methods

Cell culture. PC12 cells were obtained from the laboratory of Dr. E. M. Shooter (Department of Neurobiology, Stanford University, CA). The cells were maintained in plastic Lux tissue culture dishes (Miles Laboratories, Naperville, IL) with Dulbecco's modified Eagle's medium with or without 25 mM HEPES (GIBCO, Grand Island, NY), supplemented with 5% horse- and 5%-supplemented calf sera (Hyclone Laboratories, Logan, UT). Two different lots of each sera type produced indistinguishable results. Levels of glucocorticoids in the media were <30 nM. For most of the electrophysiological experiments, the cells were plated on borosilicate glass coverslips (Bellco Biological Glassware, Vineland, NJ). The coverslips were seeded such that cells became confluent after 3–3.5 weeks. The length of time a culture was maintained was measured from the day a new plating was made. β-NGF (200 ng/ml) and DEX (1 μM) were added at the time of the new plating and were present thereafter in the medium. The medium was changed twice per week. In some experiments, coverslips were coated with Cell-Tak (a preparation of barnacle glue shown to increase attachment of tissue culture cells: BioPolymers, Inc., Farmington, CT) or with nitrocellulose (Lagenaur and Lemmon, 1987). β-NGF was a gift from Prof. E. M. Shooter.

An adherent PC12 subclone, clone B, was obtained from Dr. D. L. Shelton (Department of Neurobiology, Stanford University, Stanford CA) and selected from the parent PC12 stock as follows: PC12 cells were plated on Costar (Cambridge, MA) dishes. After 24 hr, medium was removed and fresh medium was vigorously and repeatedly squirted into the dish in an attempt to remove all the cells. Cells which remained adherent to the dish were then fed with fresh medium. This procedure was repeated every other day. Discrete colonies of very adherent cells were apparent after 4 weeks. These adherent colonies were then grown for 2 weeks in the presence of NGF. Colonies which were well separated and exhibited vigorous neurite outgrowth were picked individually. These clones were placed in NGF-free medium and allowed to proliferate.

Electrophysiology. PC12 cells were voltage-clamped using the tight-seal whole-cell patch-clamp method (Hamill et al., 1981). Electrophysiological data were collected and analyzed essentially as described previously (Hoshi and Aldrich, 1988a, b). Capacitative and leak currents have been subtracted from the data shown. All experiments were performed at room temperature (20 ± 2°C). Unless otherwise noted, the data were obtained from the cells grown on uncoated coverslips.

The following 2 methods were used to improve voltage-clamp of NGF-treated cells. (1) NGF-treated cells were grown in suspension. The cells in suspension do not extend neurites. The cells were then plated on glass coverslips 10–20 min before recording. (2) NGF-treated cells with extensive neurites were collected with a 25 gauge needle and triturated. The cells were replated on a new coverslip 10–20 min before recording. This procedure either removed or retracted the cellular processes, making the cells suitable for voltage-clamp experiments. Recordings made from cells treated in this manner represent the ionic

currents primarily from the cell body. There were no marked qualitative differences in the capacitance measurements or the current recordings obtained from the cells using either of these 2 methods or those made from intact cells with neurites.

The cell capacitance was determined from the capacitative transient current in response to a 20 mV voltage step. The whole-cell results presented are based on recordings from >900 cells. Single-channel recordings were made from 25 cells.

Solutions. Whole-cell Ca currents were recorded with external solutions containing (in mM) 10 $CaCl_2$; 130 tetraethylammonium (TEA); 2 $MgCl_2$; 10 HEPES; 5 4-aminopyridine; internal solutions contained (in mM) 120 CsCl; 20 TEA; 2 $MgCl_2$; 11 EGTA; 1 $CaCl_2$; 10 HEPES; 10 nM free Ca^{2+}. Whole-cell Na and K currents were recorded with external solutions with 140 NaCl; 2.8 KCl; 2 $MgCl_2$; 1 $CaCl_2$; 10 HEPES (solutions contained up to 40 mM sucrose); internal solutions normally contained (in mM) 140 KCl; 2 $MgCl_2$; 11 EGTA; 1 $CaCl_2$; 10 HEPES; 10 nM free Ca^{2+}. All solutions were brought to pH 7.2 with N-methylglucamine (NMG). Single-channel recordings were made in the cell-attached configuration. The pipette was filled with (in mM) 105 $BaCl_2$; 10 HEPES; pH 7.2 with TEAOH. The bath solution contained (in mM) 140 K-gluconate; 3 $MgCl_2$; 1 EGTA; 10 HEPES; pH 7.2 with NMG. Other solutions used are described in the figure legends. Salts and DEX (cell culture grade) were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. Recordings from cells were scored on the absence or presence of transient T-type Ca current, Na current, or transient K current. Recordings made from cells within each treatment group were distributed over 30 d after plating. Data from each group, at each time point, were pooled for statistical comparison. We found that changes in the numbers of cells expressing the ionic current in question and current densities occurred gradually. These changes could be summarized by pooling the data into groups which were treated up to (and inclusive of) 21 d, over 21 d, or up to 30 d. Percentages of cells expressing either T-type Ca current or Na current were determined as a simple average of cells within a treatment group over an indicated time period. Differences in the fractions of the cells expressing the ion current of interest between different treatment groups were evaluated using the 2-tailed χ^2 test. A level of $p = 0.05$ was considered to be significant. Current densities were determined as an average of [peak current amplitude/cell capacitance].

Results

Morphological changes induced by NGF and DEX

Treatment of PC12 cells with NGF, DEX, or both NGF and DEX together (DEX/NGF) resulted in distinct morphological changes (Fig. 1). Untreated PC12 cells remained mostly round, with occasional oval and rare flat cells, over the course of 4 weeks in culture (Fig. 1A). DEX-treated cells became larger and more irregular in shape over 2–3 weeks (Fig. 1B). These DEX-treated cultures did not become confluent, suggesting that cell division had been drastically slowed (Goodman et al., 1979; Schubert et al., 1980). Cultures treated with DEX for 4 weeks showed a large proportion of cells in clumps with occasional cytoplasmic degeneration (Harris et al., 1986). Isolated cells were chosen for recording in order to increase fidelity of the voltage clamp. Cells treated with NGF ceased dividing and extended long processes within 2–3 d from the beginning of the treatment (Fig. 1C). The presence of both DEX and NGF together prevented the extensive neurite outgrowth observed in the presence of NGF alone. Only short, stubby processes were observed on DEX/NGF-treated cells during the first weeks of culture (Fig. 1D). Longer, thick processes were observed on DEX/NGF-treated cells after 4 weeks in culture. These results of DEX and NGF treatment are similar to those observed in adrenal chromaffin cells (Unsicker et al., 1978; Tishler et al., 1982b; Lillien and Claude, 1985).

Changes in membrane area, as measured by cell capacitance, were also observed with NGF and DEX. The capacitance of untreated cells, ranging from 4 to 12 pF, remained stable with
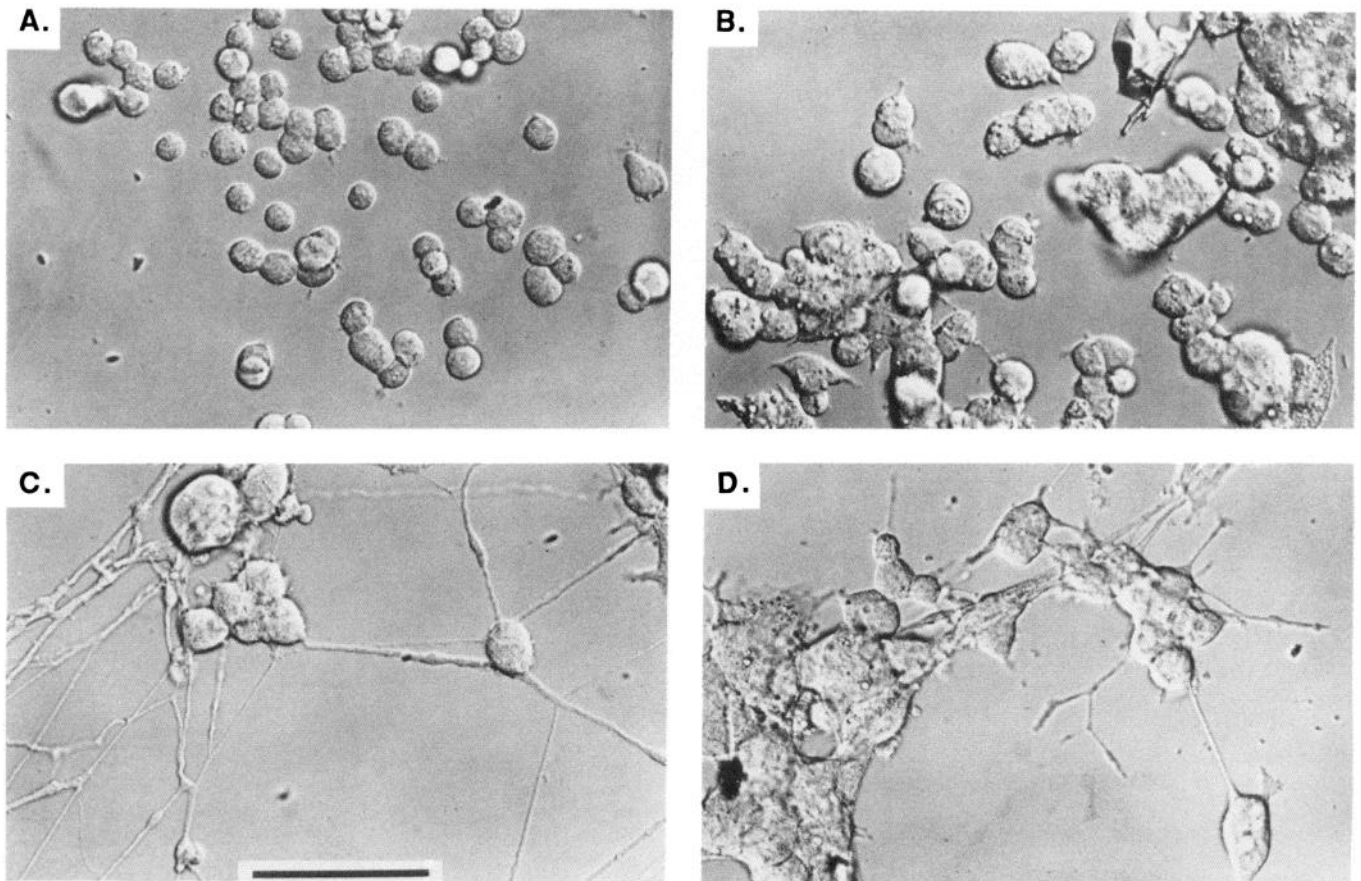
Figure 1. PC12 cells grown (*A*) untreated (12 d) or in the presence of (*B*) DEX (20 d), (*C*) NGF (12 d), and both (*D*) DEX and NGF (12 d) have distinct morphologies. Untreated cells remain round and 15–20 μm in diameter. DEX-treated cells become larger with increased time in culture, and often appear to lose distinct cellular boundaries. The extensive neurite-like processes observed within days of the application of NGF alone appeared as short, stubby processes in the presence of DEX and NGF. Scale bar, 50 μm. Photographs were taken using DIC (Nomarski) optics.

time in culture (Fig. 2*A*). DEX, or NGF alone, however, increased the capacitance in some cells by 2- to 3-fold (Fig. 2*B*, *C*). The measured capacitance of NGF-treated cells reflects primarily that of the cell bodies because most of the neurites were sheared off for more accurate electrophysiological measurements (see Materials and Methods). The capacitance of cells treated with both DEX and NGF increased 4- to 5-fold over that of the untreated population within the first week of treatment (Fig. 2*D*). It was not possible to reliably determine the capacitance of NGF- and DEX/NGF-treated cells after 2 weeks in culture because of the large size of the cells. It was also difficult to obtain high resistance seals with older NGF- and DEX/NGF-treated cells, resulting in smaller test populations for these categories. These cultures, however, remained viable for several weeks. The capacitance measurements made on DEX-, NGF-, and DEX/NGF-treated cells represent a lower estimate of the membrane area as our bias was to record currents from smaller, isolated cells in order to increase the fidelity of the voltage-clamp.

DEX antagonizes NGF-induced increase in voltage-dependent Na current density

PC12 cells without (Fig. 3*A*) and with (Fig. 3*B*) voltage-dependent Na currents were found in all treatment groups (untreated, DEX, NGF, and DEX/NGF). The peak current-voltage [*I*(*V*)] relationships of representative currents are shown in Figure 3*C*.

Cells were scored as expressing voltage-dependent Na current if transient inward currents greater than 25 pA in amplitude were recorded at −10 mV following a 1 sec hyperpolarizing prepulse to a voltage ≤ −100 mV, using Na^+-containing external solutions. In cells expressing Na current, little or no inward current was observed following a prepulse to −50 mV. In a majority of the experiments, Na currents were recorded with an internal solution containing K^+, resulting in the presence of voltage-dependent outward currents at voltages ≥ −10 mV. However, the outward currents activated much more slowly than the voltage-dependent Na currents at voltages < 0 mV (see Fig. 3). Thus, the measurements of the peak inward currents observed at −10 mV were only slightly contaminated by the outward K currents. Because the replacement of external Na^+ with NMG^+ almost completely abolished any inward currents observed, Ca currents contributed very little to the inward current measured with these solutions. The relative proportion of cells expressing Na current changed over time in response to different treatments. The properties of the voltage-dependent Na currents recorded from cells under different treatments, however, did not differ noticeably. The presence of the Na current was not correlated with any specific cellular morphology or with membrane area.

Voltage-dependent Na current was observed only in a minority of the untreated cells (24%, *n* = 39) over a 21 d period after plating (Fig. 4*A*). The number of untreated cells expressing
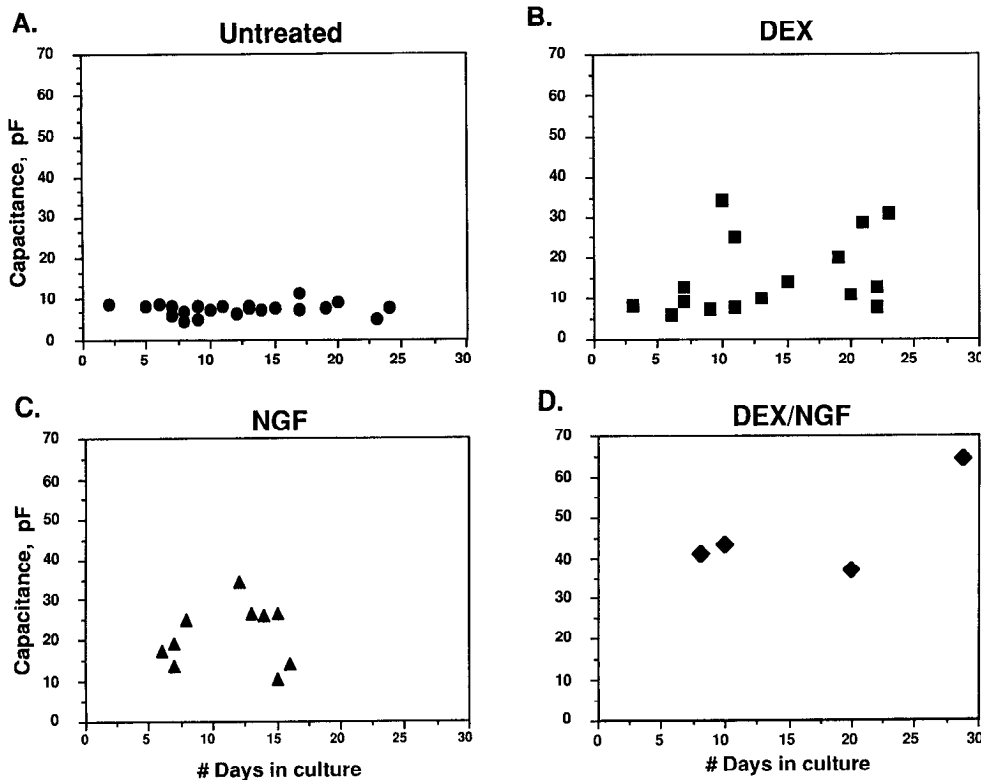
Figure 2. Capacitance of (*A*) untreated PC12 cells remained 8 ± 4 pF for 4 weeks. The capacitance of some cells increased up to 2- to 3-fold in the presence of (*B*) DEX and (*C*) NGF and 4- to 5-fold in the presence of (*D*) DEX/NGF. Each data point shown is an average capacitance of at least 3 cells.

voltage-dependent Na current also increased with time in culture (Fig. 4*A*), while the current density decreased (Fig. 4*B*). Voltage-dependent Na currents were expressed in 88% of NGF-treated cells ($p = 0.0001$, $n = 33$: Fig. 4*A*). The Na current density in NGF-treated cells increased 3-fold with respect to untreated cultures (Fig. 4*B*). Significantly more cells exhibited Na currents when incubated with DEX than untreated cells (72%, $p = 0.0004$, $n = 46$: Fig. 4*C*). This increase was not accompanied by an increase in Na current density (Fig. 4*D*), suggesting that, along with the increase in membrane area (Fig. 2*B*), there is an increase in the total number of Na^+ channels.

Treatment with both DEX and NGF also increased the percentage of cells expressing Na current ($p = 0.005$, $n = 5$: Fig. 4*C*). This increase was significant despite the small sample size. The presence of DEX, however, antagonized the increase in the Na current density induced by the presence of NGF alone. When compared with the large increase in Na current density in NGF alone, the increase in DEX/NGF-treated cells was only to 1.2-fold over untreated cells (Fig. 4*D*). This antagonistic effect of DEX on current density is at least partly due to the larger increase in membrane area of these cells (Fig. 2*D*). No significant changes in Na current density over time in culture were observed in DEX- or DEX/NGF-treated cells.

Both DEX and NGF alter voltage-dependent Ca current

Two distinct types of voltage-dependent Ca current were observed in PC12 cells; sustained high-threshold Ca current and low-threshold, transient T-type Ca current. All whole-cell Ca current experiments were done under conditions in which Ca^{2+} was the only charge carrier. The criteria for the presence of T-type Ca current was the appearance of transient inward current, greater than 5 pA in amplitude in response to a depolarizing pulse to -10 mV from a holding potential of ≤ -80 mV. Under

this protocol, L-type Ca^{2+} channels would be activated but a proportion of N-type Ca^{2+} channel were probably inactivated. We do not make a distinction between L and N Ca currents and have denoted nontransient, sustained Ca currents as high-threshold Ca currents. The L and N components of high-threshold Ca currents are difficult to separate in PC12 cells without determining dihydropyridine sensitivity (Plummer et al., 1989). In cells expressing the T-type Ca current, very little or no transient Ca current was observed following a 1 sec prepulse to -40 mV.

Untreated, DEX-, NGF-, and DEX/NGF-treated cells each fell into 2 populations: those that expressed only high-threshold Ca currents (Fig. 5*A*) and those that expressed both high-threshold and T-type currents (Fig. 5*B*). High-threshold currents were observed in virtually all cells examined. Peak $I(V)$ relationships (Fig. 5*C*) showed that high-threshold Ca current had a maximal amplitude at $+20$ mV, whereas T-type Ca current had a maximal amplitude at -20 to -10 mV following a prepulse to -90 mV. Representative openings of the single channels which underlie the T-type and high-threshold Ca currents are shown in Figure 6. In agreement with the properties of the whole-cell Ca currents, the probability of the T-type channel being open rapidly increased after a depolarization to -20 mV and then declined rapidly (Fig. 6*A*). In contrast, the probability of a single high-threshold-type channel being open did not decline noticeably during a pulse to 0 mV (Fig. 6*B*). The amplitude and gating properties suggest that these records are from an L-type channel. We did not, however, confirm this by testing dihydropyridine sensitivity. The unitary current amplitude of the T-type channel was 2–3 times smaller than that of the high-threshold channel in the range of -20 to 0 mV. The mean open duration of the T-type channel was, however, markedly greater than that of the high-threshold single channel. Both T-type and high-threshold
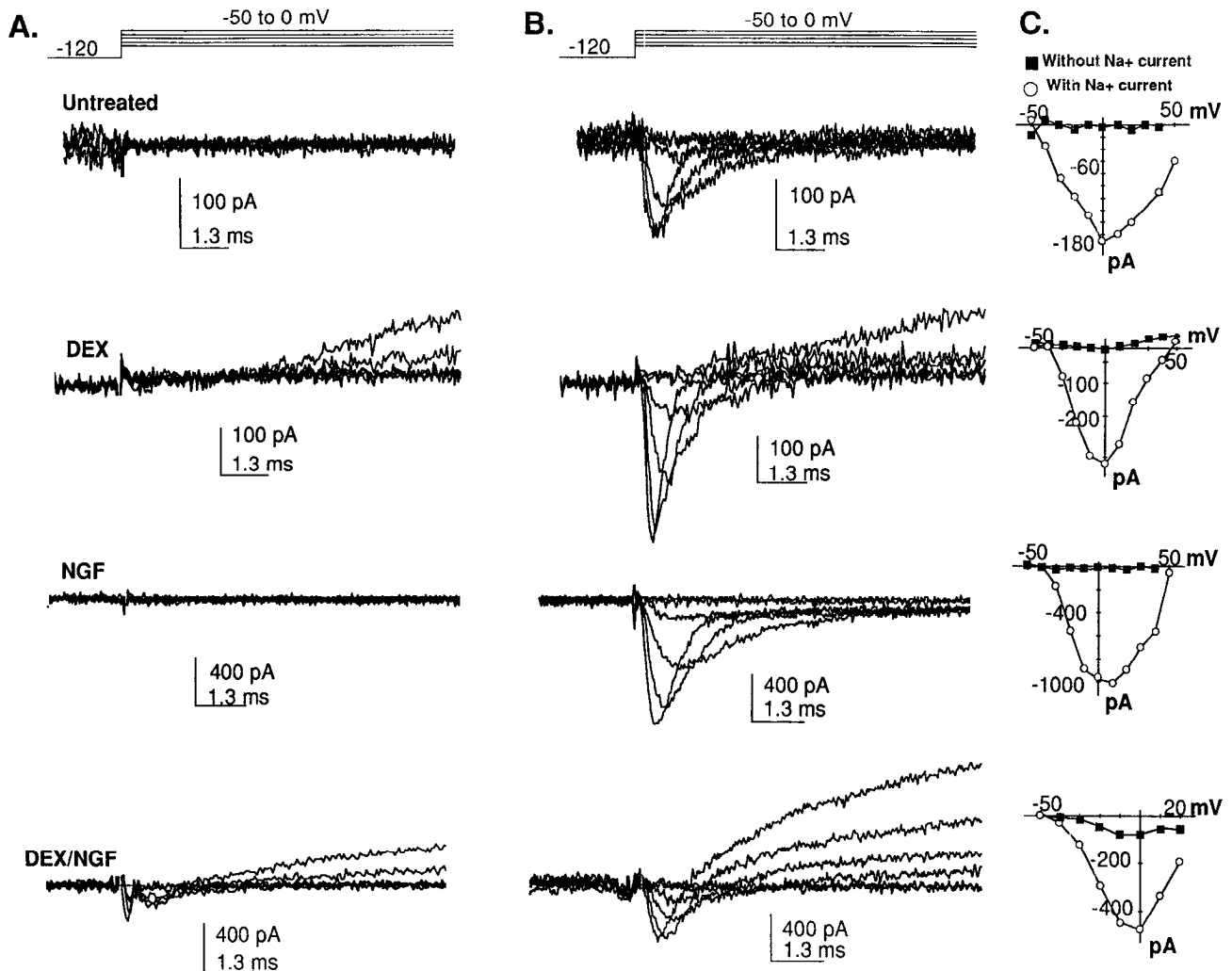
Figure 3. Representative families of the voltage-dependent Na currents from untreated, DEX-, NGF-, and DEX/NGF-treated cells. In each treatment group, some cells (*A*) expressed no or little Na current and others (*B*) expressed appreciable amounts of Na current. The currents were elicited at the voltages shown following 1 sec prepulses to voltages ≤ -100 mV. *C,* Peak $I(V)$ curves were obtained from cells which did not express the Na current (*black squares*) and those which expressed the Na current (*circles*). The voltage-dependent Na currents were often recorded using an internal solution containing K^+, thus resulting in outward voltage-dependent K currents at positive voltages. However, the voltage-dependent K currents activated much more slowly than the voltage-dependent Na current at the voltages more negative than $+10$ mV (see DEX records in *A*). In other experiments (untreated and NGF records in *A* and *B*), outward K currents were reduced substantially by using an internal solution with Cs^+ (see Materials and Methods). Some fidelity of the voltage-clamp in DEX/NGF cells was lost because of the very large cell size (see Fig. 2*D*). The apparent inward currents observed in DEX/NGF cells in *A* were artifacts of the imperfect leak current subtraction [see the corresponding $I(V)$ curve in *C*].

currents observed in this study closely resemble those described in other preparations (Tsien et al., 1987). Plummer et al. (1989) have also described single, high-threshold L and N Ca^{2+} channels in PC12 cells.

The relative proportion of cells expressing T-type current changed in response to the length of time cells were treated with DEX, NGF, or DEX/NGF. Only 20% of untreated cells ($n = 101$), grown in culture up to 3 weeks, expressed the T-type current in addition to the high-threshold current (Fig. 7*A*). In the presence of DEX, the fraction of cells expressing T-type Ca currents increased slowly over the course of 3 weeks to 40% ($p = 0.0008$, $n = 142$; Fig. 7*A*). There was a small increase in T-type current densities with the DEX treatment (Fig. 8*B*). Ca current densities did not change with time in DEX-treated cells. Treatment with NGF alone increased the fraction of cells expressing the T-type current within 7–10 d to ~60% ($p = 0.017$,

$n = 44$: Fig. 7*A*) and resulted in a 3-fold increase in the density of T-type current with respect to untreated cells (Fig. 8*C*). NGF treatment did not markedly change the high-threshold Ca current density. High-threshold currents were assayed as previously described. Under these conditions, not all the N channels may be available for opening. These experiments would not detect changes in current density of high-threshold channels which may require longer times to recover from inactivation. The presence of the T-type voltage-dependent Ca current in any of the treatment groups was not correlated with any specific cellular morphology or with cell capacitance. Voltage-dependence of T-type and high-threshold Ca currents did not differ markedly among treatment groups.

Despite the ability of either DEX or NGF to increase the fraction of cells expressing T-type Ca current, the percentage of DEX/NGF-treated cells with T-type Ca current was not signif-
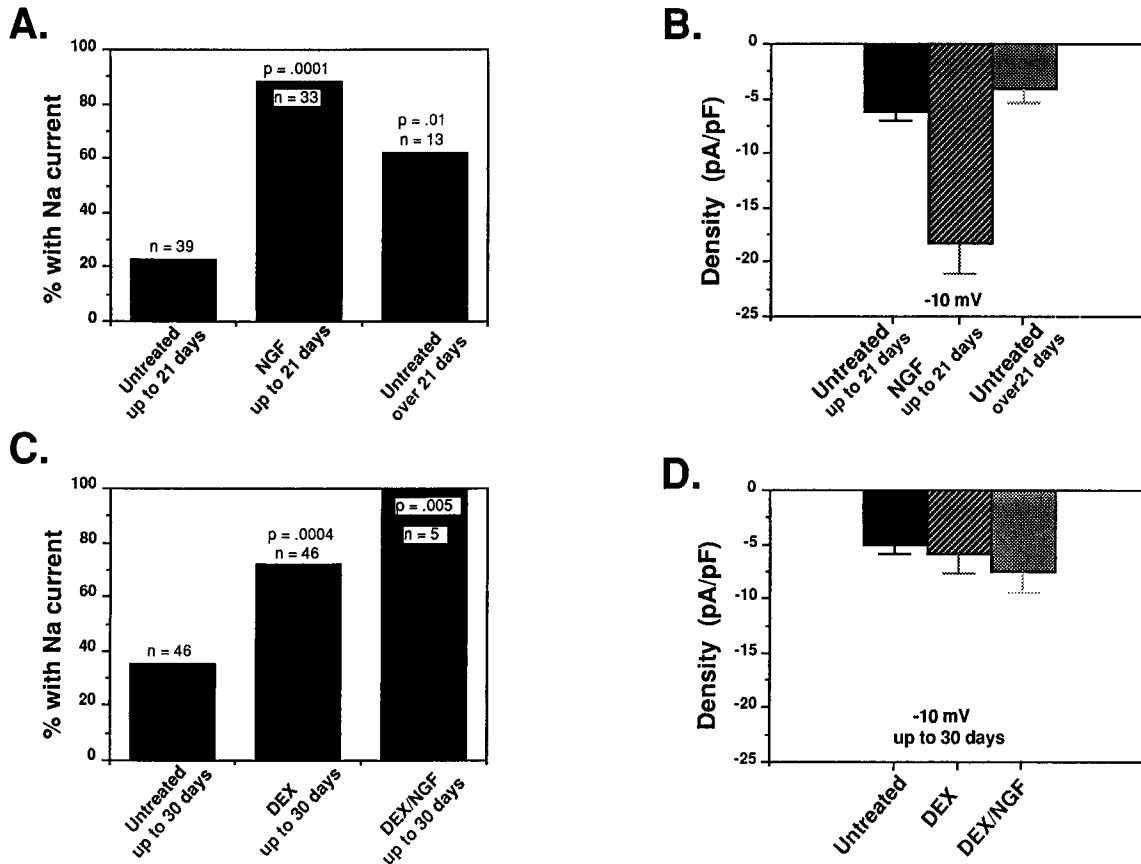
Figure 4. *A*, The fraction of untreated control cells expressing Na current grown in culture less than 21 d compared to NGF-treated cells and untreated cultures grown for over 21 d. *B*, Average densities of the voltage-dependent Na current in untreated cells grown for less than 21 d in culture compared to NGF-treated cells and untreated cells grown for more than 21 d in culture. *C*, DEX- and DEX/NGF-treated cells compared to untreated populations grown over 30 d in culture. No significant differences were observed between DEX- and DEX/NGF-treated cells grown up to and over 21 d in culture; thus, these populations were pooled for each treatment. *D*, Na current density of DEX- and DEX/NGF-treated cells compared to that of untreated cells grown in culture over a 30 d period. The total number of cells recorded in each group is given as *n*. Significant differences between untreated groups and treatment groups are given as *p* and were determined using χ^2 analysis. Average current density at -10 mV is unlikely to be contaminated by the voltage-dependent outward currents. SEM are shown as error bars.

icantly different from the control level of untreated cells over the same time period ($p = 0.35$, $n = 19$: Fig. 7*B*). The presence of DEX prevented the increase in T-type current density observed in the presence of NGF alone (Fig. 8*D*).

T-type Ca current density increases in confluent untreated cultures

As untreated cells became confluent after 3 weeks in culture, the fraction of the cells expressing T-type Ca current increased to 60% ($p = 0.001$, $n = 34$: Fig. 7*A*), compared with 20% found in the 0–21 d untreated cells. This increase is accompanied by an increase in the T-type current density in untreated cells cultured over 21 d (Fig. 8*A*). The percentage of untreated cells grown in culture for more than 21 d expressing T-type Ca current surpassed the level achieved by DEX treatment after 3 weeks and approached that induced by NGF over a shorter time period (Fig. 7*C*). Because of this increase in T-type current in >21 d untreated cells, there was no significant difference in the fraction of cells with T-type Ca current between the >21 d untreated cells and DEX-treated cells over 21 d ($p = 0.75$, $n = 45$: Fig. 7*C*). The density of T-type Ca current of the over 21 d untreated control cells decreased slightly with respect to DEX-treated cells

recorded from up to 3 weeks in culture, while that of high-threshold current increased (Fig. 8*E*).

Effect of substrate on T-type Ca current density

Recent reports have suggested that cellular functions such as neurite outgrowth in cultured cells are substrate-dependent (Lin and Levitan, 1988; Tomaselli et al., 1986). Bethea and Kozak (1984, 1985) have also shown that PC12 cells grown in the presence of extracellular matrix release more and store less dopamine then cells grown on plastic. Although coverslips in the present experiments were uncoated borosilicate glass, confluent PC12 cells are in contact with neighboring cells. This contact may provide a substrate quite different from that encountered by a PC12 cell, freshly seeded at low density. Cell–cell and cell/extracellular matrix interactions, for example, have resulted in modulation of the NGF response in PC12 cells (Doherty et al., 1987). We therefore considered the possibility that interaction with a more adherent, nonspecific substrate was responsible for the change in the fraction of cells expressing T-type Ca current between untreated cells grown less than 21 d and over 21 d in culture.

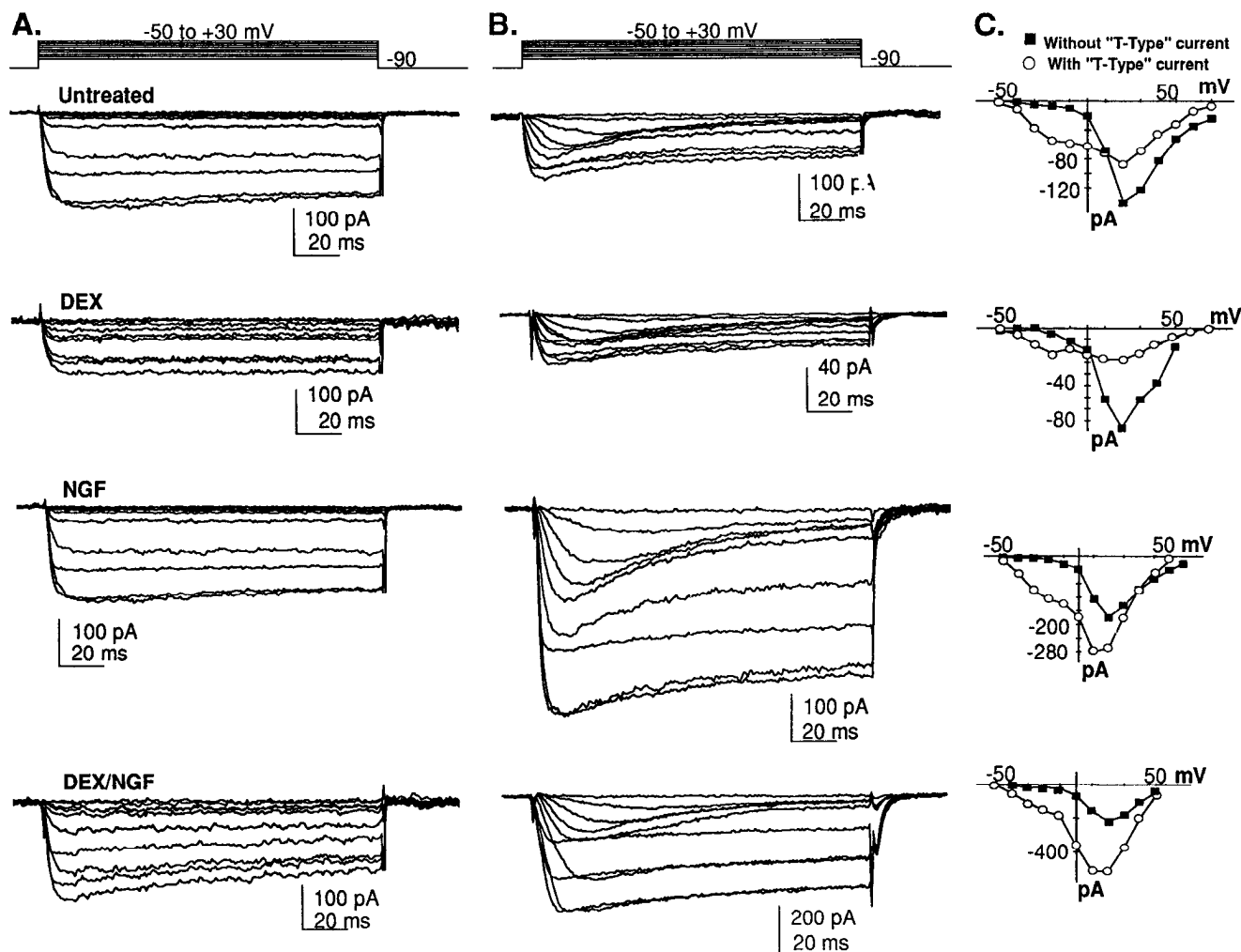Cells grown on glass coverslips coated with Cell-Tak to pro-

Figure 5. Representative families of Ca currents recorded from untreated and DEX-, NGF-, and DEX/NGF-treated cells. In each treatment group, some cells (*A*) expressed only high-threshold Ca current and others (*B*) expressed both high-threshold and T-type current. The currents shown were elicited at the voltages indicated from a holding potential of -90 mV (repetition interval = 6 sec). *C*, Peak $I(V)$ curves obtained from the cells which showed only the high-threshold current (*black squares*) and those which had both T-type and high-threshold currents (*circles*). The T-type Ca current shows up as a distinct shoulder at voltages more negative than 0 mV, with peak current occurring at -20 to -10 mV. In cells expressing T-type Ca current, high-threshold Ca current was also observed. Holding potentials of ≤ -80 mV and 1 sec prepulses to -40 mV elicited similar high-threshold currents. Some variability of the inactivation kinetics (independent of holding or prepulse potential) of low- and high-threshold currents was observed.

mote cellular adhesion to coverslips did not show a significant difference in the fraction of the cells expressing T-type Ca current ($p = 0.35$, $n = 16$: Fig. 9*A*), although those grown on nitrocellulose did ($p = 0.02$, $n = 12$: Fig. 9*A*). T-type Ca current density also did not change significantly in cells grown on Cell-Tak (Fig. 9*B*), but did increase in cells grown on nitrocellulose (Fig. 9*C*), when compared with untreated cells grown on uncoated coverslips. There was also a significant decrease in high-threshold current density in cells grown on nitrocellulose (Fig. 9*C*). Untreated, NGF-, and DEX-treated populations of cells grown in suspension did not alter the expression of the T-type Ca current compared with cultures grown on coverslips under equivalent conditions (data not shown).

To test the possibility that changes in the adherent properties of PC12 cells may also result in a corresponding change in ionic currents, we made recordings from a subcloned continuous, adherent PC12 cell line, clone B. Clone B was selected on the basis of its adherent properties to uncoated Costar culture dish-

es. These cells were generally ovoid and slightly flat in appearance. Capacitance of clone B cells was similar to that of untreated cells (data not shown). A significant fraction (77%) of these cells expressed T-type current as compared with untreated cells grown up to 21 d in culture ($p = 0.0001$, $n = 31$: Fig. 9*A*). Other equally adherent subcloned lines also exhibited a larger fraction of cells with T-type current (data not shown). The T-current density also increased, whereas the high-threshold current density decreased, with respect to untreated populations (Fig. 9*D*).

Plating on Cell-Tak-coated coverslips did not result in a significant increase in percentage of cells with Na current ($p = 0.28$, $n = 16$: Fig. 10*A*). Na current density of cells grown on Cell-Tak did not increase significantly (Fig. 10*B*). The fraction of clone B cells expressing Na current, however, was significantly greater than untreated cells ($p = 0.002$, $n = 17$: Fig. 10*A*). This was accompanied by a large increase in Na current density (Fig. 10*B*). These observations suggest that the expression of adhesion molecules and the development of certain ionic currents may
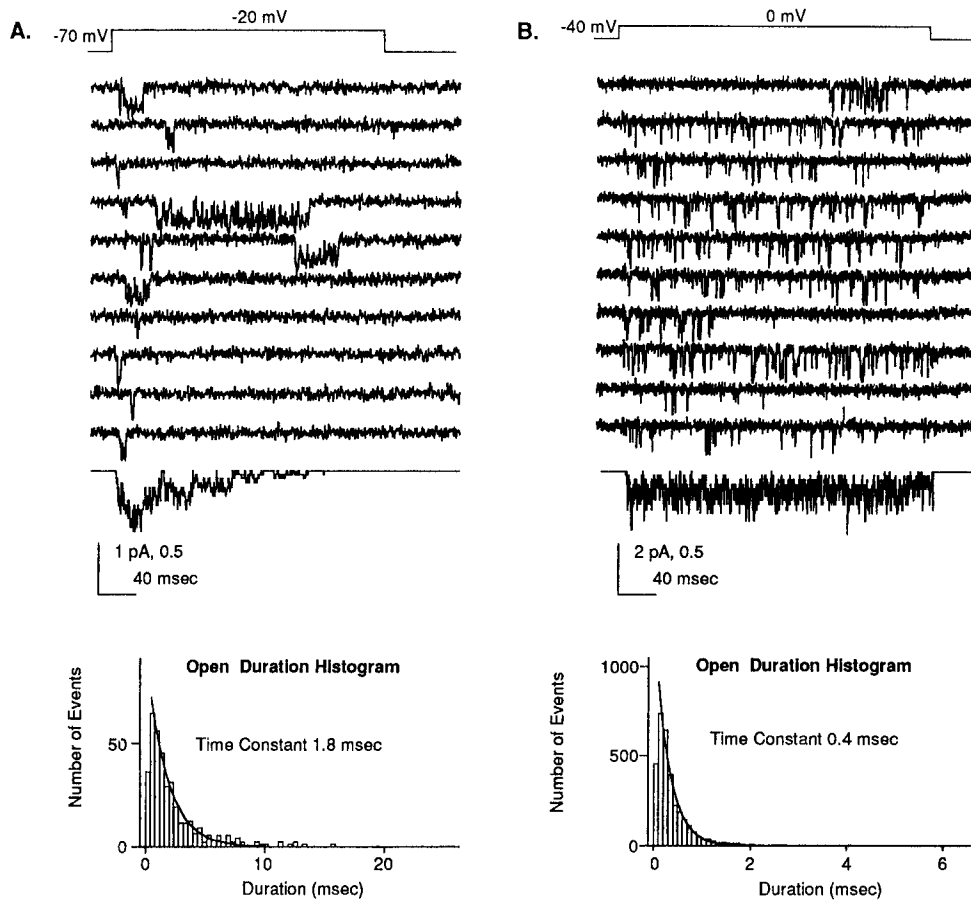
Figure 6. Activity of single voltage-dependent Ca^{2+} channels recorded from NGF-treated cells using the cell-attached recording configuration. *A,* Representative openings of the T-type Ca^{2+} channels elicited at -20 mV from a holding voltage of -70 mV. The *bottom trace* is an ensemble average of idealized records showing the time course of the probability of the channel being open. The data were filtered at 500 Hz through an 8-pole Bessel filter and digitized at 5 kHz. Pulses were applied every 5 sec. The recording was made from the cell body. The neurites of this cell were intact. Open duration histograms of a T-type Ca channel recorded at -20 mV are shown below traces. *B,* Representative openings of a high-threshold Ca channel elicited at 0 mV from a holding voltage of -40 mV. The *bottom trace* is an ensemble average of idealized records showing the time course of the probability of the channel being open. The recordings shown are probably from an L-type Ca^{2+} channel. The data were filtered at 1.2 kHz and digitized at 10 kHz. The recording was made from the cell body. The neurites of this cell were intact. Open durations of the high-threshold Ca channels shown below current traces were recorded at 0 mV.

be linked in PC12 cells. Other processes, such as increases in acetylcholinesterase activity in PC12 cells (Lucas et al., 1980; Reiger et al., 1980) and survival of rat chromaffin cells in the presence of DEX (Grothe et al., 1985), have also been shown to be dependent on substrate.

Voltage-dependent K currents were not affected by NGF or DEX

The whole-cell voltage-dependent K currents in the PC12 cells used in this study activate rapidly and inactivate very slowly, with transient K currents very infrequently observed (Hoshi and Aldrich, 1988a). DEX or NGF treatment up to 4 weeks did not increase the appearance of transient voltage-dependent K currents at $+10$ mV ($p = 0.088$, $n = 66$ and $p = 0.75$, $n = 24$, respectively) or otherwise alter the composition of the whole-cell K current. Clone B cells or cells grown on Cell-Tak or nitrocellulose did not show any marked differences in K currents. The following treatments also failed to affect these K currents; dibutryl cAMP and theophylline (2 and 5 mM, respectively, for 2 d, $p = 0.99$, $n = 5$), β-estradiol (0.4 μM for 3 d, $p = 0.29$, $n = 9$), absence of sera in the medium (for 6 d, $p = 0.47$, $n = 2$).

Discussion

Both DEX and NGF have distinct effects on the morphology and complement of voltage-dependent ionic currents in PC12 cells. In general, DEX also appears to antagonize the effects of NGF. Ionic current composition, however, can also be altered to some extent by the time in culture, substrate, and perhaps by adhesion. The results described in this report are summarized below.

Morphology

Treatment with NGF alone, DEX alone, or both NGF and DEX together, increases the capacitance, and hence the membrane area, of PC12 cells. However, the extensive neurite outgrowth observed with NGF is antagonized by DEX.

Voltage-dependent Na currents

Both NGF and DEX increase the fraction of cells which express voltage-dependent Na current. Only NGF, however, causes significant increases in the Na current density. The effect of NGF on the Na current is attenuated in the presence of DEX. Both clone B, a subcloned line selected for adherence to uncoated plastic, and confluent untreated cells showed increases in the fraction of cells expressing Na current.

Voltage-dependent Ca currents

Chronic application of DEX or NGF or confluency of untreated cells increases the occurrence and density of T-type Ca current. Clone B cells also show increased occurrence and density of T-type Ca current in the absence of either DEX or NGF. Expression of T-type Ca current may be influenced by certain substrates. A possibly related result is that fibroblasts, which become much less adherent after oncogenic transformation, also lack T-current after tranformation (Chen et al., 1988).
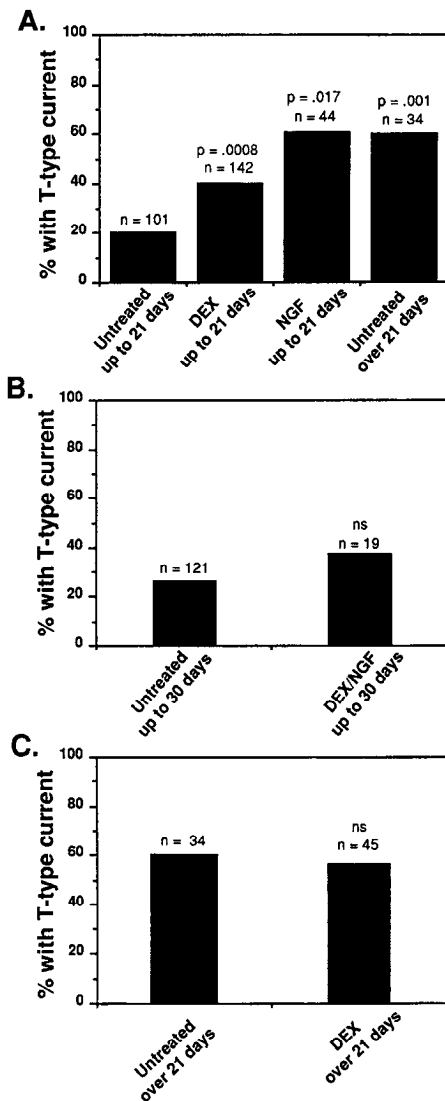
Figure 7. Percentages of cells with different treatments expressing T-type Ca current. *A,* The fraction of untreated control cells grown in culture less than 21 d compared to DEX- and NGF-treated cells and untreated cultures grown for over 21 d. *B,* DEX/NGF-treated cells compared to untreated populations grown over 30 d in culture. *C,* Untreated cells grown in culture over 21 d compared to DEX-treated cells also grown over 21 d. The total number of cells recorded in each group is given as *n*. Significant differences between untreated groups (presented on the *left side* of each graph) and treatment groups are given as *p* and were determined using χ^2 analysis. *ns* denotes $p > 0.05$.

Figure 8. *A,* Average densities of the voltage-dependent Ca current in untreated cells grown for less than 21 d in culture compared to those grown for more than 21 d in culture. Ca current density of (*B*) DEX- and (*C*) NGF-treated cells compared to that of untreated cells grown in culture for <21 d. *D,* DEX/NGF-treated cells compared to untreated cells grown over 30 d. *E,* DEX-treated cells grown in culture over 21 d compared with untreated cells of the same age. The average current density at -10 mV primarily represents the T-type Ca current density, while that at $+20$ mV consists primarily of the high-threshold Ca current density. SEM are shown as error bars.

Voltage-dependent K currents

Neither NGF or DEX markedly affect voltage-dependent K currents. These results are in contrast to those of Rudy and Pollock (1988), who observed that application of NGF to PC12 cells cause a decrease in a TEA-sensitive, transient voltage-dependent K current.

The straightforward interpretation of the observed increases in the fractions of cells expressing T-type Ca current or Na current in response to DEX and/or NGF is that there is an increase in the number of functional ion channel molecules underlying these currents. The increase in capacitance without decreases in current density suggests that new ion channel proteins are either inserted into the plasma membrane or that pre-
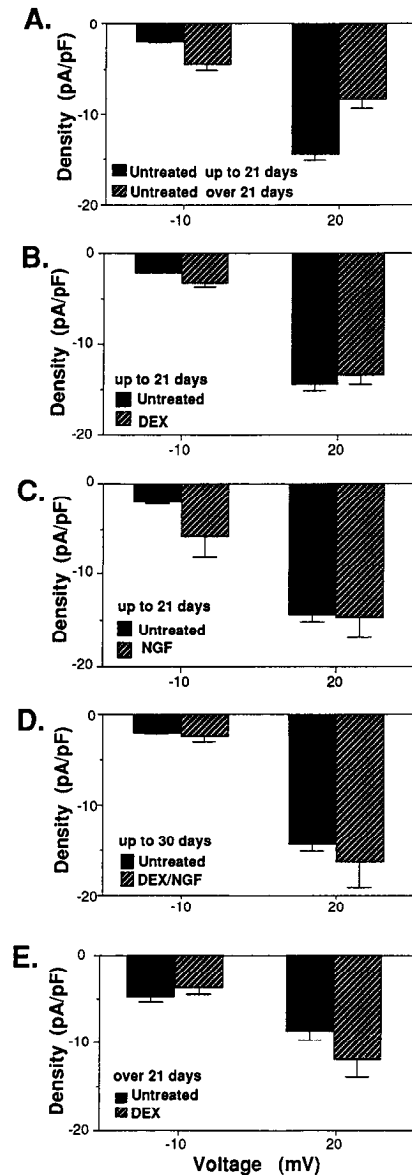
viously inserted, but nonfunctional, channels are somehow activated. Alternatively, because a small fraction of nonconfluent, untreated cells also express T-type Ca currents and Na currents, it is possible that treatment with DEX and/or NGF selectively enhances the division of cells containing these channels. This latter hypothesis is unlikely, however, because PC12 cells cease to divide or divide only very slowly in the presence of NGF and DEX (Greene and Tishler, 1976; Goodman et al., 1979; Schubert et al., 1980). It is possible that with an increase in confluence or age of cells, the numbers of dead or floating cells increased, resulting in an unintended selection of more adherent
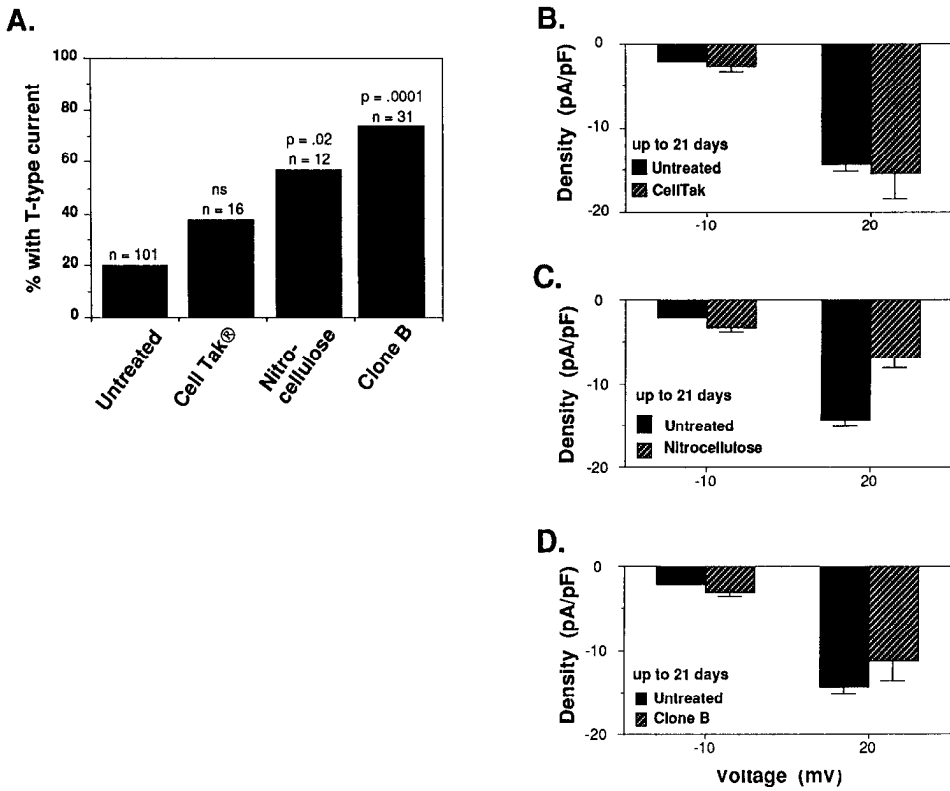
A.

B.

C.

D.

Voltage (mV)

Figure 9. *A,* Percentages of clone B, Cell-Tak, and nitrocellulose-treated cultures expressing T-type Ca current compared with untreated cultures grown up to 21 d in culture. Average densities of the voltage-dependent Ca currents of untreated cells compared to (*B*) Cell-Tak- and (*C*) nitrocellulose-treated cultures and to (*D*) clone B. The average current density at −10 mV primarily represents the T-type Ca current density, while that at +20 mV consists primarily of the high-threshold Ca current density. All test groups are compared with control cells grown on untreated borosilicate glass coverslips. The total number of cells recorded in each group is given as *n. ns* denotes $p > 0.05$. SEM are shown as error bars.

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cells with characteristics similar to that of clone B. If this were the case, however, continued passaging of PC12 cells over several months should result in cell lines with greatly increased appearance of T-type Ca current. This is not observed (unpublished results). Another possibility is that channels are synthesized at a constant rate, and are maintained at a constant turnover rate. Thus, channels would accumulate in slowly dividing or nondividing cells (such as NGF-, DEX-treated, or confluent control cells). Mandel et al. (1988) have shown that NGF selectively induces type II Na channel mRNA expression and that this induction results in an increase in voltage-dependent Na current density. This observation suggests that, at least in the case of NGF-treated PC12 cells, the increase in Na current density is in part due to increased synthesis of the channel protein. We suggest that the DEX- and NGF-induced increases in the fractions of the cells with T-type Ca currents and voltage-dependent Na currents reflect an increase in the number of active ion channel proteins in the cell membrane.

Capacitance measurements indicate that the average membrane area of the DEX- or NGF-treated PC12 cell bodies increased 2- to 3-fold. These observations suggest that both treatments result in the addition of membrane to the cell body. In the case of NGF, additional membrane may be needed for neurite extension. The increased membrane area in DEX-treated cells may be a result of membrane incorporation due to fusion of vesicles associated with the release of neurohormones. The larger increase in the capacitance of DEX/NGF-treated cells suggests that while DEX inhibits NGF-mediated neurite outgrowth, the additional membrane intended for outgrowth is still produced and is instead added to the cell body.

Previous studies have documented differential, often antagonistic, effects of DEX and NGF on cellular functions. For example, the intracellular content of neuropeptide Y (NPY) in PC12 cells increases in the presence of NGF but not DEX (Allen et al., 1984). Addition of DEX to NGF-treated cultures of PC-12 cells prevents any further increase in NPY content (Allen et
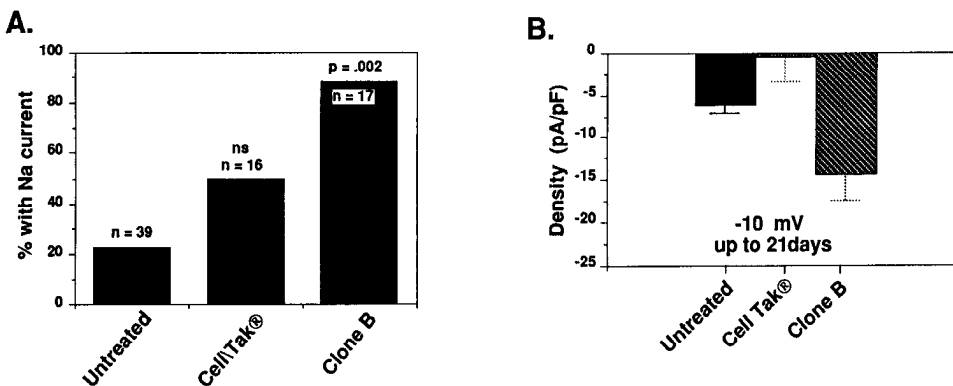
A.

B.

Figure 10. *A,* Percentages of clone B and Cell-Tak-treated cultures expressing Na current compared with untreated cultures grown up to 21 d in culture. *B,* Voltage-dependent Na current average density of untreated cells compared to clone B and Cell-Tak-treated cultures. Average current densities at −10 mV are shown. All test groups are compared with control cells grown on untreated borosilicate glass coverslips. The total number of cells recorded in each group is given as *n. ns* denotes *p* > 0.05. SEM are shown as error bars.

al., 1984). Similar observations were made concerning neurotensin and vasoactive intestinal peptide-like immunoreactivity in primary human pheochromocytoma cultures incubated with NGF or DEX/NGF (Tishler et al., 1982a, 1984, 1986). In addition, the specific activity of choline acetyltransferase increases in NGF-treated cells, whereas the specific activity of tyrosine hydroxylase increases in the presence of glucocorticoids (Greene and Rein, 1977; Edgar and Thoenen, 1978; Otten and Towbin, 1980; Schubert et al., 1980; Tishler et al., 1983). The activity of both of these enzymes, however, also increases with increased cell density (Greene and Rein, 1977; Edgar and Thoenen, 1978; Lucas et al., 1979). It is not clear whether the changes in voltage-dependent ionic currents reported here and changes in enzymatic activity are associated or simply concurrent. It is likely, however, that treatment of PC12 cells with DEX or NGF results in a cascade of effects. Two of the many possible end points of these cascades could be alterations of enzymatic or ionic current activity. It would be of interest to determine if the specific activity of these enzymes is increased in clone B.

Our results show that undifferentiated PC12 cells can express both high- and low-threshold-type Ca currents. This is true for both the untreated population of PC12 cells and of the subclone, clone B. The existence of more than one type of Ca current in PC12 cells had been suggested in the work of Takahashi et al. (1985) and Kongsamut and Miller (1986). The presence of T-type Ca current differs from the results of Streit and Lux (1987), who did not observe a T-type Ca current in PC12 cells, although high-threshold Ca currents were recorded. Streit and Lux observed, however, that these Ca currents inactivated more rapidly after the application of NGF. These differences may be caused by experimental or inherent differences between the clonal PC12 cell lines used. We have found that the fraction of cells exhibiting the low-threshold T-type Ca^{2+} current and the density of this current increased in response to NGF and DEX, with confluence, and in clone B. Plummer et al. (1989) have investigated changes in high-threshold Ca channels in PC12 cells in response to NGF and suggested that NGF causes an increase in the N-type Ca channel. Our work does not address differential changes in the high-threshold currents, as they were not distinguished from each other.

Because the T-type Ca currents were observed in at least some cells in the untreated and DEX- and NGF-treated groups, it is clear that the presence of the T-type current is not prerequisite for any one differentiated state. While some differentiated NGF-treated cells only had high-threshold current, some perfectly spherical untreated cells expressed very robust T-type currents. Although DEX-treated PC12 cells are considered similar to adrenal chromaffin cells, DEX-treated PC12 cells express both T-type and high-threshold currents, whereas chromaffin cells do not appear to have T-type currents (Fenwick et al., 1982; Hoshi et al., 1984). Thus, while it is possible that different voltage-dependent currents have different cellular functions, the relative contributions of T-type, high-threshold Ca^{2+}, and Na^+ channels to cellular physiology may also be important to cellular differentiation.

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