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Physiological and morphological studies of rat pheochromocytoma cells (PC12) chemically fused and grown in culture

(clonal cells/nerve growth factor/neurite-like outgrowth/Na^+, Ca^{2+}, and K^+ conductance mechanisms/tetanus toxin binding)

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ABSTRACT **Cell fusion induced by polyethylene glycol has been used to produce in culture giant multinucleate PC12 cells (up to 300 μm in diameter compared to 10–20 μm for unfused cells). Fused cells, like their unfused counterparts, were found to express various neuronal properties. They contained catecholamines. In the presence of nerve growth factor they extended long processes and expressed Na^+, Ca^{2+}, and K^+ conductances generally associated with excitable cells. In the absence of nerve growth factor these cells neither grew long processes nor generated Na^+-spikes. Other neuronal properties were also observed.**

Little is known about factors that influence the expression of excitable properties of vertebrate neurons during their differentiation. Several recent studies have demonstrated that the PC12 clonal cell line, isolated by Greene and Tischler (1) from a rat adrenal medullary tumor (pheochromocytoma), is a useful model for studying neuronal properties. These cells display excitable mechanisms whose expression is influenced by nerve growth factor (NGF) and they exhibit a number of properties that resemble rather closely those of sympathetic neurons. PC12 cells have mechanisms for the synthesis, storage, release, and uptake of catecholamines (CA) (1–4); they express in addition cholinergic properties under various culture conditions (4–7). In the presence of NGF many PC12 cells cease dividing, grow long (>100 μm) processes (1) similar in appearance to those seen in cultures of sympathetic neurons (e.g., ref. 8), and develop action potentials sensitive to tetrodotoxin (TTX) (5).

Studies of the electrophysiological properties are technically difficult because PC12 cells are small (10–20 μm in diameter) and are easily damaged by microelectrode penetrations. To minimize these difficulties we have been studying giant PC12 cells (up to 300 μm in diameter) produced by chemically induced fusion of individual cells and grown in the presence or absence of NGF. In this paper we report on the morphological and electrophysiological characteristics of the fused PC12 cells. These cells were found to express various neuronal properties, including action potential mechanisms for Na^+, Ca^{2+}, and K^+ conductances, as well as other K^+ conductance mechanisms. Our findings provide further support for the usefulness of PC12 cells as a neuronal model.

METHODS

Cultures. PC12 cells were obtained from Lloyd Greene. They were grown in 75-cm^2 Falcon tissue culture flasks in Dulbecco's modified Eagle's medium (GIBCO) containing heat-inactivated (56°C, 0.5 hr) 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) and 5% horse serum (Microbiological Associates, Bethesda, MD), 100 units of penicillin G (Squibb) per ml, and 0.1 mg of streptomycin (Pfizer) per ml. Cells were grown to a density of approximately 10^5–2×10^5 per cm^2 and transferred by tapping the side of the flask, vigorously triturating the resulting cell suspension, and replating at one-fifth the original density. For the electrophysiological experiments cells were plated onto the collagen-coated surface of a coverslip attached to a modified 35-mm petri dish (9). NGF (7 S) was isolated by the methods of Bocchini and Angeletti (10) and Varon *et al.* (11). Cells grown with NGF (200 ng/ml) or without NGF will be designated $^+$NGF or $^-$NGF, respectively. Cultures were maintained at 36–37°C in a CO_2 incubator (10% CO_2/90% air).

Cell Fusion. Prior to passage, fusion of attached cells was induced with polyethylene glycol (PEG) by the methods of Davidson *et al.* (12). The cells were removed with trypsin (0.125%, Difco) by standard procedures and plated as described above.

Electrophysiological Methods. These methods were described (9), except Hepes buffer [5 mM; pH 7.4 (Sigma)] replaced $NaHCO_3$ in the perfusion medium. Drugs were obtained from the following sources: TTX (Calbiochem); tetramethylammonium (Me_4N) (Baker); tetraethylammonium (Et_4N) (Pfaltz and Bauer, Stamford, CT).

CA Histofluorescence. CA were detected by the glyoxylic acid technique of de la Torre and Surgeon (13). Two control experiments were performed: (*i*) omission of glyoxylic acid from the reaction solution and (*ii*) omission of the heating steps. In a few cases prior to staining, cultures were incubated (37°C, 4 hr) with 10 μM reserpine (CIBA Pharmaceutical).

RESULTS

Morphological Characteristics of Fused Cells. After PEG treatment many cells (>10%) appeared to be multinucleate and dramatically increased in size. Unfused cells (small and with a single nucleus) did not appear to differ from untreated cells (see below). Cells not exposed to PEG rarely appeared multinucleate. Fig. 1*A* shows a multinucleate cell 1 day *in vitro* (DIV) after replating; unfused cells are shown at the same magnification in Fig. 1*A Inset*. Five observations indicated that the large cells were not aggregates of individual cells but were the result of cell fusion similar to that seen after PEG treatment of other cell types (for a review see ref. 14): (*i*) Individual nuclei were never associated with the phase halos that typically surround individual PC12 cells in an aggregate viewed

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Abbreviations: CA, catecholamine(s); DIV, day(s) *in vitro;* LAH, long after-hyperpolarization; NGF, nerve growth factor; PEG, polyethylene glycol; Et_4N, tetraethylammonium; Me_4N, tetramethylammonium; TTX, tetrodotoxin; $[X]_o$, extracellular concentration of ion X.

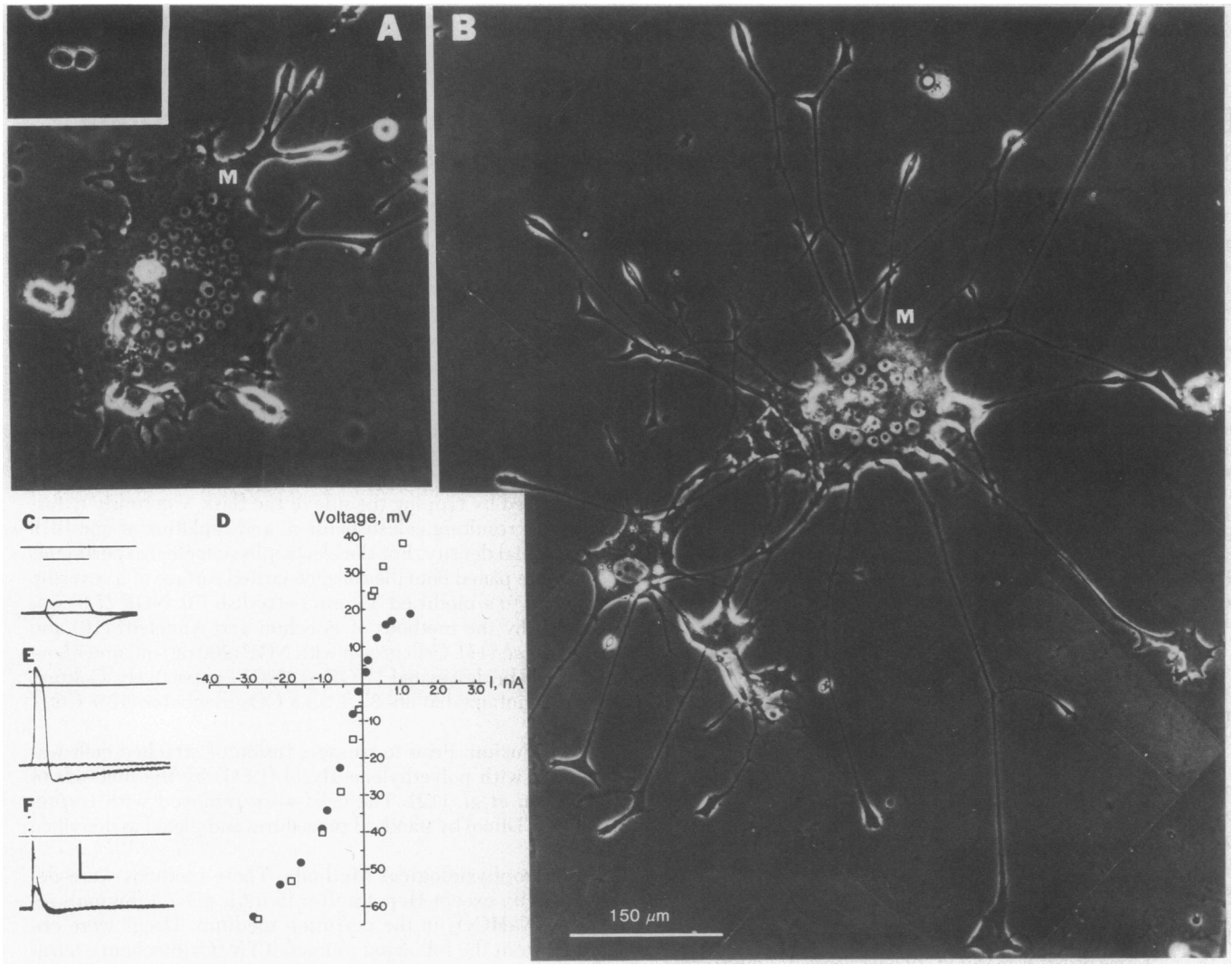

FIG. 1. Phase contrast micrographs (montages) of fused, multinucleate cells (M) from: (*A*) A 1-day-old culture without NGF (*Inset:* Two unfused cells) and (*B*) a 3-day-old culture with NGF (photo retouched). Magnification was the same for all. (*C*) Two superimposed voltage responses (lower trace) to a depolarizing current pulse (top trace, upward) and a hyperpolarizing pulse (downward). Fused cell $^{-}$NGF (7 DIV). (*D*) Steady-state current–voltage characteristics of a fused cell ($^{-}$NGF; 15 DIV) bathed in a low-$[Ca]_o$ (0.1 mM) perfusion media (see text) with 18.5 mM Et_4N (□) and without Et_4N (●). Origin is the resting potential, −55 mV. (*E*) Ca^{2+}-spike. An all-or-nothing potential (lower trace) stimulated in Et_4N (18.5 mM) by a depolarizing current pulse (upper trace); unstimulated levels are superimposed. (7 DIV; $^{-}$NGF.) (*F*) Block of Ca^{2+}-spike by 0.75 mM Cd^{2+}. Same cell as in *E*. Responses that remain are primarily electrotonic. Black vertical bar: *C*, 40 mV, 2 nA; *E* and *F*, 20 mV, 4 nA. Black horizontal bar: *C*, 40 msec; *E* and *F*, 100 msec.

in the phase contrast microscope. (*ii*) The boundaries of large cells appeared continuous, without the abrupt changes in contour commonly seen along the perimeter of a cell clump where cells abut. (*iii*) CA fluorescence of fused cells (see Fig. 2) was uniform, whereas that of cell aggregates (not shown) was highly nonuniform, presumably because individual cells have different concentrations of CA. (*iv*) The fused cells appeared to be isopotential; in every case tested (>100) with two intracellular microelectrodes placed at opposite poles of a large cell, current passed through one electrode caused a voltage deflection at that electrode in a bridge-balanced condition equal in amplitude to that measured by the other. (*v*) PC12 cells were rarely observed to form low-resistance junctions (only one in over 200 pairs tested) regardless of PEG treatment.

Multinucleate cells ranged in diameter from approximately 30 μm (a few nuclei) to 300 μm (tens of nuclei). Cells as large (200–250 μm in diameter) as that in Fig. 1*A* were common. In the presence of NGF both fused and unfused cells grew processes that extended for hundreds of micrometers (Fig. 1*B*). Growth cones of fused cells could be many times larger than those of unfused cells (Fig. 1*B*). Unusually large mononucleate cells frequently contained nuclei many times the size of unfused PC12 cell nuclei. These presumably resulted from the fusion of several nuclei, an event common in the production of cell hybrids (15).

CA Histofluorescence. Both $^{+}$NGF and $^{-}$NGF fused cells were shown to contain CA by glyoxylic acid-induced fluorescence. Fig. 2 shows a phase contrast micrograph (*A*) and a fluorescence micrograph (*B*) of a multinucleate $^{+}$NGF cell (arrows). The fluorescence intensity of this cell was typical and well above that of controls (Fig. 2*C*). In the few cases tested reserpine, known to deplete cellular CA (16), decreased the intensity to control levels.

Electrophysiological Properties of Fused PC12 Cells. Experiments were carried out on 16 separate platings of $^{-}$NGF cultures (1–20 DIV) and $^{+}$NGF cultures (1–29 DIV), usually on cells 50–100 μm in diameter [larger cells often required stimulation currents greater than could be easily passed with

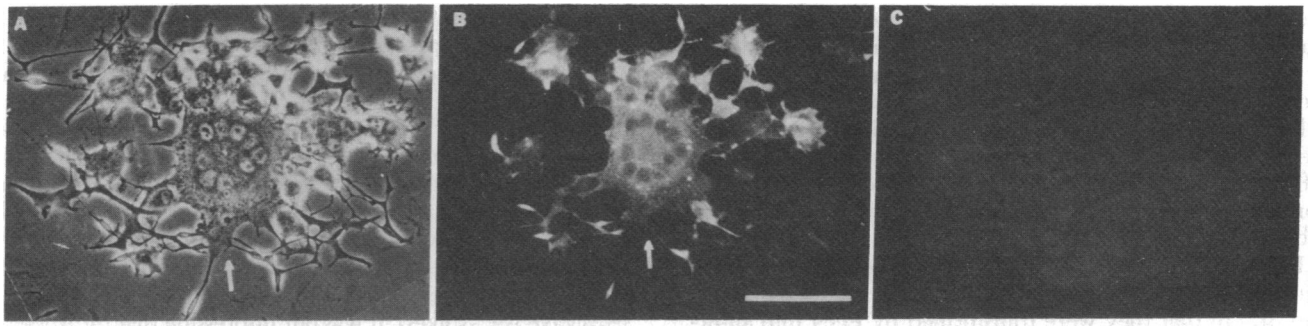

FIG. 2. CA fluorescence. A multinucleate $^{+}$NGF cell 5 DIV (arrow) together with several unfused cells as seen in the phase contrast microscope before (*A*) and in the fluorescence microscope after (*B*) glyoxylic acid treatment. (*C*) Fluorescence micrograph of a 5 DIV control culture ($^{+}$NGF) without the glyoxylic acid step. Fluorescence seen here is typical of fused and unfused cells in all control cultures. Bar, 100 μm.

the high-resistance (i.e., 150–200 MΩ) microelectrodes used in this study]. Measurements were made during continuous impalement with two microelectrodes. Resting potentials were −50 to −65 mV; similar resting potentials were recorded with a single microelectrode in unfused PC12 cells with or without PEG treatment. Input resistances (around rest) of fused cells ranged from 10 to 60 MΩ.

In all cells regardless of growth conditions membrane resistance decreased with depolarization. Fig. 1*C* shows superimposed voltage responses to one depolarizing and one hyperpolarizing current pulse. The depolarizing pulse, although larger, produced a smaller response than the hyperpolarizing pulse. This decrease in membrane resistance with depolarization was apparently due to a voltage-sensitive K^+-conductance (17) and to a Ca^{2+}-dependent K^+-conductance (18). The presence of voltage-controlled K^+-channels—i.e., delayed rectification—was indicated by the decreasing slope of the steady-state current–voltage curve in the depolarizing direction (Fig. 1*D*) of a cell bathed in low extracellular Ca^{2+} ($[Ca]_o$ = 0.1 mM) to decrease the Ca^{2+}-dependent K^+-conductance (ref. 18; see below). This decrease in slope with depolarization was reversibly decreased by Et_4N (18.5 mM, Fig. 1*D*), as expected of delayed rectification.

Another region of decreased resistance was seen at hyperpolarizations ≥40 mV (Fig. 1*D*). This region, characteristic of all cells tested ($n > 100$), is reminiscent of anomalous rectification seen in sympathetic neurons (ref. 19; unpublished data). In contrast to delayed rectification, this resistance decrease was little affected by Et_4N (Fig. 1*D*); we have not studied this region further.

The peak response to depolarization in Fig. 1*C* was typical of the "active" responses of $^{-}$NGF cells. These responses were generally small and graded in normal perfusion medium but acquired a clear threshold and became all-or-nothing in Et_4N (Fig. 1*E*). Ca^{2+} appeared to carry much of this inward current because the spike was: (*i*) little affected by 3–6 μM TTX (three trials) or total replacement of $[Na]_o$ by Me_4N^+ (four trials; not shown); (*ii*) reversibly blocked by the conventional Ca^{2+}-conductance blockers Co^{2+} (2.5–10 mM, five trials), Mn^{2+} (5.6 mM, four trials), and Cd^{2+} (0.75 mM, Fig. 1*F*); (*iii*) reversibly eliminated by decreasing $[Ca]_o$ (0.1 mM, five trials) and increased in amplitude by increasing $[Ca]_o$ (up to 11.2 mM, three trials). These results suggest that $^{-}$NGF cells express a voltage-dependent Ca^{2+}-conductance pharmacologically similar to that of other excitable cells (20) and that they do not have appreciable voltage-dependent Na^+-conductances. Ca^{2+}-spikes in unfused PC12 cells have been reported (2). The falling phase of the Ca^{2+}-spike was followed by a long after-hyperpolarization (LAH) (Fig. 1*E*; ref. 9) similar to that found in $^{+}$NGF cells (described below).

Another property of $^{-}$NGF cells was a long-lasting hyperpolarizing response following a conditioning hyperpolarization that was usually 40–80 mV more negative than the resting potential for 50–100 msec. These responses had peak amplitudes of 5–20 mV, durations of 0.1–1 sec, and conductance increases at the peak of 100–300% of resting values. They are reminiscent of those due to a fast K^+-conductance inactivated at rest, which is seen in various neurons (21–24) and will be the subject of a later paper.

Expression of Na^+-Dependent Conductance Mechanism in the Presence of NGF. In addition to the above ionic mechanisms, $^{+}$NGF cells, usually at 10 DIV or older, had a Na^+-dependent spike mechanism sensitive to TTX. Before this time few cells with a detectable Na^+-spike were found. Instead, graded responses similar to those in $^{-}$NGF cells were common (i.e., Fig. 1*C*); however, the responses of $^{+}$NGF cells had a TTX-sensitive component (not shown).

Na^+-dependent action potentials (Fig. 3 *A* and *B*), occasionally overshooting, ranged from 40 to 60 mV in amplitude,

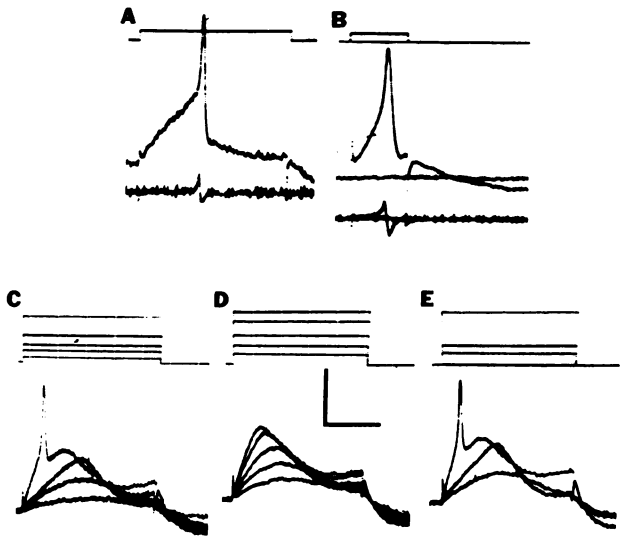

FIG. 3. Examples of Na^+-dependent action potentials and the effect of TTX. (*A* and *B*) Spikes (middle traces) evoked by depolarizing current pulses (top traces) from a cell 29 DIV (*A*) and a cell 13 DIV (*B*). *B* also shows unstimulated voltage level (middle trace). Bottom traces indicate rate of change of potential, dV/dt. (*C–E*) Superimposed voltage responses (lower traces) to depolarizing current pulses of increasing strength (top traces) before (*C*), during (*D*), and after (*E*) exposure to 3 μM TTX. Further increases in current strength during exposure to TTX (not shown) failed to evoke the spikes seen in *C* and *E*. Cell was 23 DIV. Voltage traces *D–E* were manually shifted. Vertical bar: *A–E*, 20 mV; *A* and *B*, 184 V/sec. Horizontal bar: *A*, 20 msec; *B*, 10 msec; *C–E*, 40 msec.

had rates of rise of 46–92 V/sec, and had durations at half-maximal amplitude of 2–4 msec. They were reversibly blocked by 3 μM TTX (Fig. 3 *C–E*) or total replacement of $[Na]_o$ with Me_4N^+ (not shown), but were little affected by lowering $[Ca]_o$ to levels that blocked the Ca^{2+}-spike (see below). A Na^+-spike mechanism in PC12 cells grown in the presence of NGF under different culture conditions has been reported (5). The development of the Na^+-spike mechanism in fused cells will be the subject of a later paper.

In the $^+$NGF cells TTX-resistant responses to depolarizing current pulses were similar to those of $^-$NGF cells (see Figs. 1*C* and 3*D*) in that they were transformed by Et_4N into all-or-nothing potentials (e.g., Fig. 4) sensitive to Cd^{2+}, Mn^{2+}, and Co^{2+} (not shown). Furthermore, as was true of $^-$NGF cells, the falling phase of the Ca^{2+}-spike was followed by an LAH (Fig. 4). In both cases the LAH was dependent upon $[Ca]_o$; it increased in duration as $[Ca]_o$ increased. It was associated with a conductance increase (Fig. 4) of 150–250% at the peak and was reversibly blocked by Co^{2+}, Mn^{2+}, and Cd^{2+} at concentrations that block the Ca^{2+}-spike (e.g., Fig. 1*F*).

DISCUSSION

The electrophysiological properties of fused PC12 cells appeared remarkably similar to those generally seen in neurons. Action potentials involved conventional conductance mechanisms for Na^+, Ca^{2+}, and K^+. Ca^{2+}-spikes of $^-$NGF and $^+$NGF cells were reversibly decreased or abolished by decreasing $[Ca]_o$ or by addition of the blockers, Cd^{2+} (Fig. 1*F*), Mn^{2+}, or Co^{2+}, but were little affected by TTX (3–6 μM). The Na^+-spikes of fused cells, in contrast, were little affected by lowering $[Ca]_o$ but were reversibly abolished by replacing $[Na]_o$ with Me_4N^+ or by addition of TTX (Fig. 3 *C–E*). In other respects Na^+-spikes had amplitudes, rates of rise, and durations (at half-maximal amplitude) somewhat similar to those reported for adrenal cells (ref. 25; however, see ref. 26) but were somewhat lower than those reported for rat sympathetic neurons in culture, except for their durations (9).

Cells possessed voltage-sensitive K^+-channels (Fig. 1*D*) resembling those of conventional delayed rectification (i.e., sensitive to Et_4N), a property seen in PC12 cells (5) and adrenal chromaffin cells (25, 26). The LAH, presumably also mediated by K^+, was associated with a conductance increase (Fig. 4) and was sensitive to $[Ca]_o$ and Ca^{2+}-conductance blockers (Fig. 1*F*). Long-lasting hyperpolarizations produced by a Ca^{2+}-controlled K^+-permeability have been well documented in rat sympathetic neurons (27) and other neurons (18).

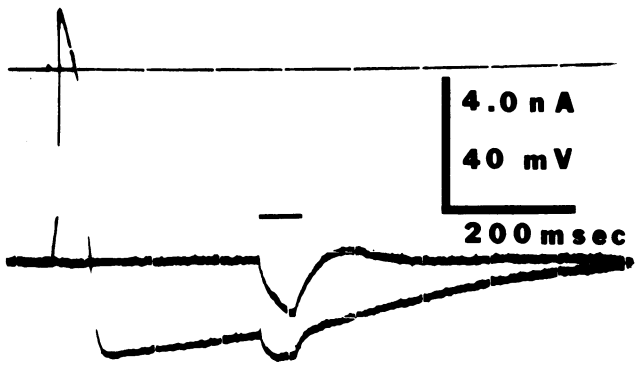

FIG. 4. LAH associated with the Ca^{2+}-spike of a fused cell grown with NGF (11 DIV). Et_4N (18.5 mM), 3 μM TTX, and 11.2 mM Ca^{2+} were present throughout. Depolarizing current pulse is shown in the top trace and superimposed voltage responses are shown in the bottom traces. Conductance increase during the LAH is shown by the response to a hyperpolarizing current pulse [0.6 nA in amplitude (not shown); duration is given by bar].

The development of the Na^+-spike mechanism in $^+$NGF cells warrants further investigation. In another study (5) on PC12 cells grown under different culture conditions, evidence was presented for the development of a Na^+-dependent spike mechanism. In our cultures we also saw evidence for such development: (*i*) In more than 100 $^-$NGF cells (1–20 DIV) we never saw evidence for such a mechanism (however, for veratridine effects see refs. 3 and 28). (*ii*) No young $^+$NGF cells tested (<7 DIV) exhibited this mechanism. (*iii*) The number of cells possessing TTX-sensitive action potentials increased thereafter (however, even in the oldest cultures studied not all cells gave Na^+-spikes). It was our impression that the appearance of the Na^+-mechanism (independent of PEG treatment) varied from cell to cell even within the same culture. Experiments are in progress to determine the timetable of the appearance of the Na^+-spike in a single fused cell.

Another neuron-like property of fused cells was their ability to bind a neuron-specific marker, tetanus toxin (e.g., ref. 29). PC12 cells grown with NGF for only 24 hr under different culture conditions have been shown to bind tetanus toxin by indirect immunofluorescence (K. Fields, personal communication). We have demonstrated by using similar techniques (29, 30) that both fused and unfused PC12 cells grown with NGF (36 hr, the earliest time we tested) bind this toxin specifically. Little membrane fluorescence for tetanus toxin binding was seen on cells grown without NGF. Therefore, both fused and unfused PC12 cells apparently express a cell surface receptor induced by NGF and shared by vertebrate neurons.

In the population of PC12 cells exposed to PEG, and apparently in contact, many cells fused, yet many did not. The source of this variability is not clear. However, under appropriate conditions both the fused and unfused cells contained CA (Fig. 2) and in the presence of NGF grew processes (Fig. 1*B*), expressed action potential mechanisms involving Na^+, K^+, and Ca^{2+} conductances, and showed neuron-specific binding of tetanus toxin. Thus, fusion does not detectably alter these important properties of PC12 cells.

Previous work by others has shown the usefulness of PC12 cells for investigations of various neuronal properties. In this paper we show that cell fusion, a technique used widely in cell biology, provides large PC12 cells suitable for electrophysiological studies. This may extend the application of PC12 cells in investigating excitable properties of developing and adult neurons and may allow various novel experimental approaches heretofore impossible with mammalian neurons.

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