Ionic Responses and Growth Stimulation Induced by Nerve Growth Factor and Epidermal Growth Factor in Rat Pheochromocytoma (PC12) Cells

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ABSTRACT Rat pheochromocytoma cells (clone PC12) respond to nerve growth factor (NGF) by the acquirement of a phenotype resembling neuronal cells. In an earlier study we showed that NGF causes an increase in Na^+,K^+ pump activity, as monitored by ouabain-sensitive Rb^+ influx. Here we show that addition of epidermal growth factor (EGF) to PC12 cells resulted in a stimulation of Na^+,K^+ pump activity as well. The increase of Na^+,K^+ pump activity by NGF or EGF was due to increased Na^+ influx. This increased Na^+ influx was sensitive to amiloride, an inhibitor of Na^+,H^+ exchange. Furthermore, no changes in membrane potential were observed upon addition of NGF or EGF. Amiloride-sensitive Na^+,H^+ exchange in PC12 cells was demonstrated by H^+ efflux measurements and the effects of weak acids on Na^+ influx. These observations suggest that both NGF and EGF activate an amiloride-sensitive, electroneutral Na^+,H^+ exchange mechanism in PC12 cells.

These findings were surprising in view of the opposite ultimate biological effects of NGF and EGF, e.g., growth arrest vs. growth stimulation. However, within 24 h after addition, NGF was found to stimulate growth of PC12 cells, comparable to EGF. In the presence of amiloride, this stimulated growth by NGF and EGF was abolished. In contrast, amiloride did not affect NGF-induced neurite outgrowth of PC12 cells. From these observations it is concluded that in PC12 cells: (*a*) NGF has an initial growth stimulating effect; (*b*) neurite outgrowth is independent of increased amiloride-sensitive Na^+ influx; and (*c*) growth stimulation by NGF and EGF is associated with increased amiloride-sensitive Na^+ influx.

Upon addition of nerve growth factor (NGF), rat pheochromocytoma cells (clone PC12) acquire within a few days a phenotype which resembles sympathetic neurons, including neurite outgrowth, electrical excitability, neurotransmitter production, and cessation of cell growth (1–5). Therefore PC12 cells provide a useful system for in vitro study of neuronal differentiation and of the action of NGF.

The first interaction of NGF with PC12 cells occurs through binding to specific high-affinity receptor sites at the cell surface (6–8). In addition to containing NGF-receptors, PC12 cells contain distinct cell surface receptors for epidermal growth factor (EGF) (9–11). Interestingly, the number of EGF-binding sites decreases drastically during NGF-induced differentiation (9, 11). Despite the opposite ultimate biological effects of NGF and EGF, i.e., neuronal differentiation and cessation of cell growth vs. growth stimulation (11), both factors display a number of similar effects as well. Among the common events evoked by NGF and EGF are the induction of ornithine decarboxylase (12) and, in another cell clone (PCG-2), also of tyrosine hydroxylase (13), the enhancement of cellular adhesion (10) and the stimulation of [^{3}H]choline and [^{32}P]orthophosphate incorporation into macromolecules (14). In addition we demonstrated recently that NGF stimulates the activity of the Na^+,K^+ pump in PC12 cells within minutes after NGF addition (15) as well as in chick embryo dorsal root ganglion cells (16), similar to the effects of EGF

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 JULY 1983 92-98

on the Na^+,K^+ pump in other cell lines (17–20). It has been shown (18–24) that the stimulation of the Na^+,K^+ pump activity is due to increased intracellular Na^+, resulting from the activation of an amiloride-sensitive, electroneutral Na^+,H^+ exchange system, and that these early ionic events are prerequisite to the stimulation of cell growth (17, 18, 20, 23).

In view of the great similarity of the early effects of NGF and EGF on the one hand and the difference in their ultimate biological effects on the other, the question arises as to the involvement of the early events in the action of NGF and EGF in PC12 cells in regulation of cell growth and differentiation. This is of particular interest for the early ionic changes, since they appear to be directly involved in the growth stimulatory response of EGF in a number of cells (17, 20, 23).

In this paper we demonstrate that both NGF and EGF stimulate Na^+,K^+ pump activity in PC12 cells through the activation of an amiloride-sensitive, electroneutral Na^+ influx system. Furthermore, we provide evidence that cessation of growth and the initiation of differentiation in PC12 cells upon exposure to NGF is preceded by an initial phase of enhanced growth rate, and that the early ionic response is a prerequisite for this growth stimulation by NGF but not for the expression of a differentiated phenotype. These results support the concept that the rapid effects of NGF and EGF on cation transport are part of their mitogenic response.

MATERIALS AND METHODS

Cell Culture: PC12 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (Flow Laboratories, Inc., Maclean, VA) and 5% heat-inactivated horse serum (Flow Laboratories), as described previously (1, 2). For tracer flux experiments, cells were plated at a density of 3.10^4 cells/cm^2 in poly-L-lysine (50 μg/ml, 60,000 mol wt) coated culture dishes (3.5-cm diameter; Costar, Cambridge, MA) and grown to a final density of $\sim 4.10^4$ cells/cm^2. For electrophysiological measurements cells were grown to confluence and fused with polyethylene glycol (PEG) (6,000 mol wt, 50% wt/vol; Sigma Chemical Corp. St. Louis, MO) by the method of Davidson et al. (25). For cell growth determination, cells were plated at a density of 7×10^3 cells/cm^2 in poly-L-lysine coated culture dishes (3.5-cm diameter; Costar). Growth medium was renewed after 48 h of growth. Cell growth was determined by cell counting after trypsinization according to standard methods.

K^+ Influx Measurements: Medium from cells grown in tissue culture dishes was replaced by 1 ml of serum-free DME at least 1 h before the measurements were started. K^+ influx was determined routinely from a 5-min labeling pulse at 37°C with $^{86}RbCl$ (Amersham International, Amersham, UK) as a radioactive tracer. $^{86}Rb^+$ was added at a final concentration of 2–5 μM (S.A.: 1 Ci/mol K^+). The uptake was terminated and radioactivity was measured as described previously (15, 26). The Na^+, K^+ pump-mediated K^+ uptake was obtained from the difference between total and ouabain-insensitive $^{86}Rb^+$ uptake, ouabain being added 1 min prior to the start of the experiment at a final concentration of 5 mM. The ouabain-sensitive uptake or active uptake was linear for at least 10 min, allowing the use of a 5-min labeling pulse to obtain active K^+ influx (27).

Na^+ Influx Measurements: For the Na^+ flux measurements, cells were treated as described above. The unidirectional Na^+ influx was determined from a 5-min labeling pulse with $^{22}Na^+$ (Amersham International) added at a final concentration of 2–5 μM (S.A.: 0.1 Ci/mol Na^+) in the presence of 5 mM ouabain. Na^+ uptake was terminated and radioactivity measured as described previously (28).

Membrane Potential Measurements: The membrane potential was measured 24 h after fusion of cells at 37°C in serum-free DME, using a conventional 3 M KCl-filled microelectrode as described (22, 26). Fused cells responded to NGF just like unfused cells; they developed neurites within a few days (29).

H^+ Production Assay: For H^+ production measurements the cells were removed from culture bottles by gentle pipetting and subsequently suspended in 3.5 ml of weakly buffered test solution containing 130 mM choline-Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.2 mM Tris-HCl (pH 7.4). The H^+ production was determined in this cell suspension essentially as described in detail previously (30).

Materials: NGF and EGF (receptor grade) were obtained from Collaborative Research Corp. (Waltham, MA), ouabain from Sigma Chemical Co., and amiloride was a gift from Merck, Sharp and Dohme B. V. (Haarlem, the Netherlands).

RESULTS

Effect of NGF on Active K^+ Influx

Active K^+ influx, i.e., K^+ influx mediated by the Na^+,K^+ pump, was determined from the accumulation of $^{86}Rb^+$ as a radioactive tracer for K^+ (15), in the absence and presence of ouabain as described under Materials and Methods. The basal active K^+ influx was ~ 3.5 nmol K^+/min/10^6 cells, being 70% of the total K^+ influx (15). Addition of NGF (50 ng/ml) to the cells resulted in a gradual increase of active K^+ influx to a maximal level of ~ 6 nmol K^+/min/10^6 cells after 30 min of incubation. This level was maintained during the next 50 min of incubation (Fig. 1*A*). NGF had no effect on ouabain-insensitive K^+ influx or on K^+ efflux (data not shown). The stimulation of active K^+ influx was dependent upon the NGF concentration, as shown in Fig. 1*B*, with half-maximal stimulation after 30 min at a NGF concentration of 3 ng/ml, and saturation at NGF concentrations >20 ng/ml.

Effect of NGF on Na^+ Influx

We showed previously (15) that active K^+ influx in PC12 cells can be increased upon addition of the Na^+ ionophore monensin, and we suggested then that the stimulation of the Na^+,K^+ pump by NGF was caused by increased Na^+ influx. To validate this hypothesis, we determined the effects of NGF on Na^+ influx directly. Unidirectional Na^+ influx was obtained from a 5-min labeling pulse with $^{22}Na^+$ in the presence of ouabain (5 mM) to prevent backflux of the tracer. The uptake of $^{22}Na^+$ in the presence of ouabain was linear over a 5-min period, and the influx estimated from this uptake was within error equal to the influx estimated from $^{22}Na^+$ uptake in the absence of ouabain during a 2.5-min labeling pulse. These findings validate the use of a 5-min labeling pulse in the presence of ouabain to determine the unidirectional Na^+ influx.

Addition of NGF (50 ng/ml) to the cells resulted in a stimulation of Na^+ influx from 4.4 to ~ 7 nmol Na^+/min/10^6 cells. The maximal stimulation was obtained 20 min after NGF addition, after which we observed no further changes (Fig. 2).

The effects of NGF on active K^+ influx and on Na^+ influx in PC12 cells resemble the effects of EGF or serum on cation fluxes in other cell lines (17–23). It has been suggested that the effects are due to an activation of an electroneutral Na^+, H^+ exchange system (18, 19, 22).

Na^+,H^+ Exchange in PC12 Cells

The Na^+,H^+ exchange system present in a number of cell lines (30–32) has been shown to be electrically silent and sensitive to the diuretic amiloride (30). Its activity depends upon the magnitude and direction of the electrochemical gradients involved.

A first line of evidence for the presence of Na^+,H^+ exchange in PC12 cells was obtained by measurements of H^+ efflux from the cells. Cells suspended for 30 min in a weakly buffered

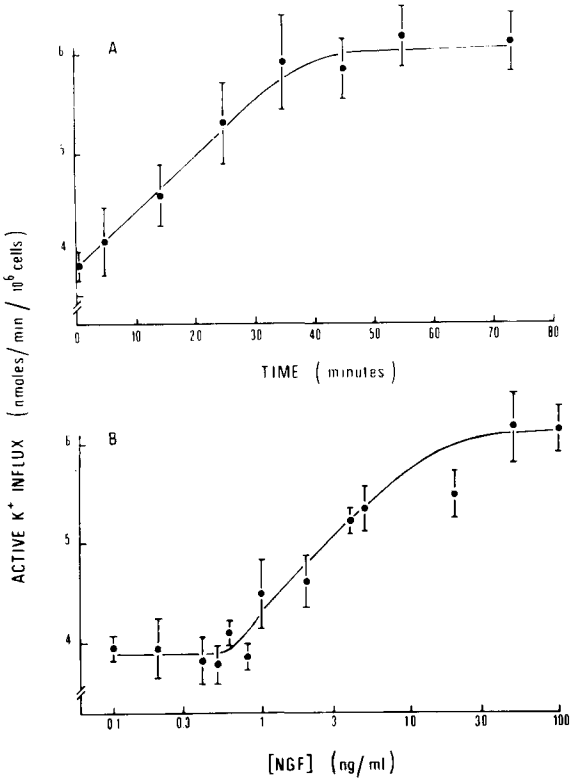

FIGURE 1 Effect of NGF on Na^+,K^+ pump mediated K^+ influx. Na^+,K^+ pump-mediated or active K^+ influx was measured at 37°C as described in Materials and Methods. Ouabain was used at a final concentration of 5 mM and added to the cells 1 min prior to the addition of the tracer. (*A*) NGF, at a final concentration of 50 ng/ml, was added to the cells at zero time, and the active K^+ influx was measured from 5-min labeling pulses at the times indicated. (*B*) Dose-response curve for the stimulation of active K^+ influx. NGF was added at various concentrations to the cells 30 min before the tracer. Data presented as mean ± SEM (n = 3).

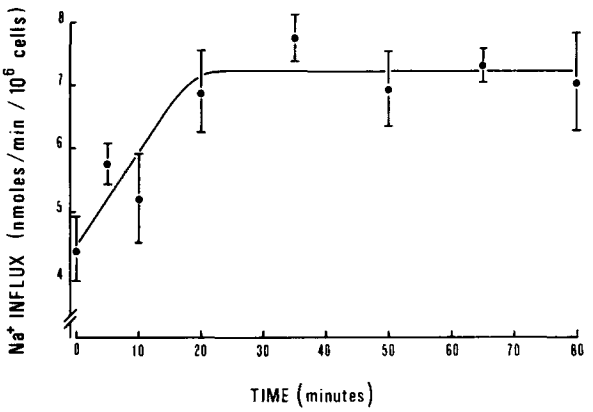

FIGURE 2 Effect of NGF on unidirectional Na^+ influx. Unidirectional Na^+ influx was measured at 37°C as described in Materials and Methods. Ouabain was present at a final concentration of 5 mM and added to the cells 1 min prior to the addition of the tracer. NGF (50 ng/ml) was added to the cells at zero time, the $^{22}Na^+$-labeling pulse of 5 min at the times indicated. Data presented as mean ± SEM (n = 5).

solution in the absence of Na^+ exhibit a basal H^+ efflux of 0.8 nmol H^+/min/10^6 cells (Fig. 3). Addition of Na^+ (20 mM), thereby generating an inward-directed Na^+ gradient (30) across the plasma membrane, resulted in a marked increase of the H^+ efflux to 3.9 nmol H^+/min/10^6 cells, the maximal rate being reached within 1 min after addition of Na^+. During the next ten minutes, the rate of H^+ release declined until a new steady level was reached which was slightly higher than the prestimulation level (Fig. 3). In the presence of 0.3 mM amiloride, however, addition of Na^+ to Na^+-deprived cells had no effect on H^+ efflux (Fig. 3). Thus, increasing the Na^+ gradient ($[Na]_o^+ > [Na]_i^+$) results in an increase in H^+ efflux from the cells via an amiloride-sensitive system. A second line of evidence for the presence of Na^+,H^+ exchange in PC12 cells was obtained by modification of the H^+ gradient across the plasma membrane. Addition of weak acids, such as acetate or 5,5-dimethyloxazolidine-2,4-dione (DMO), to the cells results in a decrease of the intracellular pH (30). Addition of acetate (10 mM) or DMO (10 mM) resulted in a stimulation of Na^+ influx, but in the presence of 0.3 mM amiloride this stimulation was completely abolished (Fig. 4). Thus, increasing the H^+ gradient across the plasma membrane ($[H]_i^+ > [H]_o^+$) results in an increase in Na^+ influx via an amiloride-sensitive system.

These results demonstrate that an increase in the magnitude of the Na^+ or H^+ gradient across the plasma membrane results in an increase in respectively H^+ and Na^+ transport in opposite direction, both fluxes being sensitive to amiloride. In analogy with a number of studies in other cell lines, these results indicate the presence of an amiloride-sensitive Na^+, H^+ exchange system in the plasma membrane of PC12 cells.

Activation of Electroneutral Na^+ Influx by NGF

The stimulation of active K^+ influx by NGF in PC12 cells has been shown to be sensitive to amiloride (15). Amiloride has hardly any effect on the basal active K^+ influx, but it abolishes completely the stimulation observed in the presence of NGF (Fig. 5*A*). Furthermore, amiloride has no effect on the basal Na^+ influx, but, again, it completely inhibits the stimulation of Na^+ influx by NGF (Fig. 5*B*).

Fusion of PC12 cells by PEG allows impalement of the cells by microelectrodes (29). The fused cells respond to NGF

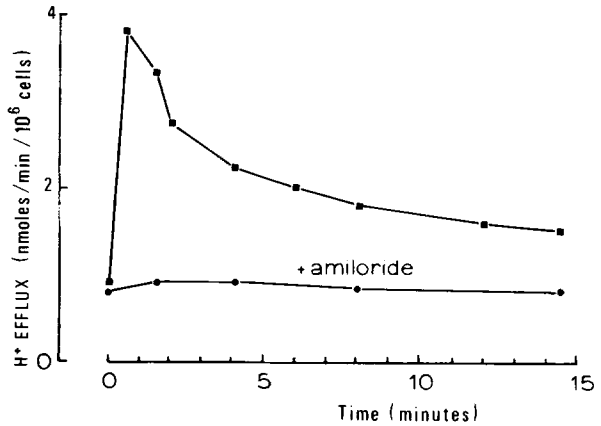

FIGURE 3 Effect of extracellular Na^+ on H^+ efflux. H^+ efflux from the cells was essentially measured as described in detail previously (30). Cells were incubated in Na-free test solution for at least 20 min, assuming that this period was sufficient to deplete the cells of intracellular Na^+. At zero time, Na^+ was added to the cells at a final concentration of 20 mM (■). In the lower curve (●) 0.3 mM amiloride was added 1 min prior to the addition of Na^+. H^+ efflux was calculated from the amount of OH^- that had to be added per unit of time to maintain the pH of the cell suspension at 7.40 ± 0.02.

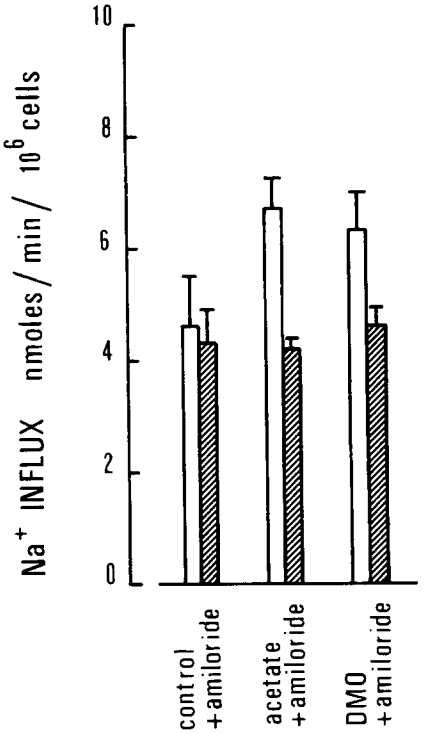

FIGURE 4 Effect of weak acids on Na^+ influx. Unidirectional Na^+ influx was measured as described in Materials and Methods. Amiloride (0.3 mM), ouabain (5 mM) and K-acetate (10 mM) or K-DMO (10 mM) was added to the cells as indicated 1.5; 1 and 0.5 min respectively prior to the addition of the tracer. Data presented as mean ± SEM (n = 5).

in a similar way as untreated cells: within a few days the cells form neurites and are electrically excitable. The cells exhibit a stable membrane potential of −54.1 ± 1.4 mV (Mean ± SEM, n = 34), 24 h after fusion, as inferred from microelectrode measurements. Addition of NGF (50 ng/ml) has no effect on the membrane potential, and also amiloride in the absence or presence of NGF did not affect the membrane potential (data not shown). Taken together, these results suggest that NGF stimulates Na^+ influx in PC12 cells by activation of an amiloride-sensitive, electroneutral system, most likely Na^+,H^+ exchange. As a result of the enhanced availability of intracellular Na^+, the Na^+,K^+ pump is being activated secondarily.

Effect of EGF on K^+ and Na^+ Influx

The effects of NGF on cation transport described above are qualitatively similar to the reported effects of EGF on cation transport in other cell lines (17–20). Addition of EGF (25 ng/ml) to PC12 cells resulted in an increase in active K^+ influx as shown in Fig. 6. The maximal stimulation occurred ~20 min after addition, followed by a gradual decrease during the next 40 min.

The stimulation of active K^+ influx by EGF was completely inhibited by amiloride (Fig. 5*A*), similar to the stimulation by NGF. In addition, EGF stimulated also Na^+ influx and again this stimulation was found to be inhibitable by amiloride (Fig. 5*B*). Furthermore, addition of EGF with or without amiloride had no significant effects on the membrane potential. In conclusion, EGF and NGF have similar stimulatory effects on Na^+,K^+ pump activity, resulting from an activation of an amiloride-sensitive, electroneutral Na^+ influx system. The temporal response of NGF and EGF on active K^+ influx is the most striking difference in the action of both factors. NGF activates the Na^+,K^+ pump activity during a prolonged time, while EGF activates the Na^+,K^+ pump transiently. On the other hand, the effects of NGF and EGF, 30 min after addition, on active K^+ influx or Na^+ influx were not additive, indicating the involvement of a common mechanism in the response of both factors (data not shown).

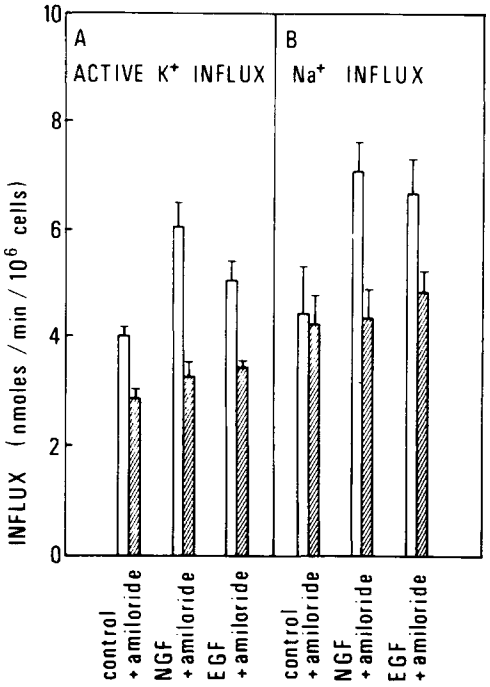

FIGURE 5 Effect of NGF and EGF on active K^+ and Na^+ influx in the presence and absence of amiloride. Active K^+ and Na^+ influx were measured at 37°C as described in Materials and Methods. NGF (50 ng/ml) and EGF (25 ng/ml) were added to the cells 30 and 20 min, respectively, prior to the addition of the tracer. Amiloride (0.3 mM) and ouabain (5 mM) were added 1 and 0.5 min respectively before the tracer. Data presented as mean ± SEM (n = 5).

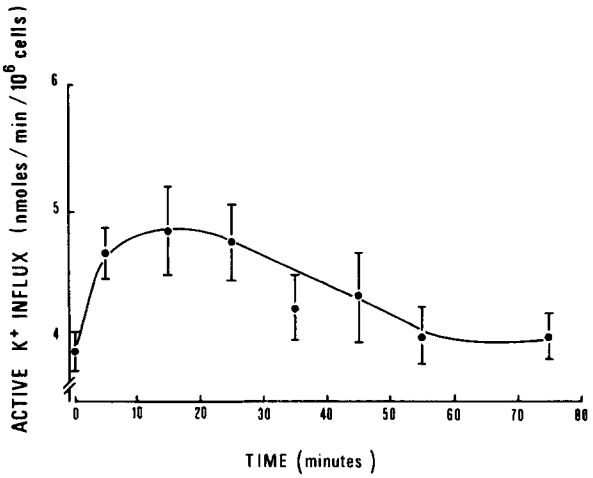

FIGURE 6 Effect of EGF on Na^+,K^+ pump mediated K^+ influx. Na^+, K^+ pump-mediated K^+ influx or active K^+ influx was measured at 37°C as described in Materials and Methods. EGF was used at a final concentration of 25 ng/ml and added to the cells at zero time. The active K^+ influx was obtained from a 5-min labeling pulse at the times indicated. Data presented as mean ± SEM (n = 3).

Effects of NGF, EGF, and Amiloride on Cell Growth and Differentiation

The observed similarities of the NGF and EGF responses on Na^+ and K^+ influx in PC12 cells are surprising in view of the opposite ultimate biological effects. To associate the rapid responses of NGF and EGF with their ultimate biological effect, we examined the effects of NGF, EGF, with and without amiloride, on neurite outgrowth and on cell growth.

NEURITE OUTGROWTH: Upon addition of NGF (50 ng/ml) the cells extend neurites from the cell body within 72 h (Fig. 7*A*). Addition of amiloride (0.3 mM) does not influence this morphological differentiation significantly (Fig. 7*B*). In the presence of EGF (25 ng/ml), the cells continue to proliferate (Fig. 7*C*) and, again, no morphological differences were observed in the presence of amiloride (Fig. 7*D*). Thus inhibition of the rapid effects of NGF on Na^+ and K^+ transport by amiloride does not influence cellular differentiation. On the other hand, stimulation of Na^+ and K^+ transport by EGF does not induce differentiation.

CELL GROWTH: Under normal growth conditions referred to as control (Fig. 8), the cells had a mean generation time of ~40 h. The presence of amiloride (0.3 mM) for 24 h did not alter the growth rate (Fig. 8*A*), but prolonged incubation with amiloride resulted in a progressive decrease of growth compared to control cultures (Fig. 8*B*). It is important to note that even after 7 d of incubation in the presence of amiloride the cells are still viable; the cell number increased from 10^5 cells/dish to 5×10^5 cells/dish over a 7-d period.

Surprisingly, in the presence of NGF (50 ng/ml) an increase in cell number was determined after 24 h from 10^5 cells/dish to 2×10^5 cells/dish, equivalent to a generation time of 24 h. Amiloride completely inhibited this growth stimulatory effect of NGF (Fig. 8*A*). Similar results were obtained in the presence of EGF, although the increase in cell number in the presence of EGF was smaller compared to NGF; the mean generation time was ~34 h, but again amiloride inhibited completely the stimulation of growth by EGF (Fig. 8*A*). Incubation of PC12 cells in the presence of NGF for 7 d resulted in a differentiated phenotype (Fig. 7*A*), and inhibition of growth was observed compared to control cultures (Fig. 8*B*). In the presence of NGF and amiloride the cell number after 7 d of incubation was equal to that of cultures with amiloride only (Fig. 8*B*). In contrast, in the presence of EGF the cells continued to grow with a mean generation time of 34 h. Again, in the presence of amiloride, growth was inhibited (Fig. 8*B*) although the cell number was found to be higher than with amiloride or NGF plus amiloride.

These results demonstrate the NGF, as well as EGF, stimulates growth of PC12 cells initially but that prolonged incubation in the presence of NGF results in an arrest of growth. In the presence of the Na^+,H^+ exchange inhibitor amiloride, the stimulation of cell growth by both NGF and EGF was abolished.

DISCUSSION

In this study we examined the early ionic effects induced by NGF and EGF in PC12 cells and studied the relevance of these events for the biological response to these polypeptide

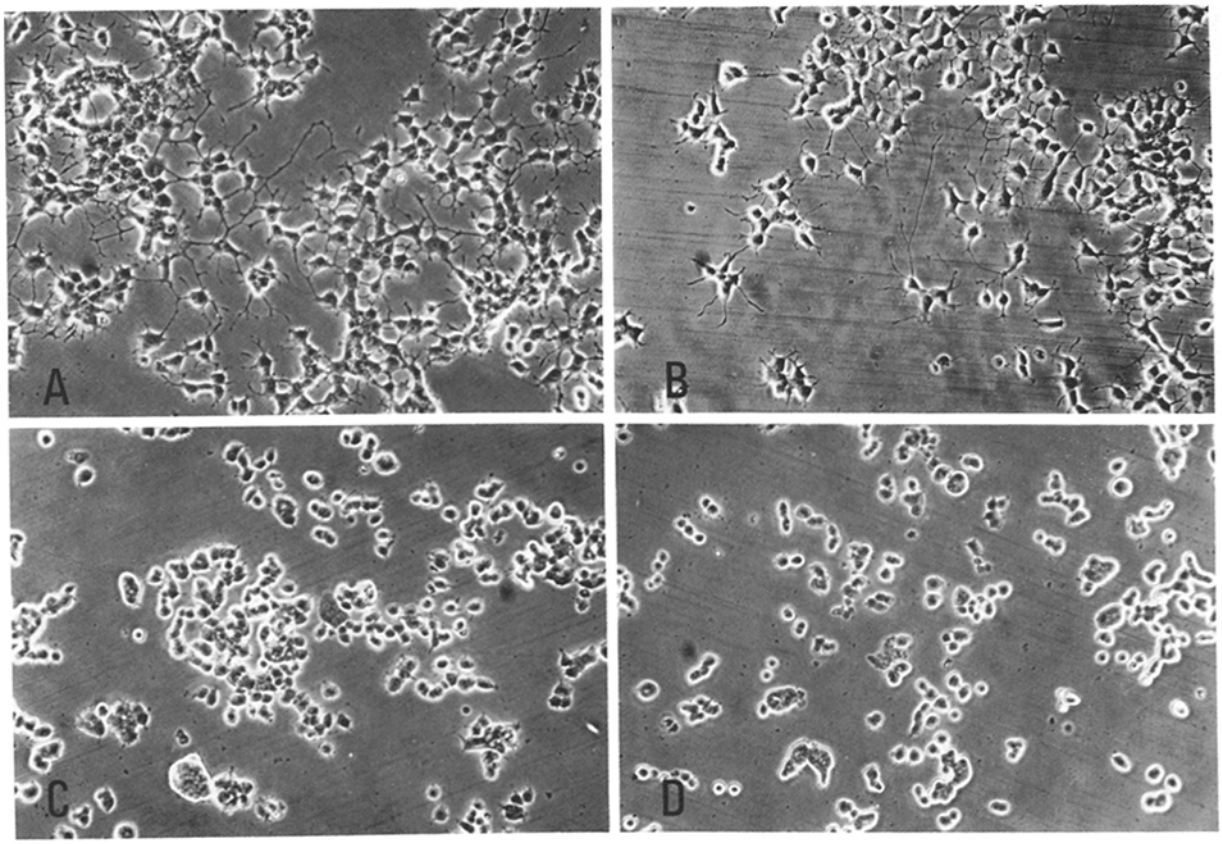

FIGURE 7 Effect of NGF, EGF, and amiloride on differentiation. Cells were grown in DME as described in Materials and Methods in the presence of 50 ng/ml NGF, 25 ng/ml EGF, and 0.3 mM amiloride as indicated. Medium was renewed after 48 h and photos were taken after 72 h after addition. (*A*) NGF. (*B*) NGF and amiloride. (*C*) EGF. (*D*) EGF and amiloride.

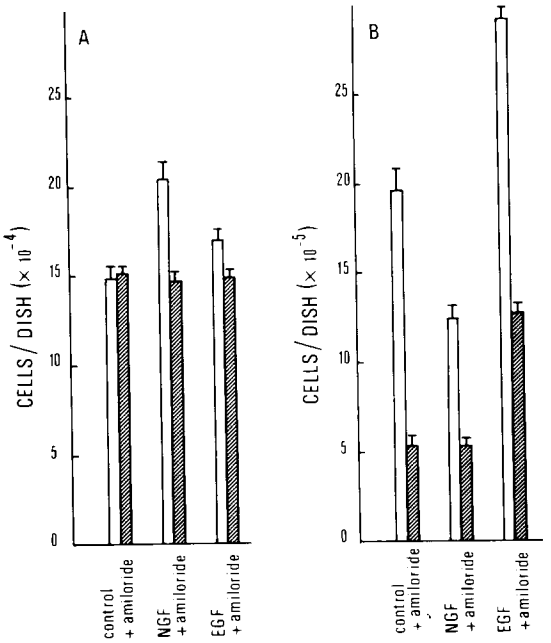

FIGURE 8 Effect of NGF, EGF, and amiloride on cell growth. Cells were grown in DME as described in Materials and Methods. After 24 h, NGF (50 ng/ml), EGF (25 ng/ml), and amiloride (0.3 mM) were added as indicated, and the cells incubated for 24 h or 7 d, after which time period the cell number per culture dish was determined as described in Materials and Methods. The growth medium, including the respective additions, was renewed after 48 h. (*A*) Cell number, 24 h after additions were made. (*B*) Cell number, 7 d after additions. Data presented as mean ± SEM (n = 3).

hormones. The results demonstrate that both NGF and EGF stimulate within minutes the Na^+,K^+ pump activity. This stimulation is evoked by the activation of an amiloride-sensitive, electroneutral Na^+ influx system, most likely Na^+,H^+ exchange. The early NGF and EGF responses in PC12 cells seem to occur through a common system, since the effects on Na^+, K^+ pump activity were not additive. On the other hand, the temporal responses of NGF and EGF differed markedly (Figs. 1 and 6). NGF caused a persistently increased Na^+,K^+ pump activity over a 50-min period, in contrast to EGF. Whether this temporal difference in the ionic response to NGF and EGF is related to their ultimate biological effects remains to be established. In their response to EGF, PC12 cells behave like a number of other target cells (17–20). In view of the ultimate opposite biological effects of NGF and EGF in PC12 cells, i.e., neuronal differentiation and growth arrest vs. growth stimulation, the similarity in the early ionic responses is surprising. However, we show here that NGF increases the growth rate of PC12 cells before commencing differentiation. This initial growth stimulation, but not the subsequent expression of a differentiated phenotype, was dependent upon the early increases in cation transport. In the presence of amiloride, the stimulation of growth by NGF (and also by EGF) was completely abolished. It should be mentioned that amiloride has been shown to affect other cellular processes as well (30, 33, 34), but in the absence of NGF or EGF it inhibits the growth rate of PC12 cells only upon exposure for more than 24 h. Recently, it has been shown that NGF stimulates cell growth also in a pheochromocytoma cell line (clone PCG2) when cultured in chemically defined medium (35). The growth-stimulating effect of NGF seems to be a general phenomenon and can be fully separated from its differentiation-inducing capacity. Thus, NGF has been shown to induce cell growth in certain neuroblastoma cells (36, 37) and to be crucial for survival of certain embryonic sympathetic and sensory neurons (38, 39). So far, in all systems measured, NGF caused an increase in Na^+, K^+ pump activity (15, 16) similar to the early effects of serum and purified growth factors (17–22, 40), indicating that activation of Na^+ and K^+ transport is an important factor in the stimulation of DNA synthesis or cell survival. In this respect it is of interest that the mechanism by which NGF influences Na^+, K^+ pump activity might differ from one cell to the other. In chick embryo dorsal root ganglion cells, NGF influences Na^+, K^+ pump activity directly (16), in contrast to PC12 cells where it activates Na^+,K^+ pump activity via increased Na^+ influx. Thus, in chick embryo dorsal root ganglion cells the Na^+,K^+ pump activity decreases in the absence of NGF to values which are too low to maintain the normal intracellular Na^+ and K^+ concentrations. Readdition of NGF to such deprived cells results in a full restoration of Na^+,K^+ pump activity and hence in normal intracellular K^+ and Na^+ concentrations (16, 41, 42). Whether other early responses to NGF, such as induction of ornithine decarboxylase (12), increased cellular adhesion (10), and phosphorylation of specific proteins (43), are involved in stimulation of growth in PC12 cells is at present not clear.

We conclude that in PC12 cells: (*a*) NGF and EGF induce an identical sequence of rapid cation transport changes, similar to the effects of EGF in other target cells, (*b*) NGF and EGF stimulate growth; (*c*) growth stimulation by NGF or EGF is coupled to increased K^+ and Na^+ transport, in contrast to the induction of differentiation; and (*d*) the effects of NGF on growth and differentiation are dissociable.

The latter observation raises the question about the differentiation-inducing factor in the NGF action in PC12 cells. It seems likely that cAMP might fulfil the role of a second messenger in the induction of differentiation in at least some target cells. NGF stimulates cAMP production in PC12 cells (44) and in dorsal root ganglion cells (45), and cAMP and cAMP analogues are able to promote neurite outgrowth in PC12 cells (44, 46, 47). Furthermore, it has been shown in chick embryo dorsal root ganglion cells that the NGF response on cAMP content is independent from the rapid ionic effects of NGF (45), and cAMP and cAMP analogues do not change Na^+,K^+ pump activity in PC12 cells (own unpublished observations). Recently, a number of neurite outgrowth-promoting factors have been identified, which stimulate neurite outgrowth even in the presence of anti NGF antiserum (48, 49), in agreement with the intention that also factors other than NGF are able to induce such a second messenger.

On the other hand, it has been demonstrated that direct introduction of NGF into the cytoplasm does not induce neurite outgrowth and that introduction of anti-NGF antibodies does not prevent NGF-induced neurite formation (50). These observations indicate that the association of NGF (or another differentiation-inducing factor) with the plasma membrane is a prerequisite for the formation of this second messenger.

Future experiments in which the effects of plasma membrane-associated NGF can be dissociated from those of intracellular NGF might clarify the nature of the action of NGF, as well as part of the regulatory circuits of cellular growth and differentiation in PC12 cells.

We would like to thank Mr. P. Meyer for skillful technical assistance.

This work was supported by the Netherlands Cancer Society (Koningin Wilhelmina Fonds).

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Received for publication 29 December 1982.

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