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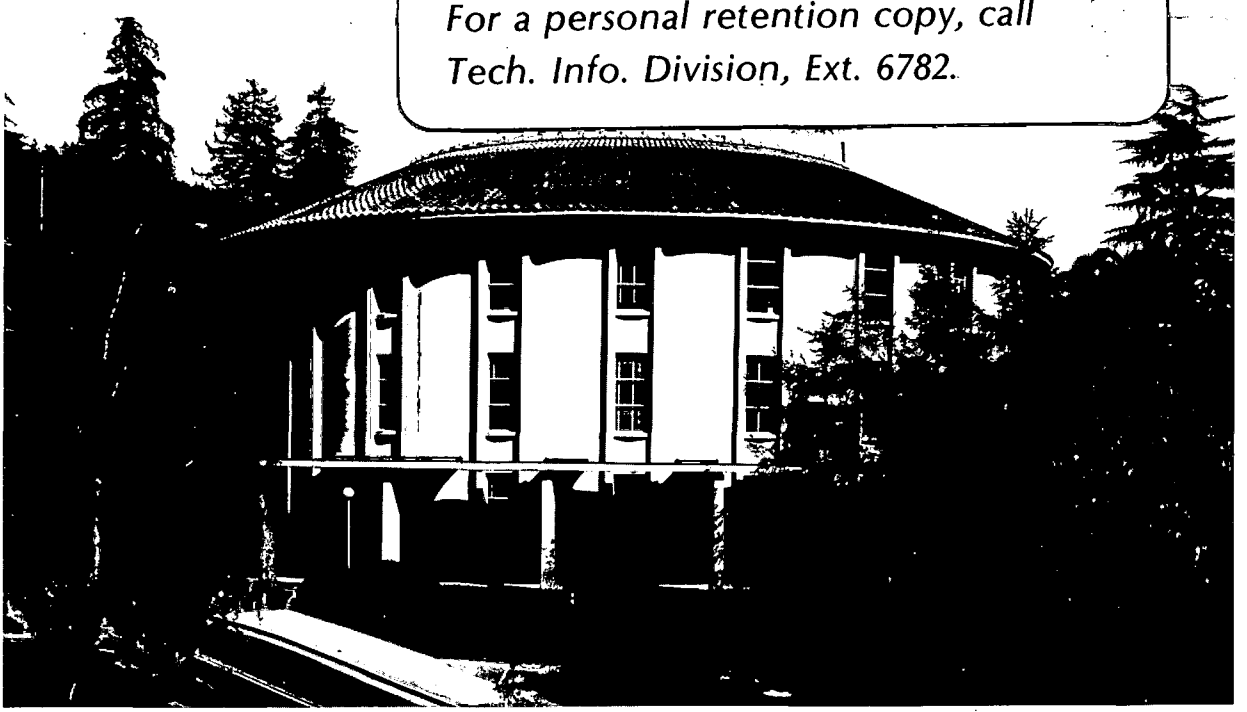

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Nerve Growth Factors Increase Sodium Ion Conductance Through the Nicotinic Acetylcholine Receptor Channel in PC12 Cells[1]

by

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ABSTRACT

$^{22}Na^+$ uptake in response to cholinergic stimulation was measured in PC12 cells exposed to either βNGF from mouse submaxillary gland or a component of Bungarus multicinctus venom (fraction 9B) which has nerve growth factor activity. Our results showed that nerve growth factors produced a relatively stable increase in the Na^+ conductance capacity of ACh receptor-linked ion channels in PC12 cells which accompanied cellular differentiation but was not linked to neurite outgrowth itself. Compared to untreated cells, Na^+ uptake was enhanced by about 40% in cells exposed for 24 to 30 hrs to either 50 ng per ml βNGF or to 5 µg per ml fraction 9B. This enhanced Na^+ flux through the ACh receptor-linked ion channels increased with time and reached a level 2- to 3-fold higher than that of untreated cells after 4 to 6 days exposure to NGFs. The generation of neurites in response to NGFs from either source occurred with the same time course as the increase in Na^+ channel conductance. Thus, βNGF from mouse submaxillary gland was 100-fold more effective than snake venom fraction 9B on a weight basis in stimulating both neurite outgrowth and enhanced Na^+ uptake, and the dose of each NGF which produced the greatest neurite outgrowth also increased cholinergically stimulated Na^+ flux to the greatest extent. This enhanced Na^+ flux capacity was shown to be inhibited by nicotinic (but not muscarinic) antagonists and was maintained for several days after NGFs were removed from cells and neurites were lost. Dibutyryl cyclic AMP did not cause an increase in Na^+ uptake in response to cholinergic agonists at concentrations which generated neurites, nor did it potentiate the effects of NGFs.

When PC12 pheochromocytoma cells in culture are exposed to nerve growth factor (NGF), these normally spherical, continuously dividing cells respond by extending neurites and by ceasing cell division within one week (Greene and Tischler, 1976). These changes are accompanied by an increase in the specific activities of choline acetyltransferase (Schubert et al., 1977), acetylcholinesterase (Greene and Rukenstein, 1981), and ornithine decarboxylase (Hatanaka et al., 1978) along with an increase in the synthesis of proteins (Garrels and Schubert, 1979) and RNA (Gunning et al., 1981a). Dichter et al. (1977) showed that, compared to untreated cells, NGF caused an increased electrical excitability in PC12 cells as well as an increased sensitivity to acetylcholine. A high percentage of PC12 cells grown for more than two weeks with NGF were depolarized when exposed to short pulses of ionotophoretically applied acetylcholine. These changes are consistant with the view that NGF stimulates the differentiation of PC12 pheochromocytoma cells into sympathetic-like neurons (Greene and Tischler, 1976).

α-Bungarotoxin has been used extensively as a ligand which specifically binds to and inhibits the normal functioning of the acetylcholine receptor-linked sodium channel in muscle cells (Devreotes and Fambrough, 1976). Patrick and Stallcup (1977a) compared the binding of α-bungarotoxin to PC12 cells with its effect on the physiological functioning of the acetylcholine receptor-linked ion channel. They found that this ligand binds to PC12 cells but does not inhibit sodium ion flux through the acetylcholine receptor in response to nicotinic stimulation. These results are consistant with other studies with non-muscle acetylcholine synapses (Brown and Fumagalli, 1977; Carbanetto et al., 1978) which suggested that α-bungarotoxin was not a specific ligand for acetylcholine receptors on central nervous system and sympathetic nerve cells.

Because of these questions concerning the specificity of α-bungarotoxin,

investigations of changes in nicotinic acetylcholine receptor function during differentiation of PC12 cells in response to NGF have been limited to electrophysiological studies of individual impaled cells (Dichter et al., 1977). Since stimulation of acetylcholine receptors opens receptor-linked channels to the influx of Na^+, we have used the $^{22}Na^+$ uptake assay developed by Catterall (1975) as a measure of the sensitivity of PC12 cells to nicotinic cholinergic stimulation and, indirectly, as an indication of the number of ion channels sensitive to ACh stimulation on the surface of PC12 cells grown under different culture conditions.

βNGF isolated from the mouse submaxillary gland has been used for most investigations of the effect of nerve growth factors on neuronal development. Polypeptide components of venoms from a number of snakes have also been found to possess similar nerve growth factor activity (Tu, 1977). In this study we have compared the effects on PC12 cell function of one such nerve growth factor from the venom of the Formosan krait Bungarus multicinctus with the effects of βNGF isolated from mouse submaxillary gland. This comparison showed that, although βNGF was effective at a much lower concentration than the snake venom component, each of these NFGs produced identical effects on cell morphology and nicotinic ACh receptor function. Our results showed that growth factor treatment enhanced the acetylcholine-stimulated influx of Na^+ into PC12 cells after a 24 hr lag period and reached a maximum level after 4 to 6 days. This increased sensitivity to cholinergic stimulation was lost slowly after the removal of nerve growth factors from cells, suggesting that there was a relatively stable increase in the number of ACh receptor-linked channels on PC12 cells treated with NGF.

MATERIALS AND METHODS

Cell growth and growth factor treatment conditions

The PC12 clone of a rat pheochromocytoma cell line was obtained from the laboratory of Eric Shooter at Stanford University and grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal calf serum and 5% horse serum. Cells were grown on polystyrene tissue culture dishes (Falcon) in a water-saturated atmosphere of 90% air, 10% CO_2.

Nerve growth factors from two different sources were added to cultures of subconfluent cells plated at a concentration of about 3×10^5 cells per ml to induce cellular differentiation characteristic of PC12 cells. βNGF from mouse submaxillary gland was obtained from the Technology Transfer Office of SUNY, Albany, New York. Venom from the Formosan krait Bungarus multicinctus obtained from the Miami Serpentarium contained a component with nerve growth factor activity. The protein species in this crude venom were separated and purified by column chromatography on CM-Sepadex C50 and Sephadex G50 (Hanley et al., 1977). This procedure separated the toxic venom components from a nontoxic protein (fraction 9B) which induced neurite outgrowth from chick ciliary ganglia (Hanley, 1978). Snake venom fraction 9B used in this study was isolated by Hanley and contained two protein components with molecular weights of approximatetly 10-14,000 when electrophoresed on 15% SDS polyacrylamide gels. Either βNGF or fraction 9B were added to subconfluent cells in normal growth medium for the periods of time indicated for each experiment.

Neurite outgrowth was observed with a Nikon Diaphot-TMD inverted microscope with phase contrast optics, and the extent of neurite production was estimated using the criteria developed by Gunning et al. (1981a). Since the cells used for $^{22}Na^+$ uptake assays were plated at very high density (see below), it was not feasible to determine the actual percentage of neurite-

bearing cells. Estimates of neurite outgrowth were therefore based on neurite production relative to maximum responses under optimal βNGF treatment conditions.

Assay for $^{22}Na^+$ Uptake

Measurements of $^{22}Na^+$ influx into PC12 cells was performed as described by Patrick and Stallcup (1977b). PC12 cells were resuspended in fresh growth medium with or without growth factor and plated on polylysine-coated 12-well cluster dishes (Costar) at a concentration of 1-2 x 10^6 cells per ml and 1 ml per well (4 cm^2 surface area) for 24 hrs prior to assay unless specified otherwise. Immediately before the assay, cells were washed briefly with assay buffer composed of 130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose and 50 mM HEPES adjusted to pH 7.4 with NaOH. Assay buffer (300 µl) supplemented with 5 mM ouabain, 1.5 µCi per ml $^{22}NaCl$ (New England Nuclear) and agonist (carbamylcholine or nicotine) was then added to each well to initiate the assay. $^{22}Na^+$ uptake was stopped by washing the cells within 5 sec with three 2-ml aliquots of wash buffer composed of 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose and 5 mM HEPES buffer (pH 7.4). Cells were dissolved in 1 ml of 0.4 N NaOH and the amount of labeled sodium taken up was determined by counting in a Tracor Analytic gamma counter. All values were normalized for protein content in each well determined by the method of Lowry et al. (1951); cell counts with a hemocytometer showed that there were approximately 240 µg of protein per 10^6 cells. Unless indicated otherwise, $^{22}Na^+$ uptake into identically treated cells assayed without agonist was subtracted to obtain net agonist-stimulated uptake. Preliminary studies showed that uptake was linear with time between 0 and 30 sec in agreement with previous $^{22}Na^+$ uptake studies with PC12 cells (Stallcup, 1979; Karpen et al., 1982). All assays were performed for 30 sec at 22°C.

The effects of cholinergic inhibitors on Na^+ uptake were tested by preincubating cells for 10 min prior to assay in Prep medium (Dulbecco's modified Eagle's medium plus 1% fetal calf serum and 5 mM HEPES, pH 7.4) containing inhibitor (Patrick and Stallcup, 1977b). Inhibitors were removed prior to assay when cells were washed with assay buffer.

To test the effect of removing growth factors from treated cultures, cells were resuspended in 15 ml of Prep medium and centrifuged at 200 g for 4 min. The supernatant was discarded, and the cell pellet was gently resuspended in Prep medium. This procedure was repeated for a total of three washes before the cells were resuspended in growth medium for replating on fresh culture dishes or on polylysine-coated dishes for Na^+ uptake assays.

Materials

Atropine and d-tubocurarine were obtained from Calbiochem and $N^6,O^{2'}$-dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP), nicotine and carbamylcholine were from Sigma.

RESULTS

←—Effects of Nerve Factors on PC12 cell morphology

Neurite outgrowth from PC12 cells exposed to nerve growth factor increased with time and with increasing βNGF concentrations. This outgrowth was maximal when the cells were treated with 50 ng βNGF per ml of medium for 4 to 6 days (see Gunning et al., 1981a). Approximately the same level of neurite production was obtained by growing cells in medium containing 5 µg of snake venom fraction 9B per ml of medium for the same period. The morphological changes in response to 9B treatment were indistinguishable from those of βNGF-treated cells, but 100-fold more 9B was necessary to elicit the response.

Hanley (1978) also reported a 100-fold difference in the amount (by weight) of mouse submaxillary βNGF and fraction 9B required to stimulate maximal neurite outgrowth from chick ciliary ganglia.

Carbamylcholine-stimulated $^{22}Na^+$ uptake

Our initial experiments were designed to determine whether both nerve growth factors increased ACh-stimulated Na^+ uptake by PC12 cells and, if so, whether this increased Na^+ uptake could be inhibited by α-bungarotoxin or other fractions from the venom of Bungarus multicinctus which were lethal when injected into mice (Hanley et al., 1977). Table 1 presents data which showed that a week-long exposure of PC12 cells to the nontoxic snake venom fraction 9B stimulated Na^+ uptake by about 80% when cells were assayed with the cholinergic agonist carbamylcholine (CCh). Under these growth conditions, neurite outgrowth was substantial. No increase in Na^+ flux was seen after treating previously untreated cells with 9B for only 1 hr prior to assay. Also, a 1 hr preincubation of cells grown in normal or growth factor-containing media Prep medium containing 9B had no effect on Na^+ flux in response to cholinergic agonists. When other pre- and post-synaptic toxins isolated from B. multicinctus venom (fraction numbers 6,7,8,9A and 10 as described by Hanley et al., 1977) were added to cells at a concentration of 1 mg per ml for 1 hr prior to assay for Na^+ flux, we found no evidence for 20% or more inhibition of CCh-stimulated Na^+ flux (data not shown). Also, α-bungarotoxin added to PC12 cells for up to 4 days did not induce neurite outgrowth. Thus, fraction 9B, when added to cells for a prolonged period, enhanced CCh-sensitive cholinergic functioning, but none of the toxic snake venom fractions showed any substantial inhibition of CCh stimulated Na^+ flux or any evidence of growth factor activity.

We next compared the cholinergic sensitivity of PC12 cells treated with

different levels of βNGF or 9B for 4 days with cells grown in normal growth medium without growth factor (Fig. 1). Since βNGF caused inhibition of cell division after 4 to 6 days (Greene and Tischler, 1976), we generally avoided longer βNGF or 9B exposure times to minimize the differences in growth conditions of treated and untreated cells. Net CCh-stimulated Na^+ uptake was about 215 to 230 nmoles per min per mg protein for untreated cells in these experiments and rose with increasing growth factor concentration to a maximum level of 650 to 680 nmoles per min per mg, almost 3 times as great. The maximum amount of CCh-stimulated ion flux was the same for each growth factor, but the amount of 9B required to achieve these levels was 100-fold greater than for βNGF on a weight basis. A 4-day exposure to 0.2 µg fraction 9B per ml of medium produced discernable neurite outgrowth while only 5 ng per ml of βNGF per ml medium produced a similar response; maximum levels of growth factor-induced morphological change required 5 µg per ml of 9B or 50 ng per ml of βNGF. Overall, the changes in CCh-sensitive Na^+ uptake closely paralleled the morphological changes induced by each growth factor after a 4-day treatment.

Carbamylcholine concentrations of 3.2 mM were maximally effective in stimulating $^{22}Na^+$ uptake into subconfluent cultures of PC12 cells grown under normal culture conditions (Fig. 2). Half maximal uptake required approximately 0.5 mM CCh. In this experiment, the maximum CCh-stimulated Na^+ uptake was 277 ± 7 nmoles per min per mg protein above the background uptake of 42 ± 3 nmoles per min per mg in cells assayed without CCh. These values for maximum and half-maximum stimulation of Na^+ uptake into PC12 cells are similar to those reported by Patrick and Stallcup (1977b) for PC12 cells in response to CCh.

PC12 cells treated with 9B or βNGF for 5 days also showed increased Na^+ uptake in response to increasing concentrations of CCh (Fig. 2). When assayed

without CCh, approximately the same low background level of Na^+ uptake was seen in untreated and growth factor-treated cells. Cells grown with each of the two growth factors had the greatest Na^+ uptake with 3.2 mM CCh and half maximal uptake at 0.4 to 0.5 mM CCh. Na^+ uptake by βNGF- and 9B-treated cells assayed with maximally effective CCh concentrations increased by at least 100% compared to untreated cells assayed under the same conditions. These results confirmed that the morphological differentiation of PC12 cells in response to nerve growth factors was accompanied by an increase in Na^+ uptake stimulated by the same maximally effective concentrations of CCh.

Nicotinic stimulation of Na^+ uptake

The nicotinic cholinergic inhibitor d-tubocurarine (dTC) has been shown to inhibit CCh-stimulated Na^+ influx (Patrick and Stallcup, 1977b) as well as electrophysiological responses to iontophoretically applied acetylcholine (Dichter et al., 1977) in PC12 cells. We therefore examined the effect of dTC on the $^{22}Na^+$ uptake into PC12 cells grown with and without each growth factor (Fig. 3A). Preincubation of cells with 400 μM dTC for 10 min prior to assay inhibited more than 90% of the Na^+ uptake stimulated by CCh. Half-maximal inhibition of Na^+ flux occurred with 20-50 μM dTC both for growth factor treated and untreated cells. In contrast, atropine, an inhibitor of muscarinic cholinergic responses, reduced Na^+ flux by less than 50% of control values in PC12 cells grown in each of the three media at the highest inhibitor concentration used (400 μM, Fig. 3B).

We also examined the extent to which nicotine could mimic the effect of carbamylcholine in stimulating $^{22}Na^+$ influx. Na^+ uptake by untreated and 9B- or βNGF-treated cells (5-day total exposure) in response to nicotine concentrations ranging from 40 to 320 μM was very similar to those results

presented in Fig. 1 for CCh-stimulated uptake. Nicotine concentrations of 160 μM were maximally effective in stimulating Na^+ uptake by untreated cells and cells treated with either βNGF or 9B. Table 2 presents a comparison of uptake by cells assayed with 160 μM nicotine compared to uptake into cells from the same experiment assayed with 3.2 mM CCh. These results showed that the Na^+ influx induced by maximally effective doses of nicotine was approximately equal to the Na^+ uptake stimulated by 3.2 mM CCh regardless of the growth conditions. Thus, nicotinic stimulation accounted for virtually all of the CCh-stimulated uptake measured in these assays, and the nicotinic antagonist dTC was more than 10-fold more effective as an inhibitor than was the muscarinic antagonist atropine on a molar basis.

Cyclic AMP effect on CCh-stimulated Na^+ uptake

N^6, $O^{2'}$-Dibutyryl cyclic AMP (dbcAMP) induces the outgrowth of short neurites from PC12 cells within 24 hrs (Schubert et al., 1977). βNGF in combination with dbcAMP greatly potentiates morphological differentiation in response to either agent alone after 24 hrs (Gunning et al., 1981b). We tested the possibility that dbcAMP would increase CCh-sensitive Na^+ uptake by PC12 cells or potentiate the effect of βNGF or 9B. The results in Table 3 showed that the net CCh-stimulated Na^+ influx into cells exposed to 1 mM dbcAMP was in fact less than into untreated cells. A high percentage of these cells had developed short neurites under these growth conditions while the outgrowth of neurites from cells exposed to βNGF or 9B for 24 hrs was very limited even though these cells showed a 30% and 44% increase in Na^+ uptake, respectively (Table 3). The addition of dbcAMP to 9B- or βNGF-treated cells during the 24 hr treatment period greatly enhanced neurite production but decreased Na^+ uptake into cells to a level lower than uptake into untreated cells. A

combination of 9B and βNGF produced an increase in Na^+ flux above the level in untreated cells approximately equal to βNGF treatment alone; neurite outgrowth from such cells was essentially the same as from cells treated with 9B or βNGF for 24 hrs. Growth of cells for more than 1 or 2 days in 1 mM dbcAMP caused a large reduction in cell number apparently due to a toxic effect of dbcAMP as previously noted by Garrels and Schubert (1979).

Time course of development of CCh-sensitivity

We next examined the time required for the development of increased CCh-sensitive Na^+ uptake by PC12 cells grown with growth factors by the procedure used for the experiment in Figure 4. Cells subcultured at the same time were exposed to maximally effective doses of fraction 9B for different intervals, then replated for Na^+ uptake measurements as described in Materials and Methods. CCh-stimulated uptake by cells grown without growth factors remained unchanged over the 4 day duration of the experiment. Whether grown with or without 9B, cells assayed without agonist showed uniformly low levels of uptake. The first increase in agonist-stimulated uptake above the control level was seen after 48 hrs when CCh-induced Na^+ influx into 9B-treated cells was 77% greater than into untreated cells. After 3 and 4 days treatment with 9B, uptake was more than 100% higher than control values.

We next compared the Na^+ uptake in response to cholinergic stimulation by cells grown in culture medium without growth factor with cells grown over the same period of time with βNGF or 9B added at approximately 12 hr intervals (Fig. 5). As in the previous experiment, the Na^+ uptake by each of the cultures assayed without a cholinergic agonist remained the same (data not shown). For cells assayed with 3.2 mM CCh, no increase in Na^+ uptake was detected after 18 hrs of exposure to either βNGF or 9B. After a 26 hr exposure

to growth factors, however, Na^+ influx into cells treated with either growth factor had increased by about 40% compared to control cells. This enhanced sensitivity to CCh continued to increase gradually over most of the rest of the 90 hr treatment. The maximum increase of about 2 times the control level was reached after about 3 days exposure to growth factor. In all other experiments performed by this same procedure, CCh-sensitivity increased only after 24-30 hrs of growth factor treatment, and this sensitivity increased gradually for 3 to 4 days thereafter.

Stability of the growth factor-induced change in CCh-sensitivity

We next tested how long the nerve growth factor-enhanced CCh-sensitivity of PC12 cells remained elevated after growth factor-treated cells were replated in normal growth medium (Table 4). Cells grown with 9B for 8 days had Na^+ uptake rates 2.5 times higher than did cells never exposed to growth factors; βNGF treatment resulted in a 2-fold increase compared to untreated cells. Cells exposed to either growth factor for the final 4 days of the 8-day period had about 75% of the increased uptake seen in cells grown in growth factor for the whole period. About 75% of the maximum uptake with each growth factor remained after βNGF or 9B was removed 24 hrs before the cells were assayed for Na^+ flux. Roughly one-third of the maximum response remained when growth factors were removed during the final 4 days of the experiment.

Neurites rapidly regenerated from cells replated with growth factor-containing media after 4 days exposure to either 9B or βNGF as described in previous studies with βNGF treatment of PC12 cells (Greene, 1977). However, when such growth factor-pretreated cells were replated without growth factors, neurites were almost completely absent. Thus, even though cell morphology had returned to the undifferentiated state, a substantial enhancement of Na^+ uptake relative to untreated cells still occurred in

response to CCh 4 days after the removal of growth factor. When cells from one such experiment were exposed to 9B or βNGF for 4 days, then grown without growth factors for 6 additional days before assay, CCh-stimulated Na^+ flux was approximately 20% higher than in untreated cells in each case (data not shown).

DISCUSSION

These results demonstrated that nerve growth factors from two different sources dramatically enhanced the flux of Na^+ through the nicotinic acetylcholine receptor-linked ion channels in PC12 cells. The passive flux of ions through these channels was measured by the uptake of radiolabeled Na^+ during the first 30 sec after the addition of cholinergic agonists to cells. Under these conditions, $^{22}Na^+$ uptake was maximally stimulated with the same concentration of the cholinergic agonists carbamylcholine or nicotine and was inhibited to the same extent by the nicotinic cholinergic antagonist d-tubocurarine whether the cells were grown with or without either NGF. These results suggest that NGF treatment increases the number of functional ion conductance channels in NGF-treated cells rather than changing the pharmacological characteristics of the receptors themselves.

Dichter et al. (1977) first showed that βNGF increased ACh sensitivity in PC12 cells by measuring the depolarization of individual impaled cells grown for 2 weeks with NGF from mouse submaxillary gland. When compared to untreated cells, a higher percentage of NGF-treated cells were depolarized in response to iontophoretically applied ACh. This response was associated with an increased membrane conductance and was inhibited by d-tubocurarine. Our experiments differed from those of Dichter et al. (1977) in focusing on changes in ACh-sensitive ion conductance changes in cells treated with NGFs for shorter periods of time as well as in the methods we used to measure these changes. Our results showed an increase in Na^+ flux into cells between 1 and 4 to 6 days after the addition of nerve growth factors which occurred before the cessation of cell division and DNA synthesis in response to NGF (Gunning et al., 1981a). Overall, our quantitative measurements of ion conductance changes in PC12 cells in response to NGF were consistant with the electrophysiological

measurements performed on individual cells by Dichter et al. (1977) indicating that $^{22}Na^+$ flux measurements accurately reflected the sensitivity of PC12 cells to cholinergic agonists.

Neither NGF from mouse submaxillary gland or from B. multicinctus venom (nor any of the other snake venom fractions tested) had any effect on CCh-stimulated Na^+ uptake after a short treatment period (see Table 1). These results confirmed the observations made by Patrick and Stallcup (1977b) from which they concluded that α-bungarotoxin has no inhibitory effect on the functioning of the nicotinic ACh receptor in PC12 cells. Our results also indicated that other venom components are ineffective as inhibitors of this receptor-linked ion flux into cells of neuronal origin and suggested that their lethal effect on mice, like the effect of α-bungarotoxin, was due to inhibition of cholinergic function at the neuromuscular junction.

Cells grown in either βNGF or 9B for more than 24 hrs showed an enhanced sensitivity to cholinergic agonists which increased so that Na^+ uptake after 4 to 6 days was 2- to 3-times the level of uptake in untreated cells. All of our results showed the same Na^+ uptake and neurite generation responses with maximum concentrations of either NGF, and a combination of maximally effective doses of each NGF did not increase Na^+ flux beyond the level seen with either growth factor alone. Though the 100-fold difference in the efficacy of the two growth factors on a weight basis showed that they were clearly different proteins, their effects on Na^+ uptake and neurite generation were indistinguishable.

In all time course experiments, cells were replated in fresh media with fresh growth factor 24 hrs before assay for Na^+ flux to assure that the cells grown with different growth factors were assayed under more nearly similar conditions. When PC12 cells subcultured on the same day were assayed on 4

successive days (Fig. 4), the uptake of Na^+ into untreated cells remained the same demonstrating that the rate of $^{22}Na^+$ uptake did not increase merely as a consequence of growth-conditioning of the medium or because of the increased cell density with age. Growth factor-treated cells subcultured and assayed by the same procedures had an uptake rate twice as great.

The amount of nerve growth factor added to PC12 cells and the length of time that the cells were exposed to NGF from either source coincided with the outgrowth of neurites in response to βNGF as described in detail by Gunning et al. (1981a). However, the generation of neurites by treatment with dbcAMP alone or in combination with either growth factor for 24 hrs was not associated with increased sensitivity to cholinergic stimulation of sodium flux through the ACh receptor-linked ion channel. Also, when cells with 9B- or βNGF-generated neurites were replated in medium without nerve growth factors, neurite regeneration was limited to a small percentage of the cells. However, the cholinergic sensitivty of these cells was largely maintained for at least 24 hrs and as much as 30-40% of the increased Na^+ flux activity remained 4 days after cells were replated without NGF and neurites were lost. These results were consistant with the results of Gunning et al. (1981b) which showed that dbcAMP and NGF induced neurite formation by different mechanisms and showed that there was no clear association between the presence of neurites and the sensitivity of the cells to CCh stimulation.

Burnstein and Greene (1978) showed that the generation of neurites in response to NGF was prevented by RNA synthesis inhibitors. However, the NGF-dependent regeneration of neurites occurred in cells treated with RNA synthesis inhibitors, suggesting that RNA synthesis had occurred during the initial exposure to NGF which facilitated the extension of neurites in response to continued exposure to NGF. Gunning et al. (1981a,b) observed an increase in

the levels of RNA and protein synthesis which began within 24 hrs after NGF was added to PC12 cells but was not seen when neurite production was induced by dbcAMP. Both the rise in CCh-sensitive Na^+ uptake after 24 hrs and the insensitivity of dbcAMP-treated cells to CCh stimulation which we have observed therefore are consistant with the idea that these effects of NGF depend upon synthesis of new mRNA and protein. However, when we treated PC12 cells with low levels of the RNA synthesis inhibitor actinomycin D (0.01 µg/ml) 30 hrs before they were assayed for Na^+ uptake, CCh-stimulated ion flux into both NGF treated and untreated cells was substantially inhibited (unpublished results). Thus, this RNA synthesis inhibitor appeared to inhibit $^{22}Na^+$ uptake directly, and we were unable to determine whether new RNA synthesis was required for the increase in carbamylcholine sensitivity by using this inhibitor.

Jumblatt and Tischler (1982) recently showed that PC12 cells possess high affinity binding sites for the muscarinic antagonist quinuclidinyl benzilate (QNB). Treatment with βNGF produced an elevation in the number of QNB binding sites on cells which was first apparent after a 2- to 5-day lag period and reached a maximum level of binding after about 2 weeks. Their results showed that the muscarinic antagonist atropine was a much better inhibitor of QNB binding than was d-tubocurarine. Our results showed that the opposite was true for CCh-stimulated $^{22}Na^+$ uptake in PC12 cells (see Fig. 3). Our data also showed that nicotine was as effective as carbamylcholine in stimulating Na^+ uptake by NGF-treated or untreated cells (Table 2). Thus, though the 5-fold increase in muscarinic binding sites described by Jumblatt and Tischler (1982) occurred over roughly the same time period as the NGF-induced effect we have described, our results clearly showed that the increased $^{22}Na^+$ uptake in response to cholinergic agonists was not due to an increase in the number of

QNB binding sites.

These results have contributed to our understanding of the cholinergic sensitivity induced by nerve growth factors in PC12 cells. This increased sensitivity to neurotransmitter is undoubtedly an important event in the differentiation of neuronal cells and is accompanied by a number of other biochemical and morphological changes when PC12 cells are exposed to NGFs in culture. These changes include a 1- to 2-fold increase in the capacity of cells to bind βNGF after 3 to 5 days of treatment with βNGF (Calissano and Shelanski, 1980). This apparent increase in the number of βNGF receptors on the surface of PC12 cells thus appears to closely parallel the NGF-induced effect we have observed. Whether either or both of these changes in PC12 cells is due to NGF-induced gene regulation leading to an increased incorporation of specific proteins in the cell surface remains to be discovered. A great deal more needs to be learned before we can expect a complete understanding of how NGF induces these developmental changes in neuronal cells.

FOOTNOTE

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TABLES

Table 1. Effect of α-bungarotoxin and 9B fractions on CCh-stimulated Na^+ uptake. Subconfluent cultures of PC12 cells were treated with 0.5 μg/ml of 9B on day 0 and at 2 day intervals thereafter. On day 7, untreated and 9B-treated cells were replated in fresh media with or without 9B (0.5 μg/ml) for $^{22}Na^+$ flux assay the next day as described in Methods. Prior to assay, the culture media were removed and the cells were preincubated with Prep medium containing no additions (control), α-bungarotoxin (0.5 μg/ml) or 9B (0.5 μg/ml) for 1 hr. The preincubation media were removed prior to assay for Na^+ uptake in response to 0.5 mM CCh. Each value is the mean ± SEM for triplicate samples, and these results are typical of 3 similar experiments.

Growth Conditions	Preincubation Conditions	$^{22}Na^+$ Uptake (nmoles/min/mg)	% Uptake into Untreated Cells
Untreated	control	158 ± 12	100
	α-Bgt	173 ± 13	109
	9B	148 ± 4	94
9B-treated	control	283 ± 3	179
	α-Bgt	275 ± 6	174
	9B	301 ± 23	190

Table 2. Nicotinic stimulation of Na^+ flux into PC12 cells. CCh and nicotine-stimulated Na^+ uptake by cells grown without added growth factor was compared to uptake by cells grown with 9B or βNGF for 4 days. Results are presented as the average of duplicate determinations from a single experiment and compared as a percentage of the uptake into control (untreated) cells assayed with either 3.2 mM CCh or 160 μM nicotine (nic); the nicotine-stimulated values are also expressed as a percentage of the CCh-stimulated uptake into cells exposed to each growth factor.

	$^{22}Na^+$ Uptake (nmoles/min/mg)		% Uptake into Untreated Cells		% Uptake into CCh-Stimulated Cells
Agonist	Nic	CCh	Nic	CCh	
Growth conditions: Untreated	302	355	100	100	85
9B (5 μg/ml)	832	746	276	210	112
BNGF (50 ng/ml)	844	852	280	240	99

Table 3. Effect of dbcAMP on CCh-stimulated Na^+ uptake with and without growth factors. Subconfluent cultures of PC12 cells were replated on polylysine-coated dishes and exposed to the indicated concentrations of dbcAMP and/or growth factors for 24 hrs prior to assay for Na^+ uptake in response to 3.2 mM carbamylcholine. Duplicate wells were assayed for each treatment condition, and these results are representative of two similar experiments.

	$^{22}Na^+$ Uptake (nmoles/min/mg)	% Uptake into Untreated Cells
Untreated	198 ± 19	100
dbcAMP (1 mM)	184 ± 1	93
9B (5 µg/ml)	257 ± 5	130
βNGF (50 ng/ml)	284 ± 14	144
dbcAMP + 9B	157 ± 9	80
dbcAMP + βNGF	175 ± 19	89
9B + βNGF	281 ± 23	142

Table 4. Reversibility of growth factor enhanced CCh-stimulated Na^+ uptake. Freshly subcultured PC12 cells were grown in media without growth factor or with 5 μg/ml of 9B or 50 ng/ml of βNGF for 4 days. Cells were then replated in fresh media either with or without growth factors for an additional 3-day period. Following this second growth period, cells were harvested and replated in media with or without growth factor on polylysine-coated dishes for Na^+ uptake assay 24 hrs later. In all cases, cells transferred from growth factor-containing to control growth medium were washed with Prep medium as described in Methods. The results are expressed as a percentage of the net growth factor-enhanced Na^+ uptake (i.e., uptake into cells treated with 9B or βNGF for the entire length of the experiment minus the uptake by cells grown in control medium throughout the experiment). Essentially the same results were obtained when this experiment was repeated.

Growth Media Changes 4 days/3 days/1 day	$^{22}Na^+$ Uptake (nmoles/min/mg)	% Growth Factor-Enhanced Uptake
Con/Con/Con	206 ± 20	0
Con/9B/9B	440 ± 14	75
9B/9B/9B	519 ± 40	100
9B/9B/Con	450 ± 13	78
9B/Con/Con	286 ± 9	26
Con/NGF/NGF	351 ± 18	73
NGF/NGF/NGF	410 ± 36	100
NGF/NGF/Con	358 ± 1	75
NGF/Con/Con	290 ± 23	42

FIGURE LEGENDS

Figure 1. Effect of 9B or βNGF on $^{22}Na^+$ uptake by PC12 cells. Subconfluent cultures of PC12 cells were plated in media containing fraction 9B (◆) at concentrations ranging from 0.1 to 10 μg per ml or βNGF (■) at concentrations of 5 to 100 ng per ml. After 3 days, cells were replated for assay in fresh media with growth factors and assayed for Na^+ uptake with or without 3.2 mM CCh 24 hrs later. Each point represents the average uptake in duplicate wells from which the values for each cell sample varied by less than 10%. Protein content per well ranged from 270 to 440 μg per well equivalent to 1.13 to 1.83 X 10^6 cells per well. The open symbols in each case represent data from control cells grown without growth factor in each experiment. These results are representative of results obtained in three separate experiments.

Figure 2. Carbamylcholine stimulation of Na^+ uptake in growth factor-treated and untreated cells. PC12 cells replated on 100 mm dishes were grown in control medium (●) or in media containing 50 ng/ml of βNGF (■) or 5 μg/ml of snake venom fraction 9B (◆). After 2 days a second dose of fresh growth factor was added to the βNGF- and 9B-treated cells. All cells were harvested after a total of 4 days and replated on polylysine-coated dishes in media containing fresh growth factors. Duplicate cell samples were assayed for Na^+ uptake without agonist and with CCh concentrations ranging from 0.1 to 6.4 mM. Uptake by individual cell samples was within 7% of each average uptake value for all assays with CCh concentrations greater than 0.4 mM and within 20% of average uptake for the remaining samples; these results are typical of two such experiments. Protein concentration in individual wells ranged from 240 to 350 μg equivalent to 1.0 to 1.46 X 10^6 cells per well.

Figure 3. d-Tubocurarine and atropine inhibition of Na^+ uptake by untreated, 9B- and βNGF-treated cells. PC12 cells plated on polylysine-coated 12-well dishes were grown without (○) or with 5 μg per ml 9B (◆) or 50 ng per ml βNGF (□) for 48 hrs. Media were then removed and replaced with Prep medium containing d-tubocuarine (A) or atropine (B) at concentrations ranging from 1 to 400 μM for 10 min. The inhibitors were then removed, and the cells were assayed for $^{22}Na^+$ uptake in response to a subsaturating concentration of CCh (1 mM). The results are expressed as the percent of activity assayed without inhibitor for each growth condition: 206 ± 2, 346 ± 13 and 364 ± 19 nmoles per min per mg protein for untreated, 9B- and βNGF-treated cells, respectively, assayed in duplicate. A second experiment of this kind gave essentially the same results.

Figure 4. Na^+ uptake by PC12 cells grown with and without 9B for 1, 2, 3 or 4 days. Cells were replated and grown for 2 days before half of the dishes were treated with 9B at a concentration of 5 μg/ml. The cells to be assayed for Na^+ uptake after 24 hrs (day 1 cells) were also replated on polylysine-coated dishes in fresh medium with (◇,◆) or without (○,●) 9B at that time. At 1 day intervals thereafter, other cells from these 9B-treated and untreated cultures were harvested and assayed as described for day 1 cells. In all cases, cells were assayed for $^{22}Na^+$ uptake without (open symbols) or with (closed symbols) 3.2 mM CCh. Results are the mean of duplicate values and the range of the duplicates are represented by error bars.

Figure 5. Sodium uptake into PC12 cells treated with βNGF or 9B for a total of 18 to 90 hrs. All cells in this experiment were subcultured from a subconfluent population of PC12 cells, and a single dose of βNGF (50 ng/ml) or 9B (5

μg/ml) was added to one group of dishes at that time and to other dishes of cells at intervals thereafter. Seventy-two hrs after the first cells were exposed to growth factor, all cell cultures were replated for assay of Na^+ uptake in response to 3.2 mM CCh as described in Methods. Fresh growth factors were added to all previously treated cells at that time as well as to previously untreated cells for the final 18 hr period prior to assay for sodium uptake. Results are expressed as nmoles of Na^+ taken up by untreated (0) cells grown for the entire 90 hr period as well as for cells treated with 9B (◆) and βNGF (□) for varying lengths of time before the assay. The error bars indicate the range of duplicate cell samples in this experiment; three other experiments of this type gave similar results.

FIGURE 1
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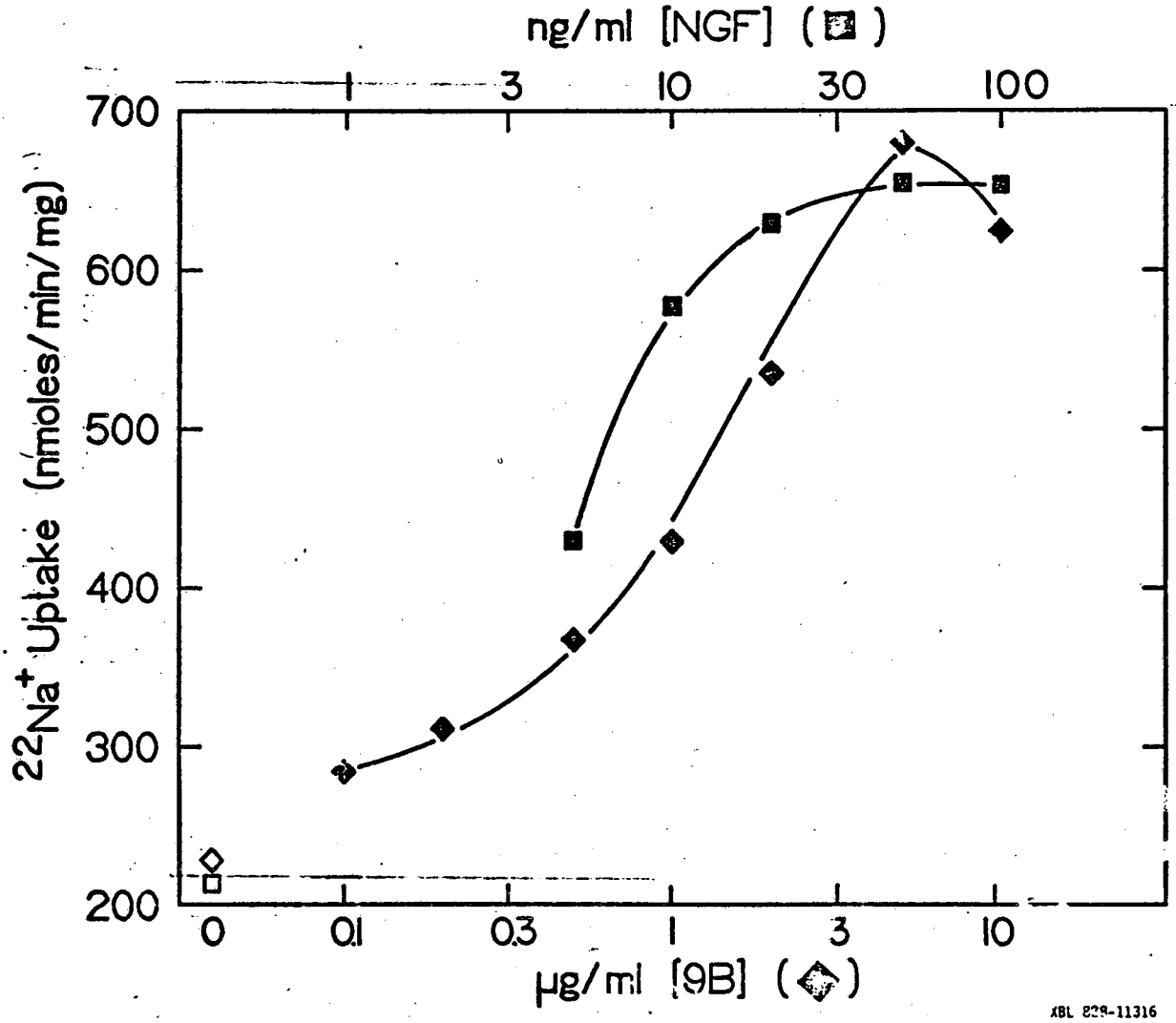

FIGURE 2
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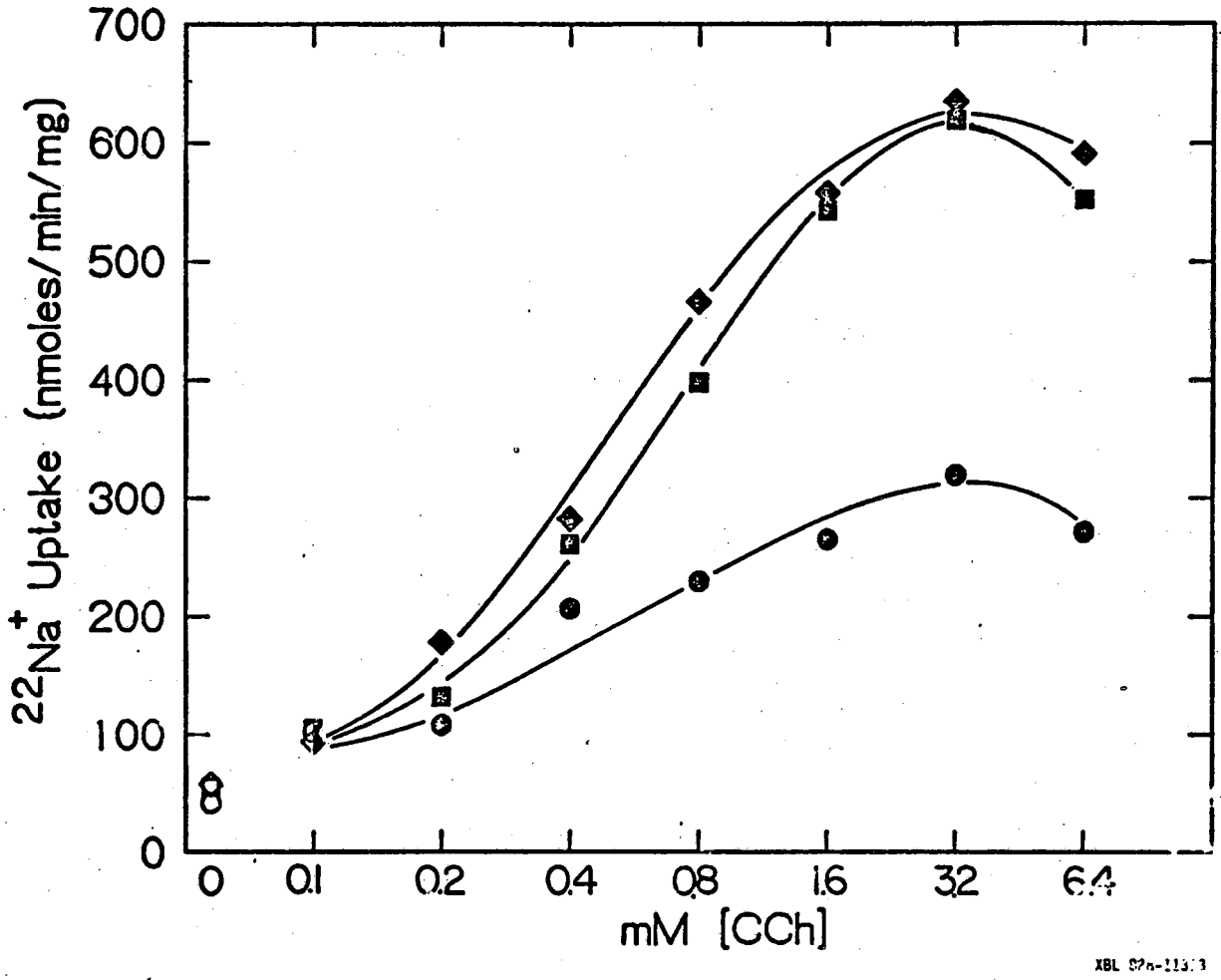

FIGURE 3
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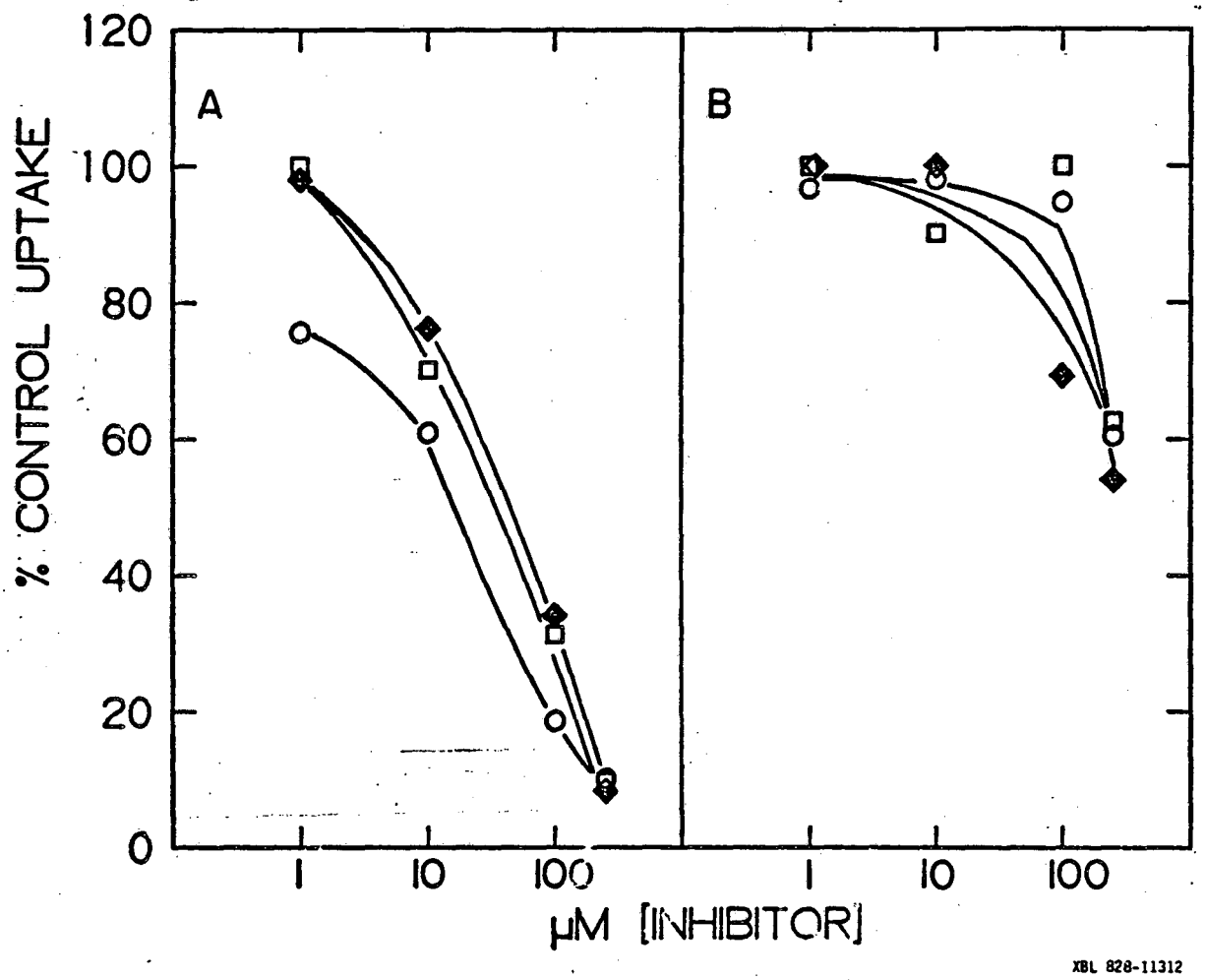

FIGURE 4
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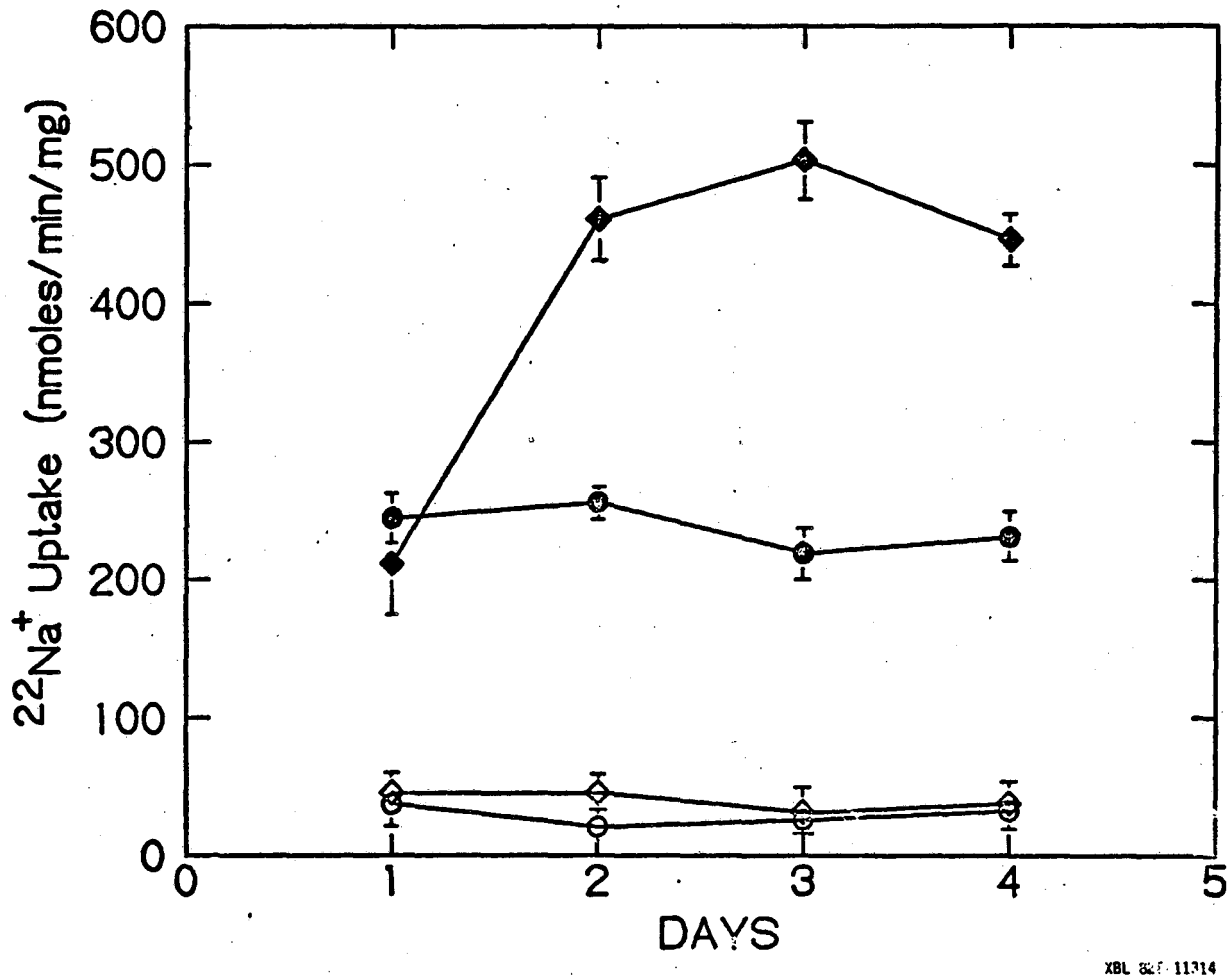

FIGURE 5
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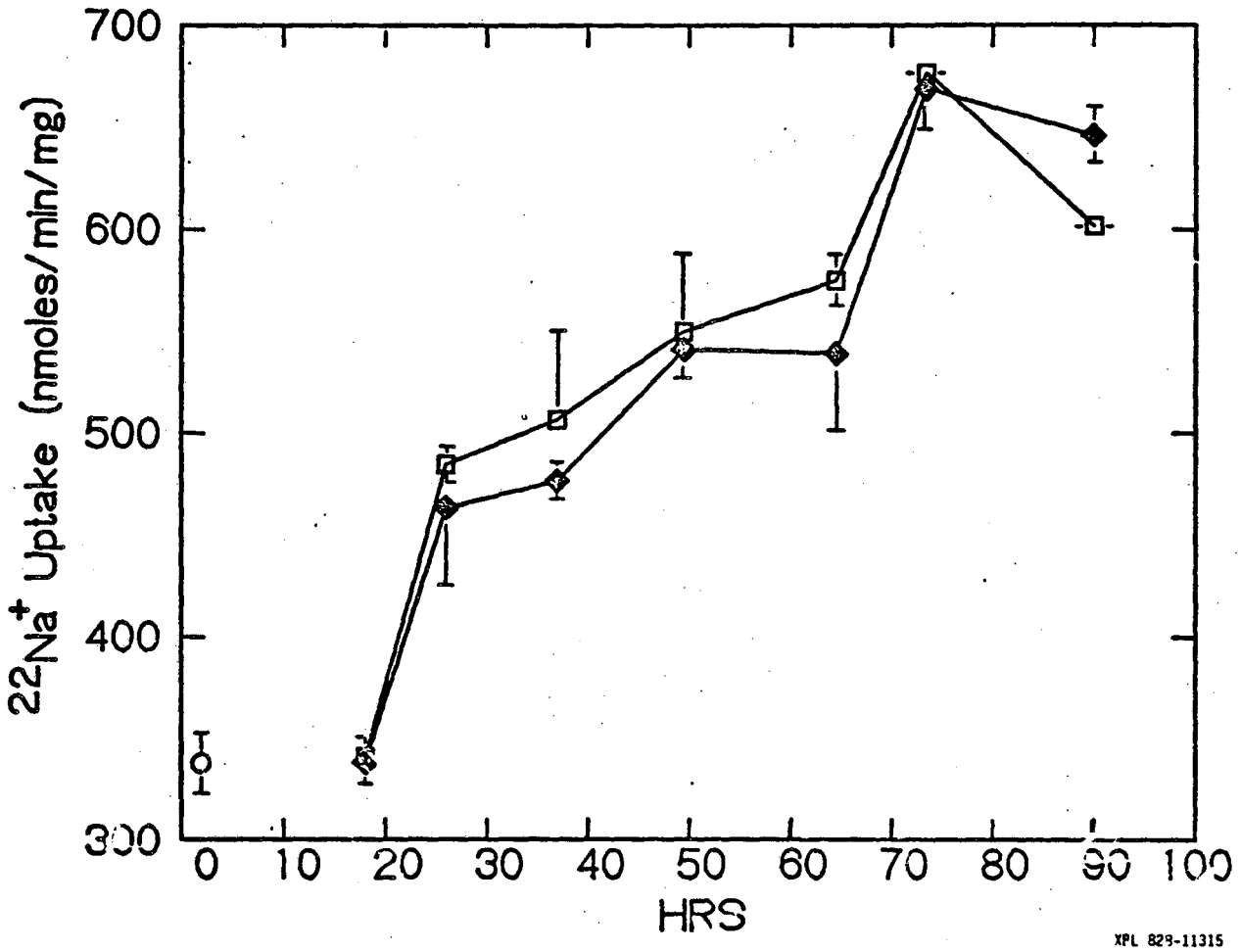

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