Voltage-Sensitive Calcium Currents Are Acutely Increased by Nerve Growth Factor in PC12 Cells

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1Laboratory of Developmental Neurobiology and 3Section on Growth Factors, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; 2William T. Gossett Neurology Labs, Henry Ford Health Sciences Center, Detroit 48202; and 4John D. Dingell Veterans Administration Medical Center, Detroit, Michigan 48201

Jia, Min, MinXu Li, Xu-Wen Liu, Hao Jiang, Phillip G. Nelson, and Gordon Guroff. Voltage-sensitive calcium currents are acutely increased by nerve growth factor in PC12 cells. J. Neurophysiol. 82: 2847–2852, 1999. Whole cell calcium currents were recorded from PC12 cells with the perforated patch technique. Currents were evoked by step depolarization from a holding potential of −90 mV. Nerve growth factor (NGF) increased calcium currents through L-type calcium channels by ≥75% within 3–5 min. This increase was inhibited by K-252a, by nifedipine, and by inhibition or down-regulation of kinase C. Brain-derived neurotrophic factor (BDNF) also increased calcium current, but to a smaller extent. Thus increases in calcium current can be linked to activation of either the high- or the low-affinity nerve growth factor receptor. Increases in presynaptic calcium uptake appear to be a crucial element in the short-term actions of the neurotrophins on neurotransmitter release leading to long-term potentiation. Also, the control of calcium uptake is likely to be an important factor in the long-term actions of the neurotrophins on neuronal survival and neuronal protection. The present data indicate that the PC12 cell may be a useful model for studying the effect of the neurotrophins on calcium uptake.

INTRODUCTION

The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3, 4/5, 6, and 7. Two classes of neurotrophin receptors have been identified, the p140trk family, which exhibits specificity in the binding of the neurotrophins, and p75NGFR, which binds all the neurotrophins relatively equally.

The long-term effects of the neurotrophins on neuronal survival and neuronal protection have been well studied in many types of neurons. Neurotrophins act to support neuronal survival during development and allow neurons to withstand damage on environmental insult. Both neuronal survival (Collins et al. 1991) and neuronal protection (Cheng and Mattson 1991) appear to require appropriate intracellular calcium levels. Increasing evidence indicates that neurotrophins are also involved, short-term, in various forms of synaptic plasticity (Lohof et al. 1993; Stoop and Poo 1996), phenomena that seem to underlie such fundamental properties of the nervous system as learning and memory (Berninger and Poo 1996). Here, also, alterations in intracellular calcium levels appear to be pivotal.

It has been shown, then, that neurotrophins can alter intracellular levels of calcium, and this action may be crucial to both their long-term and their short-term functions.

Experiments using radiolabeled 45Ca2+ have demonstrated (Nikodijevic and Guroff 1991) that NGF produces an increase in calcium uptake into PC12 cells. Such stimulation of calcium uptake involves a phosphorylation reaction (Nikodijevic and Guroff 1992) and protein kinase C (Dickens et al. 1997). Recent studies indicate that both p140trk and p75NGFR will support increased calcium uptake into the cells (Jiang et al. 1997).

The effects of the neurotrophins on synaptic efficacy may involve such changes in calcium uptake, and an increase in intracellular calcium levels has been shown to accompany the increase of transmitter output produced by BDNF at the Xenopus neuromuscular junction (Stoop and Poo 1996). Indeed, several studies have shown that both spontaneous and evoked transmitter release can be increased by increasing presynaptic calcium ion concentration (Zucker 1989).

The ion channels, if any, responsible for neurotrophin-dependent calcium uptake are unknown. We have addressed the possibility that voltage-sensitive calcium channels (VSCC) might be involved. We find that calcium currents through L-type VSCC are rapidly increased on NGF treatment of PC12 cells and that this effect is initiated by NGF binding to either p140trk or p75NGFR receptors. This is a plausible mechanism for the rapid effects of the neurotrophins in increasing transmitter output at responsive synapses, which leads to long-term potentiation and also may be involved in alternations in intracellular calcium related to neuronal survival and neuronal protection under various conditions.

METHODS

PC12 cells were plated on collagen-polylsine–coated 35-mm dishes in DMEM containing 7.5% fetal bovine serum, 7.5% donor horse serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (Life Technologies) at 37°C with 6% serum. Nystatin-perforated whole cell patch-clamp techniques were applied (Horn and Marty 1988). Whole cell currents under voltage-clamp mode were recorded and analyzed with an Axo-patch 1B amplifier and ITC-16 computer interface and Macintosh computer. Junction potential and series coupling resistance were compensated for, and this compensation did not change during the period of drug application. The Synapse software from Instrutech Corporation and Synergistic Research Systems was used for data acquisition and analysis. Patch pipettes were fire-

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polished, and the resistances of the patch pipettes were between 2.5 and 5.0 MΩ in our recording solution. Cell membrane capacitances were measured by integrating the capacitive transient at a 5 mV hyperpolarizing voltage step. Voltage-gated calcium currents were evoked by depolarization pulses from a holding potential of −90 mV. The currents were filtered at 500 Hz. The recording bath solution contained (in mM) 135 TEACl, 14-aminopyridine, 10 HEPES, 10 CaCl₂, 2 MgCl₂, and 0.001 tetrodotoxin (pH 7.3). The pipette contained (in mM) 125 CsCl, 20 TEACl, 10 HEPES, 5 EGTA, and 2 MgCl₂ (pH 7.3). Calcium (10 mM) was used to increase calcium currents.

For statistical purposes the maximal voltage-sensitive inward current was determined for each cell with and without the addition of NGF (or other agents as indicated). The mean and standard deviation of those maximal currents was used in determining the statistical significance of differences between the various experimental conditions. The voltage at which the inward current was maximal was around +20 mV.

For the determination of TrkB mRNA expression, total RNA was isolated from PC12 cells using RNA STAT-60 (Tel Test, Friendswood, TX). The TrkB Primer Pair (Hutton et al. 1992) was purchased from Promega (Madison, WI). RT-PCR reactions were carried out using the Superscript One-step RT-PCR system (Life Technologies, Bethesda, MD) for one cycle of 50°C for 30 min and 94°C for 2 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 45 s. Total RNA from rat glioma C6 cells and primers for specific isoforms of the α1D subunit of the calcium channel (Liu et al. 1996) (forward: 5'-GGAGAGGAGGCAACTACCTAGC-3', 1892–1918; reverse: 5'-CGTACACACCGGACACAGACGC-3' 2364–2388; Genebank No. M57682) were used as a positive control. The RT-PCR products were resolved on 4% agarose gels.

The down-regulation of protein kinase C in PC12 cells was accomplished by treating the cells with 1 μM phorbol 12-myristate 13-acetate (PMA) for 18 h. The down-regulation was estimated by immunoblot analysis of equal amounts of cell lysate protein with anti-PKCα antibody (Santa Cruz Biotechnology). Protein content was evaluated using a protein detection kit (Pierce).

**RESULTS**

To address the question of the types of calcium channels that might be involved in synaptic plasticity and the increased

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**FIG. 1.** Nerve growth factor (NGF)–induced increase in L-type calcium currents in PC12 cells. A: average current-voltage relationship curve, before (*) and after (■) application of NGF (200 ng/ml; n = 6). Currents at the end of a 200-ms pulse are plotted against the membrane potentials of the test pulses. The holding potential was −90 mV. Brackets indicate SE. B: whole cell calcium currents recorded from a single PC12 cell in control recording medium and in the recording medium containing NGF (200 ng/ml). Holding potential: −90 mV; command potential: +20 mV. C: increase in maximum calcium currents (mean ± SE) after perfusion with (from left to right) control medium (n = 4), NGF (200 ng/ml; n = 11), K-252a (200 nM, 30 min), then NGF (200 ng/ml; n = 5), and K-252a alone (200 nM; n = 5). Brackets indicate SE. * P < 0.005.
NGF ACUTELY INCREASES CALCIUM CURRENTS IN PC12

FIG. 2. Time course of the normalized calcium current after perfusion of NGF (200 ng/ml; n = 3). Brackets indicate SE.

calcium uptake produced by neurotrophin treatment, we have used whole cell patch-clamping in this study. As shown in Fig. 1, NGF (200 ng/ml) elicited an increase (77%) in voltage-sensitive calcium currents at a membrane potential of around +20 mV. This stimulation was substantially inhibited by preincubation of the cells with K-252a (200 nM; Fig. 1C). K-252a is a kinase inhibitor that specifically prevents the actions of NGF on PC12 cells (Koizumi et al. 1988) by inhibiting the p140\textsuperscript{trk} tyrosine kinase (Berg et al. 1992). This indicates that the increased uptake is, at least in part, supported by p140\textsuperscript{trk}.

The time course of this augmentation (Fig. 2), a maximal effect seen 3–5 min after NGF addition, is comparable with that shown in the activation of synaptic currents by BDNF in the Xenopus neuromuscular junction preparation (Lohof et al. 1993; Stoop and Poo 1996). These data are also temporally consistent with those obtained in \(^{45}\text{Ca}^{2+}\) experiments (Nikodijevic and Guroff 1991). Also consistent are the present experiments in which we find that K-252a, by itself, stimulates voltage-sensitive inward calcium currents (Fig. 1C), as it does \(^{45}\text{Ca}^{2+}\) uptake (Nikodijevic and Guroff 1992; Nikodijevic et al. 1995).

BDNF (200 ng/ml) also induced a small, but significant (24%) increase in voltage-sensitive inward calcium currents (Fig. 3). Because BDNF binds only to p75\textsuperscript{NGFR} on PC12 cells, this result is consistent with that from previous studies (Jiang et al. 1997) using \(^{45}\text{Ca}^{2+}\) that suggested that NGF-induced increases in calcium uptake can be mediated by both p140\textsuperscript{trk} and p75\textsuperscript{NGFR}. These data are supported by RT-PCR analysis of the PC12 cells used in this study, which indicates that TrkB, the specific receptor for BDNF, is not present (Fig. 4), leaving p75\textsuperscript{NGFR} the only receptor to which BDNF might bind.

The characteristics of the current elicited by NGF, high-voltage-activated and long-lasting, suggests the participation of L-type calcium channels. This suggestion is supported by the observation that nifedipine, an inhibitor of L-type calcium channels, inhibits the action of NGF on calcium currents (Fig. 5A). Also consistent with the involvement of L-channels is the finding that, in current-voltage relationship curves, the amplitudes of the currents from a holding potential of −40 mV and from a holding potential of −90 mV were almost identical (Fig. 5B). Finally, these data are consistent with observations on 3T3 cells transfected with NGF receptors (Jiang et al. 1999) showing inhibition of NGF-dependent increases in calcium levels by nifedipine. It should be noted, however, that the concentration of nifedipine needed for complete inhibition of the effect of NGF (10 \(\mu\text{M}\)) is relatively high, so the final identification of the NGF-stimulated channel as L-type must await molecular characterization.

Protein kinase C is activated by treatment of PC12 cells with NGF (Hama et al. 1986; Kondratyev et al. 1990), and the involvement of kinase C has been shown in several of the actions of NGF on its target cells (Balbi and Allen 1994; Perrone-Bizzozero et al. 1993). Protein kinase Ca, specifically, has been implicated in the NGF-stimulated uptake of \(^{45}\text{Ca}^{2+}\) into PC12 cells (Dickens et al. 1997). Figure 6A shows that chronic treatment of PC12 cells with 1 \(\mu\text{M}\) PMA, which has been shown to down-regulate protein kinase C in PC12 cells, largely prevented the NGF-induced increase in calcium current. Such treatment did not markedly alter basal calcium uptake by the cells. Support for these data were found in experiments with Go6976, a specific inhibitor of the calcium-independent isoforms of kinase C (Fig. 6A), which largely inhibited the action of NGF on calcium current, and with PMA, which, on an acute basis activates kinase C and which stimulated calcium current (Fig. 6A). Previous reports from this laboratory (Zheng et al. 1996) indicate that protein kinase Ca is the only classical form of kinase C in these PC12 cells, and the present data show that it is down-regulated by 24-h treatment with PMA (Fig. 6B). These data suggest that phosphorylation of an L-type calcium channel by protein kinase Ca is necessary for at least a large part of the NGF-induced increase in calcium current.

DISCUSSION

The data presented here indicate that NGF acutely increases voltage-sensitive calcium currents in PC12 cells, that both p140\textsuperscript{trk} and p75\textsuperscript{NGFR} can support such increases, and that phosphorylation by protein kinase Ca is at least part of the mechanism by which these increases occur. Because calcium influx is a critical step in the synaptic transmission process, the effects of NGF demonstrated here could serve as a basis for the increased synaptic efficacy produced by neurotrophins in a number of systems. The increase in peak voltage-sensitive calcium currents produced by NGF averaged 77% and that produced by BDNF averaged 24%. If such a change occurred in a presynaptic terminal, it could have a major effect on transmitter output, because of the proportional relationship between calcium current and transmitter release (Llinas et al. 1976). It needs to be noted that neither Lei et al. (1997), using adult frog sympathetic ganglion cells, nor Li et al. (1998), with cultured embryonic hippocampal neurons, observed the acute neurotrophin-induced changes in calcium currents seen here.

The dual effects of K-252a, inhibiting the actions of NGF, but having actions comparable with those of NGF when administered alone, reflects its dual role as both antagonist and partial agonist of p140\textsuperscript{trk}. Because its actions on the uptake of \(^{45}\text{Ca}^{2+}\) are transient (Nikodijevic and Guroff 1991), it may be that its actions on calcium currents are also transient, allowing a return to baseline during the 30-min preincucation before the
addition of NGF. When added alone and evaluated directly, however, the stimulation by K-252a is revealed. The ability of K-252a to inhibit the calcium currents stimulated by NGF, but to stimulate calcium currents itself is consistent with its ability to inhibit the neurotrophic actions of NGF (Doherty and Walsh 1989; Koizumi et al. 1988; Matsuda and Fukada 1988), but to exhibit neurotrophic actions of its own (Borasio 1990; Cheng et al. 1994; Glicksman et al. 1993).

The question of the possible relationship between the increase in voltage-sensitive calcium currents demonstrated here, and the increased $^{45}\text{Ca}^{2+}$ influx shown in previous studies should be addressed. The resting potential of the PC12 cells used in the present work was usually about $-60 \text{ mV}$, and at that potential there was no difference in calcium current under voltage clamp between the control and the NGF-treated cells. There was a small difference at clamping potentials more positive than $-40 \text{ mV}$, and it is possible that a portion of the NGF-sensitive channels would be activated even in the resting state. Alternatively, it may be that at least a part of the population of PC12 cells, perhaps those in a specific phase of the cell cycle, has a resting potential more positive than the average, and those cells account for the increases in $^{45}\text{Ca}^{2+}$ influx.

FIG. 3. Brain-derived neurotrophic factor (BDNF)–induced increase in L-type calcium currents in PC12 cells. A: average current–voltage relationship curve, before (*) and after (■) application of BDNF (200 ng/ml; $n = 5$). Currents at the end of a 200-ms pulse are plotted against the membrane potentials of the test pulses. Holding potential was $-90 \text{ mV}$. Brackets indicate SE. B: whole-cell calcium currents recorded from a single PC12 cell in control recording medium and in the recording medium containing BDNF (200 ng/ml). Holding potential: $-90 \text{ mV}$; command potential: $+20 \text{ mV}$. C: increase of maximum calcium currents after perfusion of control medium (left) and BDNF (200 ng/ml; right; $n = 3$). * $P < 0.01$.

FIG. 4. Determination of TrkB mRNA expression in PC12 cells. Lane 1: total RNA from C6 cells with trkB primers. Lane 2: total RNA from PC12 cells with trkB primers. Lane 3: total RNA from PC12 cells with $\alpha$ID calcium channel subunit primers. PCR products were separated on a 4% agarose gel. Molecular weight standards are shown on either side.
that NGF produces. Finally, it is possible that NGF treatment itself changes the resting potential of the cells. It is equally possible, however, that the present observation that NGF produces an increase in the activity of voltage-sensitive Ca$^{2+}$ channels and the increase in intracellular calcium shown to result from NGF action in earlier studies represent two separate effects of this trophic molecule.

Recent data obtained with cultures of dissociated hippocampal neurons from fetal rats (Li et al. 1998) indicate that the neurotrophin-induced increase in synaptic currents in that system depends on mobilization of intracellular calcium and only indirectly on increases in calcium uptake. A similar reliance on mobilization of intracellular calcium stores was observed in studies of neurotrophin-induced neurotrophin release (Canossa et al. 1997). However, more recent data (Kruttgen et al. 1998) has shown that activation of either p140$^{trk}$ or p75$^{NGF}$ will support neurotrophin-induced neurotrophin release. Because experiments with 3T3 cells transfected with NGF receptors show that p75$^{NGF}$ does not mediate mobilization of intracellular calcium but does mediate uptake of calcium from the extracellular compartment, uptake of extracellular calcium would seem a likely candidate for the support of at least some neurotrophin-dependent release processes.

Whether, in fact, these results from PC12 cells provide a useful model for neuronal synapses remains to be determined, but several observations suggest parallels between the two

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**FIG. 5.** Identification of L-type channels. A: effect of pretreatment with nifedipine (10 μM, 30 min) on the action of NGF (200 ng/ml) on calcium currents in PC12 cells (n = 4). * P > 0.69. B: current-voltage relationship curves from different holding potentials (−40 and −90 mV) recorded from a PC12 cell. Currents measured at the end of a 200-ms pulse were plotted against the membrane potentials. *, from a holding potential of −40 mV; ◦, from a holding potential of −90 mV.

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**FIG. 6.** Involvement of protein kinase Cα in the action of NGF on calcium currents in PC12 cells. A: increase of maximum calcium current (from left to right) after perfusion of control, 200 ng/ml NGF (n = 6), pretreated with PMA (1 μM, 18 h), then NGF (200 ng/ml; n = 4), pretreated with Go6976 (200 nM, 30 min), then NGF (200 ng/ml; n = 5), and treated with PMA (100 nM; n = 3). * P < 0.005; ** P < 0.02. B: down regulation of protein kinase Cα by PMA in PC12 cells.
experimental situations. The stimulation by NGF in PC12 cells requires the participation of a Trk receptor, as does the stimulation by NT-4 in the Xenopus neuromuscular junction (Wang and Poo 1997). The stimulation by NGF in PC12 cells takes between 3 and 5 min, as does the stimulation by BDNF in the Xenopus preparation (Stoop and Poo 1996) and the stimulation by NGF in molluscan neurons (Wildering et al. 1995). Finally, the stimulation by NGF in PC12 cells is linked to the release of neurotransmitter (Nikodijevic et al. 1990), as is the stimulation by BDNF in the Xenopus system (Lohof et al. 1993). Thus the characteristics of the NGF-induced increase in calcium currents in PC12 cells would seem to be a plausible model for synaptic events leading to long-term potentiation and other forms of synaptic plasticity. It seems equally reasonable, in the light of recent data on the role of L-channel activation in the survival of cerebellar granule neurons in culture (Blair et al. 1999), to regard NGF-induced L-channel activation in PC12 cells as a model for studying the changes in calcium levels underlying neuronal survival.

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