Early rise of cytosolic Ca^{2+} induced by NGF in PC12 and chromaffin cells

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A rise of cytosolic Ca^{2+} is induced by NGF in rat pheochromocytoma PC12 and bovine chromaffin cells investigated (both in suspension and while attached to polyornithine-coated glass slides) by fluorescence techniques (with quin-2 and fura-2). The effect of NGF on $[Ca^{2+}]_i$ is delayed (30–40 s of lag phase), slow ($t_{1/2} = 40$ s), relatively small ($+50$–75%) and persistent (over 10 min). It is due to Ca^{2+} influx (requires extracellular $Ca^{2+} > 10$ μM) through a pathway different from the voltage-gated Ca^{2+} channel, possibly accompanied by intracellular Ca^{2+} redistribution, and might play a messenger role in NGF action.

Nerve growth factor Ca^{2+} *Second messenger* *Phosphatidylinositol* *Quin-2* *Fura-2*

1. INTRODUCTION

Nerve growth factor (NGF) induces in target cells (among which are sympathetic and sensory neurons; chromaffin and pheochromocytoma cells) a whole host of effects. These effects, which include: surface changes, induction of enzymes, receptors, channels and transient stimulation of cell division, ultimately lead to enhanced cell survival and differentiation (reviews [1,2]). Strong evidence indicates that the effects of NGF are the consequence of its interaction with specific surface receptors, and are probably mediated by the generation of one (or more) intracellular second messengers [2,3]. In spite of recent progress in the NGF receptor structure [4], the mechanism(s) of transmembrane signalling and the nature of the second messenger(s) have so far remained mysterious. In particular, the involvement of cAMP and Ca^{2+} [5] has been seriously questioned [6,7]. By the use of two fluorescence techniques (with quin-2 and fura-2) we demonstrate here that the application of NGF causes within a short time (a few tens of seconds) an increase of cytosolic Ca^{2+} in two types of target cells, rat pheochromocytoma (PC12) and bovine chromaffin cells.

2. MATERIALS AND METHODS

PC12 cells (initially obtained from Dr P. Calissano, Rome) were cultured as described in [8]. Bovine chromaffin cells were dispersed and cultured as described by Greenberg and Zinder [9]. For the experiments, an incubation medium was used that contained, in mmol/l: 125 NaCl, 5 KCl, 1.2 KH_2PO_4 and $MgSO_4$, 2 $CaCl_2$, 25 Hepes-NaOH buffer (pH 7.4), 6 glucose. In some experiments, 3 mM EGTA was added to lower $[Ca^{2+}]_o < 10^{-7}$ M.

Suspensions of PC12 cells were prepared, loaded with quin-2 by incubation with quin-2 AM (50 μM) and analyzed in a Perkin-Elmer LS5 spec-

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Abbreviations: NGF, EGF, nerve and epidermal growth factor; $[Ca^{2+}]_i$, $[Ca^{2+}]_o$, concentration of free Ca^{2+} in the cytosol and extracellular medium

trofluorimeter as in [8]. Other PC12 cells, as well as bovine chromaffin cells, were attached onto glass slides coated with polyornithine, cultured for 2 days and then loaded with fura-2 (10 μM, at 15°C) as described in [10]. Fura-2 measurements of $[Ca^{2+}]_i$ in single cells were carried out by the ratio (340/385 nm) technique of Grynkiewicz et al. [11], using a modified Zeiss Photomicroscope III apparatus, as described in [10]. Accumulation of [^{3}H]inositol phosphates in cells preloaded with [^{3}H]inositol for 24 h, and treated with NGF in a medium supplemented with 10 mM LiCl, was carried out according to [12].

Highly purified 2.5 S NGF (prepared as in [13]) was the kind gift of Dr P. Calissano; human recombinant EGF and [^{3}H]inositol were purchased from Amersham, quin-2 and insulin from Calbiochem and fura-2 from Molecular Probes.

3. RESULTS AND DISCUSSION

Suspensions of pheochromocytoma PC12 cells were studied by the quin-2 technique. Fig.1A shows that application of either insulin (10 μg/ml) or EGF (0.1 μg/ml: the latter factor induces some early events similar to NGF [14,15]) failed to cause any changes in $[Ca^{2+}]_i$, whereas NGF did. The lowest concentration that yielded a measurable response was 0.8 nM. With optimal concentration of NGF (>3 nM) the response typically began 30–40 s after the application of the factor, and consisted of a slow ($t_{1/2}$ = 40 s) rise from a resting level of 102 ± 5 nM (n = 50) to values ranging between 115 and 170 nM (mean 152 ± 12 nM, n = 28) and that, in the continuous presence of NGF, remained elevated at a steady state for several (at least 10 min) minutes.

The NGF-induced response was dependent on $[Ca^{2+}]_o$. Below 0.1 mM the response decreased progressively, and was no longer seen at $[Ca^{2+}]_o$ < 10 μM (fig.1D). In contrast, the response was unaffected by verapamil, added either before (fig.1B) or after (not shown) NGF, at concentrations (20 μM) known to block voltage-gated Ca^{2+} channels in PC12 cells [8]. These results suggested an origin of the NGF-induced $[Ca^{2+}]_i$ rise by influx, however through a pathway different from the voltage-gated Ca^{2+} channels. In agreement with this last conclusion NGF was shown: (i) to cause no change of the membrane potential, as in-
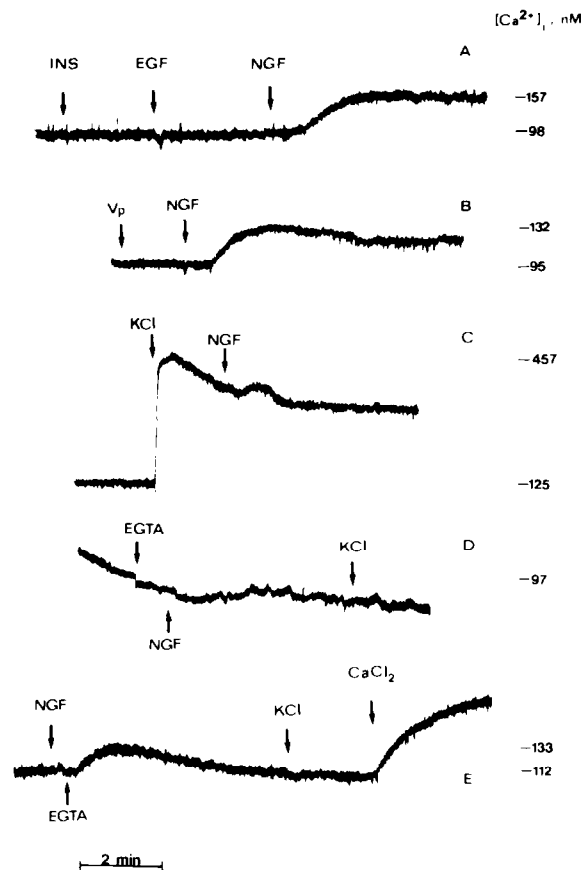

Fig.1. Quin-2 measurements of $[Ca^{2+}]_i$ in suspended PC12 cells. The concentration of cells ranged between 0.8 and 1.1 × 10^6/ml, the concentration of quin-2 between 0.8 and 1 mM. Where indicated the various agents were added at the following final concentrations: insulin (Ins), 10 μg/ml; EGF, 0.1 μg/ml; NGF, 3.5 nM; verapamil (Vp), 20 μM; KCl, 50 mM; EGTA and CaCl$_2$, 3 mM.

dicated by the specific probe, bisoxonol (not shown); (ii) to maintain a detectable effect in cells where $[Ca^{2+}]_i$ had been increased by K^+ predepolarization (fig.1C); and (iii) to leave unaffected the verapamil-inhibitable Ca^{2+} transients induced by K^+ applied after NGF (not shown).

In addition to stimulated Ca^{2+} influx, a second, intracellular component of the NGF-induced $[Ca^{2+}]_i$ rise is however possible. In fig.1E NGF was applied in the complete, Ca^{2+}-containing medium, and excess EGTA then rapidly added (to lower $[Ca^{2+}]_o$ < 10^{-7} M) during the lag phase that precedes the $[Ca^{2+}]_i$ rise. Under these conditions a

$[Ca^{2+}]_i$ rise did occur which however was smaller (~40%) than those induced in the Ca^{2+}-containing medium. The changes in $[Ca^{2+}]_i$ of fig.1E are not due to the $[Ca^{2+}]_o$ fluctuations, because they did not appear when EGTA and Ca^{2+} were added in sequence, but NGF was omitted. The different results obtained when EGTA was added before and after NGF suggest that extracellular Ca^{2+}, although not necessary for NGF binding [16], might be required for coupling receptor activation to at least part of its intracellular transduction machinery.

The NGF-induced rise of $[Ca^{2+}]_i$ was also investigated in single PC12 cells attached to glass slides and loaded with the new Ca^{2+} indicator, fura-2, using the microscopic ratio technique of Grynkiewicz et al. [11]. The results obtained (fig.2) appear to be in excellent agreement with those obtained with quin-2-loaded cells in suspension, except that in single cells the $[Ca^{2+}]_i$ rises were more transient. NGF-induced $[Ca^{2+}]_i$ rises were also recorded by the fura-2 technique in attached bovine chromaffin cells. These rises were approx. 50% greater than in PC12 cells (cf. figs 2 and 3).

The possibility that the NGF-induced $[Ca^{2+}]_i$ rises are due in part to redistribution from intracellular stores was further investigated by measuring in target cells prelabelled with [3H]-inositol the accumulation of radioactive inositol phosphates, i.e. the metabolites generated by the

Fig.3. Microscopic fura-2 measurements of $[Ca^{2+}]_i$ in attached bovine chromaffin cells. The experimental conditions were as in fig.2. Solid half arrows mark additions of NGF, 1.7 and 3.5 nM (upper and lower trace, respectively).

receptor-triggered reaction known to cause release of Ca^{2+} from an intracellular, 'microsomal' store [17]. In PC12 cells 1–15 min treatments with NGF failed to induce any detectable accumulation, whereas in chromaffin cells the concentration of inositol phosphates was increased by 50% after 15 min treatment with NGF (in the presence of Li^+ to block inositol-1-phosphatase), while a similar treatment with the cholinergic agonist carbachol increased this concentration by 130% (table 1).

In the past, metabolism of phosphoinositides and influx of Ca^{2+} were repeatedly considered as possible mediators of NGF effects. Increased incorporation of either $^{32}P_i$ or [3H]inositol into phosphoinositides or phosphatidic acid was indeed reported in sympathetic ganglia and PC12 cells, but only after long (20 min to many hours) treatments. In addition, similar effects were induc-
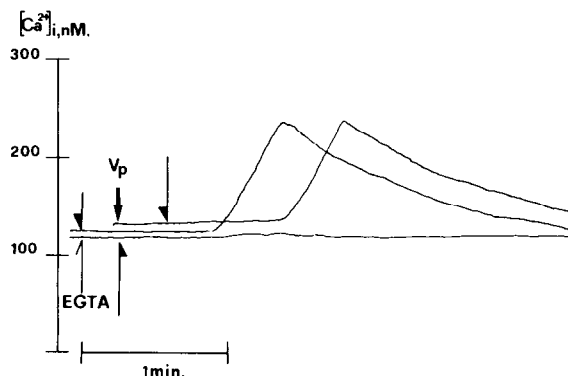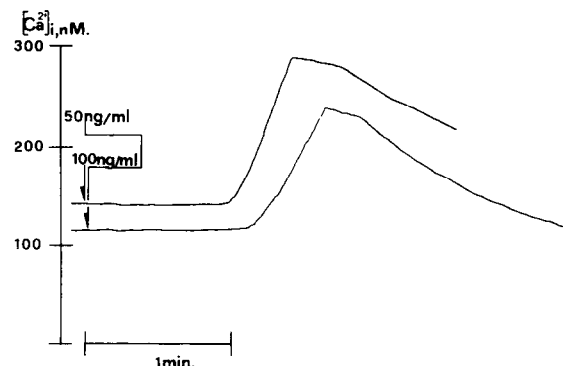

Fig.2. Microscopic fura-2 measurements of $[Ca^{2+}]_i$ in attached PC12 cells. The traces show the time course of $[Ca^{2+}]_i$ in single cells, measured by the technique described in [10], using the ratio approach of [11]. Solid half arrows indicate additions of NGF, 3.5 nM. Other indications as in fig.1.

Table 1

Accumulation of inositol phosphates induced by a 15 min treatment with either NGF or carbachol in bovine chromaffin cells

	dpm/sample
Controls	717 ± 45
NGF (3.5 nM)	1092 ± 55[a]
Carbachol (0.5 mM)	1672 ± 32[a]

[a] $p < 0.05$

Results shown are means of triplicate samples ± SD

ed by other stimulatory agents, such as high K^+ and dibutyryl cAMP [18,19]. A small stimulation of ^{45}Ca efflux from prelabelled PC12 cells by large concentrations of 7 S NGF (but also of dibutyryl cAMP) was reported by Schubert et al. [5], but not confirmed by Landreth et al. [6], who also found no effect of NGF on ^{45}Ca influx. On the other hand, recent results dealing with the effects of NGF on protein phosphorylation and protein kinase C activation [20,21] indirectly suggested an increase of $[Ca^{2+}]_i$ to be caused through the stimulation of polyphosphoinositide hydrolysis. These studies, however, were also carried out after long (1 h) treatments, and no attempt was made to establish at which step Ca^{2+} is involved in the cascade of events initiated by the NGF-receptor interaction. To our knowledge the rise of $[Ca^{2+}]_i$ demonstrated here is the earliest biochemical event ever shown to occur in target cells treated with NGF, and the only one generated early enough to be a direct consequence of (or at least closely linked to) receptor activation. At variance with other effects of NGF, those discussed above as well as cytosolic alkalinization and activation of the Na^+/K^+ ATPase [15], the rise of $[Ca^{2+}]_i$ is induced neither by cAMP analogs and cAMP-raising agents [12] nor by EGF. Its typical delayed appearance, slow rate and small size differentiate this effect of NGF from the $[Ca^{2+}]_i$ transients induced by depolarizing agents and neutrotransmitters [8,12], and might explain why it was missed by the previous investigators [6] working with radiochemical Ca^{2+} transport techniques. Whether and to what extent this early event elicited in chromaffin and PC12 cells is related to all or to only some of the later effects of NGF (e.g. the transient growth-promoting activity [15,22,23] or the differentiation effects [1,2,22]) remains to be elucidated.

ACKNOWLEDGEMENTS

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