Nerve growth factor induces mast cell degranulation without changing intracellular calcium levels

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Nerve growth factor (NGF) induces degranulation of rat peritoneal mast cells (RPMC) in a dose-dependent manner, providing direct evidence for its action on non-neuronal tissues. Activation of RPMC by NGF depends on lysophosphatidylserine and extracellular calcium. NGF-mediated RPMC degranulation is not coupled to a transient increase in intracellular free calcium ($[Ca^{2+}]_i$). It is suggested that NGF has a unique mode of action independent of $[Ca^{2+}]_i$ and presumably also without involving protein kinase C activation as indicated by the effects of phorbol esters and NGF on antigen-evoked $[Ca^{2+}]_i$ rise.

Nerve growth factor *12-O-Tetradecanoylphorbol 13-acetate* *(Rat peritoneal mast cell, Rat leukemia cell line)* *Degranulation* *Ca^{2+}*

1. INTRODUCTION

Nerve growth factor (NGF) is a well characterized neuronotrophic polypeptide which is essential for the normal development and function of sympathetic and sensory neurones [1]. This polypeptide is synthesized in target tissues innervated by sympathetic and sensory nerves [2,3]. NGF initiates its characteristic effects by interacting with specific surface receptors on the plasma membrane of neuronal target cells [4]. Furthermore, NGF has been shown to elicit biological effects on non-neuronal tissues, such as a rapid shape change of blood platelets [5], an increase in the number of mast cells in neonatal rats [6] and degranulation of mast cells [7]. The recent finding that biologically active NGF stimulates an increase in vascular permeability in the rat skin [8] and the detection of high (nanomolar) concentrations of NGF immunoreactivity in joint effusions of experimentally induced arthritis and in pleural and peritoneal exudates in rats (Weskamp and Otten, unpublished) suggest that NGF is involved in inflammatory responses [9]. In this study we show that NGF is an effective secretagogue for mast cells, an effect blocked by specific monoclonal antibodies against NGF. Unlike antigenic stimulation, NGF does not elevate $[Ca^{2+}]_i$ as measured by quin-2 fluorescence.

2. MATERIALS AND METHODS

2.1. *Preparation of NGF and its antibody*

2.5 S NGF was isolated from the submaxillary glands of adult male mice according to Suda et al. [10]. 2.5 S NGF was further purified by gel filtration on a Biogel P 30 column (2.6 by 90 cm) equilibrated with 50 mM Tris-HCl, pH 7.4. The purified NGF migrated as a single band in SDS-polyacrylamide gel electrophoresis. Biological activity was monitored by following the dose-dependent neurite outgrowth of PC 12 rat pheochromocytoma cells [11]. Monoclonal antibodies to 2.5 S NGF were generated by conventional procedures [12]. Briefly, Sprague-Dawley rats were immunized with 2.5 S mouse NGF according to the procedure of Stähli et al. [13]. Spleen cells were fused with FO myeloma cells [14] with polyethylene glycol and plated at about 10^5

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cells per well in a selection medium comprised of Dulbecco's modified Eagle's medium (hypoxanthine, aminopterine, thymidine and 10% fetal calf serum). Hybridomas producing anti-NGF antibodies were screened by a solid-phase immunoassay and cloned twice by limiting dilution [15]. Clone 23 C4 producing an antibody of the IgG2a subclass which inhibits neurite outgrowth from sensory neurones and PC 12 cells was used in our experiments.

2.2. *Preparation of cells and degranulation measurements*

Male Sprague-Dawley rats (250–300 g) were decapitated after anaesthetizing with diethyl ether. 50 ml Tyrode buffer (137.0 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 10 mM Hepes, 5 mM glucose, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) were injected into the peritoneal cavity of each rat. Peritoneal cavity was then opened by a midline incision; the fluid collected contained about 3% mast cells. Separation of mast cells was carried out by density gradient centrifugation. The gradient was composed of a lower layer of 1 ml Tyrode containing 0.26 g/ml bovine serum albumin (BSA) and an upper layer containing 0.20 g/ml BSA onto which a 5 ml suspension of peritoneal cells was placed. Mast cells were pelleted by centrifugation at 900 × *g* for 10 min. The purity of mast cells recovered from the bottom of the tube was 80%. The cells were washed twice with 10 ml Tyrode buffer before being divided into aliquots for the release experiments. RBL-2H3 cells were grown in Eagle's minimal essential medium (with Earle's salts) supplemented with 20% fetal calf serum, 1 mM glutamine, 200 IU penicillin/ml and 200 μg streptomycin/ml (supplied by Gibco). Cells were plated in 250 ml Nunc tissue culture T flasks, at 37°C, in a humid, 5% CO_2 atmosphere incubator. Cells for the experiments were taken in the early stationary growth phase, 48 h after transfer, and removed from the flask by means of a rubber policeman. Assay of serotonin release: 10^7 cells were incubated for 24 h at 37°C in 10 ml RPMI-1640 medium containing 1.2–2.5 × 10^{-6} mol [^{3}H]serotonin (5 mCi/ml, Amersham) and plated into 96-well flat-bottom microtiter plates (Nunclon; 10^5 cells in 100 μl medium per well). The cells were passively sensitized by incubation with ascitic fluid containing dinitrophenyl (DNP)-specific monoclonal IgE antibodies [16], 150 μl of a 1:50 dilution per well for 1 h at 4°C. The supernatants were removed and the cells washed twice with medium to remove unbound IgE. The cells were challenged to release [^{3}H]serotonin by adding either 0.2–1.0 ng of the antigen $(DNP)_8$-BSA (a covalent conjugate of an average of 8 DNP groups attached to BSA) or 10^{-9}–10^{-7} M NGF in 150 μl medium per well, and plates incubated for 30 min at 37°C. 100 μl samples were removed from each well into a toluene-Triton scintillation liquid for determination of [^{3}H]serotonin release. The total radiolabeled serotonin incorporated into cells was estimated by dissolving the cells in 100 μl of 1 N NaOH and counting the whole sample. Release was expressed as a percentage of the total [^{3}H]serotonin incorporated. The degranulation process was monitored by either the fluorimetric histamine method of Shore [17] or by a modification of the procedure for measuring [^{3}H]serotonin release described by Taurog et al. [18]. Briefly, aliquots of 0.9 ml of a peritoneal cell suspension (10^5 cell/ml) were equilibrated at 37°C in Tyrode solution. NGF or compound 48/80 were added in a volume of 0.1 ml and incubation was carried out at 37°C for 10 min. The reaction was stopped with 2 ml of ice-cold Ca^{2+}-free Tyrode solution. The cells were sedimented by centrifugation at 1000 × *g* for 5 min. Aliquots from the supernatants were reacted with 0.4 ml of 1 N NaOH and 0.1 ml of *o*-phthalaldehyde (10 mg/ml in methanol) for 4 min at room temperature, and then 0.2 ml of 3 N HCl was added. The fluorescence emitted by the samples at 450 nm after excitation at 360 nm was monitored on a Perkin-Elmer fluorimeter.

2.3. *Measurement of* $[Ca^{2+}]_i$

Cells were maintained as described. Attached cells were collected 48 h after passage by repeated pipetting and suspended in Tyrode buffer containing 0.1% BSA, pH 7.4. The cells (1 × 10^6/ml) were incubated with a monoclonal, DNP-specific, IgE antibody for 1 h at 37°C. After 3 washes with Tyrode buffer, aliquots of cells (each 5 × 10^7 cells/ml) were subsequently incubated with 100 μM quin-2 acetoxymethyl ester (quin-2) (AME, Ciba-Geigy, Basel) for 15 min at 37°C. Aliquots of the cell suspension (2 × 10^6 cells) were then centrifuged and the supernatants discarded.

The cell pellets were resuspended to a final volume of 2 ml each and transferred to 1-cm optical pathway quartz cuvettes, continuously stirred by an inserted electrical stirrer. Fluorescence changes were monitored on a Perkin-Elmer spectrofluorimeter (excitation 339 nm, emission 492 nm). Hydrolysis of quin-2-AME to quin-2 and its uptake into the cells were examined by following the shift in emission maximum from 435 to 492 nm. Agent-induced changes in $[Ca^{2+}]_i$ were quantified as detailed by Rink et al. [19] with modifications [20]. Cellular leakage was checked by addition of 0.5 mM $MnCl_2$ to the samples. Intracellular free Ca^{2+} concentrations were calculated using the following formula:

$$[Ca^{2+}]_i = k_D(F - F_{min})/(F_{max} - F)$$

where k_D (115 nM) represents the dissociation constant of quin-2, F the fluorescence measured in intact cells, F_{max} the fluorescence in lysed cells at 2 mM Ca^{2+} in the medium and F_{min} at 10 nM Ca^{2+}, pH 8.3.

3. RESULTS AND DISCUSSION

As shown in fig.1 NGF causes dose-dependent histamine secretion from rat peritoneal mast cells (RPMC). The effect of NGF on degranulation is dependent on the presence of lysophosphatidylserine (lysoPS) and extracellular Ca^{2+} (table 1). NGF-mediated degranulation was compared with the prototypical histamine releasing compound 48/80, low-M_r polyamine (fig.1). In the presence of lysoPS (1 μM) a significant 5% net release ($P < 0.05$) is achieved at 10^{-9} M NGF while the half-maximal effect was evident at 5×10^{-8} M, reaching saturation (60–70% histamine release) at 10^{-7} M NGF. Histamine release was completed within 5 min and was not due to cell damage as monitored by the presence of lactate dehydrogenase in the medium. The specificity of the NGF-mediated degranulation was examined by monoclonal anti-NGF antibodies. Preincubation of RPMC with monoclonal anti-NGF antibodies (10^{-6} M) markedly reduced NGF-mediated secretion by 65% (table 1).

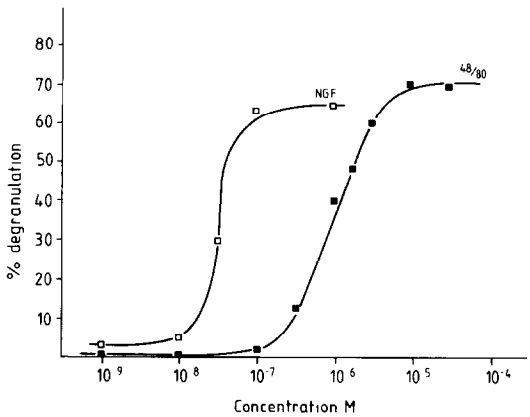

Fig.1. Histamine release from rat peritoneal mast cells. Dose dependency of compound 48/80 (■) and NGF (□). Each point represents the mean of 5 independent experiments performed in duplicates with SE < 10%. Spontaneous histamine release was <7% and was subtracted from total release.

These results prompted us to investigate in detail the mechanisms underlying NGF-mediated secretion. The following question was addressed: Does NGF (p*I* 9.3) produce its effect via stimulation of specific membrane receptors or by a more non-specific action common to basic proteins [21]? The

Table 1

Modulation of NGF-induced degranulation

Treatment	% degranulation	
	Rat mast cells	Rat basophilic leukemia cells (RBL-2H3)
Unstimulated cells + lysoPS	5–7	4–6
– lysoPS (10^{-6} M)	7	6
+ monoclonal anti-NGF antibodies (10^{-6} M)	7	4
+ EDTA (10^{-3} M)	7	6
NGF-stimulated cells + lysoPS	71	65
– lysoPS	12	36
+ monoclonal anti-NGF antibodies (10^{-6} M)	29	26
+ EDTA (10^{-3} M)	7	6
+ Mg^{2+} (1 mM; Ca^{2+}-free)	5	6

NGF-induced degranulation of mast cells and RBL-2H3 cells. Effect of lysophosphatidylserine (lysoPS), monoclonal anti-NGF antibodies, the Ca^{2+} chelator EDTA and Mg^{2+}

fact that NGF-induced histamine secretion is dependent on lysoPS and extracellular Ca^{2+} (in contrast to basic proteins) indicates that NGF elicits its effects by a more specific mechanism, presumably via specific receptors, as demonstrated by the inhibition of NGF-dependent degranulation by anti-NGF antibodies. To study the cellular mechanisms of exocytosis evoked by NGF-receptor activation we used the rat basophilic leukemia cell line RBL-2H3, increasingly used as a model system for examining the cellular mechanisms involved in stimulus-secretion coupling [22]. One of the generally accepted early events in neurotransmitter and hormone-receptor activation is the stimulation of inositol phospholipid breakdown and subsequent mobilization of $[Ca^{2+}]_i$ [23]. Therefore, changes in $[Ca^{2+}]_i$ were monitored by using the fluorecent Ca^{2+} indicator quin-2 [19] following treatment of RBL-2H3 cells with NGF. Surprisingly, NGF in concentrations up to 10^{-7} M did not affect the resting intracellular Ca^{2+} concentration (fig.2) although it caused a massive degranulation. In contrast, exocytosis of passively sensitized RBL-2H3 cells induced by antigen $(DNP)_8$-BSA led to a rapid and transient increase in $[Ca^{2+}]_i$, ranging from 90 ± 5 nM (mean ± SE, $n = 8$) in resting conditions up to an average peak value of 403 ± 21 nM ($n = 8$) (fig.2). Therefore, we conclude that the mode of NGF action differs from the immunological triggering pathway. On the other hand, the characteristics of the NGF response are very similar to mast cell degranulation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [24]. Both agents induce exocytosis without affecting $[Ca^{2+}]_i$. Since protein kinase C is the receptor for phorbol esters, the hypothesis that NGF action is mediated via this multifunctional enzyme was tested [25]. This assumption was examined by studying the regulatory effects of either TPA or NGF on the antigen-coupled $[Ca^{2+}]_i$ signals (fig.3). Upon stimulation with antigen (figs 2a and 3aI) a rapid increase in the internal Ca^{2+} concentration of RBL-2H3, from an initial value of 90 ± 5 to 403 ± 21 nM (average of 8 different experiments), was observed. Maximal increase in $[Ca^{2+}]_i$ was reached within 1 min and decreased 2 min later, returning to basal levels within 30 min after stimulation. Addition of TPA (1.5–75 nM) failed to trigger any change in the internal Ca^{2+} concentration (not shown). However, TPA at 75 nM completely blocked the antigen-induced Ca^{2+} signal (fig.3bI). When added immediately after the antigen, TPA rapidly reversed the Ca^{2+} signal (fig.3aII). In contrast, NGF (up to 10^{-7} M) had no effect on the antigen-induced $[Ca^{2+}]_i$ signal regardless of whether it was added before or after antigen stimulation (fig.3bII,aIII). These data suggest a unique mode of action of NGF operating distal from protein kinase C and $[Ca^{2+}]_i$. Such a model, however, does not exclude synergism between phorbol esters and NGF in inducing degranulation. Indeed, it has been shown that phorbol esters synergize with NGF to induce neurite outgrowth in PC 12 rat pheochromocytoma cells [26].

Our findings suggest that in peripheral targets there might be close communications between innervating sensory neurones and non-neuronal cells which are mediated by NGF. On the one hand, NGF regulates the development and function of substance P-containing sensory neurones [9,27,28] whereas on the other it elicits non-cytotoxic release of mediator substances, including histamine from peritoneal mast cells. The liberated histamine

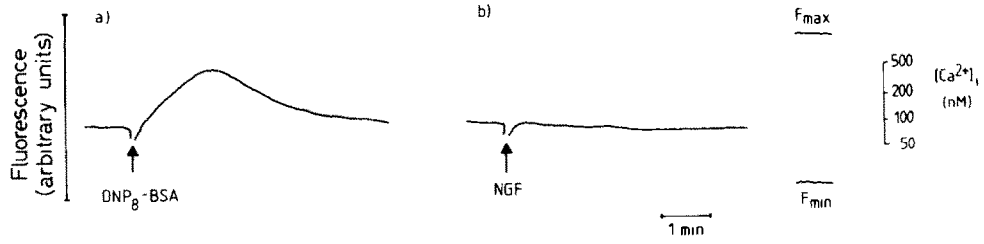

Fig.2. Fluorescence changes induced by antigen and NGF in RBL-2H3 cells loaded with quin-2, reflecting $[Ca^{2+}]_i$. Response of the cells to (a) 1 ng/ml antigen $(DNP)_8$-BSA, (b) 10^{-7} M NGF. These traces show the results of a typical experiment. Similar results were obtained in 8 similar experiments.

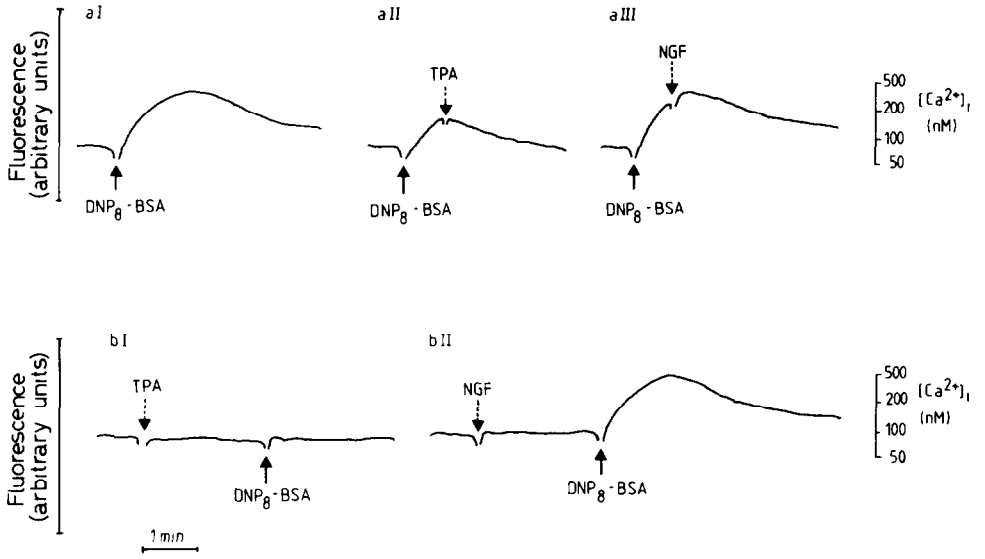

Fig.3. (a) Effect of TPA and NGF on fluorescence changes induced by antigen on RBL-2H3 cells loaded with quin-2. Effect of TPA (75 nM) (aII) and NGF (10^{-7} M) (aIII) on Ca^{2+} signal when added during the rise of the Ca^{2+} signal induced by $(DNP)_8$-BSA (1 ng/ml) (aI). (b) Effect of TPA (75 nM) (bI) and NGF (10^{-7} M) (bII) on the Ca^{2+} signal when preincubated for 2 min before the addition of $(DNP)_8$-BSA (1 ng/ml).

directly activates terminals of certain primary sensory neurones to release substance P, a potent mast cell-depleting agent [29]. A local feedback cycle controlled by NGF might be of importance in pathophysiological conditions, such as neurogenic inflammation. However it remains to be seen whether the observations reported here are applicable to other types of mast cells [30].

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