Neurotensin modulates human neutrophil locomotion and phagocytic capability

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Neurotensin (NT) was found to induce oriented locomotion and augment the phagocytic capability of human blood neutrophils over 10^{-11}–10^{-7} M. The tridecapeptide also causes Ca^{2+} extrusion from neutrophils, very likely as a result of intracellular Ca^{2+} mobilization. It is suggested that the NT-mediated functional modulation of neutrophils correlates with the capacity of NT to affect the intracellular compartmentalization of Ca^{2+}. Peripheral NT-elicited phenomena such as vasodilation, enhanced vascular permeability, mast cell degranulation and the enhancement of directional migration and phagocytosis of neutrophils described here, classify NT as a typical mediator of inflammation.

Neutrophil *Neurotensin* *Chemotaxis* *Phagocytosis* *Ca^{2+} extrusion* *Inflammation*

1. INTRODUCTION

The tridecapeptide neurotensin (NT), pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu, was first isolated from bovine hypothalami and subsequently from a variety of other tissues and species [1,2]. In the central nervous system NT participates in the regulation of a variety of physiological and behavioral processes [3,4]. Its peripheral activities include hypotension, inhibition of gastric acid secretion, stimulation of ileum contraction, vasodilation, increase in vascular permeability and mast cell degranulation [1,3]. The three latter phenomena are early components of an inflammatory response, and are followed by the recruitment of granulocytes and macrophages into the site of inflammation. NT bound specifically to murine macrophages and augments their phagocytic capability [5,6]. Here, we explore the interaction of NT with human blood neutrophils concentrating on two functions, directional migration and phagocytosis. Since a displacement of intracellular bound Ca^{2+} into the cytosol is thought to be involved in the chemoattractant-activated oriented locomotion of neutrophils [7] and tuftsin-mediated augmentation of phagocytosis [8]; we have also studied the effect of NT binding on the compartmentalization of intracellular Ca^{2+}.

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Abbreviations: NT, neurotensin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TBS, Tris-buffered saline

2. MATERIALS AND METHODS

Neutrophils were isolated from either whole blood or buffy coats of healthy donors essentially as in [9]. When used for phagocytic assays, cells were suspended in Dulbecco's modified Eagle's medium (Gibco) supplemented with 15 mM HEPES buffer (pH 7.2) (DMEM), plated onto glass coverslips (10^6 cells in 0.2 ml DMEM) and incubated for 90 min at 37°C. Neutrophils used for chemotaxis and Ca^{2+} release studies were resuspended after hypotonic lysis of residual erythrocytes [9] in either phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 8.1 mM Na_2HPO_4, 1.5

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mM KH_2PO_4 (pH 7.4) (PBS) or in Tris-buffered saline containing 122 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 20 mM Tris–HCl, pH 7.4 (TBS), respectively. Cells were >98% viable as assessed by trypan blue dye exclusion.

Neutrophil directional migration was assayed by a modified method for chemotaxis under agarose [10,11]. Glass microscope slides (Corning disposable) were pretreated with KOH/methanol, followed by 2 N HNO_3, and finally coated with gelatin (Biolife) [10]. Melted agarose (Type II, Sigma, 2% in distilled water) was mixed at about 50°C with an equal volume of prewarmed 2 × TC medium 199 (Difco), adjusted to pH 7–7.2 (37°C) with solid $NaHCO_3$, and containing 0.5% gelatin. The agarose solution (3.5 ml/slide) was then layered over the glass microscope slides and allowed to solidify for about 1 h in the cold. Six series of 3 wells, 2.5 mm in diameter and spaced 1.5 mm apart were cut in each plate using a stainless steel punch.

NT and the appropriate buffer control (10 μl) were placed in the outer wells and the slides were incubated at 37°C for 15 min in a humidified atmosphere containing 5% CO_2 in air. Neutrophils (3×10^5 in 10 μl) were then added to the central well and the incubation continued for 120 min. Cells were fixed (2% glutaraldehyde in PBS), stained (May-Grünwald-Giemsa) and examined by light microscopy. A calibrated ocular micrometer was used to measure the distance migrated by three leading cells aligned in the same plane and parallel to the margin of the well [10,11]. Directional migrations were evaluated as chemotactic differences [10,11]. In some experiments the number of migrated leukocytes was estimated by counting the cells present within the frame of a photofinder ocular in 3 adjacent strips, extending from the edge of the central well to the further point of migration.

For the chemotaxis assays, 10^{-4} M solutions of NT (Vega or Serva) were diluted in TC medium 199, containing 0.25% gelatin and 10% heat-inactivated human blood serum (56°C, 30 min). The complement system of fresh human serum was activated with baker's yeast (20 mg/ml, 30 min at 37°C) [12].

The phagocytic capability of neutrophils was assessed using starch particles (Starch, rice, BDH). Leukocyte monolayers were washed in PBS and incubated with 1.5×10^7 starch particles suspended in DMEM (1 ml) containing 2 mg/ml of fatty acid-free bovine serum albumin (Sigma) in the presence or absence of the specified concentrations of NT for 30 min at 37°C. Monolayers were washed with PBS, fixed (2% glutaraldehyde in PBS) and stained (Giemsa). Ingested starch particles in 400–1000 cells in triplicate cultures were enumerated. Within each experiment the standard error of the means (SEM) was in the range 5–15%.

Ca^{2+} net fluxes were followed by measuring changes in extracellular Ca^{2+} concentrations with the help of a very sensitive Ca^{2+}-selective electrode/amplifier/recorder system [13–15]. The assays were carried out in a thermostated (37°C) vessel containing 3 ml of TBS with 5 mM glucose, under continuous stirring. Upon equilibration of the electrode system, an aliquot (0.2–0.5 ml) of the medium was withdrawn and replaced by a suspension of 5×10^7 neutrophils. Changes in the extracellular [Ca^{2+}] were quantified by calibration with EGTA at the end of each experiment [13–15].

3. RESULTS

Exposure of human blood neutrophils to a chemical gradient of NT causes activation of their locomotion (table 1). Directional migration occurs over 10^{-10}–10^{-5} M [initial peptide], with optimal cell response at 10^{-7} M NT.

With NT as attractant, neutrophils migrate lower distances than with 10% yeast-activated serum (table 1). Furthermore, as judged from light microscopoy observations and counts of migrating cells, a lower number of neutrophils respond to NT than to activated serum (not shown).

The interaction of NT with human neutrophils also leads to an augmentation of their phagocytic capability (table 1). This effect is already seen at 10^{-11} M NT and plateaus at 10^{-9} M NT, to further increase at 10^{-7} M peptide. Some variability exists in the response to NT of different cell preparations. This very likely reflects the variability in basal phagocytic capability and NT-sensitivity of individual donors. However, the NT-mediated enhancement of phagocytosis is statistically significant, at least over 10^{-10}–10^{-7} M peptide.

When neutrophils are directly exposed to 10^{-11}–10^{-8} M NT they extrude Ca^{2+}, as indicated

Table 1

Activation by NT of directional migration and phagocytic capability of human neutrophils

	Directional migration (μm)	Ingested particles/ 100 cells	P
Control cells	4 ± 6	48 ± 11	–
+ 10^{-11} M NT	–	59 ± 18	<0.25
+ 10^{-10} M NT	52 ± 8	68 ± 12	<0.001
+ 10^{-9} M NT	94 ± 13	73 ± 20	<0.10
+ 10^{-8} M NT	115 ± 17	72 ± 17	<0.10
+ 10^{-7} M NT	148 ± 16	90 ± 14	<0.05
+ 10^{-6} M NT	139 ± 23	–	
+ 10^{-5} M NT	126 ± 16	–	
+ 10% YAS	406 ± 96	–	

Data are means of values obtained with 4 (migration) or 5 (phagocytosis) individual neutrophil preparations ± SEM. Directional migration (120 min at 37°C) was assayed in triplicate by the chemotaxis under agarose method [10,11]. Phagocytosis was assayed by enumerating ingested (30 min at 37°C) starch particles in 400–1000 neutrophils. YAS: yeast-activated serum. The significance of the differences between means (particles ingested by treated *vs* control cells) was estimated by the Student's *t*-test for paired data

by an increase in extracellular cation concentrations. Unlike the rapid Ca^{2+} release elicited by the ionophore A23187, the extrusion of the cation from neutrophil interacting with NT proceeds rather slowly (fig.1), attaining steady-state after several minutes. Dose–response curves, derived from experiments carried out with different

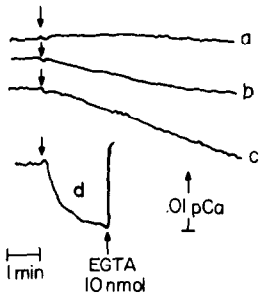

Fig.1. Kinetics of Ca^{2+} extrusion from neutrophils exposed to 3.3×10^{-9} M (b) or 6.6×10^{-10} M (c) neurotensin. Control experiments were carried out by adding buffer (a) or 2×10^{-6} M ionophore A23187 (d) to the neutrophil suspension (5×10^{7} cells/3 ml).

leukocyte preparations, indicate that maximal Ca^{2+} extrusion (0.6–1 nmol.min^{-1}.5×10^{7} cells^{-1}) occurs at about 10^{-10} M NT, with decreasing rates of cation release at higher peptide concentrations (not shown).

4. DISCUSSION

These data show that the interaction of NT with human neutrophils cause an activation of two typical functions of these cells, locomotion and phagocytosis.

NT activates neutrophil oriented migration over 10^{-10}–10^{-5} M, with an optimal cell response at 10^{-7} M peptide. The decreased response to higher NT concentrations might be ascribed to a process of cell desensitization, which is characteristic of 'down-regulation' of chemoattractant receptors [16].

In the method we have used to evaluate the neutrophil migration, the attractant molecules diffuse from wells, where they are placed at suitable initial concentrations, into the agarose gel and generate a chemical gradient. The actual NT concentrations sensed by neutrophils are thus much lower than those indicated above, with an ED_{50} very likely close to that of other peripheral activities of the peptide [1,3].

Another neuropeptide, substance P, has also been reported to be chemotactic for neutrophils [17]. However, the high ED_{50} for the chemotactic activity of substance P makes it a rather unlikely candidate for a chemotaxin of physiological significance.

When compared to the cleavage product of the fifth component of the complement system, which is the major chemoattractant present in yeast-activated serum [12,16,18], NT appears to activate a lower number of cells for enhanced migration. However, this is not surprising, because different percentages of cells migrate to different optimal concentrations of chemoattractants [19]. For example, 30–50% of neutrophils respond to C5a, but only 25–35% to leukocyte-derived chemotactic factor.

Stimulation by NT of phagocytosis of starch particles by neutrophils also occurs in a dose-dependent fashion, being detectable at 10^{-11} M and reaching a plateau level at 10^{-9} M peptide. It thus appears that this neuropeptide is effective on

neutrophils at concentrations lower than those reported for substance P ([20]; unpublished).

The additional stimulation of phagocytosis observed at 10^{-7} M NT might be ascribed to either the existence of two subpopulations of neutrophils with a different affinity for NT or the coexistence of high and low affinity sites for NT on the neutrophil surface. It is interesting that murine macrophages have been shown to have two classes of NT binding sites, one with a $K_d \sim 10^{-9}$ M and another one with a $K_d \sim 0.3 \times 10^{-7}$ M [5,6].

A wide spectrum of plasma membrane and intracellular events are triggered in leukocytes by chemoattractants as well as by phagocytosis-modulating agents. One such event is an increase in the intracellular concentration of free Ca^{2+} [7,8,18,21,22]. A rise in cytosolic Ca^{2+} can activate several Ca^{2+}-dependent proteins, whose functions are connected with cell migration and phagocytosis, as well as the plasmalemmal Ca^{2+} extrusion system [14,15]. Thus, the extent of a shift of Ca^{2+} from membrane sites to cytosol can be indirectly evaluated from the rate and the amount of cation extruded from the cell.

Our investigations have shown that NT activates Ca^{2+} extrusion from human neutrophils with maximal cation release at about 10^{-10} M peptide concentration. As discussed above, at this concentration NT provides an optimal stimulation of phagocytosis and very likely also of locomotion. However, at $>10^{-10}$ M, NT elicits a lower extrusion of Ca^{2+}. Although this might be ascribed to a decreased intracellular mobilization of the cation, the possibility exists that NT inhibits the process of active Ca^{2+} release from the cells by some yet unknown mechanism.

Ca^{2+} extrusion induced by optimal concentrations of NT proceeds much more slowly than that elicited by other neutrophil activators such as the ionophore A23187 and a phorbol diester [14,15]. This suggests that in the former case displacement of Ca^{2+} from intracellular stores does not lead to cytosolic concentrations of the cation, which maximally activate the extrusion system [14,15]. Such a conclusion is consistent with the general concepts [21,23] that:

(i) The elevation in Ca^{2+} steady-state levels in the cytosol of neutrophils interacting with chemotactic peptides is modest and very likely persists for several minutes;

(ii) A rise of Ca^{2+} concentration above the threshold levels, such as that promoted by the ionophore A23187 and the phorbol diester [14,15], does not lead to activation of leukocyte locomotion and may even inhibit it [24].

We have found that NT activates the locomotion and augments the phagocytic capability of neutrophils at hormone-like concentrations, and that these effects are likely correlated with Ca^{2+} displacement from intracellular stores. Since NT has vasodilatory properties, enhances vascular permeability, stimulates the release of histamine from mast cells [1,3], and modulates macrophage [5,6] and neutrophil functions, it may well be considered a typical mediator of inflammation.

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