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Activation of human neutrophils by tachykinins: effect on formyl-methionyl-leucyl-phenylalanine- and platelet-activating factor-stimulated superoxide anion production and antibody-dependent cell-mediated cytotoxicity

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SUMMARY

This study examines the contribution of tachykinins to the processes of inflammation. Neurokinin A (NKA), neurokinin B (NKB) and eledoisin (E) but not kassinin (K) have similar effects to substance P (SP) in priming neutrophils for increased superoxide anion (O_2^-) production in response to formyl-methionyl-leucyl-phenylalanine (FMLP). This similarity in activity may be due to the carboxy amino acid terminal end of these tachykinins being highly conserved. This was confirmed by demonstrating that SP fragment 7–11 (SP_{7-11}) had the same priming effect as the whole molecule, whereas, the amino end fragment 1–4 (SP_{1-4}) inhibited the response to FMLP. The priming effect of tachykinins was not confined to a single stimulus, such as FMLP, since NKA, NKB and SP also enhanced O_2^- production stimulated by platelet-activating factor (PAF), an important mediator of inflammation but a weak stimulus of O_2^- production on its own. In addition, all the tachykinins studied increased neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC) towards opsonized target cells. In contrast to their effects on FMLP-induced O_2^- production, both SP fragments, SP_{1-4} and SP_{7-11}, stimulated neutrophil ADCC and had a synergistic effect when used together.

INTRODUCTION

The tachykinins are a family of neuropeptides which are putative neurotransmitters in the central and peripheral nervous systems. They are defined by a common C-terminal amino acid sequence of phe-X-gly-leu-met-NH_2, where X is an aliphatic or aromatic amino acid, and an ability to cause rapid contraction of a variety of smooth muscle types. Three mammalian tachykinins have been described to date; substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). In addition, there are amino terminal (neuropeptide K and neuropeptide γ) and truncated (NKA_{3-10}) derivatives of NKA.[1] A number of non-mammalian tachykinins have also been isolated including eledoisin (E) and kassinin (K). Kassinin is the homologue of NKA as both have identical C-terminal pentapeptide sequences.

Substance P, the first of these peptides described,[2] has a variety of physiological actions such as smooth muscle contraction, vascular dilatation and plasma extravasation,[3] and has been implicated as a mediator of neurogenic inflammation. This conclusion is supported by a number of studies which have shown that SP has stimulatory effects on a variety of immune and inflammatory cells including lymphocytes,[4–6] macrophages and monocytes,[7–11] mast cells[12–14] and neutrophils.[15–22] Previously, we have shown that SP primes human neutrophils to produce increased amounts of superoxide anion (O_2^-) and leukotriene B_4 in response to the synthetic tripeptide formyl-methionyl-leucyl-phenylalanine (FMLP) and stimulates neutrophil antibody-dependent cell-mediated cytotoxicity (ADCC).[20] Information on the effects of the other tachykinins on inflammatory cells is limited,[22,23] and their potential role in neurogenic inflammation is largely unknown. Since several of these tachykinins colocalize in sensory afferents, it is possible that some of the actions ascribed to SP from *in vivo* studies may in fact be due to other peptides. Furthermore, there are several enzymes which metabolize SP to smaller fragments,[24] whose effects on neutrophils may be either stimulatory or inhibitory.[17]

To test the hypothesis that tachykinins other than SP, as well as SP metabolic fragments, may regulate human neutrophil function, we studied their effects on neutrophil O_2^- production and ADCC. We report that mammalian tachykinins, NKA and NKB as well as SP, directly stimulate human neutrophil ADCC or prime neutrophils for an enhanced response to a second stimulus such as FMLP or platelet-activating factor (PAF). We

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; E, eledoisin; FMLP, formyl-methionyl-leucyl-phenylalanine; K, kassinin; NKA, neurokinin A; NKB, neurokinin B; PAF, platelet-activating factor; SP, substance P; O_2^-, superoxide anion.

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also show that SP metabolic fragments have diverse effects and can either prime, activate or inhibit neutrophils depending on the cell function and fragment tested.

MATERIALS AND METHODS

Peptides

All peptides were purchased from AUSPEP (Melbourne, Australia). Stock solutions of 1 mM were made up in 10 mM acetic acid and aliquots were stored under nitrogen at −70° and thawed only once just before use.

Isolation of human neutrophils

Neutrophils were isolated from the peripheral blood of normal volunteers after informed consent. A leucocyte fraction was obtained by sedimenting the erythrocytes with dextran (Dextran *T*-500; Pharmacia, Uppsala, Sweden). Neutrophils were isolated by density-gradient centrifugation (400 *g*, 20 min, 22°) using Lymphoprep (Nycomed AS, Oslo, Norway) and hypotonic lysis of residual erythrocytes. The cells were always >96% pure, as determined by Grunwald–Giemsa staining, and >98% viable by trypan blue exclusion. Neutrophils were resuspended either in RPMI-1640 medium supplemented with 20 mM HEPES (Gibco, Grand Island, NY), 2 mM L-glutamine, 60 μg/ml penicillin and 8 μg/ml gentamycin for the ADCC assay, or in a modified Dulbecco's phosphate-buffered saline (DPBS) containing 138 mM NaCl, 2·7 mM KCl, 16·2 mM Na_2HPO_4, 1·4 mM KH_2PO_4, 0·5 mM $MgSO_4$, 0·6 mM $CaCl_2$ and 7·5 mM glucose, pH 7·3, for O_2^- measurement.

Superoxide anion production

Generation of O_2^- was assessed as superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*. This was determined by the addition of 10 μl of 2 mg/ml SOD (Boehringer Mannheim, Mannheim, Germany) to duplicate samples in all experiments. Neutrophils (10^6) were incubated in triplicate with 100 μM cytochrome *c* (Calbiochem, La Jolla, CA) in medium containing the varying concentrations of peptides (0·01 μM to 100 μM) in a final volume of 1·0 ml. Since the peptides were dissolved in acetic acid, our medium control included the highest possible concentration (1 mM) of acetic acid. This did not have any effect on FMLP-stimulated O_2^- production. Cells were incubated for 30 min at 37° and then either FMLP (0·1 μM final concentration), PAF (1 mM final) or medium was added and the mixture incubated for a further 6 min. The reaction was stopped by addition of SOD (10 μl of 2 mg/ml) and by placing tubes on ice. The cells were pelleted by centrifugation at 4° and O_2^- production was quantified in cell supernatants by changes in absorption at 550 nm.[25]

ADCC

The method has been described in detail previously.[26] All experiments were performed in triplicate, and SD within an experiment were <10%. The assay was performed in RPMI-1640 containing 0·1% bovine serum albumin (BSA). Briefly, 40 μl (4×10^3) of ^{51}Cr-labelled trinitrophenyl (TNP)-coupled P815 target cells (DBA/2 mastocytoma) were mixed with 80 μl ($1{\cdot}2 \times 10^5$) neutrophils as effector cells, and 25 μl of rabbit IgG anti-TNP antibody (Miles-Yeda, Rehovot, Israel) and 16 μl of the appropriate peptide in V-bottomed microtitre plates. The final assay volume was 160 μl. After incubation of the reaction mixture for 2·5 hr at 37°, 80 μl of the supernatant was removed and the radioactivity counted using a gamma-counter (LKB, 1282 Commugamma, Turku, Finland). Percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental c.p.m.} - \text{spontaneous release c.p.m.}}{\text{total c.p.m.} - \text{spontaneous release c.p.m.}}$$

where spontaneous release was the ^{51}Cr released from P815 cells in the presence of medium alone and the total count was the ^{51}Cr released from the P815 cells lysed by the addition of 4% Triton X-100. Conditioned medium (CM) from the bladder carcinoma cell line U5637 was used as a positive control, since it contains factors which maximize human neutrophil ADCC.[20]

Tachykinins (at the highest concentrations used) did not affect neutrophil viability under the different experimental conditions as demonstrated by a negative effect on trypan blue exclusion. Furthermore, all tachykinins were shown to be endotoxin free by the *Limulus* amoebocyte lysate assay.

Statistics

Data were analysed by Student's *t*-test for paired data and ANOVA, using a Macintosh computer and the STATVIEW II™ statistics package. Experiments were performed in triplicate, and the number of experiments is indicated in the figure legends. Results are expressed as means ± SEM.

RESULTS

The effect of tachykinins on FMLP-stimulated superoxide anion production

As previously reported by us,[20] SP had no direct effect on neutrophil O_2^- production alone, but primed for an increased O_2^- production in response to FMLP. To determine, whether other tachykinins could influence FMLP-stimulated O_2^- production, neutrophils were incubated with each tachykinin for 30 min, and then stimulated with 0·1 μM FMLP. None of the tachykinins (0·01–100 μM) studied stimulated significant O_2^- production on its own (data not shown). Preincubation of neutrophils with either SP, NKA, NKB or E enhanced the response to FMLP in a dose-dependent manner (Fig. 1). A maximum effect was observed at 100 μM for SP, NKA and E, when production increased from 12·7 ± 3·1 to 28·8 ± 5·1 ($P < 0{\cdot}01$), 23·3 ± 3·7 ($P < 0{\cdot}002$) and 21·1 ± 3·6 ($P < 0{\cdot}02$) nmol/10^6 cells respectively. Kassinin did not have any effect on neutrophil FMLP-stimulated O_2^- production (Fig. 1a). Although NKB significantly enhanced the response to FMLP at lower concentrations than the other tachykinins, the effect was reduced at 100 μM (Fig. 1b). For example 5 μM NKB increased O_2^- production from 15·3 ± 2·1 to 27·9 ± 1·7 nmol/10^6 cells ($P < 0{\cdot}001$), had its maximum effect at 50 μM (36·8 ± 5·1 $P < 0{\cdot}02$), and a reduced effect at 100 μM (20·5 ± 4·2 $P < 0{\cdot}04$).

The effect of SP fragments on FMLP-stimulated superoxide anion production

The activities of SP_{1-4} and SP_{7-11} on FMLP-induced neutrophil O_2^- production are compared in Fig. 2. SP and SP_{7-11} had similar effects in that they primed for an enhanced response in a dose-dependent fashion. For example, SP and SP_{7-11} increased O_2^-

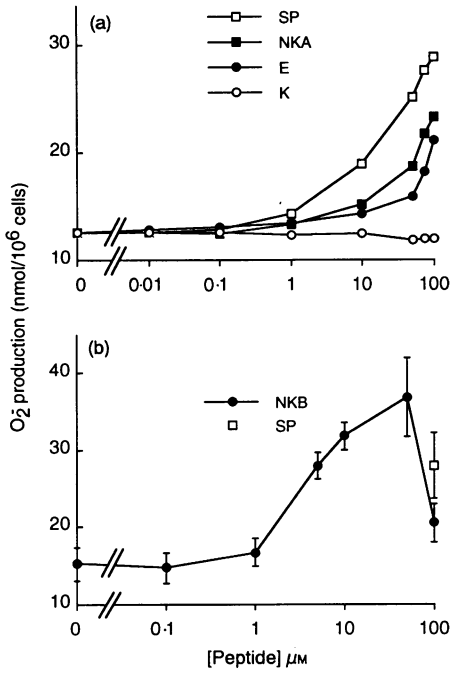

Figure 1. Effect of tachykinins (a) and NKB (b) on FMLP-stimulated O_2^- production. Neutrophils were preincubated with medium or tachykinins for 30 min at 37° and were then stimulated with 0·1 μM FMLP for an additional 6 min. Values represent means of five experiments in (a) and three experiments in (b). SEM are not shown in (a) for better graph clarity.

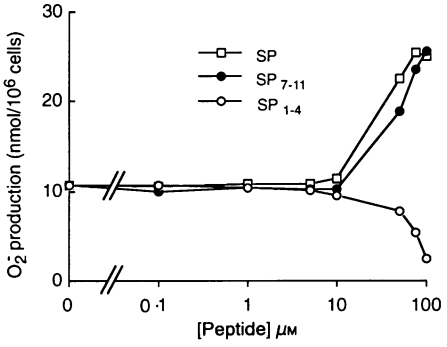

Figure 2. Effect of substance P fragments on FMLP-stimulated O_2^- production. As in Fig. 1, cells were preincubated with SP_{1-4}, SP_{7-11} or SP for 30 min before FMLP stimulation. Values represent means of three experiments.

production maximally from 10·7±1·6 to 25·0±4·7 ($P<0·03$) and from 25·6±5·2 ($P<0·03$) nmol/10^6 cells respectively. In contrast, SP_{1-4} at concentrations above 10 μM (Fig. 2) decreased the response by as much as 77%, to 2·5±1·2 nmol/10^6 cells ($P<0·01$).

The effect of tachykinins on PAF-stimulated superoxide anion production

As illustrated in Fig. 3, tachykinins also enhanced PAF-stimulated O_2^- production. Here, cells were incubated for 30 min

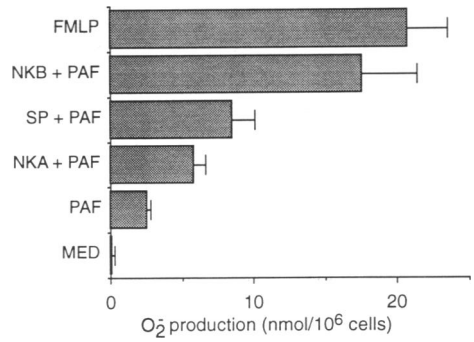

Figure 3. Effect of tachykinins on PAF-stimulated O_2^- production. Neutrophils were preincubated for 30 min with medium or 50 μM NKA, SP or NKB and then stimulated for an additional 6 min with 1 μM PAF. For comparison, results are shown for cells that were stimulated with 0·1 μM FMLP alone. Values represent means of four experiments.

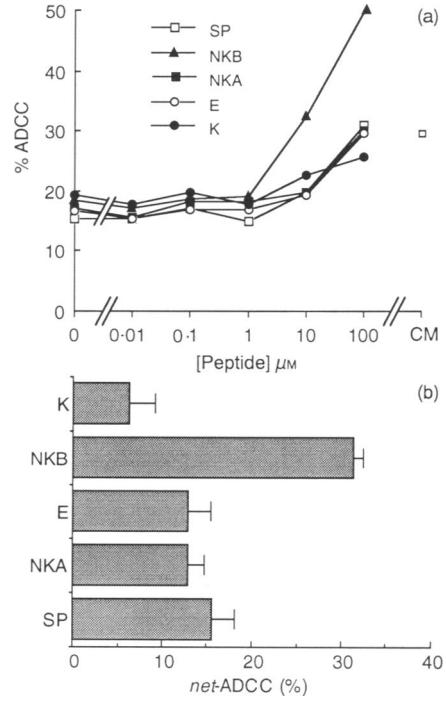

Figure 4. (a) Effect of tachykinins on neutrophil ADCC. Neutrophils were incubated for 2·5 hr with target cells, antibody and various concentrations of tachykinins. Bladder carcinoma cell line U5637 CM was used as a positive control. Data are means of three experiments. (b) Effect of tachykinins (100 μM) on *net*-ADCC (stimulated minus baseline responses). Values are derived from (a) and are means of three experiments.

with medium or 50 μM NKA, SP or NKB and then stimulated with 1 μM PAF (for 6 min). O_2^- production increased from 2·3±0·4 to 5·8±0·7 ($P<0·002$) for NKA, to 8·4±1·5 ($P<0·008$) for SP and 17·5±3·9 ($P<0·02$) nmol/10^6 cells for NKB. This priming effect of NKB was to increase O_2^- production to 766±116% of control.

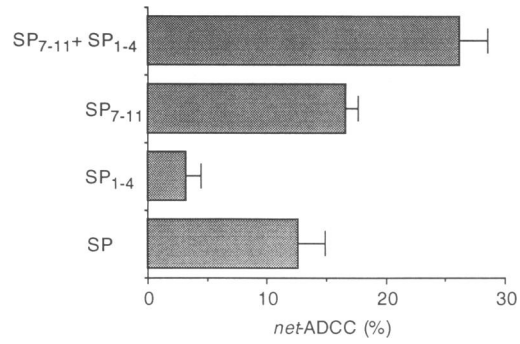

Figure 5. Effect of SP_{7-11} and SP_{1-4} on neutrophil *net*-ADCC. Cells were incubated with 100 μM SP_{7-11} or SP_{1-4} added separately or together. SP was used as a positive control. Values are means of three experiments.

The effect of tachykinins on neutrophil ADCC

We have previously reported that SP directly stimulates neutrophil ADCC in a dose-dependent fashion.[20] Here, we studied the effect of other tachykinins, using an antibody concentration of 1 μg/ml, which we found previously to be suboptimal for SP stimulation. As illustrated in Fig. 4a, in the presence of antibody alone (medium control), neutrophils killed 15–19% of target cells. Although the tachykinins had no direct cytotoxic effect on the target cells (e.g. in the absence of either neutrophils or antibody), they stimulated ADCC dose dependently within the range of 1–100 μM. We compared the maximal responses at 100 μM and the results are shown in Fig. 4b, expressed as *net*-ADCC (calculated as ADCC in presence of stimulus minus spontaneous ADCC in the presence of medium alone). NKB stimulated neutrophil *net*-ADCC was $31{\cdot}4 \pm 1{\cdot}0\%$. NKA- and E-induced *net*-ADCC was $12{\cdot}9 \pm 1{\cdot}8$ and $12{\cdot}8 \pm 2{\cdot}6\%$ respectively, which was similar to the value for SP ($15{\cdot}5 \pm 2{\cdot}5\%$). Kassinin was the weakest stimulus of all tachykinins and *net*-ADCC was only $6{\cdot}3 \pm 2{\cdot}8\%$.

The effect of SP fragments on neutrophil ADCC

In the next series of experiments, we compared the effects of SP, SP_{7-11} and SP_{1-4} on ADCC. Again we used a suboptimal concentration of antibody of 1 μg/ml. Both fragments stimulated a dose-dependent increase in ADCC in the range of 1–100 μM, and exerted their maximal effect at 100 μM (data not shown). As shown in Fig. 5, *net*-ADCC with 100 μM SP was $12{\cdot}7 \pm 2{\cdot}2\%$. *Net*-ADCC with 100 μM SP_{7-11} was $16{\cdot}7 \pm 1{\cdot}2\%$, which was not significantly different from that of SP. SP_{1-4} (100 μM) increased ADCC to a lesser extent (*net*-ADCC was $3{\cdot}3 \pm 1{\cdot}0\%$, $P < 0{\cdot}03$).

When cells were stimulated with both fragments simultaneously, there was a synergistic increase in *net*-ADCC in that their combined effect was greater than the sum of each fragment [e.g. *net*-ADCC was $26{\cdot}2 \pm 2{\cdot}5$ compared to $20{\cdot}0 \pm 1{\cdot}5$ ($P < 0{\cdot}04$)].

DISCUSSION

The present study provides evidence for a role of tachykinins in the modulation of different neutrophil functions. Our results extend our earlier findings[20] on the priming and direct effects of SP on neutrophil function, and demonstrate that other mammalian (e.g. NKA and NKB) and non-mammalian tachykinins (e.g. eledoisin but not kassinin) and SP fragments have similar modulating effects. For example, we demonstrated that although NKA, NKB and E had no direct effect on neutrophil O_2^- production, they did facilitate the response to FMLP. Although NKB produced its effects at slightly lower concentrations than the other tachykinins, at the highest concentration tested there was a reduction in the stimulation, which was not observed with the other tachykinins. Similarly, SP, NKA and NKB facilitated neutrophil O_2^- production in response to PAF, with NKB being the most potent. All the tachykinins tested increased neutrophil ADCC towards opsonized target cells, and again NKB was the most potent.

The stimulatory effect of NKB on neutrophil function is not in agreement with Brunelleschi *et al.*,[23] who found NKB to be inactive in priming human neutrophils for increased responses to both FMLP and PAF. There may be several reasons for this discrepancy. Firstly, in the experiments of Brunelleschi and coworkers the cells were primed with the tachykinins for only 3 min compared to our 30 min, and we have shown that longer preincubation times are required for SP[20] and other tachykinins to exert their effects (data not shown). Secondly, they dissolved NKB in DMSO rather than water or dilute acetic acid. Although DMSO does not interfere with FMLP- or PAF-stimulated O_2^- production, it is possible that low concentrations of DSMO (0·1%) inhibit the priming activity of NKB.

We found that high concentrations of tachykinins are needed to facilitate neutrophil responses. However, neutrophils may be exposed to high concentrations of tachykinins as they migrate through inflamed innervated tissue (e.g. synovial membrane). This is supported by evidence that in rheumatoid disease, levels of synovial fluid SP as high as 0·2 μM have been measured.[27] Since synovial fluid SP is originally released from the nerve terminals within the synovial membrane, concentrations considerably higher than 0·2 μM must occur within these inflamed synovial membranes. Furthermore, the migration of neutrophils through the synovial membrane to the synovial fluid coincides with the release of neuropeptides, both of which are early events in the establishment of an inflammatory response. Similarly in asthma, airways inflammation is characterized by cellular infiltration by neutrophils and eosinophils and the SP produced locally by axonal reflexes, and also eosinophils and mast cells, may reach sufficient concentrations to affect the function of migrating neutrophils. Furthermore, the concentrations needed to demonstrate an *in vitro* effect under our assay conditions, are similar to those needed to degranulate mast cells *in vitro*.[28]

A common feature of all tachykinins is their highly conserved carboxy terminal and it has been hypothesized that the carboxy terminal determines the priming activity observed. For example, the carboxy terminal appears to be largely responsible for the stimulatory effect of SP on lymphocyte proliferation.[6] Similarly the carboxy end of SP has been found to induce chemotaxis, increase cytosolic-free Ca^{2+} concentration and oxidative metabolism in neutrophils.[16,21] In our study, SP_{7-11} (10–100 μM) but not SP_{1-4}, enhanced O_2^- production by neutrophils stimulated with 0·1 μM FMLP in the same manner as did SP_{1-11}. Similarly, Hafstrom *et al.*[17] demonstrated that the carboxy end of SP (SP_{7-11}) but not the amino end (SP_{1-4}) stimulated neutrophil oxidative metabolism (as measured by chemiluminescence) and aggregation. Thus, the priming activity

of tachykinins on neutrophils appears to be associated with the common carboxy end.

However, the inability of kassinin to prime neutrophils as well as the different potencies of the other tachykinins tested, suggests an additional role for the amino terminus in modulating this activity. This was futher confirmed by the observation that SP_{1-4} inhibited the FMLP-stimulated neutrophil O_2^- production. Similarly the finding that both SP_{7-11} and SP_{1-4} stimulated ADCC (as well as the facilitation observed with these fragments together) suggests that tachykinin activity is not solely determined by carboxy end alone.

However, it should be noted that, in contrast to the above findings, degranulation of mast cells has been shown to be determined by the amino terminus of SP (e.g. SP_{1-4} was active but not SP_{7-11}), and that deletion of two amino acids from the carboxy terminal modulated the activity of SP—reducing the activity by 30%.[12,13]

The diverse effects of SP fragments on FMLP-stimulated O_2^- production and ADCC suggest alternative mechanisms by which tachykinins may modulate inflammation. In addition, their potential effects on inflammatory cells may further be modified by cytokines and other cell agonists that are released during inflammation. Although this study and others[15,17,21] have evaluated the effects of SP fragments on neutrophil function, enzymes that are capable of cleaving the other tachykinins have been identified on neutrophils and their effects remain to be studied.

With the exception of the effect of 100 μM NKB on neutrophil O_2^- production, we observed no biphasic responses (e.g. stimulation followed by inhibition at higher concentrations) of any of the tachykinins tested. This is in contrast to the results of Hafstrom[17] who observed that concentrations of SP and SP_{7-11} greater than 10 μM inhibited the priming effect on neutrophil O_2^- production, an effect that was overcome by increasing FMLP concentration. These differences and other differences may result from the different techniques used to quantify oxy radical production. In the studies of Hafstrom, total oxy radical production rather than O_2^- production, was monitored by luminol-amplified chemiluminescence. This method is non-specific as luminol may be oxidized by many different oxy radicals including H_2O_2 and $^1O_2$ and oxidants such as hypochlorous acid.[29-31] Furthermore, the chemiluminescence assay is very sensitive to minor changes in pH and alterations in buffer components (e.g. anti-oxidants and albumin). In contrast, the assay system we used, the superoxide dismutase-inhibitable reduction of ferricytochrome *c*, is specific for O_2^-.

Since many tachykinins have been shown to colocalize in the same sensory fibres, and SP and NKA and its derivatives are products of the same gene and arise by alternative RNA splicing and post-translational modification,[1,32] it would be of great interest to study the effects of multiple peptide stimulation on neutrophil function and activity. These, we believe, would provide important new information for assessment of the role of tachykinins in inflammation.

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