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Intracellular signaling pathway of substance P-induced superoxide production in human neutrophils

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Abstract

We examined the intracellular mechanisms of substance P-induced superoxide anion (O_2^-) production in human neutrophils. Addition of substance P (30 μM) caused O_2^- production and biphasic increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (early transient and subsequent sustained components) associated with the formation of inositol 1,4,5-trisphosphate (IP_3). O_2^- and $[Ca^{2+}]_i$ were assayed by using ferricytochrome C and fura 2-AM, respectively. These responses were abolished by tachykinin NK_1 receptor antagonists, [D-Pro^9[spiro-γ-lactam],Leu^{10},Trp^{11}]physalaemin-(1–11) (GR82334) or [D-Arg^1,D-Trp^{7,9},Leu^{11}]substance P (spantide), and an intracellular Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM). Inhibition of IP_3 formation by GTP-binding protein (G-protein) inactivators such as guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) and islet-activating protein (IAP), or a phospholipase C inhibitor, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]1*H*-pyrrole-2,5-dione (U-73122), blocked the substance P-induced O_2^- production and biphasic increases in $[Ca^{2+}]_i$. An IP_3 receptor antagonist, heparin, reduced both the substance P-induced O_2^- production and the transient increase in $[Ca^{2+}]_i$ without any significant effects on the sustained increase in $[Ca^{2+}]_i$. Protein kinase C inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and calphostin C, only slightly suppressed O_2^- production, and abolished the sustained increase in $[Ca^{2+}]_i$ without any significant effects on the transient increase in $[Ca^{2+}]_i$. A Ca^{2+} entry blocker, nicardipine, completely inhibited the sustained increase in $[Ca^{2+}]_i$ without affecting O_2^- production and the transient increase in $[Ca^{2+}]_i$. These results suggest that the tachykinin NK_1 receptor/G-protein-linked IP_3 formation with the resulting IP_3-induced transient increase in $[Ca^{2+}]_i$ is the main signal transduction pathway for substance P-stimulated O_2^- production in neutrophils.

Keywords: Substance P; Neutrophil; Superoxide production; Ca^{2+}, intracellular; Phosphoinositide

1. Introduction

Neuropeptide-mediated regulation of the immune system including inflammatory reactions has attracted attention in the last few years. The inflamed tissues contain elevated levels of neuropeptides such as tachykinin, calcitonin gene-related peptide (CGRP) and neuropeptide Y, each of which influences various patterns of inflammatory responses including vasodilation, increased vascular permeability and regional activation of immunological cells (Larsson et al., 1989; McGillis et al., 1990; Menkes et al., 1993).

Substance P is a biologically active decapeptide, and is released from the sensory C-fiber endings by noxious stimuli. This neuropeptide induces the release of histamine and prostaglandin E_2 from macrophages and synovial cells (Kimball, 1990), and has been found in the joint fluids of rheumatoid arthritis patients (Lotz et al., 1987) and in the skin tissue of patients suffering from urticaria (Brain and Williams, 1988). Thus, substance P appears to be a key molecule in the pathogenesis of neuronal inflammatory responses.

Recently, Serra et al. and our preliminary study have shown that substance P stimulates superoxide production and increases intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in human neutrophils (Serra et al., 1988; Tanabe et al., 1993). However, signal transduction pathways of the substance P-induced O_2^- production are still unclear.

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In a variety of cell types, substance P induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) through tachykinin NK_1 receptor stimulation (Watson and Downes, 1983). The degradative products of PIP_2, IP_3 and diacylglycerol, have been shown to be the second messengers that produce the Ca^{2+}-requiring cellular responses (Berridge, 1984; Abdel-Latif, 1986). In addition, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyzes single electron reduction of O_2 to O_2^- using NADPH as an electron donor, is activated in a Ca^{2+}-dependent manner (Tauber, 1987).

Based on these data, we examined the intracellular mechanisms of the substance P-induced O_2^- production in human neutrophils, especially with respect to the linkage of phosphoinositide-mediated changes in $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Materials

Substance P, ferricytochrome C (Type 3), superoxide dismutase, heparin, GDPβS, islet-activating protein (IAP), 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethyoxtbenzoate (TMB-8), calphostin C, nicardipine were obtained from Sigma Chemical Co., St. Louis, MO, USA. GR82334: [D-Pro^9[spiro-γ-lactam],Leu^{10},Trp^{11}]physalaemin-(1–11), spantide: [D-Arg^1,D-Trp^{7,9},Leu^{11}]substance P, MEN10207: [Trp^5,D-Trp^{6,8,9},Arg^{10}]neurokinin A-(4–10) and [Trp^7,β-Ala^8]neurokinin A-(4–10) were purchased from Peninsula Laboratories, Belmont, CA, USA. H-7: 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, was obtained from Seikagaku Kogyo, Tokyo, Japan. U-73122: 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]1*H*-pyrrole-2,5-dione, and U-73343: 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione, were obtained from Upjohn Co., Kalamazoo, MI, USA. Fura 2-AM was obtained from Dojindo Laboratories Co., Kumamoto, Japan. Calphostin C, U-73122 and U-73343 were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10^{-3} M and diluted with Hank's balanced salt solution (HBSS, pH 7.4). The final concentration of DMSO was less than 0.1%.

2.2. Preparation and stimulation of human neutrophils

Neutrophils were isolated from heparinized venous blood of seven healthy donors by dextran sedimentation and centrifugation on a Ficoll-Hypaque gradient (Serra et al., 1988). Neutrophils thus obtained were washed 3 times in Hank's balanced salt solution (HBSS, pH 7.4) containing 136.8 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 5.5 mM glucose and 20 mM Hepes. Contaminating erythrocytes were disrupted by hypotonic lysis for 40 s with 5 ml ice-cold distilled water, and the same volume of 2-fold concentrated HBSS was then added to restore isotonicity. After centrifugation, the resulting pellet was suspended in HBSS (pH 7.4) containing 2 mM NaN_3, an inhibitor of superoxide dismutase, and was used as a purified neutrophil preparation (96% purity). The viability of neutrophils after isolation was checked by trypan-blue exclusion and was found to be greater than 98%.

The cells (10^6 cells/ml) were allowed to equilibrate for 10 min at 37°C in the presence of the three kinds of protease inhibitors, 20 μg/ml leupeptin, 0.5 mM phenylmethyl-sulphonyl floride and 200 units/ml aprotinin. Several kinds of inhibitors to be examined were added to the buffer 10 min before the addition of substance P.

The islet-activating protein (IAP) treatment was carried out by incubating the neutrophils with 10 μg/ml IAP for 90 min at 37°C to obtain ADP-ribosylation of G-protein (Lad et al., 1985). The cells were then washed and resuspended in HBSS.

2.3. Determination of O_2^- production

O_2^- production was assayed spectrophotometrically by recording the reduction of ferricytochrome C (Type 3, Sigma) using a double-beam spectrophotometer (Type 557, Hitachi Co., Japan), equipped with a thermostatted cuvette holder, as described by Cohen and Chovaniec (1978). An aliquot (100 μl) of the cell suspension (final concentration of 10^6 cells/ml) was added to a cuvette with 1 ml HBSS containing 60 μM ferricytochrome C, 1 mM KCN (an inhibitor of superoxide dismutase), and the protease inhibitors, and the cuvette was put in a cuvette holder (37°C) of the spectrophotometer. The changes in the ferricytochrome C reduction following the administration of substance P was measured at 550 nm with a reference wavelength at 540 nm, and the difference between these absorbances (absorbance at 550 nm minus that at 540 nm) was continuously monitored with a pen-writing recorder. The amount of O_2^- produced by substance P was calculated by subtracting the amount of reduced cytochrome C in the resting state from that obtained 5 min after the administration of substance P, using an extinction coefficient of 21.1/mM/cm (Cohen and Chovaniec, 1978).

2.4. Measurement of changes in $[Ca^{2+}]_i$

The neutrophils were suspended in HBSS, and loaded for 20 min at 37°C with 3 μM fura 2-AM (Dojin Co., Japan). The cells were centrifuged ($100 \times g$, 8 min) twice, and resuspended in HBSS. Fura 2 fluorescence was measured at 37°C using a spectrofluorometer (Type F-2000, Hitachi Co., Japan) equipped with a thermostatted cuvette holder and a stirring apparatus at dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) were determined using the equation: $[Ca^{2+}]_i = K_d \times \beta(R - R_{min})/(R_{max} - R)$, here R_{max} represents the maximam

ratio of both fluorescences ($F340/F380$) after the lysis of cells with 25 μM digitonin, and R_{min} represents the minimum ratio of both fluorescences after the addition of 12.5 mM EGTA, β represents the 'scaling factor' defined as the ratio of fluorescence at 380 nm with 0 Ca^{2+} ($F380_{min}$) to saturating Ca^{2+} ($F380_{max}$) conditions, and K_d, the dissociation constant for fura 2-Ca^{2+}, is 224 nM (Borzak et al., 1990).

2.5. Measurement of IP_3

The neutrophils suspended in 2 ml of HBSS medium at a concentration of 2×10^7 cells/ml were pretreated with 10 mM LiCl, an inhibitor of inositol phosphatase, and with HBSS (vehicle) or several reagents for 10 min, and then exposed to 30 μM substance P. At 30 s after the addition of substance P, the reaction was terminated by addition of the same volume of ice-cold 15% (w/v) trichloroacetic acid, and cell debris were removed by centrifugation. The resulting supernatant was extracted 3 times with diethylether, neutralized with $NaHCO_3$ to pH 7.5. IP_3 levels in the neutralized aqueous samples were measured using an IP_3 radioreceptor assay kit obtained from Amersham International, Buckinghamshire, UK.

2.6. Permeabilization of neutrophils

For the study of the effects of membrane impermeable agents such as GDPβS and heparin on the substance P-induced responses, the neutrophils were permeabilized with saponin. The cells were first exposed to permeabilization buffer which contained 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM Hepes-K (pH 7.0), 1 mM ATP, 10 mM glucose, 2 mM NADPH, $CaCl_2$ to achieve pCa 8 (Grinstein and Furuya, 1991), and 50 μg/ml saponin for 10 min (Smith and Snyderman, 1987), and then were treated with impermeable agents or vehicle for 5 min at 37°C. After washout with the same buffer without saponin, the cells were resuspended in the normal HBSS medium and stimulated with substance P.

2.7. Statistical analysis

Student's t-test was used for statistical analyses. The differences between means giving P values of less than 0.05 were considered to be significant. The data in Fig. 2 were analyzed using Bonferroni's test.

3. Results

3.1. O_2^- production by substance P

Human neutrophils treated with 30 μM substance P produced O_2^- which was measured by ferricytochrome C reduction (Fig. 1A). This ferricytochrome C reduction was

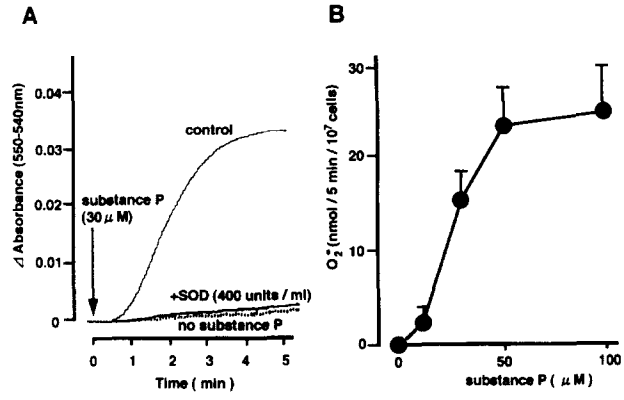

Fig. 1. (A) Representative time course of O_2^- production in human neutrophils stimulated with 30 μM substance P. O_2^- production was monitored spectrophotometrically as described in Methods. The increases in Δabsorbance induced by the peptide completely disappeared on pretreatment with superoxide dismutase (SOD; 400 units/ml). The dotted line shows the basal trace in the absence of substance P. (B) The concentration-response curve for O_2^- production in neutrophils stimulated with substance P. The human neutrophils were treated with a single addition of various concentrations of substance P (●). The production of O_2^- was calculated from the amounts of cytochrome C reduced over 5 min after the addition of the peptide. Each symbol represents the mean ± S.E.M. for 6–12 preparations.

abolished in the presence of superoxide dismutase (400 units/ml), showing that this reduction is an indicator of O_2^- production. After the stimulation of neutrophils, O_2^- was produced rapidly during the first 3 min and then slowly, reaching a plateau within 5 min. Therefore, in subsequent experiments, the amount of O_2^- released during the first 5 min was determined. Fig. 1B shows the effects of different concentrations of substance P on O_2^- production. Substance P at concentrations over 10 μM stimulated O_2^- production in a concentration-dependent manner, with the maximum effect at 50 μM, as has been shown in previous reports (Serra et al., 1988; Tanabe et al., 1993).

3.2. Effects of tachykinin NK receptor antagonists and intracellular specific Ca^{2+} chelator on the substance P-induced O_2^- production

Since substance P elicits various biological effects by stimulating three tachykinin NK_1, NK_2, and NK_3 receptors (Gether et al., 1993), we examined the effects of specific antagonists for these receptors on the substance P-induced O_2^- production. As shown in Fig. 2, tachykinin NK_1 receptor antagonists, GR82334 and spantide, dose dependently inhibited substance P (30 μM)-induced O_2^- production with apparent IC_{50} values at 90 and 250 nM, respectively. However, neither a NK_2 receptor antagonist, [Tyr^5,D-$Trp^{6,8,9}$,Arg^{10}]neurokinin A-(4–10) (MEN10207), nor a NK_3 receptor antagonist, [Trp^7,β-Ala^8]neurokinin A-(4–10), influenced the response to substance P. Furthermore, pretreatment of neutrophils with an intracellular specific Ca^{2+} chelator, BAPTA-AM (10 μM) decreased

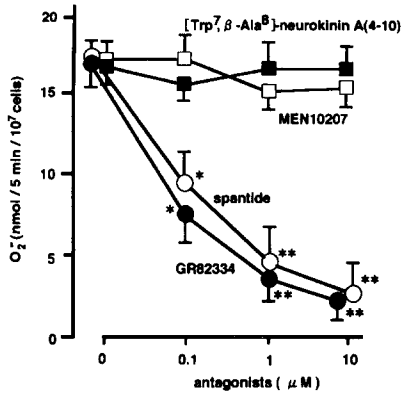

Fig. 2. Effects of tachykinin NK_1, NK_2 and NK_3 receptor antagonists on O_2^- production by neutrophils stimulated with 30 μM substance P. The human neutrophils were preincubated for 10 min with a tachykinin NK_1 receptor antagonists, 10 μM GR82334 (●) or 10 μM spantide (○), a tachykinin NK_2 receptor antagonist, 10 μM MEN10207 (□), or a tachykinin NK_3 receptor antagonist, 10 μM [Try^7,β-Ala^8]neurokinin A-(4–10) (■), then exposed to substance P. Each symbol represents the mean ± S.E.M. for six to eight preparations. * $P < 0.05$, ** $P < 0.01$ compared to the corresponding control values (Bonferroni's test).

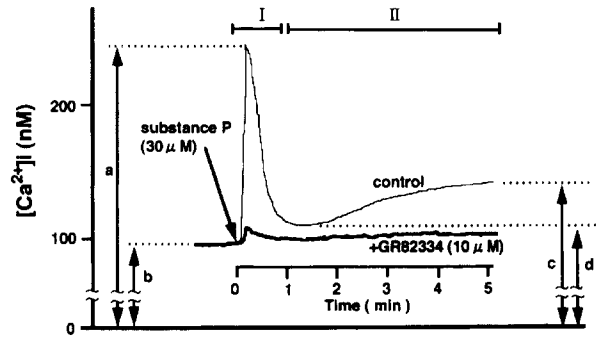

Fig. 3. Representative traces of $[Ca^{2+}]_i$ in human neutrophils stimulated with 30 μM substance P. Cells loaded with fura 2-AM were equilibrated in HBSS medium for 10 min then exposed to substance P with (thick line) or without (thin line) a tachykinin NK_1 receptor antagonist, 10 μM GR82334.

the substance P (30 μM)-induced O_2^- production from 16.0 ± 1.2 to 4.9 ± 2.8/O_2^- nmol/5 min/10^7 cells ($n = 4$, $P < 0.01$ vs. substance P alone), suggesting that substance P induces such a response via tachykinin NK_1 receptor stimulation followed by a Ca^{2+}-dependent process.

3.3. Substance P-induced changes in the intracellular Ca^{2+} level ($[Ca^{2+}]_i$)

Fig. 3 shows the typical changes in $[Ca^{2+}]_i$ in neutrophils during exposure to 30 μM substance P. The basal level of $[Ca^{2+}]_i$ was estimated to be 98.5 ± 7.8 nM (mean ± S.E., $n = 15$) in the normal HBSS solution. Addition of substance P caused an immediate and transient increase in $[Ca^{2+}]_i$, reaching a peak level (241.3 ± 12.9 nM, $n = 15$) within 40 s (termed phase I). $[Ca^{2+}]_i$ then rapidly decreased to near the basal level (108.2 ± 9.5 nM, $n = 15$) by 70–90 s after the stimulation, and again increased gradually to a plateau level (138.5 ± 9.0 nM, $n = 15$) by 5 min (termed phase II), this level being maintained for at least 10 min. A tachykinin NK_1 receptor antagonist, GR82334 (10 μM) almost completely inhibited the increases in both phase I and phase II. In the experiments following, the responses in phase I and phase II were expressed as $\Delta[Ca^{2+}]_i$ increases based on the following equations. Phase I, response (nM) = $a - b$; phase II, response (nM) = $c - d$, where b is the basal $[Ca^{2+}]_i$, a and

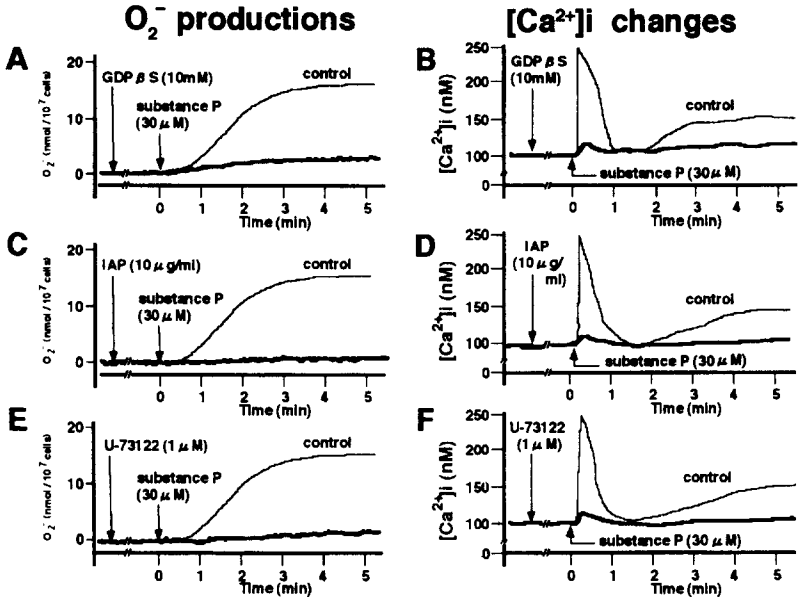

Fig. 4. Representative traces for substance P-induced O_2^- production and $[Ca^{2+}]_i$ changes in human neutrophils with or without GDPβS, islet-activating protein (IAP) and U − 73122. The human neutrophils were pretreated with 10 mM GDPβS (A,B), 1 μM U-73122 (E,F) or 10 μg/ml islet-activating protein (IAP) (C,D) for 10, 10 or 90 min, respectively, and then exposed to 30 μM substance P. GDPβS (10 mM) pretreatment was performed using saponin-permeabilized cells as described in Methods. Thick line, drug treatment; thin line, control.

c are the maximum $[Ca^{2+}]_i$ responses in phase I and phase II, respectively, and d is the $[Ca^{2+}]_i$ at the bottom point after phase I.

3.4. Effects of inhibitors of G-protein and phospholipase C on the substance P-induced O_2^- production and $[Ca^{2+}]_i$ increase

We next examined the effects of G-protein inactivators, GDPβS and islet-activating protein (IAP, pertussis toxin), and a phospholipase C inhibitor, U-73122, on substance P-induced O_2^- production and increases in $[Ca^{2+}]_i$. As shown in Fig. 4, GDPβS (10 mM), a metabolically stable analog of GDP, and IAP (10 μg/ml) abolished the substance P-induced O_2^- production (Fig. 4A,C) and biphasic $[Ca^{2+}]_i$ increase without affecting the basal $[Ca^{2+}]_i$ levels (Fig. 4B,D). GDPβS pretreatment was performed with saponin-permeabilized cells as described in Methods. After saponin permeabilization, the cells were rapidly washed and reincubated in normal HBSS solution. In such saponin-permeabilized cells, the resting $[Ca^{2+}]_i$ levels were almost the same as those in non-permeabilized cells (97.5 ± 3.9 nM and 98.5 ± 7.8 nM in saponin-treated and untreated cells, respectively, $n = 8$). Furthermore, these permeabilized cells retained the tachykinin NK receptor-coupled responsiveness, because substance P was capable of inducing O_2^- production and $[Ca^{2+}]_i$ increase to almost the same intensity as that observed in non-permeabilized cells (16.0 ± 1.2 vs. 15.8 ± 2.0 nmol/5 min/10^7 cells for O_2^-, 241.3 ± 12.9 vs. 238.9 ± 15.1 nM for phase I $[Ca^{2+}]_i$ and 138.5 ± 9.0 vs. 139.4 ± 5.5 nM for phase II $[Ca^{2+}]_i$ in saponin-treated vs. untreated cells, $n = 4–8$). In addition, substance P responses in permeabilized cells were also blocked by the tachykinin NK_1 receptor antagonist (data not shown).

U-73122 inhibits phospholipase C and prevents the degradation of PIP_2 by this enzyme (Smith et al., 1990). Pretreatment of neutrophils with 1 μM U-73122 markedly reduced the substance P-induced O_2^- production and biphasic $[Ca^{2+}]_i$ increases (Fig. 4E,F). Table 1 summarizes the average responses to substance P in the presence of these inhibitors. GDPβS (10 mM) and islet-activating protein (IAP) (10 μg/ml) reduced O_2^- production to 21.3 and 7.5% of the control, respectively, and completely abolished both components of $[Ca^{2+}]_i$ increase. Similarly, U-73122 (1 μM) caused significant decreases in substance P responses (O_2^- reduced to 15.0% of control, $[Ca^{2+}]_i$ increase reduced to 7.7 and 9.2% of control for phase I and II, respectively). U-73343, an inactive analog of U-73122, did not show any effects on the substance P-induced responses at the same concentration as U-73122, excluding the non-specific effect of U-73122. These findings suggested that the substance P-induced O_2^- production with an increase in $[Ca^{2+}]_i$ is mediated by stimulation of G-protein and phospholipase C.

3.5. IP_3 production by substance P

Exposure of neutrophils to 30 μM substance P increased the IP_3 levels from a basal level of 0.5 ± 0.1 ($n = 8$) to 3.5 ± 1.1 pmol/2 × 10^7 cells ($n = 6$) in 10 s. IP_3 formation was maximal after 30 s of stimulation (5.8 ± 0.6 pmol/2 × 10^7cells, $n = 6$), followed by a rapid decline by 50 s (3.3 ± 1.3 pmol/2 × 10^7cells, $n = 3$). Substance P in a concentration range from 1 μM to 100 μM dose dependently stimulated IP_3 production, with a half-maximal concentration at 30 μM which was close to that for superoxide production (Fig. 1B) (data not shown).

Next, we examined the effects of various agents on IP_3 formed with a 30 s stimulation (Fig. 5). Both the basal IP3 levels and substance P-induced IP_3 formation in saponin-treated cells were similar to those in normal cells (0.5 ± 0.1 vs. 0.7 ± 0.2 pmol/2 × 10^7 cells for basal IP_3 levels, and 5.8 ± 0.6 vs. 6.2 ± 0.4 pmol/2 × 10^7 cells for substance P (30 μM)-induced IP_3 formation, in saponin-untreated vs. treated cells, $n = 4–8$, $P > 0.1$). The tachykinin NK_1 receptor antagonist, GR82334 (10 μM), almost completely abolished the increase in IP_3. Both G-protein inactivators (GDPβS and IAP) and phospholipase C inhibitor (U73122) suppressed substance P (30 μM)-induced IP_3 formations by approximately 70–80% but U-73343 did not inhibit this formation. None of these inhibitors affected the basal IP_3 levels (data not shown). These findings suggest that substance P stimulates PIP_2 hydrolysis and the resulting IP_3

Table 1
Effects of G-protein inactivators and a phospholipase C inhibitor on substance P-induced O_2^- production and $[Ca^{2+}]_i$ increases in neutrophils

	O_2^-	$\Delta[Ca^{2+}]_i$ (nM)	
	(nmol/5 min/10^7 cells)	Phase I	Phase II
Substance P (30 μM) (control)	16.0 ± 1.2	142.8 ± 6.4	30.5 ± 4.7
GDPβS (10 mM) + substance P	3.4 ± 1.9 [a]	10.0 ± 2.0 [a]	3.3 ± 1.5 [a]
IAP (10 μg/ml) + substance P	1.0 ± 0.5 [a]	7.8 ± 1.0 [a]	1.4 ± 1.0 [a]
U-73122 (1 μM) + substance P	2.4 ± 0.9 [a]	11.0 ± 0.6 [a]	2.8 ± 1.2 [a]
U-73343 (1 μM) + substance P	15.4 ± 1.9	130.0 ± 9.8	30.8 ± 6.3

Each value represents the mean ± S.E.M. for 6–12 preparations. [a] $P < 0.01$ compared with substance P alone. The production of O_2^- was estimated from the amounts of cytochrome C reduced in 5 min after the addition of substance P. Changes in $[Ca^{2+}]_i$ in phase I and phase II were determined as Δincreases above the values obtained before substance P exposure and at the bottom point after phase I, respectively, as described in Results (Fig. 3).

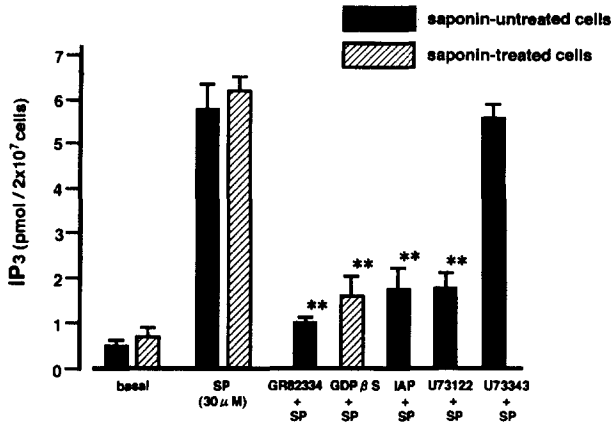

Fig. 5. Effects of a tachykinin NK_1 receptor antagonist, G-protein inactivators and a phospholipase C inhibitor on substance P (SP)-induced IP_3 formation in human neutrophils. The human neutrophils (closed column) were preincubated with GR82334 (10 μM), U-73122 (1 μM) or IAP (10 μg/ml) for 10, 10 or 90 min, respectively, then treated with 30 μM substance P. GDPβS (10 mM) pretreatment was performed using saponin-permeabilized cells (dashed column) for 10 min. IP_3 formed in 30 s after the addition of substance P was measured by a radioreceptor assay. Each value represents the mean ± S.E.M. for six preparations. ** $P < 0.01$ compared to the corresponding control responses with 30 μM substance P alone.

formation through G-protein-mediated coupling of tachykinin NK_1 receptor to phospholipase C.

3.6. Effects of inhibitors for IP_3 functions, protein kinase C activity and Ca^{2+} entry on the substance P-induced O_2^- production and $[Ca^{2+}]_i$ increase

If the breakdown of PIP_2 participates in the substance P-induced O_2^- production and $[Ca^{2+}]_i$ increase, another product of PIP_2 hydrolysis, diacylglycerol, may also be responsible for these events. To evaluate the roles of IP_3 and diacylglycerol-stimulated protein kinase C in the substance P-induced responses, reagents that affect the functions of IP_3 or protein kinase C were tested.

Fig. 6 shows representative traces of substance P-induced O_2^- production and biphasic increases in $[Ca^{2+}]_i$ in the presence of an IP_3 receptor antagonist, heparin (Meyer and Stryer, 1990) (Fig. 6A,B), or an inhibitor of Ca^{2+} mobilization from the intracellular Ca^{2+} store, TMB-8 (Hunt et al., 1990) (Fig. 6C,D). Both heparin (2 μg/ml) and TMB-8 (10 μM) markedly reduced O_2^- production and the phase I increase in $[Ca^{2+}]_i$ without any significant effects on the phase II $[Ca^{2+}]_i$ responses. The basal $[Ca^{2+}]_i$ levels were unchanged by the pretreatments. Heparin pretreatment was performed using saponin-permeabilized cells, which retained almost the same responsiveness to substance P as that observed in non-permeabilized cells (see 'GDPβS treatment').

Fig. 7 shows the representative traces of substance P-induced O_2^- production and $[Ca^{2+}]_i$ responses in the presence of protein kinase C inhibitors, 1 μM H-7 (Fig. 7A,B) and 1 μM calphostin C (Fig. 7C,D), or a Ca^{2+} entry blocker, 10 μM nicardipine (Fig. 7E,F). Both protein kinase C inhibitors, slightly and markedly reduced the O_2^- productions and phase II increases in $[Ca^{2+}]_i$, respectively. A Ca^{2+} entry blocker, nicardipine, suppressed only the phase II increases in $[Ca^{2+}]_i$. The treatment with H-7, calphostin C or nicardipine did not change significantly the basal $[Ca^{2+}]_i$ levels and phase I increase.

Table 2 summarizes the effects of these inhibitors on the substance P responses. Heparin (2 μg/ml) reduced the substance P-induced O_2^- production and phase I increase in $[Ca^{2+}]_i$ to approximately 33.1% and 13.3% of the control, respectively, but had no effect on the phase II response. TMB-8 (10 μM) also reduced the substance P-induced O_2^- production and phase I increase in $[Ca^{2+}]_i$ to an extent similar to that observed with heparin without any significant effects on phase II response.

In contrast, H-7 (1 μM) reduced the O_2^- production to

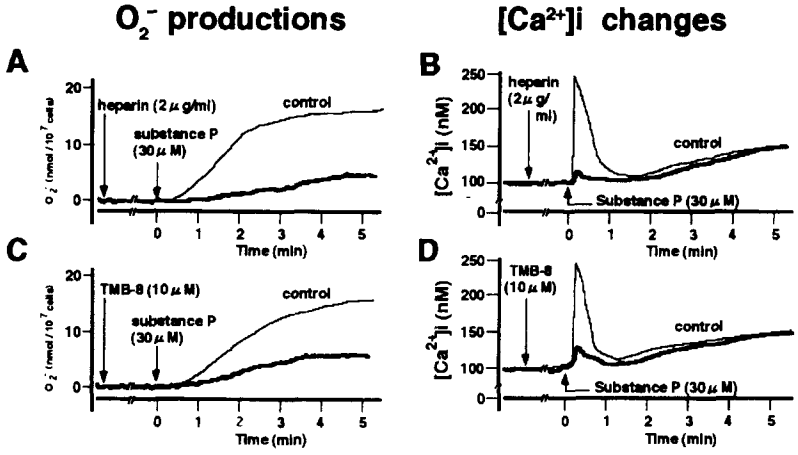

Fig. 6. Representative traces for substance P-induced O_2^- production and $[Ca^{2+}]_i$ changes in human neutrophils with or without heparin and TMB-8. The human neutrophils were pretreated with (thick line) 2 μg/ml heparin (A,B), 10 μM TMB-8 (C,D) or vehicle (control: thin line) for 10 min and then exposed to 30 μM substance P. Heparin (2 μg/ml) pretreatment was performed using saponin-permeabilized cells as described in Methods.

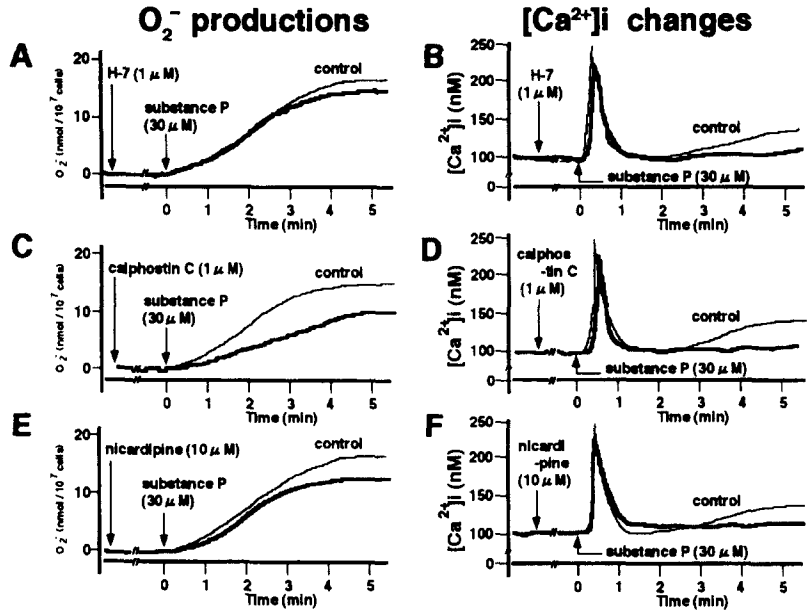

Fig. 7. Representative traces for substance P-induced O_2^- production and $[Ca^{2+}]_i$ changes in human neutrophils with or without H-7, calphostin C or nicardipine. The human neutrophils were pretreated with (thick line) 1 μM H-7 (A,B), 1 μM calphostin C (C,D), 10 μM nicardipine (E,F) or vehicle (control: thin line) for 10 min and then exposed to 30 μM substance P.

75.0% of the control, but attenuated the phase II increase in $[Ca^{2+}]_i$ to 10.8% of the control without affecting the phase I response. Considering the low specificity of H-7 for protein kinase C (Hidaka et al., 1984), we also used calphostin C as a potent and specific inhibitor of protein kinase C with a low K_i of 0.05 μM compared with the K_i of 50 μM for cAMP-dependent protein kinase or tyrosine kinase (Kobayashi et al., 1989). Calphostin C (1 μM) also reduced O_2^- production to 71.3% of the control, but caused marked inhibition only for the phase II response (3.6% of control) to a much greater extent than H-7.

Nicardipine (10 μM) completely reduced the phase II increase in $[Ca^{2+}]_i$, to 4.3% of the control. However, this Ca^{2+} channel blocker had no effect on the O_2^- production or phase I response.

Phorbol 12-myristate 13-acetate (PMA, 10 μM), which is known to induce protein kinase C-mediated O_2^- production (Tauber, 1987), stimulated O_2^- production in human neutrophils (255.5 ± 10.8 nmol/5 min/10^7 cells). In this case, H-7 and calphostin C markedly inhibited O_2^- production, by 89% and 95%, respectively, but the inhibitors for IP_3-mediated Ca^{2+} release, heparin and TMB-8, had no effect (data not shown).

4. Discussion

To clarify the intracellular mechanisms by which substance P activates human neutrophils to produce O_2^-, we evaluated the effects of tachykinin NK receptor antagonists

Table 2
Effects of reagents affecting the functions of IP_3 and protein kinase C on substance P-induced O_2^- production and $[Ca^{2+}]_i$ elevation in neutrophils

	O_2^- (nmol/5 min/10^7 cells)	Δ$[Ca^{2+}]_i$ (nM)	
		Phase I	Phase II
Substance P (30 μM) (control)	16.0 ± 1.2	142.8 ± 6.4	30.5 ± 4.7
Heparin (2 μg/ml) + substance P	5.3 ± 0.4 [b]	19.0 ± 6.1 [b]	27.5 ± 5.9
TMB-8 (10 μM) + substance P	6.4 ± 0.3 [b]	19.5 ± 16.9 [b]	26.5 ± 7.0
H-7 (1 μM) + substance P	12.0 ± 1.2	128.0 ± 8.0	3.3 ± 1.8 [b]
Calphostin C (1 μM) + substance P	11.4 ± 0.8 [a]	129.0 ± 11.0	1.1 ± 4.0 [b]
Nicardipine (10 μM) + substance P	13.4 ± 1.1	127.3 ± 7.0	1.3 ± 1.1 [b]

Each value represents the mean ± S.E.M. for 6–12 preparations. [a] $P < 0.05$, [b] $P < 0.01$ compared with substance P alone. The production of O_2^- was estimated from the amounts of cytochrome C reduced in 5 min after the addition of substance P. Changes in $[Ca^{2+}]_i$ in phase I and phase II were determined as Δincreases above the values obtained before substance P exposure and at the bottom point after phase I, respectively, as described in Results (Fig. 3).

and several kinds of agents that interfere with the signal transduction pathways coupled with phosphoinositide breakdown.

Substance P stimulated neutrophils to elicit O_2^- production (Fig. 1) and biphasic increases in $[Ca^{2+}]_i$ with the peaks at 20–30 s and 5 min (Fig. 3). Of the three tachykinin NK receptors, substance P has been reported to bind preferentially to the NK_1 rather than the NK_2 or NK_3 receptor (Gether et al., 1993). In the present study, tachykinin NK_1 receptor antagonists, GR82334 and spantide, markedly inhibited the substance P-induced O_2^- production (Fig. 2) and $[Ca^{2+}]_i$ elevation (Fig. 3). These findings indicate that tachkinin NK_1 receptor activation is involved in the occurrence of such responses to substance P. This idea was also supported by the fact that a selective tachykinin NK_1 receptor agonist, [Met-*O*-Me^{11}]substance P, but not NK_2 or NK_3 agonists, mimicked the substance P-induced O_2^- and $[Ca^{2+}]_i$ responses (data not shown). In addition, the substance P-induced O_2^- production and increases in $[Ca^{2+}]_i$ were obviously reduced by the G-protein inactivators (GDPβS and islet-activating protein (IAP)) or the phospholipase C inhibitor (U-73122) (Table 1). These findings suggest that tachykinin NK_1 receptor stimulation by substance P stimulates phospholipase C through the activation of islet-activating protein (IAP)-sensitive G-protein (presumably Gp). As observed in this study and previously reported (Serra et al., 1988), substance P stimulates neutrophils at a 10- to 100-fold higher concentration than is effective in other tissues such as airway smooth muscles (Gatto et al., 1989; Mayer et al., 1990). Two kinds of recently cloned isoforms of the human tachykinin NK_1 receptor showed that they are different from each other as to length of polypeptide chains and affinity to substance P, i.e., the short form is at least 10-fold less responsive to substance P than the long form (Fong et al., 1992). The existence of multiple forms of the tachykinin NK_1 receptor may explain the difference in substance P efficacy observed in the different types of tissue or cells.

After treatment of the neutrophils with an intracellular Ca^{2+} chelator, BAPTA-AM, substance P failed to stimulate O_2^- production, indicating that an increase in $[Ca^{2+}]_i$ is a prerequisite for the production of O_2^-. Furthermore, substance P produced the tachykinin NK_1/phospholipase C-coupled maximum increase in IP_3 level at 30 s after stimulation. These findings suggest that degradative products of PIP_2, IP_3 and/or diacylglycerol are involved in the occurrence of the early and the late events. The former product is thought to participate in a transient component of the cellular response by mobilizing intracellular Ca^{2+} from the store sites, while the latter is known to produce a sustained phase response, probably through the stimulation of extracellular Ca^{2+} influx via protein kinase C activation (Berridge, 1984; Abdel-Latif, 1986). In the present study, heparin, which is reported to competitively block IP_3 binding to its receptor on the surface of the cytosolic Ca^{2+} store (Meyer and Stryer, 1990), attenuated substance P-induced O_2^- production and a transient, but not sustained, increase in $[Ca^{2+}]_i$ (Fig. 6A and Table 2). Furthermore, when neutrophils were incubated in a Ca^{2+}-free medium containing 2 mM EGTA, only an early and transient increase in $[Ca^{2+}]_i$ and O_2^- production were observed during the exposure to substance P (data not shown). Though heparin is not a highly specific inhibitor for the IP_3 receptor, it is possible to conclude that IP_3 produces the transient increase in $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from the store site, being responsible for the O_2^- production. This idea is supported by the facts that (1) TMB-8, an inhibitor of Ca^{2+} mobilization (Hunt et al., 1990), showed almost the same inhibitory effects on O_2^- and $[Ca^{2+}]_i$ responses as did heparin (Fig. 6 and Table 2), and (2) the time (30 s) required for the maximum formation of IP_3 was coincident with the transient increase in $[Ca^{2+}]_i$. In the present study, GDPβS as well as IAP and U-73122 inhibited the transient increase in $[Ca^{2+}]_i$ almost completely, but suppressed IP_3 production by 70–80%. One possible explanation for such a discrepancy may be that, in the presence of these inhibitors, the IP_3 levels reached with substance P (1.5–2.0 pmol/2×10^7 cells which correspond to 50–70 nM per cell) are not high enough to activate IP_3-dependent Ca^{2+} channels, since IP_3 has been shown to provoke a dose-dependent Ca^{2+} release from Ca^{2+} store sites in a concentration range of 0.1–2 μM (Streb et al., 1983).

On the other hand, both protein kinase C inhibitors, H-7 and calphostin C, and the Ca^{2+} channel blocker, nicardipine, markedly suppressed the sustained, but not the transient, increase in $[Ca^{2+}]_i$. In contrast to heparin and TMB-8, substance P-induced O_2^- production was slightly inhibited by protein kinase C inhibitors or unaffected by nicardipine. These lines of evidence can be interpreted as follows. (1) Protein kinase C activation stimulates the nicardipine-sensitive Ca^{2+} channel of neutrophils, resulting in the sustained increase in $[Ca^{2+}]_i$, as shown in various tissues (Galizzi et al., 1987; Otani et al., 1988). (2) Such a protein kinase C-regulated Ca^{2+} entry pathway mostly does not participate in the O_2^- production cascade triggered by substance P. However, the protein kinase C inhibitors tended to slightly inhibit the O_2^- responses. Considering that phorbol ester induces O_2^- production independently of Ca^{2+} in neutrophils (Tauber, 1987), protein kinase C may partly contribute to the substance P-induced O_2^- production through the direct activation of NADPH oxidase. Together these findings suggest that substance P-induced O_2^- production is triggered mainly by an IP_3-induced transient increase in $[Ca^{2+}]_i$.

In conclusion, this study showed clearly, for the first time, that substance P-induced O_2^- production in neutrophils is mediated by PIP_2 hydrolysis through tachykinin NK_1 receptor/G-protein-coupled phospholipase C activation. Among the two second messengers generated, IP_3 and diacylglycerol, IP_3 appear to mainly contribute to substance P-induced O_2^- production via Ca^{2+} mobilization from the store sites, diacylglycerol probably taking only a

small part in this O_2^- production through protein kinase C-activated and Ca^{2+}-independent mechanisms.

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