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Inhibitory effects of calcitonin gene-related peptide on substance-P -induced superoxide production in human neutrophils

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Abstract

We examined the mechanisms of the inhibitory effects of calcitonin gene-related peptide (CGRP) on substance-P-induced superoxide anion (O_2^-) production in human neutrophils. Substance P (30 μM) caused O_2^- production associated with an inositol-1,4,5-trisphosphate (IP_3)-induced transient increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). CGRP (10 μM) significantly inhibited substance-P -induced O_2^- production and transient increase in $[Ca^{2+}]_i$, but it only slightly suppressed IP_3 formation. In addition, CGRP inhibited IP_3-induced O_2^- production and transient increase in $[Ca^{2+}]_i$ caused by exogenous addition of IP_3 in saponin-permeabilized neutrophils. These findings suggest that CGRP inhibits the response of neutrophils to substance P through the inhibition of IP_3-induced Ca^{2+} release from intracellular Ca^{2+} stores. The inhibitory effects of CGRP on substance P- or IP_3-induced O_2^- production and increases in $[Ca^{2+}]_i$ were abolished by pretreating the neutrophils with a CGRP receptor antagonist, CGRP-(8–37), or cyclic AMP (cAMP)-dependent protein kinase inhibitors, *N*-[2-(methylamino) ethyl]-5-isoquinoline-sulfonamide dihydrochloride (H-8) and 9-*n*-hexyl ester derivative of K-572a (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo(a,g)cycloocta(cde)trinden-1-one (KT5720). We concluded that CGRP receptor stimulation reduces substance-P-induced O_2^- production by the inhibition of IP_3-induced transient increase in $[Ca^{2+}]_i$, probably via the phosphorylation of IP_3 receptor by cAMP-dependent protein kinase.

Keywords: Substance P; CGRP (calcitonin gene-related peptide); Superoxide; Neutrophil

1. Introduction

The role of neuropeptides in inflammatory reactions has been observed in the last few years. For example, substance P, which is released from the sensory C-fiber endings in response to noxious stimuli, induces the release of histamine and prostaglandin E_2 from synoviocytes and leukocytes, thus it is a potent mediator of inflammation (Lotz et al., 1987; Kimball, 1990). Stimulation of neutrophils with substance P has been reported to induce hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) production associated with the accumulation of inositol phosphates, and this results in Ca^{2+} mobilization from intracellular Ca^{2+} stores (Serra et al., 1988; Tanabe et al., 1996).

Another sensory neuropeptide, calcitonin gene-related peptide (CGRP), a 37-amino-acid peptide formed by alternative processing of a primary calcitonin gene product, co-localizes with substance P in sensory C-fibers, and is co-released with this peptide from the nerve endings (Goodman and Iversen, 1986). Besides CGRP's own effect, such as vasodilation (Brain and Williams, 1988; Edwards et al., 1991) and stimulation of endothelial cell proliferation (Wang and Fiscus, 1989), interactions between substance P and CGRP in physiological responses have recently been studied. For example, CGRP was demonstrated to antagonize substance-P-induced bronchoconstriction, leukocyte aggregation and increase in arteriolar diameter (Ohlen et al., 1988; Gatto et al., 1989). CGRP has also been shown to increase intracellular cyclic AMP (cAMP) levels in several tissues such as human endothelial cells and atria (Wang and Fiscus, 1989; Hegerstrand et al., 1990). Elevation of intracellular cAMP

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levels and the following activation of a cAMP-dependent protein kinase is known to interfere with phosphoinositide metabolism (Kim et al., 1989; Lazarowski and Lapetina, 1989; Murthy et al., 1993), inhibiting inositol-1,4,5-trisphosphate (IP_3) production and subsequent Ca^{2+} mobilization (Smith and Snydermann, 1987; Supattapone et al., 1988). These findings raised the possibility that CGRP modulates neutrophil responses to substance P through cAMP-dependent signalling pathways. This study was designed to examine the effects of CGRP on substance-P -induced O_2^- production in human neutrophils by focussing on the ability of CGRP/cAMP-dependent protein kinase to inhibit IP_3-induced Ca^{2+} release from intracellular Ca^{2+} stores.

2. Materials and methods

2.1. Materials

Substance P and calcitonin gene-related peptide (CGRP) were obtained from Peptide Institute (Osaka, Japan). Inositol-1,4,5-trisphospate (IP_3), ferricytochrome C (Type 3), superoxide dismutase, leupeptin, phenylmethyl-sulphonyl fluoride, aprotinine, dibutyl cAMP, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-AM, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and heparin were purchased from Sigma (St. Louis, MO, USA). CGRP-(8–37) was kindly donated from Toyo Jozo (Shizuoka, Japan), and used as a CGRP receptor antagonist (Chiba et al., 1989). *N*-[2-(Methylamino)ethyl]-5- isoquinolinesulfon-amide dihydrochloride (H-8) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were purchased from Seikagaku Kogyo (Tokyo, Japan). The 9-*n*-hexyl ester derivative of K-572a (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-methoxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo(a,g)cycloocta-(cde)trinden-1-one (KT5720), an antagonist of cAMP-dependent protein kinase (Kase et al., 1987), was obtained from Kyowa Medex (Tokyo, Japan) and fura-2-AM was obtained from Dojindo Laboratories (Kumamoto, Japan).

2.2. Preparation of neutrophils

Neutrophils were isolated from the heparinized venous blood of seven healthy donors by dextran sedimentation and centrifugation in a Ficoll-Hypaque gradient (Serra et al., 1988; Hasui et al., 1989). Neutrophils were washed three times in Hanks' balanced salt solution (HBSS, pH 7.4) containing 136.8 mM NaCl, 5.0 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 20.0 mM Hepes. Contaminating erythrocytes were disrupted by hypotonic lysis for 40 s with 5 ml of ice cold distilled water, and then the same volume of 2-fold concentrated HBSS was added to restore isotonicity. After centrifugation, the resulting pellet was suspended in HBSS containing 2 mM NaN_3, a superoxide dismutase inhibitor, and was used as a purified neutrophil preparation (96% purity). The viability of neutrophils after isolation was found to be greater than 98% by Trypan blue exclusion.

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

2.3. Determination of O_2^- production

O_2^- production was assayed spectrophotometrically by recording the reduction of ferricytochrome *c* (Type 3), using a double wavelength double beam spectrophotometer (model 557, Hitachi, Japan) equipped with a thermostatically controlled cuvette holder, as described by Cohen and Chovaniec (1978). The cell suspension (final concentration of 10^6 cells/ml) was added to a cuvette with 1 ml HBSS containing 60 μM ferricytochrome *c* and 1 mM KCN, and the cuvette was put into a cuvette holder (37°C) in the spectrophotometer. After a 10 min equilibration period at 37°C, the cells were pretreated with test agents or a vehicle for the indicated periods, and then the reaction was started by the addition of substance P. Changes in the rate of ferricytochrome *c* reduction following the administration of substance P or IP_3 were measured at 550 nm with a reference wavelength of 540 nm, and the absorbance difference (absorbance at 550 nm minus that at 540 nm) was continuously monitored on a pen-chart recorder. The amount of O_2^- produced by the administration of substance P or IP_3 was calculated by subtracting the amount of reduced cytochrome *c* in the resting state from the amount obtained 5 min after the addition of each agent, using an extinction coefficient of 21.1/mM/cm (Cohen and Chovaniec, 1978).

2.4. Measurement of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$)

The neutrophils were incubated in HBSS containing 3 μM fura-2-AM (Dojindo Co., Japan) for 20 min at 3°C. The cells were centrifuged (100 × *g*, 8 min) twice, and resuspended in HBSS. Fura-2 fluorescence measurements were performed at 37°C using a spectrofluorimeter (Type F-2000, Hitachi) equipped with a thermostatically controlled 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 maximum 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+} ($F340_{max}$) conditions, and K_d is the dissociation constant for fura-2-Ca^{2+}, which is 224 nM (Borzak et al., 1990).

2.5. Measurement of inositol-1,4,5-trisphospate (IP_3) formation

The neutrophils suspended in 2 ml of HBSS medium at a concentration of 2×10^7 cells/ml were incubated with different concentrations of substance P in the presence of 10 mM LiCl, an inihibitor of inositol phosphatase, to prevent IP_3 recycling. Thirty seconds after the addition of substance P, the reaction was terminated by the addition of the same volume of ice-cold 15% (w/v) trichloroacetic acid, and the cell debris was removed by centrifugation. The resulting supernatants were extracted three times with diethylether, and 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 (Amersham, UK).

2.6. Permeabilization of neutrophils and determination of IP_3-induced responses

When studying the responses of neutrophils to membrane impermeable agents such as IP_3 and heparin, the neutrophils have to be permeabilized with saponin. The cells were exposed to a 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 7, and 85 μg/ml saponin for 10 min (Smith and Snydermann, 1987; Meyer and Stryer, 1990). After the following wash out, the cells were resuspended in the same buffer without saponin to a final concentration of 10^6 cells/ml. After an equilibration period of 10 min, the cells were pretreated with test agents or a vehicle for 10 min at 37°C, and then the reaction was started by the addition of IP_3. Changes in the rate of ferricytochrome *c* reduction and fura-2-AM fluorescence were determined as described above. Every experiment was thus performed in the absence of saponin, and saponin itself did not induce either $[Ca^{2+}]_i$ elevation or O_2^- production in the intracellular environment. We checked the changes in intracellular fura-2 fluorescence intensity of single saponin-permeabilized neutrophils using an ultrasensitive fluorescence counting imaging camera (C-2400-08H, Hamamatsu Photonics, Hamamatsu, Japan) equipped with a computer-assisted image processor. In the permeabilized neutrophils, both fluorescence intensities at 340 and 380 nm showed gradual decreases in parallel with those in non-permeabilized cells during the first 30 min of resuspension (about 24% loss at 30 min), representing the naturally occurring dye quenching without changing the fluorescence ratio (340 nm/380 nm). After 30 min, fluorescence intensities at 340 and 380 nm in the permeabilized cell decreased with higher rates as compared with those in non-permeabilized cells during the same periods, reaching values below 25% of respective original intensities at 50 min (Fig. 5, inset). Such augmented decreases in fluorescence intensities were probably caused by the dye leakage out of the cells. Thus, the permeabilized cells were considered to stably retain the dye for 30 min and to be well suited to the $[Ca^{2+}]_i$ measurement. Each experiment was finished within 25 min after permeabilization.

2.7. Statistical analysis

Student's *t*-test was used for statistical analysis. The differences between mean values with P values less than 0.05 were considered significant.

3. Results

3.1. Effects of CGRP on substance-P-induced O_2^- production, changes in $[Ca^{2+}]_i$ and IP_3 formation

Human neutrophils treated with 30 μM substance P produced O_2^- as measured by ferricytochrome *c* reduction (Fig. 1A, control). The reduction of ferricytochrome *c* was abolished in the presence of superoxide dismutase (400 units/ml), showing that reduction is an indicator of O_2^- production (Fig. 1A). After the stimulation of neutrophils, the amounts of O_2^- produced increased rapidly during the first 3 min and then slowly, reached a plateau level within 5 min. Therefore, in the following experiments the amounts of O_2^- released during the first 5 min was determined. Treatment of neutrophils with 10 μM CGRP for 10 min reduced substance-P-induced O_2^- production to approximately 56% of the control level (Fig. 1A). When the neutrophils were pre-exposed to a CGRP receptor antagonist, 10 μM CGRP-(8–37) (Chiba et al., 1989), the inhibitory effect of CGRP was abolished (Fig. 1A).

Fig. 1B 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.M., $n = 6–10$) in normal HBSS medium. Addition of substance P immediately increased $[Ca^{2+}]_i$ to a peak level (241.0 ± 12.9 nM, $n = 6–10$) within 40 s. The $[Ca^{2+}]_i$ then rapidly decreased to nearly the basal level (103.0 ± 6.5 nM, $n = 6–10$) 70–90 s after stimulation. CGRP (10 μM) completely inhibited this substance-P-induced transient elevation of $[Ca^{2+}]_i$, and CGRP-(8–37) abolished the inhibitory effects of CGRP.

Fig. 1C shows the time-course of substance-P-induced IP_3 formation in the presence or absence of 10 μM CGRP. In 30 s after exposure to 30 μM substance P, the IP_3 levels in neutrophils transiently increased from the basal level of 0.5 ± 0.3 pmol/2×10^7 cells ($n = 8$) to a maximal level of 5.7 ± 1.1 pmol/2×10^7 cells ($n = 15$). The time-course of IP_3 production induced by substance P was almost the

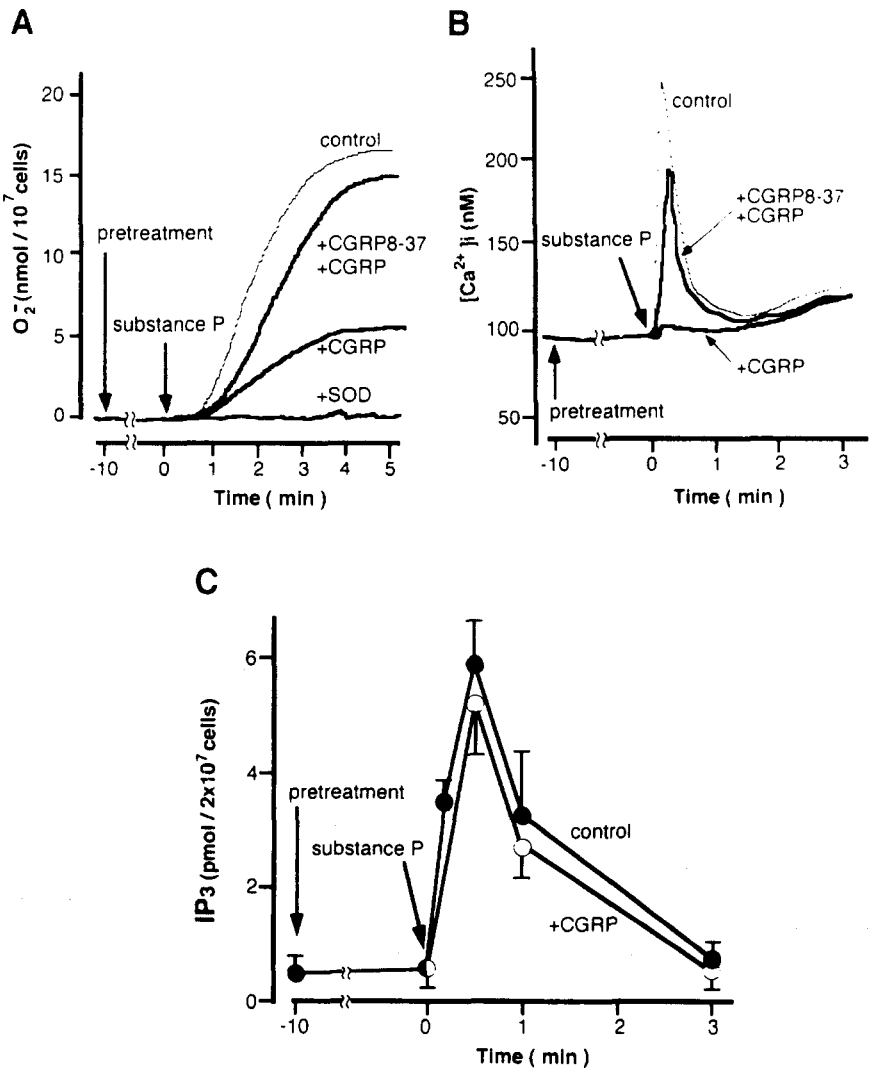

Fig. 1. Effects of CGRP on substance-P-induced O_2^- production (A), changes in $[Ca^{2+}]_i$ (B) and IP_3 formation (C) in human neutrophils. (A) A representative time-course of O_2^- production. O_2^- production was monitored spectrophotometrically as described in Materials and methods. Cells were equilibrated for 10 min in HBSS medium with or without (control) test reagents; 10 μM CGRP, 10 μM CGRP plus a CGRP receptor antagonist, 10 μM CGRP-(8–37),or 400 units/ml of superoxide dismutase (SOD), and then exposed to 30 μM substance P. (B) A representative trace of $[Ca^{2+}]_i$. Cells loaded with fura-2-AM were equilibrated for 10 min in HBSS medium with or without (control) test reagents; 10 μM CGRP or 10 μM CGRP plus 10 μM CGRP-(8–37), and then exposed to 30 μM substance P. (C) A representative time-course of IP_3 formation. IP_3 formation was measured by using an IP_3 radioreceptor-assay kit as described in Materials and methods. Cells were equilibrated for 10 min in HBSS medium with or without (control) test reagents; 10 μM CGRP, and then exposed to 30 μM substance P.

same as that observed for $[Ca^{2+}]_i$ elevation. The formation of IP_3 by substance P was not inhibited by pretreatment with CGRP (10 μM).

Fig. 2 shows the dose-dependent effects of CGRP on substance-P-induced O_2^- production, $[Ca^{2+}]_i$ elevation and IP_3 formation. CGRP (10 and 20 μM) inhibited both substance P (30 μM)-induced O_2^- production and $[Ca^{2+}]_i$ changes in a dose-dependent manner, without affecting IP_3 production. These findings suggest that CGRP may inhibit substance-P-induced O_2^- production by inhibiting IP_3-triggered Ca^{2+} mobilization rather than IP_3 formation. To test this possibility, the next experiment was done to examine the effect of CGRP on IP_3-induced responses, using saponin-permeabilized human neutrophils.

3.2. Effect of CGRP on IP_3-induced O_2^- production in permeabilized neutrophils

In saponin-permeabilized human neutrophils, IP_3 (from 1 nM to 1 μM) dose dependently produced O_2^- with a plateau level at IP_3 concentrations over 100 nM (Fig. 3), though it caused no O_2^- production in normal (non-permeable) neutrophils (data not shown). Such responses were abolished in the presence of an IP_3 receptor antagonist, heparin (2 μg/ml) (Meyer and Stryer, 1990), suggesting that IP_3 stimulates its receptors on intracellular Ca^{2+} stores to produce O_2^- in the permeabilized neutrophils. Fig. 4A shows a representative trace of IP_3 (10 nM)-induced O_2^- production in the presence or absence of 10 μM CGRP.

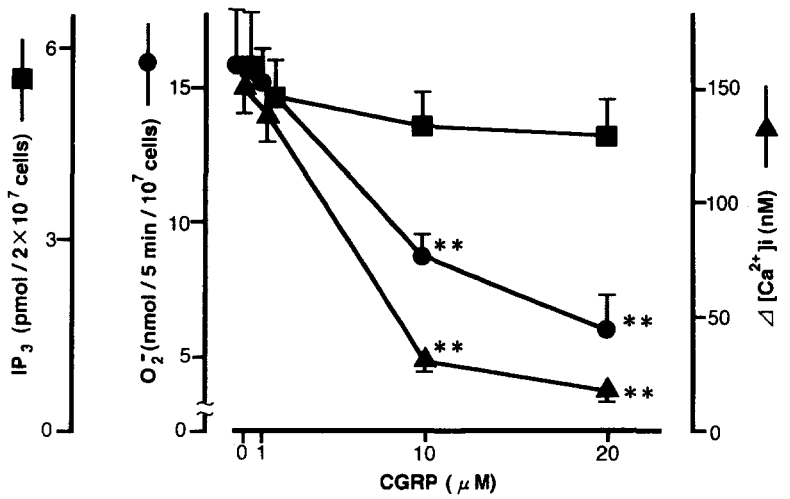

Fig. 2. Dose-dependent effects of CGRP on O_2^- production, $\Delta[Ca^{2+}]_i$ and IP_3 formation in neutrophils stimulated with substance P. Human neutrophils were preincubated for 10 min with HBSS medium with or without 1, 10 or 20 μM CGRP, and then exposed to 30 μM substance P. Each symbol represents the mean ± S.E. for 16–20 preparations. ** $P < 0.01$ compared with the value without CGRP.

After the stimulation of permeabilized neutrophils with 10 nM IP_3, the amounts of O_2^- produced increased rapidly during the first 2 min and reached a plateau level within 3 min. Ferricytochrome *c* reduction was abolished in the presence of superoxide dismutase (400 units/ml), showing that this reduction is an indicator of O_2^- production (Fig. 4A). CGRP significantly inhibited IP_3-induced O_2^- production with an apparent half maximal inhibition at 8 μM (Fig. 4A,B), and this inhibitory effect was abolished by pretreating the neutrophils with a CGRP receptor antagonist, CGRP-(8–37) (10 μM) (Fig. 4A). Since stimulation of the CGRP receptor inhibited IP_3-induced O_2^- production, the effects of CGRP on IP_3-induced Ca^{2+} mobilization was then examined.

3.3. Effects of CGRP on IP_3-induced changes in $[Ca^{2+}]_i$ in permeabilized neutrophils

Fig. 5 shows the typical changes in $[Ca^{2+}]_i$ in saponin-permeabilized neutrophils during exposure to 10 nM IP_3. The basal $[Ca^{2+}]_i$ level of permeabilized cells was estimated to be 104.5 ± 10.0 nM (mean ± S.E.M., $n = 15$) in a permeabilization buffer. Addition of IP_3 caused an immediate and transient increase in $[Ca^{2+}]_i$, with a peak level of 207.1 ± 31.1 nM ($n = 10$) being reached within 30 s. $[Ca^{2+}]_i$ then decreased to nearly the basal level of 122 ± 13.9 nM ($n = 10$), 40–50 s after stimulation. Such an IP_3-induced increase in $[Ca^{2+}]_i$ was not observed in normal (non-permeabilized) neutrophil (data not shown) as in

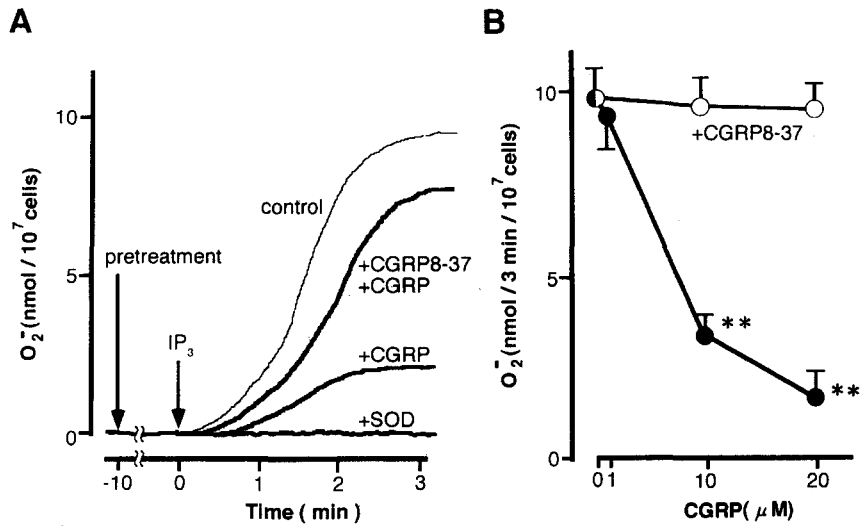

Fig. 3. The concentration-response curve for O_2^- production in permeabilized neutrophils stimulated with IP_3. The saponin-permeabilized human neutrophils were preincubated for 10 min with (○) or without (●) an IP_3 receptor antagonist, heparin (2 μg/ml), and then exposed to various concentrations of IP_3. The production of O_2^- was calculated from the amounts of cytochrome *c* reduced 3 min after the addition of IP_3. Each symbol with a bar represents the mean ± S.E. for 10–12 preparations.

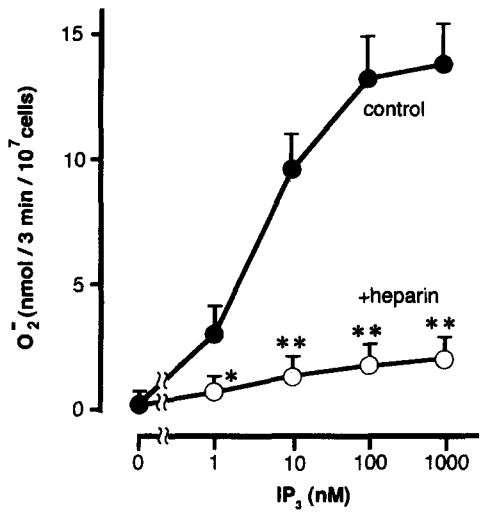

Fig. 4. Effects of CGRP on IP_3-induced O_2^- production in permeabilized human neutrophils. (A) A representative time-course of O_2^- production. O_2^- production was monitored spectrophotometrically as described in Materials and methods. Saponin-permeabilized cells were preincubated for 10 min with or without (control) 10 μM CGRP, 10 μM CGRP plus a CGRP receptor antagonist, 10 μM CGRP-(8–37) or 400 units/ml of superoxide dismutase (SOD), and then exposed to 10 nM IP_3. (B) Dose-dependent effects of CGRP on IP_3-induced O_2^- production. The permeabilized cells were preincubated for 10 min with or without different concentrations of CGRP (●) or CGRP plus 10 μM CGRP-(8–37) (○), and then exposed to 10 nM IP_3. Each symbol with a bar represents the mean ± S.E. for 16–20 preparations. ** $P < 0.01$ compared with the value of without CGRP.

the case of O_2^- production, and this response was attenuated by the pretreatment with heparin. These results suggest that IP_3 directly stimulated IP_3 receptors on the intracellular Ca^{2+} stores in the permeabilized neutrophils. CGRP (10 μM) completely inhibited this IP_3-induced $[Ca^{2+}]_i$ increase, however, the effects of CGRP were reduced again in the presence of CGRP-(8–37), suggesting that stimulation of CGRP receptors inhibits IP_3-induced Ca^{2+} mobilization. Fig. 5 (inset) shows the time-course of the changes in intracellular fura-2 fluorescence intensity of the single saponin-permeabilized neutrophil using an ultrasensitive fluorescence counting imaging camera. During the first 30 min, degree and time-course of the dye leakage in the permeabilized cells were almost the same as those in control (saponin-untreated) one. Therefore, the permeabilized cells were considered to stably retain the dye for 30 min and to be well applicable for the $[Ca^{2+}]_i$ measurement. The measurements of IP_3-induced changes in $[Ca^{2+}]_i$ were finished within 25 min after permeabilization.

3.4. Effects of inhibitors of cAMP-dependent protein kinase on the inhibitory effects of CGRP

It has been proposed that CGRP activates adenylate cyclase (Takenawa et al., 1986; Kim et al., 1989; Lazarowski and Lapetina, 1989). We recently reported that

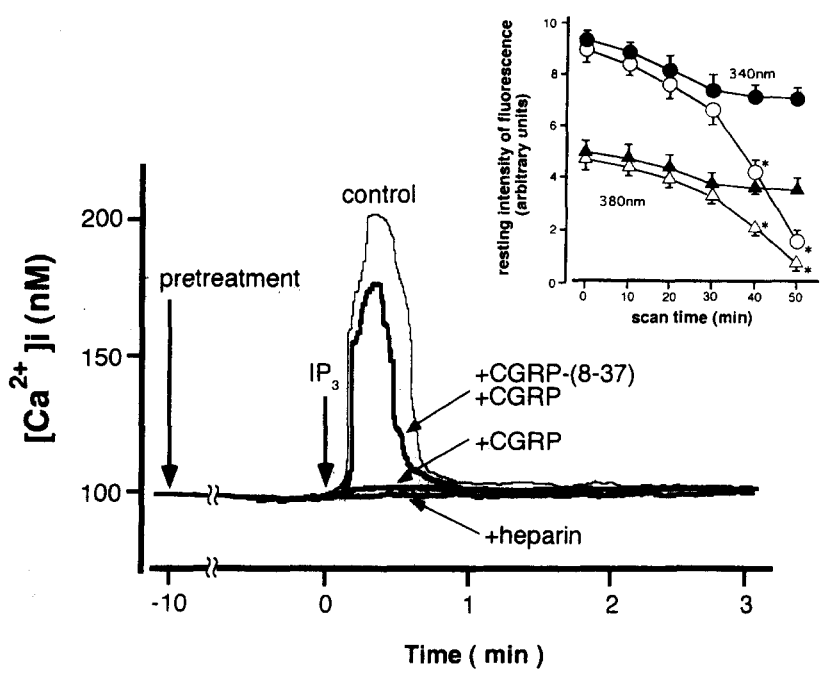

Fig. 5. Representative traces of IP_3-induced changes in $[Ca^{2+}]_i$ in permeabilized human neutrophils. $[Ca^{2+}]_i$ was measured fluorometrically as described in Materials and methods. Saponin-permeabilized and fura-2-AM-loaded cells were preincubated for 10 min with or without (control) 10 μM CGRP, 10 μM CGRP plus an CGRP receptor antagonist, 10 μM CGRP-(8–37), or IP_3 receptor antagonist, 2 μg/ml heparin, and then exposed to 10 nM IP_3. Inset: the time-course of changes in resting fura-2 fluorescence intensities at 340 nm (●, ○) and 380 nm (▲, △) of the single saponin-permeabilized (○, △) and non-permeabilized (●, ▲) cells were checked by using an ultrasensitive fluorescence counting imaging camera as described in Materials and methods. Each symbol with a bar represents the mean ± S.E. for 8–10 preparations. * $P < 0.01$ compared with the value of non-permeabilized cell.

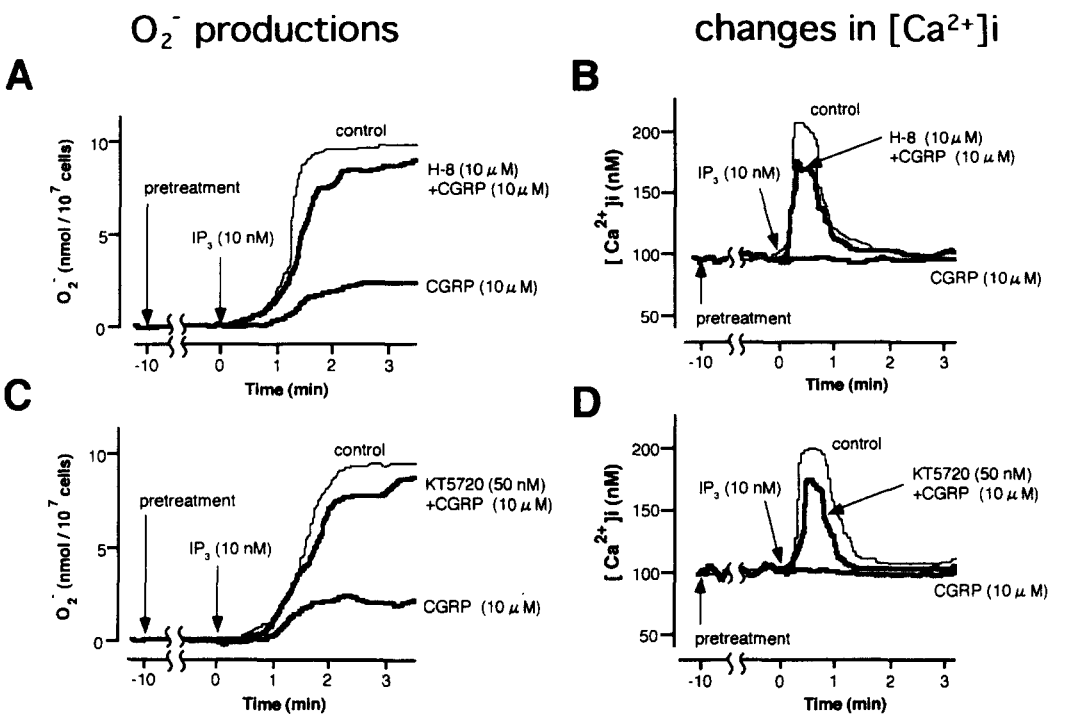

Fig. 6. Effects of cAMP-dependent protein kinase inhibitors on the inhibitory effects of CGRP and dibutyl cAMP in IP_3-induced O_2^- production and $[Ca^{2+}]_i$ changes. Saponin-permeabilized human neutrophils were pretreated for 10 min with or without (control) 10 μM CGRP or 10 μM CGRP plus either of the cAMP-dependent protein kinase inhibitors, 10 μM H-8 (A,B) or 50 nM KT5720 (C,D), and then exposed to 10 nM IP_3. Same experiments carried out by using 1 mM dibutyl cAMP (E,F). O_2^- production (A,C,E) and changes in $[Ca^{2+}]_i$ (B,D,F) were measured as described in Materials and methods.

CGRP elevated intracellular cAMP levels in human neutrophils (Tanabe et al., 1994). In addition, dibutyl cAMP (1 mM) also gave the similar inhibitory effects (85% inhibition) on IP_3-induced O_2^- production as CGRP did. Therefore, whether or not the activation of cAMP-dependent protein kinase plays a key role in the inhibitory effects of CGRP was examined (Fig. 6). Permeabilized neutrophils were pretreated with either cAMP-dependent protein kinase inhibitor, 10 μM H-8 or 50 nM KT5720, for 10 min, then treated with 10 μM CGRP for an additional 10 min, and finally exposed to 10 nM IP_3. Both inhibitors abolished the inhibitory effects of CGRP on IP_3-induced O_2^- production (Fig. 6A,C) and elevation of $[Ca^{2+}]_i$ (Fig. 6B,D). In addition, 1 mM dibutyl cAMP inhibited IP_3-induced O_2^- production and IP_3-induced $[Ca^{2+}]_i$ elevation in the permeabilized neutrophils as CGRP did, and both dibutyl cAMP-induced inhibitory effects were also inhibited by pretreatment of 50 nM KT5720 (Fig. 6E,F). As summarized in Table 1, cAMP-dependent protein kinase inhibitors, H-8 (10 μM) and KT5720 (50 nM) significantly reduced the inhibitory effects of CGRP and dibutyl cAMP on both IP_3-induced O_2^- production and IP_3-induced increase in $[Ca^{2+}]_i$, without any significant effect on the IP_3-induced response itself. These findings suggest that CGRP inhibits IP_3-induced O_2^- production and IP_3-induced increase in $[Ca^{2+}]_i$ through the stimulation of cAMP-dependent protein kinase.

Table 1
Effects of cAMP-dependent protein kinase inhibitors on the inhibitory effects of CGRP and dibutyl cAMP in IP_3-induced O_2^- production and $[Ca^{2+}]_i$ changes

	O_2^- (nmol/3 min per 10^7 cells)	$\Delta[Ca^{2+}]_i$ (nM)
IP_3 (10 nM) (control)	9.8 ± 0.8	102.6 ± 10.1
CGRP (10 μM) + IP_3	3.4 ± 1.9 [a]	25.9 ± 9.8 [a]
H-8 (10 μM) + IP_3	9.1 ± 0.8	95.7 ± 9.6
H-8 (10 μM) + CGRP + IP_3	8.6 ± 1.2	84.0 ± 5.1
KT5720 (50 nM) + IP_3	9.6 ± 1.5	98.1 ± 10.4
KT5720 (50 nM) + CGRP + IP_3	9.2 ± 0.9	91.5 ± 6.5
Dibutyl cAMP (1 mM) + IP_3	1.5 ± 0.4 [a]	14.3 ± 3.1 [a]
KT5720 (50 nM) + dibutyl cAMP + IP_3	7.7 ± 1.3	78.3 ± 2.5

Each value represents the mean ± S.E.M. for 6–12 preparations. [a] $P < 0.01$ compared with IP_3 alone. Cells described in Materials and methods. The production of O_2^- was estimated from the amounts of cytochrome *c* that had been reduced 3 min after the addition of IP_3. Changes in $[Ca^{2+}]_i$ were determined as Δ increases above the values obtained before IP_3 exposure as described in Results.

4. Discussion

Substance P and CGRP are known to be co-released from nerve endings in response to noxious stimuli, result-

ing in various inflammatory responses (McGillis et al., 1990; Goodman and Iversen, 1986). We previously reported that O_2^- is produced in human neutrophils through IP_3-induced transient increases in $[Ca^{2+}]_i$ (Tanabe et al., 1996). In this study, another neuropeptide, CGRP, inhibited substance-P-induced O_2^- production (Fig. 1A). A specific CGRP receptor antagonist, CGRP-(8–37), abolished the inhibitory effects of CGRP, suggesting the involvement of CGRP receptor stimulation in the inhibitory effect. Such interference by CGRP has also been observed in the substance-P-induced increase in guinea-pig airway tone (Gatto et al., 1989), as well as in arteriolar dilatation and leukocyte aggregation in the rabbit (Ohlen et al., 1988). Conversely, CGRP has been reported to potentiate the actions of substance P, such as biting and scratching behavior in rats (Hylden and Wilcox, 1981; Piercey et al., 1981; Seybold et al., 1982), probably via the inhibition of substance P degradation (Greves et al., 1985). Thus, the modes of interaction between substance P and CGRP differ depending on the cell type or tissue type.

In this study on neutrophils, CGRP also abolished substance-P-induced transient increases in $[Ca^{2+}]_i$ (Fig. 1B). While this finding raised the possibility that CGRP inhibits substance-P-induced IP_3 formation or IP_3-induced Ca^{2+} mobilization from the interacellular Ca^{2+} stores, CGRP did not inhibit IP_3 production (Fig. 2). Therefore, we examined the effects of CGRP on IP_3-induced Ca^{2+} mobilization using saponin-permeabilized neutrophils.

In saponin-permeabilized neutrophils, IP_3 at concentrations ranging from 1 nM to 1 μM dose dependently produced O_2^-, with a half maximal concentration of 8 nM (Fig. 3). This reaction was inhibited in the presence of 2 μg/ml heparin, which is reported to competitively block IP_3 binding to its receptors localized on the surface of intracellular Ca^{2+} stores (Meyer and Stryer, 1990). In addition, such an IP_3 response was not observed in normal (non-permeabilized) cells. These findings suggest the involvement of the direct binding of IP_3 to its receptors in IP_3-induced O_2^- production. Beside the IP_3 receptor blocking effect, however, heparin has been reported to react with a variety of proteins including a neutrophil adhesion molecule, selectin. Since K_i for heparin in IP_3 receptor blocking and ED_{50} for heparin in selectin binding have been reported to be 15 μg/ml (Timothy et al., 1987) and 200–800 μmol/l (approximately 100–400 μg/ml, Richard et al., 1993), respectively, such inhibitory actions appear to be mediated by blocking IP_3 receptor. When neutrophils were stimulated with substance P (30 μM) to produce O_2^-, the amounts of IP_3 formed were 5.7 ± 1.1 pmol $IP_3/2 \pm 107$ neutrophils (Fig. 2). Assuming a single cell volume as $(1.0–3.0) \times 10^{-9}$ cm^3, this corresponds to an intracellular IP_3 concentration of 100–300 nM. IP_3 (100 nM)-induced O_2^- production in permeabilized neutrophils thus appears to reflect the amount of O_2^- produced by 30 μM substance P. Since 100 nM IP_3 produced the maximal response in permeabilized neutrophils (Fig. 3), we chose a submaximal concentration of IP_3 (10 nM) in this study. IP_3-induced O_2^- production was completely inhibited by the pretreament of neutrophils with an intracellular Ca^{2+} chelator, 10 μM BAPTA-AM (75% inhibition), and therefore, we concluded that this is a Ca^{2+}-dependent response. Although the exact mechanism linking the IP_3 receptor to NADPH oxidase is still unknown,calmodulin is now considered to be one of candidates for Ca^{2+}-dependent modulator of this oxidase (Tauber, 1987). In fact, O_2^- production in response to IP_3 as well as substance P was inhibited by a calmodulin inhibitor, W-7 (data not shown), and thus compatible with the Tauber report. CGRP inhibited IP_3 (10 nM)-induced O_2^- production (Fig. 4A,B), and transient Ca^{2+} elevation (Fig. 5). These results indicate that CGRP interfered with IP_3-induced Ca^{2+} mobilization from the intracellular Ca^{2+} stores. In the present permeabilized cell system, the saponin-pretreated neutrophils were capable of retaining fura-2 within the cells, despite of being permeable for IP_3. Although the exact reason for such an apparent discrepancy is unclear, one possible explanation is that fura-2 may bind to intracellular proteins (Konishi et al., 1988) and form a larger molecule than the membrane-spanning pore made by saponin.

CGRP has been shown to increase intracellular levels of cAMP, a putative second messenger linked to the final effects of CGRP in several tissues (Wang and Fiscus, 1989; Hegerstrand et al., 1990). Elevation of intracellular cAMP levels by CGRP activates cAMP-dependent protein kinase (Wang and Fiscus, 1989; Hegerstrand et al., 1990), and the activation of cAMP-dependent protein kinase reportedly inhibits a signal transduction pathway linked to phosphoinositide hydrolysis in a number of cell types including neutrophils (Takenawa et al., 1986; Kim et al., 1989; Lazarowski and Lapetina, 1989). In addition, activated cAMP-dependent protein kinase has been shown to phosphorylate inositol trisphosphate receptors with a concomitant decrease in Ca^{2+} release from rat brain microsomes (Supattapone et al., 1988). In this study, dibutyl cAMP (1 mM) inhibited both IP_3-induced O_2^- production and $[Ca^{2+}]_i$ elevation as CGRP did. Furthermore, cAMP-dependent protein kinase inhibitors, H-8 (10 μM) and KT5720 (50 nM), attenuated the inhibitory effects of CGRP and dibutyl cAMP on IP_3-induced O_2^- production and $[Ca^{2+}]_i$ mobilization (Fig. 6). KT5720 was used as a specific cAMP-dependent protein kinase inhibitor with a low K_i for cAMP-dependent protein kinase (60 nM) as compared with those for cyclic GMP (cGMP)-dependent protein kinase (> 2 μM) and for Ca^{2+}-dependent protein kinase C (> 2 μM) (Kase et al., 1987), although H-8 is a rather non-specific inhibitor with K_i values for the following kinases, cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C at 1.2 μM, 0.43 μM and 14.4 μM, respectively (Hidaka et al., 1984). Thus, CGRP appears to inhibit IP_3-induced O_2^- production by interfering with IP_3-induced Ca^{2+} mobilization from intracellular stores probably through IP_3 receptor phospho-

rylation via the activation of cAMP-dependent protein kinase.

In summary, this paper describes for the first time that CGRP inhibits substance-P-induced O_2^- production in human neutrophils via CGRP receptor stimulation resulting in the attenuation of IP_3-induced Ca^{2+} mobilization. As for the main mechanism involved, IP_3-IP_3 receptor coupling and/or subsequent IP_3-induced transient Ca^{2+} release from intracellular Ca^{2+} stores are affected by CGRP probably via cAMP-dependent protein kinase-mediated phosphorylation of IP_3 receptors. Thus, CGRP, when co-released with substance P,or even when exogenously applied, may play an important role in regulating the inflammatory response of neutrophils to substance P.

References

Brain, S.D. and T.J. Williams, 1988, Substance P regulates the vasodialator activity of calcitonin gene-related peptide, Nature 335, 73.

Borzak, S., R.A. Kelly, B.K. Kramer, Y. Matoba, J.D. Marsh and M. Reers, 1990, In situ calibration of fura 2 and BCECF fluorescence in adult rat ventricular myocytes, Am. J. Physiol. 259, H973.

Chiba, T., A. Yamaguchi, T. Yamatani, A. Nakamura, T. Morishita, T. Inui, M. Fukase, T. Noda and T. Fujita, 1989, Calcitonin gene-related peptide receptor antagonist human CGRP8-37, Am. J. Physiol. 256, E331.

Cohen, H.J. and M.E. Chovaniec, 1978, Superoxide generation by digitonin-stimulated guinea pig granulocytes, J. Clin. Invest. 401, 1081.

Edwards, R.M., E.J. Stack and W. Trizna, 1991, Calcitonin gene-related peptide stimulates adenylate cyclase and relaxes intracerebral arterioles, J. Pharmacol. Exp. Ther. 257, 1020.

Gatto, C., R.C. Lussky, L.W. Erickson, K.J. Berg, J.D. Wobken and D.E. Johnson, 1989, Calcitonin and CGRP block bombesin- and substance P-induced increases in airway tone, J. Appl. Physiol. 66, 573.

Goodman E.C. and L.L. Iversen, 1986, Calcitonin gene-related peptide: novel neuropeptide, Life Sci. 38, 2169.

Greves, P.L., F. Nyberg, L. Terenius and T. Hokfelt, 1985, Calcitonin gene-related peptide is a potent inhibitor of substance P degradation, Eur. J. Pharmacol. 115, 309.

Hasui, M., Y. Hirabayashi and Y. Kobayashi, 1989, Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood, J. Immunol. Methods. 117, 53.

Hegerstrand, A., C.J. Dalsgaard, B. Jonzon, O. Larsson and J. Nilsson, 1990, Calcitonin gene-related peptide stimulated proliferation of human endothelial ells, Proc. Natl. Acad. Sci. USA 87, 3299.

Hidaka, H., M. Inagaki, S. Kawamoto and Y. Sasaki, 1984, Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C, Biochemistry 23, 5036.

Hylden, J.L.K. and G.L. Wilcox, 1981, Intrathecal substance P elicits a caudally directed biting and scratching behavior in mice, Brain Res. 217, 212.

Kase, H., K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato and M. Kaneko, 1987, K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinase, Biochem. Biophys. Res. Commun. 142, 436.

Kim, U.H., J.M. Kim and S.G. Rhee, 1989, Phosphorylation of phospholipase C-r by cAMP-dependent protein kinase, J. Biol. Chem. 264, 20167.

Kimball, E.S., 1990, Substance P, cytokines, and arthritis, Ann. NY Acad. Sci. 594, 293.

Konishi, M., A. Olson, S. Hollingworth and S.M. Baylor, 1988, Myoplasmic binding of Fura-2 investigated by steady-state fluorescence and absorbance measurements, Biophys. J. 54, 1089.

Lazarowski, E.R. and E.G. Lapetina, 1989, Activation of platelet phospholipase C by fluoride is inhibited by elevation of cyclic AMP, Biochem. Biophys. Res. Commun. 158, 440.

Lotz, M., D.A. Carson and J.H. Vaughan, 1987, Substance P activation of reumatoid synoviocytes: neural pathway in pathogenesis of arthritis, Science 235, 893.

McGillis, J.P., M. Mitsuhashi and D.G. Payan, 1990, Immunomoduration by tachykinin neuropeptides, Ann. NY Acad. Sci. 594, 85.

Meyer, T. and L. Stryer, 1990, Transient calcium release induced by successive increments of inositol 1,4,5-trisphosphate. Proc. Natl. Acad. Sci. USA 87, 3841.

Murthy, K.S. C. Severi, J.R. Grider and G.M. Makhlouf, 1993, Inhibition of IP_3 and IP_3-dependent Ca^{2+} mobilization by cyclic nucleotides in isolated gastric muscle cells, Am. J. Physiol. 264, G967.

Ohlen, A., N.P. Wiklund, M.G. Persson and P. Hedqvist, 1988, Calcitonin gene-related peptide desensitize skeletal muscle arterioles to substance P in vivo, Br. J. Pharmacol. 95, 673.

Piercey, M.F., P.J.K. Dobry, L.A. Schroeder and F.J. Einspahr, 1981, Behavioral evidence that substance P may be a spinal cord sensory neurotransmitter, Brain Res. 210, 407.

Richard, M.N., O. Cecconi, W.G. Roberts, A. Aruffo, R.J. Linhardt and M.P. Bevilacqua, 1993, Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation, Blood 82, 3253.

Serra, M.C., F. Bazoni, V.D. Bianca, M. Greskowiak and F. Rossi, 1988, Activation of human neutrophils by substance P, J. Immunol. 141, 2118.

Seybold, V.S., J.L.K. Hyden and G.L. Wilcox, 1982, Intrathecal substance P and somatostatin in rats: behaviors indicative of sensation, Peptides 3, 49.

Supattapone, P., S.K. Danoff, A. Theibert, S.K. Joseph, J. Steiner and S.H. Snyder, 1988, Cyclic AMP-dependent phosphorylation of brain inositol trisphosphate receptor decreases its release of calcium, Proc. Natl. Acad. Sci. USA 85, 8747.

Smith, C.D. and R. Snydermann, 1987, Guanin-nucleotide regulatory proteins in receptor-mediated polyphosphoinositide hydrolysis in human leukocytes, in: Methods in Enzymology: Cellular Regulators, eds. P.M. Conn and A.R. Means (Academic Press, London) p. 261.

Takenawa, T., J. Ishitoya and Y. Nagai, 1986, Inhibitory effect of prostaglandin E_2, forskolin, and dibutyryl cAMP on arachidonic acid release and inositol phospholipid metabolism in guinea pig neutrophils, J. Biol. Chem. 261, 1092.

Tanabe, T., H. Otani and C. Inagaki, 1994, Inhibitory effect of calcitonin gene-related peptide on substance P-induced superoxide production by neutrophils, Jpn. J. Pharmacol. 58 (Suppl. 1), 124.

Tanabe, T., H. Otani, L.-H. Bao, Y. Mikami, T. Yasukura, T. Ninomiya, R. Ogawa and C. Inagaki, 1996, Intracellular signalling pathway of substance P-induced superoxide production in human neutrophils, Eur. J. Pharmacol. 299, 187.

Tauber, A.I, 1987, Protein kinase C and the activation of the human neutrophil NADPH-oxidase, Blood 69, 711.

Timothy, D.H., P.-O. Berggren and A.L. Boynton, 1987, Heparin inhibits inositol trisphosphate-induced calcium release from permeabilized rat liver cells, Biochem. Biophys. Res. Commun. 149, 897.

Wang, X. and R.R. Fiscus, 1989, Calcitonin gene-related peptide increase cAMP, tension, and rate in rat atria, Am. J. Physiol. 256, R421.