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Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition

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1 We have examined the generation of intracellular reactive oxygen species (ROS) and release of histamine by rat peritoneal mast cells (RPMC) in response to stimulation with antigen (ovalbumin), compound 48/80, nerve growth factor (NGF) and substance P (SP).

2 We have also examined the effects of the non-specific nitric oxide synthase inhibitor, L-NAME (100 μM) upon the release of histamine and generation of intracellular ROS in response to the named secretagogues.

3 Ovalbumin (100–1000 μg ml^{-1}), compound 48/80 (0.1–100 μg ml^{-1}), NGF (0.1–100 μg ml^{-1}), and SP (5–50 μM), caused a concentration-dependent release of histamine from RPMC.

4 Ovalbumin (1 ng ml^{-1}–0.1 μg ml^{-1}), compound 48/80 (1–100 μg ml^{-1}), NGF (1 pg ml^{-1}–1 μg ml^{-1}), and SP (0.005–50 μM) caused a concentration-dependent generation of intracellular ROS by RPMC.

5 Pre-incubation of RPMC with L-NAME (100 μM) caused a significant enhancement of both histamine release and intracellular ROS from RPMC in response to ovalbumin, compound 48/80, NGF and SP.

6 Our data demonstrate that NGF, SP and ovalbumin are capable of causing intracellular ROS generation by RPMC at lower concentrations than those causing significant histamine release and we speculate that this may contribute to the activation of cytokine production.

7 The data also show that NO modulates histamine release, and ROS generation in response to the secretagogues used. This may have significance in pathologies where NO synthesis is decreased, leading to an increased activation of mast cells.

Keywords: Antigen; nerve growth factor; neurogenic inflammation; nitric oxide; rat peritoneal mast cells; reactive oxygen species; substance P

Abbreviations: DCF, dichlorofluoroscin; DCF-DA, dichlorofluorscein-diacetate; $EC_{1/2\ max}$, the concentration of secretagogue which causes half the maximal obtained release; NGF, nerve growth factor; OPT, o-phthaldialdehyde; PBS, phosphate buffered saline; PS, L-α-phosphatidylserine; ROS, reactive oxygen species; RPMC, rat peritoneal mast cells

Introduction

Mast cells have been implicated in many biological responses, including allergy, host responses to parasites and neoplasms, acute and chronic inflammation, tissue remodelling and wound healing (Metcalfe *et al*., 1997). Mast cells have been demonstrated to have a close microanotomical relationship with nerve fibres which stain positive for SP, calcitonin gene-related peptide (CGRP), and vasoactive intestinal peptide (VIP) in rat mesentery (Crivellato *et al*., 1991) and rat dura mater (Dimitriadou *et al*., 1997), and are found in close apposition with nerve growth factor receptor (NGFr)-positive nerve fibres in human skin (Liang *et al*., 1998). Therefore, recent interest has focused upon the apparent cross talk between mast cells and neuronal cells, which may be an important regulatory process in normal and inflammatory conditions.

Rat peritoneal mast cells (RPMC) generate intracellular reactive oxygen species (ROS) following incubation with gold compounds, D-penicillamine (in the presence of copper ions; Wolfreys & Oliviera, 1997), compound 48/80 and calcium ionophore, A23187 (Niu *et al*., 1996). Reactive oxygen species have also been shown to enhance mast cell histamine release (Gushchin *et al*., 1990; Wolfreys & Oliviera, 1997). In contrast nitric oxide (NO) has an inhibitory effect upon mast cell histamine release. Nitric oxide donors such as sodium nitroprusside inhibit mast cell histamine release (Mannaioni *et al*., 1991), and nitric oxide synthase (NOS) inhibitors, such as N^G-monomethyl-L-arginine (MeArg), increase histamine release (Mannaioni *et al*., 1991; Salvemini *et al*., 1991a,b).

Rat peritoneal mast cells (RPMC) represent a population of rat mast cell protease-1 (RMCP-1) positive cells, which release histamine in response to NGF (0.1–10 μg ml^{-1}) (Pearce & Thompson, 1986) and SP (Johnson & Erdos, 1973). However, under normal physiological conditions in humans, the plasma concentration of NGF is approximately 3 pg ml^{-1}, and this is significantly enhanced in patients suffering from allergic diseases such as asthma (Bonni *et al*., 1996). Therefore, the concentrations of NGF and SP which have been shown to cause histamine release in RPMC *in vitro* may not reflect values attained *in vivo*. In the present study we have measured intracellular ROS generation in RPMC induced by antigen, SP and NGF. We have also investigated the effects of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) upon histamine and ROS release in response to these stimuli. A preliminary account of this work has been presented to the British Pharmacological Society (Brooks *et al*., 1998).

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Methods

Collection of peritoneal cells

Male Wistar rats (300–400 g) were killed by inhalation of carbon dioxide. A mixed peritoneal cell population was obtained by lavage of the peritoneal cavity with heparinized (5 U ml^{-1}) HEPES buffered Locke's (HBL). Cells were recovered by centrifugation (200 × *g*, 4°C, 5 min) and washed twice (200 × *g*, 4°C, 5 min) with ice-cold HBL solution before use as either unpurified cell suspensions or for further purification.

Purification of RPMC

RPMC were purified by centrifugation at 450 × *g* (4°C, 15 min) on 38% w v^{-1} bovine serum albumin (BSA) in 0.9% w v^{-1} NaCl. Mast cells were recovered in the pellet, and all other cells remained at the interface between BSA and HBL. The BSA and unwanted cells were carefully removed, and the mast cells washed twice in ice cold HBL (200 × *g*, 4°C, 5 min). Following washing a sample of mast cells (97.5% purity ($n = 6$)) was stained with toluidine blue, and counted on a Neubaeur haemocytometer. Following counting RPMC were resuspended at 10^6 cells ml^{-1} in vehicle.

Sensitization procedure

In experiments where mast cells were challenged with antigen, male Wistar rats (250–300 g body weight) were sensitized with ovalbumin as described by Underwood *et al.* (1997). Briefly, rats were injected intraperitoneally with 1 ml ovalbumin (OA; 10 μg ml^{-1}) and aluminium hydroxide ($AL(OH_3)$; 100 mg ml^{-1}) on day 0. This injection was repeated on day 12. Peritoneal mast cells were harvested, as described previously, on day 21 and used without purification.

Measurement of NO release

Nitric oxide release by unstimulated RPMC was measured to monitor the efficacy of the NOS inhibitor, L-NAME. The assay was based upon a modified Griess assay (Verdon *et al.*, 1995). Briefly, samples, and nitrite standards were incubated in the presence of nitrate reductase (1 U ml^{-1}), glucose-6-phosphate dehydrogenase (200 U ml^{-1}), and glucose-6-phosphate (500 μM). NADPH (1 μM final concentration) was added to start the reaction. Following conversion of nitrate to nitrite, the 'total nitrite' was measured by the addition of 100 μl of sulphanilamide (0.1% w v^{-1} in 3 N HCl) and 100 μl of napthylethylenediamne (NED) (0.01% w v^{-1}), and the absorbance read at 540 nm on a 96 well plate reader (Labsystems).

Measurement of histamine

Histamine release from RPMC was measured using the alkaline condensation reaction of o-phthaldialdehyde (OPT) with histamine as described by Anton & Sayre (1969). Briefly, samples were incubated in the presence of NaOH (1 M), and OPT (5 mg ml^{-1}) for exactly 4 min. The reaction was quenched by the addition of 200 μl of citric acid (2 M), and the fluorescence measured on a Perkin Elmer LS-5B fluorimeter, with $\lambda_{excitation} = 345$ nm and $\lambda_{emission} = 441$ nm.

Measurement of intracellular reactive oxygen species

This was based upon the method of LeBel & Bondy (1990). Briefly, in experiments where intracellular ROS were measured, RPMC were resuspended in phosphate buffered saline (PBS), supplemented with Ca^{2+} (1 mM) and glucose (5 mM), and incubated with dichlorofluoroscein diacetate (DCF-DA; 1.25 μM final concentration) in 0.1% methanol for 15 min at 37°C. Following incubation mast cells were washed (200 × *g*, 4°C, 5 min) and resuspended in PBS prior to addition of the test compound. Basal ROS generation was measured from cells incubated in vehicle alone. Fluorescence was measured on a Perkin Elmer LS-5B fluorimeter with $\lambda_{excitation} = 488$ nm, and $\lambda_{emission} = 525$ nm, and intracellular ROS generation was expressed as the % ROS generated above the basal.

Investigation into the effects of the NOS inhibitor, L-NAME upon RPMC histamine release, and ROS generation

RPMC suspensions were incubated with L-NAME (100 μM final concentration) or vehicle alone for 30 min prior to treatment with secretagogue. Histamine and NO released by the RPMC were determined as previously described. In experiments where ROS were measured, cells were incubated for 15 min with PBS + L-NAME or vehicle alone, before the addition of DCF-DA, and incubation for a further 15 min at 37°C. Following washing (200 × *g*, 4°C, 5 min) cells were resuspended in PBS alone or PBS supplemented with L-NAME (100 μM).

Challenge of cells with compound 48/80, SP, NGF or ovalbumin

Aliquots of cells (500 μl; 250 μl for NGF studies) and secretagogue were pre-warmed separately to 37°C. Compound 48/80 (0.03–100 μg ml^{-1}; 500 μl), SP (0.005–50 μM; 500 μl), NGF (10^{-6}–10 μg ml^{-1}; 250 μl) + of L-α-phosphatidyl-serine (PS) (50 μg ml^{-1}; 50 μl) or ovalbumin (0.001–1000 μg ml^{-1}; 500 μl) were added to each sample of cells to give the final concentration of secretagogue shown. After a further 15 min incubation at 37°C, the reaction was stopped by the addition of ice cold HBL up to a volume of 2 ml, and the tubes were placed on ice. Samples were centrifuged at 200 × *g*, at 4°C for 5 min, and 1 ml of supernatant was removed into a separate tube, and diluted with 1 ml of ultra pure (Milli-Q) water. Spontaneous histamine release was measured from cells incubated with vehicle alone, and total releasable histamine was obtained from samples of cells which were sonicated for 60 s at 5 amps using a Heat Systems XL sonicator. Samples were then assayed for the presence of histamine by the OPT condensation reaction (Anton & Sayre, 1969). A separate sample was removed for measurement of NO by the Griess assay.

Challenge of cells for the measurement of intracellular ROS

In experiments where intracellular ROS were being measured 250 μl of cells (10^6 cells ml^{-1}) were pre-warmed in a 0.7 ml quartz cuvette, and the relevant test compound added in a 1 : 1 ratio with the cells as described above. DCF fluorescence was measured and compared to the fluorescence obtained from cells incubated with just vehicle alone. Results were expressed as the percentage increase in DCF fluorescence above the basal.

Statistical analysis

Results are expressed as the mean ± s.e.mean. Differences between means were tested for significance with a Student's

t-test for paired data. Differences were considered significant when the probability was $P<0.05$.

Materials

The following were obtained from Sigma-Aldrich (Poole, Dorset, U.K.): compound 48/80, SP, nerve growth factor, ovalbumin, L-α-phosphatidylserine, HEPES, $CaCl_2$, NaCl, KCl, glucose, heparin, bovine serum albumin, nitrate reductase, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, sulphanilamide, napthylethylenediamine, o-phthaldialdehyde, histamine, and phosphate buffered saline tablets. $AlOH_3$, NaOH, and citric acid were obtained from BDH chemicals (Poole, Dorset, U.K.). The NOS inhibitor, L-NAME, and its enantiomer, D-NAME, were obtained from Calibiochem, and the dichlorofluoroscein-diacetate was obtained from Molecular Probes (OR, U.S.A.).

Results

Release of histamine

Following purification mast cells were 97.5±3% pure ($n=6$), and were resuspended at 10^6 cells ml^{-1}. Basal histamine release was 6.1±1.05% ($n=6$) total releasable histamine, as determined by the OPT alkaline condensation reaction. However, basal histamine release in RPMC sensitized to ovalbumin was significantly ($P<0.05$) increased to 26.5±1.01% ($n=6$) of total histamine. Treatment of unsensitized RPMC with ovalbumin did not significantly ($P<0.05$) enhance histamine release above the basal level (6.1±1.05% ($n=6$)) for basal compared to 6.3±0.67% total histamine for ovalbumin treated cells ($n=3$). Incubation of ovalbumin sensitized cells with BSA (0.001–1000 μg ml^{-1}) did not cause any significant ($P>0.05$) release of histamine above the basal for these cells (25.2±0.45% total histamine ($n=3$)). In unsensitized cells, compound 48/80 (0.1–100 μg ml^{-1}; $n=6$), NGF (0.1–100 μg ml^{-1}; $n=6$), and SP (5–50 μM; $n=6$) caused significant ($P<0.05$) concentration-dependent release of histamine from RPMC compared to basal. Maximal release of histamine obtained was 40±4.5% ($n=6$) total histamine at 100 μg ml^{-1} 48/80 (Figure 1A), 87±1% ($n=6$) at 10 μg ml^{-1} NGF (Figure 1B), and 50±0.2% ($n=6$) total histamine at 50 μM SP (Figure 1C). $EC_{1/2\ max}$ values were 251±3 ng ml^{-1}, 708±7 ng ml^{-1}, 25±1 μM for 48/80, NGF and SP respectively (Figure 1). In antigen sensitized cells, ovalbumin caused a concentration-dependent increase in histamine release, with maximal release obtained being 60±3.8% ($n=6$) total histamine at 1000 μg ml^{-1} ovalbumin, and $EC_{1/2\ max}$=710.7±50 μg ml^{-1} (Figure 1D).

Generation of intracellular ROS

Unstimulated RPMC were found to generate a low level of basal ROS, and this was used as a baseline to which all other results were compared. Compound 48/80 (1–100 μg ml^{-1}), NGF (1 pg ml^{-1}–1 μg ml^{-1}), SP (0.005–50 μM), and ovalbumin (1 ng ml^{-1}–1 μg ml^{-1}), caused a concentration-dependent generation of ROS with maxima of 404±170% over basal ($n=6$) at 100 μg ml^{-1}, 115±14.5% over basal ($n=6$) at 0.1 μg ml^{-1}, 150.6±31% over basal ($n=6$) at 50 μM, and 260±50% over basal ($n=6$) at 1 μg ml^{-1} respectively. The $EC_{1/2\ max}$ values were 3.93±0.5 μg ml^{-1}, 0.185±0.005 ng ml^{-1}, 731±2 nm, and 0.0152 μg ml^{-1} respectively (Figure 2), and for NGF, SP, and OA, but not compound 48/80, were significantly lower ($P<0.05$) than the threshold concentrations of secretagogue required to cause histamine release from RPMC.

The effects of the NOS inhibitor, L-NAME

L-NAME (100 μM), but not its enantiomer, D-NAME (100 μM) caused a significant ($P<0.05$) reduction in basal NO release measure as total nitrite by the Griess assay from RPMC. At the basal level, total nitrite was determined as 13±0.5 μM, and was reduced to below the sensitivity of the assay in the presence of L-NAME (100 μM).

In the presence of L-NAME (100 μM), but not its enantiomer, D-NAME (100 μM), ROS generation by RPMC induced by compound 48/80, NGF, SP, and ovalbumin was significantly ($P<0.05$) increased (Figure 2). Maximal ROS generation was increased to 577±38% ($n=6$) at 100 μg ml^{-1} 48/80, 425±50% ($n=6$) at 0.1 μg ml^{-1} NGF, 335±49% ($n=6$) at 50 μM SP, and 400±15% ($n=6$) at 1 μg ml^{-1} OA. Histamine release by RPMC at the basal level, and in response to compound 48/80, NGF, SP, and ovalbumin, was also increased significantly ($P<0.05$) by pre-incubation of cells with L-NAME (100 μM), but not its enantiomer, D-NAME (100 μM) (Figure 1A–D).

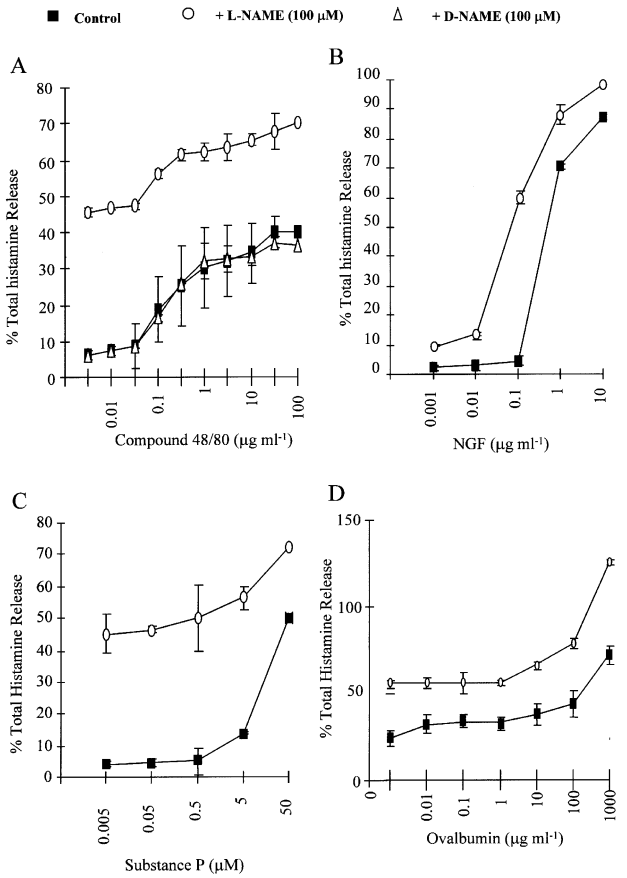

Figure 1 Concentration-response relationships for histamine release from rat peritoneal mast cells in response to (A) compound 48/80, (B) nerve growth factor, (C) substance P, and (D) ovalbumin in the absence and presence of the non-selective nitric oxide synthase inhibitor, L-NAME (100 μM) or its inactive enantiomer, D-NAME (100 μM). Each point is the mean±s.e.mean of six experiments.

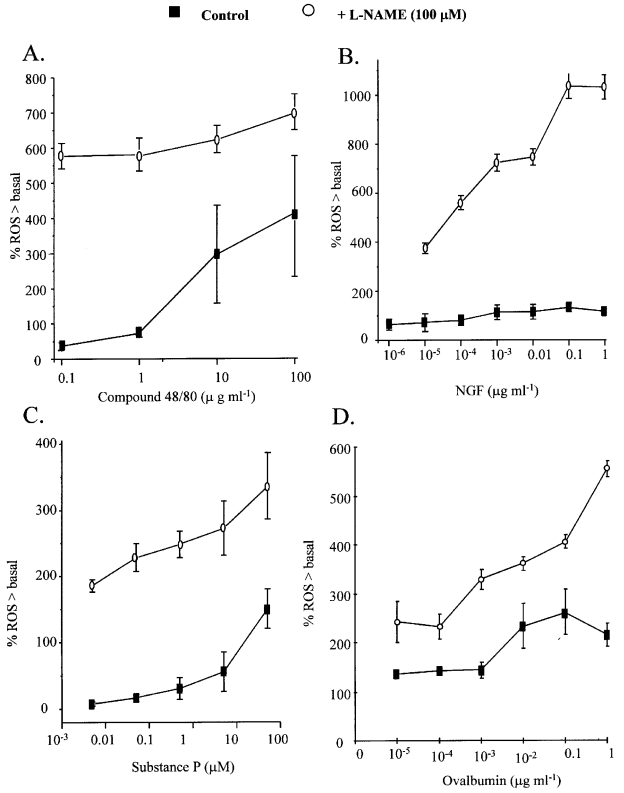

Figure 2 Concentration-response relationships for intracellular reactive oxygen species generation in the absence and presence of the non-selective nitric oxide synthase inhibitor, L-NAME (100 μM), measured as DCF fluorescence at 488 and 525 nm, from rat peritoneal mast cells in response to (A) compound 48/80, (B) nerve growth factor, (C) substance P, and (D) ovalbumin. Each point is the mean $\pm$ s.e.mean of six experiments.

Discussion

Mast cells are classically associated with hypersensitivity reactions involving the interaction of allergens and cell bound IgE (Metcalfe *et al*., 1997). While much evidence supports a role for mast cells in allergic reactions, recent research has focused upon the observation that these cells respond functionally to a large range of neuroactive compounds, including neuropeptides, and neurotrophins (Purcell & Atterwill, 1995). This has led to the theory that mast cells may play a central role in cross-talk between the immune and nervous system (Purcell & Atterwill, 1995).

In the present report we have shown that the neurotrophin, NGF, the neuropeptide, SP, the mast cell activator, compound 48/80, and the immunological stimulus, ovalbumin caused the generation of intracellular ROS from RPMC in a concentration-dependent manner. Our data extend the findings of Wolfreys & Oliviera (1997), and Niu *et al*. (1996) who have reported the generation of ROS by RPMC in response to D-penicillamine, and A23187 by demonstrating the generation of intracellular ROS in response to physiologically relevant secretagogues.

In the present study, mast cells have been shown to generate intracellular reactive oxygen species in response to a variety of secretagogues. However, the measurement of intracellular ROS using the DCF assay (LeBel & Bondy, 1990) does not provide information as to the exact species of ROS being measured in RPMC. However, a number of studies have demonstrated that histamine releasing stimuli can cause release of extracellular superoxide (Scinetti *et al*., 1984; Salvenimi *et al*., 1991b). In addition, changes in the levels of peroxidation products in RPMC have been noted following stimulation (Guschin *et al*., 1990). RPMC have been demonstrated to stain positively for components of NADPH oxidase (Fukuishi *et al*., 1997), and superoxide in the granules (Frederiks *et al*., 1998). Therefore further experimentation would be necessary to identify the specific nature of intracellular ROS generation by mast cells.

The secretagogues used in the present study also caused a concentration-dependent release of histamine, confirming published data (Johnson & Erdos, 1973; Pearce & Thompson, 1986). In preliminary experiments where ROS generation was measured in response to NGF, substance P, and ovalbumin it was found that the ROS generation was already maximal at the concentrations which caused significant histamine release. Therefore, it was decided to test the effects of lower concentrations of these secretagogues. Thus, our data demonstrate that NGF, SP, and ovalbumin, but not compound 48/80 were able to cause the generation of intracellular ROS at concentrations of secretagogue which did not cause histamine release. In contrast, compound 48/80 only caused the generation of ROS at concentrations of secretagogue which caused histamine release. These differences between compound 48/80, and the other secretagogues may reflect differences in the mechanisms by which mast cells are activated by the compounds used.

Compound 48/80 has been shown to penetrate cellular membranes and directly activates G proteins by interaction of an aromatic ring within compound 48/80 with the COOH-terminal domain of the α subunit of a G protein involved in mast cell exocytosis (reviewed in Metcalfe *et al*., 1997).

Substance P normally exerts its effects through the NK_1-receptor. However, SP induced histamine release by mast cells has been shown to be a non-receptor mediated event (Maggi, 1997). The data presented in the present study show that ROS generation is caused by lower concentrations of SP than those required for histamine release. Furthermore, we have shown that SP-induced ROS generation is mediated by NK_1-receptors (Brooks & Whelan, 1999). Therefore, we can conclude that in RPMC, SP-induced intracellular ROS generation occurs *via* a receptor mediated mechanism, whereas histamine release does not.

NGF has been shown to elicit effects in the nervous system through either the high affinity NGF receptor, TrkA, or the low affinity p75 receptor. Mast cells express the TrkA receptor (Horigome *et al*., 1994), and this receptor is thought to mediate NGF induced histamine release by mast cells. The data presented in the present report show that NGF induced ROS generation occurs at lower concentrations than those required for histamine release. These findings may indicate that ROS generation may be receptor mediated whilst histamine release is not. Like substance P, this would explain the differences seen between thresholds of secretagogue required for ROS generation and histamine release. However further experimentation would be necessary, with TrkA receptor antagonists to confirm which of the effects of NGF are mediated by this receptor. However, since these antagonists are not readily available this could not be performed at this time.

Antigen activates mast cells *via* interaction with IgE bound to $FC_\varepsilon RI$ (Metcalfe *et al*., 1997). In the present study, ovalbumin, like SP and NGF, caused ROS generation at lower concentrations than required for histamine release. Both effects were dependent upon sensitization, since ovalbumin but not bovine serum albumin caused mediator release in sensitized RPMC, and ovalbumin did not have a significant

effect upon mediator release from non-sensitized RPMC. The relatively high concentrations of antigen (1000 μg ml^{-1}) required to cause histamine release from sensitized RPMC may reflect the strain of rat used in these experiments. RPMC from Wistar rats, the strain used in the present study, have been demonstrated to express less IgE per cell than RPMC obtained from Brown Norway rats (Sugimoto *et al*., 1998), and therefore are consequently less reactive to challenge by antigen. These findings may suggest that intracellular ROS generation by RPMC is more readily detectable than histamine release. It is widely accepted in the literature that exogenous free radicals can activate mast cells (Wolfreys & Oliviera, 1997; Bello *et al*., 1998). and therefore the production of ROS by activated RPMC may represent a priming of mast cells to degranulate.

The concentrations of NGF and SP which cause ROS generation in RPMC elicit other physiological effects. Substance P (5 pM) has been shown to trigger outwardly rectified Cl^- currents in RPMC, without causing degranulation, and repeated exposure of mast cells to SP at this concentration appear to 'prime' mast cells to degranulate (Janiszewski *et al*., 1994). In man the circulating serum concentration of NGF is 3 pg ml^{-1}, in normal patients, and is significantly increased in patients with allergic conditions such as asthma (>48 pg ml^{-1}; Bonni *et al*., 1996). We have demonstrated that both NGF (1 pg ml^{-1}) and SP (5 nM) are able to cause a significant generation of intracellular ROS by RPMC, and that the concentrations used in the present study may be physiologically relevant. Therefore, ROS generation may demonstrate a mechanism of modulating mast cell function under physiological conditions, ready to respond any increases in circulating NGF or SP which may occur in pathological conditions such as during neurogenic stimulation. Increased circulating concentrations of NGF have been reported in association with the upregulation of SP production by nerve fibres (Aloe *et al*., 1997), suggesting a relationship between NGF, SP and mast cells which is important in pathological conditions (Aloe *et al*., 1997).

The present study demonstrates that pre-incubation of RPMC with the NOS inhibitor L-NAME (100 μM), but not its enantiomer D-NAME, increased the release of histamine from unstimulated RPMC and RPMC incubated with compound 48/80, NGF, SP, and ovalbumin. The generation of intracellular ROS by unstimulated and secretagogue stimulated RPMC was also enhanced markedly by pre-incubation of RPMC with L-NAME (100 μM). Concentrations of L-NAME significantly higher than the concentration used in this study (1 mM compared to 100 μM) have been previously shown to inhibit NOS (Filep *et al*., 1996). The effects of NOS inhibitors, and NO donors upon histamine release in responses to antigen (Bidri *et al*., 1997), and the non-immunological stimuli, including compound 48/80 and A23187 (Salvemini & Botting, 1993; Salvemini *et al*., 1991a,b) are well documented. The work presented in the present study further extends the phenomenon of mast cell modulation by NO to secretagogues which are relevant to pathophysiological situations. The modulation of antigen, NGF, or SP induced ROS generation shown in the present paper, by NO may have important implications in a variety of physiological/pathological states. The presence of accessory cell derived NO in the micro-environment may serve as an inhibitor of mast cell function (Eastmond *et al*., 1997), and a reduction in extracellular NO may increase the sensitivity of mast cells to other stimuli, such as neurotrophins or neuropeptides as well as antigens. However the mechanism by which NO modulates mast cell activation is still unclear. Salvenimi *et al*. (1991a) describe a significant increase in the basal levels of cyclic GMP following mechanical activation of mast cells, incubation with LPS or the NO-donor sodium nitroprusside. Incubation of mast cells with indomethacin-treated human washed platelets inhibited thrombin-induced platelet aggregation and this effect was potentiated by superoxide dismutase (SOD) (Mannaioni *et al*., 1991). However, the first of these reports used RPMC which contained high numbers of contaminating cells, used non-mast cell specific stimuli, and did not test inhibitors of guanylate cyclase. In contrast, DeSchoolmeester *et al*. (1999) have demonstrated that the action of NO upon mast cells does not occur either through cyclic GMP dependent mechanism, or through interaction with superoxide to form peroxynitrite. NO is known to interact with a variety of small G proteins (Lander, 1996) and thus may affect activation of G proteins involved in exocytosis of mast cell granules. Alternatively NO has been demonstrated to inhibit the formation of superoxide by neutrophils through a direct action upon the NADPH oxidase complex (Clancy *et al*., 1992). Therefore it is possible that this may occur in mast cells, the increase in histamine release by L-NAME treated RPMC may be mediated through the observed increased production of ROS. The inhibition of mast cell activation afforded by NO is a matter of debate, with some authors showing a lack of effect of NO donors and NOS inhibitors (Lau & Chow, 1999). However the present study clearly shows that L-NAME causes an increase in degranulation and ROS generation in response to a variety of different stimuli. Our findings are further confrimed by DeSchoolmeester *et al*. (1999), who report a direct effect of NO upon mast cell activation, with the NOS inhibitor L-NMMA enhancing secretion, and the NO donor, SNOG inhibiting anti-IgE induced secretion.

In summary the data presented in the present report show that mast cells generate intracellular ROS in response to NGF, SP, and antigen (ovalbumin) without con-commitant histamine release at concentrations of secretagogue which are effective in a wide range of physiological situations. The generation of intracellular ROS is enhanced by inhibition of NO production, and thus any condition that causes a decrease in NOS activity may contribute to either an acute or chronic inflammation *via* the increased activation of mast cells within the localized area. These findings may have some relevance in chronic inflammatory conditions involving antigen, neuropeptide or neurotrophin activation of mast cells.

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