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J Clin Invest. 1994;94(5):1974-1980. https://doi.org/10.1172/JCI117549.

Research Article

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Effects of Relaxin on Mast Cells

In Vitro and In Vivo Studies in Rats and Guinea Pigs

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Abstract

The results of the current study demonstrate that relaxin inhibits histamine release by mast cells. This effect is related to the peptide concentrations, and could be observed in both isolated rat serosal mast cells stimulated with compound 48/80 or calcium ionophore A 23187, and in serosal mast cells isolated from sensitized guinea pigs and challenged with the antigen. The morphological findings agree with the functional data, revealing that relaxin attenuates calcium ionophore–induced granule exocytosis by isolated rat serosal mast cells. Similar effects of relaxin have also been recognized in vivo by light microscopic and densitometric analysis of the mesenteric mast cells of rats which received the hormone intraperitoneally 20 min before local treatment of the mesentery with calcium ionophore. Moreover, evidence is provided that relaxin stimulates endogenous production of nitric oxide and attenuates the rise of intracellular Ca^{2+} concentration induced by calcium ionophore. The experiments with drugs capable of influencing nitric oxide production also provide indirect evidence that the inhibiting effect of relaxin on mast cell histamine release is related to an increased generation of nitric oxide. It is suggested that relaxin may have a physiological role in modulating mast cell function through the L-arginine–nitric oxide pathway. (*J. Clin. Invest.* 1994. 94:1974–1980.) Key words: relaxin • mast cells • histamine • nitric oxide • intracellular calcium

Introduction

Relaxin (RLX)[1] is a peptide hormone produced mainly by the corpus luteum of pregnancy, with well-established effects on the female reproductive organs (1) and the mammary gland (2). There is increasing evidence that RLX is more than a hormone of pregnancy, especially considering its ability to influence cardiovascular function (3). Since the earlier studies carried out in our laboratory on the mammary gland, mesenteric vessels, and pigeon crop sac (4–6), as well as studies of other authors on the rat uterus (7), RLX was shown to cause striking enlargement of microvessels. Interestingly, our findings on the mammary gland of mice given RLX systemically (8) revealed that vasodilation was not accompanied by a concurrent release of granules from mast cells (MCs), thus suggesting that the action of RLX is independent of the release of vasoactive mediators contained in the MC granules, such as histamine. Moreover, studies on the gilt uterus (9) showed that coadministration of H1 and H2 histamine receptor antagonists did not reduce the ability of RLX to increase blood vessel permeability. Therefore, a possible interaction of RLX with potential regulators of vascular function other than histamine can be postulated. In fact, it is known that MCs, when activated, can release a number of chemical mediators even in the absence of granule exocytosis (10). In this context, much interest has been focused in recent years on vasodilation induced by the release of endothelium-derived relaxing factor (11), identified by some authors (12) as nitric oxide (NO). NO is synthesized from the terminal guanidine nitrogen atom(s) of L-arginine in several cell types, including MCs (13–15). In these cells, NO produced endogenously has also been shown to inhibit histamine release (16).

The aim of this study was to investigate whether RLX influences the release of histamine from MCs and, if this is the case, to correlate the effect of RLX with granule exocytosis, generation of NO, and intracellular Ca^{2+} levels. To address these points, we carried out in vitro and in vivo studies using rat serosal MCs stimulated with different secretagogues, and guinea pig serosal MCs challenged with immunologic stimuli. The heterogeneity of MCs is a well known phenomenon (17), called to explain the differential responses to the various stimulants of MCs from different animal species, and from different organs even within the same species. For these reasons, we have selected nonimmunologic (compound 48/80 and calcium ionophore A 23187) and immunologic (antigen) stimuli in the rat and guinea pig, using the same MC type: i.e., serosal MCs.

Methods

Chemicals. The chemicals used to prepare the solution for the fluorimetric assay were of Suprapur quality (E. Merck, Darmstadt, Germany); *o*-phthaldialdehyde was obtained from BDH Chemicals, Ltd. (Poole, UK); Ficoll from Pharmacia (Uppsala, Sweden); L-arginine (L-Arg) (free base), D-arginine (D-Arg) (free base), indomethacin, calcium ionophore A23187, compound 48/80, SOD (from bovine erythrocytes), DMSO, and Quin-2 acetoxymethyl ester (Quin-2 AM) were obtained from Sigma Chemical Co. (Poole, UK); sulfanilic acid, N-(1-naphthyl)ethylenediamine dihydrochloride, sodium nitrite, and *N*ω monomethyl-L-arginine (MeArg) from Ultrafine Chemical Ltd. (Manchester, UK); BSA and egg albumin from Boehringwerke (Marburg/Lahn, Germany). *N*ω-nitro-L-arginine methyl ester (L-NAME) and *N*ω-nitro-D-arginine methyl ester (D-NAME) were from Calbiochem-Inalco (Milano, Italy). Pure porcine RLX, prepared according to Sherwood and O'Byrne (18), was a generous gift from Dr. O. D. Sherwood.

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Received for publication 14 January 1994 and in revised form 14 June 1994.

1. *Abbreviations used in this paper:* $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; D-Arg, D-arginine; D-NAME, Nω-D-arginine methylester; L-Arg, L-arginine; L-NAME, Nω-L-arginine methylester; MC, mast cell; MeArg, Nω monomethyl-L-arginine; Quin-2 AM, Quin-2 acetoxymethylester; RLX, relaxin.

J. Clin. Invest.

0021-9738/94/11/1974/07 $2.00
Volume 94, November 1994, 1974–1980

Isolation of rat serosal mast cells. Male Wistar albino rats, weighing 200–300 g, were used. Rats were anesthesized in an atmosphere of ethyl ether and air and then killed by decapitation. Saline (154 mM NaCl), adjusted to pH 6 with Sörensen phosphate buffer, was injected into the peritoneal and pleural cavity of the rats. The fluid was then collected, and isolation of MCs from other cell types in the peritoneal and pleural washings was achieved by density gradient centrifugation in Ficoll as previously described (19) or by using a Beckman elutriation system (Rotor JE-6, 4.5-ml chamber size) according to Glick et al. (20). Evan's buffer (138 mM NaCl; 2.7 mM KCl; 6.5 nM glucose; 2.5 nM Tris-HCl; and 0.1% BSA) was used as elutriation fluid. The elutriation was carried out at 5°C at a rotor speed of 2400±10 rpm for 1 h and at a flow rate of 15 ml/min. Several fractions were collected by successive increments in flow rate (21). A final yield of 90–95% pure MCs was achieved. The final count was adjusted to 10^6 cells/ml. Indomethacin (10 μM) was added to MC suspension to inhibit the formation of cyclooxygenase products. Finally, the cells were suspended in Sörensen or Evan's buffer, which were also used as incubation media.

Isolation of mesenteric mast cells from sensitized guinea pigs. Male guinea pigs, weighing 300–400 g, were sensitized by intraperitoneal injections of 1 ml of 1% egg albumin (22), given on two consecutive days. The animals were sacrificed 18–20 d after sensitization, and MCs were obtained according to the method of Pearce and Ennis (23), with modifications. In particular, the mesentery was washed thoroughly for 30 min with a solution having the following composition: 137 mM NaCl; 5.6 mM glucose; 2.7 mM KCl; 0.3 mM NaH_2PO_4; 1 mM $CaCl_2$; 10 mM Hepes; and 1 mg/ml collagenase. MCs were then isolated according to Masini et al. (24). Briefly, MCs were separated from the other cell types with Ficoll gradient and washed twice with a medium containing: 145 mM NaCl; 0.9 mM $CaCl_2$; 2.4 mM KCl; 0.45 mM $MgCl_2$; 0.1% glucose; 0.1% HSA, and adjusted to pH 7.4 with Sörensen phosphate buffer. Finally, the cells were suspended in the same fluid, added with indomethacin (10 μM), which was also used as incubation medium. The final count was adjusted to 10^6 cells/ml.

Determination of histamine release. This was performed in isolated rat and guinea pig serosal MCs. MC incubation was carried out in 10-ml test tubes at 37°C in a metabolic shaker, with air as gas-phase. The cells (5×10^4 per tube) were incubated for 30 min in 2 ml of medium alone or added with 1, 10, or 100 ng/ml RLX. The rat MCs were then challenged with compound 48/80 (0.05 μg/ml) or calcium ionophore A23187 (10^{-6} M); the guinea pig MCs were stimulated with the specific antigen (100 μg/ml egg albumin). The reaction was stopped by chilling the tubes in an ice-water bath. Cells were then separated from the medium by centrifugation (400 *g* for 5 min at 4°C) and supernatants and pellets were used for histamine assays.

Histamine was measured fluorimetrically using the method of Shore et al. (25), as modified by Kremzner and Wilson (26). In the supernatants, *o*-phthaldialdehyde was added directly to the sample after alkalinization with 0.4 M NaOH. The same procedure was used for the pelleted cells, after extraction with 0.1 M HCl according to Bergendorff and Uvnas (27). The authenticity of histamine in the samples was demonstrated by recording the excitation and emission fluorescence spectra. Histamine release was expressed as a percentage of the total present in the cells plus supernatants. Spontaneous histamine release, less than 5%, was subtracted from all values.

Determination of nitric oxide production. This was performed on isolated rat serosal MCs. The amount of nitrites (NO_2^-), which are stable end-products of NO metabolism, was determined in the supernatants of cells incubated with no RLX (control), or with 10, 30, and 100 ng/ml RLX. In some experiments, the MC suspensions were preincubated for 60 min with MeArg (10^{-4} M) that is an inhibitor of NO-synthase. The amount of nitrites was measured spectrophotometrically by the Griess reaction. Briefly, the samples were allowed to react with the Griess reagent (aqueous solution of 1% sulphanylamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) to form a stable chromophore absorbing at 546 nm wavelength, in the presence of nitrate reductase (276 mU) and $NADPH^+$ (40 μM). The values were obtained by comparison with standard concentrations of sodium nitrite and expressed as net amounts of NO_2^- per μg of protein (28). The protein concentrations were determined by the Lowry method using BSA as standard (29).

Determination of histamine release following RLX and drugs affecting the L-arg–NO pathway. This was carried out on isolated rat serosal MCs. The cell suspensions were preincubated for 60 min with the NO-synthase inhibitor MeArg (10^{-4} M) or the NO-synthase inhibitor L-NAME (10^{-4} M), that is even more active than MeArg. The cell suspensions were then exposed to RLX (1, 10, and 100 ng/ml) and finally to A23187 calcium ionophore (10^{-6} M). In some experiments, the MCs were preincubated with: MeArg plus L-Arg (10^{-4} M), which is the natural substrate of NO-synthase; or MeArg plus D-Arg (10^{-4} M), which is the inactive enantiomer of L-Arg. The cells were then incubated with RLX (10 ng/ml) and finally exposed to the calcium ionophore. Since, after MC activation by calcium ionophore, superoxide anions (O_2^-) are generated (14, 30) that inactivate NO (31–33), some MC samples were preincubated with 50 IU/ml of SOD, a scavenger of O_2^- and a potent enhancer of NO activity during treatment with RLX and calcium ionophore.

Determination of intracellular calcium concentration. Since the release of histamine by MCs, as well as granule exocytosis, are known to be calcium-dependent processes (34, 35), we assayed whether RLX causes variations in intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$). To address this point, isolated rat serosal MCs were loaded for 1 h with 50 μM Quin 2 AM in 1% DMSO and then washed. Aliquots of MCs were placed in quartz cuvettes at 37°C under constant stirring, and fluorescence was measured at 492 nm with an excitation wavelength of 339 nm, using a spectro-fluorophotometer (RF-500; Shimadzu Corp., Osaka, Japan). Fluorescence was recorded continuously (36) and RLX (10 and 100 ng/ml) was added to the cells without any interruption in the recording.

Light and electron microscopy of isolated rat serosal mast cells. MCs were incubated for 30 min at 37°C as follows: incubation medium alone; medium added with 100 ng/ml RLX; medium added with 10^{-6} M calcium ionophore A23187; and medium added with RLX before challenge with calcium ionophore at the noted concentrations. After incubation, the cells were pelleted by centrifugation, fixed in cold 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 15 min at 4°C and then for additional 60 min at room temperature, and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at room temperature for 1 h (37). The cells were then dehydrated in a graded acetone series, passed through propylene oxide and embedded in Epon 812. Semithin sections, 2 μm thick, were stained with toluidine blue–sodium tetraborate, passed through an ascending ethanol series to obtain metachromatic staining of the MC granules, mounted in Permount and viewed in the light microscope. Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under an electron microscope (Elmiskop 102; Siemens Analytical X-Ray Instruments, Madison, WI) at 80 kV.

Light microscopy and computer-assisted cytophotometry of rat mesenteric mast cells in vivo. 24 male Wistar albino rats, weighing 200–300 g were used. In a first series of experiments, 16 rats were anesthesized in an atmosphere of ethyl ether and air, and divided into four groups of four animals each. Groups 1 and 2 were injected with 0.5 ml PBS i.p., pH 7.4; groups 3 and 4 were injected by the same route with 10 μg RLX dissolved in 0.5 ml PBS. 20 min later, the rats were sacrificed by prolonged anesthesia and then underwent laparotomy. In the rats of groups 1 and 3, the mesentery, still anchored to an ileal loop at one side and to its vascular root at the other side, was gently picked up and placed on a histological slide. In the rats of groups 2 and 4, an ileal loop was gently drawn out and the mesentery exposed at the surface of the peritoneal cavity. Calcium ionophore A23187 (10^{-6} M in 1 ml PBS) was dropped over the mesentery and left to act for 5 min. In each animal, the mesentery was then placed on a histological slide and dried until it was completely attached to the slide. In a second series of experiments, two additional groups of rats, four animals each, were used. Group 5 was treated with L-NAME, and group 6 with the inactive enantiomer D-NAME (38, 39). Drug solutions (0.5 g/liter), prepared freshly every day, were given in the drinking water for 10 d. Water intake was approximately 20 ml/d per animal, with no differences be-

tween the two experimental groups. On day 10, the rats of the two groups were anesthesized, treated for 20 min with RLX (10 μg in 0.5 ml PBS i.p.), and sacrificed by prolonged anesthesia. The mesenteries were exposed, treated for 5 min with calcium ionophore A23187 (10^{-6} M in 1 ml PBS), and placed on histological slides, as described above.

In all the groups of rats, once adhesion of the mesentery to the slide was achieved, the mesenteric root and the intestinal loop were resected. The specimens were fixed in 10% formalin in PBS for 4 h at room temperature, dehydrated in ethanol, and placed in xylene to solubilize fat. Subsequently, the specimens were rehydrated, stained with 0.1% toluidine blue in distilled water, and passed through an ascending ethanol series to obtain metachromatic staining of MC granules. After mounting in Permount, the specimens were used for light microscopic examination and computer-assisted cytophotometry. The latter was carried out on MCs lying in the thin areas of the mesentery containing the microvessel network, using a light microscope with a ×40 objective. Microscopic images of the MCs were registered through a CCTV television camera (Sony, Tokyo, Japan) applied to the light microscope, and interfaced with a Apple Macintosh LC II personal computer through a Videospigot card (Supermac, Sunnyvale, CA). The card allows for the light transmitted across the microscopic image to be determined and a digitized image to be reproduced on the basis of the values estimated. Measurements of transmittance were carried out using a National Institutes of Health 1.49 image analysis program. It allows for a measurement of the staining intensity of MCs to be obtained and, therefore, the amount of MC granules to be evaluated. In each experimental group, the transmittance of 20 different, randomly chosen MCs—five from each of the four different animals—was measured, and the mean transmittance value (±SEM) was then calculated.

Statistical analysis. Significance of differences among the different experimental groups in the percent histamine release, in $[Ca^{2+}]i$, and in the transmittance values of the mesenteric MCs in vivo was determined by Student's *t* test for paired values. $P \leq 0.05$ was considered significant.

Results

Effect of RLX on histamine release by isolated MCs stimulated with different secretagogues. Rat serosal MCs preincubated for 30 min with RLX at 10 and 100 ng/ml underwent a significant, concentration-dependent inhibition of histamine release when stimulated with compound 48/80 (Fig. 1 *A*) and calcium ionophore A23187 (Fig. 1 *B*). No significant effect of RLX on basal histamine release by MCs was found after a 30-min incubation with the hormone at the noted concentrations.

A similar effect of RLX could be achieved on MCs from guinea pigs sensitized with egg albumin. These cells released histamine when exposed to the specific antigen and yielded a maximal response to 100 μg/ml of antigen. Preincubation of the MCs with RLX at 1, 10, and 100 ng/ml before challenge with the antigen caused a significant, dose-related inhibition of histamine release (Fig. 1 *C*).

Effect of RLX on NO production by isolated MCs. Rat serosal MCs incubated for 30 min with RLX at 10, 30, and 100 ng/ml resulted in a significant, concentration-dependent increase in the amount of nitrites (NO_2^-) in the supernatants of MC suspensions. 1-h preincubation with MeArg strongly depressed the RLX-induced increase in nitrite production by MCs (Fig. 2).

Effect of drugs affecting the L-Arg–NO pathway on RLX-induced inhibition of histamine release by isolated MCs. Preincubation of rat serosal MCs with MeArg depresses, and preincubation with L-NAME nearly abrogates the inhibitory effect of RLX on A23187-induced histamine release. This reduction was related to the RLX concentrations (Fig. 3), thus suggesting that the inhibitory effect of RLX on histamine release involves endogenous NO production. The involvement of the L-arg–NO pathway in the effect of RLX on MC histamine release is further evidenced by the results obtained with other drugs capable of modifying NO production and activity. In fact, preincubation of the MCs with MeArg plus L-Arg or D-Arg showed that the natural substrate L-Arg, but not the inactive enantiomer D-Arg, reversed the effect of MeArg on the RLX-induced inhibition of histamine release evoked by calcium ionophore. On the contrary, treatment of the MCs with SOD, which potentiates NO activity, enhanced significantly the RLX-induced inhibition of histamine release evoked by calcium ionophore (Fig. 4).

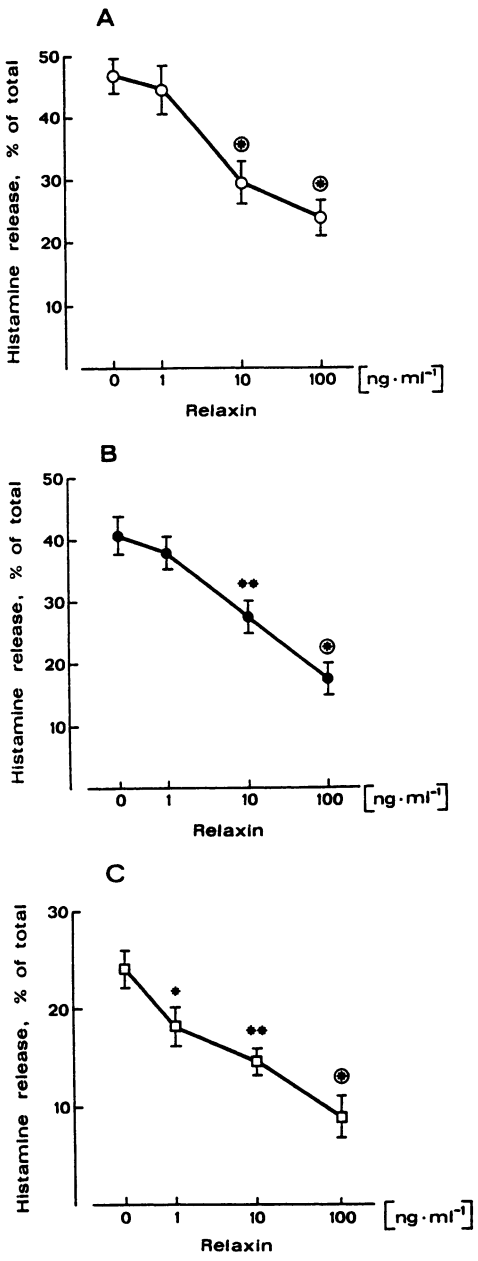

Figure 1. Effect of relaxin at different concentrations on histamine release by: (*A*) isolated rat serosal mast cells stimulated with compound 48/80 (0.05 μg/ml); (*B*) isolated rat serosal mast cells stimulated with calcium ionophore A23187 (10^{-6} M); and (*C*) isolated serosal mast cells from sensitized guinea pigs stimulated with the antigen (egg albumin, 100 μg/mL). The values are expressed as mean±SEM of eight experiments performed in duplicate. $*P < 0.01$; $**P < 0.005$; $\circledast P < 0.001$.

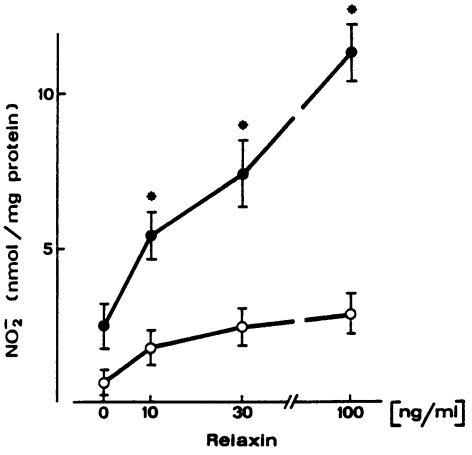

Figure 2. Effect of relaxin (●) at different concentrations on nitric oxide production by isolated rat serosal mast cells, determined by evaluation of nitrites (NO_2^-) in the supernatants. The effect of relaxin is attenuated by preincubation with MeArg (10^{-4} M) (○). The values are expressed as mean±SEM of six experiments performed in duplicate. *$P < 0.005$, **$P < 0.001$.

Effect of RLX on intracellular Ca^{2+} in isolated MCs. Rat serosal MCs stimulated with calcium ionophore A23187 underwent a striking increase in $[Ca^{2+}]i$, as shown by the results of the experiments with Quin 2 AM. When MCs were incubated with RLX at concentrations of 10 and 100 ng/ml before stimulation with calcium ionophore, a significant, concentration-related depression of $[Ca^{2+}]i$ elevation was obtained. The $[Ca^{2+}]i$ values were related positively to the percent inhibition of histamine release by the same cells (Fig. 5).

Morphology of isolated MCs upon treatment with RLX and/or calcium ionophore. By light microscopy, the rat serosal MCs incubated in medium alone usually appeared filled with metachromatic granules. Signs of granule release by the cells were uncommon. No substantial differences were observed in MCs incubated with 100 ng/ml RLX. Addition of calcium ionophore (10^{-6} M) to the MC suspensions in the absence of RLX resulted in the appearance of numerous MCs undergoing granule release (Fig. 6 *A*). Incubation of the cells with RLX before addition of calcium ionophore resulted in a clear-cut reduction in the number of degranulating MCs (Fig. 6 *C*).

By electron microscopy, the MCs incubated in medium alone usually appeared filled with electron-dense granules. Signs of secretory activation, i.e., clearing of the granule matrix and exocytosis, were sporadic. MCs incubated with RLX had ultrastructural features nearly similar to those of the untreated MCs. Addition of calcium ionophore to the MC suspensions resulted in the appearance of signs of active secretory processes in numerous MCs, i.e., electron-lucent granules often opened at the cell surface and large intracellular cavities containing solubilized granule matrix typical of sequential exocytosis, as occurs during physiologic secretory activation (Fig. 6 *B*). No images of cell damage or plasma membrane ruptures suggesting toxic granule release were ever observed, in agreement with the paraphysiologic action of this secretagogue. When challenge with the calcium ionophore was preceded by incubation with RLX, most MCs showed moderate or no signs of granule exocytosis (Fig. 6 *D*).

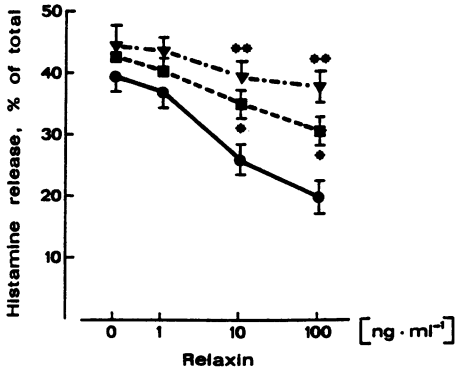

Figure 3. Effect of MeArg (10^{-4} M) (■) or L-NAME (10^{-4} M) (▼) on relaxin-induced inhibition of histamine release evoked by calcium ionophore A23187 (10^{-6} M) (●) in isolated rat serosal mast.cells. Noteworthy, treatment with MeArg depresses, and treatment with L-NAME—which is even more powerful than MeArg in inhibiting NO-synthase—nearly abrogates the effect of RLX on histamine release. The values are expressed as mean±SEM of six experiments performed in duplicate. *$P < 0.005$.

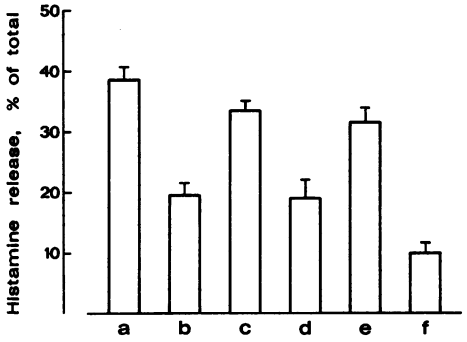

Figure 4. Histamine release by isolated rat serosal mast cells following treatment with: (*a*) calcium ionophore A23187 (10^{-6} M); (*b*) relaxin (100 ng/ml) + A23187; (*c*) MeArg (10^{-4} M) + relaxin + A23187; (*d*) L-Arg (10^{-4} M) + MeArg + relaxin + A23187; (*e*) D-Arg (10^{-4} M) + MeArg + relaxin + A23187; and (*f*) SOD + relaxin + A23187. The values are expressed as mean±SEM of six experiments performed in duplicate. Significance of differences: *b* vs. *a* and *c*: $P < 0.01$; *b* vs. *d*: not significant; *b* vs. *e*: $P < 0.01$; *b* vs. *f*: $P < 0.01$.

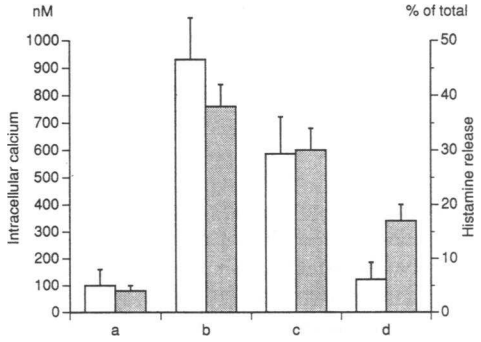

Figure 5. Effect of relaxin at different concentrations on the rise in intracellular Ca^{2+} levels (*open columns*) and on histamine release (*shaded columns*) evoked by calcium ionophore A23187 (10^{-6} M) in isolated rat serosal mast cells. (*a*) resting values; (*b*) A23187; (*c*) RLX (10 ng/ml) + A23187; and (*d*) RLX (100 ng/ml) + A23187. The values are expressed as mean±SEM of four separate experiments. Significance of differences: *c* vs. *b*: $P < 0.05$; *d* vs. *b*: $P < 0.001$.

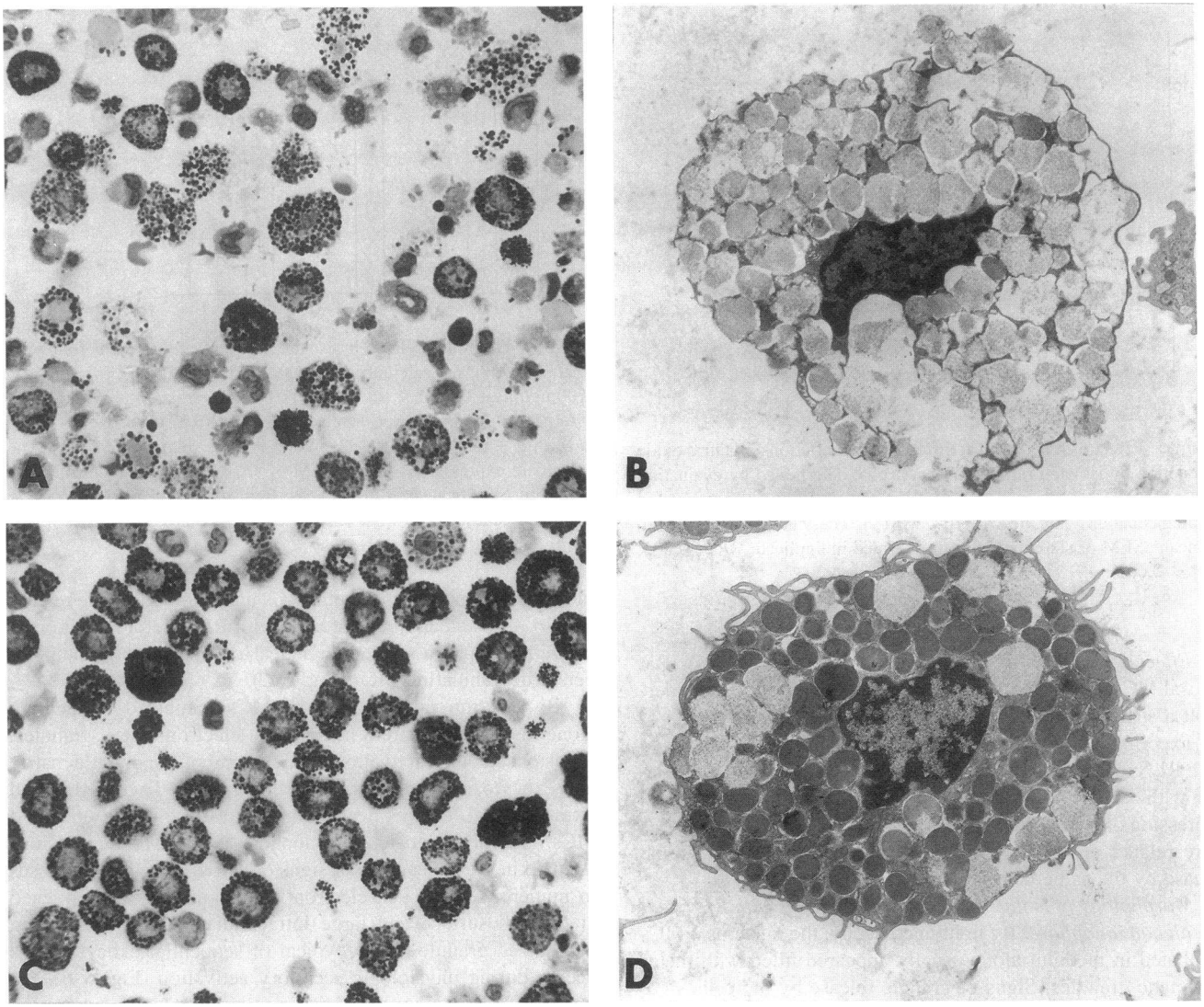

Figure 6. Light and electron microscopic features of isolated rat serosal mast cells. Mast cells incubated with calcium ionophore A23187 (10^{-6} M) are often degranulated and show clearing of the granule matrix and compound exocytosis (*A* and *B*). Mast cells preincubated with relaxin (100 ng/ml) before challenge with calcium ionophore are usually rich in secretory granules and show signs of moderate to scarce secretory activation (*C* and *D*). *A* and *C*: light microscopy, semithin sections stained with toluidine blue-sodium tetraborate, ×800. *B* and *D*: electron microscopy, ×6,000.

Morphology of mesenteric rat mast cells in vivo upon treatment with RLX and/or calcium ionophore. In the mesentery of rats injected intraperitoneally with PBS, most MCs were intensely metachromatic, only a minority of them showing a less intense staining. A similar picture was observed in the rats treated with RLX alone. After local treatment of the mesentery with calcium ionophore A23187, the MCs of the rats previously injected with PBS usually showed a weak metachromatic staining (Fig. 7 *A*). On the contrary, the MCs of the rats that received RLX intraperitoneally before local treatment with calcium ionophore were intensely stained (Fig. 7 *B*). Noteworthy, the MCs of the rats which received the inhibitor of NO synthase L-NAME before treatment with RLX and then with calcium ionophore were less intensely stained than those of the rats given RLX plus calcium ionophore or those of the rats pretreated with the inactive enantiomer D-NAME. Computer-assisted cytophotometry (Fig. 8) revealed that the transmittance level of the MCs from the rats treated with RLX alone was similar to that of the MCs from the rats given PBS. Local treatment with calcium ionophore of the mesentery of rats previously injected with PBS resulted in a marked, significant increase in the transmittance level of the MCs. Local treatment of the mesentery with calcium ionophore of the rats previously injected with RLX failed to induce any increase in the transmittance levels of the MCs as compared with those of the rats given RLX alone. Administration of L-NAME to rats before treatment with RLX and then with calcium ionophore resulted in a significant increase in the transmittance levels of the MCs compared with those of the rats given RLX plus calcium ionophore, whereas no substantial increase in the transmittance level was measured in the MCs from the rats pretreated with D-NAME.

Discussion

The results of the current study first demonstrate that RLX inhibits histamine release from MCs. This effect of RLX is concentration-related, and could be observed in isolated serosal MCs from both rat and guinea pig after stimulation with either

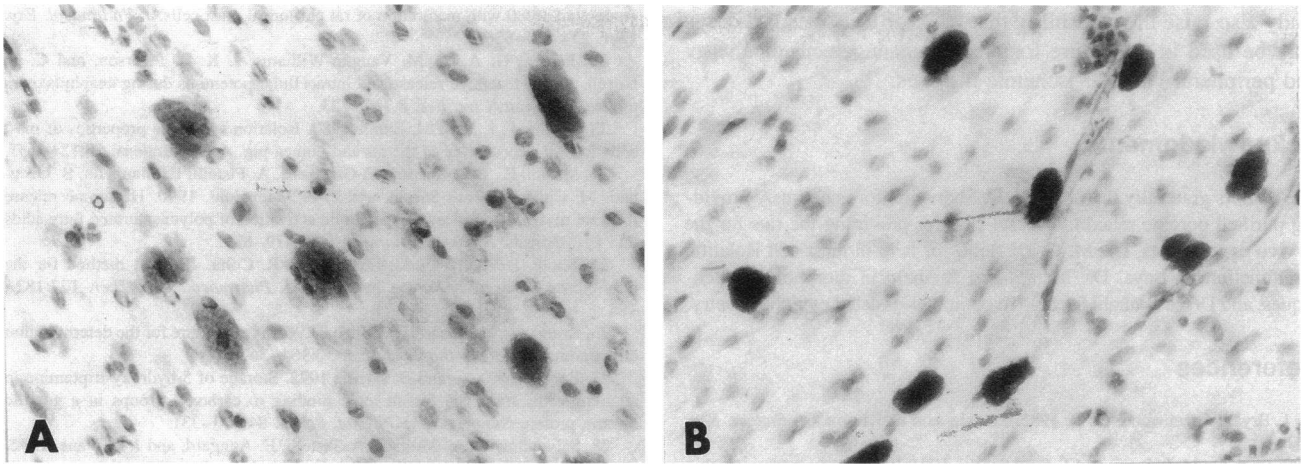

Figure 7. Light microscopy of rat mesenteric mast cells in vivo. (*A*) 20 min after intraperitoneal injection of PBS followed by 5-min local treatment with calcium ionophore A23187 (10^{-6} M). Mast cells show weak metachromatic staining. (*B*) 20 min after intraperitoneal injection of relaxin (10 μg) followed by 5-min local treatment with calcium ionophore. Most mast cells are intensely metachromatic. Toluidine blue, ×260.

nonimmunologic and immunologic stimuli. The morphological findings are in keeping with the functional data, revealing that RLX attenuates MC granule release evoked by calcium ionophore. Similar effects of RLX as inhibitor of MC granule release have also been recognized in in vivo conditions by light microscopic and densitometric analysis of rat mesenteric MCs. Therefore, the inhibition afforded by RLX on the release of histamine from MCs is not accounted for by an unspecific membrane-stabilizing effect, but rather by an intracellular event leading to a decrease in the exocytosis of MC granules.

The effect of RLX on MCs is associated with an elevation of endogenous production of NO by these cells, as shown by the concentration-related increase in nitrites, which are stable end-products of NO, upon RLX treatment. The observed increase in the amounts of nitrites roughly parallels the decrease in MC histamine release. Because NO has been shown to inhibit histamine release from MCs (16), this renders possible the hypothesis that RLX reduces the release of histamine from MCs by increasing the generation of NO. Indirect evidence in favor of this hypothesis is the antagonism of the inhibitory action of RLX afforded by the inhibitors of NO-synthase, and the potentiation of RLX's effect induced by SOD, which is known to protect NO by the inactivation produced by superoxide anions.

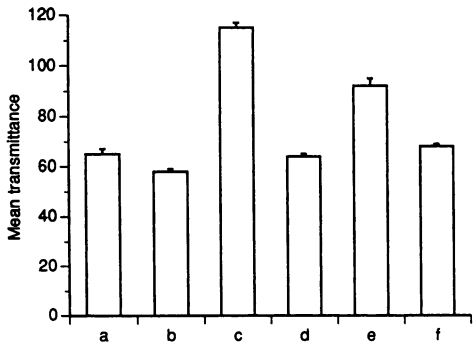

Figure 8. Histogram showing the mean transmittance (±SEM) of rat mesenteric mast cells in vivo (expressed in arbitrary units) under different treatments. (*a*) PBS; (*b*) relaxin (10 μg); (*c*) calcium ionophore A23187 (10^{-6} M); (*d*) relaxin + A23187; (*e*) L-NAME (10^{-4} M) + relaxin + A23187; (*f*) D-NAME (10^{-4} M) + relaxin + A23187. Significance of differences: *c* vs. *d*: $P \ll 0.001$; *d* vs. *e*: $P \ll 0.001$; *d* vs. *f*: not significant.

The current study also demonstrates that RLX attenuates the calcium ionophore–induced increase in $[Ca^{2+}]i$ in isolated rat MCs. This fits well with the ability of RLX to reduce the rise in $[Ca^{2+}]i$ observed in uterine smooth muscle cells by previous authors (40, 41), and by us in recent studies on platelets. It is questionable if elevation of NO and reduction of $[Ca^{2+}]i$ in the RLX-treated MCs are correlated as a cause and effect. This possibility, however, should not be ruled out, since NO can depress $[Ca^{2+}]i$ levels in other cell types, as shown in platelets (42) and postulated in vascular smooth muscle cells (43).

The RLX-induced reduction of the rise in $[Ca^{2+}]i$ evoked by calcium ionophore was concentration-dependent and was accompanied by inhibition of histamine release. This is in agreement with the fact that histamine release and granule exocytosis are calcium-mediated processes (34, 35). It is worth noting that, with 100 ng/ml RLX, the drop in $[Ca^{2+}]i$ is more pronounced than the corresponding reduction in histamine release. It is conceivable that the $[Ca^{2+}]i$ level, even if slightly exceeding the resting value, may be high enough to sustain a sharply suprabasal histamine release.

The current findings indicate that RLX enhances production of NO by MCs, and that this effect is associated with a clear-cut inhibition of MC histamine release. Therefore, it may be argued that the role of MCs in the vasodilatory response to RLX observed in earlier in vivo studies (4–7) is not exerted substantially through histamine release, but rather through local production of NO, which is known to be a powerful vasodilatory agent (10, 11). It is likely that the final effect of RLX on depression of the vascular tone is the result of its combined action on perivascular MCs and on the components of the vascular wall, on which RLX may also act through a NO-dependent mechanism. In fact, endothelium is known to be able to produce NO (12), and vascular smooth muscle cells have been shown by us to enhance NO production in response to RLX.

RLX may have a physiological role in regulating MC function. In fact, the RLX concentrations used in the experiments reported herein on rat MCs are within the range of physiological plasma levels of the hormone determined by radioimmunoassay in the rat (44). Going a step further, the results of the current

study also raise the possibility that RLX or RLX-derived drugs may be used in the future for clinical management of allergy and peripheral vascular ischemic diseases.

Acknowledgments

The authors gratefully thank Dr. O. D. Sherwood for generously providing purified porcine relaxin, Malesci Ltd. for providing the rats for the in vivo experiments, Dr. M. G. Di Bello for help in mast cell isolation and functional assays, Dr. M. Maurizi for help in morphologic techniques, and Dr. S. Bianchi for help in computer-assisted cytophotometry.

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