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Pharmacological characterization of polycation-induced rat hind-paw oedema

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1 The inflammatory response induced by poly-L-arginine in the rat hind-paw was studied both by measuring paw oedema and histologically.

2 The paw volume was measured with a hydroplethysmometer at 0.5, 1, 2, 4, 6 and 18 h after the subplantar injection of the polycation. Protein extravasation was evaluated with Evans' blue and the histology studied by light microscopy.

3 Poly-L-arginine (12, 24, 43 and 115 kD) caused dose- and molecular weight-dependent oedema which had a rapid onset and long duration. Evans' blue extravasation paralleled the oedema induced by poly-L-arginine. Microscopic examination of the paws at early stages of oedema formation showed exuberant liquid exudate with no inflammatory cells. After 18 h, a cellular infiltrate was present, consisting mainly of mononuclear cells.

4 Indomethacin, dexamethasone, BW755c or the PAF-antagonist WEB 2086 caused no significant inhibition of the poly-L-arginine-induced oedema. Cyproheptadine had inhibitory effects only on the early stages of the polycation-induced oedema. Similar results were observed with rats depleted of histamine and 5-hydroxytryptamine.

5 Heparin, a polyanion, injected in the rat paw caused a marked inhibition of the polycation-induced oedema. N^G-monomethyl-L-arginine (LNMMA), an inhibitor of EDRF synthesis, injected locally also produced a marked inhibition, but this inhibition was reversed by iloprost.

6 These results suggest that the oedema induced by polycations was due to their cationic charge. The inhibitory effect of LNMMA is probably due to a decrease in vascular flow rather than a decrease in vascular permeability.

Introduction

Several basic and acidic (but not neutral) synthetic polyamino acids increase permeability and leucocytic infiltration when injected intradermally or intraperitoneally into rats (Stein *et al.*, 1956). Polycations such as poly-L-lysine induce charge- and size-dependent local oedema formation in the rabbit skin and also the release of prostacyclin and cytoplasmic purines from pig cultured aortic endothelial cells (Needham *et al.*, 1988). In addition, basic polyamino acids rich in arginine, lysine or ornithine stimulate the formation and/or release of the endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) in perfused bovine segments of both artery and vein (Ignarro *et al.*, 1989).

In this paper we describe some pharmacological and histological characteristics of the inflammatory response induced by polycations (poly-L-arginine and poly-L-lysine) in the rat hind-paw. We have also assessed the involvement of EDRF as a possible mediator of polycation-induced oedema using an inhibitor of EDRF synthesis, N^G-monomethyl-L-arginine (LNMMA, Rees *et al.*, 1989).

Preliminary results have been communicated to the British Pharmacological Society (Antunes *et al.*, 1990).

Methods

Measurement of paw oedema

Male Wistar rats (150–200 g) were used. Oedema was induced by a single subplantar injection of the inflammatory agent (dissolved in saline) into the left hind-paw of the rat under light ether anaesthesia.

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The paw volume was measured immediately before the injection and at selected intervals thereafter with a hydroplethysmometer (model 7150, Ugo Basile, Italy). The final volume injected in the paw was always 0.1 ml. Results are expressed as increase in the paw volume (ml) calculated by subtracting the basal volume. In some cases, the area under the time-course curve was calculated (Lesser *et al.*, 1980) and the results expressed as percent of inhibition of the oedema total volume in comparison with control. The results are presented as mean ± s.e.mean.

Assessment of vascular response with Evans' blue

Evans' blue (25 mg kg^{-1} as a 2.5% solution in 0.45% NaCl) was injected intravenously immediately before subplantar injection of poly-L-arginine (24 kD, 1 mg per paw) into the rat hind-paw. At time intervals (0.5, 6 and 18 h) after poly-L-arginine injections, the animals were killed, the paws cut off and minced. The paws were then incubated with formamide (15 ml) for 72 h at 37°C. The solution was then filtered and the optical density of the filtrate assessed at 619 nm in a Uvikon 810 spectrophotometer (Lykke & Cummings, 1969; Garcia-Leme & Wilhelm, 1975).

Depletion of histamine and 5-hydroxytryptamine

Rats (Wistar, male, 180–220 g) were depleted of their stores of histamine and 5-hydroxytryptamine (5-HT) by repeated injections of compound 48/80 as previously described (Spector & Willoughby, 1959; Di Rosa *et al.*, 1971). Briefly, a 0.1% w/v solution of compound 48/80 in saline was given intraperitoneally morning and evening for eight doses, starting with an evening dose. The dose employed was 0.6 mg kg^{-1} for the first six injections and 1.2 mg kg^{-1} for the last two doses. Polycation was injected 5–6 h after the last injection of compound 48/80.

Histological studies

The foot pads were fixed in 10% formalin, embedded in paraffin and the histological sections stained by haematoxylin and eosin for light microscopical analysis.

Statistical analysis

The unpaired Student's t test was used for statistical evaluation of the results and $P < 0.05$ was taken as significant.

Drugs

Poly-L-arginine (12, 24, 43 and 115 kD), poly-L-lysine (85 kD), mepyramine, 5-HT, lambda-carrageenan, compound 48/80 and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cellulose sulphate and formamide were bought from Aldrich Chemical Co. (Dorset, UK) and Merck (FRG), respectively. Methysergide and BW 755C (3-amino-1-(*m*-(trifluoromethyl)-phenyl)-2-pyrazolone) were gifts from Sandoz Ltd (Switzerland) and the Wellcome Research Laboratories (Kent, UK), respectively. N^G-monomethyl-L(D)-arginine and WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propane) were kindly provided by Italfarmaco (Milan, Italy) and Boehringer-Ingelheim (FRG), respectively. Iloprost and heparin sodium salt were gifts from Schering (FRG) and Roche (São Paulo, Brazil), respectively.

Results

Poly-L-arginine- and poly-L-lysine-induced dose-dependent rat hind-paw oedema

Figure 1 shows the dose-dependent relationships of the oedema produced by poly-L-arginine (43 kD) and poly-L-lysine (85 kD). At the higher dose (1.0 mg per paw), both poly-L-arginine and poly-L-lysine induced oedema which had rapid onset (at 30 min, 0.88 ± 0.03 ml and 0.62 ± 0.02 ml, respectively, $n > 35$) and long duration of action (at 6 h, 0.61 ± 0.03 ml and 0.57 ± 0.02 ml, respectively, $n > 35$). Increase in molecular weight of poly-L-arginine (12 kD to 115 kD, 1.0 mg per paw, $n > 5$) induced greater oedema (Figure 2).

Poly-L-arginine-induced Evans' blue leakage

Thirty min after the poly-L-arginine (24 kD, 1 mg per paw) injection, both the oedema and protein leakage were maximal whereas at 18 h the paw volume almost reached basal values and the protein leakage was decreased (Figure 3).

Histological analysis

Histological analysis of the hind-paw 30 min after the injection of poly-L-arginine (1 mg per paw; $n = 2$) showed an exuberant liquid exudate with no inflammatory cells. The intensive plasma leakage dissociated collagen fibres and a marked dilatation of lymphatic vessels was observed. The oedema and lymphatic vessel dilatation were still present 6 h after the polycation injection when a few polymorphonuclear as well as mononuclear cells were present; 18 h after the polycation injection the oedema decreased and an inflammatory cell infiltration mainly composed of mononucler cells was observed.

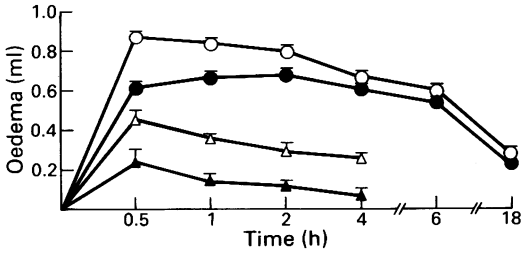

Figure 1 Poly-L-arginine 43 kD (△, 0.1 mg per paw; ○, 1 mg per paw; $n = 5$) or poly-L-lysine 85 kD (▲, 0.1 mg per paw; ●, 1 mg per paw; $n = 5$) injected into the rat hind-paw caused dose-dependent oedema. The oedema is expressed as the difference in volume (ml) of the paw compared to its basal volume. Each point represents the mean; s.e.mean shown by vertical bars.

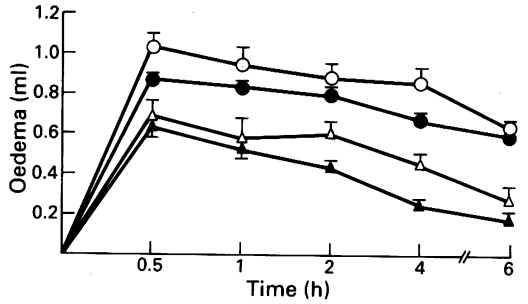

Figure 2 Molecular weight-dependent oedema formation in the rat hind-paw induced by subplantar injection of poly-L-arginine (▲, 12 kD; △, 24 kD; ●, 43 kD; ○, 115 kD; 1 mg per paw, $n > 5$). Each point represents the mean; s.e.mean shown by vertical bars.

Evaluation of histamine and 5-hydroxytryptamine participation

The involvement of 5-HT and/or histamine in the poly-L-arginine oedema was investigated in rats pre-treated with cyproheptadine ($0.5\ mg\ kg^{-1}$, i.p., 1 h), mepyramine ($0.25\ mg\ kg^{-1}$, i.v., 0.5 h) or with the mixture of the above antagonists (same doses). Rats treated with either cyproheptadine or the mixture of cyproheptadine and mepyramine showed an inhibition only 30 min later ($28.3 \pm 4.91\%$ and $34.2 \pm 6.4\%$, respectively; $n = 5$, $P < 0.05$, not shown). Mepyramine alone produced no significant inhibition of the poly-L-arginine oedema (not shown). At the doses used above, both cyproheptadine and the mixture of cyproheptadine and mepyramine (but not mepyramine alone) produced an inhibition of $45.1 \pm 8\%$ and $49.9 \pm 7\%$ of the 48/80 (10 μg per paw)-induced oedema ($n = 4$, $P < 0.05$, not shown).

In rats depleted of histamine and 5-HT stores, the oedema induced by poly-L-arginine (24 kD, 1 mg per paw) was inhib-

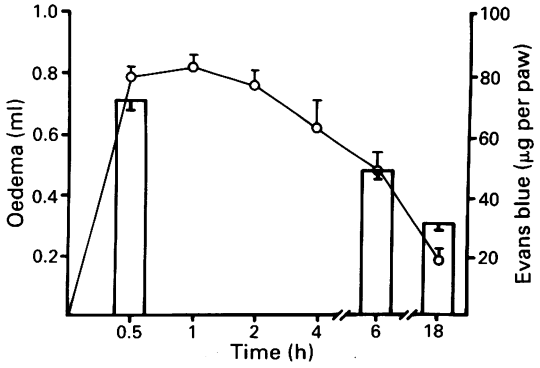

Figure 3 Protein leakage as measured by Evans' blue extravasation (columns). Poly-L-arginine (24 kD, 1 mg per paw) was injected into the hind-paw of rats pretreated with Evans' blue ($25\ mg\ kg^{-1}$, i.v.). At 30 min, 6 h and 18 h the animals were killed, the paws excised and the concentration of Evans' blue measured (see Methods). Oedema (ml) shown by (○).

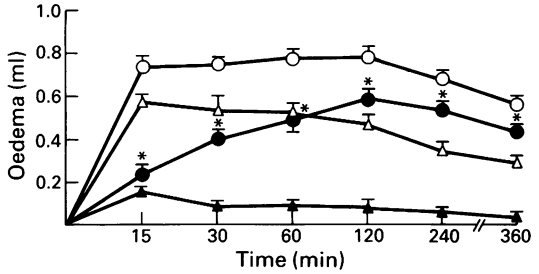

Figure 4 Effect of depletion of histamine and 5-hydroxytryptamine stores. Compound 48/80 (10 μg per paw) caused paw oedema in control rats (△, $n = 6$) but not in depleted rats (▲, $n = 8$). The poly-L-arginine (24 kD, 1 mg per paw)-induced oedema was reduced in depleted animals (●, $n = 8$) as compared to controls (○, $n = 6$). Each point represents the mean; s.e.mean shown by vertical bars.

ited by 41.6 ± 4.8% ($n = 8$, $P < 0.05$) whereas that induced by 48/80 was abolished (Figure 4).

Effect of WEB 2086, BW 755C, dexamethasone, indomethacin and heparin on poly-L-arginine-induced paw oedema

Rats treated 30 min beforehand with the PAF antagonist WEB 2086 (20 mg kg^{-1}, p.o., $n = 5$) or with the cyclo-oxygenase and lipoxygenase inhibitor BW 755 C (50 mg kg^{-1}, p.o., $n = 5$) exhibited no significant inhibition of the poly-L-arginine (43 kD, 1 mg per paw)-induced oedema (Figure 5). At the dose used above, BW 755C produced significant inhibition of the carrageenan (1 mg per paw)-induced paw oedema (49.9 ± 10.6%, $P < 0.05$, $n = 5$). Dexamethasone (1 mg kg^{-1}, s.c., 1 h before) or indomethacin (2 mg kg^{-1}, i.p., 30 min before) did not inhibit the poly-L-arginine-induced oedema ($n = 5$, not shown). Dexamethasone or indomethacin at these doses induced a marked inhibition of carrageenan (1 mg per paw)-induced oedema (67 ± 5.9%, $n = 8$ and 63.1 ± 4.1%, $n = 13$ respectively).

Heparin (50 u per paw) produced a marked inhibition of the oedema at all times (Figure 5). This compound had a dose-dependent inhibitory effect as measured by comparison of the area under the time-course curves (Table 1).

Heparin given systemically (5000 u kg^{-1}, 30 min beforehand) also caused an inhibition of the polycation-induced oedema (30.9 ± 6.7%, $P < 0.05$, not shown).

Poly-L-arginine-induced oedema is not abolished by a mixture of antagonists

Poly-L-arginine-induced oedema in rats treated 3 h before with cellulose sulphate (3 mg kg^{-1}, i.v.) and 30 min before with mepyramine (2 mg kg^{-1}, i.p.), methysergide (2 mg kg^{-1}, i.p.) and indomethacin (10 mg kg^{-1}, i.p.) was reduced by 39.9 ± 4.9% ($P < 0.001$, $n = 10$, not shown). Similar results were observed with poly-L-lysine (53.8 ± 3% inhibition, $P < 0.001$, $n = 5$, not shown).

Table 1 Inhibition by heparin of poly-L-arginine-induced oedema

Dose of heparin (u/paw)	*Inhibition* (%)	n	P
5	10.0 ± 7.41	10	NS
20	36.9 ± 7.60	10	0.01
50	62.9 ± 6.23	15	0.001

NS – not significant

N^G-monomethyl-L-arginine (LNMMA) inhibits poly-L-arginine-induced oedema

LNMMA (1 and 2 mg per paw) caused an inhibition of 27.6 ± 5.2% and 49.4 ± 5.3% ($n = 5$, $P < 0.05$, respectively) of the poly-L-arginine-induced oedema. However, LNMMA caused no reduction of the oedema when injected in the contralateral paw (2 mg per paw, $n = 5$, not shown). When injected systemically at a higher dose (100 mg kg^{-1}, 30 min before) LNMMA also exerted significant inhibition of the oedema (39.0 ± 5.8%, $n = 4$, $P < 0.05$). Inhibition of the poly-L-arginine-induced oedema was also observed with the D-form of N^G-monomethyl-arginine (DNMMA), although it was less potent than the L-isomer. At the dose of 2 mg per paw, DNMMA caused an inhibition of 25.1 ± 2.5% ($n = 5$, $P < 0.05$). In addition, iloprost (100 ng per paw) which did not induce oedema *per se*, partially prevented the inhibitory effect of the LNMMA (Figure 6).

LNMMA (4 mg per paw) produced no inhibition of carrageenan (1 mg per paw)-induced paw oedema (not shown, $n = 5$).

Discussion

Our results clearly demonstrate that the polycation poly-L-arginine injected into the rat hind-paw causes dose- and molecular weight-dependent oedema. The histological analysis of these lesions also showed a peculiar pattern of intense liquid exudation and lymphatic vessel dilatation, not charac-

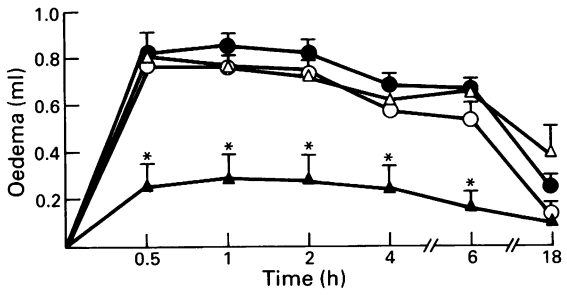

Figure 5 Effect of WEB 2086, BW 755C and heparin on oedema responses induced by subplantar injection of poly-L-arginine (43 kD, 1 mg per paw) in the rat hind paw. Rats were pretreated with WEB 2086 (●, 20 mg kg^{-1}, p.o. 30 min beforehand), BW 755C (△, 50 mg kg^{-1}, p.o., 30 min beforehand), heparin (▲, 50 U per paw) or saline (○). Each point represents the mean of $n = 5$; s.e.mean shown by vertical bars.

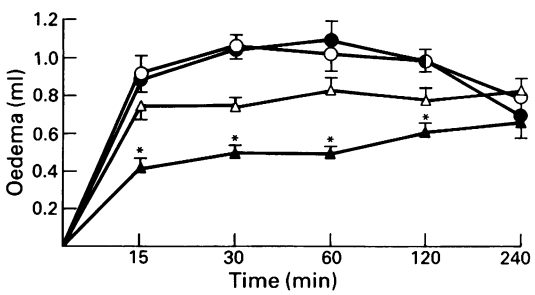

Figure 6 The inhibition caused by N^G-monomethyl-L-arginine (LNMMA) (▲, 4 mg per paw; $n = 5$) on poly-L-arginine-induced paw oedema (○, 1 mg per paw; $n = 5$) was partially reversed by iloprost (△, 100 ng per paw; $n = 5$). Iloprost (100 ng per paw) did not potentiate poly-L-arginine-induced oedema (●; $n = 5$). Each point represents the mean; s.e.mean shown by vertical bars.

teristic of other oedema producing substances, such as bradykinin, 5-HT or carrageenan (Spector & Willoughby, 1968). The increase in vascular permeability induced by the polycations was further confirmed by the extravasation of Evans' blue.

It is likely that the initial oedema was partially caused by the release of 5-HT since methysergide had an inhibitory action at this time and so did depletion of the 5-HT and histamine stores by 48/80. This is to be expected since irritant agents cause release of these mediators (Spector & Willoughby, 1968) and poly-DL-lysine (Padawer, 1970; Ennis *et al.*, 1980) and the polycation 48/80 (Kazimierczak & Diamant, 1978) degranulate mast cells. The lack of effect of mepyramine is not surprising, since histamine is not an important mediator of vascular permeability in rats (Rowley & Benditt, 1956; Wilhelm, 1962).

Although polycations induce release of prostaglandins (Shier *et al.*, 1984; Shier & DuBourdieu, 1986), it is unlikely that the persistence of the oedema was due to these mediators, since indomethacin, the dual inhibitor BW755C (Higgs *et al.*, 1979) and dexamethasone each failed to inhibit this oedema. It is interesting to note that the permeability increase induced by poly-L-lysine in the rabbit skin was sensitive to indomethacin (Needham *et al.*, 1988). The doses we used of the above antagonists were effective since they inhibited carrageenan-induced oedema.

Kinins are important modulators of vascular permeability (for review see Movat, 1985). We tried to evaluate the role of kinins by depleting the rats of kallikrein with cellulose sulphate (Di Rosa *et al.*, 1971). Our results indicate that it is unlikely that kinins play a major role in the polycation-induced oedema.

We have also investigated whether the increase of vascular permeability was due to PAF release. The failure of the PAF antagonist WEB 2086 (Casals-Stenzel *et al.*, 1986) to inhibit the poly-L-arginine-induced oedema indicates that PAF does not appear to be involved in the polycation-induced oedema.

Although the specific inhibitor of EDRF synthesis, LNMMA, caused a marked inhibition of the polycation-induced oedema, the finding that iloprost reversed the inhibition suggests that the inhibitory effect of LNMMA was due to a decrease in flow rather than a decrease in vascular permeability. It is interesting that LNMMA had no effect on carrageenan-induced paw oedema, indicating that in this oedema EDRF does not play an important role.

Polycations are naturally occurring polymers, released mainly from activated leukocytes and platelets (Peterson *et al.*, 1985; Tetta *et al.*, 1985; Camussi *et al.*, 1986). The vascular endothelium (Skutelsky *et al.*, 1975; Skutelsky & Danon, 1976; Simionescu *et al.*, 1981) and glomerular basement membrane (Barnes *et al.*, 1984) contain fixed anionic sites which confer an overall electronegative charge in these structures. The anionic sites are constituted primarily by sulphated glycosaminoglycans, most probably heparan sulphate (Simionescu *et al.*, 1981). It is likely that polycations increase vascular permeability due to electrostatic interactions with these anionic sites. Indeed, polycations of higher molecular weights have larger numbers of cationic sites, which increase their adsorption to surfaces (Hesselink, 1983). The finding that the ability of polycations to induce oedema was proportional to their molecular weights would support this conclusion. However, poly-L-arginine with lower molecular weight than poly-L-lysine was more potent in inducing paw oedema. This result indicates that other factors in addition to electrostatic forces, such as hydrophobic bonding and chain stiffness, modulate polycation-membrane interactions. Poly-L-lysine has lower hydrophobic bonding (Ichimura & Zama, 1977) and non-specific electrostatic binding ability (Ichimura *et al.*, 1978) than poly-L-arginine. There are also differences in the ability of these polycations to display helical conformation which increases chain stiffness, thus decreasing chain interaction with charged surfaces (Ichimura *et al.*, 1978).

Interestingly, heparin binds to vascular endothelium (Hiebert, 1981; Barzu *et al.*, 1985). Our results clearly demonstrated that heparin produced a marked inhibition of the polycation-induced oedema. The neutralization of the positively charged groups of the polycations could be explained if heparin after binding to the endothelial cell, competes with the anionic sites for the polycations. In this case, one might assume that polycations interact with specific endothelial anionic proteins to trigger the increase in the vascular permeability leading to oedema formation, since heparin inhibited the oedema. Alternatively, poly-L-arginine may be interacting with anionic sites on resident cells such as macrophages or mast cells.

Since there is some evidence that platelet polycations modulate glomerular vascular injury (Barnes & Venkatachalam, 1984), our results indicate that it may be worth developing heparin analogues devoid of anticoagulant activity as potential anti-inflammatory drugs.

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