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Anti-inflammatory effects of peripheral benzodiazepine receptor ligands in two mouse models of inflammation

Sandra R. Torres [a], Tânia S. Fröde [b], Geisson M. Nardi [a], Natalio Vita [c], Rennée Reeb [c], Pascual Ferrara [c], Rosa M. Ribeiro-do-Valle [a], Roseli C. Farges [a,*]

[a] *Department of Pharmacology, Centre of Biological Sciences, Universidade Federal de Santa Catarina, Rua Ferreira Lima, 82, Florianópolis, SC, 88015-420, Brazil*

[b] *Clinical Chemistry, Centre of Health Science, Universidade Federal de Santa Catarina, Rua Ferreira Lima, 82, Florianópolis, SC, 88015-420, Brazil*

[c] *Sanofi-Synthelabo Recherche, BP 137, 31676 Labègé Cedex, France*

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Abstract

In vivo treatment of mice with peripheral benzodiazepine receptor ligands exerts an inhibitory effect on the inflammatory response in two models of acute inflammation. In the first model, pretreatment of the animals (24 h) with 1-(2-chlorophenyl)-*N*-methyl-*N*(1-methyl-propyl)-3-isoquinoline carboxamide (PK11195) and 7-chloro-5-(4-Chlorophenyl)-1,3-dihydro-1-methyl-2-*H*-1,4-benzodiazepin-2 (Ro5-4864), at different doses (0.00001–10 mg/kg, i.p.) dose dependently inhibited the formation of mouse paw oedema induced by carrageenan with mean ID_{50s} of 0.009 (95% confidence limits = 0.0076–0.013) and 0.04 (95% confidence limits = 0.025–0.0086) mg/kg, respectively. Both ligands (0.1 mg/kg, i.p.) inhibited in the same way the mouse paw oedema induced by carrageenan in animals with and without adrenal glands. PK11195 and Ro5-4864 (0.1 mg/kg, i.p.) inhibited the mouse paw oedema induced by several inflammatory mediators. In the second model, the pretreatment (24 h) with peripheral benzodiazepine receptor ligands (0.1 mg/kg, i.p.) exerted an inhibitory effect on neutrophil influx and produce a marked inhibition of carrageenan-produced interleukin-13 and interleukin-6 in pleural exudation. Our results extend previous findings that peripheral benzodiazepine receptor is involved in the inflammatory response, and suggest that this action may be linked to the action of different inflammatory mediators, probably mainly by the inhibition of the release of pro-inflammatory cytokines.

Keywords: Benzodiazepine receptor, peripheral; Paw oedema; Pleurisy

1. Introduction

Benzodiazepines such as diazepam are used clinically because of their anxiolytic, anticonvulsant, muscle relaxant and hypnotic properties. These therapeutic actions are believed to involve central-type benzodiazepine receptors located exclusively or primarily in the central nervous system (Costa et al., 1975; Braestrup and Squires, 1977; Squires and Braestrup, 1977). In initial studies of these central-type sites in the brain, high densities of labelled diazepam binding sites were observed in the mitochondrial fractions from peripheral tissues used as negative control (Braestrup and Squires, 1977; Squires and Braestrup, 1977), and because of their initial identification outside the central nervous system, these sites were designated peripheral benzodiazepine receptors. Peripheral benzodiazepine receptor are distributed in several glandular and secretory tissues, such as adrenal glands, salivary glands, testis, kidney, lung and liver (Anholt et al., 1986; Parola et al., 1993). In the central nervous system, peripheral benzodiazepine receptor are enriched in the choroid plexus, ependymal linings and glia (Marangos et al., 1982; Gehlert et al., 1983; Schoemaker et al., 1983). Peripheral benzodiazepine receptor have also been identified in various types of human blood cells, and the rank order of cell expression is: monocytes = neutrophils, lymphocytes (including B cell, natural killer, CD4 and CD8 positive cells) ≫ platelets > erythrocytes (Canat et al., 1993). The mitochondrial outer membrane is particularly rich in peripheral benzodiazepine receptor (Anholt et al., 1986), but other subcellular loca-

* Corresponding author. Tel.: +55-48-331-94-91; fax: +55-48-222-41-64.

E-mail address: roseli@farmaco.ufsc.br (R.C. Farges).

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tions have been described. Diazepam, the most commonly prescribed benzodiazepine, binds with nanomolar affinity to both the peripheral and central receptors (Wang et al., 1984), but other benzodiazepines show different selectivity among themselves. For example, clonazepam and flumazenil are specific ligands for central-type benzodiazepine receptors, while peripheral benzodiazepine receptor bind, with high affinity, the 7-chloro-5-(4-Chlorophenyl)-1,3-dihydro-1-methyl-2-*H*-1,4-benzodiazepin-2 (Ro5-4864), which is the 4′-chloro derivative of diazepam (Braestrup and Squires, 1977; Squires and Braestrup, 1977). The 1-(2-chlorophenyl)-*N*-methyl-*N*(1-methylpropyl)-3-isoquinoline carboxamide (PK11195), which is structurally quite different from benzodiazepines, has much greater selectivity for peripheral benzodiazepine receptor than for central-type benzodiazepine receptors (Le Fur et al., 1983). Acute diazepam treatments have shown various peripheral actions, such as the suppression of cell proliferation in rat thymus, a decrease in Tumor Necrosis Factor-α (TNF-α), interleukin-1 and interleukin-6 release from mouse macrophages (Schreiber et al., 1993), a long-lasting immuno-suppression (Schlumpf et al., 1989), and changes in the secretion of anterior pituitary hormones in rats (Grandison, 1983), suggesting a possible role for peripheral benzodiazepine receptors in these different responses. Several studies have shown that the peripheral benzodiazepine receptors ligands, PK11195 and Ro5-4864, affect various cellular functions, the best-known examples occurring in steroid biosynthesis. In fact, many studies reveal that peripheral benzodiazepine receptor ligands stimulate steroid synthesis in steroidogenic tissues (Besman et al., 1989; Papadopoulos et al., 1990; Amsterdam and Suh, 1991), in glia (Papadopoulos et al., 1992), and in the peripheral nervous system (Lacor et al., 1999).

One line of evidence suggests that peripheral benzodiazepine receptor plays a major role in the regulation of immune function. Peripheral benzodiazepine receptor ligands have been found to modulate monocyte functions such as cellular chemotaxis (Ruff et al., 1985). Ro5-4864, PK11195 and diazepam inhibit interleukin-3-like secretory activity in human peripheral blood mononuclear cells, while interleukin-2 production is inhibited by Ro5-4864 or diazepam only (Bessler et al., 1992). Peripheral benzodiazepine receptor ligands have been found to modulate lymphoid cell proliferation (Wang et al., 1984; Laird et al., 1989). Peripheral benzodiazepine receptor levels are greatly reduced in the neutrophils of patients suffering from chronic granulomatous disease, a rare inherited disorder characterized by the inability of phagocytes to produce superoxide anion (Zavala et al., 1990a). Treatment of mice with Ro5-4864 markedly reduces the capacity of macrophages to produce key mediators of inflammation such as superoxide anion, interleukin-1, TNF-α and interleukin-6 (Zavala et al., 1990b). These data, together with the demonstration of the in vivo anti-inflammatory properties of PK11195 and Ro5-4864 (Torres et al., 1999), suggest that peripheral benzodiazepine receptor ligands might be potential anti-inflammatory agents.

Here, we report that peripheral benzodiazepine receptor ligands produce a long-lasting inhibition of the paw oedema induced by several mediators involved in inflammatory processes. We further analyzed the anti-inflammatory effects of peripheral benzodiazepine receptor ligands in a model of acute inflammation, using carrageenan-induced pleurisy in mice. In this model, carrageenan provokes an increase in exudation and accumulation of polymorphonuclear leukocytes in the pleural cavity (Lo et al., 1982).

Abnormal interleukin-6 production has been associated with a large number of diseases including tuberculous pleurisy and asthma (Virchow et al., 1996; Hoheisel et al., 1998). Other studies have demonstrated that levels of interleukin-13, which has been ascribed an anti-inflammatory role in several experimental models, are increased in the presence of inflammatory lung disease (Oh-ishi, 1997).

Therefore, we also investigated the production of interleukin-13 and interleukin-6, which induce neutrophil migration in rat pleural cavity during carrageenan-induced pleurisy (Hancock et al., 1998), in the pleural exudate of mice pretreated or not pretreated with peripheral benzodiazepine receptor ligands before pleurisy induction.

2. Material and methods

2.1. Animals

Male Swiss mice (weighing 20–25 g; $n = 5–11$) were used in this study. The animals were maintained in an environment with controlled temperature (22 ± 2°C), illuminated by daylight supplemented by electric light, from 0600 to 1800 h. In all experiments, animals were managed using the principles and guidelines for the care of laboratory animals according to Zimermann (1983).

2.2. Evaluation of the effect of peripheral benzodiazepine receptor ligands in the model of mouse paw oedema induced by carrageenan

In order to determine the effectiveness of peripheral benzodiazepine receptor ligand treatment in the modulation of responses to carrageenan, separate groups of mice received an intraperitonial (i.p.) injection of 0.1 mg/kg of PK11195 or Ro5-4864 at different times (1–48 h). Control animals received a similar volume (0.1 ml per animal, i.p.) of sterile phosphate-buffered saline (NaCl 137 μmol/l; KCl 27 mmol/l and phosphate buffer 10 mmol/l) at several time-points (1–48 h). After the pretreatments with phosphate-buffered saline or peripheral benzodiazepine receptor ligand, the animals were anaesthetized with ether and received a subcutaneous injection of carrageenan (300 μg/paw) into the right paw. The contralateral paw re-

ceived the same volume of sterile phosphate-buffered saline and served as a control. The volume of the paw was measured with a plethysmometer immediately after phosphate-buffered saline or carrageenan administration. Results are expressed as the volume difference between the carrageenan or mediator-treated paw and the saline-treated paw. Subsequent readings from the same paw were carried out at different time-points after several periods of time (30, 60, 120 and 240 min) after carrageenan or sterile phosphate-buffered saline injection. Paw oedema was expressed in milliliters as the difference between the test and the control paws. The percentage inhibition of peripheral benzodiazepine receptor ligands in the model of mouse paw oedema induced by carrageenan were determined from the area under the curve.

Later, to determine the best duration of pretreatment, several doses of PK11195 or Ro5-4864 were tested. Thus, separate groups of animals were pre-treated (24 h) with different doses of peripheral benzodiazepine receptor ligands (0.00001–10 mg/kg, i.p.) or 0.1 ml of phosphate-buffered saline (control animals). In another group of experiments, the animals were treated with indomethacin and used as positive control.

2.3. Evaluation of the effect of peripheral benzodiazepine receptor ligands in the carrageenan-induced paw oedema model in the adrenalectomized mouse

In order to investigate the possible participation of adrenal glucocorticoids in the inhibition of oedema caused by peripheral benzodiazepine receptor ligands, the animals were anaesthetized with 2,2,2 tribromoethanol (0.25 g/kg, i.p.). They were operated on as described (Flower et al., 1989), except that mice were used instead of rats. Briefly, the dorsal region was incised (approximately 2 cm), and both adrenal glands were carefully removed. The control animals had the same incision without removal of adrenal glands (sham-operated). After surgery, the animals were returned to their cages, with free access to food and drink, but water was replaced by substituted with 0.9% NaCl solution to maintain a physiological plasma sodium concentration. After 7 days, the animals received PK11195 or Ro5-4864 (0.1 mg/kg, i.p.) or saline phosphate-buffered saline (0.1 ml/10 g) 24 h before paw oedema induction with carrageenan.

2.4. Evaluation of the effect of peripheral benzodiazepine receptor ligands in the model of mouse paw oedema induced by different inflammatory mediators

In a separate series of experiments, animals were treated with peripheral benzodiazepine receptor ligands or phosphate-buffered saline as described above. Twenty-four hours after this pretreatment, the animals were anaesthetized with ether and received a subcutaneous injection of 5-hidroxytryptamine (5-HT) (10 nmol/paw), histamine (100 nmol/paw), substance P (10 nmol/paw), bradykinin (3 nmol/paw), prostaglandin E_2 (30 nmol/paw) or platelet activating factor (10 nmol/paw). Paw oedema was measured at 15 to 120, 15 to 120, 15 to 120, 10 to 120, 30 to 240 and 30 to 240 min after flogistic agent injection, respectively. The percentage inhibition by peripheral benzodiazepine receptor ligands in the model of mouse paw oedema induced by inflammatory mediators was determined from the area under the curve.

2.5. Evaluation of effect of peripheral benzodiazepine receptor ligands in the model of mouse pleurisy induced by carrageenan

On the day of the experiments, the animals were lightly anaesthetized with ether, and carrageenan dissolved in sterile phosphate-buffered saline, or sterile phosphate-buffered saline alone (control group), was injected into the right pleural space through the chest skin (final volume of 0.1 ml). According to the experimental protocol, the animals were killed with an overdose of ether, and immediately after the thorax was opened, the pleural cavity was washed with 1.0 ml of sterile phosphate-buffered saline containing heparin (20 IU per ml), the fluid leakage then being collected with automatic pipettes. All animals were injected 5 min previously with a solution of Evans blue dye (25 mg/kg, 0.2 ml, i.v.) in order to evaluate the degree of exudation in the pleural space (Saleh et al., 1996, 1998). The total leukocyte counts were performed with an automatic counting machine (Caulter, USA). Cytospin preparations of pleural washes were stained with May–Grunwald–Giemsa for the differential count of leukocytes, which was performed under immersion objective. A sample of the fluid (500 μl) collected from the pleural cavity was separated and stored in the freezer (−20°C) for subsequent determination of the concentration of Evans blue dye. For this, on the day of the experiments, a batch of samples was thawed at room temperature, and the amount of dye was estimated by colorimetry (Campu-Espectro Spectrometer, Brazil) at 600 nm by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50 μg/ml.

Considering that the inflammatory response induced by carrageenan (1%) in the pleural space of the mouse has an important inflammatory profile, peaking at 4 h with an increase in exudate and total cells (primarily consisting of neutrophils) after pleurisy induction (Saleh et al., 1996), these time-points were chosen in order to analyze the effect of PK11195 or Ro5-4864 in this experimental model.

In preliminary experiments ($n = 5$ to 11, results not shown), several doses of each drug and different intervals of pretreatment were tested in order to determine the best period for pretreatment. Based on this protocol, the animals were treated i.p., 24 h before the induction of pleurisy with either the vehicle (sterile phosphate-buffered saline), PK11195 (0.1 mg/kg) or Ro5-4864 (0.1 mg/kg). Each

experimental protocol included an equivalent number of control animals that received the same volume of sterile phosphate-buffered saline. The animals were killed 4 h later.

2.6. Evaluation of the effect of peripheral benzodiazepine receptor ligands on the levels of interleukin-6 and interleukin-13 induced by carrageenan in the mouse model of pleurisy

In another set of experiments, samples of exudate from control and treated groups were collected in polyethylene tubes to determine the concentration of interleukin-6 and interleukin-13 in the pleural exudate induced by carrageenan. Interleukin-6 and interleukin-13 levels were measured using the interleukin-6 and interleukin-13-dependent murine B-cell hybridomas B9 (Aarden et al., 1987) and B9-1-3 (Bouteiller et al., 1995), respectively. Twofold dilutions of interleukin-6 or interleukin-13 standard, or of the different samples from the mouse pleural cavity, were prepared in 96-flat-bottom well plates. 10^4 cells per well were then seeded. After 72 h of culture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO, USA) was added to each well at a concentration of 5 mg/ml. After incubation (4 h at 37°C), 100 μl of 0.04 N hydrochloric acid in isopropanol was added to all wells and mixed thoroughly. After a few minutes at room temperature, absorbance at 565 nm of each well was measured. Curves for samples and standards were made and compared using a logistic transformation. Assays were performed in triplicate and the detection limit was 5 pg/ml.

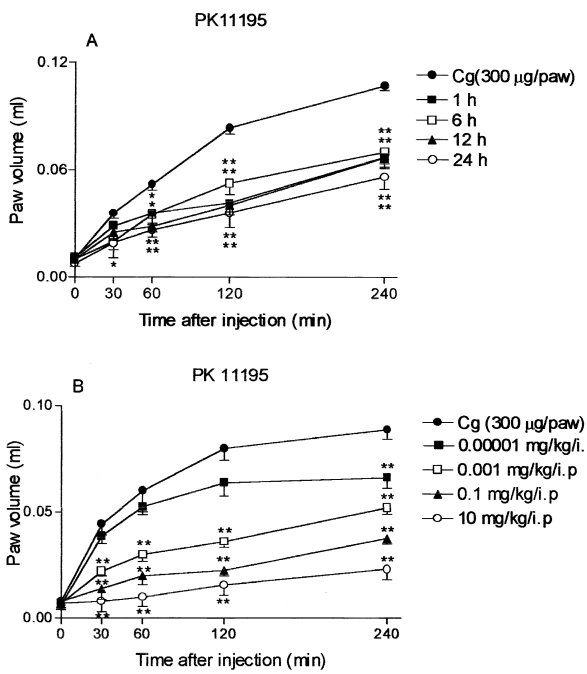

Fig. 1. Effect of PK11195 on the mouse paw oedema induced by intraplantar injection of carrageenan (Cg) (300 μg/paw): (A) time-dependent effect of PK11195 (0.1 mg/kg, i.p.) at 1–24 h prior to carrageenan injection; (B) effect of pretreatment (24 h) of PK11195 (0.00001 to 10 mg/kg, i.p.) on the paw oedema induced by carrageenan (300 μg/paw). Each point represents the mean for 6 to 10 animals and the vertical lines the S.E.M. Asterisks indicate statistically significant differences. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with respective control values.

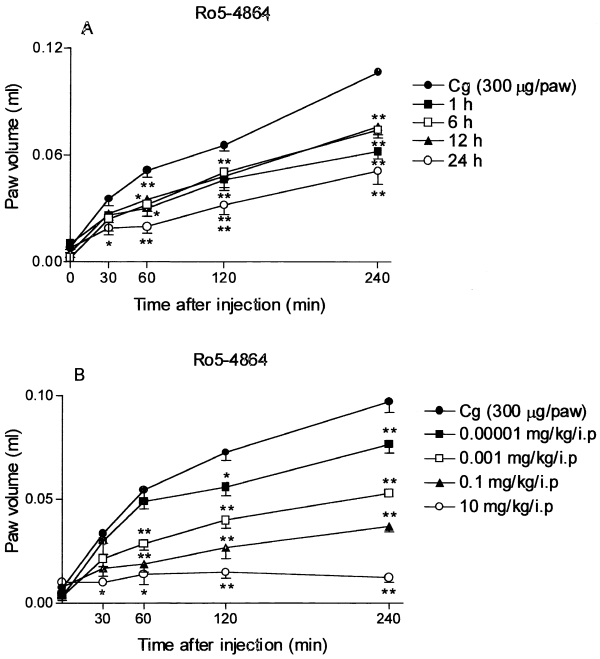

Fig. 2. Effect of Ro5-4864 on the mouse paw oedema induced by intraplantar injection of carrageenan (Cg) (300 μg/paw): (A) time-dependent effect of Ro5-4864 (0.1 mg/kg, i.p.) at 1–24 h prior to carrageenan injection; (B) effect of pretreatment (24 h) of RO5-4864 (0.00001 to 10 mg/kg, i.p.) on the paw oedema induced by carrageenan (300 μg/paw). Each point represents the mean for 6 to10 animals and the vertical lines the S.E.M. Asterisks indicate statistically significant differences. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with respective control values.

2.7. Drugs

The following drugs were used: PK11195 and Ro5-4864 were kindly supplied by Sanofi Recherche Labègé (France), bradykinin, carrageenan degree IV, substance P, histamine, 5-HT, prostaglandin E_2, platelet activating factor, indomethacin, methylene blue, Evans blue, sterile saline solution (NaCl 0.9%), May–Grunwald–Giemsa dye and phosphate buffered saline (pH 7.6: composition mmol/l: NaCl 137, KCl 2.7 and phosphate buffer salts 10) were purchased from the Sigma. Heparin (Liquemine®, Roche, Brazil). All salts used were Merck high purity grade reagents. Recombinant mouse interleukin-13, Dulbecco's modified essential medium, RPMI 1640 and foetal calf serum were from Life Technologies (Paisley, Scotland). Cell lines were purchased from the American Type Culture Collection.

Table 1
Time course study of peripheral benzodiazepine receptor ligands on the model of mouse paw oedema induced by carrageenan

Sample	Time of pretreatment	Oedema induced by carrageenan (300μg/paw) (Mean ± S.E.M.) (values 10^{-3} ml)			
		30 min	60 min	120 min	240 min
Control		35.7 ± 2.9	51.7 ± 3.2	83.5 ± 3.5	107.0 ± 2.5
PK 11195 0.1 mg/kg, i.p.	1 h	28.6 ± 7.9	35.7 ± 6.4*	41.3 ± 6.7**	67.1 ± 3.6**
	6 h	20.0 ± 9.1	35.0 ± 5.0*	52.5 ± 6.2**	70.0 ± 9.1**
	12 h	25.0 ± 3.0	28.3 ± 3.0**	40.0 ± 4.0**	66.7 ± 5.0**
	24 h	19.2 ± 4.0*	26.3 ± 4.0**	36.0 ± 8.1**	56.0 ± 6.8**
Ro5-4864 0.1 mg/kg, i.p.	1 h	26.0 ± 4.0	30.0 ± 4.4**	46.0 ± 6.0**	62.0 ± 4.0**
	6 h	24.0 ± 6.7	32.0 ± 3.7*	50.0 ± 3.1**	74.0 ± 4.5**
	12 h	27.0 ± 3.6	35.0 ± 3.2*	48.0 ± 6.4**	76.0 ± 4.7**
	24 h	18.8 ± 3.8*	19.8 ± 3.9**	31.8 ± 5.3**	51.0 ± 7.4**

The parameters were analysed 30 to 240 min after administration of carrageenan or buffered saline solution in the mouse paw. The flogistic agent was given by the intraplantar route. Each group represents the mean for 6 to 10 animals.
* $P < 0.05$, statistical analysis by ANOVA test complemented by Dunnett's test.
** $P < 0.01$, statistical analysis by ANOVA test complemented by Dunnett's test.

2.8. Statistical analysis

Data are reported as means ± S.E.M., except for the ID_{50} values in each experiment (i.e. dose of peripheral benzodiazepine receptor ligands needed to cause reduction in paw oedema induced by carrageenan by 50% in relation to its respective control values), which are presented as geometric means accompanied by their respective 95% confidence limits. Differences between groups were evaluated by analysis of variance (ANOVA) complemented with Dunnett's test or Student's unpaired *t*-test when indicated. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of peripheral benzodiazepine receptor ligands in the model of mouse paw oedema induced by carrageenan

In order to evaluate the anti-inflammatory effect of different times of pretreatment with peripheral benzodiazepine receptor ligands, a time course study was carried out. Figs. 1A and 2A show that paw oedema formation in response to carrageenan (300 μg/paw) was reduced by PK11195 and Ro5-4864 at different time intervals. The treatment of animals with peripheral benzodiazepine receptor ligands (0.1 mg/kg; i.p.) at 1, 6, 12 and 24 h inhibited paw oedema formation after carrageenan injection, respectively, in 40.6%, 35.6%, 44.1% and 51.5% for PK11195 and 35.7%, 29.4%, 28.1% and 52.5% for Ro5-4864. The percent inhibition was analysed using the area under the curve. The raw data from this experiment are shown in Table 1. For both ligands, the capacity of inhibition of paw oedema disappeared 48 h after treatment (results not shown). The time course studies indicated that the response to both ligands was optimal in animals injected 24 h earlier with peripheral benzodiazepine receptor ligands, so for this reason we chose this time of pretreatment for the dose–response studies. Paw oedema induced by carrageenan was inhibited in a dose-related manner by PK11195 and Ro5-4864 (Figs. 1B and 2B). The calculated mean ID_{50} values were 0.009 (95% confidence limits = 0.0076–0.013) mg/kg and 0.004 (95% confidence limits = 0.0025–0.0086) mg/kg for PK11195 and Ro5-4864, respectively.

3.2. Effect of peripheral benzodiazepine receptor ligands in the carrageenan-induced paw oedema model in the adrenalectomized mouse

Peripheral benzodiazepine receptor ligands administered at the dose of 0.1 mg/kg i.p., 24 h before carrageenan injection, significantly inhibited the paw oedema in animals with or without adrenal glands (Fig. 3) ($P < 0.01$).

3.3. Effect of peripheral benzodiazepine receptor ligands in the model of mouse paw oedema induced by different inflammatory mediators

Results presented in Fig. 4 reveal that PK11195 (0.1 mg/kg, i.p.), when administered 24 h before oedema

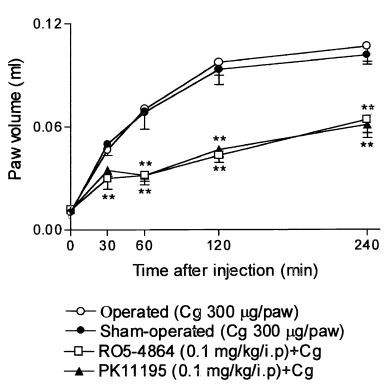

Fig. 3. Effect of PK11195 and Ro5-4864 (0.1 mg/kg, i.p.), administered 24 h earlier, on the model of adrenalectomized mouse paw oedema induced by carrageenan (Cg) (300 μg/paw). Each point represents the mean for six animals and the vertical lines the S.E.M. Asterisks indicate statistically significant differences. * $P < 0.05$ and ** $P < 0.01$ when compared with respective control values.

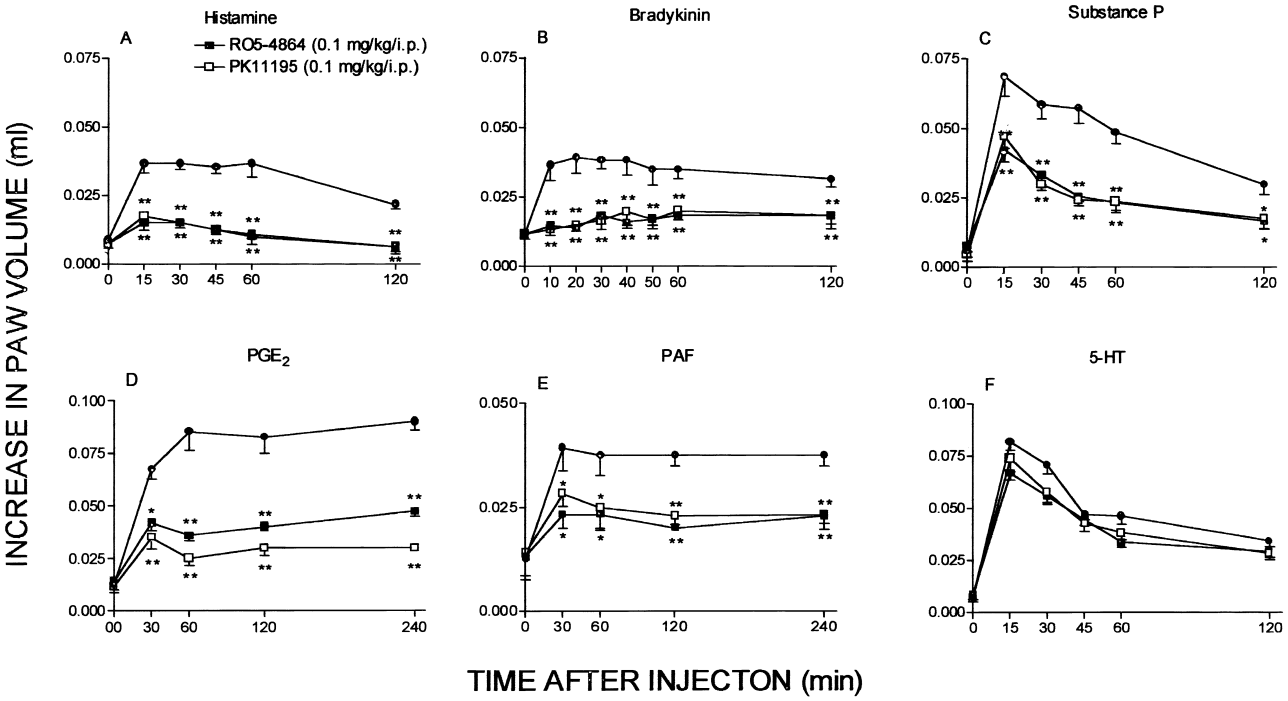

Fig. 4. Effect of Ro5-4864 (■) and PK11195 (□) (0.1 mg/kg, i.p.), administered 24 h prior, on mouse paw oedema induced by intraplantar injection of histamine (100 nmol/paw) (A), bradykinin (3 nmol/paw) (B), substance P (10 nmol/paw) (C), PGE_2 (prostaglandin E_2) (30 nmol/paw) (D), PAF (platelet activating factor) (10 nmol/paw) (E), and 5-HT (10 nmol/paw) (F). Each point represents the mean for 6 to 10 animals and the vertical lines the S.E.M. Asterisks indicate statistically significant differences. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with respective control values.

induction, induced marked inhibition of mouse paw oedema induced by histamine (100 nmol/paw), bradykinin (3 nmol/paw), substance P (10 nmol/paw), prostaglandin E_2 (30 nmol/paw) or platelet activating factor (10 nmol/paw), causing an inhibition of 65.2%, 48.3%, 46.3%, 63.7% and 34.9%, respectively. Pretreatment with Ro5-4864 (0.1 mg/kg, i.p.) 24 h before, induced an inhibition of 65.4%, 50.1%, 46.6%, 49.4% and 40.9% in oedema induction by histamine, bradykinin, substance P, prostaglandin E_2 or platelet activating factor, respectively. The percent inhibition was analysed using the area under the curve. The raw data are shown in the Tables 2 and 3. No

Table 2
Effect of peripheral benzodiazepine receptor ligands at a dose of 0.1 mg/kg i.p., 24 h before mouse paw oedema induced by histamine and bradykinin

Sample	Oedema induced by mediators (Mean ± S.E.M.) (values 10^{-3} ml)						
	Histamine (100 nmol/paw)						
	15 min	30 min	45 min	60 min	120 min		
Control	36.7 ± 3.3	36.7 ± 2.1	35.5 ± 2.4	36.7 ± 5.0	21.7 ± 1.7		
PK 11195	17.5 ± 2.5**	15.0 ± 1.9**	12.5 ± 1.6**	10.0 ± 3.0**	6.30 ± 1.8**		
Ro5-4864	15.0 ± 2.7**	15.0 ± 1.9**	12.5 ± 1.6**	10.9 ± 1.6**	6.25 ± 2.6**		
	Bradykinin (3 nmol/paw)						
	10 min	20 min	30 min	40 min	50 min	60 min	120 min
Control	36.7 ± 5.6	39.2 ± 5.6	38.3 ± 3.0	38.3 ± 5.4	35.0 ± 5.6	35.5 ± 3.4	31.7 ± 3.0
PK 11195	13.3 ± 2.1**	15.0 ± 2.2**	16.7 ± 3.3**	19.7 ± 4.8**	17.0 ± 3.4**	20.0 ± 0.0**	18.3 ± 4.7**
Ro5-4864	14.6 ± 3.3**	14.0 ± 0.0**	18.3 ± 3.0**	16.0 ± 2.2**	17.0 ± 2.2**	18.3 ± 1.7**	18.3 ± 3.3**

The parameters were analyzed 10 to 120 min after administration of histamine and bradykinin or buffered-saline solution in the mouse paw. The flogistic agent was given by the intraplantar route. Each group represents the mean for 6 to 10 animals.

$^{**}P < 0.01$, statistical analyses, ANOVA complemented by Dunnett's test.

Table 3
Effect of peripheral benzodiazepine receptor ligands at a dose of 0.1 mg/kg i.p., 24 h before mouse paw oedema induced by substance P, prostaglandin E_2 and platelet activating factor

Sample	Oedema induced by mediators (Mean ± S.E.M.) (values 10^{-3} ml)				
	Substance P (10 nmol/paw)				
	15 min	30 min	45 min	60 min	120 min
Control	68.6 ± 7.0	58.6 ± 5.0	57.1 ± 5.2	48.6 ± 4.0	30.0 ± 3.8
PK 11195	47.1 ± 4.2**	30.0 ± 2.1**	24.2 ± 2.0**	23.7 ± 3.2**	17.5 ± 3.6*
Ro5-4864	42.2 ± 4.3**	33.3 ± 3.3**	25.6 ± 1.7**	23.3 ± 3.7**	16.6 ± 2.9*
	Prostaglandin E_2 (30 nmol/paw)				
	30 min	60 min	120 min	240 min	
Control	67.5 ± 4.8	85.5 ± 8.6	82.5 ± 7.5	90.0 ± 4.0	
PK 11195	35.0 ± 5.6**	25.0 ± 3.4**	30.0 ± 3.6**	30.0 ± 0.0**	
Ro5-4864	42.0 ± 3.7*	36.0 ± 2.4**	40.0 ± 0.0**	47.5 ± 2.5**	
	Platelet activating factor (10 nmol/paw)				
	30 min	60 min	120 min	240 min	
Control	39.2 ± 5.3	37.5 ± 4.8	37.5 ± 2.5	37.5 ± 2.5	
PK 11195	28.3 ± 3.0*	25.0 ± 5.5*	23.0 ± 2.1**	23.3 ± 2.1**	
Ro5-4864	23.3 ± 3.3*	23.3 ± 3.3*	20.0 ± 0.0**	23.0 ± 3.3**	

The parameters were analyzed 15 to 240 min after administration of histamine and bradykinin or buffered-saline solution in the mouse paw. The flogistic agent was given by the intraplantar route. Each group represents the mean for 6 to 10 animals.
* $P < 0.05$, statistical analysis by ANOVA test complemented by Dunnett's test.
** $P < 0.01$, statistical analysis by ANOVA test complemented by Dunnett's test.

significant inhibition by PK11195 or Ro5-4864 was observed in 5-HT (10 nmol/paw) paw oedema (data not shown).

3.4. Effect of peripheral benzodiazepine receptor ligands in the mouse model of pleurisy induced by carrageenan

Injection of carrageenan (1%) into the pleural space of mice induced both an influx of cells, which was primarily composed of neutrophils, and an increase of the fluid leakage, 4 h after pleurisy induction. Fig. 5 shows that maximal cell influx (mean ± S.E.M.) was induced by carrageenan (total cells = $4.5 \pm 0.3 \times 10^6$) in comparison to sterile phosphate-buffered saline-treated animals ($1.3 \pm 0.1 \times 10^6$) ($P < 0.01$). Significant differences were observed in relation to the exudate values (mean ± S.E.M.) induced by carrageenan (8.0 μg/ml) and sterile phosphate-buffered saline-treated animals (0.025 ± 0.1 μg/ml). No significantly changed values were obtained for the exudate induced by carrageenan in animals treated or untreated with peripheral benzodiazepine receptor ligands (Fig. 5A) ($P > 0.01$). Mice treated with PK 11195 (0.1 mg/kg), 24 h prior to pleurisy induction, showed a significant inhibition of total and differential cell recruitment (% of inhibition: total cells: 68 ± 8 and neutrophils: 57 ± 7) induced by carrageenan, with no change in exudate levels. Treatment of animals with 0.1 mg/kg Ro5-4864, 24 h before pleurisy induction, also resulted in a significant inhibition of cell influx evoked by carrageenan (% inhibition: total cells: 53 ± 8 and neutrophils: 52 ± 2) (Fig. 5B).

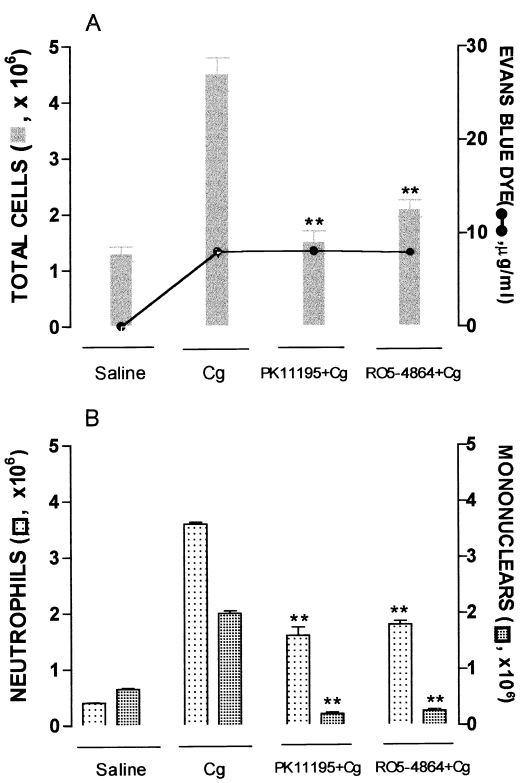

Fig. 5. Effect of PK11195 and Ro5-4864 (0.1 mg/kg, i.p.), administered 24 h prior, on the model of mouse pleurisy induced by carrageenan: (A) total cell and exudate levels; (B) differential cells. Each point represents the mean for six animals and the vertical lines the S.E.M. Asterisks indicate statistically significant differences. ** $P < 0.01$ when compared with respective control values.

Table 4
Effect of peripheral benzodiazepine receptor ligands on the interleukin-6 and interleukin-13 levels of fluid leakage in the mouse model of pleurisy induced by carrageenan (1%, 4 h)

Group	Interleukin-6 (pg/ml)	Interleukin-13 (μg/ml)
Saline-treated	0	0.045 ± 0.002
Carrageenan-treated	667 ± 0.3	8.0 ± 0.6
Ro5-4864-treated	0	0.036 ± 0.0025
PK11195-treated	0	0.023 ± 0.002
Ro5-4864 + carrageenan	0**	0.050 ± 0.0035**
PK11195 + carrageenan	0**	0.049 ± 0.003**

The parameters were analyzed 4 h after administration of carrageenan or saline solution and evaluated by means of interleukin-6 and interleukin-13 levels in the exudate of the mouse pleural cavity. Animals were treated 24 h before pleurisy induction with 0.1 mg/kg of PK11195 or Ro5-4864. The values represent the means ± S.E.M. ($n = 6–10$ animals per group). **$P < 0.01$, statistical analysis by ANOVA test complemented by Dunnett's test.

3.5. Effect of peripheral benzodiazepine receptor ligands on the levels of interleukin-6 and interleukin-13 in the fluid leakage induced by carrageenan in the mouse model of pleurisy

Table 4 shows that the injection of carrageenan induces an increase in the release of interleukin-13 and interleukin-6 in the mouse pleural cavity, as compared with sterile phosphate-buffered saline-treated animals. Interestingly, pretreatment (24 h) of the animals with PK11195 or Ro5-4864, at the dose of 0.1 mg/kg i.p., resulted in a marked and almost complete inhibition of carrageenan-produced interleukin-13 and interleukin-6 in the mouse pleural cavity fluid leakage ($P < 0.01$).

4. Discussion

These results demonstrate that in vivo treatment of mice with peripheral benzodiazepine receptor ligands exerts an inhibitory effect on the inflammatory response in the different models of acute inflammation. In the first model, we showed that PK 11195 and Ro5-4864 strongly inhibited mouse paw oedema formation induced by carrageenan in a time- and dose-dependent manner. The first phase (1–2 h) of *rat* paw oedema formation by carrageenan is characterized by release of histamine, 5-HT, bradykinin, platelet activating factor and substance P (Di Rosa et al., 1971; Hwang et al., 1986; Gilligan et al., 1994). The second phase (3–4 h) is mainly sustained by prostaglandin release (Di Rosa and Willoughby, 1971) and seems to be modulated by nitric oxide (Sautebin et al., 1995). Very few studies exist on mediators implicated in carrageenan mouse paw oedema, but it has been demonstrated that bradykinin, substance P and prostaglandin are implicated (Campos et al., 1999; Cao et al., 2000; Ueno et al., 2000). Both paw oedema models seem to be very similar because screening tests show similar profiles for different anti-inflammatory drugs in the mouse and rat paw oedema induced by carrageenaan (Sugishita et al., 1981). Histological analysis of the subplantar area of the mouse paw after injection of carrageenan reveals a diffuse cellular infiltrate with predominance of neutrophils (Henriques et al., 1987). We have now shown that peripheral benzodiazepine receptor ligands are able to decrease the paw oedema induced by different inflammatory mediators such as histamine, substance P, bradykinin, prostaglandin E_2 and platelet activating factor. Only the paw oedema induced by 5-HT was unaffected by PK11195 or Ro5-4864. To understand the exact mechanism of action of anti-inflammatory molecules, it is necessary to know the precise role of each mediator in the inflammatory process. Despite the complexity of interpreting all data about the interdependence of effects among the different mediators in inflammation, many studies have been carried out in attempts to clarify this question. For example, histamine, 5-HT, bradykinin, prostaglandin E_2 and platelet activating factor have been shown to be potent vasoactive mediators, and some of these mediators exert their effect by causing neutrophil migration also. Many of these inflammatory regulators are able to activate the vascular endothelium, partly by enhancing the release of pro-inflammatory cytokines and partly by inducing the expression of adhesion molecules. For example, bradykinin is able to up-regulate the production of interleukin-6, involved in vascular endothelium permeability (Maruo et al., 1992; Ishizu et al., 1995; Paegelow et al., 1995; Modeer et al., 1998). Some of the effects of bradykinin and substance P are due to their capacity to induce histamine release from mast cells by release of Ca^{2+} from the intracellular calcium store. Histamine interacts directly with endothelial cells and increases permeability, platelet activating factor, TNF-α and interleukin-6 secretion. The TNF-α released by vascular endothelial cells induces the expression of adhesion molecules such as ICAM-1 (intracellular adhesion molecule-1) and E-selectin (Miki et al., 1996). Substance P, which mediates vasodilatation and augments vascular permeability, is also an important modulator of cytokines, including TNF-α from mast cells, macrophages, and others (Cocchiara et al., 1997; Preval and Cantagrelm, 1998). TNF-α seems to be implicated in substance P-induced leukocyte migration and histamine release, and adds potency to the effect of other pro-inflammatory cytokines in the expression of adhesion molecules (Saban et al., 1997; Brzezinska-Blaszczyk and Pietrzak, 1997; Lambert et al., 1998). Substance P influences various functions of polymorphonuclear leukocytes, including phagocytosis and the respiratory burst. Enhancement of substance P or O_2 production was demonstrated when neutrophils were exposed to platelet activating factor, which by itself did not elicit the respiratory burst, suggesting that platelet activating factor might be capable of amplifying neutrophil oxidative responses at sites of

inflammation (Gay et al., 1986; Dianzani et al., 1994). Platelet activating factor, a phospholipid product of neutrophils, alveolar macrophages, monocytes and platelets, seems to elicit oedema by a mechanism dependent on extracellular calcium, but independent of histamine, 5-HT or prostaglandin activity (Goldenberg and Meurer, 1984). Platelet activating factor induces intravascular accumulation of neutrophils (Archer et al., 1985). Furthermore, platelet activating factor and TNF seem to act synergistically in order to increase neutrophil–endothelial adhesion by stimulating endothelial expression of ICAM-1 and E-selectin (Sterner-Kock et al., 1996). TNF and interleukin-1 promote the synthesis of platelet activating factor and this agent is able to modulate interleukin-6 production in endothelial cells (Bussolino et al., 1990; Lacasse et al., 1997). The release of platelet activating factor may be involved in the biosynthesis of prostaglandins, which initiates the second phase of paw oedema (Hwang et al., 1986). Prostaglandin formation is regulated by constitutive (COX-1) or inducible (COX-2) isoforms of cyclooxygenase. The expression of COX-2 is induced by different inflammatory stimuli such as endotoxins, interleukin-1, TNF and interleukin-13 in macrophages, fibroblasts, endothelial cells and neutrophils (Anderson et al., 1996; Yu et al., 1998). Carrageenan also enhances the expression of COX-2 in epidermis, skeletal muscle and inflammatory cells in paw oedema models, suggesting that prostaglandin E_2 production is linked through the expression of COX-2 (Nantel et al., 1999). The increase in prostaglandin E_2 synthesis causes induction of interleukin-6, and the expression of COX-2 seems to mediate this process (Hinson et al., 1996). Interleukin-1 causes induction of ICAM-1 and COX activity in human vascular smooth muscle (Bishop-Bailey et al., 1998). 5-HT oedema cannot be inhibited by indomethacin, suggesting that arachidonate metabolites do not participate in this model (Amico-Roxas et al., 1989). TNF-α enhances 5-HT uptake at inflammatory sites where the 5-HT rate is high, suggesting that TNF-α may act to re-normalize 5-HT levels (Mossner et al., 1998). Nothing is known about the mechanism by which peripheral benzodiazepine receptor ligands exert their anti-inflammatory action. Activation of steroid biosynthesis is the best-characterized activity of peripheral benzodiazepine receptor. Peripheral benzodiazepine receptor ligands stimulate steroid synthesis in adrenal, placenta, testes, ovaries and nervous cells by enhancing the translocation of cholesterol from outer to inner mitochondrial membranes, the rate-limiting step in steroidogenesis (Krueger, 1995). In the brain, peripheral benzodiazepine receptor is increased in several pathological situations where inflammatory reaction of nervous tissue is present (Messmer and Reynolds, 1998; Raghavendra et al., 2000). The peripheral benzodiazepine receptor density and the levels of its endogenous ligand, the diazepam-binding inhibitor derived-peptide, were highly increased in rat sciatic nerve degeneration, and the administration of Ro5-4864 (i.p.) induced a significant increase in pregnenolone levels in sciatic nerve and plasma, suggesting that Ro5-4864 might stimulate pregnenolone synthesis not only in steroidogenic organs such as gonads and adrenal glands, but also by local synthesis in the sciatic nerve (Lacor et al., 1999). Peripheral benzodiazepine receptor ligands reduce the macrophage secretion of interleukin-1, interleukin-6 and TNF-α (Zavala et al., 1990b). Other than nervous cells and classical steroid tissues, there are additional sites of steroidogenesis. For example, the synthesis of steroids has been demonstrated in thymocyte and vascular wall where peripheral benzodiazepine receptor and *P*450scc, the enzyme responsible for the transformation of cholesterol into pregnenolone, were detected (French and Matlib, 1988; Takeda et al., 1994; Hirsch et al., 1998; Jenkinson et al., 1999). Thus, it could be hypothesized that peripheral benzodiazepine receptor ligands exert an anti-inflammatory action via steroid synthesis, which may down-regulate different pro-inflammatory interleukin releases and consequently inhibit the action of different inflammatory mediators. This possible steroid synthesis stimulation may be from the site of the inflammation and different cells that carry out steroid synthesis. In paw oedema, peripheral benzodiazepine receptor ligands probably have the capacity to inhibit the migration of neutrophils, which are the main cells present in the mouse paw after injection of carrageenan. If this hypothesis is true, the ability of peripheral benzodiazepine receptor ligands to decrease the release of pro-inflammatory cytokines such as TNF-α, which enhances the expression of adhesion molecules like ICAM-1, could be implicated in their anti-inflammatory action. Many studies have shown that interleukin-6, a cytokine that enhances vascular permeability, has its production induced by prostaglandin E_2, histamine, bradykynin and platelet activating factor (Delneste et al., 1994; Paegelow et al., 1995; Portanova et al., 1996; Hinson et al., 1996; Lacasse et al., 1997). These findings suggest that interleukin-6 might contribute to the vascular permeability caused by these different mediators. Thus, it could be postulated that peripheral benzodiazepine receptor ligands may inhibit the oedema induced by bradykinin, histamine, platelet activating factor and prostaglandin partly via inhibition of the release of interleukin-6. The potential of peripheral benzodiazepine receptor ligands to inhibit the release of TNF-α could partially explain the inhibition of the oedema induced by substance P. The inability of peripheral benzodiazepine receptor ligands to inhibit the oedema induced by 5-HT could be explained by the down-regulation of the TNF release by peripheral benzodiazepine receptor ligands.

In the mouse model of pleurisy that is characterized by an enhancement of cell migration, due mainly to marked neutrophil migration 4 h after carrageenan injection, the peripheral benzodiazepine receptor ligands were able to exert an inhibitory effect on neutrophil influx. Regarding mouse model of pleurisy induced by carrageenan, there is little evidence for participation of inflammatory mediators,

but it is well known that, in this model, bradykinin and substance P are involved in fluid leakage formation, and the kinin effect is associated with activation of bradykinin B_2 receptor and constitutive bradykinin B_1 receptor (Saleh et al. 1996; Vianna and Calixto, 1998). Neutrophil migration in the rat pleurisy induced by carrageenan seems to be caused by cytokines such as interleukin-1 and interleukin-6 (Oh-ishi, 1997), while kinin and prostaglandin I_2 must be the main mediators for plasma exudation (Oh-ishi et al., 1989). Neutrophils are an important source of interleukin-6 in acute inflammatory reactions, and anti-inflammatory actions of glucocorticoids are attributable in part to inhibition of interleukin-6 production by these cells (Mianji et al., 1996), probably by inhibition of interleukin-1 that induces interleukin-6 production (Zitnik et al., 1994). If interleukin-6 has a similar role in rat and mouse pleurisy models, it may be supposed that peripheral benzodiazepine receptor ligands cause inhibition of neutrophil migration in pleurisy, and that this is at least partly mediated via stimulation of steroid synthesis that inhibits interleukin-1-induced interleukin-6 production. The inability to induce fluid leakage would be caused by the incapacity of peripheral benzodiazepine receptor ligands to down-regulate the mediators involved in the vascular exudation into the pleural cavity in carrageenan-induced mouse pleurisy. The mediators implicated in inhibition of paw oedema, and plasma leakage in pleurisy seem to be different, explaining the different inhibition profiles of exudation in the two models. Furthermore, neutrophils seem unable to produce interleukin-13 (Reglier et al., 1998). The exudate release in the pleural cavity of mice seems to come, not only from mediators released from the neutrophils, but also from mast cells that have the ability to produce interleukin-13. Glucocorticoids suppress gene expression and production of interleukin-13 by lung mast cells (Fushimi et al., 1998). Our studies demonstrate that PK11195 and Ro5-4864 are potent inhibitors of interleukin-13 production in pleural exudation. Interleukin-13 has been ascribed anti-inflammatory roles in several experimental models by induction of production and proliferation of B-cells, and by inhibition of the production of inflammatory cytokines (Hancock et al., 1998). However, interleukin-13 causes both inflammation when expressed selectively in the murine lung, and interleukin-13 mRNA increases in alveolar macrophages in subjects with fibrotic lung disease, suggesting a role for interleukin-13 in the development of these diseases (Zhu et al., 1999), also enhancing neutrophil phagocytosis (Yu et al., 1998). Furthermore, our data shows that peripheral benzodiazepine receptor ligands induce an almost complete inhibition of both cytokines, interleukin-6 and interleukin-13, in pleural exudation, and this inhibition seems to contribute to the inhibition of the inflammatory process, without inhibition of exudation, suggesting that, in our model, interleukin-13 has a pro-inflammatory role because its inhibition does not up-regulate the inflammatory process.

Results presented here extend previous findings that peripheral benzodiazepine receptor is involved in inflammatory responses. The collected data obtained in this study suggest that peripheral benzodiazepine receptor ligands have anti-inflammatory properties, based on their capacity to inhibit the action of different inflammatory mediators. This action could be mainly due to inhibition of the release of pro-inflammatory cytokines that presumably causes a decrease in the expression of adhesion molecules and consequently the inhibition of neutrophil migration. We propose that the whole mechanism of action could be regulated in part by glucocorticoid synthesis in local injuries and tissues able to synthesize steroids, but the validity of this hypothetical mechanism of anti-inflammatory action of peripheral benzodiazepine receptor ligands needs to be confirmed.

In conclusion, peripheral benzodiazepine receptor ligands might be of pharmacological interest as potential anti-inflammatory agents, for example in pulmonary infections such as fibrotic lung disease that is associated with excessive influx of neutrophils and enhancement of cytokines such as interleukin-6 and interleukin-13.

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