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N-(2-Hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation

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Abstract

Mast cells play a key role in inflammatory reactions triggered by tissue injury or immune perturbations. Little is known about endogenous molecules and mechanisms capable of modulating inappropiate mast cell activity. *N*-(2-Hydroxyethyl)hexadecanamide (palmitoylethanolamide), found in peripheral tissues, has been proposed to act as a local autacoid capable of negatively regulating mast cell activation and inflammation – hence the acronym Autacoid Local Inflammation Antagonism (ALIA). Recently, *N*-(2-hydroxyethyl)hexadecanamide (LG 2110/1) has been reported to down-modulate mast cell activation in vitro by behaving as an agonist at the peripheral cannabinoid CB_2 receptor. Here, we have characterized and functionally correlated the anti-inflammatory actions of LG 2110/1 with its ability to control mast cell activation, when given orally in a battery of rodent models of inflammation. LG 2110/1 diminished, in a dose-dependent and correllated manner, the number of degranulated mast cells and plasma extravasation induced by substance P injection in the mouse ear pinna. In addition, LG 2110/1 reduced dose dependently plasma extravasation induced by passive cutaneous anaphylaxis reaction. In adult rats LG 2110/1 decreased, in a dose-dependent manner, carrageenan-induced hindpaw edema and hyperalgesia, but not phospholipase A_2-induced hindpaw edema. Further, anti-edema effects were observed when utilizing dextran and formalin, known to also cause mast cell activation. Locally administered LG 2110/1 was likewise effective in minimizing dextran-induced hind paw edema. In contrast, equivalent amounts of palmitic acid plus ethanolamine were ineffective against plasma extravasation provoked by substance P. LG 2110/1 did not decrease plasma extravasation induced by the substance P fragment, substance P-(6–11), known to be inactive on mast cells. These results indicate that orally administered *N*-(2-hydroxyethyl)hexadecanamide is effective in: (a) directly down-modulating mast cell activation in vivo; (b) suppressing pathological consequences initiated by mast cell activation independently of the activating stimuli; (c) exerting an anti-inflammatory action distinguishable from that of classical steroidal and non-steroidal anti-inflammatory agents. These findings raise the possibility that *N*-(2-hydroxyethyl)hexadecanamide and related saturated *N*-acylamides ('ALIAmides') represent novel therapeutic agents useful in the management of inflammatory disease conditions.

Keywords: *N*-Acylethanolamide; Palmitoylethanolamide; Autacoid local inflammatory antagonism; Cannabinoid receptor, peripheral; Neurogenic inflammation; Passive cutaneous anaphylaxis

1. Introduction

Inflammation often accompanies tissue injury and the pathogenesis of many chronic disease states, including those of an autoimmune nature (Dinarello, 1991). Regardless of etiology or localization, inflammation involves changes in vascular permeability, with concomitant recruitment of components of the immune system (Payan et al., 1984). Innervating primary afferents also play a major role (Couture and Cuello, 1984; Janskó et al., 1967). Although the peripheral terminals of these neurons are directly or indirectly activated by noxious insults or tissue injury, mediators generated at the site of inflammation are responsible for the nociceptor sensitization (Levine et al., 1993). Such phenomena not only act to relay information to the central nervous system but also actively contribute, via neuropeptide release (most notably substance P) from their peripheral terminals, to tissue inflammation. Local functional interplay between the nervous and the immune

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systems can thus be expected to intimately regulate the entity and duration of the inflammatory response. Dysregulation of neuroimmune interactions may well underlie the propagation and exacerbation of inflammatory reactions.

Mast cells, long known to be involved in inflammatory reactions, have been suggested to function as intermediate sensors and effectors in the communication between the nervous and the immune systems (Stead and Bienenstock, 1990). Mast cells occur in many peripheral tissues in perivascular regions in close apposition to innervating fibers and also within peripheral nerves (Olsson, 1968). Stimulation of nerves can affect the state of mast cell activation in vivo (Bani-Sacchi et al., 1986). Nerve and immune mediators such as substance P, IgE and anaphylatoxins C3a and C5a cause mast cell activation in vitro and in vivo (Galli, 1993; Johnson et al., 1975; Lowman et al., 1988). Activated mast cells release secretory products which, in turn, stimulate or facilitate antidromic nerve activity (Janiszewski et al., 1990), vascular permeability changes and immune cell recruitment (Galli, 1993). Further, mast cells are reported to synthesize, store and release nerve growth factor (Leon et al., 1994a), suggesting that they may also control long-term adaptive/reactive modifications of the nervous system in response to noxious tissue perturbations. Little is known about local physiological down-modulators of mast cell activity.

N-(2-Hydroxyethyl)hexadecanamide (palmitoylethanolamide) has been hypothesized to behave as an autacoid capable of locally modulating mast cell activation in response to neurogenic inflammatory stimuli such as substance P (Aloe et al., 1993) – hence the acronym Autacoid Local Inflammation Antagonism (ALIA). This *N*-acylamide has recently been reported to down-modulate IgE-triggered activation of cultured mast cells, an effect apparently mediated by peripheral cannabinoid receptors (CB_2) present on these cells (Facci et al., 1995). The present study was undertaken to characterize and functionally correlate the anti-inflammatory actions of orally administered *N*-(2-hydroxyethyl)hexadecanamide with its capacity to control mast cell activation in various rodent models of inflammation.

2. Materials and methods

2.1. Animals and reagents

Male Wistar rats (200–250 g, Charles River, Calco, Italy) and female BALB/c mice (20–22 g, Modelli Biologici Sperimentali, Treviso, Italy) were used throughout. Animals were kept on a 12 h-12 h light-dark cycle (20–22°C) at least 6 days before being used. Rats utilized for measurement of paw volume and hyperalgesia were fasted overnight with free access to water. Mice used for measurement of plasma extravasation and mast cell degranulation were fed ad libitum until beginning the experiment. All treatments were carried out under light ether anethesia and in accordance with the declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals.

Indomethacin, cromolyn sodium salt, formalin, formamide, carboxymethylcellulose, carrageenan lambda, substance P fragment, substance P-(6–11), venom phospholipase A_2 from *Naja mocambique mocambique*, rabbit anti-human albumin IgG and human albumin were from Sigma; substance P from Clinalfa, Switzerland; dextran (70000 MW) from Sifra Spa, Italy; Evans blue from Fluka. Sterile and pyrogen-free saline (0.9% NaCl) was used to prepare all solutions. *N*-(2-Hydroxyethyl)hexadecanamide, coded LG 2110/1, was synthesized as previously described (Roe et al., 1952) and its purity, as assessed by high pressure liquid chromatography (Nakae and Kunihiro, 1987), was greater than 99.5%.

2.2. Mast cell degranulation in the mouse ear pinna

Mast cell degranulation in vivo was evaluated in the mouse ear pinna 10 min after either local injection of substance P or vehicle (3 μl saline). Animals were killed and the injected pinna was rapidly excised and placed into methacarne fixative (methanol, acetic acid, chloroform, 1 : 1 : 1 v/v). The specimens were paraffin embedded, cut into 5 μm sections, and stained with toluidine blue. Mast cell numbers (total and degranulated) were evaluated at the site of injection, 500 μm below, and 500 μm above this landmark. The frequency of degranulated mast cells for each animal was calculated from the mean frequency of degranulated mast cells obtained from these three sections.

2.3. Substance P-induced plasma extravasation in the mouse ear pinna

Plasma extravasation was induced by subcutaneous injection of substance P (1 pmol) in a volume of 3 μl in the ear pinna; control animals received the same volume of saline only. Mice were immediately injected intravenously with Evans blue (100 mg/kg) and killed 2 h later. Extravasation was evaluated as the quantity of Evans blue present in the mouse ear pinna. The dye was extracted by homogenizing the injected ear pinna in 2 ml of formamide using a Polytron homogenizer, and then incubating at 50°C for 2 h. After centrifugation (18000 rpm, $\times$15 min), the amount of Evans blue present in the supernatant was measured by optical absorbance at 620 nm according to Saria and Lundberg (1983).

2.4. Passive cutaneous anaphylaxis reaction

Passive cutaneous anaphylaxis was induced in the mouse ear pinna by injecting subcutaneously 7 μg of rabbit anti-human albumin IgG in 3 μl saline; control animals received 3 μl saline only. Twenty-four hours later mice were injected intravenously with human albumin (40

mg/kg) plus Evans blue (100 mg/kg) and sacrificed 30 min later. Extravasation of plasma proteins was evaluated as described above. Preliminary experiments indicated that under these conditions passive cutaneous anaphylaxis-induced Evans blue extravasation increased linearly with the amount of rabbit anti-human albumin IgG ($ED_{50} \cong 7$ μg/3 μl).

2.5. Rat paw edema

Hind paw edema was induced by subplantar injection of 1% (w/v) carrageenan, 5% formalin, 0.8% (w/v) dextran in 100 μl of saline or 3 μg of phospholipase A_2 from *Naja mocambique mocambique* in 100 μl of saline containing 1 mM of calcium chloride; the contralateral paw was injected with the same volume of vehicle. Hind paw volumes were measured by plethysmometry (Basile Ugo, Varese, Italy) according to Di Rosa and Willoughby (1971). Edema formation for each animal was estimated from the difference in hind paw volumes.

2.6. Carrageenan-induced hyperalgesia

Hyperalgesia was produced by subplantar injection of 100 μl of 1% (w/v) carrageenan into the hind paw. The mechanical nociceptive threshold, defined as the pressure required to elicit the withdrawal response expressed in g, was measured using an analgesimeter (Basile Ugo, Varese, Italy) according to Randall and Selitto (1957). The pressure was applied to the hindpaw at a linearly increasing rate of 16 g/s with a cut off value of 250 g.

2.7. Drug treatment

Indomethacin and LG 2110/1 were mechanically dispersed in 1.5% carboxymethylcellulose in saline using a Polytron homogenizer and were given orally by gavage, 1 h before the induction of edema or extravasation (unless specified otherwise). Cromolyn (sodium salt) was dissolved in saline and was locally injected together with dextran in rats. In the extravasation experiments, cromolyn was given intravenously (10 ml/kg) 30 min before Evans blue.

2.8. Calculations and statistical analysis

Values reported are means ± S.E.M. Statistical evaluation was performed by one-way ANOVA followed by Student-Newman-Keuls *t*-test for multiple comparisons; the maximal effect (E_{max}) was calculated with a computer-assisted program (Tallarida and Murray, 1987). Inhibition of mast cell degranulation and Evans blue extravasation were calculated as a percentage of the maximal possible effect (% MPE) according to the formula:

$$\%MPE = \left(E_{veh} - E_{drug}/E_{veh} - E_{baseline}\right) \times 100$$

were E_{veh} = values observed following substance P injection and vehicle administration; E_{drug} = values observed following substance P injection and LG 2110/1 treatment; $E_{baseline}$ = values observed following saline injection and vehicle administration.

3. Results

3.1. Substance P-induced mast cell degranulation and extravasation

Injection of substance P in the mouse ear pinna produced a highly significant increase in the number of locally degranulated mast cells (15.1 ± 2.4 and 39.2 ± 2.8 in saline- and substance P-injected ear pinnas, respectively; $P < 0.001$, $n = 5$). Orally given LG 2110/1 prevented, in a dose-dependent manner, mast cell degranulation by substance P already 10 min after the stimulus (Fig. 1A). These findings with adult mice confirm earlier experiments in neonatal rats treated subcutaneously with LG 2110/1 (Aloe et al., 1993).

Substance P injected in the mouse ear pinna produced a linear log-dose-dependent increase of Evans blue extravasation, being maximal at 1–3 pmol (data not shown). Prevention of substance P-induced mast cell degranulation by oral LG 2110/1 resulted in the reduction of substance P-induced plasma extravasation. The effect of subcutaneous injection of 1 pmol substance P was reduced in a dose-dependent fashion by oral LG 2110/1 (Fig. 1B). Importantly, inhibition by LG 2110/1 of substance P-induced mast cell degranulation and Evans blue extravasation are linearly correlated (Fig. 1C). Doses resulting in 50% of MPE calculated by probit analysis were 0.65 mg/kg and 0.86 mg/kg for mast cell degranulation and Evans blue extravasation, respectively. The effect of a higher dose of substance P (3 pmol) was equally antagonized by LG 2110/1 at 10 mg/kg (−84.9 ± 9.6%; $P <$ 0.001 vs. vehicle, $n = 10$). In contrast, palmitic acid plus ethanolamine, given orally in amounts equivalent to 1 and 10 mg/kg LG 2110/1 failed to diminish extravasation induced by 1 pmol of substance P (data not shown), implying a direct action of the intact LG 2110/1 molecule.

Substance P-induced extravasation reflects a direct activation of both mast cells and endothelial cells, whereas the substance P fragment (substance P-(6–11)) appears to cause extravasation independent of mast cell activation (Devillier et al., 1989; Tomoe et al., 1992). LG 2110/1 (1 and 10 mg/kg) was unable to prevent plasma extravasation induced by the substance P fragment, substance P-(6–11) (1 pmol) (data not shown), confirming that the *N*-acylamide did not influence a substance P-induced modification of vascular endothelium permeability.

The anti-inflammatory agents indomethacin and cromolyn inhibited substance P-induced extravasation. Maximal effects were observed after oral administration of 5

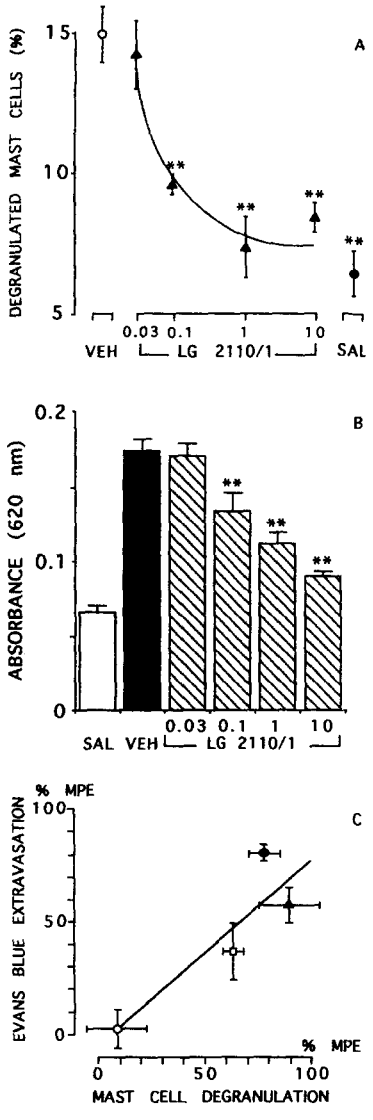

Fig. 1. LG 2110/1 reduces substance P-induced mast cell (MC)-dependent effects in the mouse ear pinna. (A) Dose dependence of LG 2110/1 in reducing MC degranulation induced by substance P injection in the mouse ear pinna. The percentage of degranulated MCs present in 5 μm tissue sections was evaluated 10 min after saline (●) (3 μl) or substance P (1 pmol/3 μl) injection in the ear pinna of mice pretreated orally for 1 h with vehicle (○) or different doses (mg/kg) of LG 2110/1 (▲). ** $P < 0.001$ vs. the substance P + vehicle-treated group; $n = 5$. (B) Dose-dependence of LG 2110/1 in reducing Evans blue extravasation after injection of substance P. The quantity of Evans blue in the ear pinna was measured by absorbance at 620 nm, 2 h after injection of saline (open column) (3 μl) or substance P (1 pmol/3 μl) in mice pretreated orally for 1 h with vehicle (black column) or different doses (mg/kg) of LG 2110/1 (hatched columns). ** $P < 0.001$ vs. the vehicle-treated group ($n = 8$). (C) Correlation between inhibitory activity of LG 2110/1 on MC degranulation and Evans blue extravasation induced by injection of substance P. % MPE (see calculations and statistical analysis) was calculated for different doses of LG 2110/1: (○) 0.03; (□) 0.1; (▲) 1 and (●) 10 mg/kg; orally. The linear regression line for mean values was calculated ($r = 0.788$).

mg/kg indomethacin (−60.7%) (see also Khalil and Helme, 1989a) or intravenous administration of 0.1 mg/kg cromolyn (−73.0%) (data not shown).

3.2. Passive cutaneous anaphylaxis-induced extravasation

Evans blue extravasation induced by passive cutaneous anaphylaxis was dose dependently diminished by oral LG 2110/1 (Fig. 2). A dose of 1 mg/kg LG 2110/1 was maximally effective (−65.4%); higher doses (10 mg/kg) produced no greater inhibition. This effect on passive cutaneous anaphylaxis-induced extravasation demonstrates that LG 2110/1 is able to reduce mast cell activation mediated by the anaphylatoxins C3a and C5a which are liberated by complement activation after immune complex formation. Passive cutaneous anaphylaxis-induced extravasation is mediated at least in part by mast cell activation, since it was inhibited by intravenously given cromolyn, a well-known mast cell stabilizer (−57.0% at 0.3 mg/kg) (Table 1). That cyclooxygenase products do not mediate passive cutaneous anaphylaxis-induced extravasation (Inagaki et al., 1986) was shown by the inability of indomethacin to inhibit this form of plasma extravasation. Indomethacin (5 mg/kg) administered orally actually enhanced this extravasation (+94.6%) (Table 1).

3.3. Carrageenan-induced edema and hyperalgesia

LG 2110/1 was orally effective in reducing carrageenan-induced edema, in a time- and dose-dependent manner (Fig. 3), in contrast to an earlier report (Perfík and Mašek, 1973). Edema at 60, 120 and 180 min after carrageenan injection was significantly decreased by 3 and 10 mg/kg LG 2110/1, while 1 mg/kg LG 2110/1 was effective at 120 and 180 min. E_{max} values, calculated as percent reduction of paw volume versus control were: −44.5%, −31.6%, and −24.5% at 60, 120 and 180 min, respectively. The time course of the anti-edema effect of LG 2110/1 is in agreement with a reduction of mast cell

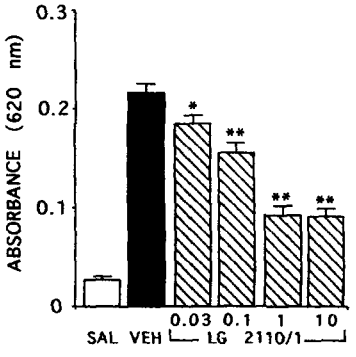

Fig. 2. LG 2110/1 dose dependently decreases Evans blue extravasation induced by passive cutaneous anaphylaxis (PCA) reaction in the mouse ear pinna. The quantity of Evans blue in the ear pinna was measured by absorbance at 620 nm. Mice were first treated for 24 h with saline (open column) (3 μl) or anti-human albumin IgG (7 μg/3 μl) followed by human albumin plus Evans blue for 30 min. Animals were pretreated orally for 1 h before antigen administration with vehicle (black column) or different doses (mg/kg) of LG 2110/1 (hatched columns). * $P < 0.05$ and ** $P < 0.001$ vs. the vehicle-treated group ($n = 10$).

Table 1
Dose dependence of cromolyn and indomethacin on passive cutaneous anaphylaxis-induced Evans blue extravasation in mouse ear pinna

Treatment	$A_{620} \times 10^{-2}$
Saline + H.Alb./EB + vehicle ($n = 17$)	41.5 ± 3.3
IgG + H.Alb./EB + vehicle ($n = 15$)	161.5 ± 11.6
IgG + H.Alb./EB + 0.1 mg/kg cromolyn ($n = 9$)	145.7 ± 17.3
IgG + H.Alb./EB + 0.3 mg/kg cromolyn ($n = 9$)	93.1 ± 8.8 **
IgG + H.Alb./EB + 2 mg/kg indomethacin ($n = 7$)	250.9 ± 7.3 **
IgG + H.Alb./EB + 5 mg/kg indomethacin ($n = 7$)	275.0 ± 16.7 **

Values are means ± S.E.M.; ** $P < 0.001$ vs. IgG + H.Alb./EB + vehicle group PCA was induced injecting 7 μg/3 μl of anti-human albumin IgG; 24 h later human albumin (H.Alb.) plus Evans blue (EB) were injected intravenously and mice were killed 30 min later to measure the amount of Evans blue present in the ear pinna. Cromolyn was given intravenously and indomethacin orally 30 min and 1 h, respectively, before H.Alb./EB.

activation. In fact carrageenan-induced edema formation, during its early stages, is dependent on the release of mast cell mediators (Di Rosa et al., 1971; Al-Haboubi and Zeitlin, 1983). This anti-edema effect of LG 2110/1 remained in the later phase of the inflammatory reaction known to include also leukocyte infiltration, suggesting a long-lasting efficacy.

Mast cell mediators may cause activation/sensitization of innervating primary afferent nociceptors either directly, or indirectly through sympathetic fibers (Cunha et al., 1992; Ferreira et al., 1988; Lewin and Mendell, 1993; Nakamura-Craig and Smith, 1989). A self-reinforcing loop between mast cells and sensory/sympathetic nerves may

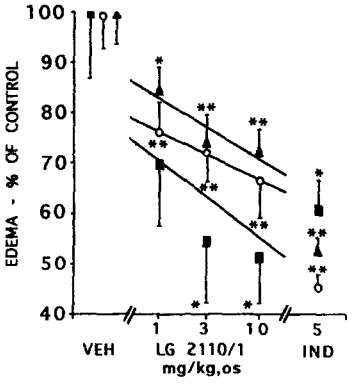

Fig. 3. LG 2110/1 dose dependently reduces rat paw edema induced by carrageenan. Edema formation was measured at different time points following subplantar injection of 100 μl of carrageenan (1% w/v): (■) 60; (○) 120 and (▲) 180 min, respectively. Data obtained following pretreatment (1 h) with LG 2110/1 (1, 3 and 10 mg/kg; orally) and indomethacin (IND) (5 mg/kg; orally) ($n = 15$–16) are reported as percentages of mean edema values observed on the vehicle-treated group ($n = 30$) at respective time points. * $P < 0.05$ and ** $P < 0.001$ vs. the vehicle-treated group.

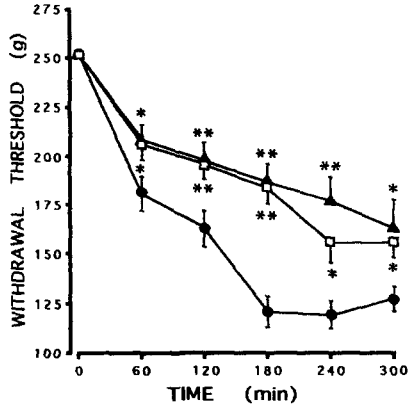

Fig. 4. LG 2110/1 decreases mechanical hyperalgesia in the rat hind paw induced by carrageenan. The pressure (g) required to elicit the withdrawal response was measured at different times (min) after subplantar injection of carrageenan (1% w/v) in rats pretreated orally for 1 h with: (●) vehicle; (□) LG 2110/1 (10 mg/kg); (▲) indomethacin (5 mg/kg). * $P < 0.05$ and ** $P < 0.001$ vs. the vehicle-treated group ($n = 14$–15).

thus act to sustain both the neurogenic inflammatory component (Khalil and Helme, 1989a, Khalil and Helme, 1989b, Khalil and Helme, 1990; Khalil et al., 1988), and the hyperalgesia typically associated with tissue injury/noxious perturbations. The anti-edema effects of oral LG 2110/1 also encompassed an anti-nociceptive action against noxious stimuli such as carrageenan. Orally administered LG 2110/1 (10 mg/kg) significantly reduced the mechanical hyperalgesia provoked by carrageenan (Fig. 4). Indomethacin also displayed anti-edema and anti-nociceptive effects (Figs. 3 and 4).

3.4. Formalin-induced edema

Formalin-induced edema involves mast cell activation (Rosland et al., 1990). LG 2110/1 significantly decreased paw edema over the first 1–3 h after injection of 5% formalin (Fig. 5). Indomethacin was without effect, as seen

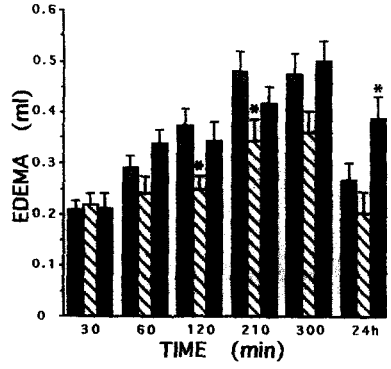

Fig. 5. LG 2110/1 diminishes formalin-induced rat hind paw edema. Rats were pretreated orally for 1 h before subplantar injection of 100 μl of 5% formalin or saline. (Black columns) vehicle; (hatched columns) LG 2110/1 (10 mg/kg); (black columns) indomethacin (10 mg/kg). * $P < 0.05$ vs. vehicle-treated group ($n = 13$–14).

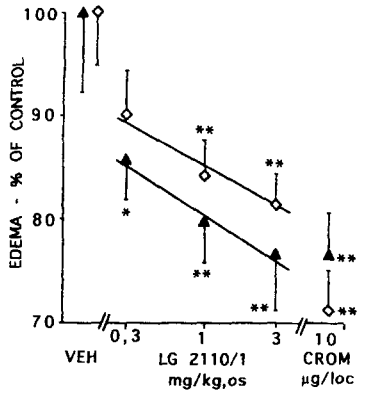

Fig. 6. LG 2110/1 dose dependently reduces dextran-induced rat hind paw edema. Edema formation was measured at different time points following subplantar injection of 100 µl of dextran (0.8% w/v): (▲) 30 min and (◇) 60 min, respectively. Data, obtained following pretreatment (1 h) with LG 2110/1 (0.3, 1, 3 mg/kg; orally) and local subplantar with dextran and cromolyn (CROM) (10 µg) are reported as percentages of mean edema values observed in the vehicle-treated group at each time point ($n = 15$–16 for each group). * $P < 0.05$ and ** $P < 0.001$ vs. the vehicle-treated group ($n = 14$–15).

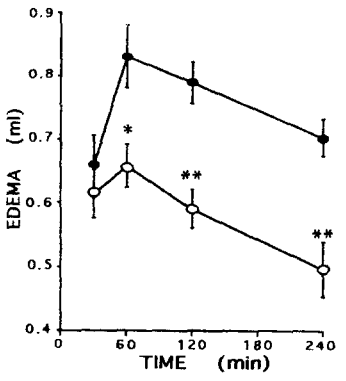

Fig. 7. Locally administered LG 2110/1 reduces rat hind paw edema induced by dextran. LG 2110/1 or vehicle (DMSO, 10 µl) were given by subplantar injection 30 min before dextran (0.8%, w/v) or saline. (●) Vehicle; (○) LG 2110/1: 0.125 mg. * $P < 0.05$ and ** $P < 0.001$ vs. the vehicle-treated group ($n = 9$–10).

by others (Winter et al., 1963). The anti-edema activity of LG 2110/1 still tended to be evident 24 h after formalin injection, whereas indomethacin-treated animals actually fared worse than did the vehicle-treated ones (Fig. 5). This clearly differentiates the activity of LG 2110/1 from the anti-inflammatory action of classical non-steroidal anti-inflammatory drugs such as indomethacin, which act by inhibiting cyclooxygenase.

3.5. Dextran-induced edema

Oral administration of LG 2110/1 was dose dependently active in reducing dextran-induced hind paw edema formation (Fig. 6). The effect was already significant at 30 min with 0.3 mg/kg (Fig. 6), in keeping with the inhibition of release of mast cell amines which are a major factor underlying dextran-induced edema formation (Nishida et al., 1978; Nishida and Tomizawa, 1980). The anti-edema effects of LG 2110/1 (3 mg/kg) persisted for up to 120 min (not shown) after induction of edema. E_{max} values calculated as percentage reduction of paw volume versus control were −24.5% and −19.3% at 30 and 60 min, respectively. The effect of LG 2110/1 (3 mg/kg) was also evident when administered 3 h rather than 1 h prior to dextran injection (−16.3 ± 4.3% at 120 min; $P < 0.001$ vs. vehicle, $n = 15$), suggesting a long-lasting modulation of mast cell activation. When injected directly in the hind paw LG 2110/1 was still highly effective in minimizing dextran-induced edema (Fig. 7), pointing to a local, direct effect of the *N*-acylamide. Cromolyn (10 µg), a blocker of mast cell activation, inhibited dextran effects when locally co-injected (Fig. 6).

3.6. Phospholipase A_2-induced edema

Phospholipase A_2 may be involved in mast cell activation. Peritoneal mast cell degranulation is activated in vitro by exogenous phospholipase A_2 (Murakami et al., 1993), and mast cell degranulation induced by FcϵR1 cross-linking is blocked by phospholipase A_2 inhibitors (Bronner et al., 1990). LG 2110/1 was therefore tested in an in vivo model of phospholipase A_2-induced mast cell activation. Rat paw edema provoked by phospholipase A_2 from the venom of *Naja mocambique mocambique* (Cirino et al., 1989) was monitored. As Table 2 shows, maximally effective doses of LG2110/1 towards other types of mast cell

Table 2
Effect of LG 2110/1 on phospholipase A_2-induced rat paw edema

Treatment	30 min	60 min	90 min	150 min
Phospholipase A_2 + vehicle	0.62 ± 0.06	0.57 ± 0.08	0.52 ± 0.06	0.48 ± 0.06
Phospholipase A_2 + LG 2110/1				
1 mg/kg	0.57 ± 0.05	0.53 ± 0.04	0.48 ± 0.04	0.36 ± 0.04
10 mg/kg	0.59 ± 0.05	0.55 ± 0.06	0.53 ± 0.04	0.49 ± 0.04

Values are means ± S.E.M. obtained from groups of 7–8 animals. Edema (ml) was induced by subplantar injection of 3 µg of phospholipase A_2 from *Naja mocambique mocambique*. LG 2110/1 was given orally 1 h before phospholipase A_2.

activation were ineffective in this paradigm, indicating that in vivo LG 2110/1 does not modify phospholipase A_2 activity.

4. Discussion

The experiments described here demonstrate that LG 2110/1 is orally active in preventing mast cell degranulation provoked by exogenous substance P, as well as different aspects of the acute inflammatory response functionally correlated to mast cell activation produced by a number of inflammatory stimuli. Noteworthy is that the capability of LG 2110/1 to suppress extravasation was, in the case of substance P, highly correlated with the reduced number of degranulated mast cells. The latter phenomenon was observed almost immediately following substance P injection, whereas plasma extravasation was evaluated at a dose of substance P (1 pmol) producing modest, if any, granulocyte infiltration (Yano et al., 1989). In contrast, no effect of LG 2110/1 was observed in the case of the substance P fragment (substance P-(6–11))-induced endothelial cell-mediated plasma extravasation. In addition, immunogenic (passive cutaneous anaphylaxis) complement and mast cell-dependent extravasation was strongly suppressed by this saturated *N*-acylamide at a time point when the protein leakage is mainly due to the release of mast cell mediators (therefore cromolyn sensitive) rather than neutrophil infiltration (Ramos et al., 1992; Zhang et al., 1991). Edema formation associated with cutaneous inflammatory reactions induced by carrageenan or formalin – the latter being irritants known to activate resident mast cells prior to, or with scarse, leukocyte infiltration (Al-Haboubi and Zeitlin, 1983; Di Rosa and Willoughby, 1971; Rosland et al., 1990) – was sensitive to pharmacological modulation by LG 2110/1. Similar results were obtained with dextran, another established mast cell secretagogue causing edema formation with a poor leukocyte infiltration (Di Rosa and Willoughby, 1971). Further, LG 2110/1 prevented inflammatory mechanical hyperalgesia induced by carrageenan. Taken together, these findings, although not totally exclusive of possible effects of LG 2110/1 on cell types other than the mast cells, indicate that this saturated *N*-acylamide is, upon oral administration, efficacious in negatively modulating the mast cell itself as well as the pathogenetic correlates that are initiated by the mast cell activation.

The anti-inflammatory actions of LG 2110/1 can be clearly distinguished from those of classical non-steroidal anti-inflammatory drugs (i.e. indomethacin), corticosteroids, and cannabinoids like Δ^9-tetrahydrocannabinol. In contrast to indomethacin, LG 2110/1 prevented extravasation by passive cutaneous anaphylaxis, formalin and dextran-induced (Di Rosa et al., 1985) edema, indicating an action clearly separate from cyclooxygenase inhibition. Further, LG 2110/1 was inactive against rat hind paw edema induced by venom phospholipase A_2 ruling out either direct or indirect enzyme inhibitory effects via increased lipocortin-1 synthesis typical of dexamethasone, recently shown to exert anti-edema effects independently of the reduction in mast cell degranulation (Wershil et al., 1995). Accordingly, LG 2110/1 reduced lipocortin-1-insensitive dextran-induced edema (Carnuccio et al., 1987). The failure of LG 2110/1 to prevent a phospholipase A_2-associated edema, which is sensitive to serotonergic antagonists (e.g. methysergide) (Cirino et al., 1989), argues against local anti-serotonergic effects of LG 2110/1. An anti-histaminergic action can also be excluded since LG 2110/1 (dose range 0.1–10 mg/kg; orally) did not inhibit Evans blue extravasation induced by histamine dihydrochloride (250 pmol/3 μl $\cong ED_{90}$) injected in the mouse ear pinna (data not shown). Unlike LG 2110/1, Δ^9-tetrahydrocannabinol is reported to not inhibit carrageenan-induced edema and inflammatory rat-paw hyperalgesia (Kosersky et al., 1973). This is consistent with the described lack of affinity of *N*-(2-hydroxyethyl)hexadecanamide for the brain type cannabinoid CB_1 receptor (Felder et al., 1993), and with a recent study showing that the *N*-acylamide can down-modulate mast cell activation in vitro through the peripheral cannabinoid CB_2 receptor (Facci et al., 1995). Finally, and in contrast to the well-known mast cell stabilizer cromolyn, LG 2110/1 is orally active.

Local modulatory effects of LG 2110/1 toward mast cell activation may be linked to its presence in inflamed tissues. LG 2110/1 was locally active in reducing dextran-induced edema. Unlike *N*-(2-hydroxyethyl)hexadecanamide, a mixture of palmitic acid/ethanolamine failed to decrease substance P-induced extravasation. Preliminary pharmacokinetic studies demonstrated the presence of *N*-(2-[^{14}C]hydroxyethyl)hexadecanamide in blood as early as 30 min after an oral load of 30 mg/kg, with the plasma concentration (3×10^{-5} M) remaining almost constant for at least the next 6 h (our unpublished observations). Similar concentrations of LG 2110/1 are effective in reducing IgE-mediated [^{3}H]serotonin release from cultured mast cells (Facci et al., 1995).

These results raise several important questions concerning the presence and actions of the endogenously occurring *N*-acylethanolamide(s). The trace amounts of *N*-(2-hydroxyethyl)hexadecanamide normally found in tissues were originally attributed by some investigators to artifactual causes. Two enzymatic pathways producing *N*-acylethanolamides, including *N*-(2-hydroethyl)hexadecanamide are now known. Brain homogenates contain an *N*-acylamide synthase, capable of forming various *N*-acylethanolamides, whose activity is highly enriched in the hippocampal synaptic fraction (Devane and Axelrod, 1994). A similar activity has been reported in liver (Schmid et al., 1985), suggesting a wide distribution for this biochemical pathway. Interestingly, *N*-acylethanolamide synthesis and its regulation by excitatory amino acids recently has been

reported in cultured neuronal cells (Di Marzo et al., 1994; Hansen et al., 1995). In addition, *N*-(2-hydroxyethyl)hexadecanamide and other related *N*-acylamides may accumulate in relatively large amounts in pathological conditions involving cardiac injury (Epps et al., 1979), presumably via a reaction involving phosphodiesterase-mediated cleavage of *N*-acylglycerophospholipids (Natarajan et al., 1982). A variety of organs thus are able to synthesize *N*-acylethanolamides.

The regulation and functional role(s) of *N*-acyl4-ethanolamide biosynthetic activities by physiological or pathological events remain unknown. Different biological actions have been suggested for the *N*-acylamide(s) produced endogenously or given exogenously (Schmid et al., 1990). In particular, exogenous *N*-(2-hydroxyethyl)hexadecanamide has been reported to exert a protective action in a model of chronic inflammation (i.e. Freund's adjuvant-induced arthritis; Perfik et al., 1971) as well as in acute inflammatory processes involving mast cells (this paper). In addition, *N*-(2-hydroxyethyl)hexadecanamide and related (saturated) *N*-acylamides ('ALIAmides') represent, unlike the unsaturated *N*-acylamide arachidonylethanolamide (anandamide), potential functional agonists for CB_2 receptors on mast cells (Facci et al., 1995). The CB_2 receptor plays a role in down-modulating mast cell activation and consequently related inflammatory processes. Although anandamide, originally identified as a putative endogenous ligand for the CB_1 receptor (Devane et al., 1992; Felder et al., 1993), binds CB_2 receptors on mast cells, it does not inhibit, in contrast to *N*-(2-hydroxyethyl)hexadecanamide, IgE-induced degranulation of cultured mast cells, and actually antagonizes functional actions of the mast cell CB_2 receptor (Facci et al., 1995). Mast cells thus express a CB_2 receptor with differential sensitivity to structurally distinct *N*-acylethanolamides, supporting the possibility that locally produced saturated *N*-acylamides, such as *N*-(2-hydroxyethyl)hexadecanamide, could be selectively targeted towards controlling tissue inflammation by down-modulating mast cell activation. Conversely, deficits or alterations in their local metabolism, could be implicated in mast cell-mediated amplification and/or progression of inflammatory tissue responses.

In conclusion, the ability of oral *N*-(2-hydroxyethyl)hexadecanamide to down-modulate mast cell activation in vivo suggests that exogenous *N*-(2-hydroxyethyl)hexadecanamide may, in fact, provide the mast cell with quantities of its physiological negative regulator sufficient to exert anti-inflammatory actions. This pharmacological effect (ALIA) occurs via an action distinct from that of cannabinoids, steroidal, and existing non-steroidal anti-inflammatory drugs, and may lead to new therapeutic strategies for the management of inflammatory disease states, including nerve inflammation (Mazzari et al., 1995) and autoimmune diseases (Leon et al., 1994b).

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