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A comparative study of the ability of calcitonin gene-related peptide and adrenomedullin$_{13-52}$ to modulate microvascular but not thermal hyperalgesia responses

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1 Calcitonin gene-related peptide (CGRP), a neuropeptide, is a potent vasodilator. Adrenomedullin (ADM) is suggested to be produced by vascular cells in inflamed tissue. ADM shares some structural homology with CGRP. We have compared the ability of CGRP and ADM to modulate microvascular and thermal hyperalgesic responses in rat skin. Vasodilator activity was assessed by laser Doppler flowmetry, inflammatory oedema by the extravascular accumulation of intravenously-injected labelled albumin, and neutrophil accumulation by tissue myeloperoxidase, in dorsal skin. Hyperalgesia was assessed by a thermal hyperalgesimeter in paw skin.

2 ADM (10–300 pmol) was 3 fold less potent than CGRP (3–100 pmol) as a direct vasodilator. CGRP (30 pmol) potentiated oedema formation induced by mediators of increased microvascular permeability, as expected ($P<0.01$). However, ADM (30–100 pmol) was without a potentiating effect, although ADM (300 pmol) was effective ($P<0.01$). By comparison ADM (100 pmol) potentiated neutrophil accumulation induced by interleukin-1β ($P<0.05$), whereas CGRP (30 pmol) did not. No thermal hyperalgesia was observed to either CGRP or ADM, when given as single or repeated treatments.

3 Thus despite a dilator activity neither CGRP nor ADM appears to mediate hyperalgesic activity in the periphery. However ADM, like CGRP, has the ability to potentiate inflammatory oedema formation and, in addition, ADM can potentiate neutrophil accumulation.

4 ADM may, as suggested for CGRP, act as a modulator of the vascular phases of inflammation. The property of the two compounds of evoking differential microvascular responses and neutrophil accumulation may be due to differing mechanisms of action.

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Abbreviations: ADM, adrenomedullin; BK, bradykinin; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; HTAB, hexadecyltrimethyl-ammonium bromide; i.d., intradermal; IL-1β, interleukin-1β; i.p., intraperitoneal; i.pl., intraplantar; MPO, myeloperoxidase; PMN, polymorphonuclear leukocytes; SP, substance P; TMB, 3,3′,5,5′-tetramethyl benzidine

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide with high structural homology among species (Wimalawansa, 1997). Immunohistochemical studies have shown CGRP to be produced in both the central and peripheral nervous system in rats (Rosenfeld *et al*., 1983). Circulating CGRP is thought to be predominantly released from sensory nerve terminals of finely myelinated A delta fibres and unmyelinated C fibres. CGRP-containing fibres are associated with vascular smooth muscle, indicating a role in the regulation of the cardiovascular system. Indeed, CGRP is well known as an extremely potent and long lasting microvascular vasodilator (Brain *et al*., 1985). CGRP can also act to potentiate inflammatory oedema in skin induced by mediators of increased microvascular permeability (Brain & Williams 1985). This is thought to be a consequence of its action as a microvascular vasodilator. In addition, in the rabbit, CGRP was found to potentiate neutrophil accumulation induced by agents that mediate neutrophil accumulation, (such as interleukin-1β; IL-1β, Buckley *et al*., 1991a,b). Evidence from a variety of sources indicate that the synthesis and release of CGRP can be upregulated soon after induction of the inflammatory response, thus CGRP released from sensory nerves is in an ideal location to play a role in inflammatory and hyperalgesic responses.

Adrenomedullin (ADM) was discovered as a 52 amino-acid peptide isolated from human phaeochromocytoma tissue by Kitamura *et al*. (1993). ADM is a member of the calcitonin family of peptides (which includes CGRP). The peptide fragment ADM$_{13-52}$ possesses vasodilator activity and has approximately 24% homology with CGRP (Richards *et al*., 1996). From hereon ADM$_{13-52}$ is referred to as ADM. ADM, by comparison with CGRP, is a vasodilator mediator that is primarily produced by non-nervous tissues. ADM mRNA has been found in the heart, lung and kidney (Ichiki *et al*., 1995); areas where abundant microvascular vessels exist. It is suggested that although low levels of ADM may be found in normal tissue, and be constitutively secreted (Isumi *et al*., 1998), substantially higher levels may be induced in stimulated cells. The synthesis and secretion of ADM has been reported *in vitro* in vascular endothelial cells (Sugo *et al*., 1994) and smooth muscle cells (Sugo *et al*., 1995; Richards *et al*., 1996) after stimulation with inflammatory mediators such as cytokines. Thus ADM is present at an ideal location to

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participate in the inflammatory responses. However, results to date to support this hypothesis have either been obtained from the study of tissues in culture, or from the measure of samples (especially plasma and serum levels) obtained from human disease. Interestingly it has been suggested that in the cerebrovascular circulation plasma ADM concentration correlates with degree of endothelial damage (Kuwasako *et al*., 1997). Furthermore, studies have detected ADM in various disease states such as septic shock and arthritis, (e.g. 16.4 ± 5.4 fmol ml^{-1} in plasma of rheumatoid arthritis patients compared with 2.8 ± 1.6 fmol ml^{-1} for normal volunteers; Yudoh *et al*., 1999 and 107 ± 139 fmol ml^{-1} for septic shock patients; Hirata *et al*., 1996). Levels have been suggested to be raised in proportion to disease severity (Richards *et al*., 1996).

The release of inflammatory mediators in peripheral tissues such as skin can cause sensitization of primary afferent nerve terminals, leading to hyperalgesia. It has been shown that although a single subplantar injection of low amounts of CGRP had no hyperalgesic effect (Nakamura-Craig & Gill, 1991), multiple doses of CGRP did cause significant hyperalgesia. Thus it is suggested that CGRP can sensitize nociceptors and possibly participate in peripheral inflammatory hyperalgesia. Furthermore, anti-CGRP serum has been shown to suppress both adjuvant- and carrageenan-induced hyperalgesia in the paw (Kawamura *et al*., 1989). In keeping with these results, the intrathecal administration of $CGRP_1$ antagonist, $CGRP_{8-37}$, has been shown to increase hindpaw withdrawal latencies in both mechanical and thermal nociceptive tests (Yu *et al*., 1994; 1998); although intrathecal injection of CGRP had no effect on nociceptive responses, (Jolicoeur *et al*., 1992; Yu *et al*., 1994). In addition, Menard *et al*. (1996) have demonstrated an interaction between CGRP and the development of tolerance in the spinal antinociceptive effects of morphine. The hyperalgesic role of ADM has not been previously investigated.

The detection of raised levels of ADM in diseases that include arthritis (Yudoh *et al*., 1999) is indicative that ADM may be induced in inflammatory disease and be present in the microvasculature to influence microvascular events. The aim of this study was to investigate the possibility that ADM possesses pro-inflammatory activity, by possessing an ability to modulate the inflammatory process. Thus a study was designed to learn more about the comparative actions of CGRP and ADM, with respect to their actions as putative mediators of microvascular responses and hyperalgesia in skin. Experiments were carried out to determine the comparative ability of CGRP and ADM to potentiate inflammatory oedema formation, modulate neutrophil accumulation and induce hyperalgesia; in addition to increasing blood flow in rat skin. It was also intended that a comparative study would allow a discussion of possible receptor-mediated mechanisms.

Methods

Animals

Male Wistar rats (from A. Tuck, Essex, U.K.) were used for microvascular (200–300 g) and hyperalgesia studies (65–105 g). Experiments were carried out under U.K. law. The rats were housed in a temperature-controlled environment, and given access to food and water *ad libitum*. The effect of agents on thermal hyperalgesia was examined in the conscious rat. The effect of agents on microvascular responses induced by test agents injected intradermally into the dorsal skin was examined in anaesthetized rats. Rats were anaesthetized with sodium pentobarbitone (Sagatal), with an intraperitoneal (i.p.) injection of 60 mg kg^{-1}. Maintenance doses were administered as and when required *via* the tail vein cannulae. The dorsal skin was shaved, and in the case of blood flow experiments it was also depilated with a commercial cream (Boots). Rats were then left for 20 min after which injection sites were marked out (in duplicates in oedema experiments) according to a balanced randomized site pattern.

Measurement of dorsal skin blood flow

Cutaneous blood flow measurements were recorded using Moor laser Doppler flow meters (Moor Instruments, Devon, U.K.) as previously described (Ridger *et al*., 1997). After the animals' body temperature had stabilized to approximately 37°C, a laser Doppler probe was placed one over each of the two sites under study. Basal blood flow was recorded prior to the intradermal (i.d.) administration of agents (100 μl $site^{-1}$). Test agents were made up in modified Tyrode solution and kept on ice until use. Immediately after i.d. injections the laser probes were replaced onto the holders, and flux was read, taken as value at time 0 min. Subsequent flux readings were taken at 2 min, 5 min and at every 5 min time points thereafter over a continuous 60 min recording period. Results were recorded as blood cell flux (arbitrary values which are the number of moving cells detected by the beam multiplied by their velocity), and subtracted from the flux value at time 0. Results were represented as change in flux compared to flux at 0 min.

Measurement of skin oedema formation

The animals were anaesthetized as previously described, the dorsal skin was shaved, and a balanced site pattern was marked out, according to Brain *et al*. (1985). Oedema formation was measured by the extravascular accumulation of ^{125}I-bovine serum albumin (^{125}I-BSA, 92.5 kBq). ^{125}I-BSA and the visual marker, Evans blue dye (100 μl of 2.5% w v^{-1} solution) were injected *via* a tail vein cannulae. Test agents were made up as for blood flow experiments and injected (100 μl) i.d. in duplicates. After an accumulation period of 30 min, (or for 3 h in a set of experiments involving IL-1β) a 5 ml blood sample was collected by cardiac puncture, and rats were killed by barbituate overdose and cervical dislocation. The dorsal skin was removed, and injection sites punched out (16 mm diameter). Blood samples were centrifuged ($8000 \times g$ for 4 min) in a microcentrifuge (Micro Centaur, MSE, U.K.) and plasma collected. Radioactivity was counted in skin and plasma samples. Results were expressed as the amount of plasma extravasated (μl) in each site by comparing radioactivity in skin sites with that in a known volume of plasma.

Measurement of neutrophil accumulation

Animals were prepared for i.d. injection as described in plasma extravasation experiments, but no radiolabel or Evan's blue dye was administered. In addition, due to the duration of these experiments, the trachae was cannulated to aid the ease of breathing. Here, 50 μl of IL-1β (0.1% BSA) and Tyrode (0.1% BSA) was injected intradermally at 0 h. Two hours later 100 μl of the agents under investigation were injected (i.e. CGRP and ADM) into the same site as IL-1β. Then at 3 h the experiment was terminated as described earlier, and skin sites (16 mm diameter) punched out, weighed and stored in the freezer, ready for the homogenization process. This process enables cell lysis and lysis of the PMN granules to release myeloperoxidase (MPO).

Skin sites were kept on ice and homogenized as previously described (Pintér *et al*., 1999) in 4 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) using a Polytron homogenizer (Brinkman, New York, NY, U.S.A.). After homogenizing, samples were centrifuged twice, once in a Coolspin centrifuge (MSE, U.K.), at $3500 \times g$ for 30 min at 0–4°C, then 1.5 ml supernatant removed and re-centrifuged at $8000 \times g$ for 5 min in a microcentrifuge (Micro Centaur, MSE, U.K.). The supernatant (approximately 1 ml) was removed and frozen ready for the measurement of MPO activity as first described by Schierwagen *et al*. (1990) and modified by Pintér *et al*. (1999). The MPO was assayed in triplicate in a 96-well plate at room temperature. Equal volumes of MPO source (25 μl) and phosphate buffer solution (pH 6, containing 0.5% HTAB) was added to the reaction wells along with 100 μl 'K-blue' substrate (stabilized TMB; 3,3′, 5,5′-tetramethyl benzidine, and H_2O_2; hydrogen peroxide). Measurements were taken at 620 nm in an automated plate reader (Anthos HTIII), at 5 min intervals for 30 min. The number of neutrophils per site was determined by using a standard neutrophil (rat) preparation. The initial rate of the reaction (O.D./time) was calculated from the initial slope of the curve. This value was then used to extrapolate from the rat standard calibration curve to allow conversion to number of neutrophils accumulating per site.

Measurement of thermal hyperalgesic responses and paw volume

Thermal hyperalgesic thresholds were determined according to Hargreaves *et al*. (1988), as adapted by Bennett *et al*. (1998). Animals were placed singly into behavioural boxes that had a glass floor and transparent walls and tops (Ugo Basile, Italy). The adjacent walls connecting the boxes were opaque and thus prevented interactions between the animals. After an habituation period of 10 min, control (basal) measurements of hyperalgesic threshold were taken after shining an automatic heat source (Ugo Basile, Italy) onto the plantar surface of the paw. All measurements were performed in triplicate and the mean time taken for the rat to react to the light beam was automatically recorded as the hyperalgesic threshold. Fifteen minutes later the animals were transiently sedated by exposure to an increasing concentration of CO_2 for about 1 min. Then, one hindpaw was injected intraplantarly with agent under test (100 μl of either carrageenan, ADM, CGRP or the respective vehicle). The other acted as an uninjected control. Intraplantar (i.pl.) injections were made with a 1 ml syringe and a 30 G hypodermic needle. The needle was inserted into the pad region of the glabrous skin and moved 6–8 mm proximal towards the tarsal region. All experiments were performed under blind conditions to prevent any bias. Hyperalgesic thresholds were measured at 30, 90, 180 and 270 min post-injection for both paws. After the final measurements, the animals were killed by CO_2 overdose followed by cervical dislocation. Results were expressed as the difference in withdrawal latency (in seconds) compared with equivalent saline paw. The protocol was adapted to enable repeated treatments of CGRP and ADM to be investigated as follows: Rats were divided into three groups and each group received one of the following treatments i.pl. saline (vehicle control, 100 μl), CGRP (100 pmol site^{-1}) or ADM (300 pmol site^{-1}). The treatments were given at each of the following time points −48 h, −24 h and 0 h. Measurements of paw withdrawal latency were taken before the 0 h injection (basal) and post-injection as described above. In a further set of experiments rats were anaesthetized and agents injected i.pl. into one paw, whilst the other remained uninjected as described above. Paw oedema was then measured at 30 min by water displacement plethysmography. Results were expressed as paw weight (g) of injected paw and contralateral uninjected paw.

Materials

Human αCGRP was purchased from Bachem, Essex, U.K. Human ADM_{13-52} was purchased from Phoenix Pharmaceuticals, Belmont, CA, U.S.A. Human recombinant IL-1β was purchased from R&D Systems, Oxon, U.K. Sodium pentobarbitone (Sagatal) was purchased from May & Baker, Essex, U.K. Depilatory cream was from Boots, Nottingham, U.K. ^{125}I-BSA was from ICN, Asse-Relegem, Belgium. 'K-blue' substrate was from Bionostics (Skybio Ltd), Bedfordshire, U.K. Carrageenan lambda, Evan's blue dye, bradykinin (BK), substance P (SP) and HTAB were from Sigma-Aldrich Company Ltd, Dorset, U.K. Stock interleukin-1β (IL-1β) was made up in 0.1% BSA (bovine serum albumin) saline, stock ADM in N_2 free water, and the other stock drugs were dissolved in ultrapure water or saline and kept frozen in aliquots until use. Intradermal test agents for skin assays were dissolved in modified Tyrode's solution (mM) NaCl 136.89, KCl 2.68, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $MgCl_2$ 1.05 and glucose 5.5, pH 7.4 before injection.

Statistical analysis

Results are presented as mean ± s.e.mean (or s.d.). Data, raw and when required logged (Figure 2A), were analysed using ANOVA followed by Dunnett's, Bonferroni's (modified) or Student-Newman-Keuls post-test. For the comparison of CGRP (30 pmol site^{-1}) and ADM (100 pmol site^{-1}) responses (in Figure 1A) an unpaired Students *t*-test was used. Data were considered significant if $P < 0.05$.

Results

The effect of intradermally-injected (i.d.) CGRP and ADM on blood flow is shown in Figure 1A. CGRP (3–100 pmol site^{-1}) induced a dose-dependent increase in skin blood flow as previously described by Brain *et al*. (1985). ADM (10–300 pmol site^{-1}) also acted to increase blood flow. A similar magnitude of response was observed with CGRP (30 pmol site^{-1}) and ADM (100 pmol site^{-1}) suggesting that ADM was approximately three times less potent than CGRP. Higher doses of CGRP and ADM were not tested, due to the probability of the locally-injected higher doses having systemic effects and affecting the validity of the *in vivo* assay. The time-dependent increase in blood flow for ADM (100–300 pmol site^{-1}) and CGRP (30 pmol site^{-1}) is shown in Figure 1B. This graph confirms that ADM was approximately three times less potent than CGRP in increasing blood flow in that the responses of CGRP at 30 pmol site^{-1}, and ADM at 100 pmol site^{-1} were similar in maximal response and duration over the 60 min experimental period. These respective doses were chosen for further experiments in skin.

In keeping with previous findings (Brain & Williams, 1985) CGRP at vasodilator doses had no effect on basal plasma extravasation when compared with the effect of Tyrode vehicle, as shown in Figure 2A,B. Similarly, ADM at vasodilator doses had no effect on basal plasma extravasation. By comparison, the well established acute mediators of increased microvascular permeability, SP and BK induced

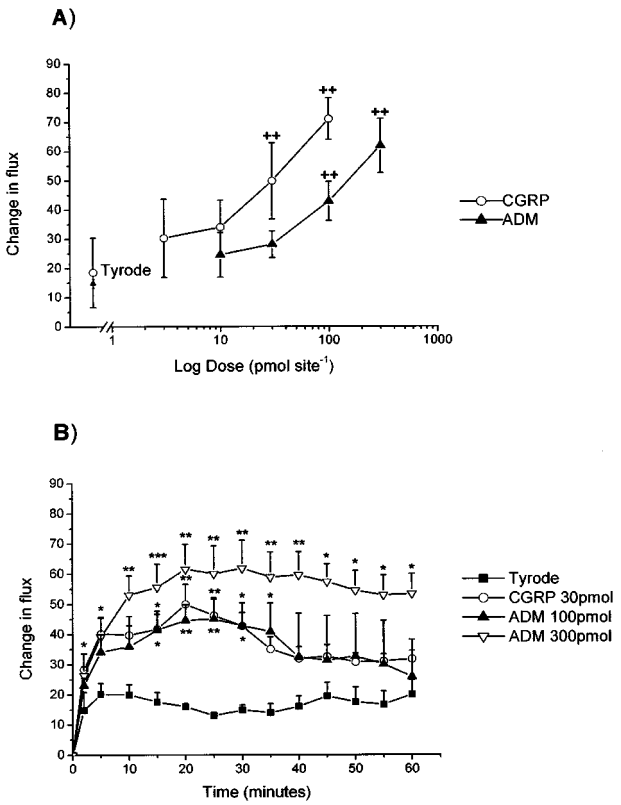

Figure 1 Effect of CGRP and ADM on blood flow. A comparison between (A) the log dose response effect at the 30 min time point, and (B) time course effect of calcitonin gene-related peptide (CGRP) and adrenomedullin (ADM) after i.d. injection into rat dorsal skin. Results are expressed as mean blood flow measurements (in arbitrary flux units–see Methods for explanation)$\pm$s.e.mean. In (A) results differing significantly from time 0 (flux 0) are represented as$^{++}P<0.01$, $n=4-6$ (CGRP) and $n=6-7$ (ADM), as assessed by ANOVA followed by Dunnett's t-test. CGRP 30 pmol site^{-1} and ADM 100 pmol site^{-1} were compared and the magnitude of the responses were found to be not significantly different to each other (unpaired Students t-test). In (B) results differing significantly from Tyrode at each time point are denoted by $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as assessed by ANOVA followed by Bonferroni's modified t-test.

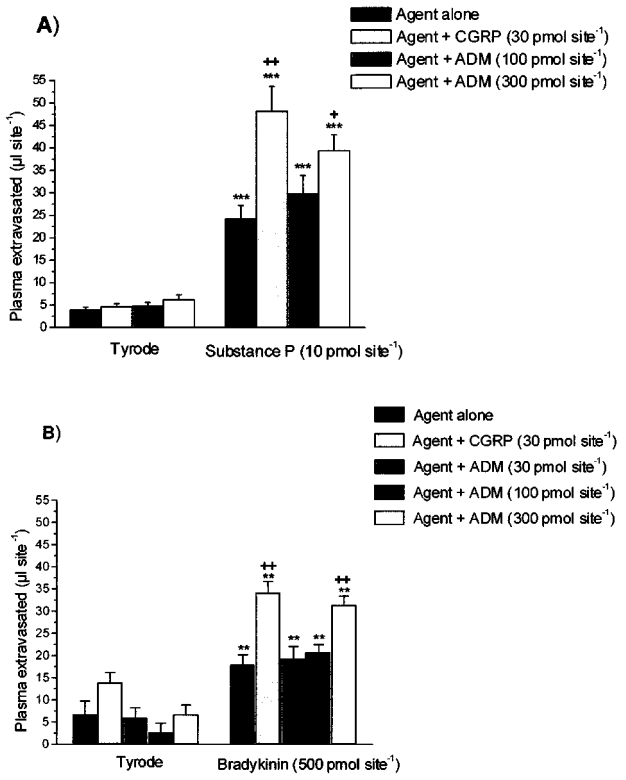

Figure 2 Effect of CGRP and ADM on (A) SP- and (B) BK-induced oedema formation in rat dorsal skin. Results are expressed as μl plasma extravasated per site, mean$\pm$s.e.mean, $n=10$ and 6 respectively. Results differing significantly from Tyrode sites are denoted by $^{***}P<0.001$ and $^{**}P<0.01$, and those differing from the respective mediator of increased microvascular permeability alone (i.e. SP and BK) are denoted with $^{+}(P<0.05)$ and $^{++}(P<0.01)$, as assessed by ANOVA followed by Bonferroni's modified t-test.

plasma protein extravasation at doses chosen to give a submaximal effect over a 30 min experimental period; see Figure 2A,B respectively. CGRP (30 pmol site^{-1}) potentiated oedema formation induced by these mediators as expected (see Brain & Williams, 1985). Interestingly ADM at a similar vasodilator dose (100 pmol site^{-1}) did not have a significant potentiating role in oedema formation, although an effect was observed at a higher dose of ADM (300 pmol site^{-1}, $P<0.01$).

However, IL-1β is a potent mediator of neutrophil accumulation in skin (see Figure 3A,B). IL-1β (1.5 pmol site^{-1}) induces a slow time-dependent neutrophil accumulation as expected (see Bennett *et al.*, 1998 for data in rat skin), due to a protein synthesis dependent effect. Thus experiments measuring neutrophil accumulation were carried out over a 3 h experimental period and CGRP was injected at two time points, once at 0 h and once at 2 h, in order that it would be active during both the protein synthesis and neutrophil-dependent stages. When injected alone, the peptides CGRP and ADM caused no significant increase in neutrophil accumulation, see Figure 3A,B. Furthermore, the co-injection of either CGRP or ADM with IL-1β at 0 h had no potentiating effect on neutrophil accumulation (data not shown). However, in the results shown in Figure 3B, CGRP and ADM were injected 2 h after IL-1β, so as to enable their presence in skin during the phase when neutrophils were accumulating. A significant potentiation of neutrophil accumulation by ADM (100 pmol site^{-1}), but not CGRP (30 pmol site^{-1}) was observed. IL-1β, unlike substance P and other mediators, is not known as an acute or potent mediator of increased microvascular permeability/oedema formation in rat skin and no oedema was observed at the doses used. Results are as follows, Tyrode 13.3$\pm$2.5 μl; IL-1β (1.5 pmol site^{-1}) 6.7$\pm$7.1 μl plasma extravasated, mean$\pm$s.d., $n=3$, measured over the 0–30 min time period; Tyrode 14.7$\pm$9.1; IL-1β (1.5 pmol site^{-1}) 13.0$\pm$5.3 μl plasma extravasated, mean$\pm$s.d., $n=3$, measured over the 2–3 h time period after IL-1β administration at 0 min. Thus oedema formation could not have influenced the lack of ability of CGRP to potentiate IL-1β-induced neutrophil accumulation.

Carrageenan (2%, i.pl.) elicited significant hyperalgesia at 180 and 270 min compared to saline, and was used as positive control in thermal hyperalgesia experiments. CGRP and ADM were tested at doses similar to those used in Figure 1A, where ADM and CGRP caused significant vasodilatation. There was no significant difference observed between the different CGRP doses and the equivalent saline time point, or pre-injection basal levels (Figure 4A) in the thermal hyperalgesia assay. ADM was also shown to have no significant effects with either dose (Figure 4B). In a separate set of experiments paw oedema was measured at 30 min by water displacement plethysmography. This was to enable the possibility that plasma extravasation may be induced by CGRP and ADM in the paw to be evaluated. However, as expected from the results

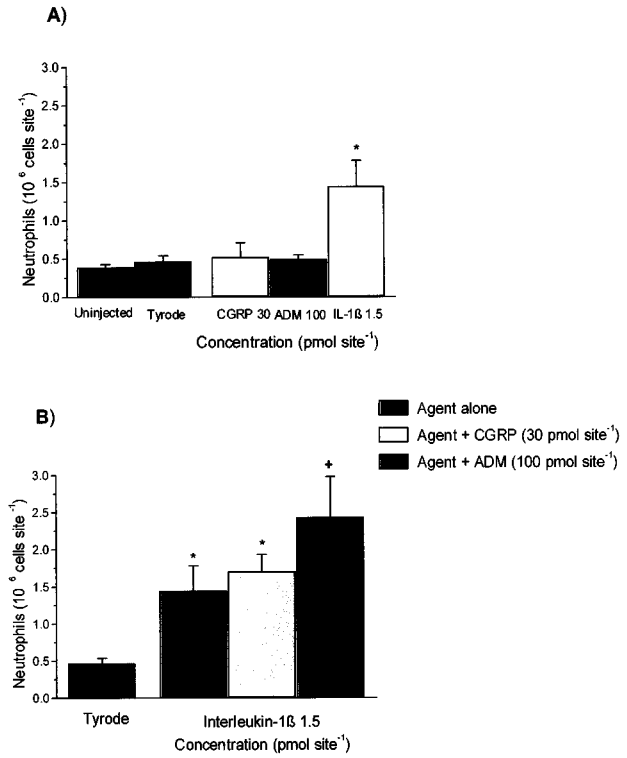

Figure 3 Effect of (A) CGRP, ADM and interleukin-1 (IL-1)β alone and (B) co-injection of CGRP (or ADM) and IL-1β on neutrophil accumulation in rat dorsal skin. Results are expressed as mean number of neutrophils accumulated per site, $\pm$s.e.mean, $n=6$. Statistically significant results are denoted by $^{*}P<0.05$, compared with Tyrode, and $^{+}P<0.05$ compared with IL-1β, after analysis with ANOVA followed by Bonferroni's modified t-test.

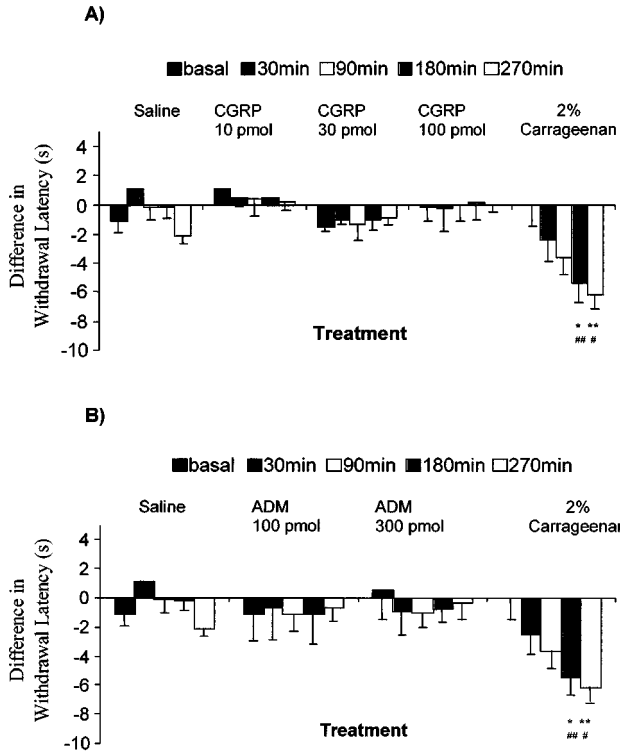

Figure 4 Effect of (A) CGRP and (B) ADM on rat hind paw thermal hyperalgesia. The response to carrageenan (2%) is shown for comparison. Measurements of paw withdrawal latency were taken pre-injection (basal) and post-injection (i.pl.) of agent at 30, 90, 180 and 270 min. Results are expressed as the mean difference (s) in withdrawal latency compared to equivalent saline paw, at each time point, $\pm$s.e.mean, $n=6-11$ for CGRP and 5–11 for ADM. Statistically significant results are denoted by $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared to equivalent saline paw, as assessed by ANOVA followed by Student-Newman-Keuls test. Measurements differing significantly from those at the basal time point are denoted by $^{*}P<0.05$, $^{**}P<0.01$, as assessed by ANOVA followed by Student-Newman-Keuls test.

shown in Figure 2 for dorsal skin, no oedema formation was observed. Results, shown as weight (g) of injected paw and contralateral uninjected paw are as follows: uninjected 0.96 ± 0.08 g, saline (vehicle, 100 μl) 1.08 ± 0.02 g; uninjected 1.08 ± 0.06 g, CGRP (100 pmol site^{-1}) 1.17 ± 0.02 g; uninjected 1.08 ± 0.05 g, ADM (300 pmol site^{-1}) 1.17 ± 0.09 g; expressed as mean $\pm$s.e.mean, $n=4$ for each group. In further experiments, the ability of CGRP (100 pmol site^{-1} i.pl.) and ADM (300 pmol site^{-1} i.pl.) to influence hyperalgesia when given as repeated injections at 48, 24 and 0 h pretreatments was investigated. However, as for a single treatment repeated injections of CGRP and ADM had no effect on thermal hyperalgesia (Figure 5).

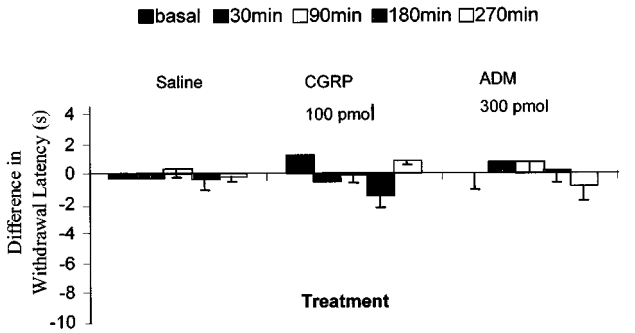

Figure 5 Effect of repeated treatments of CGRP and ADM on rat hind paw thermal hyperalgesia. Rats were divided into three groups and each group received one of the following i.pl. treatments: saline (vehicle control, 100 μl), CGRP (100 pmol site^{-1}) or ADM (300 pmol site^{-1}). The treatments were each given at each of the following times:- -48, -24 and 0 h. Measurements of paw withdrawal latency were taken before the 0 h injection (basal) and post-injection of agents at 30, 90, 180 and 270 min. Results are expressed as the mean difference(s) in withdrawal latency compared to equivalent uninjected paw, at each time point, $\pm$s.e.mean, $n=4-9$ for each group.

Discussion

The results presented demonstrate the activity of ADM and CGRP as potent microvascular vasodilator mediators and potentiators of increased microvascular permeability. The study also demonstrates the ability of ADM, but not CGRP, to potentiate neutrophil accumulation under the experimental conditions chosen. Finally it is shown that neither CGRP nor ADM, given as single or repeated injections were able to influence the hyperalgesia response to a thermal stimulus. Thus the results indicate that if ADM levels are raised at an inflammatory site, the peptide has the potential to act in a pro-inflammatory manner; but not as a prohyperalgesic mediator. However, it should be pointed out that only one hyperalgesic measurement technique was employed in this study.

It is interesting that human synthetic αCGRP was found to be approximately 3 fold more potent than ADM (commercial human synthetic ADM_{13-52}), in increasing rat skin blood flow,

as measured by laser Doppler flowmetry in this study. This contrasts with findings from an earlier paper from this laboratory (Hall *et al*., 1995) where human αCGRP was found to be 10–30 times more potent, as measured by ^{133}xenon clearance, than human synthetic ADM_{13-52}, (obtained as a gift from a co-author). The apparent comparative change in potency of CGRP and ADM as vasodilators could be due to either the improved storage of the relatively unstable ADM (stock and aliquots stored at −70°C, rather than −20°C) or improved technical methods (ADM stock made up in N_2 free water, rather than saline). Alternatively the small difference could be due to the fact that the ^{133}xenon clearance technique measures changes in blood flow over the entire injected site (see Hughes *et al*., 1994), rather than in a small area of superficial skin as measured by the laser Doppler technique (see Methods). However, having determined the fact that CGRP is approximately 3 fold more potent than ADM in stimulating increased blood flow, it is then interesting to determine that CGRP is approximately 10 times more active than ADM in potentiating oedema formation. This result is further complicated by the finding that ADM, but not CGRP at the doses used, potentiates neutrophil accumulation induced by IL-1β. The results clearly indicate that, although both CGRP and ADM are potent microvascular vasodilators, it is not possible to assume that all vascular activities of CGRP are mirrored, with slightly less activity, by ADM. The reason for the differences may be due to altered bioavailability in different inflammatory states. In this context it has been shown that tryptase and chymase released from activated mast cells degrade CGRP to inactive peptide fragments (Brain & Williams, 1988), and mast cells may well be activated after IL-1β administration in rat skin (Hogaboam *et al*., 1993). However it is also possible that CGRP and ADM exert their effects at the microvascular level *via* different receptors, depending on microvascular circumstances.

CGRP receptors are widely distributed and the use of $CGRP_{8-37}$, as a competitive antagonist, has led to the general understanding that two types of CGRP receptors exist (Dennis *et al*., 1990). Evidence indicates that CGRP acts on $CGRP_1$ receptors in the microvasculature and $CGRP_{8-37}$ antagonizes CGRP vasodilator responses in tissues that include rat skin (Escott & Brain, 1993). ADM receptors have a structural homology with $CGRP_1$ receptors and, like $CGRP_1$, have seven transmembrane domains and coupled to adenylate cyclase *via* a G-protein (Richards *et al*., 1996). Some studies have led to suggestions that the vasodilator effect of ADM is due to its interaction with CGRP receptors, since vasodilator responses in the rat isolated mesentery evoked by ADM *in vitro* and rat skin *in vivo* were inhibited by $CGRP_{8-37}$ (Nuki *et al*., 1993; Hall *et al*., 1995). Most interestingly, McLatchie *et al*. (1998) have cloned single-transmembrane proteins called receptor-activity-modifying proteins (RAMPs) that are considered to work with a cloned seven transmembrane structure calcitonin receptor-like receptor (CRLR), to create a family of CGRP and ADM receptors. The seven transmembrane, G-protein coupled CRLR (which has a 55% homology with the calcitonin receptor) may act as either a CGRP or ADM receptor depending on the presence of either $RAMP_1$ or $RAMP_2$ respectively. The role of the RAMPs is considered to be to control the transport and glycosylation of the CRLR. McLatchie *et al*. (1998) suggest that $RAMP_1$ presents the receptor at the cell surface as a mature glycoprotein and a CGRP receptor. $RAMP_2$-transported receptors are core-glycosylated and are ADM receptors. Little is known about the activity of these receptors in the microvasculature. However, one possibility is that the nature of the receptor can change depending on inflammatory conditions and that inflammatory mediators such as cytokines can influence these responses. This could be relevant to the findings presented in this manuscript.

A role for ADM in the regulation of blood pressure has been suggested and this may be related to the peripheral vasodilator activity observed here. ADM levels have been reported to be increased in a range of cardiovascular diseases (Richards *et al*., 1996) and in septic shock (Ehlenz *et al*., 1997; Hirata *et al*., 1996). The ADM levels have been suggested to be sufficiently high to play a pathological role in modulating vascular responses in septic shock (Nishio *et al*., 1997). However, in a rat model of septic shock it was suggested that CGRP, rather than ADM plays a vasoactive role (Gardiner *et al*., 1999). In the latter study, experiments were carried out where both $CGRP_{8-37}$ and a weak ADM antagonist (ADM_{22-52}) were used. Thus it remains to be clarified as to whether ADM is upregulated sufficiently in disease to influence blood flow locally or released to modulate blood pressure in a systemic manner in sepsis.

The results of the present study indicate that under the experimental conditions chosen ADM, rather than CGRP, acts to potentiate neutrophil accumulation. These results are surprising when taken in the context of previous studies, carried out in rabbit skin, where CGRP clearly and significantly potentiated neutrophil accumulation induced by a range of mediators of neutrophil accumulation (Buckley *et al*., 1991b). Furthermore, in the murine air pouch IL-1β induced neutrophil migration was blocked by $CGRP_{8-37}$ and potentiated by added CGRP (Ahluwalia & Perretti, 1994). The most obvious conclusion is that CGRP and ADM, as vasodilator peptides, can act by potentiating inflammatory oedema formation and neutrophil accumulation in certain circumstances (presumably when microvascular blood flow is low). However, other modulating activities may be present. The peptides are known to mediate events *via* an increase in cyclic AMP that may lead to increased blood flow and vasodilatation. On the other hand, an increase in cyclic AMP levels can have a differing effect on the inflammatory process, depending on circumstance. There is evidence from several *in vitro* studies that ADM can act to inhibit components in the inflammatory processes *via* cyclic AMP-dependent processes. For example, Isumi *et al*. (1999) have recently demonstrated that adrenomedullin (ADM) exhibited a potent effect in inhibiting interleukin-1β-induced tumour necrosis factor-alpha (TNF-alpha) secretion and gene transcription in Swiss 3T3 fibroblasts. Indeed CGRP has been shown to have an inhibitory effect on the activation of human neutrophils *in vitro* (Tanabe *et al*., 1996). The inhibitory effects of CGRP were abolished by pretreating the neutrophils with $CGRP_{8-37}$, or cyclic AMP-dependent protein kinase inhibitors. The authors suggested that CGRP receptor stimulation reduces neutrophil activation *via* mechanisms involving cyclic AMP-dependent protein kinase. Thus, in the present study, CGRP may have a greater ability to act in an anti-inflammatory manner than ADM in IL-1β-treated rat skin: possibly acting *via* a neutrophil-dependent manner. This will be the subject of further study.

In conclusion our results support the hypothesis that CGRP and ADM can play a pro-inflammatory role in the cutaneous microvasculature, in mediating the vasodilation and promoting the oedema formation most commonly associated with the acute inflammatory process. To our knowledge this study is the first to investigate for an hyperalgesic effect of adrenomedullin in the periphery, and a clear lack of effect is shown. The discussion highlights the complex nature of interactions of the

CGRP-like peptides with their receptors and in the inflammatory process as a whole, where CGRP and ADM may possess pro- and anti-inflammatory activities. This could well be relevant to the present observation that ADM, but not CGRP, potentiated neutrophil accumulation in the rat cutaneous microvasculature. The results and discussion highlight the need for further research in both *in vitro* and *in vivo* systems to determine the comparative roles of CGRP and ADM in inflammation.

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