

RESEARCH ARTICLE

Open Access



Differential inhibitory response to telcagepant on α CGRP induced vasorelaxation and intracellular Ca^{2+} levels in the perfused and non-perfused isolated rat middle cerebral artery

André Erdling^{1*} , Majid Sheykhzade² and Lars Edvinsson³

Abstract

Background: Calcitonin gene-related peptide (CGRP) is one of the most potent endogenous vasodilators identified to date. The present study elucidates the differential interaction of CGRP, its receptor and the effect of the CGRP-receptor antagonist telcagepant on intracellular Ca^{2+} -levels and tension in rat middle cerebral arteries (MCA) by pressurized arteriography, FURA-2/wire myography and immunohistochemistry.

Methods: A pressurized arteriograph system was used to evaluate changes in MCA tension when subjected to CGRP and/or telcagepant. Intracellular calcium levels were evaluated using a FURA-2/wire myograph system. Localization of the CGRP-receptor components was verified using immunohistochemistry.

Results: Abluminal but not luminal α CGRP (10^{-12} - 10^{-6} M) caused concentration-dependent vasorelaxation in rat MCA. Luminal telcagepant (10^{-6} M) failed to inhibit this relaxation, while abluminal telcagepant inhibited the relaxation (10^{-6} M). Using the FURA-2 method in combination with wire myography we observed that α CGRP reduced intracellular calcium levels and in parallel the vascular tone. Telcagepant (10^{-6} M) inhibited both vasorelaxation and drop in intracellular calcium levels. Both functional components of the CGRP receptor, CLR (calcitonin receptor-like receptor) and RAMP1 (receptor activity modifying peptide 1) were found in the smooth muscle cells but not in the endothelial cells of the cerebral vasculature.

Conclusions: This study thus demonstrates the relaxant effect of α CGRP on rat MCA. The vasorelaxation is associated with a simultaneous decrease in intracellular calcium levels. Telcagepant reduced relaxation and thwarted the reduction in intracellular calcium levels localized in the vascular smooth muscle cells. In addition, telcagepant may act as a non-competitive antagonist at concentrations greater than 10^{-8} M.

Keywords: CGRP, Calcitonin gene related peptide, Telcagepant, Pressurized arteriography, FURA-2, Middle cerebral artery

* Correspondence: andre.erdling@med.lu.se

¹Department of Clinical Sciences, Division of Experimental Vascular Research, Lund University, BMC A13, 221 84 Lund, Sweden

Full list of author information is available at the end of the article

Background

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide belonging to the calcitonin family of peptides, which also includes calcitonin, amylin and adrenomedullin. The peptide, which was first identified in 1982 [1], exists in two forms, α CGRP and β CGRP of which α CGRP is more abundant in the circulation and in the nervous system [2]. CGRP is widely distributed throughout the central, peripheral and enteric nervous systems [3–5] and has strong vasodilatory properties, especially in the microvasculature and in cerebral arteries [6–8]. CGRP is commonly co-localized with substance P in sensory neurons [9]. Cerebral α CGRP is stored in perivascular sensory C-fibers that originates in the trigeminal ganglion. The vasomotor actions of α CGRP are implied in a variety of both physiological and pathological processes, including migraine and other primary headaches as well as subarachnoid hemorrhage (SAH) [10, 11].

The CGRP receptor is a complex consisting of the G-protein coupled calcitonin receptor-like receptor (CLR) and a single transmembrane receptor activity modifying protein (RAMP1) [12]. CLR may form complexes with other RAMPs with receptor affinity modulation as a result. CLR/RAMP2 has, for instance, a high affinity for adrenomedullin (AM) and constitutes the AM1 receptor [13] while CLR/RAMP3 forms the AM2 receptor with reported affinity for both CGRP and adrenomedullin [14].

There is also evidence of a third, intracellularly located, receptor component protein (RCP), which is thought to stabilize the interaction between the CLR/RAMP1-complex and the G-proteins [15, 16]. In most tissues, receptor activation causes interaction with G_s which activates adenylate cyclase (AC) and stimulates the generation of cyclic adenosine monophosphate (cAMP) with subsequent activation of protein kinase A (PKA) leading to opening of K_{ATP}^+ [17] and K_{Ca}^{2+} channels [18] and hyperpolarization of smooth muscle cell membrane. A recent study demonstrates that a major portion of CGRP induced vasodilation in MCAs is attributed to activation of $Kv7.4$ channels [19]. In some vascular beds, for instance in the rat aorta [6], CGRP-induced vasodilation is to some extent mediated via release of nitric oxide from the endothelium rather than only a direct effect on the smooth muscle cells (SMC). The dilatory effect on feline cerebral arteries is however clearly endothelium independent and is associated with activation of AC [20]. The same is true for human cerebral, meningeal and temporal arteries [21–23].

In the present study, we have evaluated in more detail the functional effects of high concentrations of the CGRP-receptor antagonist telcagepant (MK-0974) on α CGRP-induced vasodilatation in isolated middle cerebral arteries (MCA) of rat, using (i) pressurized myography and (ii) FURA-2-AM for measurement of intracellular calcium performed simultaneously with recordings of

isometric tension development. We also evaluated the distribution of the CGRP receptor components using immunohistochemistry. The results suggest (i) presence of CGRP receptors on the smooth muscle cells of the MCA, (ii) absence of CGRP receptors on the vascular endothelium, (iii) coupling of receptor-ligand interaction to tension and intracellular calcium and (iv) concentration-dependent antagonism of CGRP-induced vasodilation by telcagepant.

Methods

The experimental protocol was reviewed and approved by the Animal Protocol Review committee at Lund University, Sweden (M111-04).

Male Sprague-Dawley rats (250 – 350 g; $n = 20$) were anaesthetized with CO_2 and sacrificed by decapitation. The brain was immediately removed, and placed in oxygenated (95% O_2 and 5% CO_2), cold ($\sim 4^\circ C$) buffer solution of the following composition: 119 mM NaCl, 15 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4 , 1.5 mM $CaCl_2$ and 5.5 mM glucose.

The middle cerebral artery (MCA) was carefully isolated from the brain under a stereomicroscope. Each MCA was cut into smaller segments, beginning at the circle of Willis and extending 6–8 mm distally. The dissection was done in cold and oxygenated buffer solution (see composition above).

Pressurized arteriography experiments

4–6 mm long segments of the MCA were mounted between glass micropipettes and fitted in a pressurized arteriography system (Living Systems, Burlington, VT, USA) as previously described [24]. The artery segments were immersed in buffer solution which was kept at $37^\circ C$ and continuously aerated with a gas mixture containing 95% O_2 and 5% CO_2 with a pH of 7.4. Transmural pressure and luminal perfusion was adjusted to 85 mmHg and 100 $\mu L/min$ (range 70–100 μL), respectively, by adjusting two fluid reservoirs, which were connected to the afferent (inlet) and efferent (outlet) micropipettes and placed at the appropriate height above the perfused artery segment.

The setup was visualized by a microscope (at 600-fold magnification) connected to a digital camera (Axis, Lund, Sweden). Images recorded by the camera were exported to and stored on a computer. Outer vessel diameter was measured every second and evaluated in the computer software Mary[®] (Nihil KB, Lund, Sweden).

Following mounting, the perfused vessels were allowed to equilibrate and attain a stable tone. The mean diameter of the vessel segments immediately after mounting was $197.7 \pm 4.6 \mu m$ and the mean diameter after gaining tone was $132.4 \pm 2.0 \mu m$ which equals contraction by

33.0 ± 2.0%. Failure to contract at least 10% within one hour disqualified the vessel segment from further experiments. The remaining segments had ATP (10⁻⁵ M) added to their luminal perfusate in order to evaluate endothelial viability [25]. A dilatatory response of at least 10% was considered indicative of a functional endothelium and a structurally intact blood-brain barrier [24, 26]. The mean diameter of the vessel segments increased by 36.1 ± 4.8% upon ATP exposure. The vessel segments were once again allowed to equilibrate (i.e. attain stable tone) before any further experiments were conducted.

Subsequently, αCGRP was added in increasing concentrations (10⁻¹² to 10⁻⁶ M) to either the luminal or the abluminal perfusate in order to evaluate the vascular response.

In a separate experiment, the CGRP antagonist telcagepant was added to either the luminal (10⁻⁶ M) or the abluminal perfusate (10⁻⁷-10⁻⁶ M) 30 minutes before administration of increasing concentrations of abluminal αCGRP.

At the end of each experiment, the vessels were treated with calcium free buffer solution in order to elicit maximal dilatation. The diameter increased by 30.7 ± 4.4% after treatment with calcium free buffer.

Intracellular calcium measurements

Intracellular calcium measurements were conducted in the dark on middle cerebral artery segments mounted in a wire myograph placed on the stage of an inverted microscope (Leica DMIRBE, Germany). The arteries were loaded with the fluorescent [Ca²⁺]_i indicator dye (FURA-2/AM) by incubation in an oxygenated buffer solution (10 μM FURA-2/AM, 0.2% (V/V⁻¹), anhydrous dimethylsulphoxide (DMSO), 0.01% (V/V⁻¹), pluronic F-127 and 0.03% (V/V⁻¹) cremophore EL) for 45 minutes at 37° C. Cremophore EL and pluronic F-127 (dispersing agents or non-ionic detergents) improved the loading with FURA-2/AM by promoting dye dispersion and by preventing FURA-2/AM from precipitation [27]. The loading procedure was performed twice before the vessels were washed and equilibrated for 15 minutes in buffer solution. This also allowed the FURA-2/AM to be converted to active FURA-2 by intracellular esterases.

Determination of intracellular calcium levels was performed by illuminating the vessel segments with light at 340 and 380 nm, respectively. A xenon arc lamp provided high intensity continuous broadband illumination. The emitted light was passed through filters (500–530 nm) and detected by a photomultiplier (PTI: Photon Technology International, Germany). During the experiments, in which the vessels were precontracted with U44619 (10⁻⁷ M) and then subjected to incremental concentrations of αCGRP ± telcagepant, fluorescence signals

and force signals were continuously captured and processed by a computer (FeliX32 program, Photon Technology International, Monmouth Junction, NJ, U.S.A.). The actual calcium concentrations were calculated according to the equation; [Ca²⁺]_i = K_d × β × [(R - R_{min}) / (R_{max} - R)], where K_d is the dissociation constant of the FURA-2-Ca²⁺ complex (224 nM at 37° C [28]), R is the measured background-corrected ratio between emission at 340 nm excitation and emission at 380 nm excitation, R_{max} and R_{min} are background-corrected ratios under Ca²⁺-saturating and Ca²⁺-free conditions, respectively. The β is the ratio of emission signals when excited at 380 nm during determination of R_{min} and R_{max}, respectively. R_{min} and R_{max} were determined in each vessel at the end of the experiment by adding 40 μM ionomycin in calcium free buffer solution and by using buffer-solution containing 5 mM Ca²⁺, respectively [29]. Before calculating the ratio (R) between emission after 340 nm illumination and emission after 380 nm illumination, background fluorescence signals were obtained by quenching the calcium-sensitive FURA-2 fluorescence with 20 mM Mn²⁺ at the end of each experiment [27, 29]. The mean values (n = 15) of R_{min}, R_{max} and β were 1.12 ± 0.02, 4.59 ± 0.19 and 2.11 ± 0.18, respectively. The values in Ca²⁺-free buffer and the plateau phases were designated to be 0 and 100% for both the [Ca²⁺]_i and tension, respectively.

Immunohistochemistry

MCAs from male Sprague-Dawley rats were carefully dissected from the brain as described above, fixed in 4% paraformaldehyde in phosphate buffer for 1 h, followed by rinsing in Soerensen's phosphate buffer with increasing concentrations of sucrose. The fixed specimens were then placed into Tissue TEK (Gibo, Invitrogen A/S, Taastrup, Denmark), frozen on dry ice and sectioned into 10-μm-thick slices, which were rehydrated in phosphate buffer solution (PBS) containing 0.25% Triton X-100 for 15 minutes before incubation overnight at +4° C

Table 1 Details of antibodies used for immunohistochemistry

| Primary antibodies, Immunohistochemistry | | | | |
|--|--------------------------|--------|----------|--|
| Product ID | Detects | Host | Dilution | Supplier |
| RAMP1 3158 | C-terminal of rat RAMP1 | Rabbit | 1:200 | Merck & Co. Inc, USA |
| CLR 132 | C-terminal of rat CRLR | Sheep | 1:100 | Merck & Co. Inc, USA |
| Secondary antibodies, Immunohistochemistry | | | | |
| Product ID | Conjugate and host | | Dilution | Supplier |
| 10006588 | FITC, goat anti-rabbit | | 1:100 | Cayman chemical, Ann Arbor, MI, USA |
| 713-516-147 | Dy594, donkey anti-sheep | | 1:200 | Jackson ImmunoResearch, Europe Ltd., Suffolk, UK |

with the following primary antibodies: rabbit polyclonal to RAMP1 diluted 1:200 and sheep polyclonal to CLR (details for the antibodies are summarized in Table 1). All primary antibodies were diluted in PBS containing 0.25% Triton X-100. Sections were subsequently incubated for 1 hour at room temperature with secondary FITC-conjugated donkey anti-rabbit or Dy594-conjugated donkey anti-rabbit antibodies diluted 1:200 in PBS containing 0.25% Triton X. The sections were subsequently washed with PBS and mounted with Vectashield mounting medium (Vector laboratories, Inc. Burlingame, CA, USA). Immunoreactivity was visualized and photographed using a light- and epifluorescence microscope (Nikon 80i; Tokyo, Japan) at the appropriate wavelength. The same procedure was used for the negative controls except that the primary antibodies were omitted resulting in no staining in the tissue except for auto-fluorescence in the internal elastic lamina.

Drugs

Human α -calcitonin gene-related peptide (α CGRP) (Sigma-Aldrich, Germany) was dissolved in 0.1% bovine serum albumin (BSA) to form a stock solution of 10^{-4} M. 9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α} (U46619, Sigma-Aldrich, Germany), a thromboxane A₂ agonist, was delivered in methyl acetate (10 mg/ml). A stock solution of 10^{-3} M was prepared by diluting the substance in sterile water. Telcagepant (MK-0974) was synthesized and supplied by the Medicinal Chemistry Department (Merck, West Point, PA, USA) and dissolved in dimethylsulphoxide (DMSO, Sigma-Aldrich, Germany) at a concentration of 10^{-2} M. All stock solutions were stored at -20° C until utilized.

Data analysis

Relaxations are expressed as percentage of the pre-tension or $[Ca^{2+}]_i$ level induced by 100 nM U46619. All concentration-response curves were analyzed by iterative non-linear regression analysis using GraphPad Prism 6.03 (GraphPad Corp, San Diego, CA, USA). Each regression line was fitted to a sigmoid equation: $E/E_{max} = A[M]^{n_H} / (A[M]^{n_H} + EC_{50}[M]^{n_H})$, where E_{max} is the maximal response developed to the agonist, A $[M]$ is the concentration of agonist and n_H is a curve-fitting parameter, the Hill coefficient [30]. Sensitivity to agonists is expressed as pD_2 value, where $pD_2 = -\log(EC_{50} [M])$, and $EC_{50} [M]$ is the molar concentration of agonist required to produce half-maximal response. Data are expressed as mean \pm SEM (n = number of rats) unless otherwise indicated. For experiments performed on the pressurized arteriograph, changes in measured diameters of the vessel segments are expressed as a percentage of the resting diameter. All concentrations expressed indicate the final concentration in

the luminal or abluminal compartments of the pressurized arteriograph.

The Kruskal-Wallis test or one-way ANOVA (with Dunn's or Dunnett's multiple comparison test) was used when determining differences in E_{max} and pD_2 between treatment groups. P -values < 0.05 were considered significant. NS = not significant.

Results

Pressurized arteriography

Telcagepant, luminal or abluminal, did not alter the baseline tone in the pressurized MCAs, indicating no CGRP tone in resting MCAs.

The administration of α CGRP caused concentration-dependent relaxation of the MCA when given abluminally (E_{max} $41.4 \pm 7.4\%$, pD_2 7.73 ± 0.24 , $n = 12$). Luminal administration of α CGRP failed to elicit any response at concentrations up to 10^{-6} M (E_{max} $3.1 \pm 1.3\%$, $pD_2 = 7.37 \pm 0.65$, $n = 9$). Luminal telcagepant (10^{-6} M) failed to inhibit the vasodilatory properties of abluminally applied α CGRP (E_{max} $21.85 \pm 4.59\%$, pD_2 7.75 ± 0.27 , $n = 5$, NS for E_{max}). Abluminal telcagepant reduced the dilatory response to abluminal α CGRP significantly when given at high concentration (10^{-6} M) (E_{max} $10.10 \pm 1.84\%$, pD_2 7.72 ± 0.30 , $n = 4$, $p = 0.014$ for E_{max}) while lower concentration of telcagepant (10^{-7} M) failed to do so (E_{max} $19.74 \pm 11.06\%$, pD_2 7.65 ± 0.69 , $n = 4$, NS for E_{max}) (Fig. 1).

Intracellular calcium measurements

Incremental administration of α CGRP caused vasodilatation and reduction of intracellular calcium levels. The addition of 10^{-6} M telcagepant significantly offset both the dilatory response and the reduction in intracellular calcium mediated by CGRP (pD_2 tension 9.63 ± 0.14 vs 8.03 ± 0.18 , $n = 5-6$, $p = 0.02$; pD_2 calcium 9.27 ± 0.11 vs 8.25 ± 0.09 , $n = 4$, $p = 0.03$). In contrast, telcagepant at the concentration of 10^{-7} M had no significant inhibitory effect on α CGRP-induced responses (pD_2 tension 9.63 ± 0.14 vs 8.97 ± 0.20 , $n = 5-6$, NS; pD_2 calcium 9.27 ± 0.11 vs 9.16 ± 0.14 , $n = 3-4$, NS) (Fig. 2).

Immunohistochemistry

MCA segments were first stained with hematoxylin (HTX) and inspected for general morphology; this revealed a thin adventitia, a smooth muscle cell layer, an internal elastic membrane and a single layer of endothelial cells. Both RAMP1 and CLR immunoreactivity could be detected within the smooth muscle cells of the MCA, some RAMP1 was also detected within the endothelial cells but no CLR. Thus, both components of the functional CGRP receptor complex are present in SMC cytoplasm, but not in the endothelial cells (Fig. 3).

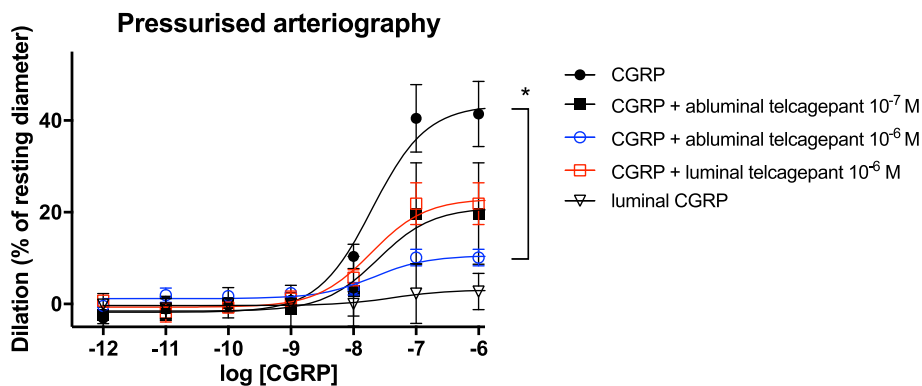


Fig. 1 Pressurised arteriography experiment demonstrating the vasodilatory properties of abluminal CGRP on rat MCA and the concentration-dependent inhibition of this relaxation by telcagepant. Values given are means \pm SEM, $n = 4-12$. * = $p < 0,05$ for CGRP vs CGRP + telcagepant 10^{-6} M. Lum = luminal application of telcagepant

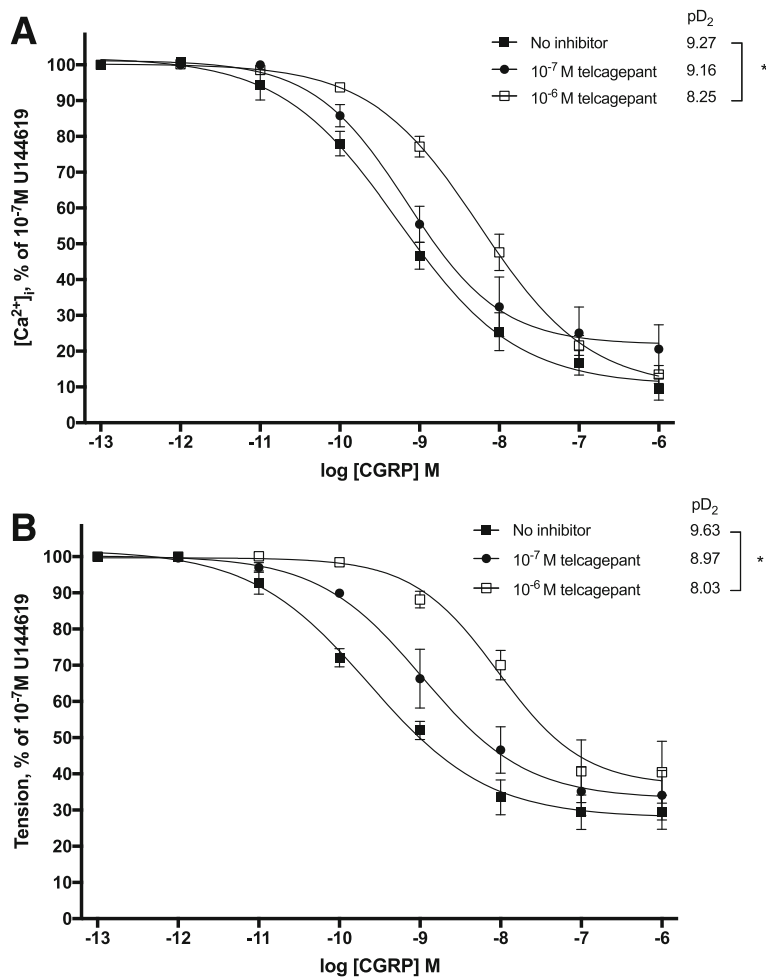


Fig. 2 Effect of 0,1 μ M and 1 μ M telcagepant on CGRP-induced reduction in [Ca²⁺]_i (a) and tension (b) of rat MCAs pre-contracted with 0,1 μ M U46619. Relative tension and [Ca²⁺]_i are given as percentages of the initial steady-state levels induced by 0,1 μ M U46619. Points represent mean values of $n = 3 - 6$ separate experiments and vertical bars indicate \pm SEM

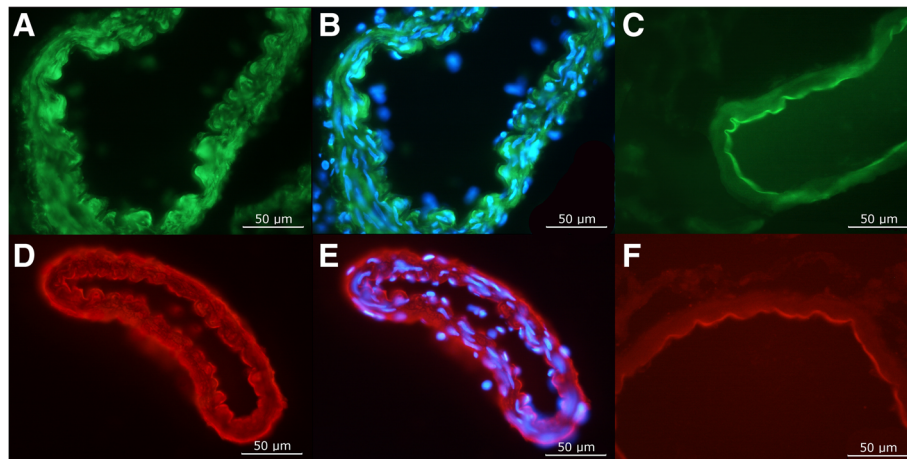


Fig. 3 Cross-sections of middle cerebral arteries showing CLR and RAMP1 expression in the smooth muscle cells of the MCA walls. Both receptor antibodies revealed positive staining in the smooth muscle cell layer. A 50 µm marker is inserted. Figure 3a and d demonstrates the expression of CLR and RAMP1 in the smooth muscle cells of the MCA walls. DAPI, a nucleus stain has been added to picture 3b and e. 3c and e are negative controls where the fluorescence staining around the lumen is due to autofluorescence of the lamina elastica interna

Discussion

The present study has localized the two CGRP receptor elements to the cerebrovascular smooth muscle cells and demonstrates the functional effects of the CGRP-receptor antagonist telcagepant on α CGRP-induced reduction in tension and intracellular calcium concentration in isolated rat MCA segments. We investigated in some detail the dilatory response of α CGRP on precontracted cerebral vessels and found that pre-treatment with telcagepant causes a concentration-dependent inhibition of the dilatory response induced by abluminal α CGRP. This was not seen when telcagepant was administered luminally. Telcagepant did not affect basal tone, hence speaking against CGRP tone in the resting or precontracted MCA. In addition, α CGRP failed to induce dilation when given luminally. This, in combination with the histological localization of the CGRP receptor components in tunica media part of the vasculature, support the opinion that CGRP-receptors are present only in the cerebrovascular smooth muscle cells rather than on the endothelium in the brain vasculature. The finding of weak expression of RAMP1 but no CLR expression could indicate that endothelial cells might express e.g. amylin receptors, but this needs further analysis. The study thus supports once again the important physiological role of CGRP as neuropeptide, namely its presence in perivascular sensory nerve endings and its local release to affect postsynaptic receptors on adjacent vascular smooth muscle cells and subsequent diffusion through the vessel into the blood stream. We also demonstrated a direct correlation between α CGRP-induced dilatory response and alterations in the intracellular calcium levels. Telcagepant was for the first time shown to inhibit the reduction in both tension and in intracellular

calcium levels at a given α CGRP concentration. However, some results are a bit contradictory. Telcagepant failed to shift the α CGRP concentration-response curve to the right in the pressurized arteriography experiments. There was, however, a reduction in CGRP-induced maximal vasodilatation (E_{max}) when the vessels were subjected to telcagepant, indicating non-competitive/unsurmountable antagonism. In contrast, the wire myography accompanying the measurement of intracellular calcium levels failed to show a drop in E_{max} , but could demonstrate a rightward shift of the log [CGRP]-response curve (change in potency) when subjected to telcagepant. The difference between tension development and receptor antagonism in the wire myographs and pressurized arteriography system is interesting. The cause for the discrepancy between results from the two methods could be due to the presence of endothelial CGRP receptors. This scenario seems unlikely since we failed to detect CLR within the endothelium. Difference in vessel diameter could be an issue, but all vessel segments had similar lumen diameter and were taken from the same anatomical location. The advantage of pressurized arteriography lies in its mimicry of actual physiological conditions such as shear stress on the endothelium caused by flow through the vessel lumen and the separation of the luminal and abluminal sides of the vessel wall. It has been demonstrated that vessels in a wire myograph are less sensitive to and respond differently to a variety of agonists (i.e. difference in maximum response and slope of the curve) when compared to vessels in a pressurized system or in vivo [31, 32]. This might be due to the need for precontraction when studying vasorelaxation in the wire myograph, but it may also be due to misinterpretation of actual vasorelaxation as measured in the pressure myograph and reduction of wall tension as measured in the wire myograph model.

The results from the pressurized arteriography are consistent with a recent study where the effect of different concentrations of telcagepant was evaluated on human subcutaneous arteries [33]. Telcagepant seemed to act as a competitive antagonist at relatively low concentrations up to 10^{-8} M but higher concentrations of telcagepant caused a decrease in E_{\max} and failed to further shift the agonist log [concentration]-response curve to the right, indicating unsurmountable antagonism. Similar findings were reported when the effect of the CGRP receptor antagonist BIBN4096BS (olcegepant) was tested on human subcutaneous arteries [34]. Further investigation of this phenomenon is needed, especially since the small sample size and the rather large variability might be an issue.

CGRP and its receptor have been under scrutiny for the last decade because of their implied role in migraine and regulation of vasomotor tone [35]. CGRP-containing trigeminal nerves have been shown to surround the major cerebral and cortical arteries and stimulation of these nerves releases CGRP which in turn causes direct vasodilation via receptors located on the vascular smooth muscle cells. It has also been suggested that its action on second order neurons may facilitate the transmission of pain to higher cortical areas. There is an ongoing debate regarding whether migraine pain originates in the perivascular nerves, in the CNS or both [10, 36].

Several publications have implied a role for CGRP in the development and prevention of cerebral vasospasm following SAH. Levels of CGRP in cerebrospinal fluid (CSF) and jugular vein blood are increased following SAH [11, 37] as well as during migraine attacks [38].

Telcagepant and olcegepant started as promising agents for the treatment of migraine. Incidence of liver toxicity in chronic studies was, however, the main reason that the projects were eventually terminated just before entry on the market. Despite this, both agents remained as important tools for the understanding of CGRP and its role in cerebrovascular regulation and there are ongoing trials with other small molecules of the "gepant" group, possibly lacking liver toxicity. Since telcagepant is a rather large, hydrophilic substance, this feature might impair its penetration through the blood-brain barrier into the CNS. The neurogenic inflammation induced by substance P and CGRP may, however, aid the translocation of telcagepant into the CNS through increased vascular permeability and reduced integrity of the blood-brain barrier and thus enable interaction with CGRP receptors on the abluminal surface of vessels as well as on perivascular nerves and higher cortical structures.

Low penetration through the blood-brain barrier is one explanation to why high plasma concentrations of telcagepant are needed to treat migraine attacks while vascular effects are seen at lower concentrations in vitro

[39]. This is somewhat supported by our study where we compare the effect of CGRP and telcagepant in two different myograph systems. The pressurized arteriography mimics the situation *in vivo* to a greater extent than the wire-myograph setup where telcagepant has direct access to both the luminal and abluminal surfaces of the blood vessel. A previous attempt at comparing the differences in *in vivo* and *in vitro* potency of olcegepant and telcagepant [40] reported a large difference in doses needed for *in vivo* and *in vitro* effect. Other factors that may explain this discrepancy between clinically effective plasma concentrations and *in vitro* effects include a high degree of binding to plasma proteins and delayed distribution to the effect compartment.

CGRP acts as an agonist not only at the CGRP receptor, but also on the amylin and adrenomedullin receptors. A functional study on the potency of CGRP, adrenomedullin and amylin at their corresponding receptors report 100-fold lower potency of CGRP at the AM1 and AM2 receptors than at the CGRP receptor [41]. CGRP effects mediated through interaction with the AM receptors cannot be ruled out at the high concentrations used, but the presence of functional AM or amylin receptors on the rat cerebral vasculature seems limited as both of amylin and adrenomedullin only caused weak dilatation when compared to CGRP [42].

Conclusions

This study demonstrates in detail the relaxant effect of α CGRP on rat MCA. The vasorelaxation is for the first time shown to be associated with a simultaneous decrease in intracellular calcium levels in the cerebral circulation. Telcagepant thwarted the CGRP-induced reduction in tension and intracellular calcium levels of MCAs. When investigated in the pressurized arteriography system, α CGRP produced relaxation only when given abluminally. This observation, together with immunohistochemistry results, strongly suggests an expression of the CGRP receptor complex on the smooth muscle cells in the media rather than on the endothelium. It also implies that CGRP antagonists such as telcagepant have to cross the blood-brain barrier in order to block CGRP induced vasorelaxation in the cerebral circulation. In addition, this study also supports earlier findings suggesting that telcagepant *may* act as an unsurmountable antagonist at concentrations greater than 10^{-8} M. Furthermore, parameters such as wall tension (i.e. transmural pressure), flow, shear stress, myogenic tone, and longitudinal stretch can be confounding factors affecting vascular responses to both agonists and antagonists *in vivo*. Therefore, our results provide additional evidences for exerting caution when extrapolating the *in vitro* results to the *in vivo* situation.

Abbreviations

AM: Adrenomedullin; BSA: Bovine serum albumin; CGRP: Calcitonin gene-related peptide; CLR: Calcitonin receptor-like receptor; DMSO: Dimethyl sulphoxide; MCA: Middle cerebral artery; NS: Not significant; PKA: Protein kinase A; RAMP1/2/3: Receptor activity modifying peptide 1/2/3; RCP: Receptor component protein; U46619: 9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α} , a thromboxane A₂ agonist

Acknowledgements

Supported by the Swedish Research Council (grant no 5958).

Authors' contributions

LE designed the study. AE drafted the manuscript and conducted the myograph experiments and the immunohistochemistry. MS conducted the intracellular calcium measurements. All three authors participated in the analysis of the results and all authors read, revised and approved the final manuscript.

Competing interests

None of the authors have any competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Department of Clinical Sciences, Division of Experimental Vascular Research, Lund University, BMC A13, 221 84 Lund, Sweden. ²Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. ³Department of Clinical Sciences, Division of Experimental Vascular Research, Lund University, Lund, Sweden.

Received: 17 March 2017 Accepted: 19 May 2017

Published online: 30 May 2017

References

- Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 298:240–244
- Mulderry PK, Ghatei MA, Spokes RA, Jones PM, Pierson AM, Hamid QA et al (1998) Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience* 25:195–205
- Fernandes ES, Schmidhuber SM, Brain SD (2009) Sensory-nerve-derived neuropeptides: possible therapeutic targets. *Handb Exp Pharmacol* 194:393–416
- Sternini C (1992) Enteric and visceral afferent CGRP neurons. Targets of innervation and differential expression patterns. *Ann N Y Acad Sci* 30:170–186
- Uddman R, Edvinsson L, Ekblad E, Håkanson R, Sundler F (1986) Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. *Regul Pept* 15:1–23
- Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313:54–56
- Edvinsson L, Chan KY, Eftekhari S, Nilsson E, de Vries R, Säveland H et al (2010) Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist telcagepant in human cranial arteries. *Cephalalgia* 30:1233–1240
- Edvinsson L, Ekman R, Jansen I, McCulloch J, Uddman R (1987) Calcitonin gene-related peptide and cerebral blood vessels: distribution and vasomotor effects. *J Cereb Blood Flow Metab* 7:720–728
- Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ, Girgis S (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci Lett* 57:125–130
- Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S (2017) Pathophysiology of Migraine: A Disorder of Sensory Processing. *Physiol Rev* 97:553–622
- Schebesch KM, Herbst A, Bele S, Schödel P, Brawanski A, Stoerr EM et al (2013) Calcitonin-gene related peptide and cerebral vasospasm. *J Clin Neurosci* 20:584–586
- McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N et al (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393:333–339
- Hay DL, Poyner DR, Sexton PM (2006) GPCR modulation by RAMPs. *Pharmacol Ther* 109:173–197
- Hay DL, Pioszak AA (2016) Receptor Activity-Modifying Proteins (RAMPs): New insights and roles. *Annu Rev Pharmacol Toxicol* 56:469–487
- Egea SC, Dickerson IM (2012) Direct interactions between calcitonin-like receptor (CLR) and CGRP-receptor component protein (RCP) regulate CGRP receptor signaling. *Endocrinology* 153:1850–1860
- Evans BN, Rosenblatt MI, Mnaver LO, Oliver KR, Dickerson IM (2000) CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J Biol Chem* 275:31438–31443
- Nelson MT, Huang Y, Brayden JE, Hescheler J, Standen NB (1990) Arterial dilations in response to calcitonin gene-related peptide involve activation of K⁺ channels. *Nature* 344:770–773
- Sheykhzade M, Berg Nyborg NC (2001) Mechanism of CGRP-induced relaxation in rat intramural coronary arteries. *Br J Pharmacol* 132:1235–1246
- Chadha PS, Jepps TA, Carr G, Stott JB, Zhu HL, Cole WC et al (2014) Contribution of kv7.4/kv7.5 heteromers to intrinsic and calcitonin gene-related peptide-induced cerebral reactivity. *Arterioscler Thromb Vasc Biol* 34:887–893
- Edvinsson L, Fredholm BB, Hamel E, Jansen I, Verrecchia C (1985) Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. *Neurosci Lett* 58:213–217
- Olesen IJ, Gulbenkian S, Valença A, Antunes JL, Wharton J, Polak JM et al (1995) The peptidergic innervation of the human superficial temporal artery: immunohistochemistry, ultrastructure, and vasomotility. *Peptides* 16:275–287
- Jansen-Olesen I, Mortensen A, Edvinsson L (1996) Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. *Cephalalgia* 16:310–316
- Jansen-Olesen I, Jørgensen L, Engel U, Edvinsson L (2003) In-depth characterization of CGRP receptors in human intracranial arteries. *Eur J Pharmacol* 481:207–216
- Bryan RM, Eichler MY, Swafford MW, Johnson TD, Suresh MS, Childres WF (1996) Stimulation of alpha 2 adrenoreceptors dilates the rat middle cerebral artery. *Anesthesiology* 85:82–90
- You J, Johnson TD, Marrelli SP, Bryan RM (1999) Functional heterogeneity of endothelial P2 purinoceptors in the cerebrovascular tree of the rat. *Am J Physiol* 277:H893–H900
- You J, Johnson TD, Childres WF, Bryan RM (1997) Endothelial-mediated dilations of rat middle cerebral arteries by ATP and ADP. *Am J Physiol* 273:H1472–H1477
- Roe MW, Lemasters JJ, Herman B (1990) Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium* 11:63–73
- Gryniewicz G, Poenie M, Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450
- Jensen PE, Mulvany MJ, Aalkjaer C (1992) Endogenous and exogenous agonist-induced changes in the coupling between [Ca²⁺]_i and force in rat resistance arteries. *Pflügers Arch* 420:536–543
- Kenakin T (1997) Analysis of Drug-Receptor Interaction, 3rd edn. Lippincott-Raven Publishers, New York
- Buus NH, VanBavel E, Mulvany MJ (1994) Differences in sensitivity of rat mesenteric small arteries to agonists when studied as ring preparations or as cannulated preparations. *Br J Pharmacol* 112:579–587
- Dunn WR, Wellman GC, Bevan JA (1994) Enhanced resistance artery sensitivity to agonists under isobaric compared with isometric conditions. *Am J Physiol* 266:H147–H155
- Edvinsson L, Ahnstedt H, Larsen R, Sheykhzade M (2014) Differential localization and characterization of functional calcitonin gene-related peptide receptors in human subcutaneous arteries. *Acta Physiol Scand* 210:811–822
- Sheykhzade M, Lind H, Edvinsson L (2004) Noncompetitive antagonism of BIBN4096BS on CGRP-induced responses in human subcutaneous arteries. *Br J Pharmacol* 143:1066–1073
- Russel FA, King R, Smillie S-J, Kodji X, Brain SD (2014) Calcitonin Gene-Related Peptide: Physiology and Pathophysiology. *Physiol Rev* 94:1099–1142
- Ho TW, Edvinsson L, Goadsby PJ (2010) CGRP and its receptors provide new insights into migraine pathophysiology. *Nat Neurol Rev* 6:573–582
- Juul R, Hara H, Gisvold SE, Brubakk AO, Fredriksen TA, Waldemar G et al (1995) Alterations in perivascular dilatory neuropeptides (CGRP, SP, VIP) in the external jugular vein and in the cerebrospinal fluid following subarachnoid haemorrhage in man. *Acta Neurochir (Wien)* 132:32–41

38. Goadsby PJ, Edvinsson L, Ekman R (1990) Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol* 28:183–187
39. Salvatore CA, Hershey JC, Corcoran HA, Fay JF, Johnston VK, Moore EL et al (2008) Pharmacological characterization of MK-0974 [N-[(3R,6S)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifluoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-1-yl)piperidine-1-carboxamide], a potent and orally active calcitonin gene-related peptide receptor antagonist for the treatment of migraine. *J Pharmacol Exp Ther* 324:416–421
40. Tfelt-Hansen P, Olesen J (2010) Possible site of action of CGRP antagonists in migraine. *Cephalalgia* 31:748–750
41. Hay DL, Christopoulos G, Christopoulos A, Poyner DR, Sexton PM (2005) Pharmacological discrimination of calcitonin receptor: receptor activity-modifying protein complexes. *Mol Pharmacol* 67:1655–1665
42. Edvinsson L, Nilsson E, Jansen-Olesen I (2007) Inhibitory effect of BIBN4096BS, CGRP8–37, a CGRP antibody and an RNA-Spiegelmer on CGRP induced vasodilatation in the perfused and non-perfused rat middle cerebral artery. *Br J Pharmacol* 150:633–640

Submit your manuscript to a SpringerOpen[®] journal and benefit from:

- ▶ Convenient online submission
- ▶ Rigorous peer review
- ▶ Open access: articles freely available online
- ▶ High visibility within the field
- ▶ Retaining the copyright to your article

Submit your next manuscript at ▶ springeropen.com
