Desensitisation of calcitonin gene-related peptide responsiveness but not adrenomedullin responsiveness in vascular smooth muscle cells

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Abstract

Adrenomedullin (ADM) and calcitonin gene-related peptide (CGRP) are distantly related peptides. Both act through G protein-coupled receptors on vascular smooth muscle cells to increase intracellular cAMP concentrations, causing vasorelaxation. Recent evidence suggests that both peptides bind to a common heptahelical receptor, with specificity for each peptide being determined by a receptor activity modifying protein (RAMP). This hypothesis predicts that each peptide should desensitise the cellular response to subsequent stimulation by the other. We have studied the patterns of desensitisation of ADM/CGRP receptors in rat aortic vascular smooth muscle cells. Cells were incubated for 20 min in either serum free medium (SFM), alone (control) or in SFM containing vasoactive agonist (e.g. ADM 10^{-8} M, CGRP 10^{-7} M, angiotensin II 10^{-9} M or isoproterenol 10^{-6} M). Cells were then washed and incubated for a further 20 min in SFM containing a second agonist and 1 mM isobutyryl methyl xanthine. Cells were harvested and assayed for cAMP. Pre-exposure of cells to CGRP, isoproterenol, angiotensin II or ADM, decreased cAMP generation in response to subsequent stimulation with CGRP by 84% (± 5), 66% (± 18), 45% (± 5) and 60% (± 10) respectively (mean ± S.D.). Pre-incubation of cells with 100 nM H-89, a protein kinase A (PKA) inhibitor, abolished the desensitisation of CGRP by itself, implying that this desensitisation was mediated through PKA. In contrast, there was no attenuation of the cAMP response to stimulation with ADM by pre-exposure to ADM and all other agonists tested. Identical results were seen with or without PKA inhibition by H-89. These results indicate that the ADM receptor does not desensitise over this time period in RAVSMCs, in contrast to the CGRP receptor, which is desensitised by pre-exposure to CGRP and other vasoactive agonists. These data also suggest that ADM and CGRP act through separate receptors in these cells.

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Introduction

Adrenomedullin (ADM) and calcitonin gene-related peptide (CGRP) are distantly related, potent vasodilatory peptides, sharing structural similarity in the form of a conserved N-terminal disulphide loop. Both act on vascular smooth muscle cells (VSMC) to stimulate adenylate cyclase and hence increase intracellular cAMP levels, resulting in vasorelaxation (Nuki *et al.* 1993). Although much is known about the distribution of CGRP, ADM and their receptors in man and other mammals (Richards *et al.* 1996, Wimalawansa 1996), it remains unclear whether they act through a common receptor or separate receptors, and what the respective roles of these peptides are in health and disease.

A recent report (McLatchie *et al.* 1998) suggested that both peptides bind to the same seven transmembrane domain receptor (termed the calcitonin receptor-like receptor, CRLR), with receptor specificity being conferred by the presence of receptor activity modifying proteins (RAMPs). RAMPs are single transmembrane domain proteins that appear to be important in the N-linked glycosylation of CRLR and in the binding of peptide ligands. According to the proposed model, coexpression of CRLR with RAMP1 in A293 cells results in a CGRP receptor, whereas when CRLR is expressed with RAMP2 in the same cells, ADM receptor pharmacology is acquired. Earlier studies with GTPγS (Yamaguchi *et al.* 1988, Eguchi *et al.* 1994) indicated that the receptors for CGRP and ADM are G protein coupled receptors (GPCRs), stimulating adenylate cyclase through G_s. Most members of the GPCR 'superfamily' display the phenomenon of desensitisation (Tsuga *et al.* 1994, Barker *et al.* 1995, Freedman *et al.* 1995), in which the size of the intracellular signal generated by the agonist-occupied receptor is attenuated in response to repeated exposure of

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the receptor to the ligand. If ADM and CGRP act through a common receptor (the CRLR) then one would predict that they should 'cross-desensitise', such that pre-exposure to either peptide would attenuate the size of the intracellular signal generated by subsequent exposure to the other. We have previously shown, in the neuroblastoma SK-N-MC cell line, that the desensitisation patterns to each peptide are markedly different (Drake *et al.* 1999). In order to further these studies in a more physiological system, we studied, *in vitro*, the patterns of desensitisation of ADM and CGRP receptors in primary cultures of rat aortic vascular smooth muscle cells (RAVSMCs). This was achieved by measuring the quantities of cAMP produced by sequential stimulation of these cells with different combinations of vasoactive agonists.

Materials and Methods

RAVSMCs

Primary cultures of RAVSMCs were prepared according to the protocol described by Clapp & Gurney (1991). The aorta was removed from a dead male Wistar rat, cut into strips, incubated in dissociation medium containing 0·16 mM Ca^{2+} and subsequently incubated overnight at 4 °C in 0·2 mg/ml papain. Following re-warming and dispersion, single cells were then isolated and grown in Dulbecco's modified eagle medium (Sigma, Poole, Dorset, UK) containing 10% fetal bovine serum, 100 mU/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 95% air, 5% CO_2. Subcultured VSMCs (passages 4–13) were used.

Experimental protocol

Cells were grown to confluency in six well plates and, after washing in serum-free medium (SFM), were incubated at 37 °C for 20 min in either SFM alone (control) or SFM containing agonists at the following concentrations: isoproterenol 10^{-6} M, CGRP 10^{-7} M, ADM 10^{-8} M, angiotensin II 10^{-9} M (isoproterenol and angiotensin, Sigma, UK; CGRP and ADM, Peninsula Labs Europe, Merseyside, UK). The concentrations of CGRP and ADM used were based on dose–response curves for these peptides (data not shown). After the initial 20 min incubation, cells were washed in SFM and incubated for a second 20 min in SFM containing a second agonist. For the second incubation step isobutyryl methylxanthine (Sigma, UK) was added at a concentration of 1 mM to inhibit phosphodiesterases. At the end of the second incubation period, cells were harvested, boiled for 5 min to inactivate phosphodiesterases, centrifuged at 10 000 r.p.m. for 3 min to remove cell debris and the cAMP content of the supernatant assayed. Cyclic AMP production during the second ('collection') step was compared with production by cells that were incubated for 20 min in SFM alone and then with agonist for 20 min, and which therefore underwent a single stimulation only.

Protein kinase A (PKA) inhibition was achieved by pre-incubating cells for 30 min at 37 °C in SFM containing 100 nM N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide (H-89, CalBiochem, Nottingham, UK). Cells were then incubated, as above, in SFM containing 100 nM H-89 and agonist. Between incubations, cells were washed in SFM containing 100 nM H-89.

Cyclic AMP assay

Cyclic AMP was measured by a competitive binding assay as described previously (Brown *et al.* 1971). Tritiated and non-tritiated cAMP were purchased from Amersham, Bucks, UK and Sigma, UK respectively.

Statistical analysis

Results are expressed as pmol/well confluent cells and represent the mean ± S.D. of three separate, duplicated experiments, each assayed in duplicate for cAMP. Data were compared using Student's unpaired *t*-test.

Results

The concentrations of agonist used in subsequent desensitisation experiments were based on a calculated EC_{50} of $3·9 \times 10^{-9}$ and $6·6 \times 10^{-8}$ for ADM and CGRP respectively (data not shown). In Figs 1 and 2, cAMP production is presented as a percentage of that produced during a simultaneously run single incubation with agonist. Pre-exposure of cells to CGRP, isoproterenol, angiotensin II or ADM at the above concentrations attenuated the subsequent CGRP-stimulated generation of cAMP by 84% (± 5), 66% (± 18), 45% (± 5) and 60% (± 10) respectively (Fig. 1A). Attenuation of cAMP production by pre-exposure of cells to CGRP, isoproterenol and ADM was completely abolished by inhibition of PKA with 100 nM H-89 (Fig. 1A). Pre-exposure of cells to isoproterenol attenuated the subsequent isoproterenol-stimulated generation of cAMP by 50% (± 12); mean (± S.D.). This effect was unchanged by inhibition of PKA with H-89 (Fig. 1B). In contrast, pre-exposure of cells to CGRP, ADM and angiotensin II had no effect on subsequent ADM-induced cAMP generation and was unaffected by pre-incubation with H-89 (Fig. 2). Identical results were seen during a single experiment using the same protocol with rat renal VSMCs prepared by the same technique.

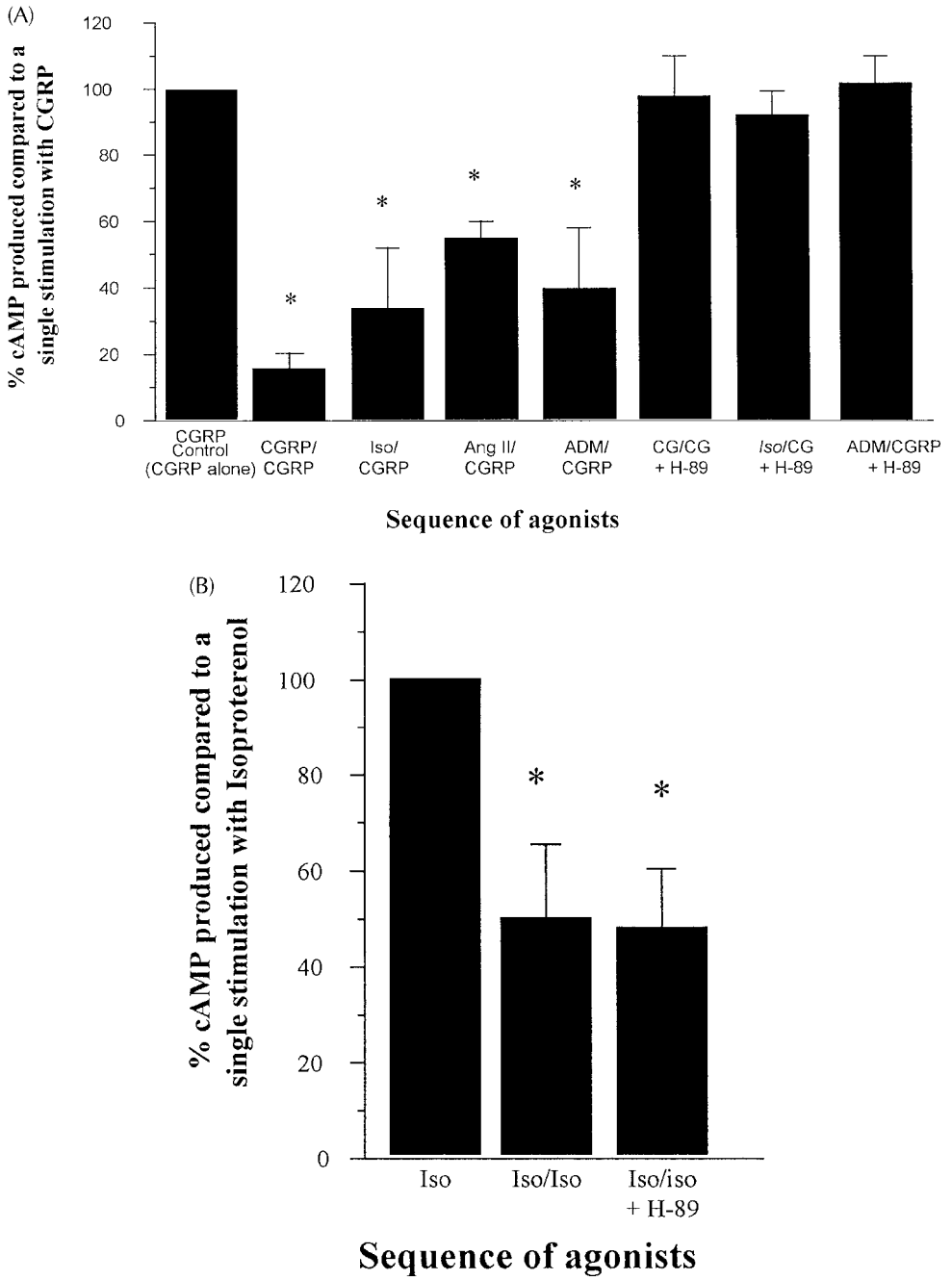

Figure 1 (A) CGRP-mediated cAMP generation by RAVSCMs in response to sequential stimulation with vasoactive agonists. cAMP generated by a single stimulation with CGRP was 93 ± 23 pmol/well, **P*<0·001. (B) cAMP generation by RAVSMCs in response to stimulation with isoproterenol (iso) alone, iso/iso and iso/iso in the presence of H-89. cAMP generated by a single stimulation with CGRP was 73 ± 12 pmol/well, **P*<0·001.

Discussion

Since the discovery of CGRP in 1983 (Rosenfeld *et al.* 1983) and ADM in 1993 (Kitamura *et al.* 1993), much has been learnt about the distribution of these related peptide hormones and their receptors in various mammals (Richards *et al.* 1996, Wimalawansa 1996). The presence of ADM in human plasma, its synthesis and release by

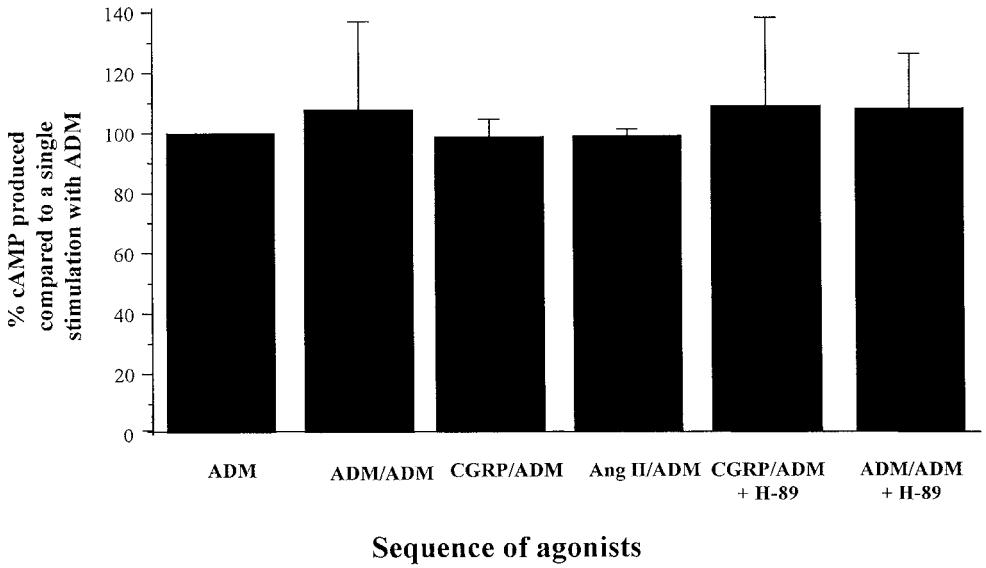

Figure 2 ADM-mediated cAMP generation by RAVSMCs in response to sequential stimulation with vasoactive agonists. cAMP generated by a single stimulation with ADM was 153 ± 21 pmol/well.

endothelial cells and its elevation in conditions such as hypertension (Ishimitsu *et al.* 1994) point to a possible role in the control of vascular tone. Studies with GTPγS (Yamaguchi *et al.* 1988, Eguchi *et al.* 1994) have shown that ADM and CGRP both act through GPCRs. Other GPCRs found on cells of the vascular system (such as β-adrenergic and angiotensin II receptors) exhibit the phenomenon of desensitisation, in which the amplitude of the intracellular signal generated by an agonist-occupied receptor decreases despite repeated exposure of the cells to agonist (Barker *et al.* 1995, Freedman *et al.* 1995). By analogy, we reasoned that the receptors that mediate the intracellular response to ADM and CGRP would exhibit desensitisation and that if these two peptides acted through a common receptor then pre-exposure of cells to one peptide would attenuate the size of the intracellular signal generated by subsequent exposure to the other. We have previously shown, in the human neuroblastoma SK-N-MC cell line, that the desensitisation patterns of ADM and CGRP responsiveness are markedly different (Drake *et al.* 1999). Given the postulated role of these peptides in the control vascular tone, we have attempted to study the patterns of desensitisation in a more physiological system, using primary cultures of RAVSMCs. Using an identical protocol to the one described here, the ability of cells to generate cAMP in response to stimulation with ADM is not altered by a 20-min pre-exposure of cells to either ADM or CGRP. In contrast, the responsiveness of cells to CGRP diminished significantly when cells were pre-exposed to either peptide.

This study provides good evidence that ADM and CGRP act through separate receptors in RAVSMCs. Generation of cAMP by stimulation with CGRP was reduced by pre-exposure of the cells to various vaso-active agonists. The desensitisation effect of pre-exposure to CGRP and ADM was completely abolished by H-89 at a concentration that selectively inhibits PKA without affecting protein kinase (PK)C activity (Chijiwa *et al.* 1990), indicating that this process was probably PKA mediated. In contrast, generation of cAMP by stimulation with ADM could not be attenuated by pre-exposure of the cells to CGRP, ADM or angiotensin II.

Alternative explanations for these data include the possibility that the CRLR/RAMP hypothesis is correct, and that RAMP2 interferes with the desensitisation process. A further possibility is that different RAMPs in association with CRLR, in addition to influencing ligand binding, lead to activation of different intracellular second messenger pathways which in turn result in different patterns of desensitisation. These alternative models seem less likely, but should be readily testable.

Desensitisation has been particularly well studied in β-adrenergic receptors (βAR), in which the responsiveness of cells to stimulation with catecholamines is, to some extent, determined by the phosphorylation state of critical amino acid residues in the third intracytoplasmic loop and the C-terminal tail of the receptor (Hausdorff *et al.* 1990). This, in turn, is controlled by two intracellular enzymes, β-adrenergic receptor kinase (βARK/GRK 2) and PKA, both of which are stimulated following binding of ligand to the βAR. Homologous desensitisation phosphorylation of specific serine and threonine residues in the C-terminal tail of the receptor by GRKs leads to binding of a β-arrestin molecule. This inhibits further

signal transduction by preventing interaction of the receptor with the linked G protein. Heterologous desensitisation of the βAR is mediated through activation of PKA which phosphorylates amino acid residues on the C-terminal tails and/or cytoplasmic loops of a variety of receptors. In the case of the βAR, desensitisation occurs via a combination of homologous and heterologous processes. We therefore hypothesised that the CGRP/ADM receptor would desensitise by more than one mechanism. However, it appears that desensitisation of CGRP receptor(s) in RAVSMCs in the model described is mediated entirely by activation of PKA, as selective inhibition of PKA during pre-exposure of cells to CGRP or ADM did not attenuate cAMP production during subsequent stimulation with CGRP. Homologous desensitisation is clearly intact in our experimental system, as shown by the fact that pre-exposure of cells to isoproterenol attenuates subsequent cAMP generation by the same agonist even when PKA is inhibited by H-89.

The effect of pre-exposure to ADM and other agonists on subsequent ADM responsiveness of RAVSMCs has recently been reported (Iwasaki *et al.* 1998). In that study, ADM responsiveness was dramatically attenuated 2 h after pre-exposure to ADM, a situation that persisted for at least 24 h. In a separate set of experiments by the same workers, pre-exposure of cells to isoproterenol and forskolin for 5 h did not alter subsequent ADM responsiveness. These findings do not necessarily conflict with our own. The time course over which ADM responsiveness was lost in their experiments is likely to have been due to receptor internalisation and sequestration rather than phosphorylation and desensitisation which, for βAR at least, takes place over seconds or minutes after even the briefest encounter with ligand (Hausdorff *et al.* 1990). Binding studies using radiolabelled ADM, with or without pre-exposure to 'cold' peptide, would be informative in this regard.

The finding that ADM receptors in these cells appear to avoid rapid desensitisation raises interesting questions about their function in health and disease. ADM causes relaxation of rat cerebral arterioles (Nishimura & Suzuki 1997) and has been shown to limit ischaemic brain injury following cerebral artery occlusion in rats (Dogan *et al.* 1997). The site of production of most ADM is endothelial cells, and it seems highly probable that ADM acts in a paracrine manner on vascular smooth muscle cells (VSMCs) to increase local blood flow around areas of ischaemic tissue, where the presence of a non-desensitising receptor would ensure that the vasodilatory signal would not be lost or attenuated. It is tempting to speculate that, in man, the lack of desensitisation of the ADM receptor that we have observed in RAVSMCs is a protective mechanism, limiting already ischaemic tissues from further insult.

In summary, we have shown evidence that ADM and CGRP act through separate receptors in RAVSMCs: findings that do not fit easily with the CRLR/RAMP hypothesis as presently stated. Furthermore, the ADM receptor does not show the desensitisation that is characteristic of almost all other G-protein coupled receptors thus far studied. Further work is required to elucidate the mechanisms by which ADM receptors avoid desensitisation and to clarify the physiological significance of these findings in health and disease.

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