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# Characterization and effects on cAMP accumulation of adrenomedullin and calcitonin gene-related peptide (CGRP) receptors in dissociated rat spinal cord cell culture

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**1** Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) have structural similarities, interact with each others receptors (calcitonin receptor-like receptor (CLR)/receptor-activity-modifying proteins (RAMPs)) and show overlapping biological activities. AM and CGRP receptors are chiefly coupled to cAMP production. In this study, a method of primary dissociated cell culture was used to investigate the presence of AM and CGRP receptors and their effects on cAMP production in embryonic spinal cord cells.

**2** Both neuronal and non-neuronal CLR immunopositive cells were present in our model.

**3** High affinity, specific [$^{125}$I]-AM binding sites ($K_d$ 79 ± 9 pM and $B_{max}$ 571 ± 34 fmol mg$^{-1}$ protein) were more abundant than specific [$^{125}$I]-CGRP binding sites ($K_d$ 12 ± 0.7 pM and $B_{max}$ 32 ± 2 fmol mg$^{-1}$ protein) in embryonic spinal cord cells.

**4** Specific [$^{125}$I]-AM binding was competed by related molecules with a ligand selectivity profile of rAM > $hAM_{22-52}$ > rCGRPα > $CGRP_{8-37}$ ≫ [*r*-(*r**,*s**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]-carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-piperidinecarboxamide (BIBN4096BS).

**5** Specific [$^{125}$I]-CGRP binding was competed by rCGRPα > rAM ⩾ $CGRP_{8-37}$ ⩾ BIBN4096BS > $hAM_{22-52}$.

**6** Cellular levels of cAMP were increased by AM ($pEC_{50}$ 10.2 ± 0.2) and less potently by rCGRPα ($pEC_{50}$ 8.9 ± 0.4). rCGRPα-induced cAMP accumulation was effectively inhibited by $CGRP_{8-37}$ ($pA_2$ 7.63 ± 0.44) and $hAM_{22-52}$ ($pA_2$ 6.18 ± 0.21) while AM-stimulation of cAMP levels was inhibited by $CGRP_{8-37}$ ($pA_2$ 7.41 ± 0.15) and $AM_{22-52}$ ($pA_2$ 7.26 ± 0.18). BIBN4096BS only antagonized the effects of CGRP ($pA_2$ 8.40 ± 0.30) on cAMP accumulation.

**7** These pharmacological profiles suggest that effects of CGRP are mediated by the $CGRP_1$ (CLR/RAMP1) receptor in our model while those of AM are related to the activation of the $AM_1$ (CLR/RAMP2) receptor subtype.
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**Abbreviations:** AM, adrenomedullin; BIBN4096BS, [*r*-(*r**,*s**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-piperidinecarboxamide; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle medium; $H_2O_2$, hydrogen peroxide; IBMX, 3-isobuthyl-1-methyl xanthine; KCl, potassium chloride; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; NaOH, sodium hydroxide; RAMP, receptor-activity-modifying protein; RP, relative potency; TBS, Tris-buffered saline; TBS + T, Tris-buffered saline containing 0.05% Tween 20

## Introduction

Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) are structurally related and belong to the calcitonin family of peptides (Jacques *et al*., 2000; Poyner *et al*., 2002; Dumont *et al*., 2004). Both peptides are multifunctional and have overlapping biological activities particularly in terms of their potent vasodilating properties (Hinson *et al*., 2000). AM and CGRP along with their receptors are widely distributed in

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peripheral tissues as well as in the central nervous system (Owji *et al*., 1995; Van Rossum *et al*., 1997; Jacques *et al*., 2000). Pharmacological and physiological studies have suggested the existence of multiple subtypes of AM and CGRP receptors (for recent reviews, see Juaneda *et al*., 2000; Poyner *et al*., 2002). For example, the $CGRP_1$ subtype is particularly sensitive to CGRP receptor antagonists such as $CGRP_{8-37}$ (Dennis *et al*., 1990) and [*r*-(*r**,*s**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-piperidinecarboxamide (BIBN4096BS) (Doods *et al*., 2000; Wu *et al*., 2000) while the putative $CGRP_2$ subtype is usually less sensitive to these antagonists (Dennis *et al*., 1989, 1990; Doods *et al*., 2000; Wu *et al*., 2000). It is now well established that the pharmacology of $CGRP_1$ and AM receptors is determined by a protein complex that includes a G-protein-coupled receptor, the calcitonin receptor-like receptor (CLR), and one of three receptor activity modifying proteins (RAMPs) (Poyner *et al*., 2002). The coexpression and association of CLR with RAMP1 generates the $CGRP_{8-37}$-sensitive $CGRP_1$ receptor (McLatchie *et al*., 1998), which can also be activated by high concentrations of AM (Nagoshi *et al*., 2002). The combination of CLR with either RAMP2 or RAMP3 produces the $AM_1$ and $AM_2$ receptor subtype, respectively. The $AM_1$ receptor has low affinity for CGRP and is particularly sensitive to the antagonistic properties of $AM_{22-52}$ while the $AM_2$ subtype is also sensitive to $AM_{22-52}$ but shows higher affinities for CGRP and $CGRP_{8-37}$ (Poyner *et al*., 2002).

CGRP and AM play important roles in the spinal cord. The presence of AM mRNA (Owji *et al*., 1996; Shan & Krukoff, 2001) and AM-like immunoreactivity (Serrano *et al*., 2000) have been reported in the rat spinal cord while that of CGRP is very well established, both in sensory and motor areas (Gibson *et al*., 1984; Jacques *et al*., 2000). Early studies have shown that spinal cord membranes are comparatively enriched with specific [$^{125}$I]-CGRP binding sites (Goltzman & Mitchell, 1985; Owji *et al*., 1995). High levels of specific [$^{125}$I]-AM binding sites have also been detected in rat spinal cord microsomal preparations when compared with various brain regions. In fact, the apparent maximal capacity of specific [$^{125}$I]-AM binding was found to be 40 times greater than that of specific [$^{125}$I]-CGRP sites in this preparation (Owji *et al*., 1996). CLR-like immunoreactivity has very recently been shown to be present in rat spinal cord neurons (Cottrell *et al*., 2005). However, the molecular identity of AM and CGRP receptors expressed in the rat spinal cord remains to be established as the expression of CLR in this tissue is still controversial. While Northern blot data (Chakravarty *et al*., 2000) suggested the expression of CLR mRNA in the rat spinal cord, *in situ* hybridization results were negative (Oliver *et al*., 1998). There is also controversy over the second messenger system activated by CGRP and AM in the spinal cord (Goltzman & Mitchell, 1985; Owji *et al*., 1996). We have established that both AM and CGRP induced, in a $CGRP_{8-37}$-sensitive manner, the expression of c-fos in the rat spinal cord (Takhshid *et al*., 2004). However, owing to the limited specificity of $CGRP_{8-37}$, further work is required to establish the nature of the receptors that are activated by the peptides.

In the present study, the existence of single or multiple populations of specific AM and CGRP receptors was investigated in the rat spinal cord using an embryonic cell culture model. Receptor binding and functional (cAMP production) data suggest the presence of specific $AM_1$ (CLR/RAMP2) and $CGRP_1$ (CLR/RAMP1) receptors in rat embryonic spinal cord culture.

## Methods

### *Materials*

All cell culture materials were from Invitrogen Canada Inc. (Burlington, ON, Canada), except for poly-D-lysine and bacitracin purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). All other chemicals were of analytical grade and purchased from Fisher Scientific (Nepean, ON, Canada) or Sigma-Aldrich Canada Ltd. Rat [$^{125}$I]-AM, human [$^{125}$I]-α-iodohistidyl$^8$-CGRP and [$^3$H]-cAMP were from Amersham Biosciences (Baie d'Urfé, QC, Canada). Rat CGRPα and rat AM were obtained from Bachem California Inc. (Torrance, CA, U.S.A.). Human $CGRP_{8-37}$ and $AM_{22-52}$ were synthesized by one of us (AF) as described earlier (Dennis *et al*., 1989; 1990). BIBN4096BS was a gift from Dr H. Doods (Boehringer Ingelheim, Germany). A polyclonal antibody raised against a peptide mapping near the carboxy terminus of human CLR (CLR (V20)) and the blocking peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit antibodies to neuron-specific enolase (NSE, Dako Carpenteria, CA, U.S.A.), glial fibrillary acidic protein (GFAP, Dako Carpenteria, CA, U.S.A.) and cAMP response element binding protein (CREB) phosphorylated at Ser$^{133}$ (Phospho-CREB, Cell Signaling Technology, Inc., Danvers, MA, U.S.A.) were polyclonal while anti-mouse neuron-specific nuclear protein (NeuN, Chemicon International, Temecula, CA, U.S.A.) was monoclonal. Goat anti-rabbit conjugated to Alexa Fluor 488 and goat anti-mouse conjugated to Alexa Fluor 568 were from Molecular Probes (Eugene, OR, U.S.A.). Antifade mounting medium (Vectashield), normal horse serum (NHS) and Vectastain Elite ABC kit were obtained from Vector Laboratories (Canada) Inc. (Burlington, ON, Canada).

### *Cell isolation and primary culture*

Spinal cord cells were prepared from fetuses (embryonic day 17–18) obtained from pregnant Sprague–Dawley rats (Charles River Breeding Laboratories, St-Constant, QC, Canada). Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care. After being aseptically dissociated into small pieces, spinal cords were dissociated enzymatically at 37°C with 0.25% trypsin in Hanks' balanced salt solution (HBSS) for 10 min. Tissues were then triturated with a thin flame-polished pipette in Dulbecco's modified Eagle medium (DMEM) containing 1% HEPES buffer solution, penicillin/streptomycin (1 : 100), 5% heat-inactivated fetal bovine serum and 5% NHS. Cells were next subjected to density gradient centrifugation at $400 \times g$ for 10 min. The resulting pellet was resuspended with DMEM and cell suspension filtered through a cell strainer (75 μM, Becton Dickinson Labware, Frankin Lakes, NJ, U.S.A.). Spinal cord cells were seeded in poly-D-lysine coated 48-well culture plates, yielding a density of $1 \times 10^5$ cells ml$^{-1}$. Cells were cultured in

a humid incubator at 37°C with 5% $CO_2$ and 95% air. The culture medium was changed on the day after seeding and every day thereafter. Experiments were performed after 7 days in culture. The presence of NSE and GFAP immunopositive cells (Figure 4c and d) showed that both neuronal cells and cells with a glial cell phenotype survive up to 7 days in this model.

### *Receptor binding assays*

For competition binding studies (Coppock *et al*., 1999), dissociated spinal cord cells were plated out at a density of $2 \times 10^5$ cells per well into 24-well culture plates and grown under the same conditions as above. Cells were incubated for 60 min at 4°C in 0.5 ml of binding buffer (20 mM HEPES, pH 7.4, 5 mM magnesium chloride, 10 mM sodium chloride, 4 mM potassium chloride, 1 mM EDTA, 1 $\mu$M phosphoramidon, 0.25 mg ml$^{-1}$ bacitracin and 0.3% bovine serum albumin (BSA)) containing 700 Bq (16 pM) rat [$^{125}$I]-AM. After the incubation, binding buffer was removed by aspiration and cells were washed twice with 0.5 ml of ice-cold assay buffer and then dissolved in 1 M sodium hydroxide (NaOH) for counting bound [$^{125}$I]-AM. For [$^{125}$I]-CGRP binding assays, [$^{125}$I]-CGRP (1000 Bq; 55 pM) was incubated for 60 min at 22°C and unbound ligand was removed as described above. Thus, [$^{125}$I]-AM and [$^{125}$I]-CGRP represented specific bindings of $80 \pm 5$ and $72 \pm 7$%, respectively. For saturation binding experiments, cells grown in a $150 \times 25$ mm dish were washed with and then resuspended in the binding buffer without BSA ($2 \times 10^6$ cells ml$^{-1}$). 100 $\mu$l of the cell suspension was incubated in a final volume of 0.5 ml of binding buffer containing increasing amounts of the labeled peptide under binding conditions as above. Nonspecific binding was measured in the presence of excess (200 nM) unlabeled rAM or rCGRP$\alpha$. The binding reaction was terminated by rapid filtration through Schleicher and Schuell #32 glass filters (previously soaked in 1.0% polyethyleneimine) using a cell harvester filtering apparatus (Brandel Instruments, Gaithersburg, MD, U.S.A.). Filters were rinsed three times with 3 ml cold binding buffer and the radioactivity retained on the filter was measured using a gamma counter with 85% efficiency (Packard Instruments, Cobra II; Mississauga, ON, Canada). Protein concentrations were determined using BSA as standard (Bradford, 1976). All binding experiments were performed in triplicate and repeated at least three times.

### *Assay of cyclic AMP accumulation*

Growth medium was removed from cells and replaced with serum-free DMEM containing 500 $\mu$M of the phosphodiesterase inhibitor IBMX (3-isobuthyl-1-methyl xanthine dissolved in 0.1 M NaOH) for 30 min. Cells were then stimulated with agonists (AM or CGRP) for 10 min in serum-free DMEM containing IBMX. The reaction was terminated by removing the agonist-containing medium and adding ice-cold ethanol (100%). In experiments where antagonists were used, cells were preincubated with antagonist for 15 min before the 10 min agonist incubation period. cAMP was measured using a radio-receptor assay as previously described (Poyner *et al*., 1992).

### *Immunocytochemistry*

Spinal cord cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. Cells were then pretreated with 0.3% hydrogen peroxide for 15 min and with 10% normal goat serum (NGS) for 1 h in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS + T) to block endogenous peroxidase and nonspecific staining. Cultured cells were then incubated in any of the primary antibodies (anti-NSE 1 : 150 and anti-GFAP 1 : 1500) for 12 h at 4°C. Thereafter, cultured cells were incubated in biotinylated goat anti-rabbit IgG and further processed using Vectastain *Elite* ABC kit according to the manufacturer's instructions. Between each incubation, cells were washed in PBS + T twice. Finally, immunoprecipitates were developed using the nickel–glucose oxidase-3,3′-diaminobenzidine method (Shu *et al*., 1988). All cultured cells were washed thoroughly before being kept in 70% glycerol for microscopic investigation. For CLR immunocytochemistry, cultured cells were incubated in a primary goat polyclonal antibody raised against a peptide mapping near the carboxy terminus of human CLR (CLR (V20); 1 : 400) and NHS (10%) was used to block the nonspecific staining. CLR-like immunostaining was abolished when the primary antibody was adsorbed by fivefold higher concentration of the blocking peptide corresponding to the amino-acid sequence used as immunogen. Photomicrographs were acquired using a Nikon Diaphot TMD inverted microscope coupled to a Nikon CoolPix 950 digital camera and digitally processed using Adobe Photoshop software.

### *Double-immunofluorescence staining*

For double staining of NeuN and phospho-CREB, an immunofluorescence staining method was used. Cells grown on eight-well chamber slides were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min and then incubated with 10% NGS for 1 h in (TBS + T). The slides were then incubated at 4°C for 12 h in a cocktail solution containing the primary antibody to phospho-CREB (1 : 400) and NeuN (1 : 200). The cultured cells were next incubated for 2 h with goat secondary antibodies (1 : 200) conjugated to Alexa Fluor 488 and Alexa Fluor 568. Between each incubation, cultured cells were rinsed thoroughly with TBS + T. Finally, cultured cells were covered with antifade mounting medium. Immunofluorescent images were obtained under a confocal laser scanning microscope (Nikon PCM2000 equiped with the Nikon Eclipse TE300, NY, U.S.A.) and digitally processed using Adobe Photoshop software.

### *Data analysis*

Curve fitting was performed using the GraphPad Prism program (version 3.03; GraphPad Software Inc., San Diego, CA, U.S.A.). For cAMP studies, data from each concentration–response curve were fitted to a sigmoidal curve to obtain the maximum response, Hill coefficient and $EC_{50}$. From individual curves, dose ratios were calculated. When only one or two antagonist concentrations were used, an apparent $pA_2$ was calculated from the formula log(antagonist)-log(dose ratio-1), after first confirming that there were no significant differences in Hill coefficient or maximum response between concentration–response curves obtained in the presence and

absence of the antagonist. Binding data were analyzed by nonlinear regression to calculate $IC_{50}$, the apparent dissociation constant ($K_d$) and maximal binding capacity ($B_{max}$). Statistical analysis was performed using the *F*-test to analyze whether the data were fit better to a one- or two-site model ($P<0.05$). As radioligands were used at concentrations well below their $K_d$, $IC_{50}$ values were effectively identical to $K_i$ values.

## Results

### *Saturation binding studies*

Whole-cell binding assay data demonstrated the existence of specific binding sites for [$^{125}$I]-AM and [$^{125}$I]-CGRP in cultured embryonic rat spinal cord. Specific [$^{125}$I]-AM binding represented 65% of totally bound ligand at a concentration of 80 pM (Figure 1a). Isotherm saturation binding experiments demonstrated that [$^{125}$I]-AM specifically bound with high affinity ($K_d$ of $79\pm9$ pM) to a saturable amount of sites ($B_{max}$ of $571\pm34$ fmol mg$^{-1}$ protein) (Figure 1a). Nonlinear analysis indicated that [$^{125}$I]-AM binds to an apparent single population of sites. In the case of [$^{125}$I]-CGRP, specific binding represented 80% of totally bound radioligand at 10 pM (Figure 1b). Binding parameters derived from saturation isotherms (Figure 1b) demonstrated that [$^{125}$I]-CGRP bound with high affinity ($K_d$ of $12\pm0.7$ pM) to an apparent single class of saturable sites ($B_{max}$ of $32\pm2$ fmol mg$^{-1}$ protein) in cultured embryonic spinal cord cells.

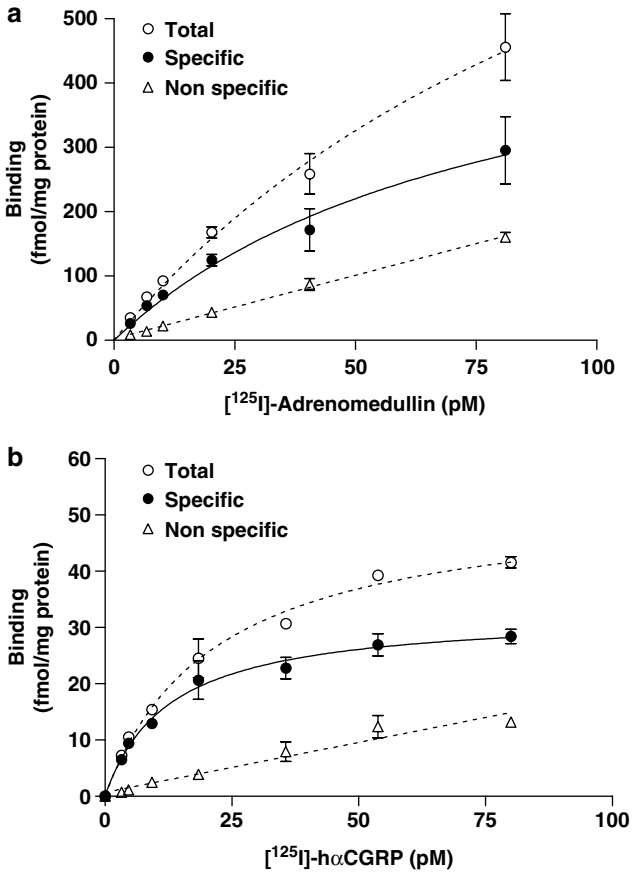


**Figure 1** Typical saturation curves and their corresponding Scatchard plots of [$^{125}$I]-AM (a) and [$^{125}$I]-CGRP (b) binding in embryonic rat spinal cord cells. Cells (80 $\mu$g protein ml$^{-1}$) were incubated with 1–80 pM [$^{125}$I]-AM and/or [$^{125}$I]-CGRP in the presence or absence of 200 nM of the nonradioactive peptide.

### *Competition binding studies*

The ligand selectivity profile of binding sites targeted by [$^{125}$I]-AM and [$^{125}$I]-CGRP was investigated next using various agonists and antagonists. The ligand selectivity profile for specific [$^{125}$I]-AM binding was: rAM > $hAM_{22-52}$ > rCGRP$\alpha \geqslant CGRP_{8-37} \gg$ BIBN4096BS (Figure 2a; Table 1). As expected, rAM competed for specific [$^{125}$I]-AM binding with the highest affinity. The purported AM receptor antagonist, $hAM_{22-52}$ was next followed by rCGRP$\alpha$ and $CGRP_{8-37}$ (Table 1). The nonpeptide antagonist, BIBN4096BS showed very low affinity for specific [$^{125}$I]-AM binding sites (Table 1) and it was not possible to construct a full competition curve.

The relative affinities of various CGRP/AM-related fragments and analogs in competing for specific [$^{125}$I]-CGRP binding was rCGRP$\alpha$ > rAM $\geqslant CGRP_{8-37} \geqslant$ BIBN4096BS > $hAM_{22-52}$ (Figure 2b). Under our experimental conditions,

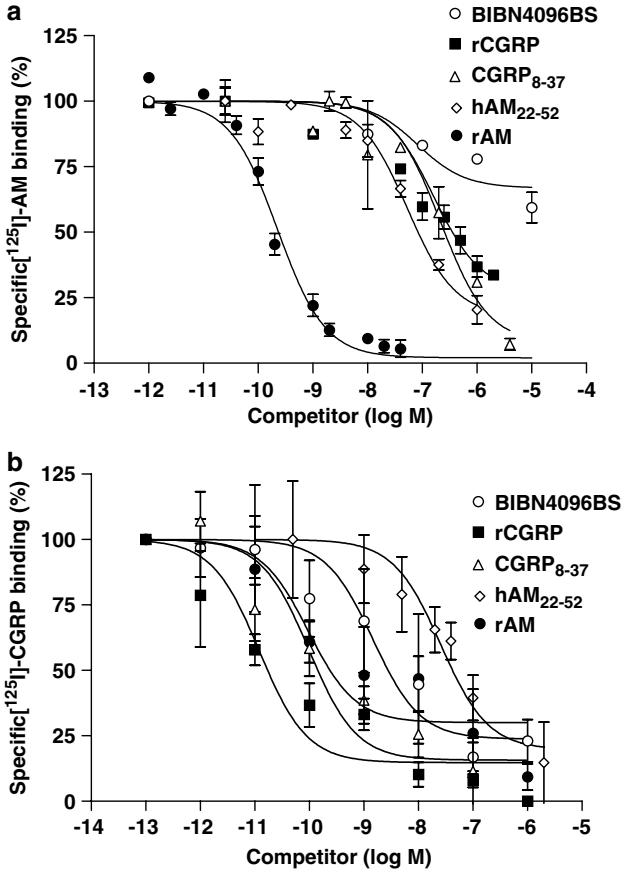


**Figure 2** Competition curves against specific binding of [$^{125}$I]-AM (a) and [$^{125}$I]-CGRP (b) in embryonic rat spinal cord cells. Whole-cell binding assays were carried out as described in Methods using 16 pM [$^{125}$I]-AM or 55 pM [$^{125}$]-CGRP and increasing concentrations of competitors. Data are from three experiments, each performed in triplicate and are expressed as percentage of maximal specific binding defined by difference between ligand binding in the absence and the presence of 200 nM of AM or CGRP.

**Table 1** Comparative affinities of various AM and CGRP-related molecules in [$^{125}$I]-AM and [$^{125}$I]-CGRP binding assays in embryonic rat spinal cord cells

| | *[$^{125}$I]-AM binding* | | *[$^{125}$I]-CGRP binding* | |
|---|---|---|---|---|
| | *$pIC_{50}$* | *RP (%)* | *$pIC_{50}$* | *RP (%)* |
| rAM | 9.37±0.58 | 100 | 9.86±0.27 | 100 |
| $hAM_{22-52}$ | 7.15±0.78 | 0.60 | 7.60±0.51 | 0.56 |
| rCGRPα | 6.65±0.38 | 0.21 | 10.54±0.15 | 483 |
| $hCGRP_{8-37}$ | 6.48±0.61 | 0.14 | 9.71±0.27 | 72 |
| BIBN4096BS | <5 | <0.004 | 9.05±0.35 | 16 |

Data are mean±s.e.m. from at least three independent experiments, each performed in triplicate. Relative potency (RP) is as compared with rAM for both radioligands. For all curves where it was possible to estimate a $pIC_{50}$, an *F*-test indicated that the data were best fitted to a single site.

rCGRPα was the most potent competitor followed by rAM, $CGRP_{8-37}$, BIBN4096BS and finally by $hAM_{22-52}$ (Figure 2b; Table 1).

### *Functional characterization of AM and CGRP receptors*

Basal levels of cAMP in spinal cord cells were 18±2.1 pmol mg$^{-1}$ protein. The adenylyl cyclase activator, forskolin, at $10^{-6}$ M, induced a marked increase in the production of cAMP (18-fold over basal level). AM increased levels of cAMP in a concentration-dependent manner with a $pEC_{50}$ value of 9.1±0.2. The maximal response obtained with AM was observed at 100 nM and was 22-fold over baseline. CGRP also increased cAMP levels with a $pEC_{50}$ of 8.3±0.2. The maximal response was obtained at 1 μM and was sevenfold over the basal level.

We examined next the effects of $hAM_{22-52}$, $CGRP_{8-37}$ and BIBN4096BS on cAMP production induced by various concentrations of AM and CGRP. The concentration–response curve to AM was significantly shifted to the right in the presence of $hAM_{22-52}$ or $CGRP_{8-37}$ with apparent $pA_2$ values of 7.26±0.18 and 7.41±0.15 for $hAM_{22-52}$ and $CGRP_{8-37}$, respectively (Figure 3a; Table 2). The $pA_2$ for $CGRP_{8-37}$ was estimated from the shift produced in the presence of 0.1 μM $CGRP_{8-37}$; at 1 μM it apparently produced a larger $pA_2$ estimate of 7.91. However, there was evidence of depression of the maximum response at this concentration and so the result with the lower concentration may more accurately reflect the true value. BIBN4096BS at 1 μM failed to produce any significant effect on AM-stimulated cAMP response (Table 2). On the other hand, all three antagonists caused significant inhibition of the concentration–response curve to CGRP (Figure 3b; Table 2). Apparent $pA_2$ values of 6.18±0.21, 7.63±0.44 and 8.40±0.30 were calculated for $hAM_{22-52}$, $CGRP_{8-37}$ and BIBN4096BS, respectively (Figure 3b; Table 2). The antagonistic effect of $hAM_{22-52}$ was more potent against AM-induced cAMP production while BIBN4096BS was markedly more effective against CGRP-stimulated cAMP production.

### *Expression of CLR-like immunoreactivity in rat spinal cord cells*

Under cell culture conditions used in the present study, CLR-like immunoreactive (IR) material was detected in embryonic

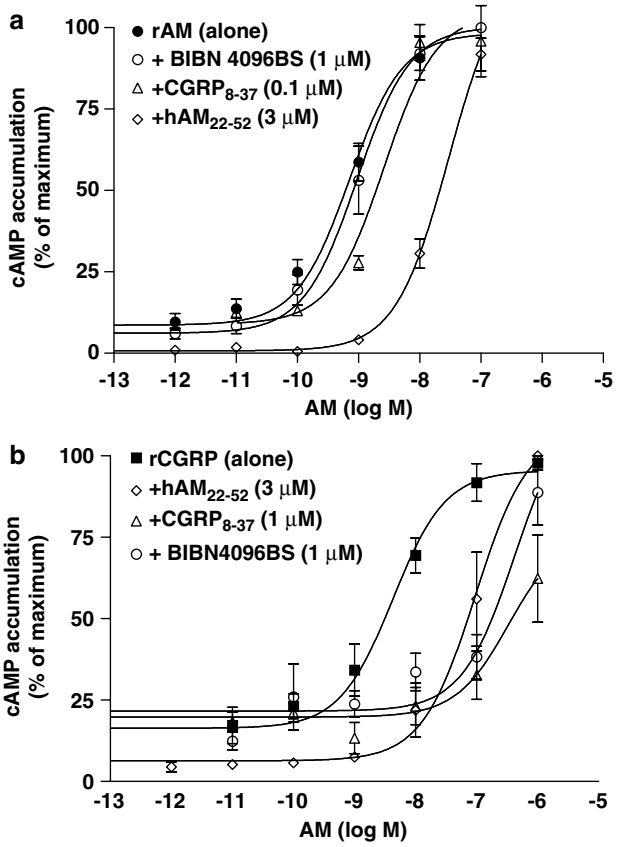


**Figure 3** Concentration–response curves for cAMP production induced by AM or CGRP in the absence or presence of $hAM_{22-52}$, $CGRP_{8-37}$ or BIBN4096BS. Cells were incubated with various concentrations of AM (a) or CGRP (b) in the absence or presence of 3 μM $hAM_{22-52}$, 0.1 or 1 μM $CGRP_{8-37}$ or 1 μM BIBN4096BS for 10 min. Data are mean±s.e.m. from at least three independent experiments, each performed in triplicate, and expressed as percentage of maximum cAMP production, estimated by fitting each line to a Hill equation as described in Methods. Maximum cAMP values induced by AM and CGRP were 369±33 and 140±19 pmol mg$^{-1}$ protein, respectively.

**Table 2** Antagonistic effects ($pA_2$) of various blockers on cAMP accumulation induced by CGRP and AM in embryonic rat spinal cord cells

| *Antagonist* | *CGRP* | *AM* |
|---|---|---|
| BIBN4096BS | 8.40±0.30 | <6 |
| $CGRP_{8-37}$ | 7.63±0.44 | 7.41±0.15 |
| $hAM_{22-52}$ | 6.18±0.21 | 7.26±0.18 |

Data are mean±s.e.m. from at least three independent experiments, each performed in triplicate.

rat spinal cord cells (Figure 4a). The immunostaining was clearly absent after preadsorption of the primary antibody with the synthetic peptide used for immunization (2.5 μg ml$^{-1}$ diluted antiserum) (Figure 4b). The specific antibody used here labeled various cell types of both neuronal and non-neuronal origin. To further investigate the nature of the cells present in the cultures, immunostaining was carried out for neuronal-specific enolase (a neuronal marker) and GFAP (an astrocyte

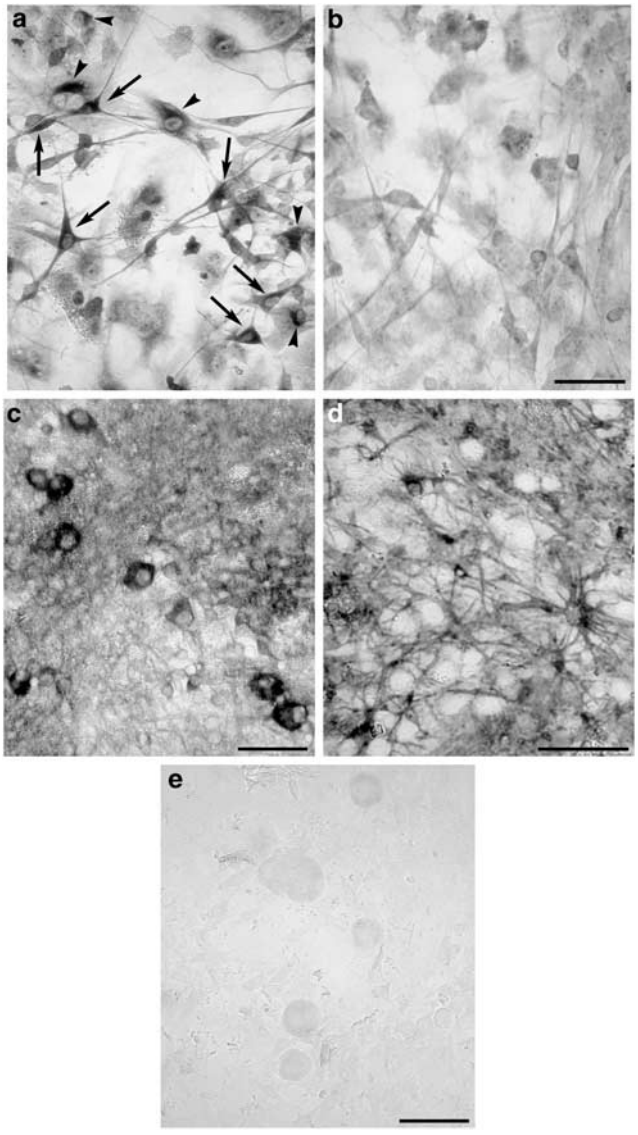


**Figure 4** Cellular expression of CLR in cultured embryonic rat spinal cord cells. (a) Neuron-like (arrows) and glial-like cells (arrowheads) display CLR immunostaining. (b) Preadsorption control of CLR antiserum. CLR-like immunoreactivity is absent when cells are treated with preadsorbed CLR antibody. (c) Neuronal cells stained for NSE. (d) Glial cells stained for GFAP. (e) Representative microphotograph showing the absence of immunostaining when the NSE or GFAP antibody is omitted. Scale bars: 50 $\mu$m.

marker) (Figure 4c and d). The presence of both neuronal and glial cells was observed and this suggested that the CLR immunoreactivity was expressed in both populations.

### *AM and CGRP receptors in primary culture cells*

To characterize cell types expressing the receptor of interest, we examined the effects of AM on the expression of phospho-CREB. DAB based immunostaining of phospho-CREB immunoprecipitates in cells treated with 1 $\mu$M AM (a concentration known to stimulate both CGRP and AM receptors) revealed that the number phospho-CREB IR cells was significantly increased ($180 \pm 20\%$; $P<0.01$, Student's *t*-test) relative to control cells. Double-immunofluorescence staining with NeuN as neuronal marker was then conducted to characterize AM responsive cells. As shown in Figure 5, neuronal cells are clearly expressed in our culture. The phospho-CREB immunoreactivity in both neuronal and non-neuronal cells was increased by 1 $\mu$M AM, suggesting the expression of CGRP and AM receptors in various cell types.

## Discussion

The main findings of the present study include the demonstration that embryonic spinal cord cells are enriched with specific AM and CGRP receptor binding sites, AM binding sites being particularly abundant. These two classes of receptors are functional as revealed by cAMP accumulation following their respective stimulation with relevant agonists. They are likely to be $AM_1$ (CLR/RAMP2) and $CGRP_1$ (CLR/RAMP1) subtypes as suggested by their ligand selectivity profile and the potencies of peptide and nonpeptide antagonists to inhibit agonist-induced cAMP production (although we cannot rule out some contribution from $AM_2$ receptors). Finally, CLR-like immunostaining is present in neuronal and non-neuronal cells in our model. Taken together, these results suggest functional role(s) for both AM and CGRP, acting *via* their respective receptor subtype, in the organization and physiology of the embryonic spinal cord.

### *Specific AM and CGRP binding sites in cultured rat embryonic spinal cord cells*

We confirmed first the presence of abundant specific [$^{125}$I]-AM binding sites in rat embryonic spinal cells. The apparent maximal binding capacity of specific [$^{125}$I]-AM sites is $\sim$17 times greater than that of specific [$^{125}$I]-CGRP binding. The ligand selectivity profile of related molecules in competing against specific [$^{125}$I]-AM binding is rAM > $hAM_{22-52}$ > rCGRP$\alpha \geqslant CGRP_{8-37} \gg$BIBN4096BS. This is in accordance with the pharmacological profile of the $AM_1$ (CLR/RAMP2) receptor observed in COS-7 cells (Poyner *et al*., 2002; Hay *et al*., 2003; Husmann *et al*., 2003). There was some evidence that BIBN4096BS was unable to displace all the [$^{125}$I]-AM. Caution is needed here as it was not possible to obtain a full inhibition curve. However, the data raises the possibility that the AM binding sites might be heterogeneous, perhaps composed of both $AM_1$ and $AM_2$ receptors. As reported earlier (Entzeroth *et al*., 1995; Owji *et al*., 1995; Zimmermann *et al*., 1995), rCGRP$\alpha$ was more potent than rAM in competing for specific [$^{125}$I]CGRP binding in our model. Interestingly, the affinity of rAM in the current preparation was found to be eight times lower than that of CGRP, in comparison to about 200 times less in rat spinal cord membrane preparations (Owji *et al*., 1996). The reason for this apparent discrepancy is not clear but may relate to assay conditions. However, these studies clearly demonstrate the existence of $CGRP_1$ receptors (CLR/RAMP1) in the rat spinal cord, in addition to the $AM_1$ subtype as discussed above.

### *Effects of AM and CGRP on cAMP accumulation in rat embryonic spinal cord cells*

AM and CGRP are well known to stimulate cAMP production as a second messenger in many tissues (Poyner *et al*., 2002).

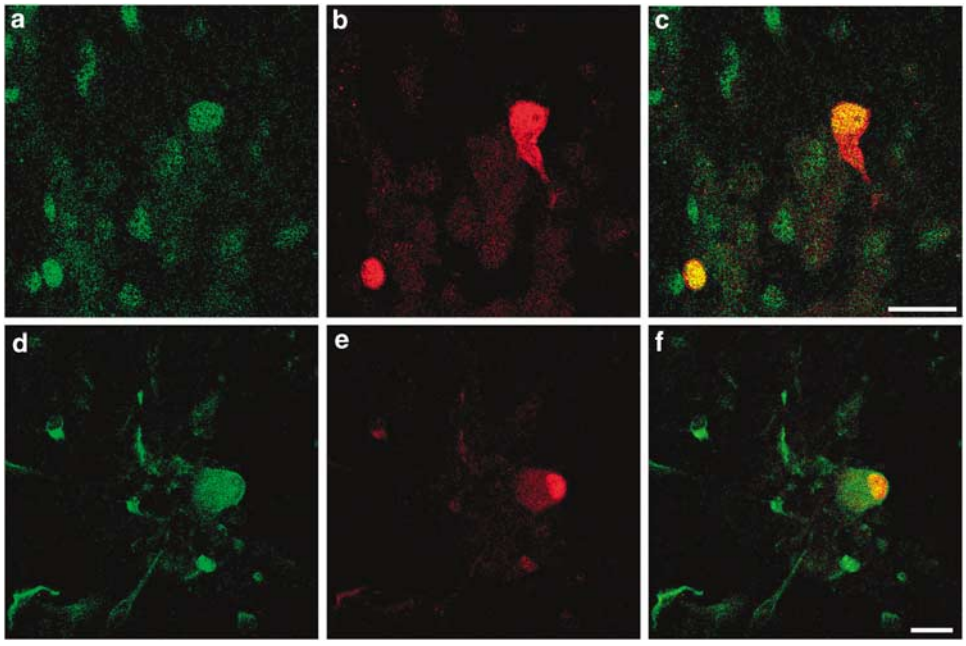


**Figure 5** Confocal color photomicrographs of double-immunofluorescence staining of cultured rat embryonic spinal cord cells expressing phospho-CREB (green) and NeuN (red) in AM-treated (0.1 $\mu$M for 20 min) cells (a–c) and in control medium-treated cells (d–f). The cells were starved in serum-free medium for 120 min before stimulation with AM. Colocalization of Neu-N and phospho-CREB appears as yellow (c, d) demonstrating cells with a neuronal phenotype subjected to the stimulatory effects of AM on phospho-CREB expression. Scale bar: 20 $\mu$m.

Our data showed that both AM and CGRP-induced cAMP accumulation in cultured spinal cord cells with rAM being at least five times more potent than rCGRP$\alpha$. Earlier on, Parsons & Seybold (1997) reported that CGRP increased cAMP accumulation in neonatal neuronal rat spinal cord cells. In their model, cAMP response to CGRP was biphasic while ours is linear, at least in the range of concentrations tested here. Interestingly, earlier reports failed to demonstrate significant changes in cAMP production when using microsomes or homogenates prepared from adult rat spinal cords exposed to AM (Owji *et al*., 1996) or CGRP (Goltzman & Mitchell, 1985; Owji *et al*., 1996). We have clearly shown here that both AM and CGRP can stimulate cAMP production in dispersed embryonic whole-rat spinal cord cells. Accordingly, the integrity of spinal cord cells may be a prerequisite for the activation of adenylyl cyclase by AM and CGRP receptors in this tissue with effects being particularly evident in embryonic tissue.

### *Comparative effects of $CGRP_{8-37}$, BIBN4096BS and $hAM_{22-52}$ on agonist-induced cAMP production*

The expression of mRNAs for all three RAMPs in the rat spinal cord has been documented (Chakravarty *et al*., 2000; Oliver *et al*., 2001) and we reported here on the presence of CLR-like IR in embryonic rat spinal cord cells. Therefore, it was important to establish which receptor subtype(s) mediated the effects of CGRP and AM on cAMP production in our model. This was addressed using various antagonists including $CGRP_{8-37}$, BIBN4096BS and $hAM_{22-52}$. $hAM_{22-52}$ inhibited AM-stimulated cAMP accumulation with a p$A_2$ of 7.26. This value is similar to that obtained in $AM_1$ (CLR/RAMP2) receptor expressing COS-7 cells (p$A_2 = 7.34$) as well as in Rat 2 (p$A_2 = 7.28$) and L6 (p$A_2 = 7.0$) cell lines which endogenously express the CLR/RAMP2 complex (Hay *et al*., 2003; 2004a). Consistent with the low affinity of BIBN4096BS for specific [$^{125}$I]-AM binding in embryonic rat spinal cord cells, BIBN4096BS failed to significantly antagonize the effects of AM on cAMP production, indicating that indeed, AM activated specific $AM_1$ (CLR/RAMP2) receptors to produce its effects. The AM-induced cAMP production was also inhibited by $CGRP_{8-37}$. There was evidence for noncompetitive behavior of $CGRP_{8-37}$ at high concentrations against AM. The p$A_2$ estimate at 0.1 $\mu$M $CGRP_{8-37}$ is both closer to the pKi estimated from our radioligand binding data and to p$A_2$ values in the literature (e.g. $6.72 \pm 0.08$, Hay *et al*., 2003). The finding that $CGRP_{8-37}$ was equal or more potent than $hAM_{22-52}$ to block the effects of AM (Table 2) is in agreement with a study in the human oligodendroglial KG1C cell line (Uezono *et al*., 2001) These cells, like embryonic rat spinal cord cells, express all three RAMPs and functional responses induced by AM are inhibited by both $CGRP_{8-37}$ and $AM_{22-52}$, while those induced by CGRP are antagonized only by $CGRP_{8-37}$ (Uezono *et al*., 2001). However, most studies find it is less potent than $hAM_{22-52}$ when acting on CLR/RAMP2 or 3 complexes (see Poyner *et al*., 2002, for a summary). Indeed, this is seen in our binding data.

BIBN4096BS and $CGRP_{8-37}$ antagonized the effects of CGRP with p$A_2$ values of $8.40 \pm 0.30$ and $7.63 \pm 0.44$, respectively. $hAM_{22-52}$, an AM receptor antagonist usually inactive on the $CGRP_1$ receptor subtype (Hay *et al*., 2003), also inhibited the effect of CGRP in our model, albeit with lower potency (p$A_2 = 6.18$). This finding may suggest that a certain proportion of the effects of CGRP on cAMP production are mediated by an AM receptor, although it may also reflect a weak action of $AM_{22-52}$ on $CGRP_1$ receptors. The high potency exhibited by $CGRP_{8-37}$ against CGRP-mediated cAMP accumulation (p$A_2 = 7.63$) corresponds very well to values reported for this antagonist at the $CGRP_1$ receptor subtype (Dennis *et al*., 1989; 1990; Juaneda *et al*., 2000;

Kuwasako *et al*., 2004). BIBN4096BS was more potent ($pA_2 = 8.40$) than $CGRP_{8-37}$ in accordance with recent studies on the $CGRP_1$ subtype (Doods *et al*., 2000; Juaneda *et al*., 2000; Wu *et al*., 2000). Taken together, these results suggest that CGRP increases cAMP content in embryonic spinal cord cells mostly by acting *via* the $CGRP_1$ receptor subtype and to some extent an $AM/AM_{22-52}$ sensitive receptor. However, as with the AM receptors, the actions of $CGRP_{8-37}$ were not fully straightforward as its affinity in radioligand binding studies was much higher than would be predicted from the $pA_2$. This observation has been seen before (Dennis *et al*., 1990; Poyner *et al*., 1992). The reason is not clear; it may relate to the different experimental conditions used in functional and radioligand binding experiments (Poyner *et al*., 1992). This study illustrates the problems of working with $CGRP_{8-37}$ on native cells and demonstrates the need for the use of a variety of techniques and antagonists to provide a reliable pharmacological profile of a receptor (Hay *et al*., 2004b).

### *Possible functional consequences of increased cAMP production following AM1 and $CGRP_1$ receptor activation in embryonic rat spinal cord cells*

Embryonic rat spinal cord cultures include a variety of neuronal and non-neuronal cells, most likely of sensory (mostly dorsal horn) and motor (ventral horn) nature. CGRP and CGRP receptors are enriched in both sensory and motor neurons (Gibson *et al*., 1984; Jacques *et al*., 2000) as well as in some non-neuronal spinal cells (Ma & Quirion, 2006). Moreover, CGRP receptors have been shown to be located pre- or postsynaptically in the spinal cord (Jacques *et al*., 2000). Not as much information is available concerning the presence of AM and AM receptors in these preparations. Similarly, only limited information is currently available on the possible role of AM in embryonic and mature spinal cord tissues. In other preparations, AM has been shown to stimulate DNA synthesis and cell proliferation *via* the cAMP/PKA pathway models (Belloni *et al*., 2001; Semplicini *et al*., 2001; Miyashita *et al*., 2003). It would thus be of interest to investigate if AM, acting on $AM_1$ receptors, could have similar effects in the embryonic spinal cord. In the case of CGRP, various studies have shown that this peptide can modulate the activity of spinal cord motoneurons (Uchida *et al*., 1990), the formation of functional synapses and the turnover of cholinergic receptors, in a cAMP-dependant manner, at the level of the neuromuscular junction (Fontaine *et al*., 1986; New & Mudge, 1986), and to regulate glycolysis in skeletal muscles (Leighton & Cooper, 1988) (for a review see Jacques *et al*., 2000). Hence, it would appear that CGRP and CGRP receptors are involved in various aspects of the organization and function of the motoneurons.

At the sensory level, various studies have clearly demonstrated the role of CGRP as a pain-related peptide in the developing and adult spinal cord of numerous species (Menard *et al*., 1995a, b; 1996; Cridland & Henry, 1988; Xu *et al*., 1990; Salmon *et al*., 1999, 2001; Ma *et al*., 2000; Powell *et al*., 2000; Mogil *et al*., 2005). CGRP and related peptides, including AM (Owji *et al*., 2004), induced nociception when injected intrathecally in the spinal cord (Cridland & Henry, 1988; Xu *et al*., 1990; Mogil *et al*., 2005). These effects are related to the activation of receptors located both pre and postsynaptically to primary afferent nerve terminals thus being relevant in the context of the present model (receptors expressed by cells postsynaptic to primary afferents). Interestingly, CGRP receptor antagonists ($CGRP_{8-37}$ and BIBN4096BS) have been shown to inhibit the development of tolerance to morphine-induced analgesia and to even reverse established tolerance (Menard *et al*., 1996; Powell *et al*., 2000; 2003). It mostly remains to be established if AM and $AM_1$ receptors can have similar roles in pain perception and in tolerance to the antinociceptive action of opiates. We have very recently reported preliminary data on pain-inducing effects of AM acting *via* an AM receptor subtype in the rat (Owji *et al*., 2004). More extensive investigations are currently underway in our laboratories.

In summary, embryonic rat spinal cord cells are enriched with both specific $CGRP_1$ (CLR/RAMP1) and $AM_1$ (CLR/RAMP2) receptor subtypes associated with increased production of cAMP upon their stimulation. This relatively simple model may prove useful to dissect comparative intracellular process and effects modulated by the respective activation of $CGRP_1$ and $AM_1$ receptors in the spinal cord.

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