A Rat skeletal muscle cell line (L6) expresses specific adrenomedullin binding sites but activates adenylate cyclase via calcitonin gene-related peptide receptors

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We have previously demonstrated specific binding sites for adrenomedullin, a novel member of the calcitonin family of peptides, in rat muscles. It is unclear whether these receptors are vascular or muscular. Receptors for the structurally similar calcitonin gene-related peptide (CGRP) are present on myocytes and might be involved in the regulation of myocyte glucose metabolism and control by motor neurons. We investigated whether adrenomedullin binding sites were present on L6 myocytes. Specific [^{125}I]adrenomedullin binding sites were demonstrated where adrenomedullin competed with an IC_{50} of 0.22 ± 0.04 nM (mean $\pm$ S.E.M.) and a concentration of binding sites (B_{max}) of 0.95 ± 0.19 pmol/mg of protein (mean $\pm$ S.E.M.). CGRP and the specific CGRP receptor antagonist CGRP(8–37) competed weakly at this site ($IC_{50} > 10$ and 601 ± 298 nM respectively). Binding studies with [^{125}I]CGRP revealed a binding site for CGRP ($IC_{50} = 0.13 \pm 0.01$ nM; $B_{max} = 0.83 \pm 0.10$ pmol/mg of protein) where both CGRP(8–37) and adrenomedullin competed with [^{125}I]CGRP with IC_{50} values of 1.15 ± 0.12 and 8.68 ± 0.98 nM respectively. Chemical cross-linking showed the CGRP and adrenomedullin binding site–ligand complexes to have approximate molecular masses of 82 and 76 kDa respectively. Both CGRP and adrenomedullin increased adenylate cyclase activity with similar potencies. In both cases adenylate cyclase activation was blocked by CGRP(8–37). Stimulation with 10 nM adrenomedullin or CGRP caused an increase in the percentage of total activated cellular cAMP-dependent protein kinase from 38 % in resting cells to 100 % and 98 % respectively. Therefore in L6 cells adrenomedullin can bind to CGRP receptors, activating adenylate cyclase and cAMP-dependent protein kinase.

INTRODUCTION

Adrenomedullin was isolated as a 52-amino-acid peptide from human phaeochromocytomas in 1993 by monitoring its ability to increase platelet cAMP [1]. The rat adrenomedullin cDNA encodes a 50-amino-acid peptide with two deletions and six substitutions compared with the human peptide [2]. Both peptides have an N-terminal ring (formed by an intramolecular disulphide bridge) and a C-terminal amidated residue, relating them structurally to the calcitonin family of peptides, calcitonin, calcitonin gene-related peptide (CGRP) and islet amyloid polypeptide (IAPP) [3]. CGRP and IAPP have multiple well-established effects on muscle glucose metabolism but the receptors involved are yet to be defined [4].

Adrenomedullin is found in low levels in the circulation but the peptide and its mRNA are abundant in adrenal medulla, heart, lung and kidney in both rat and human [1,2,5]. Powerful vasodilator effects of adrenomedullin have been demonstrated in the pulmonary [6], renal [7,8], mesenteric [9] and hindquarters [10] vascular beds. Both vascular endothelial and vascular smooth muscle (VSMC) cells release adrenomedullin [11,12], with endothelial cells showing mRNA levels 20 times greater than any tissue, possibly indicating a paracrine mechanism for the vasodilation.

The exact mechanism of adrenomedullin-induced vasodilation is controversial. However, increases in cAMP have been demonstrated in rat VSMC [13] and bovine aortic endothelial cells [14]. Adrenomedullin binds to vascular CGRP receptors with high affinity [3,15], which can mediate vasodilation via increased cAMP (reviewed in [16]). Indeed, early reports suggested that adrenomedullin-mediated vasodilation and increased cAMP could be blocked by the specific CGRP receptor antagonist fragment, CGRP(8–37) [9,15]. Recently, specific adrenomedullin binding sites that do not bind CGRP or CGRP(8–37) have been demonstrated in rat tissues [17], endothelial cells [14] and rat VSMC [13]. We have demonstrated considerable specific adrenomedullin binding in muscles, i.e. heart, soleus, gastrocnemius and diaphragm. These binding sites might be vascular or present on the myocytes themselves. CGRP receptors have already been demonstrated on muscle cells [18] and muscle cell lines (L6 myocytes) [19,20]. These receptors might be involved in communication between motor neurons and skeletal muscle [21] and in the control of muscle glucose metabolism. CGRP inhibits insulin-stimulated soleus muscle glycogen synthesis [22] and insulin-stimulated glucose uptake in L6 cells [20]. It is not yet known whether adrenomedullin can interact at myocyte CGRP receptors.

To determine whether specific adrenomedullin binding sites are present on myocytes we examined the binding of [^{125}I]adrenomedullin in the rat skeletal muscle cell line, L6G8C5 (L6 cells). After demonstration of specific adrenomedullin binding and confirmation of the expression of CGRP receptors, we examined

Abbreviations used: B_{max}, concentration of receptors; BSOCOES, bis[2-(succinimido-oxycarbonoxyloxy)ethyl] sulphone; CGRP, calcitonin gene-related peptide; IAPP, islet amyloid polypeptide; IBMX, 3-isobutyl-1-methyl xanthine; PKA, cAMP-dependent protein kinase; PKI, PKA inhibitor; VSMC, vascular smooth-muscle cells.

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whether the peptides mediated an increase in adenylate cyclase activity and whether this effect was mediated by CGRP receptors by using the CGRP receptor antagonist CGRP(8–37).

MATERIALS AND METHODS

Materials

L6G8C5 (L6) cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wilts., U.K.). All media for cell culture were supplied by Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Rat α-CGRP was from ASG University (Szedgel, Hungary); rat adrenomedullin from Peptide Institute (Osaka, Japan); CGRP(8–37) from Bachem (Saffron Walden, Essex, U.K.) and rat [tyr^0]α-CGRP from Peninsula (St. Helens, Merseyside, U.K.). Na^{125}I, ^{3}H-cAMP, [α-^{32}P]ATP and [γ-^{32}P]ATP were from Amersham International (Little Chalfont, Bucks, U.K.). bis[2-(Succinimido-oxycarbonoxyloxy)ethyl] sulphone (BSOCOES) and Iodogen reagent were supplied by Pierce (Rockford, IL, U.S.A.). Chemicals were from Sigma (Poole, Dorset, U.K.) or Merck (Poole, Dorset, U.K.).

Cell culture and membrane preparation

Cells were grown in high-glucose Dulbecco's modified Eagle's medium with 10 % (v/v) fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. Confluent cells were cultured for a further 7 days (to allow myotube formation) with medium changes every 3 or 4 days before membrane preparation. Cells were washed with ice-cold PBS and scraped into 50 mM Hepes, pH 7.6, containing 0.25 M sucrose, 15 μg/ml aprotinin, 0.5 μg/ml pepstatin, 0.25 μg/ml leupeptin, 0.25 μg/ml antipain, 0.1 mg/ml benzamidine and 0.1 mg/ml bacitracin. Cells were homogenized and centrifuged at 1000 ***g*** for 15 min at 4 °C. The supernatants were centrifuged at 100000 ***g*** for 1 h at 4 °C. The final pellets were resuspended in sucrose-free homogenization buffer and the protein concentration was measured [23].

Receptor binding assays and chemical cross-linking of receptor–ligand complexes

Rat adrenomedullin was iodinated by the Iodogen method as previously described [17,24]. Fractions showing binding were sampled, freeze-dried and stored at −80 °C. The specific radioactivity of the label was 10 Bq/fmol. Iodination of rat [tyr^0]α-CGRP was also as previously described [25]. The specific radioactivity of the CGRP label was 36 Bq/fmol. For the adrenomedullin binding assay, membranes (100 μg of protein) were incubated for 30 min at 4 °C in binding buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl, 4 mM KCl, 1 mM EDTA, 1 μM phosphoramidon, 0.25 mg/ml bacitracin and 0.3 % BSA) containing 500 Bq (100 pM) of ^{125}I-rat adrenomedullin as previously described [17]. In equilibrium competition experiments the concentration of unlabelled peptides was varied from 0 to 1.0 μM. Binding data were analysed by nonlinear regression with 'Receptor-Fit' (Lundon Software, Cleveland, Ohio, U.S.A.) to calculate the IC_{50} and concentration of binding sites (B_{max}). [^{125}I]CGRP binding (1000 Bq, 27.8 pM) was performed for 45 min at 22 °C as previously described [25]. Chemical cross-linking with BSOCOES followed by SDS/PAGE was performed as previously described [17,26,27]. Membranes were incubated with 12000 Bq (1.9 nM) of ^{125}I-rat adrenomedullin or 10000 Bq (278 pM) of [^{125}I]CGRP [28]. Autoradiographs were exposed to Kodak X-Omat film (IBI Ltd, Cambridge, Cambs., U.K.) at −80 °C for 1–10 days before development.

Adenylate cyclase assay

Adenylate cyclase activity in L6 membranes (10 μg of protein) was assayed with the method of Salomon [29] as previously described [30]. Briefly, 10 μg of protein was added to a final volume of 100 μl of assay buffer containing 100 μM GTP, 100 μM ATP, 1 mM cAMP, 20 μM creatine phosphate, 25 mM Tris HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM 3-isobutyl-1-methyl xanthine (IBMX), 200 μg/ml creatine kinase, 238 Bq of [^{3}H]cAMP and 56 000 Bq of [α-^{32}P] ATP. The reaction mixture was incubated for 10 min at 30 °C and stopped by the addition of 10 μl of 62.5 % trichloroacetic acid. cAMP was separated by sequential elution through Dowex 50W-X4 (200–400 mesh), H^+ form (Bio-Rad, Hemel Hempstead, Herts., U.K.) and alumina (Sigma) columns. Isoprenaline (10 μM) was used as a positive control for these membranes.

cAMP-dependent protein kinase (PKA) assay

Cells were exposed to control medium, CGRP (1–100 nM) or adrenomedullin (1–100 nM) in the presence of the phosphodiesterase inhibitor IBMX (50 μM). After treatment, cells were washed with PBS, scraped into 1.5 ml of extraction buffer (10 mM NaH_2PO_4, 10 mM EDTA, 0.5 mM IBMX, 100 mM NaCl, pH 6.8) and homogenized [31]. PKA activity was assayed by measuring [^{32}P]ATP incorporation into Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly). Extract (10 μl) was added to triplicate tubes containing 50 mM Tris, pH 7.5, alone or plus: 5 μM PKA inhibitor (PKI) (6–22) amide [32]; 10 μM cAMP; 5 μM PKI (6–22) amide and 10 μM cAMP. The reactions were started by adding 50 μM Kemptide, 100 μM ATP and 7400 Bq of [γ-^{32}P] ATP to tubes and incubating for 5 min at 30 °C. The reaction was stopped by adding 10 μl of 1 M HCl followed by spotting (20 μl) on to phosphocellulose discs (Gibco BRL). PKA activation was calculated as percentage maximum specific radioactivity. The radioactive counts in tubes containing PKI were subtracted from the corresponding tubes without PKI to give the total and activated PKA.

RESULTS AND DISCUSSION

Effects of adrenomedullin and CGRP on binding of [^{125}I]adrenomedullin and [^{125}I]CGRP in L6 cell membranes

We have previously demonstrated binding sites for adrenomedullin in several muscles including heart, gastrocnemius, soleus and diaphragm [17], but it was not clear whether these sites were on vascular or muscle cells. Therefore we examined [^{125}I]adrenomedullin binding in the rat skeletal muscle cell line L6, which has already been shown to exhibit CGRP binding sites [20]. Initial results demonstrated the presence of both [^{125}I]CGRP- and [^{125}I]adrenomedullin-specific binding sites with specific binding accounting for 91 ± 1 % and 76 ± 3 % ($n = 6$) of total binding respectively (values here and throughout are the means ± S.E.M.). Construction and analysis of competition curves for [^{125}I]CGRP and [^{125}I]adrenomedullin (Figure 1) against the corresponding unlabelled peptide gave similar IC_{50} values [0.13 ± 0.01 and 0.22 ± 0.04 nM respectively ($n = 3$)] and B_{max} values [0.83 ± 0.10 and 0.95 ± 0.19 pmol/mg of protein respectively ($n = 9$)]. Therefore, to confirm that the CGRP and adrenomedullin binding sites were distinct from each other, competition curves were constructed for each of the ^{125}I-labelled peptides by using CGRP, CGRP(8–37) and adrenomedullin. [^{125}I]Adrenomedullin binding ($n = 3$) was weakly inhibited by CGRP(8–37) (IC_{50} 601 ± 298 nM) and CGRP ($IC_{50} > 10$ μM). Adrenomedullin binding was determined at 4 °C, which is the optimal binding temperature [17]. However, the adenylate cyclase experiments

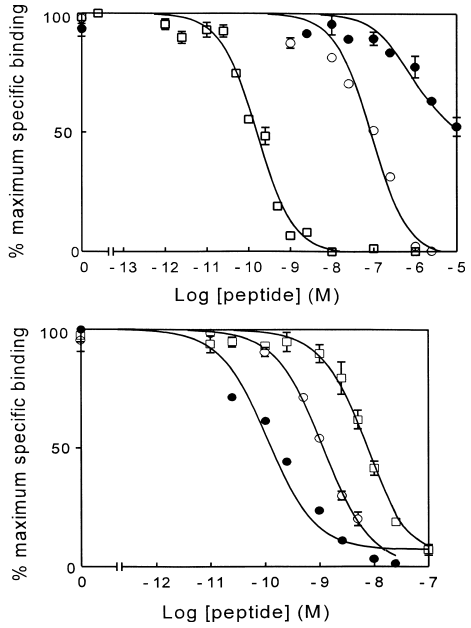

Figure 1 Competition curves for ^{125}I-labelled peptides in L6 membranes

[^{125}I]Adrenomedullin (upper panel) and [^{125}I]CGRP (lower panel) competition curves in L6 membranes. Values are expressed as a percentage of the maximal specific binding and are the means $\pm$ S.E.M. for three experiments with assays in triplicate. Symbols: ●, CGRP; ○, CGRP(8–37); □, adrenomedullin.

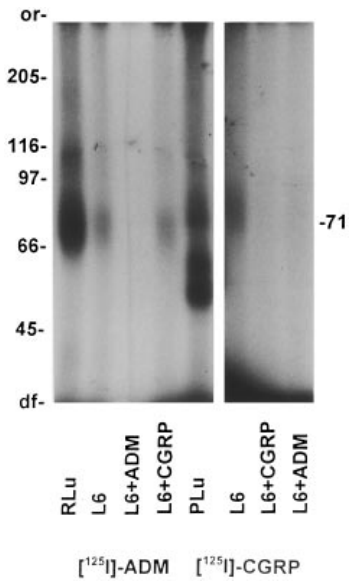

Figure 2 Chemical cross-linking of ^{125}I-labelled peptides to L6 membranes

Autoradiograph showing the effects of adrenomedullin and CGRP on chemical cross-linking of [^{125}I]adrenomedullin (lanes 1–4) and [^{125}I]CGRP (lanes 5–8) binding site complexes in L6 membranes. Cross-linking was performed with BSOCOES as the cross-linking reagent, as described in the Materials and methods section. Unlabelled peptides were added at 1 μM in the initial binding assay. Molecular masses (kDa) of the protein standards are shown at the left. Protein standards are: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa. Abbreviations: or, origin; df, dye front; ADM, rat adrenomedullin; CGRP, rat α-calcitonin gene-related peptide. All lanes are from the same representative experiment but lanes 1–5 were exposed for 3 days and lanes 6–8 for 12 days. Rat lung membranes (RLu) were used as a positive control for [^{125}I]adrenomedullin cross-linking and pig lung (PLu) for [^{125}I]CGRP.

were performed at 30 °C. To compare the affinities for adrenomedullin, CGRP and CGRP(8–37) and adenylate cyclase activity directly, we repeated the competition curves at 30 °C. There was no difference in affinity of any of the peptides at the higher temperature (results not shown). [^{125}I]CGRP binding ($n = 3$) was inhibited by CGRP(8–37) and adrenomedullin with high affinity (IC_{50} 1.15 ± 0.12 and 8.68 ± 0.98 nM respectively). These results indicate that in L6 cells the adrenomedullin and CGRP binding sites are pharmacologically distinct from each other. The calculated IC_{50} and B_{max} of the L6 adrenomedullin binding site and the very low affinity for CGRP are similar to those demonstrated by Owji et al. [17] for rat heart (IC_{50} 0.2 nM; B_{max} = 0.47 pmol/mg pf protein).

Chemical cross-linking of rat [^{125}I]adrenomedullin and CGRP to L6 membranes

Cross-linking of the binding-site–ligand complexes in L6 membranes showed that, in this cell line, specific CGRP and adrenomedullin binding sites have molecular masses of 77–96 and 73–89 kDa respectively ($n = 3$) (Figure 2). After subtracting the molecular mass of the peptides this gives molecular masses of 82 ± 5 and 76 ± 4 kDa for the CGRP and adrenomedullin binding sites respectively. Incubation with excess unlabelled adrenomedullin, but not CGRP, resulted in the loss of the band corresponding to [^{125}I]adrenomedullin binding. Incubation with excess CGRP or adrenomedullin removed the [^{125}I]CGRP band. This confirms that adrenomedullin binds to both adrenomedullin and CGRP binding sites. The calculated molecular mass for the adrenomedullin binding site in L6 cells is considerably different from that calculated for heart and soleus (94 ± 3 kDa) or lung and diaphragm (83 ± 1.4 kDa). These differences in molecular mass might be due to different glycosylations, as seen with the corticotrophin-releasing-hormone receptor [33], or it is possible that there is more than one subtype of adrenomedullin binding site. Alternatively the differences might be due to comparisons between different experiments.

Adenylate cyclase activation by adrenomedullin and CGRP

It has been previously shown that CGRP is capable of elevating intracellular levels of cAMP in L6 cells [19,20]; therefore we examined the ability of adrenomedullin and CGRP to activate adenylate cyclase in L6 membranes. CGRP gave a dose-dependent increase in membrane adenylate cyclase activity over the concentrations 0.1–1000 nM, reaching a plateau between 100 and 1000 nM (Table 1). Adrenomedullin produced a similar pattern with approximate EC_{50} values between 1 and 10 nM for both peptides. The CGRP receptor antagonist CGRP(8–37) had no effect by itself at concentrations up to 2 μM. Membranes were also treated with either 10 nM CGRP or 25 nM adrenomedullin and increasing concentrations of CGRP(8–37). CGRP(8–37) gave a dose-dependent inhibition of adenylate cyclase activation when stimulated with CGRP or adrenomedullin (Table 1). Concentrations of CGRP(8–37) exceeding 25 nM were sufficient to inhibit CGRP- or adrenomedullin-stimulated adenylate cyclase activity completely. At this low concentration of CGRP(8–37) there would be no expected interaction with adrenomedullin binding sites, strongly suggesting that adrenomedullin is acting via the CGRP binding site.

An earlier study claimed that adrenomedullin exerts its physiological effects in cultured rat VSMCs via specific adrenomedullin binding sites coupled with increased intracellular cAMP [34]. However, in that study CGRP(8–37) did inhibit the increase in cAMP mediated by adrenomedullin, albeit at high concentrations (100 nM or more). This problem was not discussed in the above

Table 1 Effect of adrenomedullin, CGRP and CGRP(8–37) on adenylate cyclase activity in L6 membranes

Activation of adenylate cyclase in L6 cell membranes was assayed in the presence of 2 mM IBMX as described in the Materials and methods section. In experiment 1, membranes were stimulated with 10 μM isoprenaline or increasing concentrations of CGRP, adrenomedullin or CGRP (8–37). In experiment 2, membranes were stimulated with submaximal concentrations of CGRP (10 nM) or adrenomedullin (25 nM) in the presence of increasing concentrations of the CGRP receptor antagonist CGRP (8–37). Activities are expressed as means ± S.E.M. ($n = 6$) and are percentages of the control activity.

Experiment 1		Experiment 2	
Conditions	cAMP production (%)	Conditions	cAMP production (%)
Control	100 ± 4	Control	100 ± 3
10 μM isoprenaline	307 ± 8	10 nM CGRP	292 ± 4
0.1 nM CGRP	154 ± 4	+ 2 nM CGRP(8–37)	232 ± 21
10 nM CGRP	334 ± 14	+ 10 nM CGRP (8–37)	211 ± 30
100 nM CGRP	460 ± 22	+ 25 nM CGRP(8–37)	163 ± 9
1 μM CGRP	412 ± 13	+ 100 nM CGRP(8–37)	113 ± 6
0.1 nM adrenomedullin	135 ± 8	+ 1 μM CGRP(8–37)	105 ± 8
10 nM adrenomedullin	238 ± 17	25 nM adrenomedullin	167 ± 5
100 nM adrenomedullin	367 ± 7	+ 2nM CGRP(8–37)	179 ± 4
1 μM adrenomedullin	367 ± 15	+ 10 nM CGRP (8–37)	146 ± 4
100 nM CGRP(8–37)	91 ± 11	+ 25 nM CGRP(8–37)	113 ± 10
1 μM CGRP(8–37)	101 ± 5	+ 100 nM CGRP(8–37)	93 ± 10
2 μM CGRP(8–37)	106 ± 13	+ 1 μM CGRP(8–37)	84 ± 8

paper and the lack of binding data for CGRP(8–37) in these cells makes the experiment difficult to interpret. Adrenomedullin is reported to increase cytoplasmic Ca^{2+} and inositol 1,4,5-trisphosphate concentrations by a cholera-toxin-sensitive G-protein in bovine aortic endothelial cells [14] and to decrease the cytoplasmic Ca^{2+} concentration via a G-protein-linked receptor in pig coronary arteries [35]. In the study on endothelial cells, specific adrenomedullin binding sites were demonstrated but the second-messenger effects were not differentiated from those mediated via CGRP receptors known to be present on endothelial cells. Hence it is not clear from these experiments what the true signal transduction mechanisms of the specific adrenomedullin binding site are. In L6 cells adrenomedullin is certainly able to activate adenylate cyclase, but this activation can be inhibited by CGRP(8–37) with a low IC_{50}, which in combination with the binding data indicates that this effect is mediated by CGRP receptors. Clearly what is required is an examination of signal transduction by adrenomedullin binding sites without the complicating influences of CGRP receptors. We have shown in Swiss 3T3 cells, which express adrenomedullin but not CGRP binding sites, that adrenomedullin potently increases intracellular cAMP [36]. The definitive answer might now have been provided by Kapas et al. [37], who reported the cloning and expression of a cDNA coding for a rat adrenomedullin receptor. Expression of the cDNA in COS-7 cells showed a pharmacology similar to our binding data in tissues [17], and stimulation of transfected cells with adrenomedullin increased cAMP production. The specific adrenomedullin binding site in L6 cells does not seem to be involved in the increase in cAMP and might be coupled to different signalling systems in L6 cells from those in COS-7 cells, be present as an uncoupled form or even be a different subtype of adrenomedullin binding site from that cloned by Kapas et al. [37].

PKA activation by CGRP and adrenomedullin

Elevation of intracellular cAMP is associated with increased phosphorylation of intracellular proteins by PKA [38]. Extracts of control cells that were assayed for PKA activity incorporated 6.8 ± 0.1 nmol of ^{32}P/min per mg of protein into the substrate, Kemptide, 15 % of which was accounted for by non-PKA kinase activity, i.e. it was not inhibited by the PKI fragment. When maximally activated by cAMP, incorporation rose to 26.2 ± 4.1 nmol of ^{32}P/min per mg of protein, 4 % by kinases other than PKA ($n = 3$). Stimulation of intact L6 cells with CGRP or adrenomedullin caused a dose-dependent increase in PKA activity, which was significant at 5 nM for both peptides (Figure 3). Approximate EC_{50} values for both peptides were between 1 and 5 nM, with 100 % activation achieved at 10 nM. These results are qualitatively similar to the adenylate cyclase results in terms of EC_{50} but with the maximum activation of PKA occurring at a lower concentration than that of adenylate cyclase. This could be explained by amplification, i.e. one molecule of agonist binds to one receptor, activating adenylate cyclase, producing many cAMP molecules and activating many PKA holoenzymes. Compared with the levels of PKA activation described by Murray et al. [31] for different tissue extracts, the control level of PKA activity in L6 cells is three times greater.

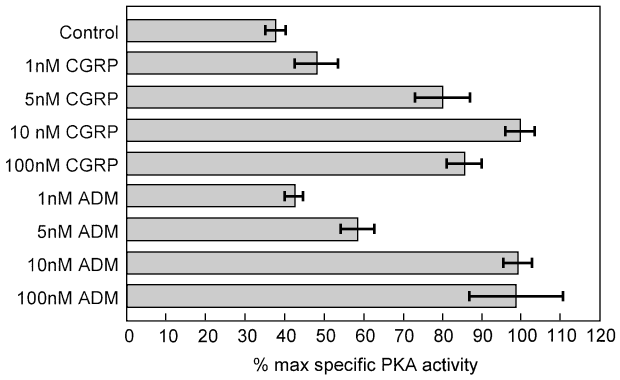

Figure 3 Activation of PKA activity in L6 cells by CGRP and adrenomedullin

Cells were stimulated with increasing concentrations of CGRP and adrenomedullin. PKA activity was assayed by measuring [^{32}P]ATP incorporation into Kemptide. Activities are expressed as percentage maximum specific activities, means ± S.E.M. ($n = 6$–9).

This might be due to an increased basal level of adenylate cyclase activity.

Activation of the cAMP cascade by adrenomedullin leading to PKA activation might have several effects on cellular function. These include altering the activity of enzymes involved in glycogen metabolism (e.g. phosphorylase kinase or glycogen synthase) [39] and insulin-stimulated glucose uptake in a similar way to CGRP and IAPP [4,20]. Adrenomedullin has been immunologically localized in the canine heart [40] and, as expected, to the vasculature, but also to the atrial and ventricular myocytes. This opens the interesting possibility of a paracrine/autocrine role for adrenomedullin in muscle cells. It has now been demonstrated that L6 cells also secrete immunoreactive adrenomedullin [0.12 pmol per 48 h per mg of protein (P. D. Upton and M. A. Ghatei, personal communication).] This possible role of endogenously produced adrenomedullin in muscle metabolism merits further investigation, even if the effects are via CGRP receptors.

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