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The CGRP receptor can couple via pertussis toxin sensitive and insensitive G proteins

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Abstract Swiss 3T3 cell lines were constructed co-expressing receptor activity modifying protein (RAMP) 1 with the calcitonin receptor-like receptor (CRLR), and showed ^{125}I-calcitonin-gene-related peptide (CGRP) 1 binding indicative of a type I CGRP receptor. Application of CGRP1 led to an increase in cAMP, which in 2/5 cell lines was augmented following pertussis toxin (PTX) pre-treatment. In *Xenopus* oocytes, expression of RAMP1, which potentiates an endogenous CGRP receptor, led to constitutive activation of co-expressed GIRK potassium channels. This potassium current was increased following CGRP application or co-expression of CRLR, but decreased by PTX or co-expression of transducin. We conclude that the CGRP receptor can signal to both PTX sensitive and insensitive G proteins.

Key words: Calcitonin-gene-related peptide receptor; Receptor activity modifying protein 1; G protein; Potassium channel

1. Introduction

The calcitonin-gene-related peptides (CGRP1 and CGRP2) are 37 amino-acid neuropeptides that differ by three amino acids and are members of a larger family that also contains adrenomedullin, amylin and calcitonin. In addition to its activity in the cardiovascular system, where it is a potent vasodilator, CGRP also mediates anti-inflammatory and neurotrophic effects [1]. In a variety of cell lines and tissues CGRP application leads to an increase in intracellular cAMP [2–4], effects which are consistent with coupling to the heterotrimeric G protein G_s. This is supported by the observation that antiserum against the amino-terminal region of G_s alpha immunoprecipitated a significant fraction of soluble CGRP receptors prepared from the rat cerebellum [5]. There is also evidence that the CGRP receptor can activate guanylate cyclase and phospholipase C, as well as calcium and potassium channels [6–10]. In some instances these responses occur via pertussis toxin (PTX)-sensitive mechanisms [9,10], suggesting the involvement of heterotrimeric G proteins in the G_i and G_o family [8].

We have recently established that in transient expression systems co-expression of the calcitonin receptor-like receptor (CRLR) [11,12] with a second protein, which we have named the receptor activity modifying protein (RAMP1), confers novel cAMP responses to CGRP and high affinity ^{125}I-CGRP1 binding [13]. We have now generated Swiss 3T3 cell lines that stably express CRLR and RAMP1 and in this study we report the characteristics of the CGRP receptor that results from this co-expression. In some cell lines cAMP responses to CGRP were potentiated by pre-treatment with PTX, suggesting that the recombinant CGRP receptor may signal through both G_i and G_s. This hypothesis was examined in *Xenopus laevis* oocytes that can be manipulated to provide a sensitive readout for receptor activation via PTX sensitive and insensitive G proteins.

2. Materials and methods

2.1. Construction of cell lines co-expressing CRLR and RAMP1

Swiss 3T3 cells were chosen as hosts because they are robust, adherent and showed no detectable cAMP responses to CGRP, calcitonin or adrenomedullin. Cells were maintained at 37°C and 95% humidity in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 2 mM glutamine. The human sequences for RAMP1 and CRLR (modified to provide a consensus Kozak sequence) [11] were cloned into the expression vectors pCIN4 and -5, respectively, which contain IRES elements with neomycin and hygromycin resistance genes downstream and in frame. Cells were transfected with 5 μg of linearised DNA (as a mixture of both constructs) per T75 cm^2 flask of 80% confluent cells using a lipofectamine procedure (Life Technologies). Dual neomycin and hygromycin selection was imposed and after a number of weeks individual colonies were isolated and subjected to dilution cloning prior to expansion. At a free radioligand concentration of 1 nM (which is approximately the K_d for ^{125}I-CGRP1 at the human receptor [2]) we were unable to distinguish between ^{125}I-CGRP1 binding to the different cell lines (data not shown) and chose to analyse the line that grew fastest in culture.

2.2. Radioligand binding

SK-N-MC cells (which endogenously express CGRP1 receptors [3]) and CRLR/RAMP1 double stable Swiss 3T3 cell lines were pelleted by centrifugation at $500\times g$ for 10 min at 4°C and then homogenised in buffer containing 50 mM HEPES-KOH, 1 mM EDTA, 100 μM leupeptin, 25 μg/ml bacitracin; pH 7.6. Immediately before the first homogenisation 1 mM PMSF and 2 μM pepstatin A were added. After centrifugation at $500\times g$ for 20 min the supernatant was removed and centrifuged at $48\,000\times g$ for 30 min. The final pellet was resuspended in homogenisation buffer (without PMSF or pepstatin A) and the protein content was measured.

Membranes (50 μg) were incubated for 90 min at 25°C in binding buffer (50 mM HEPES-KOH, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4) which contained 30 pM ^{125}I-CGRP1 (Amersham), with or without competing ligand (3 pM to 10 μM), in a total volume of 200 μl. Incubation was terminated by rapid filtration through GF/C filters soaked in 0.1% polyethylenimine using a Tomtek (Wallac) cell harvester. Competition binding data were analysed by non-linear regression using the computer program 'Excel Robosage' (Microsoft).

2.3. cAMP assay

Cells were washed with PBS and pre-incubated in DMEM containing 300 μM IBMX (Sigma) for 30 min at 37°C. Human CGRP1 (1–1000 nM) was added for a further 30 min at 37°C and the cells washed with ice cold PBS. cAMP levels were determined using Scintillation Proximity based Assays (SPA, Amersham).

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2.4. Oocytes

Adult female *Xenopus laevis* (Blades Biologicals) were anaesthetised using 0.2% tricaine (3-aminobenzoic acid ethyl ester), killed and the ovaries rapidly removed. Oocytes were then de-folliculated by collagenase digestion (Sigma type I, 1.5 mg/ml) in divalent cation-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 5 mM HEPES; pH 7.5 at 25°C). Single stage V and VI oocytes were transfered to ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate; pH 7.5 at 25°C) which contained 50 μg/ml gentamycin and stored at 18°C. Co-expression of the G-protein regulated potassium channels GIRK1 (Kir 3.1 [14]) and GIRK4 (or CIR, Kir 3.4 [15]) (injected in equal amounts to generate a heteromeric channel) was used to assay for activation of PTX sensitive G proteins [16]. A high potassium solution (90K) containing 90 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES (pH 7.5 at 25°C) was used in all GIRK experiments to increase potassium current amplitude.

RAMP1, CRLR, GIRK1, GIRK4 (all in pcDNA$_3$, Invitrogen) and CFTR (in pBluescript, Stratagene) were linearised and transcribed to RNA using T7 or T3 polymerase (Promega Wizard kit). m′G(5′)pp(5′)GTP capped cRNA was injected into oocytes (20–50 ng per oocyte) and whole-cell currents were recorded using two-microelectrode voltage-clamp (GeneClamp Amplifier, Axon Instruments) 3–7 days post-RNA injection. Microelectrodes had a resistance of 0.5–2 MΩ when filled with 3 M KCl.

2.5. Chemicals

Human CGRP peptides, calcitonin and amylin were obtained from Bachem; adrenomedullin (13–52) was from Peninsula Labs. and CGRP1 was synthesised at GlaxoWellcome. All other chemicals were of Analar grade and were obtained from Sigma.

3. Results

3.1. Characterisation of the CGRP receptor in Swiss 3T3 cells co-expressing RAMP1 and CRLR

Five Swiss 3T3 cell lines were isolated that grew under neomycin and hygromycin selection inferring expression of both CRLR and RAMP1. Each cell line expressed high affinity and specific ^{125}I-CGRP1 binding sites, whereas there was no specific binding of ^{125}I-CGRP1 to native Swiss 3T3 cells. Some Swiss 3T3 lines have been shown to express adrenomedullin receptors [17]; however, we were unable to detect specific ADM binding in the cell line we used (data not shown). Fig. 1A shows the ability of CGRP, CGRP analogues, adrenomedullin, salmon calcitonin, amylin and calcitonin to compete for ^{125}I-CGRP1 binding to SK-N-MC cells and to one of the Swiss 3T3 cell lines (number 5). The rank order of potency was the same in each case: CGRP1 > β-turn derived CGRP > $CGRP_{8-37}$ = Pro-8 $CGRP_{8-37}$ > Ala-16 $CGRP_{8-37}$ > adrenomedullin 13–52 ≫ amylin/calcitonin. Table 1 shows the pIC_{50} values that correspond to these curves. These data are consistent with a type 1 CGRP receptor pharmacology [2], as seen previously in transient expression systems [13].

In each Swiss 3T3 cell line application of CGRP1 (1–1000 nM) led to a small, dose-dependent increase in intracellular cAMP, which reached a maximum value of about 20–50 pmol cAMP per well at 1000 nM (Fig. 1B). Interestingly, cell lines 4 and 5 showed a significant increase in their intracellular cAMP responses to CGRP1 following pre-treatment with PTX (Fig. 2). This data could be explained if the CGRP receptor couples to both G_i and G_s, which have opposing actions on adenylate cyclase, and pre-treatment with PTX relieves a G_i-mediated, CGRP-dependent inhibition of adenylate cyclase.

3.2. Constitutive coupling of the CGRP receptor via PTX-sensitive G proteins to the GIRK1/4 heteromeric potassium channel in Xenopus oocytes

A number of G-protein coupled receptors have been shown to activate the inward rectifier potassium channels GIRK1 and GIRK4 when co-expressed in oocytes from *Xenopus laevis* (illustrated using the activity of adenosine on the adenosine A1 receptor, see Fig. 2A, middle panel). This activation is inhibited by pre-treatment with PTX and expression of G-protein regulated potassium channels in oocytes has been used extensively as a read-out for G_i-coupled receptors [16]. We therefore tested the hypothesis that the CGRP receptor can signal through PTX-sensitive G proteins by co-expressing RAMP1 (with or without CRLR) with GIRK1/4 in oocytes. To facilitate the recording of inward potassium currents, the extracellular solution was switched from ND96 (2 mM K^+) to 90K (90 mM K^+) solution prior to application of CGRP.

As shown in Fig. 2A (upper panel), in uninjected oocytes switching from ND96 to 90K solution led to a small inward shift in holding current (105.5 ± 29.7 nA at −60 mV, $n = 7$). This was completely reversed by application of 1 mM $BaCl_2$. A similar shift in holding current was recorded in oocytes expressing the adenosine A1 receptor plus GIRK1/4 (Fig. 2A, middle panel). However, in oocytes which expressed RAMP1 plus GIRK1/4 a 3-fold larger shift in holding current was recorded (305.6 ± 42.4 nA, $n = 7$) after switching to the high potassium solution (Fig. 2A, lower panel). This current was completely blocked by application of $BaCl_2$ (1 mM) at a concentration shown to selectively block inward rectifier potassium channels (Fig. 2B) [15]. Furthermore, the current-voltage curve for an oocyte expressing RAMP1 plus GIRK1/4 showed strong inward rectification, consistent with activation of the GIRK1/4 potassium channel (Fig. 2B). It therefore appears that expression of RAMP1 in oocytes leads to acti-

Table 1
Comparison of the CGRP receptor in SK-N-MC cells and CRLR/RAMP1 double stable cell lines using [^{125}I]αCGRP

Compound	SK-N-MC (pIC_{50} ± S.E.M.)	CRLR/RAMP1 (pIC_{50} ± S.E.M.)	n
CGRP	9.0 ± 0.1	9.0 ± 0.1	8
ADM	5.5	6.2	2
$CGRP_{8-37}$	7.9 ± 0.1	8.2 ± 0.3	3
Pro-8 $CGRP_{8-37}$	7.9 ± 0.2	8.2 ± 0.2	3
Ala-16 $CGRP_{8-37}$	6.9 ± 0.1	7.6 ± 0.03	3
BTD CGRP	8.2 ± 0.2	8.4 ± 0.02	3

Human calcitonin, salmon calcitonin and human amylin gave no significant displacement ($n = 3$ in each case).

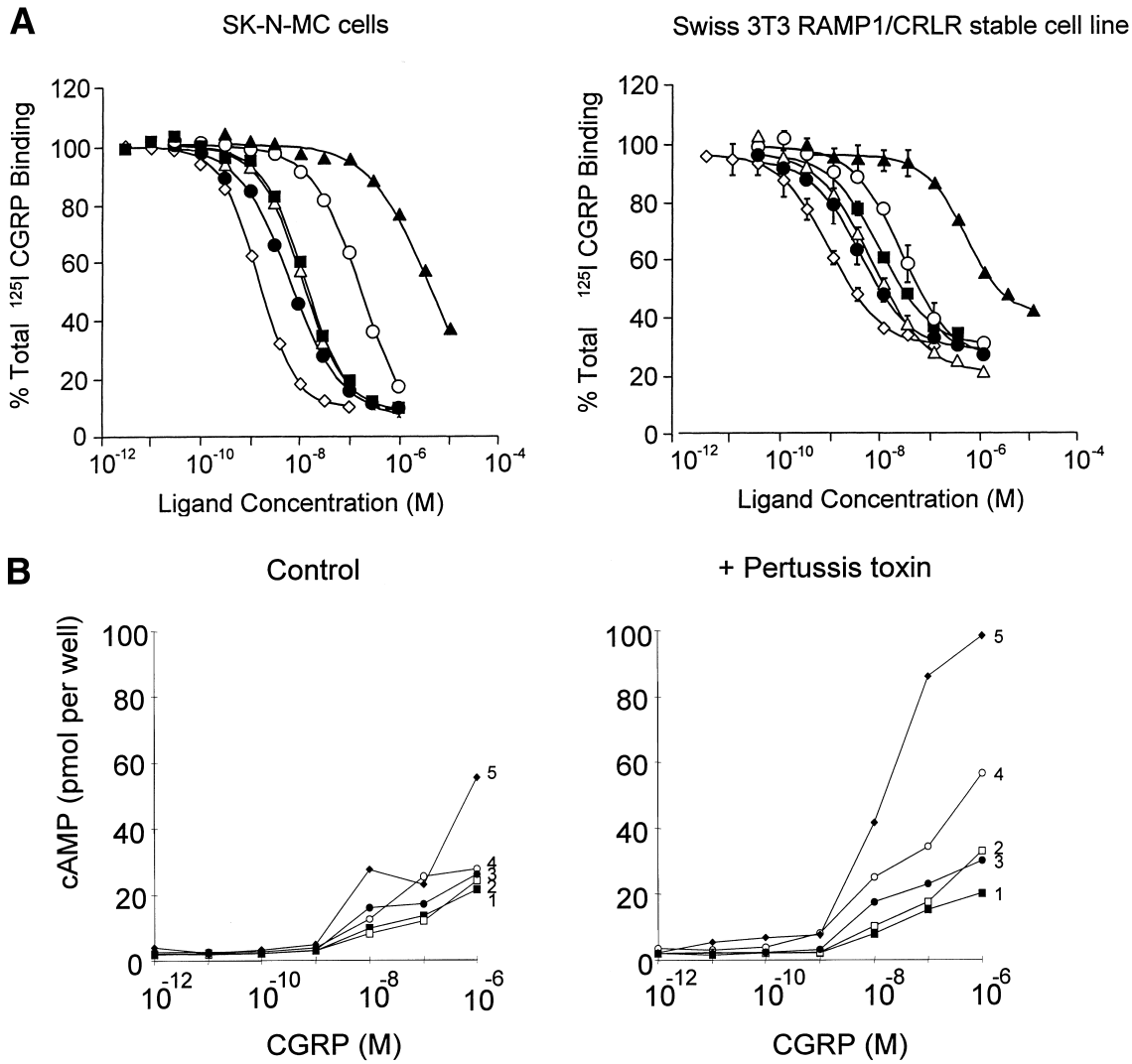

Fig. 1. Characterisation of Swiss 3T3 cell lines expressing RAMP1 plus CRLR. A: Binding curves were constructed for a single Swiss 3T3 cell line (number 5), and for native SK-N-MC cells using 30 pM ^{125}I-CGRP1 as the radioligand, and various concentrations of competing ligand (3 pM to 10 μM). Ligands used were CGRP1 (◇), β-turn derived CGRP (●), $CGRP_{8-37}$ (△), Pro-8 $CGRP_{8-37}$ (■), Ala-16 $CGRP_{8-37}$ (○) and adrenomedullin 13–52 (▲). No competition was seen in either cell type with amylin or calcitonin. B: CGRP-dependent changes in cAMP production were measured in the same Swiss 3T3 cell lines, in control conditions (left panel) and following pre-treatment with pertussis toxin (right panel). A separate curve is shown for each individual cell line. Note the increase in CGRP-dependent cAMP production following PTX pre-treatment in cell lines 4 and 5.

vation of the GIRK1/4 potassium channel, and to the generation of an inwardly rectifying potassium current.

Expression of RAMP1 in *Xenopus* oocytes potentiates the endogenous oocyte CGRP receptor, such that a 100-fold increase in CGRP responses are seen when the cystic fibrosis transmembrane regulator (CFTR) is used as a reporter [13] (CFTR is responsive to changes in intracellular cAMP and its activation is consistent with the CGRP receptor activating the oocyte equivalent of G_s [18]). The most obvious explanation for the RAMP1-induced stimulation of GIRK1/4 seen in this study is therefore that RAMP1 facilitates the oocyte CGRP receptor, and that this receptor activates the GIRK1/4 potassium channels in a ligand-independent manner. We have performed several experiments that address this hypothesis:

1. Application of 1 μM CGRP1 (in 90K solution) led to a further increase in potassium current amplitude (10.6% ± 3.3%, $n = 7$) supporting the view that RAMP1 expression activates GIRK1/4 through its actions on the endogenous oocyte CGRP receptor (Fig. 2A, lower panel).
2. Increasing the number of functional CGRP receptors by co-expression of CRLR with RAMP1 led to a significant increase in potassium current amplitude from 305.6 ± 42.4 nA ($n = 12$) to 501.5 ± 63.0 nA ($n = 23$, $P < 0.05$) (Fig. 3A).
3. Pre-treatment of oocytes with PTX led to a large decrease in potassium current amplitude (59.4% ± 12.7%, $P < 0.05$, $n = 9$, Fig. 3B, left panel) suggesting that activation of the potassium current is dependent upon signalling via G proteins of the $G_{i/o}$ class.
4. Co-expression of transducin, which is known to bind G-protein βγ subunits, led to marked (74.2 ± 5.5%, $n = 5$, $P < 0.05$) reduction in potassium current amplitude (Fig. 3B, right panel).

While we cannot exclude the possibility that RAMP1 acti-

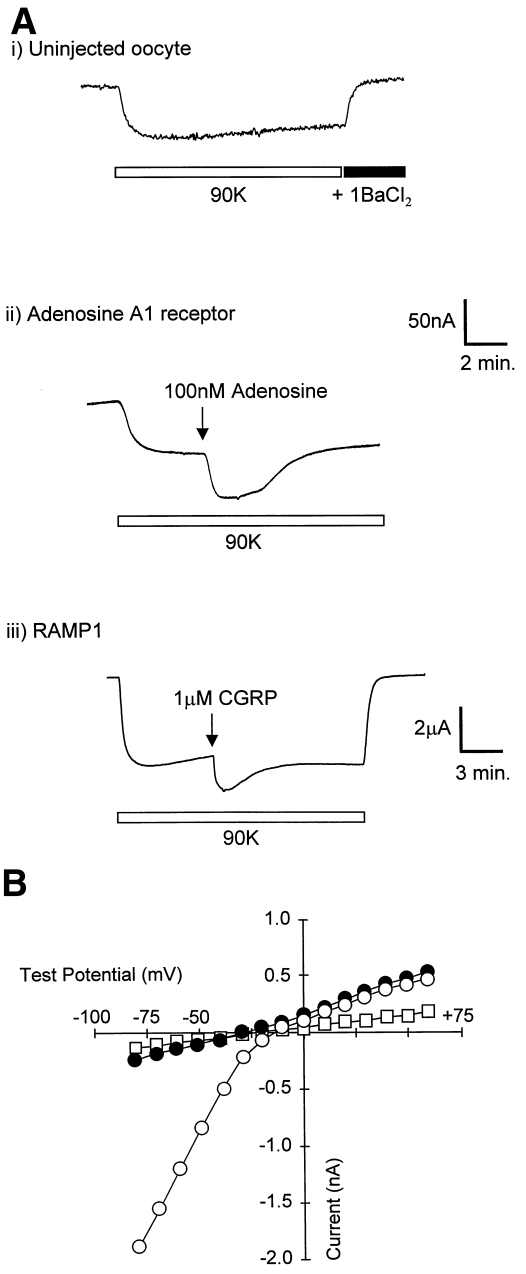

Fig. 2. Expression of RAMP1 plus GIRK1/4 in *Xenopus* oocytes. A: Current traces are shown for an uninjected oocyte (top panel), an oocyte expressing the adenosine A1 receptor plus GIRK1/4 (middle panel) and for an oocyte expressing RAMP1 plus GIRK1/4 (lower panel). In each case, switching to high potassium (90K) solution led to an inward shift in holding current. Note the far larger inward current recorded in an oocyte expressing RAMP1, and the further increase in potassium current amplitude recorded following application of 1 μM CGRP. B: Current-voltage curve (in 90K) from an oocyte expressing RAMP1 plus GIRK1/4. Curves are shown in ND96 (●), in 90K solution (○) and in 90K plus 1 mM $BaCl_2$ (□).

vates an endogenous oocyte receptor other than that for CGRP, and that this receptor shows constitutive activity, two lines of evidence suggest that this is not the case. First, RAMP1 has no significant activity on responses mediated by endogenous oocyte adenosine, vasointestinal polypeptide or β-adrenoreceptors suggesting that it acts specifically on CRLR and its oocyte equivalent [13]. Second, the increase in potassium current seen following CGRP application or co-expression of CRLR is consistent with an action of RAMP1 on the endogenous oocyte CGRP receptor. The most elegant demonstration that RAMP1 activates GIRK1/4 through constitutive activation of the oocyte CGRP receptor awaits the development of a CGRP receptor inverse agonist.

4. Discussion

When human embryonic kidney (HEK293T) cells are transiently transfected with CRLR and RAMP1 they gain high affinity and specific ^{125}I-CGRP1 binding as well as novel cAMP responses to CGRP1 [13]. We have extended these studies by constructing Swiss 3T3 cell lines that stably express RAMP1 and CRLR. All five cell lines tested (isolated and

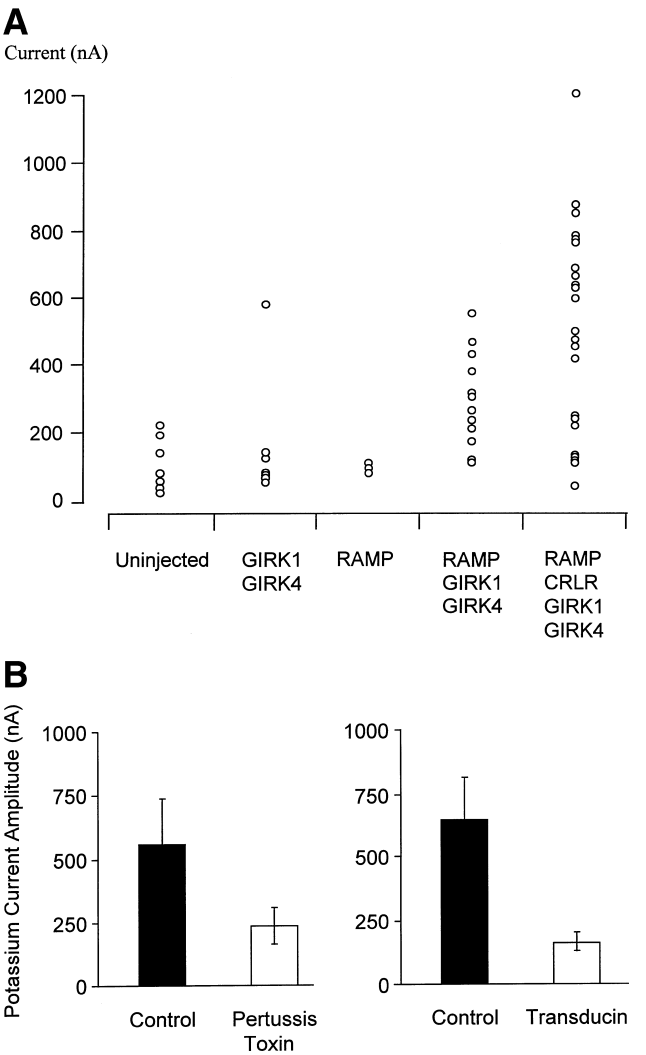

Fig. 3. The CGRP receptor couples to GIRK1/4 via a PTX-sensitive pathway. A: Dot plot. The shift in holding current recorded on switching from ND96 to 90K was measured for uninjected oocytes, and for oocytes expressing RAMP1 alone, GIRK1/4 alone, RAMP1 plus GIRK1/4 and RAMP1, CRLR and GIRK1/4. Each circle represents an individual oocyte. B: Effects of pertussis toxin pre-treatment (left panel) and transducin co-expression (right panel) on potassium current amplitude (measured as described above). Both treatments led to a reduction in potassium current amplitude suggesting that, in oocytes expressing RAMP1, GIRK1/4 is constitutively activated through G_i and the release of $G_{\beta\gamma}$ subunits.

expanded from individual clones) were found to have high affinity ^{125}I-CGRP1 binding and a detailed analysis of one clone showed that the pharmacology of the CGRP receptor expressed is very similar to the native SK-N-MC cell CGRP receptor.

We have demonstrated that the human and *Xenopus* CGRP receptors can signal via PTX sensitive and insensitive G proteins following expression in Swiss 3T3 cells or *Xenopus* oocytes. It is interesting to note that the CGRP receptor in SK-N-MC cells (which contain RAMP1 [13] and CRLR) may also signal via opposing pathways, as CGRP-stimulated cAMP production reaches a plateau at about 100 nM, with higher concentrations of CGRP being less effective (data not shown). This effect may be due to activation of G_i at high CGRP concentrations. There is also good evidence that the CGRP receptor can signal through G_i in native tissues, with coupling to both calcium and potassium channels reported [9,10]. Recently, Drissi and co-workers reported that in an osteoblastic cell line CGRP can activate PLC-β1 and increase intracellular Ca^{2+} via the activation of $G_{q/11}$ [8]. However, at least in *Xenopus* oocytes, we find no evidence for G_q-coupling which would be detected as a transient chloride ion flux through endogenous calcium-activated chloride channels. This may reflect the fundamentally different cell background in the two sets of experiments, or alternatively the receptor characterised by Drissi et al. may be a different molecular species to CRLR or the endogenous oocyte CGRP receptor. With reference to the latter, it is notable that no cAMP responses to CGRP were observed in the osteoblastic cell line.

In many studies receptors have been reported to couple through unforeseen and sometimes opposing pathways in recombinant systems, particularly if receptors are expressed at artificially high levels [19]. However, we do not appear to be over-expressing the CGRP receptor in the present study since our Swiss 3T3 cells show a comparable number of receptors to those seen in non-transfected SK-N-MC cells (B_{max} = 15 fmol per mg membrane protein) and in native tissues [1,2]. The degree to which the CGRP receptor would utilise different signalling pathways would depend upon the equilibrium constants inherent to interactions between receptor, ligand and G protein, as well as to the available concentrations of the various components [20]. Further experiments are required to elucidate the roles and relative importance of CGRP signalling through these various pathways.

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