Bombesin-like peptides depolarize rat hippocampal interneurones through interaction with subtype 2 bombesin receptors

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1. Whole-cell patch-clamp recordings were made from visually identified hippocampal interneurones in slices of rat brain tissue *in vitro*. Bath application of the bombesin-like neuropeptides gastrin-releasing peptide (GRP) or neuromedin B (NMB) produced a large membrane depolarization that was blocked by pre-incubation with the subtype 2 bombesin (BB_2) receptor antagonist [D-Phe^6,Des-Met^{14}]bombesin-(6–14)ethyl amide.

2. The inward current elicited by NMB or GRP was unaffected by K^+ channel blockade with external Ba^{2+} or by replacement of potassium gluconate in the electrode solution with caesium acetate.

3. Replacement of external NaCl with Tris–HCl significantly reduced the magnitude of the GRP-induced current at −60 mV. In contrast, replacement of external NaCl with LiCl had no effect on the magnitude of this current.

4. Photorelease of caged GTPγS inside neurones irreversibly potentiated the GRP-induced current at −60 mV. Similarly, bath application of the phospholipase C (PLC) inhibitor U-73122 significantly reduced the size of the inward current induced by GRP.

5. Reverse transcription followed by the polymerase chain reaction using cytoplasm from single hippocampal interneurones demonstrated the expression of BB_2 receptor mRNA together with glutamate decarboxylase (GAD67).

6. Although bath application of GRP or NMB had little or no effect on the resting membrane properties of CA1 pyramidal cells *per se*, these neuropeptides produced a dramatic increase in the number and amplitude of miniature inhibitory postsynaptic currents in these cells in a TTX-sensitive manner.

Bombesin is a 14 amino acid-containing peptide first isolated from the skin of the frog *Bombina bombina* (Anasti *et al.* 1971). Since its isolation, two related mammalian neuropeptides, neuromedin B (NMB) and gastrin-releasing peptide (GRP), have been isolated and shown to have a widespread distribution (Wada *et al.* 1990). Recently two distinct receptor subtypes for mammalian bombesin-like peptides have been cloned: the BB_1 receptor is the preferred receptor for NMB whilst the BB_2 receptor preferentially binds GRP (Spindel *et al.* 1990; Wada *et al.* 1991).

In situ hybridization analysis has shown that mRNAs encoding these two receptor subtypes exhibit a widespread and distinct distribution throughout the CNS (Battey & Wada, 1991). In agreement with this, autoradiographical studies demonstrate that NMB and GRP binding sites within the CNS are differentially distributed (Ladenheim *et al.* 1990). Areas rich in NMB-preferring binding sites include the ventromedial hypothalamus and dentate gyrus whilst the supraoptic nucleus, arcuate nucleus and outer rim of the hippocampal oriens layer exhibit dense amounts of GRP binding.

Since previous studies have demonstrated that bombesin-like peptides mediate potent biological effects in several of the above nuclei (Piggins & Rusak, 1993; Pinnock *et al.* 1994) in the present study we have investigated the actions of these peptides on GABAergic interneurones in the stratum oriens of the hippocampus. Although these interneurones represent a relatively small proportion of the hippocampal neurone population (Freund & Buszsáki, 1996) they are believed to exert a powerful tonic inhibition of pyramidal neurones in addition to powerful feedforward and feedback inhibitory postsynaptic potentials (IPSPs) after stimulation

(Lacaille *et al.* 1987). As such, these neurones represent an important site in the regulation of hippocampal excitability.

METHODS

Preparation

Wistar rats (14–28 days old) were killed by cervical dislocation. The brains were removed and 300 μm coronal slices containing the hippocampus prepared in ice-cold physiological saline (see below for composition) using a vibratome. A Zeiss Axioskop microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a ×64 water-immersion objective lens was used to view slices using light in the infrared range.

Recording and analysis

Whole-cell recording electrodes were pulled from borosilicate glass capillaries and had resistances of 3–6 MΩ when filled with electrolyte. Electrophysiological signals were detected using an Axopatch-1D patch-clamp amplifier and were recorded onto digital audiotape for later figure production. Following formation of the whole-cell configuration, series resistance was partially compensated using the amplifier and cellular conductance was continually monitored via the injection of hyperpolarizing current (current-clamp mode: −100 pA in amplitude, 300 ms in duration at 0·1 Hz) or voltage (voltage-clamp mode: −10 mV in amplitude, 300 ms duration at 0·1 Hz). Membrane signals were filtered at 1 kHz and digitized at 5 kHz through a Digidata 1200B A/D converter using pCLAMP 6.0 software (Axon instruments). In voltage-clamp recordings, neurones were clamped at −60 mV and current–voltage relationships were examined using a voltage ramp protocol between −140 and −60 mV (20 mV s^{-1}). Analysis of the frequency and amplitude of inhibitory postsynaptic currents (IPSCs) was performed using Jaejin mini analysis software (v3.0.1. Jaejin Software, NJ, USA). All data in the text and figures are presented as means ± S.E.M. unless otherwise stated.

Solutions and drugs

The physiological saline contained (mM): 125·0 NaCl, 25·0 $NaHCO_3$, 20·0 glucose, 2·5 KCl, 1·25 NaH_2PO_4, 2·0 $CaCl_2$, 1·0 $MgCl_2$ and was bubbled with a 95% O_2–5% CO_2 gas mixture. The intracellular (pipette) solution comprised (mM): 120·0 potassium gluconate, 10·0 NaCl, 2·0 $MgCl_2$, 0·5 K_2EGTA, 10·0 Hepes, 4·0 Na_2ATP, 0·3 Na_2GTP, pH adjusted to 7·2 with KOH. In some experiments, the ammonium salt of 500 μM guanosine 5′-*O*-(3-thiotriphosphate) P^3-(*S*)-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (caged GTPγS) was added to the pipette solution whilst in others, the potassium salt of Lucifer Yellow (1 mg ml^{-1}) was included. To investigate the effects of NMB or GRP on IPSCs in CA1 pyramidal neurones, the electrode solution contained (mM): 120·0 KCl, 10·0 NaCl, 2·0 $MgCl_2$, 1·0 2(triethylamino)-*N*-(2,6-dimethylphenyl)acetamide (QX-314), 0·5 K_2EGTA, 10·0 Hepes, 4·0 Na_2ATP, 0·1 Na_2GTP, pH adjusted to 7·2 with KOH.

Throughout the course of an experiment, slices were continuously perfused at a rate of 3–4 ml min^{-1} with physiological saline by means of a gravity feed system. GRP and other drugs used in this study were applied by changing the solution which superfused the slice to one which contained the drug. In experiments designed to examine the role of various currents in the observed effect, $BaCl_2$ was used to directly replace $CaCl_2$ in the Ba^{2+}-containing saline whilst caesium acetate directly replaced potassium gluconate in the electrode solution. When low Na^+ saline was used, NaCl was completely replaced by either LiCl or Tris–HCl. All experiments were conducted at 30–33 °C.

The following drugs were used: diazo-2 and caged GTPγS were obtained from Molecular Probes (Eugene, OR, USA). 3,5-Dihydroxyphenylglycine (DHPG), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxalone (NBQX), D-2-amino-5-phosphovalerate (APV) and (*S*)-α-methyl-4-carboxyphenylglycine (MCPG) were from Tocris Cookson (Bristol, UK). 1-(6-((17β-3-Methoxyoestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122) and 1-(6-((17β-3-methoxyoestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrolidine-2,5-dione (U-73343) were obtained from Calbiochem–Novabiochem (Nottingham, UK) whilst [D-Phe^6, Des-Met^{14}]bombesin-(6–14)ethyl amide was obtained from Peninsula laboratories. TTX and all other chemicals were obtained from Sigma Chemicals. All drug stocks used were made up in distilled water except NBQX, U-73122 and U-73343, which were made up as 10 mM stock solutions in DMSO whilst NMB and GRP were prepared as 1 mM stock solutions in DMSO.

Single cell RT-PCR

The cytoplasm from hippocampal neurones was gently aspirated under visual control into the recording electrode until at least 40% of the somatic cytoplasm had been collected (Lee *et al.* 1998). The electrode was then withdrawn from the cell, forced into a microtube and the RNA reverse transcribed using an anchored oligo dT primer and 200 Units of MMLV reverse transcriptase (BRL) according to the manufacturer's recommendations. After 60 min at 37 °C the cDNA was stored frozen at −20 °C prior to processing. After amplification of the cDNA using Taq polymerase (Dixon *et al.* 1998), the expression of specific genes was measured using primers designed to amplify products of between 150 and 250 base pairs in length, close to the 3′ ends of the mRNA transcripts.

The primers used were as follows. For cyclophilin (accession number M19533): forward primer (bases 388–407), ACTGCCAAGACTGAGTGGCT; reverse primer (bases 575–554), AATGGTTTGATGGGTAAAATGC. For BB_2 receptor (accession number X56661): forward primer (3′ untranslated), GGGGGAAAAGAGCTAAGCC; reverse primer (3′ untranslated), TATTCAAAGCACCGAAAGCC; For glutamate decarboxylase (GAD67, accession number X57573): forward primer (bases 2912–2933), ATCTTGCTTCAGTAGCCTTTGC; reverse primer (bases 3131–3110), TGTCTTCAAAAACACTTGTGGG. These PCR reactions were run for 50 cycles at 92 °C (denaturing, 2·5 min), 55 °C (annealing, 1·5 min) and 72 °C (extension, 1 min), followed by a final extension of 10 min at 72 °C. The PCR products were separated on 2·5% agarose gels and the product sizes were as predicted from the sequences. Confirmation of the nature of the bands was achieved using restriction enzyme analysis after reamplification with the appropriate gene specific primers.

RESULTS

Identification of stratum oriens interneurones

This study was performed on a total of 102 visually identified interneurones present in hippocampal slices taken from 76 animals. Only neurones located in the stratum oriens of the hippocampus were chosen for this study. These neurones were typically present in low density and were found to exhibit a heterogeneous morphology. Following formation of the whole-cell configuration, these neurones displayed electrophysiological characteristics similar to those previously attributed to GABAergic interneurones in this area (Zhang & McBain, 1995). For example, in current-clamp recordings, these neurones had a resting membrane

potential of $-53{\cdot}6 \pm 4{\cdot}4$ mV ($n = 34$) and often fired spontaneous action potentials at a rate of 2–10 Hz which were of short duration ($1{\cdot}1 \pm 0{\cdot}1$ ms, $n = 34$, measured at half-amplitude) and which were followed by a rapid after-hyperpolarization ($16{\cdot}1 \pm 1{\cdot}3$ mV in amplitude, $151{\cdot}4 \pm 11{\cdot}3$ ms in duration). Immediately after membrane breakthrough these neurones had an input resistance of $486{\cdot}9 \pm 23{\cdot}2$ MΩ ($n = 34$) and exhibited a characteristic reduction in apparent input resistance in response to hyperpolarizing current injection, which has previously been attributed to the non-selective cation channel current I_H (Maccaferri & McBain, 1996).

To confirm the GABAergic nature of these neurones, in five cells, following electrophysiological identification, cytoplasm was harvested and processed for reverse transcription and cDNA amplification. The resultant cDNA was subjected to PCR analysis with primers specific for the enzyme glutamate decarboxylase (GAD67). Each neurone examined was positive for this enzyme (Fig. 9).

Effect of bombesin-like peptides on resting membrane properties

In current-clamp recordings, bath application of GRP or NMB at concentrations previously shown to excite neurones (Pinnock & Woodruff, 1991; Pinnock *et al.* 1994) was found to depolarize interneurones within the stratum oriens. Thus, 100–200 nM GRP produced a $21{\cdot}2 \pm 3{\cdot}2$ mV depolarization ($n = 5$ of 8 neurones; Fig. 1*A* and *B*) whilst 100–200 nM NMB induced a $21{\cdot}4 \pm 4{\cdot}2$ mV depolarization ($n = 16$ of 22 cells). In each case these effects were usually associated with pronounced oscillations in membrane potential. These large oscillations in membrane potential were unaffected by the subsequent addition of 1 μM TTX together with the glutamate receptor antagonists NBQX (10 μM) and D-APV (50 μM) and the $GABA_A$ receptor antagonist bicuculline (10 μM, $n = 3$ for each; Fig. 1*B*). In the remaining neurones these peptides induced either a small depolarization ($n = 3$ of 9 cells) or had no apparent effect on resting membrane properties ($n = 6$ of 9) (not shown).

To determine the nature of neurones sensitive to these neuropeptides, some cells were filled with Lucifer Yellow via the recording electrode and after fixation subjected to epifluorescence examination of cell morphology (Grace & Llinás, 1985). Five of the seven cells examined had a morphology similar to the horizontal cells described by McBain and coworkers (McBain *et al.* 1994). Thus each neurone possessed a large cell body (30–50 μm) that was horizontally orientated with respect to the pyramidal cell layer. Each interneurone had several dendrites which extended through the stratum oriens whilst the axons of these cells crossed through the stratum pyramidale and into the stratum lacunosum. The remaining two GRP-sensitive neurones displayed a morphology reminiscent of vertically orientated neurones. These cells possessed dendrites which projected both horizontally and vertically through the stratum oriens. The axons of these cells formed a branched network through the stratum oriens and stratum pyramidale.

Nature of the current induced by bombesin-like peptides

When neurones were voltage clamped at −60 mV, GRP and NMB were found to induce an inward current of similar magnitude and with comparable concentration dependence to each other. Thus 1 nM of either ligand had no effect on the magnitude of the holding current ($n = 5$), 50 nM NMB or GRP induced an inward current of $65{\cdot}5 \pm 12{\cdot}5$ pA ($n = 6$) whilst 100 and 200 nM NMB or GRP induced a noisy inward current $125{\cdot}2 \pm 8{\cdot}5$ pA ($n = 28$) in magnitude.

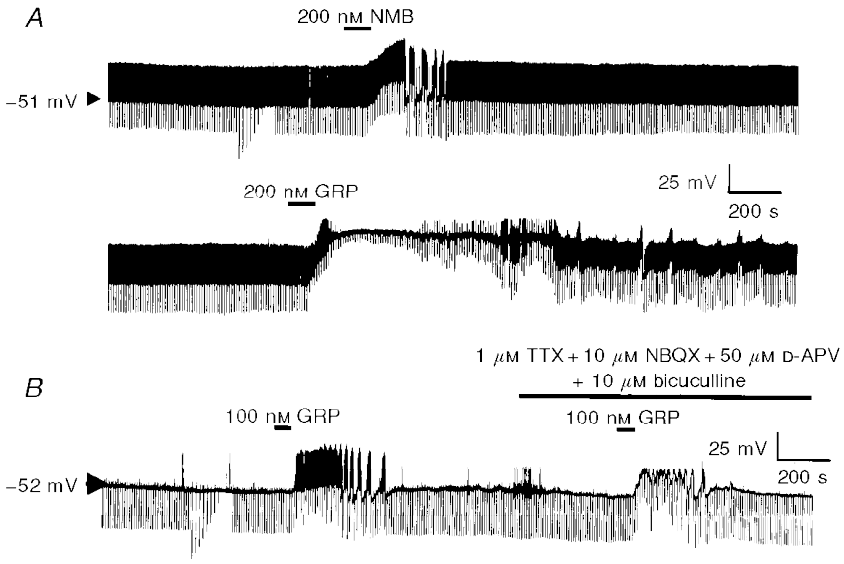

Figure 1. Bombesin-like peptides depolarize rat hippocampal stratum oriens interneurones

A, continuous whole-cell current-clamp recording trace illustrating the effect of NMB or GRP on resting membrane properties of a stratum oriens interneurone. *B*, the effect of GRP is retained in the presence of TTX and blockers of ionotropic glutamate and GABA receptors.

In each case, the inward current displayed a similar time course to the depolarization observed under current-clamp conditions.

To investigate the nature of this inward current, voltage ramps from −140 to −60 mV were applied in the absence and presence of the induced current (see Fig. 2*B*). Using this approach, it was possible to extrapolate the current plots using linear regression and thus determine the point at which the currents intersect. According to this protocol, the extrapolated reversal potential of the NMB- or GRP-induced current was −5·2 ± 4·1 mV (Fig. 2*B*; $n = 6$).

To discount the possibility that NMB or GRP might be acting presynaptically via the release of other transmitters, Ca^{2+}-free solutions containing 5 mM Mg^{2+} were used. Under these conditions, although the Ca^{2+}-free solution reduced the level of baseline noise, the size of the NMB- or GRP-induced current, together with associated noise, was unaffected (control, 124·3 ± 17·5 pA; Ca^{2+}-free, 126·1 ± 14·1 pA; $n = 4$ at −60 mV; Fig. 2*A*).

To determine the type of bombesin receptor responsible for these effects, slices were pre-incubated with the BB_2 receptor-selective antagonist [D-Phe^6,Des-Met^{14}]bombesin-(6–14)ethyl amide (Jensen & Coy, 1991). In each case examined 1 μM [D-Phe^6,Des-Met^{14}]bombesin-(6–14)ethyl amide was found to completely block the inward current induced by either 200 nM NMB or 200 nM GRP ($n = 3$ for each; Fig. 3).

Since previous studies have shown that stimulation of group I metabotropic glutamate receptors (mGluRs) elicits a similar response in stratum oriens interneurones to that observed with GRP (McBain *et al.* 1994; Woodhall *et al.* 1999), we examined the effects of group I mGluR agonists and antagonists on these GRP-sensitive interneurones. The group I mGluR agonist DHPG (50 μM; Schoepp *et al.* 1994) was found to elicit an inward current 143·4 ± 11·1 pA in magnitude in eight of the nine neurones on which it was tested. In five of these neurones, 200 nM GRP induced an inward current of 119·5 ± 14·5 pA, whilst the remaining four were insensitive to this peptide. Preincubation of slices

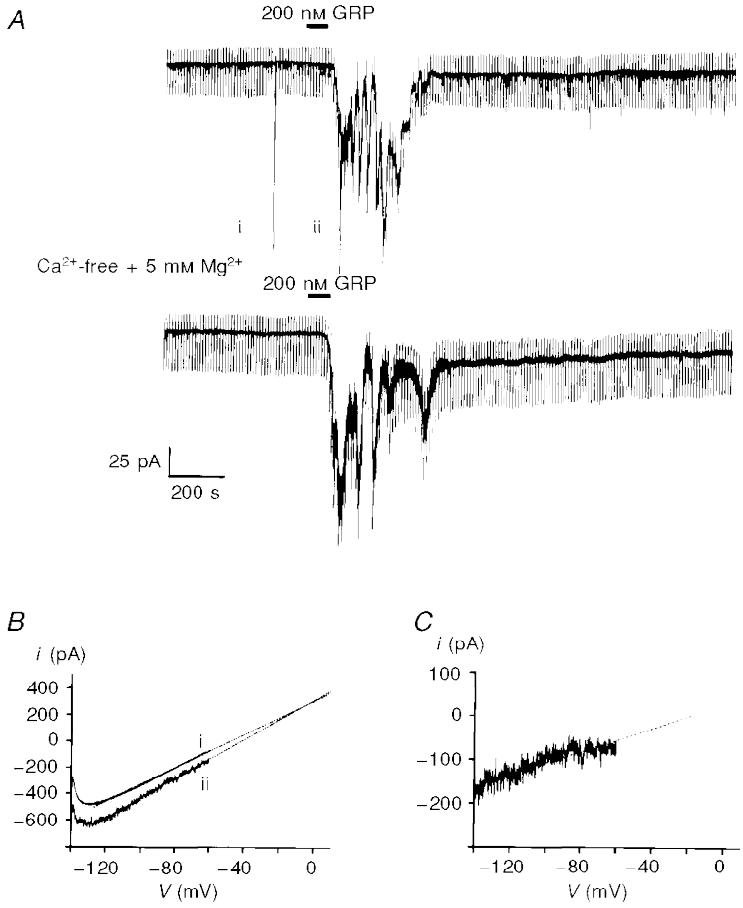

Figure 2

A, GRP activates an inward current in neurones voltage clamped at −60 mV. The magnitude of this inward current was unaffected by removal of extracellular Ca^{2+} and addition of 5 mM $MgCl_2$. The numerals beside the upper trace correspond to depolarizing ramps (from −140 to −60 mV at 20 mV s^{-1}). *B*, expanded traces of the current responses to the voltage ramps shown in *A*. The numbers beside these currents correspond to those shown in *A*. The dotted lines are the lines of best fit by linear regression. *C*, subtraction of the control current from the current observed in the presence of GRP yields the GRP-induced current.

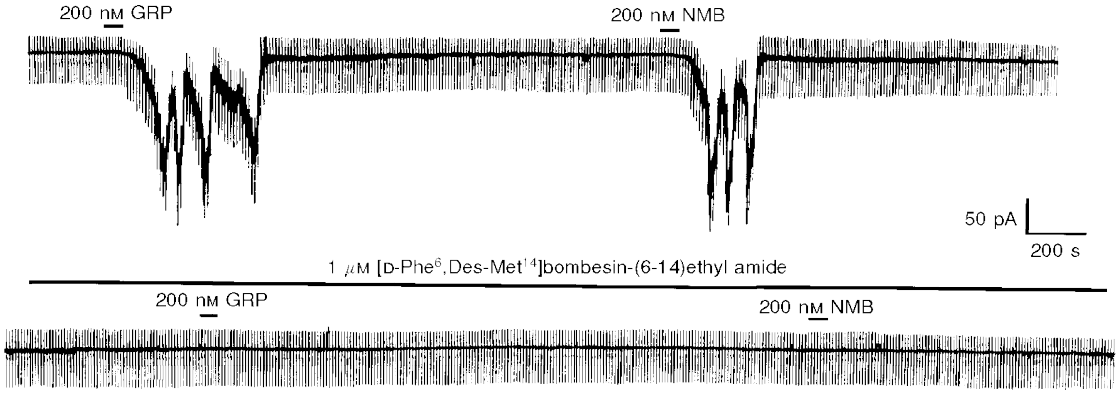

Figure 3

The inward current induced by the bombesin-like peptides GRP and NMB is inhibited by preincubation with the BB_2 receptor antagonist [D-Phe^6,Des-Met^{14}]bombesin-(6–14)ethyl amide. The recording is a continuous trace made at −60 mV.

with the group I mGluR antagonist MCPG (500 μM; Watkins & Collingridge, 1994) was found to inhibit the response to DHPG (by 82·3 ± 5·4 %, $n = 7$) without affecting the magnitude of the response to GRP ($n = 5$, Fig. 4).

The inward current elicited by 200 nM GRP was not affected by K^+ channel blockade with either extracellular Ba^{2+} (control, 144·3 ± 15·5 pA; 2 mM Ba^{2+}, 138·1 ± 15·1 pA; $n = 4$ at −60 mV) or by the inclusion of caesium acetate in the electrode solution (105·3 ± 11·1 pA at −60 mV; $n = 4$), which indicates that a K^+ conductance is not involved in the generation of the GRP-induced current.

To determine whether Na^+ ions are involved in the generation of this current, the external NaCl was replaced with Tris–HCl. Under these conditions, the amplitude of the GRP-induced current was reduced to 46·2 ± 6·3 % of its control value ($n = 4$, Fig. 5*A*). In contrast replacement of

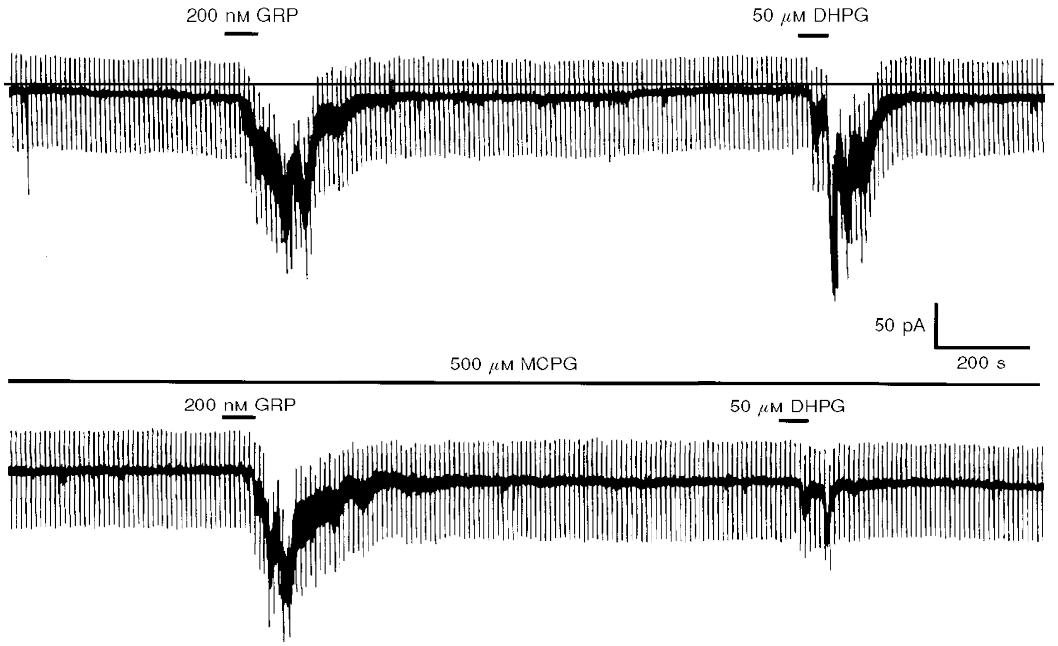

Figure 4. The GRP-induced current is unaffected by application of the mGluR antagonist MCPG

Continuous recording performed at −60 mV. In the upper panel, bath application of GRP or DHPG are shown to induce a marked oscillatory inward current. In the lower panel, preincubation with MCPG is shown to have no effect on the magnitude of the GRP-induced current whilst causing a large reduction in the size of the DHPG-induced current.

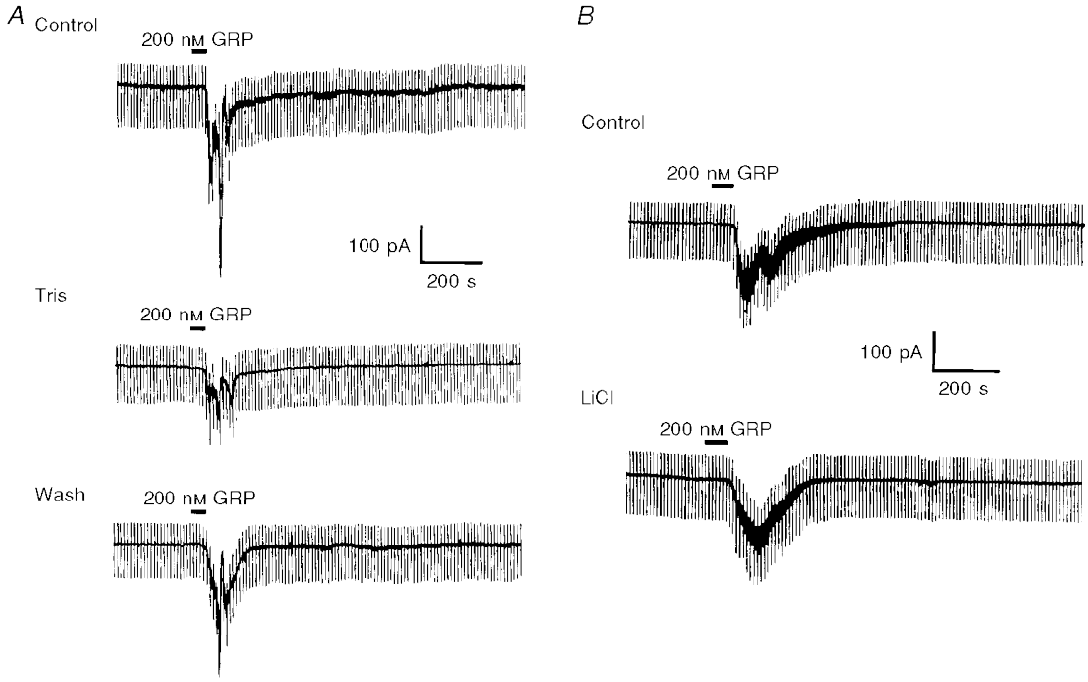

Figure 5. The GRP-induced inward current is dependent on monovalent cations in the extracellular solution

A, top trace shows the control response to GRP at −60 mV. Middle trace shows the diminished response to the same concentration of GRP 10 min after replacing all the extracellular NaCl with Tris–HCl. Bottom trace, 30 min after returning to normal physiological saline, the response to GRP had partially returned. *B*, replacement of extracellular NaCl with LiCl had no effect on the magnitude of the GRP-induced current at −60 mV.

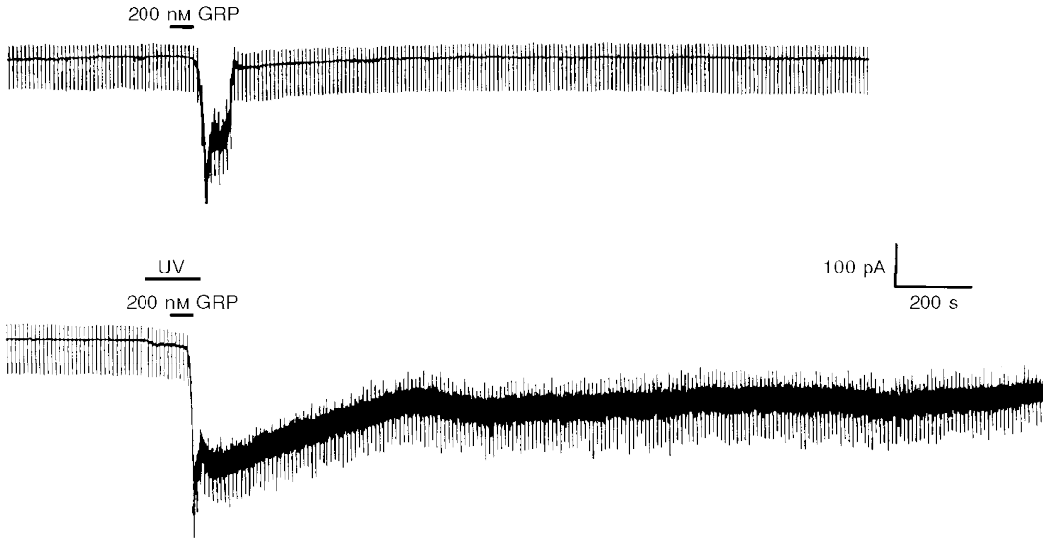

Figure 6. Continuous whole-cell voltage-clamp recording trace showing that caged GTPγS irreversibly potentiates the GRP-induced inward current at −60 mV when photolysed by UV irradiation

In this continuous recording 500 μM GTPγS was included in the electrode solution at the start of the experiment. From this caged precursor, free GTPγS was liberated by the application of UV light onto the neurone for the period indicated.

external NaCl with LiCl failed to affect the magnitude of the GRP-induced current at −60 mV (control, 98·3 ± 12·5 pA; LiCl, 97·4 ± 14·6 pA; $n = 4$; see Fig. 5*B*).

Sequence of intracellular events

Molecular cloning studies have shown that bombesin receptors belong to the G-protein-coupled receptor superfamily (Battey & Wada, 1991). To address the possible involvement of G-proteins in the generation of this inward current by GRP, 500 μM caged GTPγS was incorporated into the electrode solution. Once the control response to 200 nM GRP was established, the release of the free GTPγS was initiated by exposure to UV light. Using this system, the inward current induced by 200 nM GRP at −60 mV was increased (to 154·2 ± 7·2%, $n = 4$, $P < 0·05$) and was not readily reversed following washout of GRP (Fig. 6). In contrast, in the absence of caged compounds, exposure to UV light for a similar length of time had no effect on the current induced by GRP at −60 mV ($n = 4$, not shown).

The involvement of PLC in the present study was demonstrated using the PLC inhibitor U-73122 (Bleasdale *et al.* 1990). Application of 10 μM U-73122 was found to significantly reduce the GRP-induced current by 78·3 ± 8·3% at −60 mV ($n = 5$, $P < 0·05$, Fig. 7). These effects of U-73122 were poorly reversible on washout (not shown). In contrast, 10 μM U-73343, an inactive analogue of U-73122, did not affect the magnitude of this current ($n = 6$, Fig. 7).

To establish that an increase in intracellular calcium was a prerequisite in the generation of the GRP-induced current, 10 mM diazo-2, a caged Ca^{2+} chelator was incorporated into the electrode solution. Once the control reponse to GRP was established, UV light was directed onto the neurone to produce photolysis of the calcium chelator. Using this protocol, it was possible to reduce the magnitude of the GRP-induced current by 66·5 ± 8·7% at −60 mV ($n = 4$, $P < 0·05$, Fig. 8).

Molecular identification of the bombesin receptor involved

To confirm the identity of these GRP-sensitive cells the cytoplasm of five neurones sensitive to GRP or NMB was harvested and subjected to single cell RT-PCR analysis

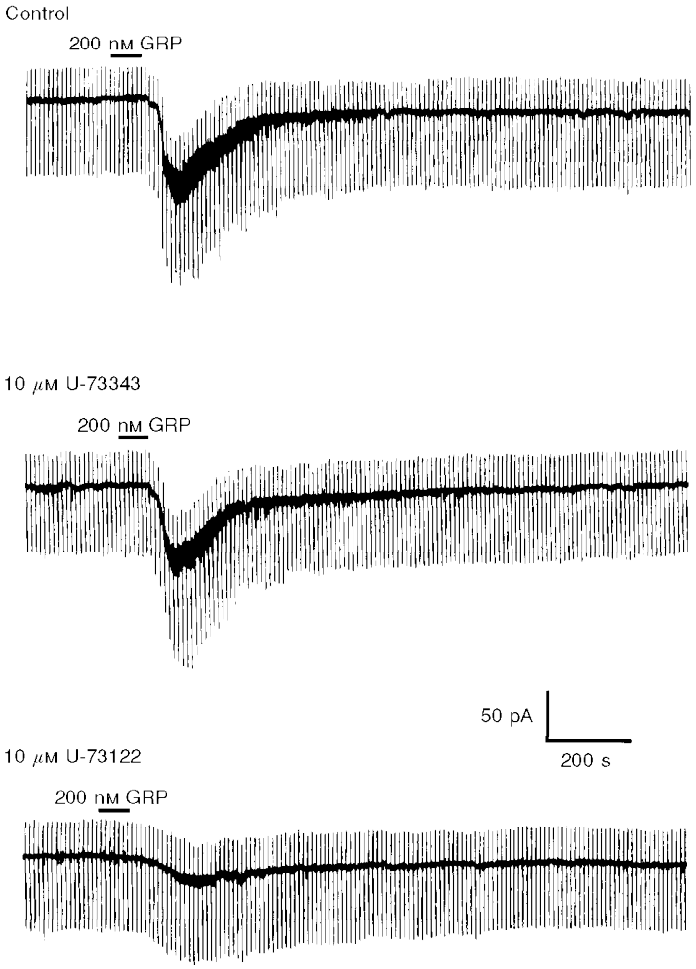

Figure 7. PLC is important in the generation of the GRP-induced inward current at −60 mV
The inward current induced by GRP was diminished by bath application of the PLC inhibitor U-73122. In contrast, this effect is not mimicked by the inactive analogue U-73343.

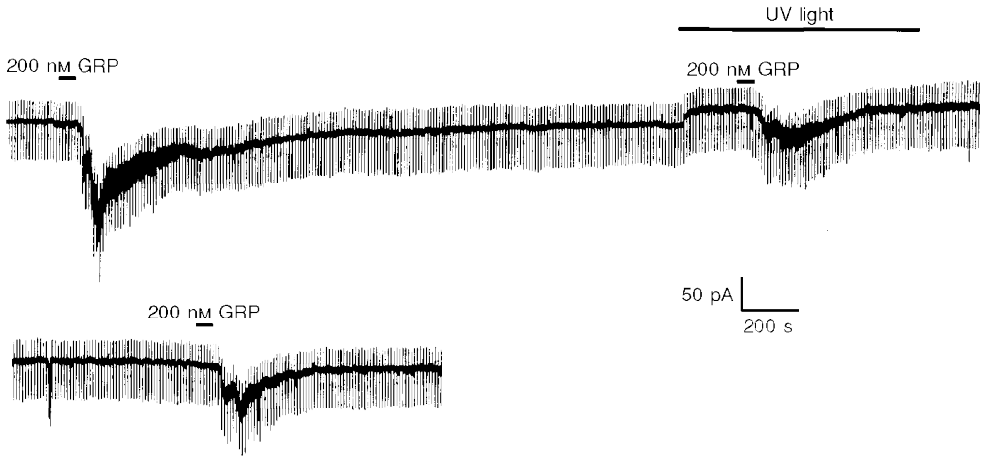

Figure 8. UV photolysis of diazo-2 inside a hippocampal interneurone reduces the magnitude of the GRP-induced current

In this continuous recording 10 mM diazo-2 was included in the electrode solution at the start of the experiment. From this caged precursor, free diazo-2 was liberated by the application of UV light onto the neurone for the period indicated. The release of free diazo-2 was associated with a reduction in the magnitude of the GRP-induced current which was partially reversible on removal of the UV light source.

using primer oligonucleotides specific for the cytoplasmic marker gene, cyclophilin and the enzyme GAD67. In each case, mRNA encoding the enzyme GAD67 was detected to indicate the GABAergic nature of these neurones ($n = 5$). In addition to detecting GAD67, mRNA for the BB_2 receptor was also detected (Fig. 9*A*). In contrast, in five CA1 pyramidal neurones, although mRNA for the housekeeping gene was detected, no expression of GAD67 and BB_2 receptors was found (Fig. 9*B*).

Effect of bombesin-like peptides on membrane properties of CA1 pyramidal neurones

NMB or GRP (100 nM to 300 nM) were tested on 14 visually identified CA1 pyramidal neurones obtained from eight separate animals. These neurones were easily distinguished from hippocampal interneurones both morphologically and electrophysiologically. For example, these neurones exhibited a more negative resting membrane potential

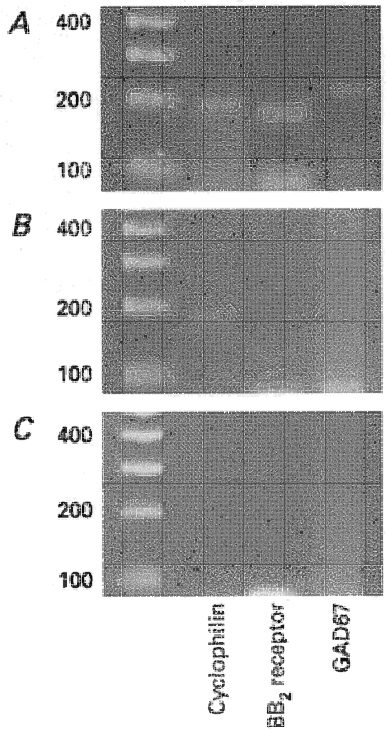

Figure 9. Molecular identity of the GRP-sensitive interneurones

A, using the cytoplasmic contents harvested from a single GRP-sensitive interneurone, the expression of GAD67 mRNA together with the housekeeping gene cyclophilin, and the BB_2 receptor was detectable. *B*, using the cytoplasmic contents harvested from a single CA1 neurone only the housekeeping gene cyclophilin was detectable. *C*, under the same conditions, replacement of cytoplasmic contents with the same volume of ACSF from the bath solution failed to yield any products. Left-hand column is the DNA ladder, units are base pairs (bp).

(−64·3 ± 1·2 mV, $n = 11$) and had a lower input resistance (150·1 ± 5·2 MΩ, $n = 11$). In two of the nine neurones on which it was tested, 300 nM NMB was found to produce a small depolarization (2·1 and 2·3 mV) whilst in the remaining seven neurones, NMB was without apparent effect (not shown). Similarly 300 nM GRP had no effect on the resting membrane potential of the CA1 neurones on which they were tested ($n = 5$). In contrast, carbachol, which has previously been shown to excite CA1 pyramidal neurones (Bernado & Prince, 1982) produced a 22·1 ± 3·2 mV depolarization following bath application at a concentration of 10 μM ($n = 4$; not shown).

Since previous studies have demonstrated that axons of stratum oriens interneurones arborize with CA1 pyramidal neurones (Lacaille *et al.* 1987; McBain *et al.* 1994), we hypothesized that bombesin-related neuropeptides should increase the number of spontaneous IPSCs recorded from CA1 pyramidal neurones. In the presence of 50 μM D-APV and 10 μM NBQX to block glutamatergic transmission, spontaneous IPSCs were observed under control conditions at a rate of 3·7 ± 1·2 Hz with a mean amplitude of 64·3 ± 15·6 pA ($n = 6$). In three neurones, IPSCs were abolished by bath application of 10 μM bicuculline to indicate that they were elicited via the $GABA_A$ receptor. Under these conditions bath application of 100–200 nM NMB or GRP produced a significant increase in both the frequency of these IPSCs (to 24·3 ± 4·3 Hz, $n = 6$, $P < 0·05$) and their amplitude (to 135·5 ± 38·5 pA, $n = 6$; Fig. 10).

These effects were abolished by the addition of 10 μM bicuculline (Fig. 10*A*) which indicates that these effects were again mediated by the $GABA_A$ receptor. Furthermore pre-incubation with 1 μM TTX was also found to prevent these effects indicating that these actions were not due to a direct action at the nerve terminal or due to some change in responsiveness to the released GABA ($n = 3$).

DISCUSSION

Bombesin-like peptides depolarize hippocampal interneurones

In the present study, we demonstrate that the bombesin-like peptides NMB and GRP depolarize interneurones in the stratum oriens of the hippocampus by the induction of an inward current at −60 mV. This inward current has an estimated reversal potential of −5·2 ± 4·1 mV and is often associated with large oscillations in the current amplitude.

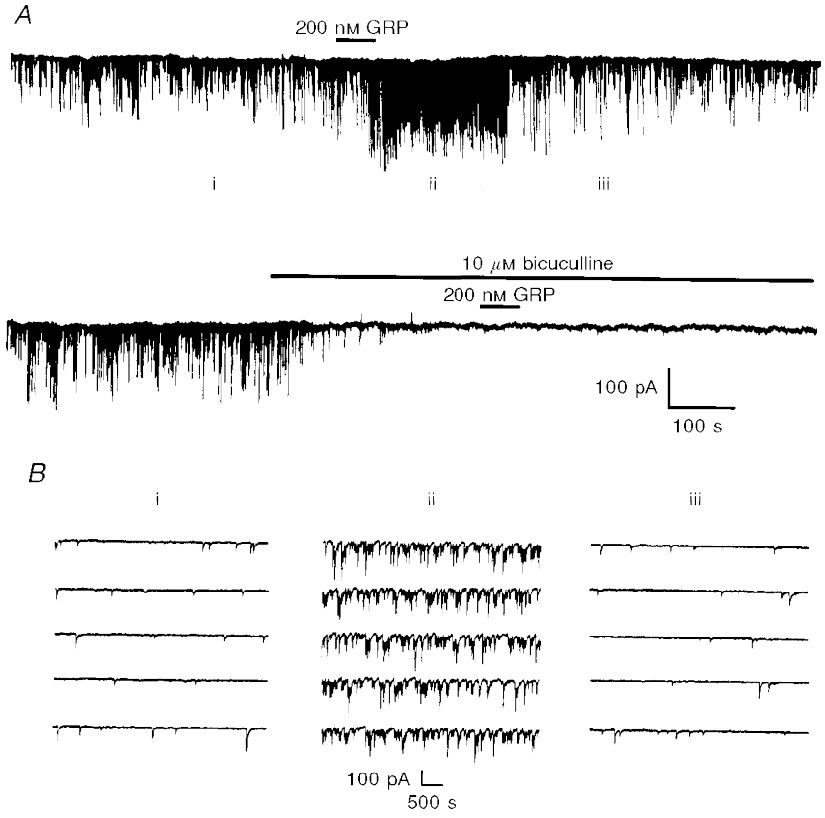

Figure 10

A, bath application of GRP increases the frequency and amplitude of spontaneous IPSCs in a CA1 pyramidal neurone held at −60 mV. In the lower part of the recording, these effects of GRP are shown to be bicuculline sensitive. *B*, expanded traces of the currents in *A*. The numbers above these currents correspond to those shown in *A*.

These findings support and extend the study of Dreifuss & Raggenbass (1986) who used extracellular recording techniques to show that bombesin and related ligands excite non-pyramidal neurones in hippocampus.

The reversal potential of this current suggests the possible involvement of a non-selective cation current in the generation of this response. This hypothesis is supported by the Na^+ dependence of the current. Replacement of extracellular Na^+ ions with Tris–HCl reduced the size of the GRP-induced current, which indicates that Na^+ ions are involved in this effect. However, it is unlikely that TTX-sensitive Na^+ channels participate in the generation of this current since TTX was present in the external solution. Another possibility is that this inward current is mediated through activation of a non-selective cation channel although any contribution from ionotropic glutamate receptors can be ruled out since neither NBQX nor D-APV affects the magnitude of the GRP-induced inward current.

The inward current was also shown to be independent of Ca^{2+} influx since it was unchanged in Ca^{2+}-free physiological saline. In contrast, the cytoplasmic concentration of Ca^{2+} was shown to be important in the generation of this inward current using the caged Ca^{2+}chelator diazo-2 (Adams *et al.* 1989). In the present study when diazo-2 was photolysed inside the neurone, the size of the GRP-induced response was significantly reduced. These findings indicate that a significant component of this response is dependent on a rise in the concentration of cytoplasmic Ca^{2+}.

The profile of this GRP-activated inward current exhibits characteristics similar to that of the current induced by (1*S*,3*R*)-aminocyclopentane dicarboxylic acid (ACPD) in the same neurones (McBain *et al.* 1994). In this former study, the authors suggest that the ACPD-induced current could arise through a Na^+–Ca^{2+} exchange mechanism. To investigate whether such a mechanism could be involved in the present study we replaced the extracellular NaCl with LiCl. Li^+ is a small monovalent cation which would be expected to pass through any putative non-selective cation channel (Partridge & Swandulla, 1988) but which is not utilized by the Na^+–Ca^{2+} exchanger (Ledvora & Hegyvary, 1983). Consequently, the lack of effect Li^+ substitution on the magnitude of the GRP-induced current suggests that the present effect is likely to be due to the activation of a calcium-activated non-selective cation channel rather than any Na^+–Ca^{2+} exchange mechanism.

In a recent study, the oscillatory nature of the mGluR-induced current was shown to coincide with rises in intracellular Ca^{2+} through a coupling of mGluR receptors to voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular stores (Woodhall *et al.* 1999). Given the similarity of the mGluR response to that seen with BB_2 receptor stimulation, it is possible to envisage that the two receptors may converge upon a common signalling pathway akin to that described for mGluR5 receptors (Kawabata *et al.* 1996). However, although it appears that these transmitters evoke a similar response in certain interneurones, differences seem to exist in the populations of neurones sensitive to these agents since only five of the eight DHPG-sensitive neurones examined were sensitive to GRP.

Pharmacology of the current induced by bombesin-like peptides

Previous studies have demonstrated the presence of mRNA for both BB_1 and BB_2 receptors in the mammalian hippocampus (Wada *et al.* 1991). In the present study, the effects of both NMB and GRP were inhibited by pre-incubation with the BB_2-selective antagonist [D-Phe^6,Des-Met^{14}]bombesin-(6–14)ethyl amide to suggest that the observed response was mediated solely via the BB_2 receptor (Jensen & Coy, 1991). This finding agrees with the results obtained from single cell RT-PCR analysis demonstrating the expression of BB_2 receptor mRNA in these neurones and previous autoradiographical studies where a predominance of GRP binding was observed in this region of the hippocampus (Ladenheim *et al.* 1990). The ability of NMB to interact with the BB_2 receptor with a similar efficacy to GRP is reminiscent of previous studies performed in both the suprachiasmatic nucleus and periventricular nucleus (Pinnock *et al.* 1994).

Sequence of intracellular events leading to activation of the GRP-induced current

The involvement of a G-protein in the present sequence of events was demonstrated using caged GTPγS. When exposed to light of the appropriate wavelength, this compound undergoes a photochemical reaction with the liberation of free GTPγS (Lee & Boden, 1997). Using this technique, it is possible to demonstrate that when free GTPγS was generated inside the cell from its caged precursor the magnitude of the GRP-induced current was greater and more prolonged than the control current.

In previous studies, bombesin-like peptides have been reported to be coupled to phosphoinositide turnover resulting from the activation of PLC (Hari-Prasad & Moody, 1988; Hollingsworth, 1989). PLC was shown to play a role in the generation of the GRP-induced current using the PLC inhibitor U-73122. This compound has been used in a number of preparations to inhibit PLC activity and thus inositol 1,4,5-trisphosphate (IP_3) formation (Bleasdale *et al.* 1990). In the present study, U-73122 inhibited the GRP response indicating the involvement of PLC in the generation of this current. Furthermore the related compound U-73343 was unable to mimic this inhibitory effect discounting the possibility that U-73122 mediates this inhibition through some non-specific mechanism.

Physiological significance

In contrast to their dramatic effects upon stratum oriens interneurones, NMB and GRP were found to exert relatively

little or no effect on the resting membrane properties of CA1 pyramidal neurones under these conditions (i.e. potassium gluconate-filled electrodes with resting potential close to the chloride equilibrium potential). However, under conditions optimized for the detection of IPSCs, these neuropeptides were shown to elicit a marked increase in the numbers of $GABA_A$-mediated IPSCs received by these neurones in a TTX-sensitive manner. These findings imply that the GABAergic interneurones depolarized by bombesin-like peptides in the stratum oriens make synaptic connections with CA1 pyramidal neurones. This scenario is similar to that recently reported by others both for the metabotropic glutamate agonist ACPD (McBain *et al.* 1994) and also for adrenergic agonists (Bergles *et al.* 1996). However, whilst we demonstrate that bombesin-like peptides have virtually no effect on the resting membrane properties of CA1 neurones themselves, the excitatory actions of both ACPD (e.g. Stratton *et al.* 1989) and noradrenaline (Nicoll *et al.* 1990) upon these neurones are well established.

The role performed by local interneurones in the modulation of neuronal excitability in the hippocampus as well as other regions of the CNS is now well documented. For example, agents which hyperpolarize interneurones can increase the excitability of their target neurones through a reduction in the frequency and amplitude of IPSCs (Zieglgansberger *et al.* 1979; Johnson & North, 1992). Thus it is tempting to suggest that agents which excite interneurones such as NMB or GRP may exert potent inhibitory effects within the hippocampus *in vivo*. In support of this, it is noteworthy that noradrenaline has been shown to reduce the firing rate of CA1 neurones *in vivo* despite its ability to directly excite CA1 neurones *in vitro* (e.g. Segal & Bloom, 1974).

At present, little is known regarding the exact role performed by bombesin-like peptides within the hippocampal formation. Previous studies have revealed that substantial amounts of immunoreactivity for bombesin-like peptides reside in the hippocampus where it appears to be localized in synaptosomes (Moody *et al.* 1986). Further studies using *in situ* hybridization have revealed many layers of the hippocampus exhibit considerable GRP mRNA (Zoeller *et al.* 1989; Wada *et al.* 1990). However, little is known as to the site of action of these peptides although it is possible to speculate that the release of GRP may act upon these interneurones to provide a powerful form of feedback inhibition under normal conditions and may ultimately be neuroprotective under excitotoxic conditions (Freund & Buzsáki, 1996).

In view of these findings, in future studies it will be important to characterize the nature of these GRP-sensitive neurones more fully using morphological and neurochemical techniques in addition to examining the effects of such peptides in other groups of hippocampal neurones.

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