*Journal of Physiology* (1991), **440**, *pp.* 55–65
*With 5 figures*
*Printed in Great Britain*



# BOMBESIN EXCITES A SUBPOPULATION OF 5-HYDROXYTRYPTAMINE-SENSITIVE NEURONES IN THE RAT DORSAL RAPHE NUCLEUS *IN VITRO*

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(*Received 1 November 1990*)

SUMMARY

1. The effects on dorsal raphe neurones of the peptides bombesin, gastrin-releasing peptide and neuromedin B were studied using intracellular recording techniques from slices of rat brain maintained *in vitro*. The peptides were added to the solutions perfusing the slices.

2. The peptides bombesin, gastrin-releasing peptide and neuromedin B depolarized neurones in the dorsal raphe nucleus. The same neurones were depolarized by phenylephrine and hyperpolarized by 5-hydroxytryptamine (5-HT) but were insensitive to sulphated cholecystokinin octapeptide (CCK).

3. The responses to the peptides were not blocked by $CCK_A$, $CCK_B$ and $\alpha_1$-adrenoreceptor antagonists.

4. The response to the peptides persisted in the presence of tetrodotoxin (TTX) and low-calcium, high-magnesium-containing artificial cerebrospinal fluid (ACSF).

5. Under voltage clamp conditions the peptides caused a decrease in membrane conductance accompanied by an inward current. The reversal potential for the event was the same as that for 5-HT.

6. The results of the present study demonstrate that bombesin and the structurally related peptides gastrin-releasing peptide (GRP) and neuromedin B depolarized a subpopulation of raphe 5-HT neurones by acting on a postsynaptically located receptor linked to potassium channels.

## INTRODUCTION

The dorsal raphe nucleus is the largest of the raphe nuclei and contains the highest density of 5-hydroxytryptamine (5-HT)-containing neurones in the rat brain. 5-HT receptors are present on the soma and dendrites of raphe 5-HT neurones and the activation of these receptors by either release of 5-HT from nerve terminals or bath application of 5-HT agonists causes an increase in an inwardly rectifying potassium conductance (Yoshimura & Higashi, 1985; Williams, Colmers & Pan, 1988; Pan,

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Colmers & Williams, 1989) leading to hyperpolarization of the neuronal membrane and a reduction in action potential firing.

The same neurones receive a direct synaptic noradrenergic input (Baraban & Aghajanian, 1981) and are excited by noradrenaline and phenylephrine (Aghajanian, 1985). Recently we have shown that a subpopulation of 5-HT-sensitive neurones in

TABLE 1. Structures of bombesin and related peptides

Bombesin
Glp-Gln-Arg-Leu-**Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-MetNH$_2$**

Neuromedin B
**Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-MetNH$_2$**

Gastrin-releasing peptide
Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-**Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-MetNH$_2$**

Neuromedin C
**Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-MetNH$_2$**

D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P
D-Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH$_2$

The conserved region of the peptides is indicated in bold and the differences within the conserved regions are underlined.

the rat dorsal raphe nucleus were excited by sulphated cholecystokinin octapeptide (CCK) (Boden, Woodruff & Pinnock, 1991). Furthermore, the CCK receptors in the raphe were of the $CCK_A$ subtype and thus had a similar pharmacological profile to the CCK receptors in the pancreas (Boden *et al.* 1990). Pancreatic acinar cells have been shown to be depolarized by both CCK and bombesin acting at different receptors to increase a similar cation conductance (Philpott & Petersen, 1979; Petersen & Maruyama, 1983). Bombesin is a fourteen amino acid-long peptide derived from the skin of the frog *Bombina bombina* (Erspamer, Erspamer, Inselvini & Negri, 1972). The mammalian counterparts of bombesin are gastrin-releasing peptide (GRP) and neuromedin B (NMB) (Spindel, Chin, Price, Roes, Besser & Habener, 1984) in which the C terminal amino acids are similar (Table 1). In the present study the actions of bombesin, GRP and NMB were investigated on neurones in the rat dorsal raphe.

Part of this work was presented in preliminary form to the Physiological Society (Pinnock & Woodruff, 1991).

## METHODS

*Preparation of slices and recording from neurones*

Intra- and extracellular recordings were made using conventional techniques from dorsal raphe neurones in the slice preparation made from rat pons–mesencephalon. Male rats (50–150 g) were killed by cervical dislocation and the brain rapidly removed. Coronal slices (350 $\mu$m) were cut with a vibratome in cold (4 °C) physiological saline. A single slice was placed in a tissue bath through which flowed physiological artificial cerebrospinal fluid (ACSF) (2 ml min$^{-1}$) at 35 °C. The content of the ACSF was as follows (mM): NaCl, 126; KCl, 5·0; $NaH_2PO_4$, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·4; glucose, 11; $NaHCO_3$, 25; the solution was gassed with 95% $O_2$–5% $CO_2$ at 35 °C. Magnesium chloride (10–20 mM) was substituted for calcium chloride to obtain calcium-free ACSF.

*Data storage and analysis*

The raw DC signal was stored in digitized form on videotape and replayed for analysis (Modified Sony PCM (Fentronics) and Sony Betamax video-recorder). The current–voltage curves obtained under voltage clamp conditions were constructed using a CED 1401 interface board, IBM AT-equivalent computer and a specially designed suite of software (VCAN, provided by Dr John Dempster, University of Strathclyde UK). Rate-meter histograms were constructed using a CED 1401 interface board, IBM AT-equivalent computer and a specially designed suite of software (MRATE, Cambridge Electronic Design, Cambridge).

*Drugs and chemicals*

Drugs dissolved in ACSF were applied to the slice by superfusion. Bombesin, GRP, NMB and CCK were obtained from either Peninsula Laboratories or Cambridge Research Biochemicals. L-364,718 (3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide), L-365,260 (3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-*N'*-(3-methylphenyl)urea), PD 134308 (butanoic acid.4-[[2-[[3(-1H-indole-3-yl)-2 - methyl - 1 - oxo - 2 - [[(tricyclo[3.3.1.1.7]dec - 2 - yloxy)carbonyl]amino]propyl]amino] -1-phenylethyl]amino]-4-oxo[R-(R+.R+)]-.*N*-methyl-D-glucamine, and CI-977 [5R-(5a,7a,8b)]-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-4-benzofuranacetamide monohydrochloride were synthesized in Medicinal Chemistry, Parke–Davis, Cambridge. All other drugs were obtained from either Sigma or Aldrich.

## RESULTS

*Types of neurones responding to 5-HT in the raphe slice*

Previous studies (Williams *et al.* 1988) demonstrated two major populations of neurones in the raphe nucleus. One population of neurones did not respond to either 5-HT or phenylephrine, whereas the second group, which were identified as 5-HT-containing neurones, were hyperpolarized by 5-HT. In the present study neurones which did not respond to either 5-HT or phenylephrine did not respond to bombesin ($n = 7$). The second group of neurones ($n = 29$) was hyperpolarized by application of 5-HT (1–10 $\mu$M, 60 s), identifying them as 5-HT-containing raphe neurones. These neurones were used in studies of bombesin, NMB and GRP action on raphe neurones.

*Response of neurones to bombesin, gastrin-releasing peptide and neuromedin B*

Of the nineteen neurones which responded to bombesin (0·01–100 nM), none was found to be inhibited by it. A 60 s application of bombesin produced a fully reversible membrane depolarization which lasted for between 5 and 15 min. The amplitude of the bombesin response was not diminished when a subsequent application of the peptide was made some 10–20 min after the first application, indicating that little or no desensitization had occurred. Similar effects were seen with 10–1000 nM-GRP ($n = 5$) and 0·01–100 nM-NMB ($n = 6$). The effects of all the peptides was dose dependent and typical responses are shown in Figs 1, 2 and 3.

*Effects of other receptor ligands*

The bombesin-, NMB- and GRP-sensitive neurones were either insensitive ($n = 14$) or very weakly excited ($n = 2$) by 0·1–1·0 $\mu$M-CCK. Bath application of the specific $CCK_A$ and $CCK_B$ receptor antagonists L-364,718 (10 nM) and either L-365,260 (10 $\mu$M) or PD 134308 (10 $\mu$M) had no effect on the bombesin response ($n = 5$). Similarly the effects of bombesin, NMB and GRP were not changed by the $\alpha_1$-receptor antagonist prazosin (10 $\mu$M, $n = 3$) (Fig. 1). Bath application of the mixed

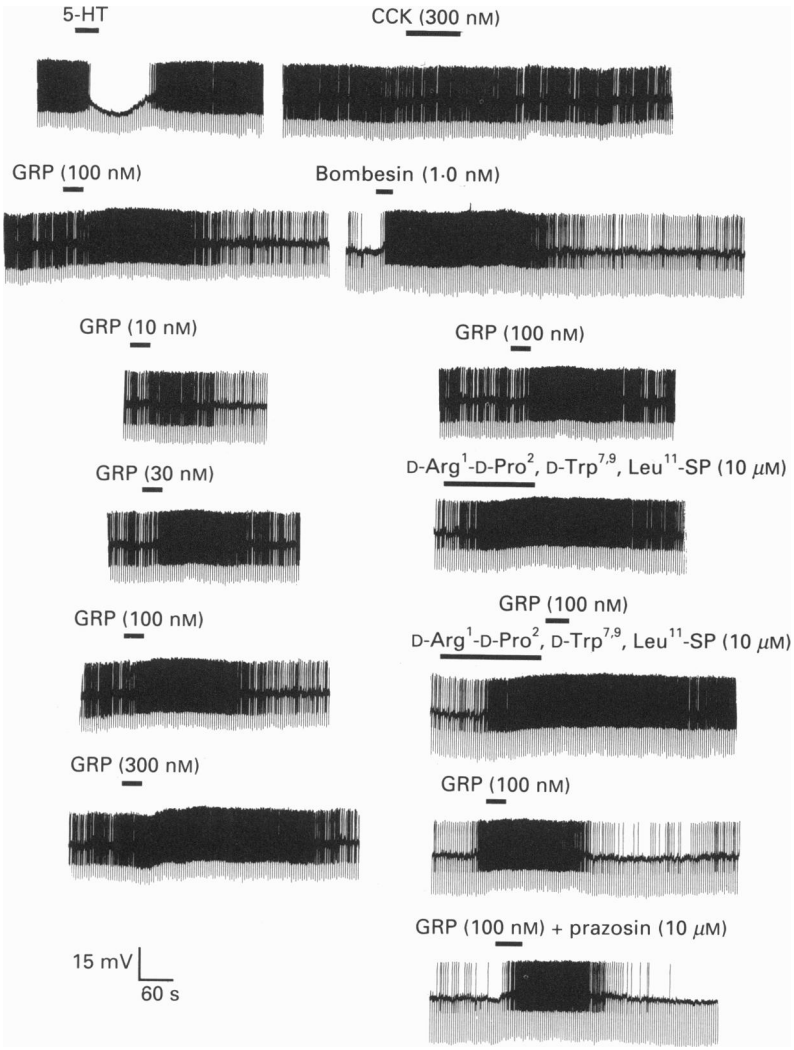


Fig. 1. Intracellular recording from a 5-HT-sensitive neurone in the dorsal raphe nucleus of the rat showing dose dependence of the GRP response and insensitivity of the GRP response to the mixed bombesin and substance P (SP) antagonist D-Arg¹,D-Pro²,D-Trp⁷,⁹,Leu¹¹-substance P and the adrenoreceptor antagonist prazosin. The neurone was hyperpolarized by bath application of 5-HT. The recording shows that CCK was inactive on this neurone whereas both bombesin and GRP caused a depolarization and increase in firing. Traces on the lower left show the response to GRP was dose dependent. Traces on the lower right show that exposure of the preparation to the bombesin antagonist D-Arg¹,D-Pro²,D-Trp⁷,⁹,Leu¹¹-substance P for 10 min caused a depolarization and an increase in firing. The response to a submaximal concentration of GRP (100 nM) was not occluded by this effect of D-Arg¹,D-Pro²,D-Trp⁷,⁹,Leu¹¹-substance P; immediately after the D-Arg¹,D-Pro²,D-Trp⁷,⁹,Leu¹¹-substance P response, the GRP response was still the same size. Perfusion of 10 μM-prazosin (the $\alpha_1$-adrenoreceptor antagonist) had no effect on the submaximal response to GRP.

substance P and bombesin antagonist, D-Arg[1],D-Pro[2],D-Trp[7,9], Leu[11]-substance P (10 $\mu$M), produced a depolarization without blocking the response to a submaximal dose of either GRP or bombesin ($n = 3$) (Figs 1 and 3). CI-977, a specific $\kappa$-opiate receptor agonist, had no effect on the bombesin response ($n = 3$).

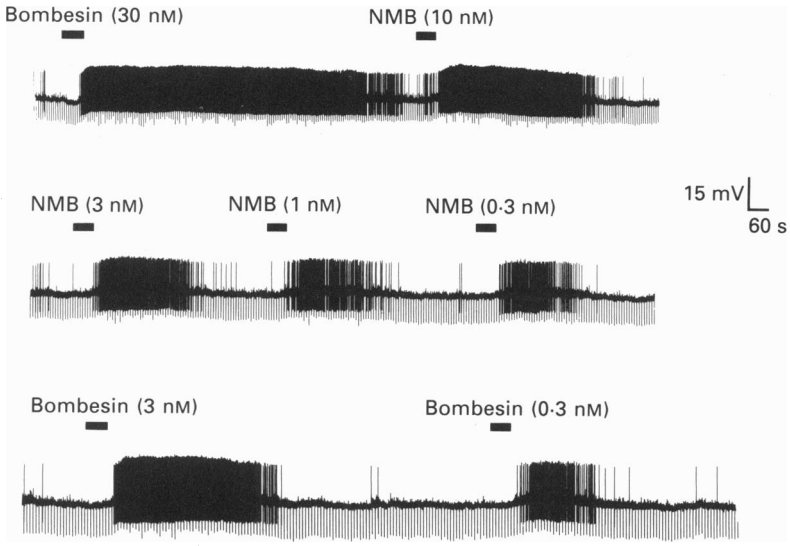


Fig. 2. Intracellular recording showing that both bombesin and NMB excite the same 5-HT-sensitive neurone in a dose-dependent manner. The three traces under current clamp conditions show the effects of different concentrations of bombesin and NMB on the same neurone.

*Effects of tetrodotoxin and 25 mM-magnesium ions*

Depolarizing responses to bombesin were not significantly different from control when recorded from neurones in slices treated with tetrodotoxin (TTX, 1 $\mu$M, for 10 min) and from neurones in slices perfused with TTX and calcium-free ACSF containing 10–25 mM-magnesium (Fig. 4).

*Conductance change associated with peptide action*

Under current clamp conditions the depolarizing response to the peptides was not consistently accompanied by a decrease in membrane conductance. However, under voltage clamp conditions, bombesin, NMB and GRP produced an inward current accompanied by a decrease in membrane conductance. The inward current produced by the peptides reversed at the same potential as the outward current evoked by 5-HT (Fig. 5).

## DISCUSSION

*Populations of neurones within the raphe nucleus*

Bombesin, GRP and NMB all produced a depolarization of the same 5-HT-sensitive raphe neurones. If a neurone did not respond to one of these three peptides, it was also insensitive to the other two peptides. Some 5-HT-sensitive neurones

responded to CCK but not to bombesin, NMB and GRP. Other 5-HT-sensitive neurones did not respond to either CCK or bombesin. Neurones which responded to bombesin but were insensitive to 5-HT were not encountered. Thus the bombesin-sensitive neurones represent a separate population of 5-HT-sensitive raphe neurones to those excited by CCK and those insensitive to both CCK and bombesin.

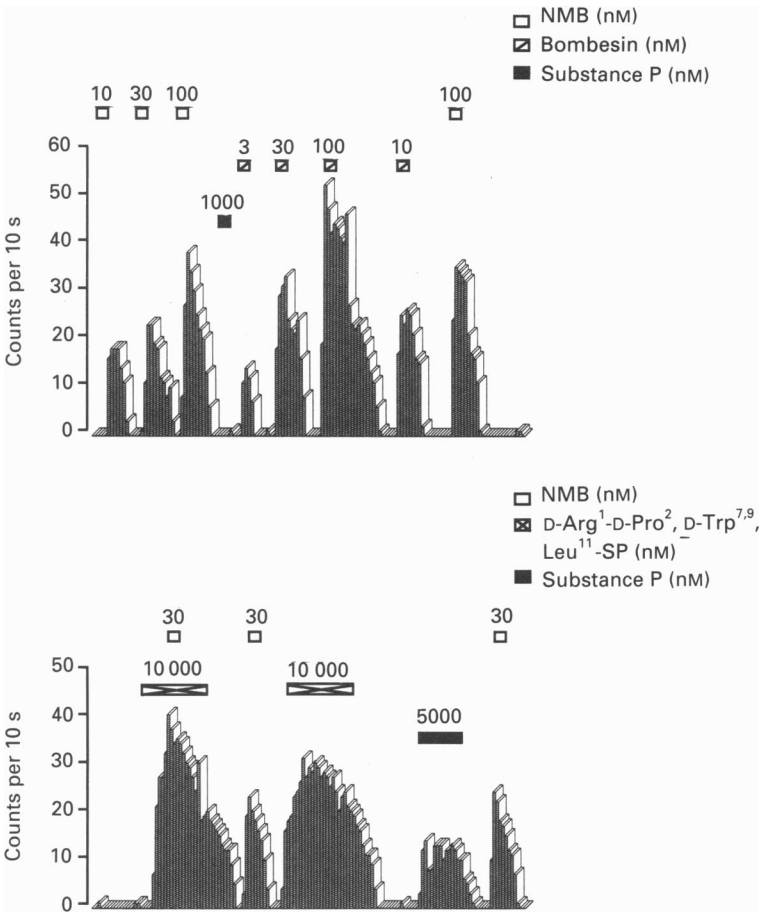


Fig. 3. Rate-meter recording from a neurone in the raphe nucleus showing the dose dependence of NMB and bombesin responses and the inability of D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P to antagonize the excitatory action of a submaximal dose of NMB. This neurone was silent at resting membrane potential and hyperpolarized by 5-HT. The top record shows that the responses to NMB (open bar) and bombesin (hatched bar) were dose dependent. Substance P had no effect at low concentrations (filled bar). The lower record shows that D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P applied for 10 min had an excitatory action of its own. The response to NMB was still present during this D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P response. The NMB response was the same size as that seen at the beginning of the experiment 6 min after ending the bath perfusion of D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P. A high dose of substance P applied for 6 min also weakly excited this neurone. The NMB response also remained intact after exposure to substance P.

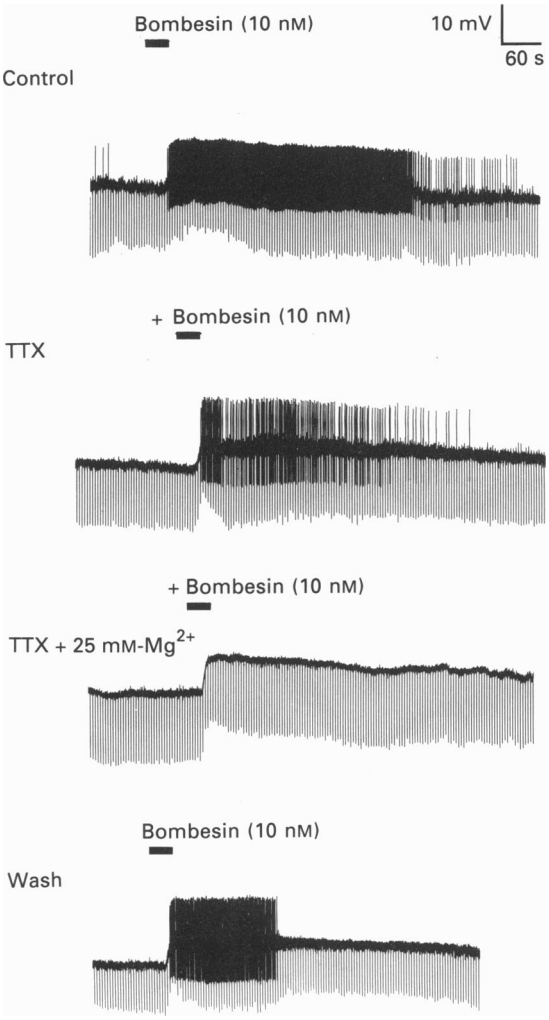


Fig. 4. Intracellular recording showing that the bombesin response is mediated by receptors on the postsynaptic membrane of the 5-HT-sensitive neurones. The top trace shows that bombesin depolarizes and increases the firing rate of the 5-HT neurone. The next trace shows that the response to bombesin remains in the presence of 1 $\mu$M-TTX. The neurone still fires action potentials in the presence of TTX, these action potentials are assumed to be calcium spikes since they do not occur during the bombesin response in the presence of 25 mM-$Mg^{2+}$ (third trace). The bottom trace shows that the effects of bombesin are still present after washing out the TTX and returning to normal ACSF containing 1·2 mM $Mg^{2+}$.

*Postsynaptic site of action for peptide actions*

The effects of bombesin, GRP and NMB persisted in the presence of TTX and low-calcium, high-magnesium solutions suggesting that the excitatory actions of the peptides were mediated by postsynaptic receptors on the membrane of 5-HT-containing neurones.

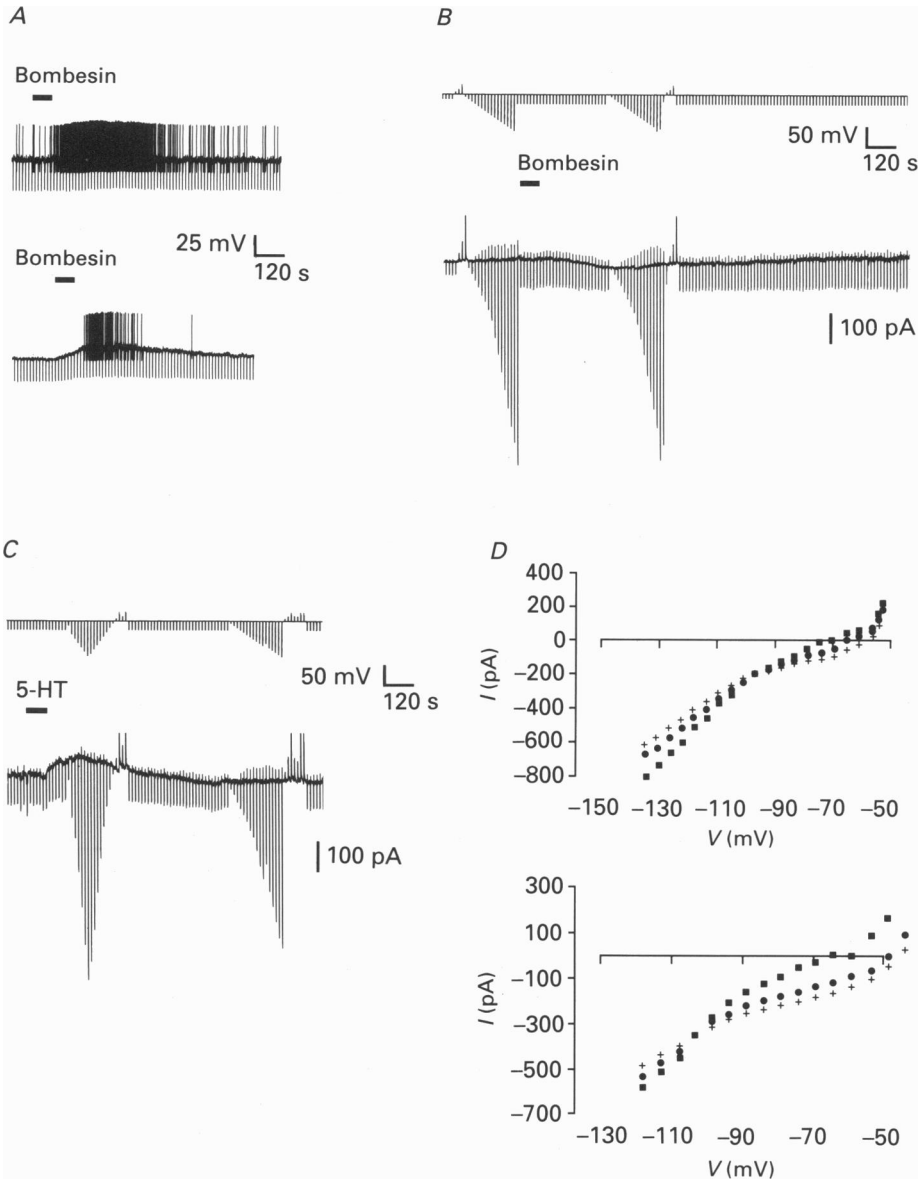


Fig. 5. Intracellular recording showing that the bombesin response and 5-HT response reverse at the same membrane potential. *A* shows the response to 10 nM-bombesin when membrane potential was held at −63 and −78 mV under current clamp conditions where the external potassium concentration was 3·5 mM. *B* is from the same neurone under voltage clamp conditions; the deflections in the traces are the currents required to produce the voltage steps in membrane potential. Bombesin causes an inward current with a decrease in membrane conductance of similar duration to that seen under current clamp conditions. *C* is from the same neurone as *A* and *B* showing the response to 5-HT under voltage clamp conditions. In *D* the current–voltage relationship for the neurone used in *A*, *B* and *C* is plotted in the top graph, whilst the lower graph is from another

*Conductance change associated with peptide responses*

No consistent change in membrane conductance was seen to accompany the depolarizing responses to the peptides under current clamp conditions. This was probably due to the non-linear current–voltage relationship of the membrane, since under voltage clamp conditions a decrease in conductance always accompanied the inward current. The reversal potential for the peptide actions was at the same potential as that described for 5-HT by Williams *et al.* (1988), indicating that a closure of potassium channels was responsible for the depolarization. The direction of the response was the same using either potassium chloride- or potassium acetate-filled recording electrodes, suggesting that it was unlikely that chloride was involved in mediating the response.

*Effects of other receptor ligands in the raphe nucleus*

Both the $\alpha_1$-adrenoreceptor agonist phenylephrine and CCK have been shown to excite dorsal raphe neurones (Aghajanian, 1985; Boden *et al.* 1991). Although two bombesin-sensitive neurones were weakly excited by high doses of CCK, CCK receptors were thought unlikely to be involved in the response to GRP, NMB and bombesin since the responses to GRP, NMB and bombesin were insensitive to the $CCK_A$ and $CCK_B$ receptor antagonists L-364,718, L-365,260, and PD 134308 at concentrations which block these receptors in both the raphe and other central nervous system (CNS) preparations (Kemp, Marshall & Woodruff, 1989; Boden *et al.* 1991; Hughes, Boden, Costall, Domeney, Kelley, Horwell, Hunter, Pinnock & Woodruff, 1990). Although all 5-HT-sensitive raphe neurones are known to be excited by noradrenaline, the responses to GRP, NMB and bombesin were insensitive to the $\alpha_1$-adrenoreceptor antagonist prazosin at concentrations which block $\alpha_1$-adrenoreceptors in the raphe (Aghajanian, 1985). In the guinea-pig ileum preparation, the substance P antagonist D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P, when applied in low micromolar concentrations, has been shown to produce a tenfold shift to the right of the bombesin dose–response curve (Leighton, Hill & Hughes, 1988). Similar concentrations of D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P did not have this action on the response to the peptides in the raphe suggesting that their action is more like that observed for bombesin in the colon, where bombesin-induced contractions were not affected by D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P (Leighton *et al.* 1988). Previous studies (Dreifuss & Raggenbass, 1986) have also found bombesin and substance P responses on non-pyramidal hippocampal neurones to be insensitive to D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P. In the present study both substance P and D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$-substance P had a weak excitatory action on the bombesin-sensitive neurones; however, this effect was unlikely to be mediated by substance P receptors since no response was achieved until micromolar concentrations of both agents were used. Although $\kappa$-opioid

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neurone where the external potassium concentration was 2·5 mM. The current–voltage curves for both bombesin (+) and 5-HT (■) intersect the control curve (●) at the same point in each case although the reversal potential is about −95 mV in the first case (external $K^+$, 3·5 mM) and about −105 mV (external $K^+$, 2·5 mM) in the second.

receptor agonists have been shown to antagonize bombesin-induced scratching behaviour (Gmerek & Cowan, 1988) there was no evidence that CI-977, a potent $\kappa$-opioid receptor agonist in other tests (Halfpenny, Horwell, Hughes, Hunter & Rees, 1990) reduced the effects of bombesin at the single neurone level in the raphe.

*Concluding remarks*

The present study has demonstrated that bombesin and the structurally related peptides GRP and NMB depolarize a subpopulation of raphe 5-HT neurones. These peptides depolarize neurones by acting on postsynaptically located receptors which results in a closure of potassium channels. The response to these peptides is independent of the actions of either phenylephrine or CCK.

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