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Characterisation of an endogenous bombesin receptor in CHO/DG44 cells

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Abstract

Bombesin and its receptors have been shown to have a role regulating circadian rhythms in the hamster suprachiasmatic and dorsal raphe nuclei and have been implicated in the regulation of sleep. We have identified and characterised a bombesin receptor endogenously expressed in a Chinese hamster ovary cell line (CHO/DG44). Using a range of bombesin-like peptides, we demonstrate that this receptor displays bombesin BB_2 receptor-like pharmacology. We also show that this receptor signals through inositol-[1,4,5]-trisphosphate and protein kinase C and thus provides a useful model system to aid in the interpretation of hamster suprachiasmatic nucleus studies of mammalian circadian rhythm.

Keywords: Bombesin; FLIPR (Fluorescent imaging plate reader); Chinese hamster ovary

1. Introduction

Bombesin is a tetradecapeptide originally isolated from the skin of the frog *Bombina bombina* (Anastasi et al., 1971). It is one of a family of related synthetic and natural peptides which have been widely used to pharmacologically characterise four distinct bombesin receptor subtypes (Fathi et al., 1993; Benya et al., 1994; Nagalla et al., 1995). These are the bombesin receptor 1 (BB_1), also known as the neuromedin B receptor (NMB receptor); the bombesin receptor 2 (BB_2), also known as the gastrin-releasing peptide receptor (GRP receptor); the bombesin receptor subtype 3 (BRS-3); and a receptor expressed only in amphibians known as bomesin receptor 4 (BB_4).

In the mammalian central nervous system bombesin receptors are involved in the regulation of homeostasis, thermoregulation, metabolism and behaviour (Kroog et al., 1995). In particular, bombesin receptors have been shown to act in the suprachiasmatic and dorsal raphe nuclei to regulate circadian rhythms, and thus have been implicated in the regulation of sleep (Piggins et al., 1995). The role of bombesin receptors in controlling circadian rhythm has also been extensively studied in hamster suprachiasmatic nuclei, both in vivo (Piggins et al., 1995) and in vitro (Piggins et al., 1994). The agonist pharmacology of these electrophysiological responses indicates that they are mediated by a bombesin BB_2 receptor (Piggins et al., 1994, 1995). However, these and subsequent studies have been hindered by the failure to isolate and clone the hamster bombesin receptor responsible for the activity observed.

Therefore, in the present study we have used a range of pharmacological tools which differentiate between the different bombesin receptor subtypes to identify and characterise a bombesin receptor endogenously expressed in Chinese hamster ovary (CHO/DG44) cells.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO/DG44) cells (ATCC) were routinely maintained as monolayers in minimal essential medium (MEM) supplemented with 10% fetal calf serum (Life Technologies, Paisley, UK) at 37°C.5%CO_2 in air. Cells were passaged every 3–4 days and the highest passage number used was 20.

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2.2. Intracellular calcium measurement

Prior to assay in FLIPR™ (Fluorometric Imaging Plate Reader, Molecular Devices, Sunnyvale, CA) CHO/DG44 cells were seeded at 30,000 cells per well in 96 well black wall, clear bottomed tissue culture plates (Costar) overnight. The cells were then incubated with the acetoxymethyl ester form of the Ca^{2+} sensitive dye Fluo-3 (4 μM; Teflabs, TX, USA) at 37°C.5%CO_2 in air for 60 min and subsequently washed four times with Tyrodes buffer containing 2.5 mM probenecid. Cells were then incubated in Tyrodes medium in the presence of either antagonist or Tyrodes buffer at 37°C.5% CO_2 in air for 30 min. Basal fluorescence was determined prior to agonist addition at 37°C by FLIPR (λ_{ex} = 488 nm, λ_{EM} = 540 nm, Sullivan et al., 1999). For each response the peak increase in fluorescence was calculated and iteratively curve-fitted using a four parameter logistic model (Bowen and Jerman, 1995). Antagonist affinities were calculated using the following equation:

$$K_B = \frac{IC_{50}}{1 + \frac{[Agonist](M)}{EC_{50}}}$$

Data are presented as mean ± S.E.M. and statistical comparisons were made, where appropriate, using the Student's *t*-test.

3. Results

All the agonists tested caused a large, rapid (peaking at 8–10 s post addition) and transient increase in intracellular

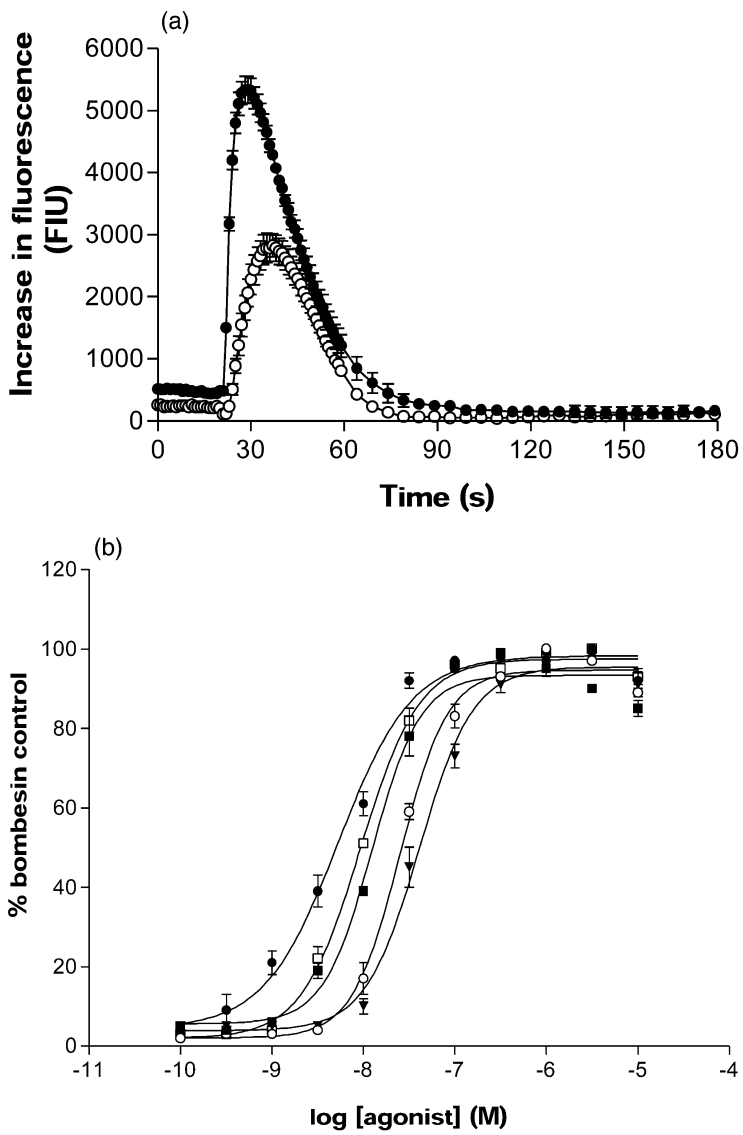

Fig. 1. (a) Timecourse of response to bombesin [1 μM] in the presence (●), and absence (○), of 2.5 mM extracellular Ca^{2+}. Data are means from eight determinations, error bars show S.E.M. (b) Concentration response curves for a range of bombesin-like peptides in CHO/DG44 cells. Ranatensin (●), acetyl neuromedin B (□), bombesin (■), neuromedin C (○) and neuromedin B (▼).

Ca^{2+} concentrations, as measured by fluorescence, which returned to baseline after 1 to 2 min (Fig. 1a).

In the absence of extracellular Ca^{2+}, the maximum Ca^{2+} response produced was reduced to 53.0 ± 1.4% (n = 8) of control (Fig. 1a) and agonist potencies were reduced approximately five-fold, for example bombesin (pEC_{50} ± S.E.M. (n)) 7.79 ± 0.05 (n = 11) reduced to 7.39 ± 0.08 (n = 8).

All the bombesin-like peptides tested were full agonists (Fig. 1b), with pEC_{50} values of: bombesin 7.79 ± 0.05 (n = 11); neuromedin B 7.48 ± 0.03 (n = 11); neuromedin C 7.77 ± 0.06 (n = 10); ranatensin 8.28 ± 0.05 (n = 5); PG-L (*Pseudophyrine guntheri* litorin-like peptide) 8.18 ± 0.05 (n = 4); bombesin [D-Phe^6,βAla^{11},Phe^{13},Nleu^{14}]-(6-14) 7.81 ± 0.07 (n = 8); bombesin [D-Tyr^6, βAla^{11},Phe^{13},*N*-leu^{14}]-(6-14) 7.88 ± 0.04 (n = 11); acetyl neuromedin B-(3-10) 7.99 ± 0.03 (n = 10); bombesin [D-Phe^6,Phe^{13}]-(6-13) propylamide 7.61 ± 0.03 (n = 8).

Substance P-[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}], *H*-D-2-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-2-Nal-NH_2 and bombesin-(6-13) methyl ester inhibited the bombesin induced response with pK_B values of 6.33 ± 0.06 (n = 5), 5.63 ± 0.06 (n = 5) and 9.47 ± 0.04 (n = 10), respectively (Fig. 2a).

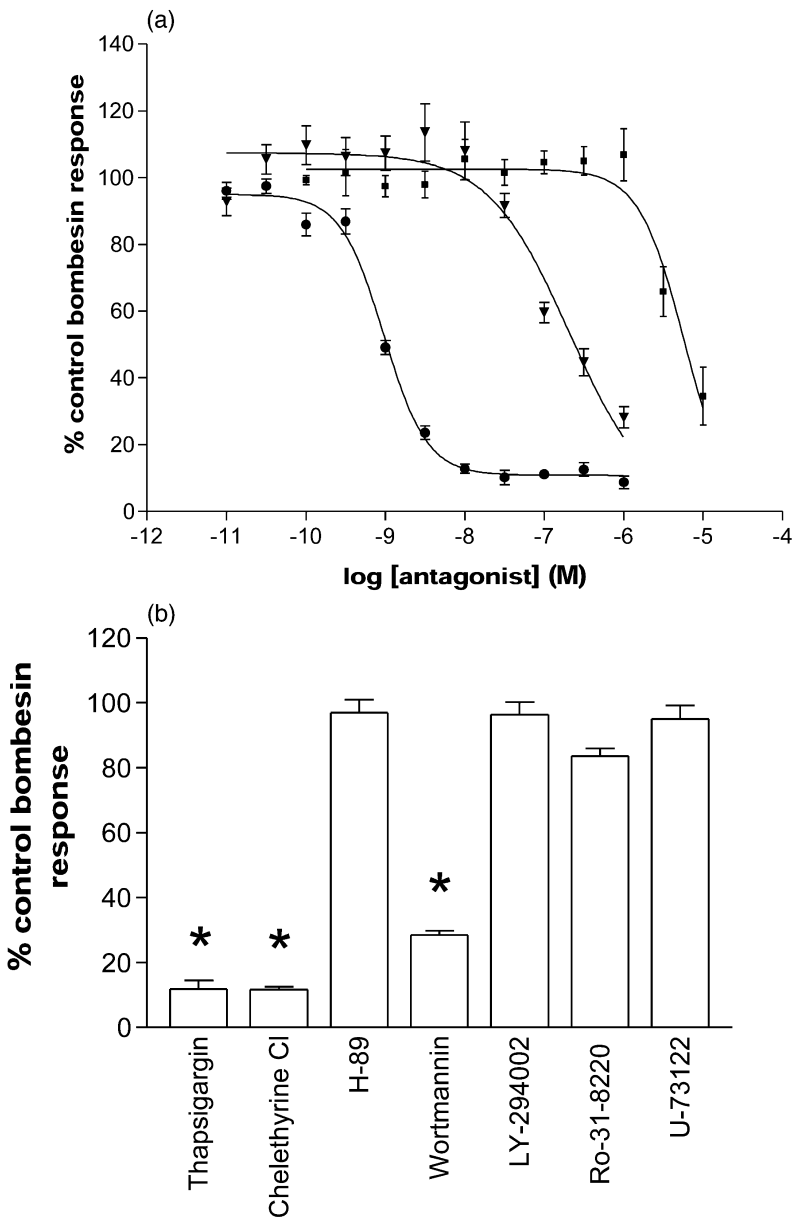

Fig. 2. (a) Inhibition by bombesin (6-13) methyl ester (●), *H*-D-2-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-2-Nal-NH_2 (■) and Substance P-[D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}] (▼) of a 30 nM bombesin response in CHO/DG44 cells. Data are expressed as percentage bombesin control response. Error bars show S.E.M. (n = 5–11). (b) The effect of various signal transduction modifying agents [10 μM] on the response to bombesin [30 nM] in CHO/DG44 cells. Bars are mean of 7–12 determinations, error bars show S.E.M. $^*P < 0.05$.

The signal transduction mechanism involved was examined using a range of agents. Thapsigargin, chelethyrine chloride and wortmannin inhibited the bombesin-induced response, with pK_B values of 7.68 ± 0.04 ($n = 12$), 6.04 ± 0.01 ($n = 12$), and 6.01 ± 0.07 ($n = 7$), respectively. {3-[1-[3-(Amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide, methane sulphonate} (Ro-31-8220) (10 μM) inhibited the bombesin-induced response by $17.5 \pm 2.5\%$ ($n = 8$) while {*N*-[2-((Bromocinnamyl)-amino)ethyl]-5-isoquinolinesulphonamide, HCl} (H-89), [2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] (LY-294002) and {1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione} (U-73122) (all 10 μM) were without significant effect ($n = 8$) (Fig. 2b).

4. Discussion

The principal pacemaker of circadian rhythm in mammals is located in the suprachiasmatic nucleus of the hypothalamus (Meijer and Reitveld, 1989). Bombesin-like peptides have been shown to influence the activity of the hamster suprachiasmatic nucleus both in vivo (Piggins et al., 1995) and in vitro (Piggins et al., 1994). It has also been shown electrophysiologically that the bombesin BB_2 receptor selective antagonist bombesin-(6-13) methyl ester blocks the physiological response to the bombesin-like peptides in hypothalamic slice preparations (Piggins et al., 1994). However, to date the bombesin receptor subtype responsible for this activity has not been cloned. Using the hamster derived immortalised cell line CHO/DG44 as a model system, an endogenous hamster bombesin BB_2 receptor subtype has been identified using a variety of natural and synthetic agonist and antagonist tools based around the structure of bombesin, and the receptor signalling pathway characterised.

The rank order of the agonist family members (ranatensin > PG-L > bombesin = bombesin[D-Phe^6,βAla^{11}, Phe^{13},Nleu^{14}](6-14) = bombesin [D-Tyr^6, βAla^{11},Phe^{13},*N*-leu^{14}]-(6-14) = neuromedin C > bombesin [D-Phe^6,Phe^{13}]-(6-13) propylamide > neuromedin B) extends that found by Benya et al. (1995) and indicates that CHO/DG44 cells express an endogenous bombesin BB_2 receptor. This tentative delineation of the bombesin BB_2 receptor subtype was confirmed by the relative activity against a bombesin induced agonist response of the non-selective antagonist Substance P-[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}] (Jensen et al., 1984), the selective bombesin BB_1 receptor/bombesin BRS-3 receptor antagonist *H*-D-2-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-2-Nal-NH_2 (Orbuch et al., 1993; Smart and Ranson, 2000) and the bombesin BB_2 receptor selective antagonist bombesin-(6-13) methyl ester (Benya et al., 1994).

Signalling of bombesin-like peptides occurs through coupling of the receptors to heterotrimeric G-proteins (Woodruff et al., 1996). The signalling mechanism utilised by the endogenous bombesin BB_2 receptor in the CHO/DG44 cell was examined using a range of signal transduction modifying agents (Smart and Wood, 2000). The inhibition by thapsigargin suggests that signalling via the endogenous BB_2 receptor involves the mobilisation of Ca^{2+} from inositol trisphosphate (IP_3) sensitive intracellular stores. Blockade of the response by chelethyrine chloride indicates that the response mediated by the bombesin BB_2 receptor also activates protein kinase C. Taken collectively, the blockade of the response by wortmannin and the absence of any effect with LY 294002 suggests a role for Phospholipase D, but not phosphoinostide-3-kinase, in the signalling cascade. The lack of effects of U-73122 and H-89 indicate that phospholipase C, and protein kinase A are not obviously involved in the Ca^{2+} response. However, the reduced agonist response in terms of both potency and maximum signal in the absence extracellular Ca^{2+} suggests that receptor activation results in Ca^{2+} influx. This examination of the intracellular signalling cascade confirms that the endogenous bombesin receptor expressed in CHO/DG44 cells signal via inositol trisphosphate sensitive intracellular stores and protein kinase C as described by Kroog et al. (1995).

In conclusion, the immortalised hamster CHO/DG44 cell line is a useful model for assessing the pharmacology of bombesin ligands at the hamster bombesin BB_2 receptor, and this will aid the interpretation of hamster suprachiasmatic nucleus model studies.

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