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SPECIAL REPORT

Potent antagonism by BIM-23056 at the human recombinant somatostatin sst_5 receptor

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We have investigated the effects of somatostatin (SRIF) and the linear octapeptide BIM-23056 on changes in intracellular calcium ion concentration ($[Ca^{2+}]_i$) and on the formation of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) in CHO-K1 cells transfected with the human recombinant SRIF sst_5 receptor. SRIF elicited concentration-dependent increases in $[Ca^{2+}]_i$, with a pEC_{50} of 7.02 ± 0.06, while BIM-23056 (1×10^{-7} M) behaved not as an agonist but as a potent, surmountable antagonist of these increases in $[Ca^{2+}]_i$. The SRIF concentration-effect curve for increases in $[Ca^{2+}]_i$ was shifted rightward producing an estimated pK_B for the antagonist of 8.0. BIM-23056 (1×10^{-7} M) also significantly attenuated $Ins(1,4,5)P_3$ increases due to SRIF, but had no effect on either basal or uridine 5'-triphosphate (UTP) (1×10^{-4} M) stimulated increases in the levels of $[Ca^{2+}]_i$ or $Ins(1,4,5)P_3$.

Keywords: Somatostatin receptors; sst_5; calcium; inositol-1,4,5-trisphosphate; antagonist; BIM-23056

Introduction Somatostatin (SRIF) is a tetradecapeptide which exerts its effects via activation of G-protein coupled seven transmembrane receptors. To date genes for five distinct SRIF receptors (sst_1 to sst_5) have been cloned (see Hoyer *et al.*, 1995, for review). When transfected into cell lines, only the sst_2 and sst_5 receptors have been shown to mediate marked elevations in inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) formation and intracellular calcium ion concentration ($[Ca^{2+}]_i$), effects involving pertussis toxin-sensitive mechanisms (Akbar *et al.*, 1994).

Characterization of the functional properties of (SRIF) receptors has been hindered by a paucity of potent and specific antagonists. In this paper we describe the ability of SRIF to induce elevations in ($[Ca^{2+}]_i$) and to stimulate the formation of ($Ins(1,4,5)P_3$), in CHO-K1 cells expressing the human recombinant sst_5 receptor and describe the novel observation that BIM-23056, a ligand shown to displace agonist binding at sst_3, sst_4 and sst_5 receptors (Patel & Srikant, 1994), displays potent and specific antagonism of these SRIF-induced responses.

Methods CHO-K1 cells ($CHOsst_5$) stably expressing the human recombinant sst_5 receptor were produced by transfection of the sst_5 DNA, after subcloning into the vector pCIN4, a derivative of the high expression vector pCIN1 (see Rees *et al.*, 1996). The sst_5 DNA within this plasmid was shown by double stranded sequence analysis to be identical to that described by Yamada and colleagues (Yamada *et al.*, 1993). The cells were maintained in Dulbecco's modified Eagles Medium/Hams F-12 nutrient (1:1) mix supplemented with Glutamax, 10% foetal calf serum (Life Technologies, Paisley, U.K.) and G418 (0.5 mg ml^{-1}). Cultures were maintained at 37°C in a 5% CO_2/humidified air atmosphere. Experiments were performed in a HEPES buffered salt solution (HBSS) of the following composition (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, NaH_2PO_4 1, $MgSO_4$ 0.8, D-glucose 5.5, HEPES 20, $CaCl_2$ 1.3 and buffered to pH 7.4 with NaOH. For calcium imaging experiments, 0.1% (w:v) bovine serum albumin (BSA) was added to the buffer.

To measure changes in $[Ca^{2+}]_i$, cells were plated onto glass coverslips and after being left overnight, were incubated with 4 μM Fura-2AM for 30 min at 37°C. The cells were then washed and incubated for a further 60 min at 37°C in the presence of either vehicle as a control or BIM-23056 (1×10^{-7} M) before addition of SRIF or uridine 5'-triphosphate (UTP). Fluorescence imaging measurements were made using an Axiovert 135 TV inverted microscope connected to an ISIS CCD camera (Photonic Science) at 400 × magnification. Calcium ion concentration was determined at 22°C from the ratio of fluorescence intensities at excitation wavelengths of 340 and 380 nm, with an emission wavelength of 510 nm.

$Ins(1,4,5)P_3$ formation was determined in confluent cells after incubation with either vehicle as a control or BIM-23056 (1×10^{-7} M) for one hour at 37°C. The cells were stimulated with either vehicle or agonist, in the presence or absence of BIM-23056, for 10 s. Incubations were terminated by the addition of ice-cold 1.0 M trichloroacetic acid (TCA). Aliquots were taken and 10 mM EDTA added. TCA was extracted with a 1:1 (v:v) mixture of tri-n-octylamine and 1,1,2-trichlorofluoroethane and samples were neutralised with 25 mM $NaHCO_3$. Measurement of $Ins(1,4,5)P_3$ mass was made with a standard radioreceptor assay kit (Amersham, U.K.).

Concentration-effect curve data were fitted to a four parameter logistic equation using Graphpad. Agonist concentration ratios were used to estimate the equilibrium dissociation constant (pK_B) for the antagonist by use of the Gaddum equation (see Jenkinson *et al.*, 1995).

All values are expressed as the mean ± s.e.mean or the geometric mean (95% confidence limits) of n determinations.

Drugs SRIF (Peninsula Laboratories), UTP (Sigma). BIM-23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2) was custom synthesized by Peptide and Protein Research Consultants, University of Exeter, U.K. Stock solutions of drugs were dissolved in deionised water (18 MΩ) (except for BIM-23056, which contained 10% (v/v) DMSO) and were then made up in HBSS.

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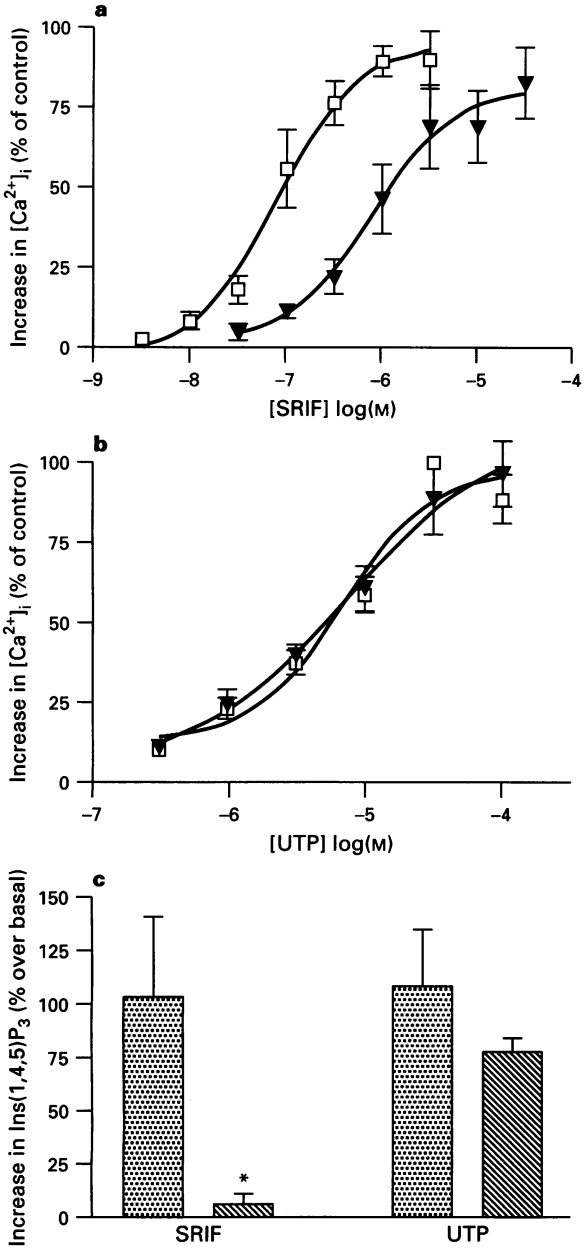

Figure 1 Responses to somatostatin (SRIF) and UTP in CHO-K1 cells, expressing human recombinant sst_5 receptors. Concentration-effect curves are shown for increases in intracellular Ca^{2+} ion concentration produced by (a) SRIF and (b) UTP in the absence (□) and presence of 1×10^{-7} M (▼) BIM-23056. Peak increases in $[Ca^{2+}]_i$ were measured and corresponding basal values deducted before being expressed as a percentage of its own curve maximum. The maximal increases in $[Ca^{2+}]_i$ in response to SRIF in the absence or presence of BIM-23056 were not significantly different from one another being 307.3 ± 62.5 and 279.8 ± 55.3 nM ($n=6$), respectively. Data are the mean ± s.e.mean of six separate experiments in which the response from 6 to 12 individual cells per coverslip was averaged for each concentration of SRIF. A separate coverslip was used for each determination. (c) Percentage increase over basal of formation of inositol-1,4,5-trisphosphate in response to SRIF (1×10^{-6} M) or UTP (1×10^{-4} M) after pretreatment for 1 h with either vehicle (stippled columns) or BIM-23056 (1×10^{-7} M) (hatched columns). Data are the mean ± s.e.mean of 3 to 6 triplicate experiments. Significant differences ($P<0.05$) from vehicle in the presence of BIM-23056 are indicated by the asterisk.

Results *Effects on increases in intracellular calcium ion concentration* SRIF increased $[Ca^{2+}]_i$ in $CHOsst_5$ cells, in a concentration-dependent manner (pEC_{50} 7.02 ± 0.06), from resting levels of 39.7 ± 3.6 nM to 347.0 ± 67.0 nM at 10^{-6} M ($n=6$) and had no effect on untransfected cells. BIM-23056 (1×10^{-7} M) produced a significant rightward shift of the concentration-effect curve to SRIF (Figure 1a), equivalent to a SRIF concentration ratio of 13.1 (2.2–77), which corresponds to an estimated pK_B for BIM-23056 at the sst_5 receptor of 8.03 ± 0.33 ($n=6$). BIM-23056 (1×10^{-7} M) had no effect on resting $[Ca^{2+}]_i$ levels or on repsonses to UTP (Figure 1b).

Effects on inositol - 1,4,5 - trisphosphate formation Incubation of $CHOsst_5$ cells with SRIF (1×10^{-6} M) or UTP (1×10^{-4} M) produced peak increases in $Ins(1,4,5)P_3$ between 10 and 15 s (data not shown). $Ins(1,4,5)P_3$ levels in unstimulated cells were 10.3 ± 1.2 pmol per well ($n=6$), the maximum levels after 10 s were 11.3 ± 0.9, 22.3 ± 3.2 and 23.6 ± 2.1 pmol per well ($n=6$) for vehicle, SRIF and UTP, respectively. Preincubation with BIM-23056 (1×10^{-7} M) for 1 h attenuated responses to SRIF (15.2 ± 1.6 pmol per well, $P<0.05$, $n=3$), while responses to vehicle and UTP were not significantly affected, being 14.3 ± 0.6 and 23.3 ± 0.9 pmol per well ($n=3$), respectively (Figure 1c).

Discussion SRIF caused an increase in both $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ in $CHOsst_5$ cells with a potency similar to that obtained by Akbar *et al.* (1994) for increases in inositol phosphate formation in COS-7 cells.

There have been few studies of selective antagonism at SRIF receptors (Hoyer *et al.*, 1995). In the present study we have demonstrated for the first time that BIM-23056 behaves as a potent and surmountable antagonist at the human recombinant sst_5 receptor. This antagonism was specific for the sst_5 receptor as BIM-23056 did not inhibit $[Ca^{2+}]_i$ or $Ins(1,4,5)P_3$ increases in response to UTP. The estimated pK_B value for BIM 23056 was similar to its equivalent affinity estimate for binding to this receptor expressed in CHO-K1 cells (Patel & Srikant, 1994; W. Feniuk, unpublished observations). Patel & Srikant (1994) have shown in radioligand binding studies that BIM-23056 has a high affinity for sst_3 and sst_4 as well as sst_5 receptors. It remains to be determined whether it behaves as an agonist or an antagonist at sst_3 and sst_4 receptors.

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