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Homologous and heterologous desensitisation of somatostatin-induced increases in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate in CHO-K1 cells expressing human recombinant somatostatin sst_5 receptors

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Abstract

The mechanisms responsible for somatostatin (SRIF)-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent desensitisation were studied in CHO-K1 cells expressing human sst_5 receptors (CHOsst_5 cells). To study the nature of the desensitisation, interactions with uridine triphosphate (UTP) were examined. SRIF (pEC_{50} 7.10) and UTP (pEC_{50} 5.14) caused concentration-dependent increases in $[Ca^{2+}]_i$ but the SRIF maximum was about 40% of that to UTP. SRIF-, but not UTP-, induced increases in $[Ca^{2+}]_i$ were transient and abolished by pertussis toxin. SRIF and UTP caused sustained increases in Ins(1,4,5)P_3 but the SRIF maximum was about 30% of that to UTP. Removal of $[Ca^{2+}]_e$ attenuated the SRIF-induced peak rise in $[Ca^{2+}]_i$ but had no effect on the peak increases in Ins(1,4,5)P_3. UTP-induced increases in $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 were attenuated in the absence of $[Ca^{2+}]_e$. Following pre-exposure to SRIF (1 μM) or UTP (100 μM) for 5 min, subsequent SRIF responses were desensitised. Similar results were obtained in the absence of $[Ca^{2+}]_e$. Pre-exposure to SRIF had no effect on subsequent responses to UTP but in the absence of $[Ca^{2+}]_e$, responses to UTP were attenuated. The results suggest that SRIF but not UTP-induced increases in $[Ca^{2+}]_i$ in CHOsst_5 cells are mediated by pertussis toxin sensitive G proteins and are caused by an entry of extracellular Ca^{2+} and release from an Ins(1,4,5)P_3 sensitive Ca^{2+} store. Homologous or heterologous desensitisation of agonist-induced increases in $[Ca^{2+}]_i$ could be demonstrated in the presence or absence of extracellular Ca^{2+} respectively, and the latter appeared to involve depletion of a common intracellular Ca^{2+} store. © 1997 Elsevier Science B.V.

Keywords: Somatostatin; Somatostatin sst_5 receptor; Desensitisation; CHO-K1 cell; Ca^{2+}

1. Introduction

The tetradecapeptide somatostatin (SRIF) exerts a wide range of effects in the brain and periphery. Some of these effects, e.g. inhibition of gastric acid secretion (Wyatt et al., 1996) and inhibition of electrogenic ion transport in the colonic mucosa (McKeen et al., 1994) are well maintained in the continuous presence of agonist and show no evidence of desensitisation. Other responses, such as contraction of the rat distal colon (McKeen et al., 1994) and contraction of the human saphenous vein which are dependent upon the external Ca^{2+} concentration (Dimech et al., 1995) are susceptible to agonist-induced desensitisation. Indeed, in the absence of specific SRIF receptor blocking drugs, agonist-induced receptor desensitisation has been used, not only to characterise specific SRIF receptor binding sites (Rens-Domiano et al., 1992) and to determine the specificity of SRIF receptor ligands (Feniuk et al., 1995) but also to discriminate between SRIF receptor subtypes in functional studies in vitro (Liapakis et al., 1996). For example, the coupling of the sst_2 receptor to L-type Ca channels in AtT-20 cells was resistant to agonist induced desensitisation whilst the sst_5 receptor which is also expressed in these cells was readily desensitised (Liapakis et al., 1996). The mechanisms responsible for SRIF induced desensitisation are poorly understood although SRIF receptors on S49 lymphoma cells which are coupled to inhibition of adenyl cyclase have been shown to be phosphorylated by a β-ARK-like kinase (Mayor et al., 1987).

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Activation of human recombinant sst_5 receptors expressed in CHO-K1 cells (CHOsst5 cells) results in an activation of phosphoinositide metabolism and an increase in intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) (Akbar et al., 1994; Wilkinson et al., 1996a,b, 1997). This increase in intracellular ($[Ca^{2+}]_i$) is subject to marked desensitisation in the continuous presence of SRIF (Wilkinson et al., 1996a). In the present study we sought to investigate the mechanism responsible for SRIF-induced increases in $[Ca^{2+}]_i$ in $CHOsst_5$ cells in an attempt to gain some insight into the mechanisms responsible for desensitisation of the Ca^{2+} response. For comparison, increases in $[Ca^{2+}]_i$ and phosphoinositide metabolism following activation of endogenous P_{2U} receptors were also studied in order to determine the specificity of the SRIF-induced desensitisation.

A preliminary account of some of these finding has been reported to the British Pharmacological Society (Wilkinson et al., 1996a).

2. Materials and methods

2.1. Cell culture

CHO-K1 cells stably expressing human recombinant sst_5 receptors ($CHOsst_5$) (Wilkinson et al., 1996b) at approximately 3.0 pmol mg^{-1} protein (Williams et al., 1997), were grown in Dulbecco's modified Eagles Medium/Hams F-12 nutrient (1:1) mix supplemented with Glutamax, 10% foetal calf serum and G418 (0.5 mg ml^{-1}) and maintained at 37°C in a 5% CO_2/humidified air atmosphere. Increases in intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) were measured in individual or small populations (8–12 cells) of cells plated onto the centre of a 24 mm diameter glass coverslip (Chance Propper, UK). $Ins(1,4,5)P_3$ formation was measured in confluent cell monolayers subcultured into 24-well multiplates.

2.2. Measurement of $[Ca^{2+}]_i$ in single cells and small populations of cells.

Cells grown overnight on 24 mm diameter glass coverslips were washed twice with 2 ml of buffer and then incubated for 30 min at 37°C with 2 ml of buffer containing 4 μM fura-2AM (Grynkiewicz et al., 1985). The buffer containing fura-2AM was subsequently removed and the cells were again washed twice with 2 ml of buffer and left for a further 30 min at 37°C to allow de-esterification of the dye. Coverslips were mounted in a teflon coverslip holder (Digitimer, UK) which was placed onto the stage of a Zeiss Axiovert 135TV inverted microscope connected to an ISIS CCD camera (Photonic Science) and perfused with buffer at 0.5 ml min^{-1} at room temperature. The volume of perfusate covering the cells was maintained at 0.5 ml by continuous aspiration of the effluent. Drugs or vehicle were added onto the cover slip in a volume of 0.5 ml and the perfusion was stopped whilst recordings were made. Preliminary studies showed that there was no difference in the magnitude of the responses obtained by either conditions of continuous drug perfusion or under conditions when flow was stopped. A 75 W xenon arc lamp was used to illuminate the cells. After placing the coverslip on the microscope stage all further procedures were carried out in the dark to avoid photobleaching of the dye. Excitation wavelengths were 340 and 380 nm and emission was recorded at 510 nm. Successive recordings were made at either every 1 or 4 s and data from four frames were averaged at each excitation wavelength. Autofluorescence was determined using a part of the coverslip covered by buffer without cells. Optical density filters were adjusted for individual coverslips to compensate for differences in fura-2 loading. Filter wheel changes and fluoresence imaging measurements were made using 'IonVision' software (Improvision, UK) controlled by an Apple Macintosh Quadra 840 computer.

Graphical representations of $[Ca^{2+}]_i$ changes are described using raw 340/380 emission ratio data. However, because of the non-linearity of the increase in 340/380 ratio values at high $[Ca^{2+}]_i$, some data has been quantified by converting the 340/380 ratios to absolute Ca^{2+} concentrations by comparison to a standard curve generated from known Ca^{2+} concentrations (Molecular Probes, USA).

2.3. Measurement of D-Ins(1,4,5)P_3 formation

$CHOsst_5$ cells in 24-well multiplates were washed twice with 250 μl of Krebs/HEPES buffer of the following composition (mM): NaCl (125), KCl (5.4), $NaHCO_3$ (16.2), D-Glucose (5.5), HEPES, (20), NaH_2PO_4 (1), $CaCl_2$ (1.3), buffered to pH 7.4 with NaOH (5 M) and gassed with 95% O_2/5% CO_2 and left for 10 min at 37°C in 250 μl of buffer per well. When the effects of removal of extracellular Ca^{2+} were studied, the cells were washed as above but in Ca^{2+}-free buffer containing 1 mM EGTA, which resulted in a free Ca^{2+} ion concentration of 100–150 nM.

Experiments were initiated by the removal of the wash buffer and its immediate replacement with 150 μl of buffer with or without agonist. Incubations were terminated by the addition of 150 μl of ice cold 1.0 M trichloroacetic acid (TCA), except for the zero time point where the TCA was added at the same time as the buffer. Multiwell plates were then put on ice for 15 min. A 160 μl aliquot of the incubate was added to 40 μl of 10 mM EDTA. The TCA was extracted with a 1:1 (v/v) mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoro-ethane (200 μl), followed by vortexing for 15 s. The samples were then left at room temperature for 15 min and subsequently centrifuged at $13000 \times g$ for 3 min. The upper aqueous

phase (100 μl) was taken and 50 μl of 25 mM $NaHCO_3$ was added.

Ins(1,4,5)P_3 mass was measured using a modification of the method described by Challiss et al. (1990). Aliquots (50 μl) of experimental samples were mixed with equal volumes of [^{3}H]Ins(1,4,5)P_3 (44 Ci mmol^{-1}) in water and assay buffer (100 mM Tris–HCl, 4 mM EDTA). The incubation was initiated by the addition of 50 μl of bovine adrenal cortical membranes and was allowed to proceed at 4°C for 15 min. Incubations were terminated by centrifugation for 10 min at 5000 × *g* at 4°C. After aspiration of the supernatant, the protein pellet was dissolved with 250 μl of 0.1 M NaOH to which 4 ml of scintillation cocktail was added. Ins(1,4,5)P_3 levels were determined by comparison with a standard calibration curve generated using known amounts of D-Ins(1,4,5)P_3; non-specific binding was typically 10–15% of total.

2.4. Interaction studies

In some experiments the effect of pre-exposing cells to either SRIF or UTP on subsequent agonist-induced increases in $[Ca^{2+}]_i$ was studied. After preincubation with media in either the presence or absence of extracellular Ca^{2+}, vehicle or agonist was added for 5 min followed by a second challenge with either SRIF or UTP. In some experiments, varying concentrations of SRIF were added in the preincubate. In another series of experiments, the initial SRIF challenge was removed and the cells continually perfused with buffer for varying times to assess the recovery of the response.

2.5. Drugs and reagents

Unless otherwise stated, all reagents were purchased from Sigma. Tissue culture media were from Life Technologies and tissue culture ware were obtained from Costar. Somatostatin was from Peninsula Laboratories, ethyleneglycol-*bis* (β-aminoethylether) *N,N,N′,N′*-tetracetic acid (EGTA) was from BDH, fura-2AM (penta-acetoxymethylester) and pertussis toxin were from Calbiochem and D-*myo*-inositol-1,4,5-trisphosphate assay kits were from Amersham, UK.

2.6. Analyses

Concentration–effect curves were analyzed by Graph Pad Prism, fitting the data to a four parameter logistic function, pEC_{50} values thus determined are presented as the mean $-\log_{10}$ M ± S.E.M. All other data are expressed as the mean ± S.E.M. of *n* separate determinations (experiments). Differences between values were tested for statistical significance using Student's *t*-test, with significance accepted at $P < 0.05$.

3. Results

3.1. Effect of SRIF and UTP on changes in $[Ca^{2+}]_i$ and dependency on $[Ca^{2+}]_e$

Both SRIF (3 nM–10 μM) and UTP (0.3–100 μM) caused concentration-dependent increases in $[Ca^{2+}]_i$ in $CHOsst_5$ cells loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2 (Fig. 1). However, the magnitude of the change, their kinetics and relative potency were markedly different. Whilst SRIF (pEC_{50} 7.27 ± 0.15) was approximately 100 times more potent than UTP (pEC_{50} 5.14 ± 0.09), the maximum increase in $[Ca^{2+}]_i$ produced by SRIF was significantly ($P < 0.05$) smaller than that produced by UTP. A maximally effective concentration of SRIF (1 μM) increased $[Ca^{2+}]_i$ from a resting value of 43.2 ± 5.2 nM to 326.0 ± 49.0 nM ($n = 12$) whilst a maximally effective concentration of UTP (100 μM) increased $[Ca^{2+}]_i$ from 59.7 ± 5.2 to 775.0 ± 172.0 nM ($n = 7$). Both SRIF and UTP-induced increases in $[Ca^{2+}]_i$ were rapid in onset, peaking 10–15 s after agonist addition. However responses to SRIF waned in the continuous presence of agonist (Fig. 1A) returning to baseline within 150 s whilst responses to UTP were more sustained and were still elevated some 300 s after agonist addition (Fig. 1B).

SRIF (1 μM)-induced increases in $[Ca^{2+}]_i$ were abolished when cells were pre-incubated for 18 h with pertussis toxin (100 ng ml^{-1}) ($n = 6$). In contrast, pertussis toxin pretreatment had no effect on UTP-induced increases in $[Ca^{2+}]_i$ (peak level in the presence of pertussis toxin was, 772 ± 118 nM, $n = 5$).

In further experiments, SRIF (1 μM) and UTP (100 μM)-induced changes in peak $[Ca^{2+}]_i$ were studied in the presence and absence of external Ca^{2+}. SRIF- and UTP-induced increases in peak $[Ca^{2+}]_i$ were attenuated in the absence of extracellular Ca^{2+} (Fig. 2). In the presence of Ca^{2+}, SRIF and UTP increased $[Ca^{2+}]_i$ from basal values of 56.0 ± 6.5 and 62.0 ± 7.2 nM to 314 ± 79 and 627 ± 145 nM, respectively ($n = 6$ and 5). In the absence of Ca^{2+}, the SRIF and UTP increases in $[Ca^{2+}]_i$ from basal values of 43 ± 6 and 61 ± 6 nM to stimulated levels of 174 ± 56 and 327 ± 98 nM, respectively ($n = 6$ and 4). Whilst removal of extracellular Ca^{2+} had little effect on the time course of SRIF-induced increases in $[Ca^{2+}]_i$ (Fig. 2A), the sustained phase of the UTP-induced rise in $[Ca^{2+}]_i$ was markedly attenuated (Fig. 2B).

3.2. SRIF and UTP-induced increases in Ins(1,4,5)P_3 formation

SRIF (0.1–30 μM) caused concentration-dependent increases in peak Ins(1,4,5)P_3 formation (pEC_{50} 5.8 ± 0.3, $n = 4$). The potency of SRIF for increases in Ins(1,4,5)P_3 were approximately 30 times less than for stimulating increases in $[Ca^{2+}]_i$ (see above).

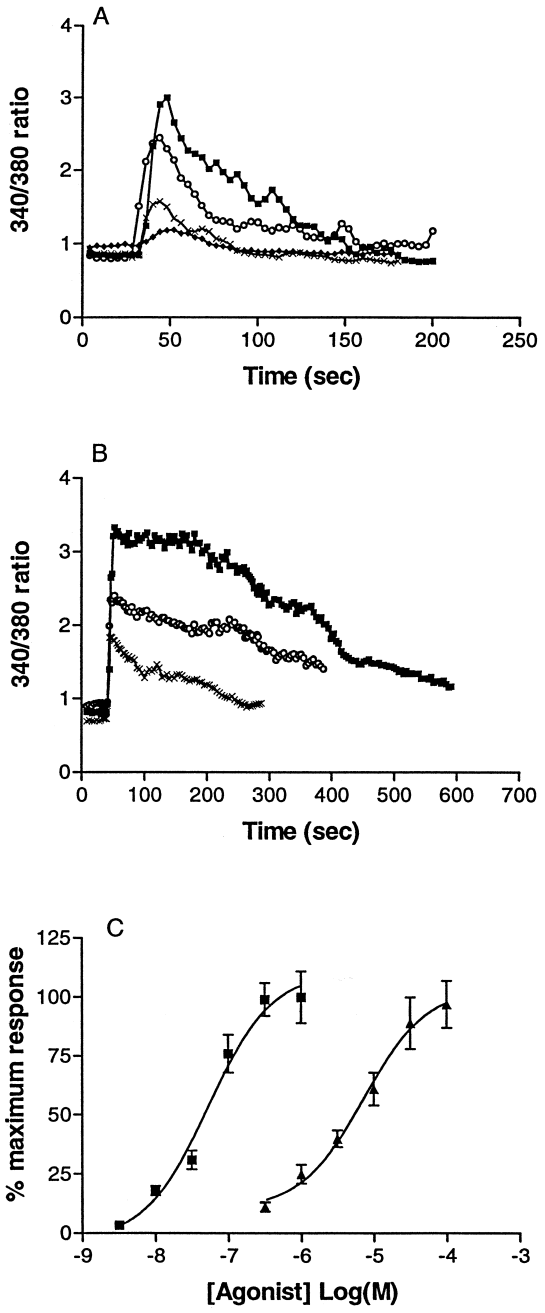

Fig. 1. Time- and concentration-dependent increases in intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) in fura-2 loaded $CHOsst_5$ cells. (A) cells were stimulated with 10 nM (♦), 100 nM (×), 1 μM (○) or 10 μM (■) SRIF, or (B) 1 μM (×), 10 μM (○) or 100 μM (■) UTP and subsequent changes in 340/380 ratio monitored. Each trace is the meaned response from 8–12 individual cells and is representative of at least 4 separate experiments. Agonist was added at about 35s and remained in contact with the cells throughout the recording period. (C) concentration-effect curves for peak $[Ca^{2+}]_i$ changes in response to SRIF (■) or UTP (▲). Data are expressed as a percentage of their individual maximum response to each agonist following conversion of 340/380 ratio values to $[Ca^{2+}]_i$ changes and after deduction of corresponding basal values and are the mean ± S.E.M., $n = 6–7$.

The time courses of SRIF (10 μM) and UTP (100 μM)-induced $Ins(1,4,5)P_3$ formation were studied (Fig. 3A). Peak levels measured 15 s after agonist addition were significantly higher for UTP than for SRIF (320.4 ± 26.5 and 105.4 ± 7.9 pmol mg^{-1} protein, $n = 4$ and 6, respectively). Despite some decline with time (Fig. 3A), levels of $Ins(1,4,5)P_3$ were still elevated 300 s following treatment with either UTP or SRIF (132.6 ± 17.7 and 69.2 ± 11.8 pmol mg^{-1} protein, $n = 4$ and 6, respectively).

In other experiments, the effect of SRIF (10 μM) and UTP (100 μM) on $Ins(1,4,5)P_3$ formation was studied in both control cells and cells incubated in the absence of extracellular Ca^{2+} (Fig. 3B and C). Removal of extracellular Ca^{2+} (in the presence of 1 mM EGTA) caused a small decrease in basal $Ins(1,4,5)P_3$ from 33.1 ± 8.9 to 14.0 ± 0.45 pmol mg^{-1} protein ($n = 6$) but had no effect on SRIF-induced peak increases in $Ins(1,4,5)P_3$ levels. Peak

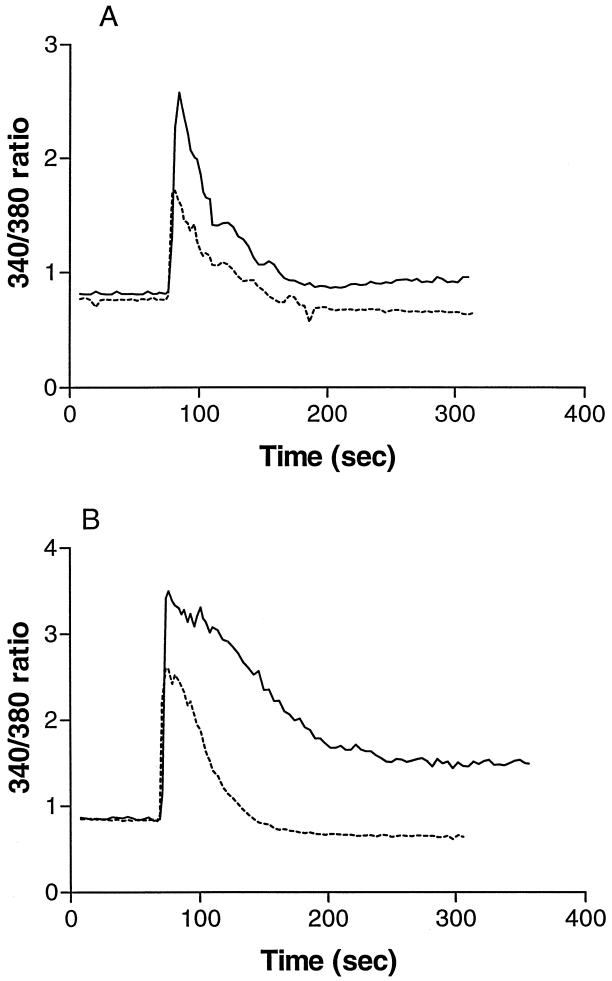

Fig. 2. Effect of removal of extracellular Ca^{2+} on $[Ca^{2+}]_i$ increases in fura-2 loaded $CHOsst_5$. $CHOsst_5$ cells were stimulated with either SRIF (1 μM) (A) or UTP (100 μM) (B) and changes in the 340/380 ratio measured. Experiments were performed either in the presence of 1.3 mM Ca^{2+} (solid lines) or in Ca^{2+}-free buffer, with 1 mM EGTA (dashed lines). Agonist was added at about 75s and remained in contact with the cells throughout the subsequent recording period. Each trace is representative of 4 separate experiments and is the meaned response from 8–12 individual cells.

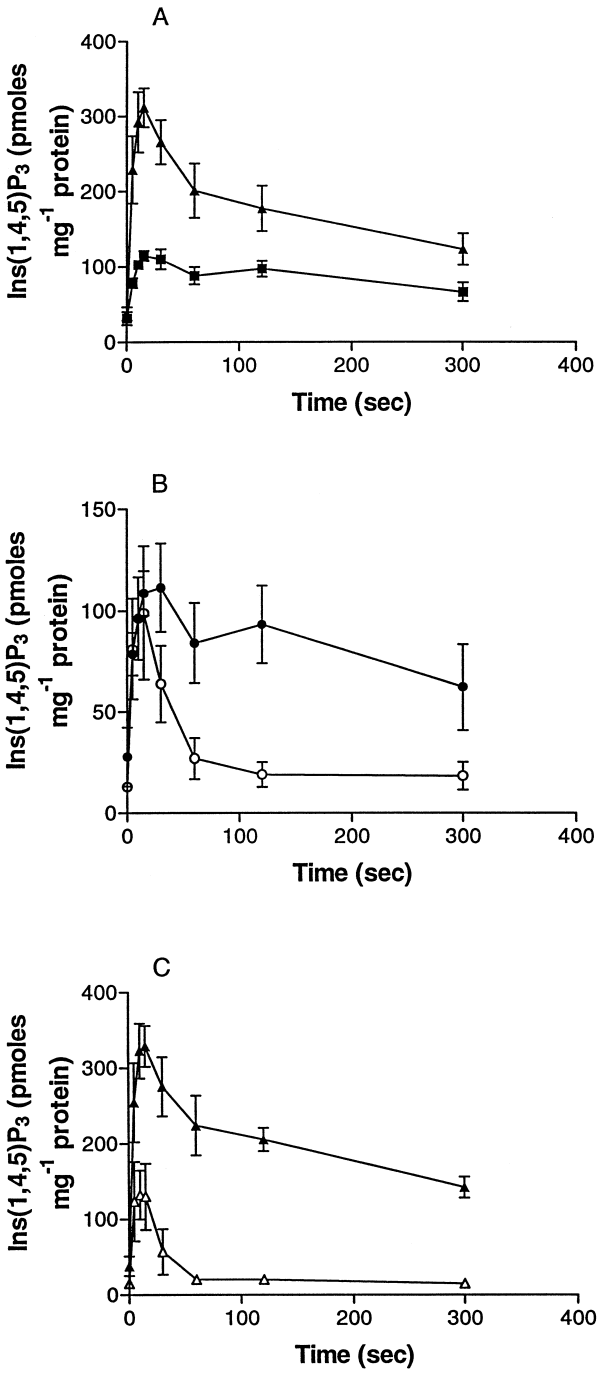

Fig. 3. Time course for mass inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) formation in $CHOsst_5$ cells. (A) $CHOsst_5$ cells were incubated for the times indicated with SRIF (10 μM) (■) or UTP (100 μM) (▲), data are the mean ± S.E.M., $n = 4$. (B) and (C) effect of removal of extracellular Ca^{2+} on $Ins(1,4,5)P_3$ formation in response to (B) SRIF (10 μM) or (C) UTP (100 μM). Experiments were performed either in the presence of 1.3 mM Ca^{2+} (filled symbols) or in the absence of Ca^{2+} with 1 mM EGTA (open symbols), agonist additions were made after a 10 min preincubation with the appropriate buffer. Data are the mean ± S.E.M., $n = 3$.

concentrations of $Ins(1,4,5)P_3$ following SRIF treatment were 108.8 ± 10.9 and 98.9 ± 33.0 pmol mg^{-1} protein, in the presence and absence of extracellular Ca^{2+}, respectively ($n = 3$). In contrast, removal of extracellular Ca^{2+}, significantly attenuated peak $Ins(1,4,5)P_3$ levels following UTP stimulation (329.0 ± 27.2 and 130.0 ± 43.7 pmol mg^{-1} protein, $n = 3$, in the presence and absence of Ca^{2+}, respectively). Removal of external Ca^{2+}, markedly affected the time course of both SRIF and UTP-induced increases in $Ins(1,4,5)P_3$. Elevated $Ins(1,4,5)P_3$ levels following both SRIF and UTP returned to basal levels within 60 s of agonist addition when Ca^{2+} was removed from the buffer (Fig. 3B and C).

3.3. Homologous desensitisation of SRIF-induced increases in $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$

In $CHOsst_5$ cells, continuous exposure to SRIF (1 μM) for 5 min caused an increase in $[Ca^{2+}]_i$ which returned to basal levels within the 5 min period (Fig. 4). Peak responses to a subsequent SRIF (1 μM) challenge were markedly attenuated (Fig. 4). Compared with vehicle pretreated cells, peak responses to SRIF (1 μM) were reduced by 96 ± 5% ($n = 3$). The degree of inhibition of subsequent SRIF (1 μM)-induced increases in $[Ca^{2+}]_i$ was highly dependent upon the concentration of the initial SRIF challenge. In vehicle pretreated cells, SRIF (1 μM) elevated $[Ca^{2+}]_i$ to 341 ± 69 nM. When cells were pre-exposed to SRIF (0.01, 0.1 and 1 μM) for 5 min, subsequent responses to SRIF (1 μM) were 296 ± 165, 172 ± 24 and 48 ± 8 nM respectively ($n = 3$).

SRIF-induced increases in $Ins(1,4,5)P_3$ levels were also subject to homologous desensitisation. Following a 5 min pre-exposure to SRIF (10 μM), subsequent peak SRIF-induced increases in $Ins(1,4,5)P_3$ levels (measured at 15 s)

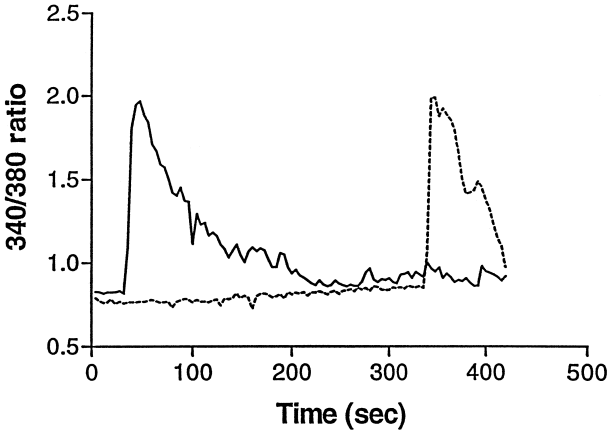

Fig. 4. Homologous desensitization of SRIF induced $[Ca^{2+}]_i$ increases in fura-2 loaded $CHOsst_5$ cells. Cells were exposed to either SRIF (1 μM) (solid line) or vehicle (dashed line) and after 5 min both sets of cells were subsequently exposed to a second challenge with SRIF (1 μM). Time points of agonist administration are indicated (△). Increases in $[Ca^{2+}]_i$ are expressed as the change in 340/380 ratio, each trace is the meaned response from 8–12 individual cells and is representative of 3 similar experiments.

were reduced by 68 ± 3% ($n = 3$) compared with vehicle pretreated cells.

The time course of the recovery of SRIF-induced increases in $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 levels following pre-exposure to SRIF was also studied (Fig. 5). After washout of the initial SRIF challenge, SRIF-induced increases in $[Ca^{2+}]_i$ had fully recovered by 15 min, whilst SRIF-induced increases in Ins(1,4,5,)P_3 levels had still not fully recovered 30 min after washout (Fig. 5).

3.4. Heterologous desensitisation of SRIF-induced increases in $[Ca^{2+}]_i$

In CHOsst$_5$ cells, continuous exposure to UTP (100 μM) for 5 min caused an increase in $[Ca^{2+}]_i$ which declined to a lower but sustained level (Fig. 6A and see above). SRIF (1 μM) had no effect on $[Ca^{2+}]_i$ following pre-exposure to UTP (100 μM). Responses to UTP (100 μM) were also abolished (data not shown) following pre-exposure to UTP. In marked contrast, responses to UTP (100 μM) were not modified when CHOsst$_5$ cells were pre-exposed to SRIF (1 μM) (Fig. 6A). UTP-induced increases in $[Ca^{2+}]_i$ following pre-exposure to SRIF or vehicle were 432 ± 34 and 472 ± 38 nM, respectively ($n = 4$).

In the absence of extracellular Ca^{2+}, continuous exposure to UTP (100 μM) for 5 min caused an increase in $[Ca^{2+}]_i$. As previously described, the sustained rise observed in the presence of extracellular Ca^{2+} was abolished (Fig. 6B). SRIF (1 μM) had no effect on $[Ca^{2+}]_i$ following pre-exposure to UTP (100 μM). In the absence of extracellular Ca^{2+}, responses to UTP (100 μM) were, however, reduced (45 ± 5% of the initial UTP challenge; $n = 5$) following pre-exposure to SRIF (1 μM) (Fig. 6B).

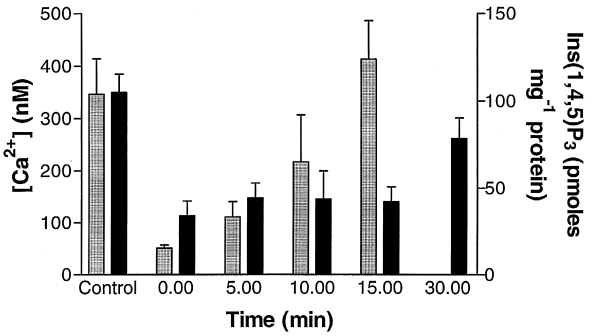

Fig. 5. Time course of recovery of peak $[Ca^{2+}]_i$ (shaded bars) and Ins(1,4,5)P_3 responses (filled bars) after pre-exposure of CHOsst$_5$ cells to SRIF. Cells were preincubated with SRIF (either 1 μM for $[Ca^{2+}]_i$ increases or 10 μM for Ins(1,4,5)P_3 formation) for 5 min and subsequently stimulated with the same concentrations of SRIF, respectively, after various wash periods. The effect of no recovery time on peak $[Ca^{2+}]_i$ rises was determined by addition of SRIF 5 min after the first challenge but in the continuous presence of the first challenge. For longer recovery periods, the first SRIF challenge was removed by continuous perfusion for the times indicated followed by the second SRIF challenge. When recovery of peak Ins(1,4,5)P_3 formation (determined at 15 s) was examined, the SRIF preincubate was removed and the cells washed twice with 200 μl of buffer and left for the times indicated and then followed by the addition of the second SRIF challenge. Control Ins(1,4,5)P_3 levels were determined after exposure of cells to a 5 min vehicle preincubation. Data are the mean ± S.E.M. of 3 experiments performed in triplicate for the Ins(1,4,5)P_3 response and 4 individual experiments for the $[Ca^{2+}]_i$ increase.

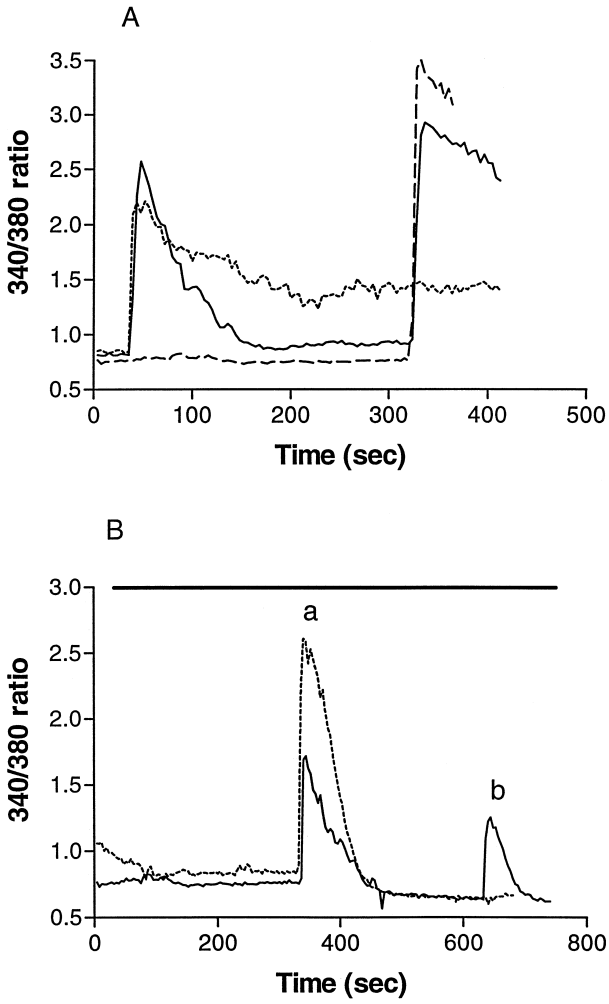

Fig. 6. Heterologous desensitization of SRIF induced $[Ca^{2+}]_i$ increases in fura-2 loaded CHOsst$_5$ cells. (A) cells were preincubated with either SRIF (————), UTP (---) or vehicle (— — — —) and after 5 min exposed to a second challenge with either UTP (————), SRIF (---) or UTP (— — — —). (B) cells were incubated in Ca^{2+}-free buffer as indicated by the solid bar and after 5 min exposed at (a) to either SRIF (solid line) or UTP (dashed line). After a further 5 min the cells were subject to a second agonist challenge (b), with the alternate agonist. Each experiment is the meaned response from 8–12 individual cells and is representative of 4 or 5 separate experiments. Agonist concentrations were SRIF, 1 μM and UTP, 100 μM.

4. Discussion

The operational characterisation of somatostatin (SRIF)-receptor subtypes has been hampered by the lack of specific and selective receptor blocking drugs. How-

ever, the phenomenon of agonist-induced receptor desensitisation has not only been used to study the specificity of action of other SRIF-agonists (eg McKeen et al., 1994, Feniuk et al., 1995) but also as a means of discriminating between SRIF-receptor subtypes (Liapakis et al., 1996). Thus sst_2 receptors endogenously expressed in AtT-20 cells are resistant to agonist-induced receptor desensitisation whilst sst_5 receptors expressed in the same cells are readily desensitised (Liapakis et al., 1996). The mechanisms responsible for desensitisation are poorly understood (see below). In preliminary studies (Wilkinson et al., 1996a) on CHOsst_5 cells, it was observed that SRIF-induced increases in $[Ca^{2+}]_i$ were very transient and recovered to basal levels in the continuous presence of agonist. Furthermore the sst_5 receptors appeared to become desensitised since subsequent challenges with SRIF were ineffective. In the present study we sought to investigate SRIF-induced Ca^{2+} signalling in CHOsst_5 cells in more detail and to examine the specificity of this desensitisation by investigating UTP-induced increases in $[Ca^{2+}]_i$ which are consequent upon activation of endogenous P_{2U} purinoceptors expressed in this cell line (Iredale and Hill, 1993).

Both SRIF and UTP caused rapid concentration-dependent increases in $[Ca^{2+}]_i$ in fura-2 loaded CHOsst_5 cells. However, responses to SRIF were transient, returning to pre-stimulated values within 150 s in the continued presence of the agonist, whilst those to UTP were sustained. Our single cell Ca^{2+}-imaging studies demonstrated that both agonists elevate $[Ca^{2+}]_i$ in the same cell, thus indicating co-expression of their respective receptors. The time course of the initial elevation of $[Ca^{2+}]_i$ in response to SRIF was extremely rapid and similar to that described for human recombinant sst_5 receptors expressed in COS-7 cells (Akbar et al., 1994), and is also similar to changes described for other receptor systems (Putney and Bird, 1993). It has been argued that in non-excitable cells, such as CHO-K1 cells, the peak increase in $[Ca^{2+}]_i$ following application of an agonist is driven predominantly by the ability of Ins(1,4,5)P_3 to release Ca^{2+} from Ins(1,4,5)P_3 sensitive intracellular stores (Berridge, 1993). The maintained plateau is the result of Ca^{2+} influx across the plasma membrane, presumably due to a capacitative entry mechanism (Putney and Bird, 1993). In keeping, in the present study, peak increases in $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 occurred at similar time points. However, in the absence of extracellular Ca^{2+}, the peak $[Ca^{2+}]_i$ responses to both SRIF and UTP were attenuated. These data suggest that capacitative Ca^{2+} influx is also a significant contributing factor to the peak $[Ca^{2+}]_i$ rise. The sustained plateau to UTP was abolished in the absence of extracellular Ca^{2+}, which is consistent with this being due to Ca^{2+} entry across the plasma membrane.

The time courses of the Ins(1,4,5)P_3 responses were similar to those reported for activation of other receptors, such as muscarinic m_3-mediated activation of SH-SY5Y cells (Willars and Nahorski, 1995a) and P2Y purinoceptor-mediated activation of bovine aortic endothelial cells (Purkiss et al., 1994). However, SRIF-induced increases in Ins(1,4,5)P_3 formation were still maintained when the $[Ca^{2+}]_i$ transient had returned to basal levels. The lack of a maintained $[Ca^{2+}]_i$ response to SRIF in the face of persistent Ins(1,4,5)P_3 formation was a surprising finding since other peptides, e.g., bradykinin (Chueh et al., 1995; Willars and Nahorski, 1995a) cause only transient increases in Ins(1,4,5)P_3 and $[Ca^{2+}]_i$. Possible explanations for these findings with SRIF may include differential regulation of Ca^{2+} release from intracellular stores following sst_5 receptor activation or some degree of homologous desensitisation of the Ca^{2+} release mechanism following Ins(1,4,5)P_3 generation.

Ca^{2+} is a key regulator of the activity of various PLC isoforms (Rhee and Choi, 1992). In our work, the absence of extracellular Ca^{2+} caused a reduction in basal Ins(1,4,5)P_3 levels, suggesting a constitutively active Ca^{2+}-dependent PLC in these cells. Peak UTP-stimulated Ins(1,4,5)P_3 formation was decreased in the absence of extracellular Ca^{2+}, whereas peak responses to SRIF were unaffected. Similar decreases in the peak Ins(1,4,5)P_3 response have been described for bradykinin stimulation of adrenal chromaffin cells (Challiss et al., 1991) and SH-SY5Y cells (Willars and Nahorski, 1995a). In the absence of extracellular Ca^{2+}, the peak Ins(1,4,5)P_3 response to UTP was similar in magnitude to that elicited by SRIF. This suggests the presence of at least two pools of PLC accessible to these receptors; one sensitive to Ca^{2+}, responsible for a portion of the peak response to UTP and the sustained response to both agonists. The remaining PLC pool, which appears insensitive to Ca^{2+}, may be involved in the peak increase to SRIF and the remaining component of the peak increase to UTP. It is clear from these findings that SRIF and UTP induced Ins(1,4,5)P_3 and Ca^{2+} signals in CHOsst_5 cells are subject to differential regulation.

The reduction of peak Ins(1,4,5)P_3 and Ca^{2+} responses in the continued presence of an agonist has been attributed to partial desensitisation of a component of the receptor/G protein/PLC complex while the return to basal levels of such responses is indicative of full desensitisation (Wojcikiewicz et al., 1993; Willars and Nahorski, 1995a). Clearly, using such criteria, sst_5, but not P_{2U}, mediated increases in $[Ca^{2+}]_i$ were fully desensitised under the present experimental conditions. This was particularly evident for the sst_5 receptor as a second challenge with SRIF was unable to elicit a further rise in $[Ca^{2+}]_i$. This also excludes the possibility that the transient nature of the SRIF-induced increase in $[Ca^{2+}]_i$ was a consequence of metabolism of the peptide. However we cannot exclude the possibility that the differential desensitisation of the SRIF- and UTP-induced increases in $[Ca^{2+}]_i$ may be related to the levels of expression of either their respective receptors or G proteins. We do know, however, that sst_5 receptor mediated responses are via pertussis toxin sensitive $G_{i/o}$ G proteins,

unlike those to UTP which are pertussis toxin insensitive, thus indicating that these two receptors mediate increases in $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 via different G protein types (Thurlow et al., 1996; Williams et al., 1996; Wilkinson et al., 1997).

SRIF induced $[Ca^{2+}]_i$ increases were subject to both homologous desensitisation following exposure to SRIF and heterologous desensitisation following exposure to UTP. Regarding the former, we showed that after the first SRIF challenge, an increasing wash period prior to addition of the second challenge resulted in a time dependent recovery of the response to the second agonist application. There was also a concomitant recovery of peak Ins(1,4,5)P_3 formation. However, the latter response exhibited a considerable lag behind the recovery of the $[Ca^{2+}]_i$ rise. A comparison of the concentration–effect curves for increases in peak Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ indicate that SRIF was approximately 30 times more potent at stimulating peak $[Ca^{2+}]_i$ increases than Ins(1,4,5)P_3 formation thus maximal $[Ca^{2+}]_i$ release can be achieved at low Ins(1,4,5)P_3 concentrations.

The mechanism(s) responsible for SRIF-induced homologous desensitisation are poorly understood but receptor phosphorylation (see below) or receptor internalisation may play a role. Indeed we have shown [^{125}I]-BIM-23027-sst_2 receptor internalisation in the neuroblastoma cell line, Neuro2A (Koenig et al., 1997). More germane, Hukovic et al. (1996) have also demonstrated ligand associated internalisation of sst_5 receptors expressed in CHO-K1 cells. However, whilst the kinetics of the internalisation were similar in both studies, they were too slow to account for the rapid desensitisation observed in the current study. There is now compelling evidence that phosphorylation of receptors, such as the muscarinic m_1 (Haga et al., 1996), muscarinic m_3 (Tobin and Nahorski, 1993) and NK_1 tachykinin receptors (Kwatra et al., 1993), may play a role in desensitisation phenomena. This phosphorylation may be due to the activity of a kinase such as the β-adrenoceptor-kinase (βARK) or another related G protein receptor kinases (Hausdorff et al., 1990; Inglese et al., 1993) or by PKC kinases (Lefkowitz et al., 1990). SRIF receptors on S49 lymphoma cells, which are coupled to the inhibition of adenylyl cyclase, are known to be phosphorylated by a β-ARK-like kinase (Mayor et al., 1987) and it is, therefore, possible that the sst_5 receptors in this study may undergo similar regulatory events.

The heterologous desensitisation observed in the present study may be due to depletion of a common Ca^{2+} store. Thus, in the absence of $[Ca^{2+}]_e$, Ca^{2+}-store depletion by UTP abolished the SRIF response, while the addition of SRIF reduced the ability of UTP to cause a $[Ca^{2+}]_i$ increase. Such Ca^{2+}-dependent desensitisation events have been described for other receptor interactions (Merrit and Rink, 1987; Smit et al., 1992; Boyd et al., 1994; Chueh et al., 1995) and it has been suggested that depletion of the Ins(1,4,5)P_3 sensitive Ca^{2+} stores may be a common feature of heterologous desensitisation (Willars and Nahorski, 1995b). The abilities of agonists to cause heterologous desensitisation is presumably dependent upon persistent receptor occupancy, thus keeping the Ca^{2+} stores in a state of partial or permanent emptiness.

In summary, we have described the kinetics of $[Ca^{2+}]_i$ signalling and Ins(1,4,5)P_3 formation in response to SRIF and UTP in CHO-K1 cells expressing human recombinant sst_5 receptors. We have shown that both homologous and heterologous desensitisation is a feature of responses mediated via sst_5 receptor activation. The latter appears to involve depletion of a common intracellular Ca^{2+} store. The extent to which these findings can be extrapolated to desensitisation of endogenous SRIF receptors is as yet unclear. Stimulation of the inositol tri-phosphate-Ca^{2+} pathway has been described in cultured rat hippocampal neurones (Miyoshi et al., 1989) and in various regions of the rat brain (Munoz-Acedo et al., 1995). Desensitisation appears to occur since SRIF-induced increases in Ins(1,4,5)P_3 in hypothalamus and striatum were poorly maintained. However the identity of the SRIF receptor types mediating these effect are unknown.

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