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The Pivotal Role of Phosphoinositide-3 Kinase in the Human Somatostatin sst_4 Receptor-Mediated Stimulation of p44/p42 Mitogen-Activated Protein Kinase and Extracellular Acidification

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We have previously demonstrated in CHO-K1 cells expressing recombinant human sst_4 receptors that somatostatin-induced increases in extracellular acidification are susceptible to a marked desensitisation after pretreatment with somatostatin, but not the somatostatin analogue, L-362855. In the present study, we have examined the human sst_4 receptor-mediated stimulation of p44/p42 mitogen-activated protein (MAP) kinase to determine whether this response is susceptible to a similar agonist-specific desensitisation. Western analysis using phosphospecific antibodies revealed that both somatostatin and L-362855 induced a transient stimulation of MAP kinase which could be desensitised by pretreatment with somatostatin, but not L-362855. The selective phosphoinositide (PI) 3-kinase inhibitor, LY 249002, blocked both the somatostatin-induced increase in MAP kinase phosphorylation and extracellular acidification. However, the MEK1 inhibitor, PD 98059, blocked only the sst_4 receptor-mediated stimulation of MAP kinase and not the extracellular acidification response. In summary, the human sst_4 receptor is selectively desensitised by somatostatin and not by L-362855 and signals through two different PI 3-kinase linked pathways.

Somatostatin (Somatotrophin release inhibitory factor, SRIF) is a tetradecapeptide neurotransmitter which transduces its intracellular effects via interaction with a family of G protein-linked somatostatin receptors (sst_1-sst_5) (1–3), which are often expressed heterogeneously within tissues as well as by a variety of tumour cell types (1–3). Northern blotting, RT-PCR and *in situ* hybridisation studies have revealed the presence of sst_4 receptor mRNA in the CNS, lung, eye, and placenta (4–8), but the physiological significance of these transcripts is still unknown. Until recently (9), investigative work on sst_4 receptors has been hampered by a lack of selective ligands, and pharmacological characterisation of this receptor has been restricted to radioligand binding studies performed on membrane homogenates from recombinant cell lines (10).

Previously, we have demonstrated a prolonged and marked desensitisation of the human recombinant sst_4 receptor-stimulated increases in extracellular acidification rates using microphysiometry (11) after treatment with somatostatin, but not the somatostatin analogue, L-362855 (12). However, the nature of the intermediary signalling pathways and mechanism of the desensitisation was not elucidated.

Recombinant rat sst_4 receptors expressed in CHO-K1 cells have been shown to couple to a number of signalling pathways, including the stimulation of p44/p42 mitogen-activated protein kinase (MAP kinase), the inhibition of adenylate cyclase, and the release of arachadonic acid (13–14). However, the rat sst_4 receptor, unlike its human counterpart, is not susceptible to agonist-induced desensitisation, or internalisation (15–17). The aim of the present study was to determine if the activation of MAP kinase shown to be mediated by human sst_4 receptors (18) was linked to increases in extracellular acidification rates (EAR) and to determine if somatostatin-induced increases in MAP kinase phosphorylation were also susceptible to agonist-induced desensitisation.

MATERIALS AND METHODS

MAP kinase stimulation studies. CHO-K1 cells expressing the human recombinant sst_4 receptor (CHOsst_4 cells) (GlaxoWellcome, UK) were seeded at a density of 200,000 cells per well into 12-well plates (Costar, UK) and incubated overnight in Dulbecco's Modified Eagles medium/Hams F:12 (1:1) mix supplemented with 10% FCS, 1

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mM glutamax and 500 μg ml^{-1} G418 (all from Gibco, UK). In time course studies, somatostatin (Peninsula Laboratories, UK) (10 nM) or L-362855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]) (Peptide Research Consultants, UK) (100 nM) were added to CHOsst$_4$ cells for increasing periods of time. All experiments were terminated by the aspiration of media and the addition of ice-cold phosphate-buffered saline (PBS). Cells were challenged with a range of concentrations of somatostatin or L-362855 (10^{-10} M–10^{-6} M) for 10 min. Studies were also designed to investigate the effects of a variety of inhibitors (all from Calbiochem, UK) upon somatostatin-induced MAP kinase stimulation. In these experiments, drugs were added to the media 30 min before a 10 min somatostatin challenge. Desensitisation of the human sst$_4$ receptor mediated MAP kinase response was investigated by pretreating cells for 30 min with somatostatin (1 nM), before exposure to a range of L-368255 concentrations (10^{-10} M–10^{-6} M) for 10 min. In another series of experiments, cells were treated with somatostatin (1 nM) for 30 min and then stimulated with a range of concentrations of uridine tri-phosphate (UTP) (10^{-6} M–10^{-3} M).

Western analysis. Experiments were terminated with the aspiration of media and washed by the addition of ice-cold PBS. Cells were solubilised in 100 μL SDS-PAGE sample buffer (4% sodium dodecyl sulphate [SDS], 5% glycerol, 60 mM Tris pH 6.8; 0.01% bromophenol blue and 50 mM mercaptoethanol), and heated to 95°C for 5 min. Whole cell protein extracts were separated on 10% SDS-polyacrylamide gels. Proteins were electrically transferred onto 0.22 μM nitrocellulose membranes, washed in Tris-buffered saline (TBS; 100 mM Tris-HCl; pH 7.5 and 150 mM NaCl) before being blocked overnight at 4°C in TBS containing 0.1% Tween-20 and 5% milk (TBST-milk). Antibody incubations were for 1 h at 21°C in TBST-milk, with an anti-phospho specific p44/p42 MAP kinase antibody as the primary (1:1000 dilution; New England Biolabs), and an anti-rabbit horseradish peroxidase conjugated secondary antibody (1:3000 dilution; Biorad, UK). Immunocomplexes were visualised using the enhanced chemiluminescence (ECL) system and detected on Amersham Hyperfilm MP (Amersham, UK). Images were scanned using Adobe photoshop software.

Microphysiometry. Microphysiometry experiments were performed as previously described (11). Briefly, 500,000 cells were plated into microphysiometer cups (Molecular Devices, UK), 18 h before experimentation. Cells were perfused with a bicarbonate-free DMEM solution (pH 7.4) at a rate of 120 μL min^{-1}. Extracellular acidification rates (EAR) were measured as described previously (11). After an initial period of equilibration and a control UTP challenge (3 μM), cells were left to equilibrate for a further 90 min. Inhibitor drugs were continuously perfused for 30 min prior to the addition of agonist. The increases in EAR responses are expressed as a percentage of the initial challenge to UTP (3 μM). Statistical comparisons of responses were performed using Student's t-Test, results were noted to be significantly different when $P < 0.05$.

RESULTS

Stimulation of CHOsst$_4$ cells with somatostatin (10 nM) and L-362855 (100 nM) led to a time-dependent increase in the amount of phosphorylated p44/p42 MAP kinase (Fig. 1). Onset of phosphorylation was rapid and detectable 2 min after stimulation with agonist. Maximal phosphorylation was reached at 4-10 min and reduced to basal levels after 30 min. Equal loading of protein between samples was confirmed by detection with phosphorylation state-independent anti-p44/p42 MAP kinase antibodies (Fig. 1C). Both somatostatin and L-362855-induced increases in the phosphorylation of MAP kinase observed at 10 min were concentration-dependent. The threshold concentration for somatostatin and L-362855 was 10^{-9} M and 10^{-8} M, respectively (Fig. 2).

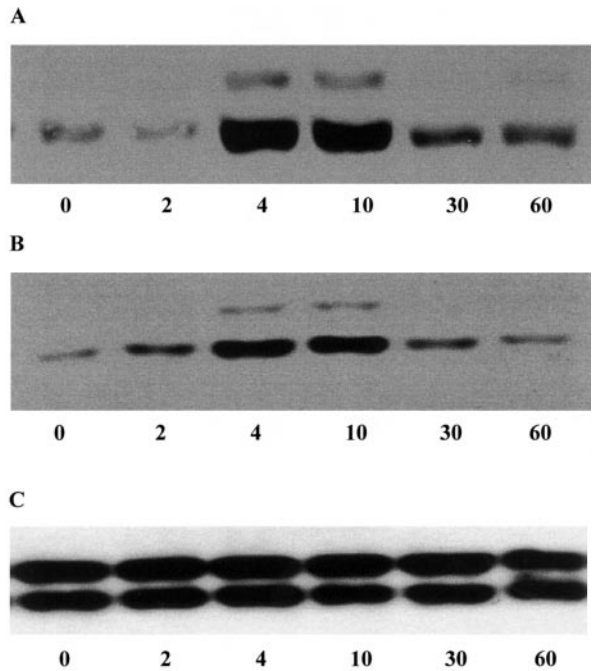

FIG. 1. Time course (in min) of the stimulated phosphorylation of p44/p42 MAP kinase in CHO-K1 cells expressing human recombinant sst$_4$ receptors by either (A) SRIF (10 nM) or (B) L-362855 (100 nM). Cells were incubated in the presence of the analogues for the times shown in min and analysed by Western blotting using an antibody specific for the dually phosphorylated forms of MAP kinase (at Thr^{202} and Tyr^{204}) (A and B). C shows the immunoreactivity obtained for samples as in A with antibodies specific for p44/p42 MAP kinase independent of their phosphorylation state. All blots are representative of at least three separate experiments and each panel has been taken from a single immunoblot.

Following treatment with somatostatin (1 nM, for 30 min), all subsequent increases in phosphorylation to L-362855 were blocked (Fig. 3A). However, in a separate set of studies, somatostatin pretreatment had no effect upon the increases in MAP kinase phosphorylation to UTP (Fig. 3B).

Cells were pretreated with a number of inhibitors prior to somatostatin stimulation in an attempt to delineate the signalling pathways involved in MAP kinase activation (Fig. 4). An 18 h preincubation of the cells with pertussis toxin (100 ng ml^{-1}) abolished the somatostatin-induced stimulation of MAP kinase. Responses were also blocked after a 30 min exposure to either the MEK1 inhibitor, PD 98059 (10 and 50 μM) or the phosphoinositide 3-kinase (PI 3-kinase) inhibitor LY 294002 (10 and 50 μM). Inhibitors of Src (PP-1) (200 nM) and protein kinase C (Calphostin-C at 50 nM and Ro-31-8220 at 1 μM) were without effect on the somatostatin-induced MAP kinase stimulation.

A series of microphysiometry studies were undertaken to establish the involvement of MEK1 and PI

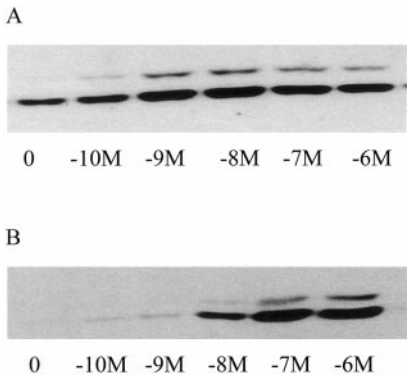

FIG. 2. Changes in the phosphorylation status of MAP kinase in response to a 10 min exposure to increasing log concentrations of either (A) somatostatin or (B) L-362855. Western blots show the immunoreactivity detected with antibodies to the dually phosphorylated forms of MAP kinase (at Thr^{202} and Tyr^{204}). All blots are representative of at least three separate experiments and each panel has been taken from a single immunoblot.

3-kinases in the somatostatin-induced increase in EAR. After equilibration, basal EAR rates were 100-300 $\mu V\ s^{-1}$ and the mean increases in EAR in response to an initial UTP challenge (3 μM) was 128.6 ± 14.7 $\mu V\ s^{-1}$ ($n = 4$). Application of a single concentration of somatostatin (10 nM) led to a robust increase in extracellular acidification, determined as 84.7 ± 6.4% of the UTP (3 μM) challenge. Infusion of either PD 98059 or LY 249002 led to small decreases in the basal acidification rates of 38.6 ± 4.3 and 46.0 ± 3.1%, respectively. A 30 min pretreatment with PD 98059 (50 μM) had no significant effect upon a somatostatin challenge (10 nM) (74.9 ± 15.1% of UTP response). However, pretreatment with the PI 3-kinase inhibitor LY 294002 (50 μM) significantly reduced the magnitude of the somatostatin-induced EAR response to 36.4 ± 10.4% of the UTP response ($P < 0.05$).

DISCUSSION

We have previously shown in microphysiometry studies, that somatostatin, but not L-362855, can induce desensitisation of the human sst_4 receptor (12). Here we have shown that the ability of the human sst_4 receptor to stimulate p44/p42 MAP kinase is also sensitive to agonist-dependent desensitisation. The sst_4 receptor-mediated MAP kinase stimulation was transient and showed similar kinetics following activation by either somatostatin or L-362855. However, the time course for somatostatin-induced phosphorylation was in marked contrast to the sustained (~4 h) phosphorylation observed in a study using re-populating $CHOsst_4$ cell monolayers (18). The difference in the intensity and the duration of the MAP kinase activation

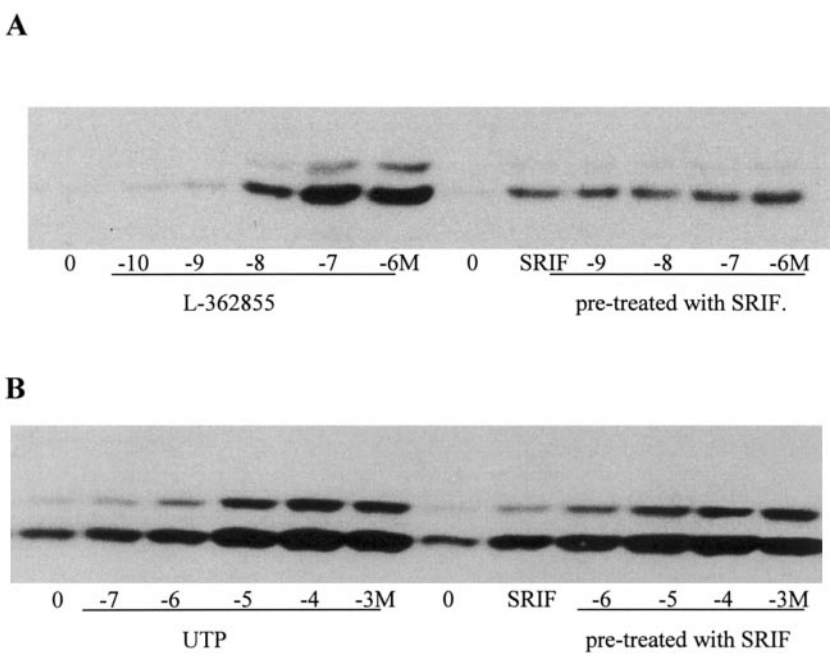

FIG. 3. Changes in the phosphorylation status of MAP kinase in response to agonist stimulation and demonstration of somatostatin-selective desensitisation of the human sst_4 receptor. (A) $CHOsst_4$ cells were stimulated with a range of concentrations of L-362855 (10 min), either with or without pretreatment with somatostatin (SRIF, 1 nM, 30 min). (B) $CHOsst_4$ cells were stimulated with a range of concentrations of UTP, either with or without pretreatment with somatostatin (SRIF, 1 nM, 30 min). Western blots show the immunoreactivity detected with an antibody to the dually phosphorylated forms of p44/p42 MAP kinase (at Thr^{202} and Tyr^{204}). All blots are representative of at least three separate experiments, and each panel has been taken from a single immunoblot.

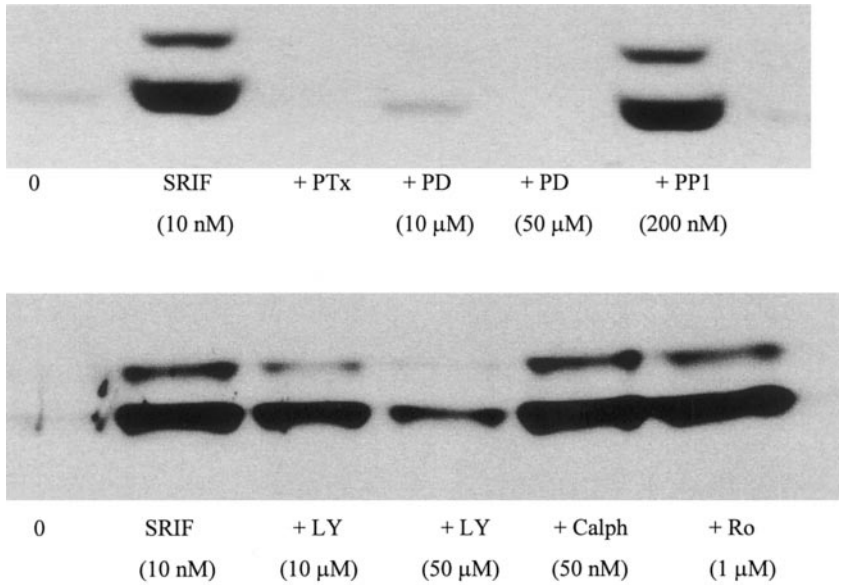

FIG. 4. Effect of pertussis toxin and various kinase inhibitors on somatostatin-induced phosphorylation of MAP kinase in CHO-K1 cells recombinantly expressing human sst_4 receptors. CHOsst_4 cells were incubated in the presence (SRIF, 10 nM) and absence of somatostatin (labelled 0) for 10 min either with or without preincubation with pertussis toxin (PTx, 18 h, 100 ng ml^{-1}), PD 98059 (PD at 10 and 50 μM), PP1 (200 nM), LY 294002 (LY at 10 and 50 μM), calphostin-C (Calph at 50 nM) and Ro-21-2880 (Ro at 1 μM) (all 30 min). Western blots show the immunoreactivity detected with a phosphospecific antibody to p44/p42 MAP kinase. All blots are representative of 3 separate experiments. None of the treatments had any effect on the basal level of MAP kinase phosphorylation (data not shown).Each panel represents a single immunoblot.

between these studies possibly reflects the application of somatostatin to cells in different phases of the cell cycle. In this study, cells will be in log phase of growth compared to partially denuded monolayers of cells synchronised in early G_1 of the cell cycle as used in the re-population model (18).

The sst_4 receptor mediated phosphorylation of p44/p42 MAP kinase exhibited concentration-dependency to both somatostatin and L-362855 and thresholds for activation of the analogues corresponded well with the relative differences in pEC_{50} values for somatostatin and L-362855 reported in microphysiometry studies. Treatment of cells with low concentrations of somatostatin (1 nM) blocked the ability of L-362855 to induce any further increases in MAP kinase phosphorylation, although pretreatment of cells with low concentrations of L-362855 (10 nM) did not effect the ability of higher concentrations of L-362855 (100 nM) to further induce p44/p42 MAP kinase phosphorylation (data not shown). In contrast, pretreatment of cells with somatostatin had no effect upon UTP-induced increases in MAP kinase phosphorylation, suggesting that the desensitisation observed is homologous. The mechanism for this desensitisation is unknown at present but is unlikely to involve internalisation (19) and data from this study suggests that the desensitisation is taking place upstream of MAP kinase. These findings highlight important species differences between the rat and human sst_4 receptors. Whereas, the human sst_4 receptor desensitises rapidly, and for prolonged time periods (>3 h) (12), the rat sst_4 receptor does not appear to be susceptible to desensitisation even after prolonged exposure to somatostatin (60 min) (16).

The somatostatin-induced phosphorylation of MAP kinase is transduced by G proteins of the Gi/Go family, as shown by sensitivity of the response to pertussis toxin, in agreement with previous observations (18). Increases in the phosphorylation of p44/p42 MAP kinase were blocked by the selective MEK1 inhibitor, PD 98059 (23) and blocked by the PI 3-kinase inhibitor, LY 249002. The involvement of PI 3-kinase in the activation of MAP kinase has been reported for a number of Gi/Go-coupled receptors via calcium-independent protein kinase C (PKC) isoforms (14, 20–22). However, in this study MAP kinase phosphorylation appeared to be PKC independent. In addition, the sst_4 receptor mediated MAP kinase activation was not inhibited following Src blockade, in contrast to reports showing that Gi-coupled receptors utilise Src to feed into the Ras-MAP kinase cascade (24).

Pretreatment of cells with the PI 3-kinase inhibitor LY 294002 significantly inhibited somatostatin-induced increases in extracellular acidification rate, demonstrating an important role for PI 3-kinase in human sst_4 receptor signalling. Interestingly the MEK1 inhibitor, PD 98059, had no effect upon somatostatin-induced EAR responses, even at concentrations that abolished MAP kinase stimulation (50 μM). This suggests that somatostatin-induced increases in EAR are

independent of MAP kinase activation, and are transduced, at least in part, through a PI 3-kinase linked pathway. Although PD 98059 and LY 249002 both reduced basal acidification rates, PD 98059 had no effect upon somatostatin-induced increases in EAR. This suggests that the ability of LY 249002 to block somatostatin-induced increases in EAR are not related to its effects upon basal metabolism.

A number of studies have suggested that MAP kinase activation is important in the agonist-induced activation of the Na^+/H^+ exchanger (25–26), but this does not appear to be the case for the human sst_4 receptor (present study) or indeed the 5-HT_{1A} receptor expressed in CHO-K1 cells (20). In this latter study, LY 294002 blocked both the 5-HT_{1A} receptor-induced increases in MAP kinase activation and extracellular acidification, whilst PD 98059 only blocked the activation of MAP kinase (20), again suggesting that p44/p42 MAP kinase does not play an important role in Na^+/H^+ exchanger regulation. However, it should be appreciated that other mechanisms may also be involved in the extracellular acidification response measured in the microphysiometer (11).

In summary, PI 3-kinase plays a pivotal role in the signalling of the human sst_4 receptor, stimulating both p44/p42 MAP kinase and increasing extracellular acidification rates through distinct pathways. Furthermore, this study has highlighted important species differences between the rat and human sst_4 receptor, with the human sst_4 receptor being susceptible to agonist-specific receptor desensitisation.

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