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Somatostatin-induced control of cytosolic free calcium in pituitary tumour cells

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1 In rat pituitary tumour cells (GC cells), spontaneous oscillations of the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) induce growth hormone (GH) secretion that is inhibited by octreotide, a somatostatin (SRIF) agonist which binds to SRIF subtype (sst) receptor 2. The effects of its functional activation on the control of $[Ca^{2+}]_i$ were investigated using fluorimetric measurements of $[Ca^{2+}]_i$.

2 SRIF decreases the basal $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ rise in response to forskolin (FSK) through the inhibition of L-type voltage-dependent Ca^{2+} channels.

3 Pretreatment with octreotide or with L-Tyr^{8}Cyanamid 154806, a sst_2 receptor antagonist, abolishes the SRIF-induced inhibition of $[Ca^{2+}]_i$. Octreotide is known to operate through agonist-induced desensitization, while the antagonist operates through receptor blockade.

4 sst_1 and sst_2 receptor-immunoreactivities (-IRs) are localized to cell membranes. sst_2, but not sst_1 receptor-IR, internalizes after cell exposure to octreotide.

5 SRIF-induced inhibition of basal $[Ca^{2+}]_i$ or FSK-induced Ca^{2+} entry is blocked by pertussis toxin (PTX).

6 FSK-induced cyclic AMP accumulation is only partially decreased by SRIF or octreotide, indicating that sst_2 receptors are coupled to intracellular pathways other than adenylyl cyclase (AC) inhibition.

7 In the presence of H-89, an inhibitor of cyclic AMP-dependent protein kinase (PKA), SRIF-induced inhibition of basal $[Ca^{2+}]_i$ is still present, although reduced in amplitude.

8 SRIF inhibits $[Ca^{2+}]_i$ by activating sst_2 receptors. Inhibition of AC activity is only partly responsible for this effect, and other transduction pathways may be involved.

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Abbreviations: AC, adenylyl cyclase; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; FSK, forskolin; GC cells, rat tumour somatotrophs; Ω-CgTX, Ω-conotoxin GVIA; GH, growth hormone; GHRH, growth hormone-releasing hormone; H-89, H-89 dihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidyl inositol; PKA, cyclic AMP-dependent protein kinase; PKG, cyclic GMP-dependent PK; PLC, phospholipase C; PTP, phosphotyrosine phosphatase; PTX, pertussis toxin; SRIF, somatotrophin release inhibitory factor; SRIF receptor-IR, SRIF receptor-immunoreactivity; sst receptor, SRIF subtype receptor

Introduction

Modulation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is critical for somatotroph function (Lussier *et al*., 1991a; Cuttler *et al*., 1992). Indeed, the release of pituitary growth hormone (GH) appears to be critically dependent on changes in $[Ca^{2+}]_i$. In particular, in rat somatotrophs, growth hormone-releasing hormone (GHRH) generates $[Ca^{2+}]_i$ oscillations that may periodically trigger GH release (Kwiecien *et al*., 1997). Somatostatin (SRIF, somatotrophin release inhibitory factor) plays an important role in inhibiting rhythmic $[Ca^{2+}]_i$ transients (Kwiecien *et al*., 1997) either by directly decreasing extracellular Ca^{2+} influx (Lussier *et al*., 1991c) or by increasing K^+ conductance, and thereby secondarily decreasing Ca^{2+} influx (Lussier *et al*., 1991b). SRIF ability to lower $[Ca^{2+}]_i$ is responsible for its inhibitory action on GH release (Lussier *et al*., 1991c).

The GC cell line is derived from a rat pituitary tumour (subclone of the GH_3 mammosomatotroph strain) and represents a homogeneous *in vitro* model of tumour somatotrophs (Mounier *et al*., 1995; Kwiecien *et al*., 1998). In contrast to GH_3 cells, GC cells release GH but not prolactin. As normal somatotrophs, GC cells exibit rhythmic $[Ca^{2+}]_i$ oscillations resulting mainly from Ca^{2+} entry through L-type Ca^{2+} channels (Kwiecien *et al*., 1998). In contrast to normal somatotrophs, however, $[Ca^{2+}]_i$ transients do not depend on GHRH, but they occur spontaneously. The function of pacemaker activity in GC cells allows GH secretion that is inhibited by the application of octreotide, a long-lasting SRIF agonist (Mounier *et al*., 1995).

SRIF has been shown to play its multiple roles by interacting with specific SRIF subtype (sst) receptors (see for review Meyerhof, 1998). Five receptors have been identified to date and designated sst_1 through sst_5 receptors, each originating from a distinct gene (see for reference Hoyer *et al*., 1995). Splice variants of the mouse sst_2 receptor, $sst_{2(a)}$ and $sst_{2(b)}$, have been cloned (Vanetti *et al*., 1992). These two isoforms that originate from alternative splicing of the sst_2 receptor mRNA differ in their coupling efficiency to adenylyl cyclase (AC) and in agonist-induced receptor desensitization (Vanetti *et al*., 1993). In contrast, equivalent rat splice variants display broadly similar pharmacological properties (Schindler *et al*., 1998).

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In normal rat pituitary somatotrophs, relatively high levels of sst_3, sst_4 and sst_5 receptor mRNAs have been found (O'Carroll & Krempels, 1995). In addition, confocal immunofluorecence experiments have shown that sst_5 receptor is the predominant SRIF receptor subtype expressed in virtually all GH-producing cells while sst_1 receptor is the least expressed subtype (Kumar *et al*., 1997). In contrast, the membranes of tumour somatotrophs express mostly sst_1 and sst_2 receptor mRNAs (Mounier *et al*., 1995; Traina *et al*., 1998) and little is known on the effects of their functional activation.

Among the synthetic SRIF analogues that bind to SRIF receptors with different affinities, des-AA1,2,5-[D-Trp^8, IAmp^9]SRIF (CH-275) and octreotide bind to sst_1 and $sst_{2/3/5}$ receptors, respectively (Raynor *et al*., 1993; Bruns *et al*., 1996; Liapakis *et al*., 1996; Reubi *et al*., 1998; see for reference. Marbach *et al*., 1998). The SRIF agonist CGP-23996 has high affinity for $sst_{3/5}$ receptors (see for reference Viollet *et al*., 1995). The ligand BIM-23052 also selectively binds to sst_3 receptors (Raynor *et al*., 1993), it displays moderate affinity for $sst_{1/4/5}$ receptors, but it does not bind specifically to sst_2 receptor (Viollet *et al*., 1997). The SRIF agonist, BIM-23056 displays high affinity for sst_3 receptor (Raynor *et al*., 1993), although its selectivity has been questioned by Viollet *et al*. (1997). BIM-23056 has been reported to also display some antagonist activity with sst_5 receptor (Wilkinson *et al*., 1996). Another agonist, BIM-23066, binds to sst_2 and sst_3 receptors (Raynor *et al*., 1993) and its cyclic octameric descendant, the L-Tyr^8 isomer of Cyanamid 154806, has been shown to antagonize the SRIF response by binding tightly to sst_2 receptor (Bass *et al*., 1996; erratum, Bass *et al*., 1997; see for reference Hocart *et al*., 1998). This peptide has been more fully characterized by Feniuk *et al*. (1998) as a potent and selective sst_2 receptor antagonist. In addition, a linear hexapeptide SRIF antagonist has been recently shown to block SRIF activity in GH_4C_1 pituitary cells and to influence the GH release in rats (Baumbach *et al*., 1998).

Although there is a high degree of sequence and structural homology among different SRIF receptors, they differ in their pharmacological and functional properties. For instance, the sst_2 receptor displays high affinity for the SRIF agonist octreotide, internalization and desensitization after exposure to the agonist, while the sst_1 receptor has a low affinity for octreotide and it does not internalize or desensitize (see for reference Meyerhof, 1998). These diversities may reflect different modes of transmembrane signalling for sst_1 and sst_2 receptors.

SRIF receptors are coupled to specific G proteins sensitive to pertussis toxin (PTX) that, in turn, activate molecular cascades generally resulting in an inhibition of AC activity and/or the activation of phospholipase activity (Lachowicz *et al*., 1994; see for review Schindler *et al*., 1996). In GC cells, for instance, sst_2 receptor is coupled to the inhibition of AC activity (Traina *et al*., 1998). In particular, octreotide and SRIF are nearly equipotent at inhibiting forskolin- (FSK-) stimulated AC activity while CH-275 has no effect.

There is experimental evidence that SRIF may exert some of its physiologic roles by regulating $[Ca^{2+}]_i$ through an action on voltage-gated Ca^{2+} channels. This regulation is known to occur through the activation of SRIF receptors that are generally coupled to the inhibition of AC activity, although other transduction mechanisms have also been proposed (see for reference Schindler *et al*., 1998). In the present investigation, fluorimetric methods were applied to rat GC cells with the aim of evaluating the effects of the functional activation of sst_1 or sst_2 receptors by SRIF on $[Ca^{2+}]_i$ control. In addition, confocal immunofluorescence was used to localize sst_1 and sst_2 receptors in GC cells. Finally, the effects of SRIF and its sst_2 receptor agonist or antagonist were investigated on the basal or the FSK-stimulated cyclic AMP accumulation.

Methods

Cell culture

GC cells were a gift of D. Gourdji (Paris, France) and were maintained routinely as a monolayer in a complete medium composed of Dulbecco's modified Eagle's Medium-Ham F-12 medium (DMEM-F12) supplemented with 15% heat-inactivated horse serum, 2.5% foetal calf serum, 2 mM glutamine, 100 UI ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested by 0.05% trypsin-EDTA. Experiments were performed after 2–8 days of incubation.

$[Ca^{2+}]_i$ measurements

Cell monolayers (11–15 passages) at about 75% confluence were detached from Petri dishes by applying a gentle flow of incubation medium Krebs-Ringer-HEPES (KRH) containing (mM l^{-1}): NaCl, 140; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2; glucose, 6; HEPES-NaOH buffer, 25, pH 7.4. Cells (1×10^6 ml^{-1}) were then washed in the same medium, dissociated and loaded for 30 min at 37°C in agitation with Fura-2 acetoxymethylester (Fura2/AM, 2 mM), a membrane permeant fluorescent $[Ca^{2+}]_i$ indicator. Loaded cells were resuspended in the incubation medium supplemented with 250 mM sulfinpyrazone, to prevent dye leakage (Fasolato *et al*., 1988), transferred to a quartz thermostatted cuvette (37°C) and maintained under continuous stirring. The fluorescence intensity of Fura-2 was quantified with a Perkin-Elmer LS-50B Luminescence Spectrometer (Norwalk, CT, U.S.A.), with a double excitation wavelength set at 340 and 380 nm, respectively and an emission wavelength monitored at 510 nm. The photomultiplier was coupled to a personal computer for data acquisition. Data were stored in sequential files and collected every 40 ms. At the end of each experiment, data were plotted and calibrated in terms of $[Ca^{2+}]_i$ according to the method of Grynkyewicz *et al*. (1985). Details on the procedure are given in Traina *et al*. (1996) and Traina & Bagnoli (1999). Briefly, per cent $[Ca^{2+}]_i$ reduction was defined as the per cent difference between the steady-state level of $[Ca^{2+}]_i$ at the time at which the ligand was applied and the $[Ca^{2+}]_i$ level 5 s after its application.

Membrane potential measurements

According to a previous study (Traina *et al*., 1996), the fluorescent dye bisoxonol was used to detect changes in the membrane potential. Its properties, in fact, allow one to correlate the fluorescence signal with membrane potential variations (Koch *et al*., 1988). Cells were allowed to equilibrate for 30 min at 37°C in KRH, washed and resuspended at a concentration of about $2-4 \times 10^6$ cells ml^{-1} in KRH medium without bovine serum albumin (BSA). Bisoxonol was added to the medium to a final concentration of 2×10^{-8} M, and the fluorescence (excitation and emission wavelengths, 540 and 580 nm, respectively) was monitored at 37°C in a well-stirred cuvette. The excitation and emission slits were 5 and 10 nm, respectively.

Materials

Octreotide was a gift of Novartis, Ltd. (Basel, Switzerland), BIM 23056 and BIM 23052 were obtained from Ipsen Beaufour (Paris, France). CH-275 was a gift of Carl Hoeger and Jean Rivier (La Jolla, CA, U.S.A.). CGP 23996 was purchased from RBI (Amersham, Italy). The sst_2 receptor antagonist, L-Tyr^{8}Cyanamid 154806 was a gift of Glaxo Institute of Applied Pharmacology (Cambridge, U.K.). Fura-2/AM, H-89 dihydrochloride, an inhibitor of cyclic AMP-dependent protein kinase (PKA), and PTX were obtained from Calbiochem-Novabiochem Intl. (La Jolla, CA, U.S.A.). Bisoxonol was purchased from Molecular Probes (Junction City, OR, U.S.A.). (G-^{3}H)adenosine 3′, 5′-monophosphate (27 Ci mmol^{-1}) was purchased from Amersham Italia Srl. (Milano, Italy). When not specified, chemicals and reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

The data are expressed as means ± s.e.mean. Error bars in Figures represent the s.e.mean. Where error bars are not present, they were too small to plot. Means were compared by one-way analyses of variance (ANOVA). Where $P<0.05$, differences were considered significant (Fisher's test for significance).

Immunocytochemistry

Cells were incubated with an anti-$sst_{2(a)}$ receptor antiserum (K-230, obtained from Marcus Schindler, Cambridge, U.K.; 1 : 200). K-230 was raised in the sheep against the carboxy-terminal peptide of the human $sst_{2(a)}$ receptor isoform and it was used to localize $sst_{2(a)}$ receptor-immunoreactivity (-IR) in distinct regions of the rat brain (Schindler *et al*., 1997). In other experiments, cells were incubated with an anti-sst_1 receptor antiserum (obtained from Lone Helboe, Copenhagen, Denmark; 1 : 10,000). This antiserum was raised in the rabbit against the carboxy-terminal part of the human sst_1 receptor (Helboe *et al*., 1997) and allowed localization of the sst_1 receptor-IR in the rat hypothalamus (Helboe *et al*., 1998). Both antisera were diluted in 0.1 M phosphate buffer saline (PBS) containing 1% BSA and 0.3% Triton X-100 overnight at 4°C. Cells were then washed in 0.1 M PBS and incubated in the presence of the appropriate affinity-purified secondary IgGs conjugated with fluorescein isothiocyanate (FITC) (1 : 50 and 1 : 100, respectively; Vector Laboratories, Burlingame, CA, U.S.A.) in 0.1 M PBS for 1 h at room temperature. Subsequently, cells were washed in 0.1 M PBS and cover-slipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). Specificity of the immune reactions was assessed by adsorbing the primary antiserum with 100 μM synthetic peptide corresponding to sst_1 or sst_2 receptor, overnight at 4°C. Further controls included the omission of the primary antibody and the use of pre-immune serum instead of the primary antibody. No immunostaining was observed in control cells.

Confocal microscopy

SRIF receptor-IR was examined with a Leitz Orthoplan fluorescence microscope equipped with a Plan Neofluar ×63 1.4na oil objective or a PlanApo ×100 1.4na oil objective. FITC fluorescence was visualized with a Leitz L2 filter cube. The distribution of SRIF receptor-IR was also examined with a Leica Laser Scanning Microscope TCS-NT 1.6.551 (LASER Technik GmbH, Heidelberg, Germany) equipped with a krypton-argon laser (OMNICHROME Corp., Chino, CA, U.S.A.) and attached to a Leica DMRBE microscope with Plan Neofluar ×63 1.4na or ×100 1.4na oil objectives. Generally, 10–12 optical sections were taken with a z-axis resolution of 0.3 μm through the immunolabelled cells. Images were processed by using Adobe Photoshop (version 4.0; Adobe Systems, Inc., Mountain View, CA, U.S.A.). Processing of images included both adjustment of brightness and contrast levels and scaling to final size.

In this paper, we use the term 'SRIF receptor-IR' in place of 'SRIF receptor-like IR'.

cyclic AMP measurements

Cells were seeded into 6-well plates at 3×10^6 and grown to confluence. Media were then replaced with fresh serum-free DMEM-F12 for 24 h. The effects of either SRIF-14 or its long-lasting agonist, octreotide (10^{-7} M in fresh serum-free DMEM-F12) were investigated on basal or forskolin- (FSK-) stimulated (10^{-6} M) cyclic AMP accumulation in the presence or in the absence of 3-isobutyl-1-methylxanthine (IBMX, 10^{-4} M, 30 min incubation). The effects of SRIF or octreotide on cyclic AMP intracellular levels were also evaluated in cells exposed or not to the sst_2 receptor antagonist (2×10^{-7} M, 30 min preincubation). The reaction was then terminated by a rapid removal of the medium and the addition of 5% ice-cold trichloroacetic acid (1.5 ml) into each well; cells were then scraped, collected and frozen at −80°C until cyclic AMP assay. The samples were centrifuged for 10 min at $2000\times g$ and the supernatants were estracted five times with aqueous ethyl ether. Neutralized supernatants were assayed for cyclic AMP content, according to the method of Brown *et al*. (1972). Data are expressed as pmoles cyclic AMP 10^6 cells^{-1} 10 min^{-1} and are means ± s.e.mean of at least five different experiments (s.e.mean run in duplicate). They were analysed by ANOVA for variance and Fisher's test for significance.

Results

Effects of SRIF-14 on $[Ca^{2+}]_i$

Levels of $[Ca^{2+}]_i$ were measured in Fura-2 loaded cells suspended in KRH medium. Basal levels of $[Ca^{2+}]_i$ ranged from 50 to 130 nM and remained relatively constant; samples showing higher levels of $[Ca^{2+}]_i$ were discarded. As shown in Figure 1A, SRIF (10^{-8} M) significantly decreased baseline $[Ca^{2+}]_i$ by 23.3 ± 4.2% ($n=7$). Initially, $[Ca^{2+}]_i$ decreased rapidly and then progressively returned to the baseline level within about 3 min. The addition of K^+ (3×10^{-2} M), transiently increased $[Ca^{2+}]_i$ to a peak level 280 ± 28% ($n=5$) greater than the pre-K^+ baseline. This increase was not significantly different from the 245 ± 26% observed with high K^+ applied to cells incubated in the presence of SRIF. The application of SRIF, 5 min after K^+ depolarization, did not significantly influence the maintained Ca^{2+} influx in response to high K^+ ($n=6$, Figure 1B). Similar results were obtained with 10^{-2} M K^+. The addition of K^+ (6×10^{-3} M), transiently increased $[Ca^{2+}]_i$ to a peak level 66 ± 1.7% ($n=5$) lower than that induced by 3×10^{-2} M K^+. The application of SRIF, after K^+ depolarization, significantly decreased $[Ca^{2+}]_i$ rise by 66.8 ± 4.5% ($n=9$; Figure 1C). As shown in Figure 1D, the addition of FSK (10^{-6} M), an alkaloid known to cause an increase in cyclic AMP intracellular level, increased $[Ca^{2+}]_i$ to a peak level 74 ± 8.0% ($n=6$) greater than the pre-FSK baseline.

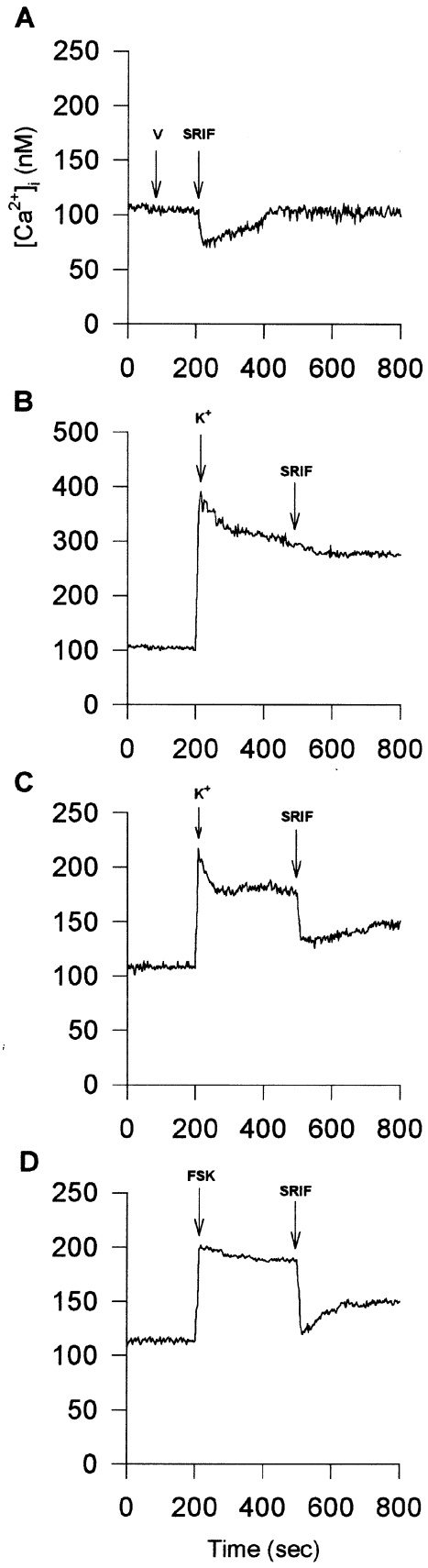

Figure 1 Effects of SRIF (10^{-8} M) on $[Ca^{2+}]_i$. SRIF application significantly decreased baseline $[Ca^{2+}]_i$ (A), whereas it did not significantly influence the maintained Ca^{2+} influx in response to 3×10^{-2} M K^+ (B). The application of SRIF significantly decreased the $[Ca^{2+}]_i$ rise in response to 6×10^{-3} M K^+ (C) or to forskolin (D). Each trace shown is typical of the recordings made in four to 11 other experiments. V: vehicle.

The FSK-induced $[Ca^{2+}]_i$ increase was maintained during the subsequent 30 min and it was not significantly different from the $92\pm7.0\%$ observed when FSK was applied to cells incubated in the presence of SRIF. FSK had no detectable effect on $[Ca^{2+}]_i$ when the cells were incubated in Ca^{2+}-free medium containing 5×10^{-4} M EGTA (data not shown). This result indicates that FSK increased the Ca^{2+} influx from the extracellular space into the cytosol. The application of SRIF, 5 min after FSK, significantly reduced the $[Ca^{2+}]_i$ rise in response to FSK by $91.9\pm5.4\%$ ($n=12$). $[Ca^{2+}]_i$ decreased rapidly and then reached a level that was $34\pm5.0\%$ higher then the pre-FSK baseline. This $[Ca^{2+}]_i$ level was maintained over the following 40 min. The SRIF-induced inhibition of $[Ca^{2+}]_i$ rise in response to FSK was significantly higher than the SRIF-induced inhibition of $[Ca^{2+}]_i$ rise in response to low K^+.

To determine whether the SRIF-induced reduction of $[Ca^{2+}]_i$ rise in response to FSK was secondary to a decrease of the level of the membrane potential, experiments with bisoxonol, a membrane potential indicator were performed. As shown in Figure 2A, no significant variations of membrane potential were observed after SRIF application ($n=6$). In addition, SRIF application did not change either the low K^+-(Figure 2B, $n=6$) or the FSK-induced depolarization (Figure 2C, $n=6$). This result indicates that the cells were neither depolarized nor hyperpolarized by SRIF application.

As shown in the inset of Figure 3A, application of nifedipine (10^{-5} M), a blocker of voltage-dependent channels of the L-type, decreased significantly baseline $[Ca^{2+}]_i$ by $27.4\pm3.2\%$. Subsequent addition of SRIF did not influence baseline $[Ca^{2+}]_i$ ($n=6$). Nifedipine application 5 min after FSK reduced the $[Ca^{2+}]_i$ rise in response to FSK by $137\pm18\%$. Subsequent SRIF application, 2 min after nifedipine, did not significantly affect baseline $[Ca^{2+}]_i$ (Figure 3A; $n=4$). As shown in Figure 3B, SRIF application before nifedipine administration decreased significantly the $[Ca^{2+}]_i$ rise in response to FSK by $92.5\pm3.0\%$. Nifedipine application 1 min after SRIF decreased significantly baseline $[Ca^{2+}]_i$ by $22.4\pm0.4\%$ ($n=4$). Cell pretreatment in Ω-conotoxin GVIA (Ω-CgTX, 10^{-6} M, 60 min), a blocker of N-type channels, did not influence either baseline $[Ca^{2+}]_i$ or the $[Ca^{2+}]_i$ rise in response to FSK that was inhibited by SRIF administration by $89\pm6.0\%$ ($n=6$; Figure 3C). As shown in Figure 3D, the application of Ω-CgTX 5 min after FSK did not influence the $[Ca^{2+}]_i$ rise. SRIF application, 3 min after Ω-CgTX, significantly decreased baseline $[Ca^{2+}]_i$ ($n=4$). This SRIF-induced reduction of $[Ca^{2+}]_i$ was not significantly different from that observed in the absence of Ω-CgTX.

SRIF receptor mediating the SRIF-induced inhibition of $[Ca^{2+}]_i$

Figure 4 shows that SRIF and SRIF agonists reduced $[Ca^{2+}]_i$ in a concentration-dependent manner ($n=6$ for each value). SRIF and its agonists were applied 6 min after FSK. At a concentration of 10^{-9} M, SRIF significantly reduced the Ca^{2+} influx in response to FSK. At this concentration, SRIF agonists used in the present study did not influence the FSK-induced Ca^{2+} entry. Application of higher concentrations of SRIF or octreotide produced a significant reduction of $[Ca^{2+}]_i$ that reached the same maximum at a concentration of 10^{-7} M. At lower concentrations, $[Ca^{2+}]_i$ reductions induced by SRIF were significantly higher than those induced by octreotide. CH-275 or BIM-23056 did not affect $[Ca^{2+}]_i$ at any concentration used. Half-maximal effective concentration (EC_{50}) values of SRIF and its agonists were as follows: SRIF,

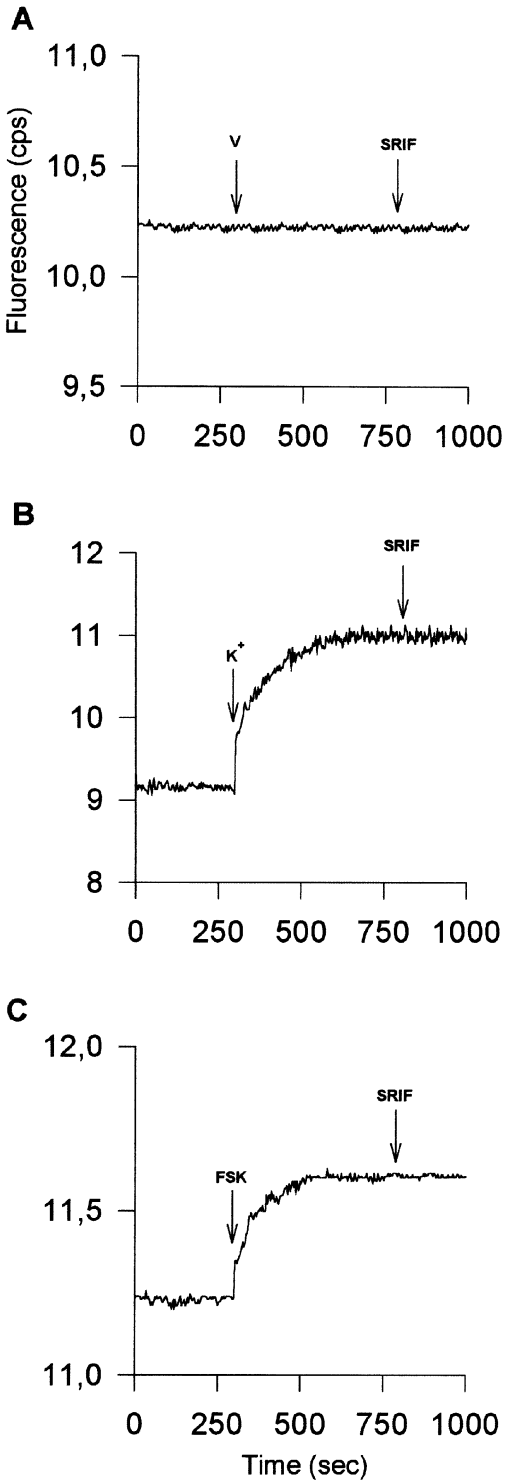

Figure 2 Effects of SRIF (10^{-8} M) on the membrane potential both at resting level (A) and depolarized in response to K^+ (6×10^{-3} M) or forskolin (B and C). The fluorescence intensity was obtained by subtracting the fluorescence background of the cells in the absence of 2×10^{-8} M bisoxonol from the fluorescent signal recorded in the presence of the dye. Each trace shown is typical of the recordings made in five other experiments. cps: counts per second; V: vehicle.

1.3 nM; octreotide, 7.5 nM; CGP-23996, 28 nM; BIM-23052, 40 nM.

The effects of SRIF application on $[Ca^{2+}]_i$ rise in response to FSK were investigated in cells preincubated in the sst_2 receptor antagonist L-Tyr^8 Cyanamid 154806 (Bass *et al*., 1996; erratum, Bass *et al*., 1997; Feniuk *et al*., 1998). In the diagram of Figure 5, the effects of SRIF application (10^{-8} M) are shown as percentages of the SRIF-induced inhibition of the $[Ca^{2+}]_i$ rise in response to FSK. Increasing duration of cell pretreatment with the antagonist (2×10^{-7} M) significantly decreased the percentage of the SRIF-induced $[Ca^{2+}]_i$ reduction, until $[Ca^{2+}]_i$ was inhibited by $11.7 \pm 1.2\%$ after 30 min preincubation ($n = 6$). Representative traces from experiments with antagonist pretreatment of increasing duration are shown in Figure 5A–C. Cell pretreatment with the antagonist also abolished the $[Ca^{2+}]_i$ inhibition induced by octreotide (10^{-8} M, $n = 4$, data not shown). In addition, preincubation in the antagonist was effective in preventing the $[Ca^{2+}]_i$ inhibition induced by low concentrations of SRIF or octreotide (2×10^{-9} M and 5×10^{-9} M, respectively, $n = 4$, data not shown). Moreover, antagonist pretreatment prevented the SRIF-induced inhibition of $[Ca^{2+}]_i$, when Ca^{2+} entry was induced by the application of low K^+ ($n = 6$, data not shown).

The effects of SRIF application on FSK-induced $[Ca^{2+}]_i$ increase were also investigated in cells preincubated for 60 min in SRIF agonists (10^{-6} M) displaying different affinities for sst_1 or sst_2 receptors (Raynor *et al*., 1993; Liapakis *et al*., 1996; see for review Marbach *et al*., 1998). When cells were incubated in octreotide, the percentage of the SRIF-induced reduction of the $[Ca^{2+}]_i$ rise decreased by $79.6 \pm 4.7\%$ (open columns in the diagram of Figure 6, $n = 6$). In contrast, the percentage of the SRIF-induced reduction of $[Ca^{2+}]_i$ was not significantly different from that obtained in control cells when cells were either incubated in CH-275 or preincubated in the sst_2 receptor antagonist and then treated with octreotide (dashed and grey columns, respectively, $n = 6$). Sixty minutes after octreotide removal, the percentage of the SRIF-induced reduction of $[Ca^{2+}]_i$ rise in response to FSK was not significantly different from that obtained in control cells (cross-hatched columns, $n = 6$). Representative traces are shown in Figure 6A–D.

Immunocytochemical localization of sst_1 and $sst_{2(a)}$ receptors

As shown in Figures 7A and 8A, both sst_1 and $sst_{2(a)}$ receptor immunofluorescence was localized to the cell surface and a close association of receptor-IR with the cell plasma membrane could be established in 0.3 μm optical sections through the centre of the immunolabelled cells.

In order to test the possibility that cell exposure to octreotide might induce an agonist-mediated reduction of $sst_{2(a)}$ receptor-IR on the cell surface, cells were exposed to 10^{-6} M octreotide at 37°C for 60 min. Three different types of control experiments were performed: (i) cells were incubated with octreotide at 0°C; (ii) cells were incubated with the sst_2 receptor antagonist (2×10^{-7} M, 30 min) before octreotide exposure; (iii) cells were exposed to octreotide at 37°C and then repeatedly washed for 60 min following octreotide removal. Following treatment, all cells were fixed and then processed for $sst_{2(a)}$ receptor-IR. As shown in Figure 7B, octreotide treatment at 37°C resulted in a redistribution of the $sst_{2(a)}$ receptor immunofluorescence which accumulated in a peri-nuclear location. In contrast, $sst_{2(a)}$ receptor immunofluorescence was seen at the cell surface when: (i) cells were incubated with octreotide at 0°C (Figure 7C), and (ii) cells were pretreated with the antagonist before octreotide (Figure 7D). When cells were washed for 60 min after octreotide removal, $sst_{2(a)}$ receptor immunofluorescence was restricted largely to the surface of the cell bodies (Figure 7E). As shown in Figure 8B, sst_1 receptor immunofluorescence was seen at the cell surface when cells were incubated with octreotide at 37°C, then fixed and processed for sst_1 receptor-IR.

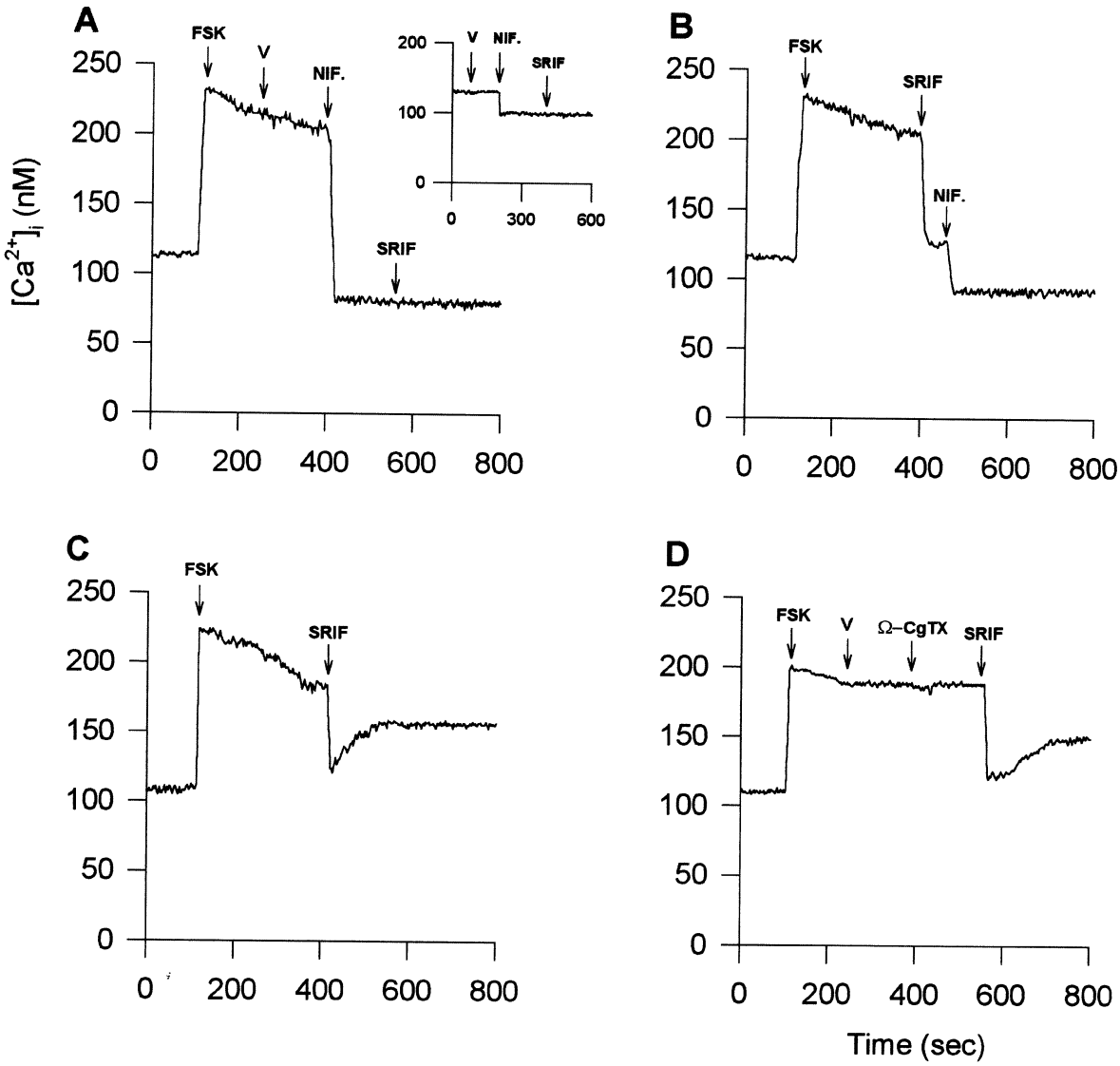

Figure 3 Effects of SRIF (10^{-8} M) on voltage-dependent Ca^{2+} channels. Application of nifedipine (10^{-5} M) induced a drastic reduction of either baseline $[Ca^{2+}]_i$ (inset in A) or $[Ca^{2+}]_i$ rise in response to forskolin (A). Addition of SRIF did not further decrease $[Ca^{2+}]_i$. (B) When nifedipine administration followed SRIF application, a significant reduction in $[Ca^{2+}]_i$ could be obtained. Ω-conotoxin GVIA (Ω-CgTX, 10^{-6} M) pretreatment (60 min) did not abolish the SRIF-induced inhibition of the $[Ca^{2+}]_i$ rise in response to forskolin (C). This inhibition also persisted when SRIF was added after Ω-CgTX application (D). Each trace shown is typical of the recordings made in 3–5 other experiments.

Signal transduction mechanisms

Cell preincubation in PTX (18 h; 100 ng ml^{-1}) did not significantly influence baseline $[Ca^{2+}]_i$ or the $[Ca^{2+}]_i$ rise in response to FSK. Neither baseline $[Ca^{2+}]_i$ nor the $[Ca^{2+}]_i$ rise were significantly affected by SRIF application in the presence of PTX ($n=6$). A representative trace is shown in Figure 9A.

To evaluate the transduction mechanisms mediating the effects of sst_2 receptor activation, we tested possible involvement of cyclic AMP-dependent pathways. To this aim, effects of SRIF or octreotide on basal or FSK-stimulated cyclic AMP accumulation in GC cells were studied in the presence or in the absence of the sst_2 receptor antagonist (2×10^{-7} M, 30 min preincubation). Intracellular cyclic AMP levels were measured in cells pretreated or not with the phosphodiesterase inhibitor IBMX (10^{-4} M, 10 min incubation). In Table 1 the effects of SRIF or octreotide at 10^{-7} M are reported. Indeed, at this concentration both SRIF and octreotide were effective in reducing $[Ca^{2+}]_i$. The data are expressed as percentages of cyclic AMP basal levels. In the presence of IBMX, SRIF or octreotide significantly inhibited both the basal or the FSK-stimulated cyclic AMP levels. In the absence of IBMX, SRIF or octreotide did not significantly influence the basal cyclic AMP level. Both peptides, in contrast, significantly decreased the FSK-stimulated cyclic AMP accumulation. SRIF- or octreotide-induced inhibition of cyclic AMP levels was abolished by cell pretreatment with the sst_2 receptor antagonist.

To further clarify to what extent cyclic AMP-dependent pathways mediate SRIF effects on $[Ca^{2+}]_i$, GC cells were incubated in the presence of H-89, a potent and widely used PKA inhibitor (Engh *et al*., 1996; Penn *et al*., 1999). It was recently used in order to investigate transduction mechanism mediating the SRIF-induced inhibition of $[Ca^{2+}]_i$ in PC12 cells (Traina & Bagnoli, 1999). H-89 (10^{-5} M) was applied 60 min before the addition of 10^{-8} M SRIF. Incubation with H-89 did not produce any significant effect on the basal $[Ca^{2+}]_i$. In H-89 incubated cells, baseline $[Ca^{2+}]_i$ was still decreased by SRIF application, but the SRIF-induced inhibition was significantly

reduced by 56±2.8% ($n=4$). A representative trace is shown in Figure 9B.

Discussion

The release of GH from the somatotrophs of the anterior pituitary appears to be critically dependent on changes in $[Ca^{2+}]_i$. In particular, the inhibitory effect of SRIF on GH release might be dependent on the reported ability of SRIF to decrease, or prevent, an increase in $[Ca^{2+}]_i$. In normal somatotrophs, GHRH triggers Ca^{2+} oscillations and raises $[Ca^{2+}]_i$ by facilitating Ca^{2+} influx (Lussier *et al*., 1991a; Cuttler *et al*., 1992; Kato *et al*., 1992; Kwiecien *et al*., 1997). Both Ca^{2+} transients and $[Ca^{2+}]_i$ increase are lowered by application of SRIF that inhibits Ca^{2+} influx (Lussier *et al*., 1991c). Rat tumour somatotrophs (GC cells) exibit a spontaneous pacemaker activity mostly resulting from Ca^{2+} entry through L-type channels (Kwiecien *et al*., 1998). These cells do not express receptors for GHRH (Lin *et al*., 1992) but display functional SRIF receptors (Mounier *et al*., 1995; Traina *et al*., 1998). In keeping with these observations, SRIF or octreotide applications result in inhibition of basal GH release from GC cells (Mounier *et al*., 1995). This effect is consistent with a SRIF-induced inhibition of $[Ca^{2+}]_i$ *via* sst_2 receptor.

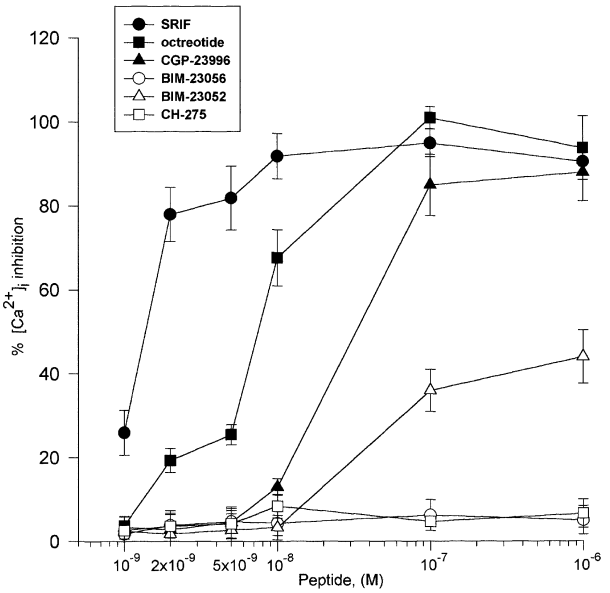

Figure 4 Concentration-dependence of the effects of SRIF and its agonists on the percentage of $[Ca^{2+}]_i$ reduction. In the ordinate, per cent $[Ca^{2+}]_i$ reduction is defined as the per cent difference between the steady-state level of $[Ca^{2+}]_i$ at the time at which the ligand was applied and the $[Ca^{2+}]_i$ level 5 s after its application.

Control of $[Ca^{2+}]_i$

In GC cells, SRIF reduces both the basal $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ rise in response to low K^+ concentration whereas it does not influence the $[Ca^{2+}]_i$ rise in response to high K^+. Also in rat somatotrophs, SRIF cannot overcome the effect of high K^+ (Thorner *et al*., 1988). The lack of SRIF effects in the presence of high K^+ is likely to be due to saturation of Ca^{2+} uptake mechanisms caused by high depolarization levels and resulting in desensitization of Ca^{2+} channels. Indeed, the massive entrance of Ca^{2+} into the cell would activate Ca^{2+}/calmodulin-dependent phosphatases which would de-phosphorylate L channels. De-phosphorylation of L channels would make them unable to close and therefore they could not be modulated by SRIF.

In our experiments, $[Ca^{2+}]_i$ increased when the intracellular cyclic AMP content was elevated by activation of cellular AC with FSK. Indeed, increased cyclic AMP intracellular levels may induce Ca^{2+} influx by activating Ca^{2+} channels (Holl *et al*., 1989; Gronroos *et al*., 1998). For instance, in clonal pituitary cells, cyclic AMP-dependent pathways lead to phosphorylation of voltage-dependent Ca^{2+} channels and allow their opening after depolarization (Armstrong & Eckert, 1987). Similarly, in rat somatotrophs, FSK-induced AC activation determines Ca^{2+} influx and stimulates GH release through an increase of the intracellular cyclic AMP concentration (Holl *et al*., 1989; Cuttler *et al*., 1992). This same mechanism is likely to underlie the FSK effects observed in our experiments, since the FSK-induced $[Ca^{2+}]_i$ rise is abolished in the absence of extracellular Ca^{2+}. More importantly, our results show that SRIF drastically inhibits the Ca^{2+} influx induced by FSK and $[Ca^{2+}]_i$ almost recovers the pre-FSK baseline. SRIF decreases $[Ca^{2+}]_i$ through the inhibition of dihydropyridine-sensitive L-type voltage-sensitive Ca^{2+} channels since SRIF application to nifedipine-treated cells does not significantly influence either the basal $[Ca^{2+}]_i$ or the $[Ca^{2+}]_i$ rise in response to FSK, thus suggesting that nifedipine and SRIF share a common mechanism of action. The SRIF-induced inhibition of Ca^{2+} influx was shown to be mediated by voltage-dependent L-type Ca^{2+} channels in different cells (Tallent *et al*., 1996; Traina *et al*., 1996). In normal and tumour somatotrophs, Ca^{2+} oscillations result, at least in part, from Ca^{2+} entry through L-type Ca^{2+} channels (Kwiecien *et al*., 1997; 1998). On the other hand, in our model, the SRIF-induced inhibition of L-type Ca^{2+} channels appears to be incomplete since nifedipine

Table 1 Effects of SRIF or octreotide on basal and FSK-stimulated cyclic AMP levels

cyclic AMP	*−IBMX* %	*s.e.mean* ±	*+IBMX* %	*s.e.mean* ±	*+sst_2 receptor antagonist* %	*s.e.mean* ±
Basal	100	6.1	100	7.9	100	4.8
SRIF	106	6.8	61	5.4*	99	6.3
Octreotide	108	3.8	70	6.6*	99	7.9
FSK	983	8.3*	984	4.7*	906	4.7*
FSK + SRIF	686	9.0*°	674	6.2*°	918	8.9*
FSK + octreotide	776	7.4*°	689	8.6*°	898	6.5*

Data are expressed as % of basal values and are means of five experiments run in duplicate. Basal values of cyclic AMP levels expressed as pmoles 10^6 cells^{-1} 10 min^{-1} are as follows: 9.7±0.6 (−IBMX), 15.2±1.2 (+IBMX), 16.2±1.2 (+IBMX, + sst_2 receptor antagonists). Intracellular cylic AMP levels were measured in the absence or in the presence of 10^{-4} M IBMX. Cells were pretreated with the sst_2 receptor antagonists (2×10^{-7} M, 30 min) in the presence of IBMX. SRIF or octreotide were applied at 10^{-7} M, FSK was applied at 10^{-6} M. *$P<0.05$ versus the respective basal values. °$P<0.05$ versus forskolin.

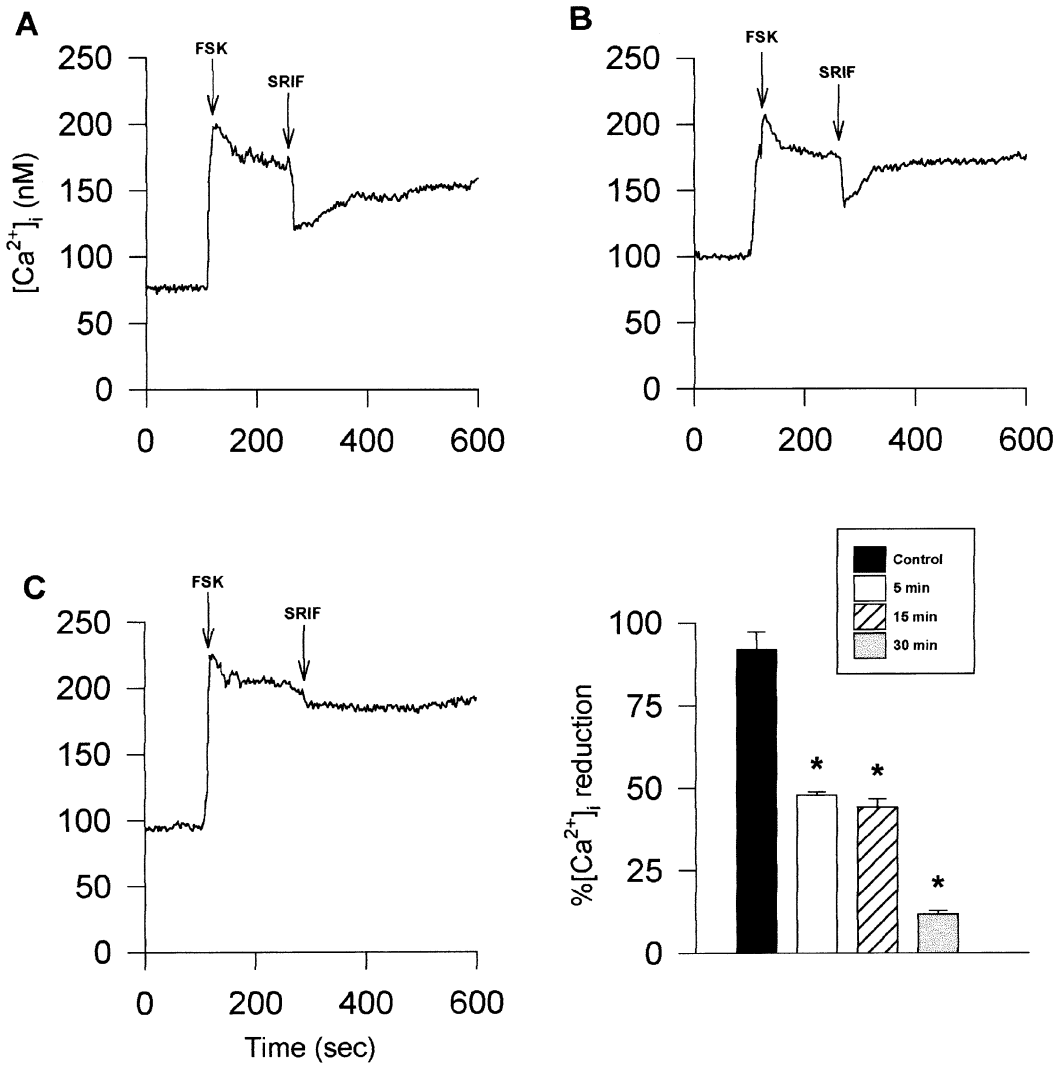

Figure 5 Effects of SRIF application (10^{-8} M) on the percentage of $[Ca^{2+}]_i$ reduction in cells pretreated with L-Tyr^8 Cyanamid 154806 (2×10^{-7} M) at increasing duration. Data in the histogram are mean ± s.e.mean (bars) values from six experiments and analysed by ANOVA for variance and Fisher's test for significance ($P<0.05$). Traces are representative of the effects obtained following 5 (A), 15 (B) and 30 (C) min pretreatment and are typical of the recordings made in five other experiments. *$P<0.05$ versus the respective control value.

application after SRIF significantly reduces $[Ca^{2+}]_i$. This observation suggests that SRIF either has an efficacy lower than that of nifedipine or it may facilitate the inactivation process, whereas nifedipine blocks the activation mechanism.

Involvement of sst_2 receptor

In different cells, the application of SRIF agonists is known to exert specific functional influences that may depend on the presence of specific SRIF receptors (Tallent *et al*., 1996; see for review Meyerhof, 1998). GC cell membranes express a relatively large amount of membrane-associated SRIF receptor mRNA, 64% of which is represented by sst_1 receptor mRNA. sst_2 receptor mRNA accounts for 29% whereas a very low percentage is represented by sst_5 receptor mRNA (Mounier *et al*., 1995; Traina *et al*., 1998).

sst_2 receptor activation has been suggested to modulate GH release from the anterior pituitary *in vitro* (Raynor *et al*., 1993). In rat somatotrophs, for instance, SRIF ability to lower $[Ca^{2+}]_i$ appears to be responsible for its inhibitory action on GH release (Lussier *et al*., 1991b). sst_2 receptor has been reported to mediate the SRIF-induced inhibition of Ca^{2+} influx in different cells. For instance, in rat insulinoma RIN cells, sst_2 receptor mediates the SRIF-induced inhibition of voltage-gated Ca^{2+} channels which, in turn, inhibits insulin secretion (Degtiar *et al*., 1996). In addition, endogenously expressed sst_1 receptor is coupled to L-type Ca^{2+} channels in AtT-20 cells (Tallent *et al*., 1996). In PC12 cells, SRIF agonists specific for sst_2 and sst_3 receptors reduce Ca^{2+} influx with an efficiency comparable to that of the native peptide (Traina *et al*., 1996; Traina & Bagnoli, 1999).

In the present study, SRIF agonists that bind to sst_1 and sst_2 receptors with different affinities have been used in order to establish whether sst_1 and/or sst_2 receptor activation mediates the SRIF-induced inhibition of Ca^{2+} channels in GC cells. Among them, we have used the long-lasting agonist octreotide, a relatively selective agonist for sst_2 receptor, that has been also reported to bind to $sst_{3/5}$ receptors (Raynor *et al*., 1993; Bruns *et al*., 1996; see for review Marbach, 1998). Octreotide is being used in the symptomatic therapy of several neuroendocrine tumours (Lamberts *et al*., 1995; see for reference Pollak & Schally, 1998). In particular, octreotide treatment reduces both GH secretion and tumour growth in rats bearing an ectopic somatotroph

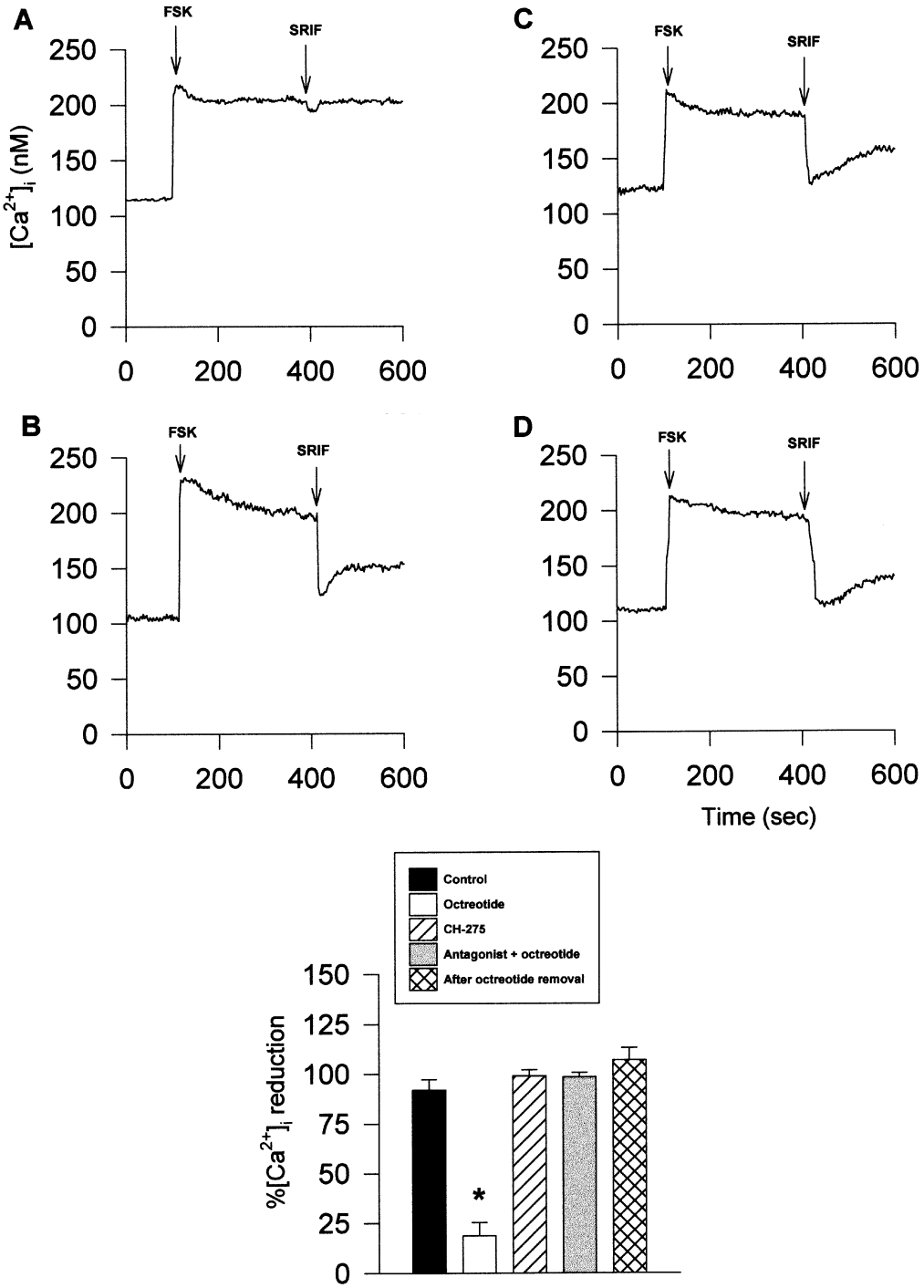

Figure 6 Effects of SRIF application (10^{-8} M) on the percentage of $[Ca^{2+}]_i$ reduction in cells pretreated with the SRIF agonists, octreotide and CH-275 (10^{-6} M, 60 min). Data in the histogram are mean ± s.e.mean (bars) values from six experiments and analysed by ANOVA for variance and Fisher's test for significance ($P<0.05$). Traces shown in A–D are representative of the experiments summarized in the histogram and are typical of the recordings made in five other experiments. *$P<0.05$ versus the respective control value.

tumour (Mounier *et al*., 1995). In addition, two tumour cell lines derived from GH-secreting pituitary adenomas and pheochromocytomas, are responsive or not responsive, respectively, to octreotide therapy depending on the presence or not of sst_2 receptor (Kubota *et al*., 1994; Broson-Chazot *et al*., 1997).

Other SRIF agonists used in the present study include CGP-23996 or BIM-23052 that have been reported to bind to $sst_{2/3/5}$ receptors with different affinities (Raynor *et al*., 1993; Siehler *et al*., 1998). In addition, BIM-23056 displays high affinity for sst_3 receptor (Raynor *et al*., 1993) and CH-275 has been shown to selectively bind to sst_1 receptor (Liapakis *et al*., 1996). However, the selectivity of this compound for sst_1 receptor appears to be less pronounced than previously shown (Viollet *et al*., 1997).

As shown by the present results, SRIF agonists that bind to sst_1 or sst_2 receptors with different affinities differentially reduce $[Ca^{2+}]_i$. SRIF and octreotide inhibit $[Ca^{2+}]_i$ in a concentration-dependent manner with EC_{50} values that are in the range of the affinity values reported for sst_2 receptors (Raynor *et al*., 1993). This result suggests that sst_2 receptors

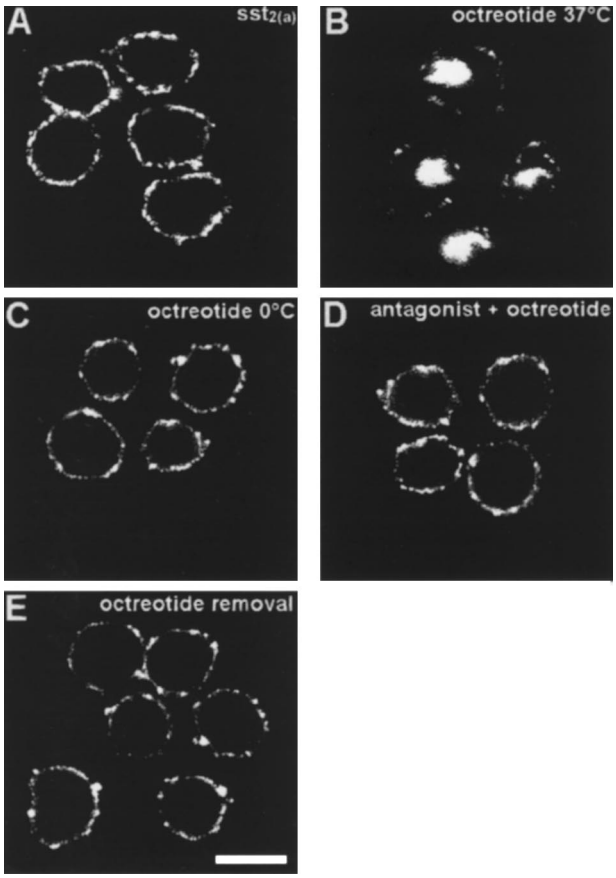

Figure 7 Confocal images (0.3 μm thick) depicting immunocytochemical localization of $sst_{2(a)}$ receptors. $sst_{2(a)}$ receptor immunofluorescence was localized to the cell plasma membrane (A). Following cell pretreatment with octreotide (10^{-6} M, 60 min, 37°C), $sst_{2(a)}$ receptor immunofluorescence accumulates in a perinuclear location (B), whereas it could be seen at the cell surface when: (i) cells were incubated with octreotide at 0°C (C), and (ii) cells were pretreated with the antagonist (2×10^{-7} M, 30 min) before octreotide (D). Sixty minute wash after octreotide removal, $sst_{2(a)}$ receptor immunofluorescence was seen at the cell surface (E). Calibration bar = 13 μm.

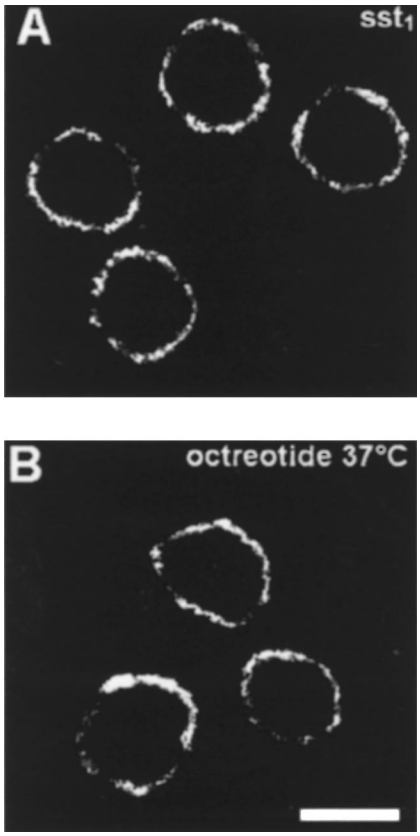

Figure 8 Confocal images (0.3 μm thick) showing immunocytochemical localization of sst_1 receptors. sst_1 receptor immunofluorescence was localized to the cell plasma membrane (A). Following cell pretreatment with octreotide (10^{-6} M, 60 min, 37°C), sst_1 receptor immunofluorescence could be seen at the cell surface (B). Calibration bar = 13 μm.

likely mediate the SRIF-induced inhibition of $[Ca^{2+}]_i$. SRIF and octreotide induce the same maximum $[Ca^{2+}]_i$ inhibition, although, at low concentrations, SRIF appears to exert an effect that is more potent than that induced by octreotide. This finding may be explained by the fact that the native peptide displays binding properties different from those of its synthetic analogue (see for review Meyerhof, 1998). The possibility that other SRIF receptors such as sst_1 receptor, may mediate the SRIF-induced inhibition of $[Ca^{2+}]_i$ could be excluded by the fact that CH-275 does not affect $[Ca^{2+}]_i$. The possibility that CH-275 may exert an antagonist activity can be excluded by the fact that its application before SRIF does not abolish the SRIF-induced inhibition of $[Ca^{2+}]_i$.

Among the SRIF agonists used in this study, BIM-23056 does not affect $[Ca^{2+}]_i$, whereas CGP-23996 or BIM-23052, at concentrations higher that those specific of their binding range, inhibit $[Ca^{2+}]_i$ although to a lesser extent than SRIF or octreotide. At these concentrations, however, the effects of CGP-23996 or BIM-23052 may be non specific (see for review Meyerhof, 1998).

In the present investigation, to antagonize SRIF effects, we have used the L-Tyr^{8}Cyanamid 154806 that is as a potent and selective sst_2 receptor antagonist (Feniuk *et al*., 1998; see for reference Hocart *et al*., 1998). As shown by our results, cell preincubation in the sst_2 receptor antagonist prevents the SRIF- or the octreotide-induced inhibition of $[Ca^{2+}]_i$, thus suggesting that SRIF inhibits Ca^{2+} channels through specific interactions with sst_2 receptor.

The confocal immunocytochemical observations reported in the present study show that GC cell membranes express both sst_1 and $sst_{2(a)}$ receptors although our data are not indicative of their relative quantity. This result is in agreement with the expression of sst_1 and sst_2 receptor mRNAs by pituitary tumour somatotrophs (Mounier *et al*. 1995; Traina *et al*., 1998). Similarly, sst_1 and sst_2 receptors have been reported to be expressed in the anterior pituitary cell line GH_3 (Raynor & Resine, 1993). In contrast, a relatively low expression of sst_1 receptor mRNA has been reported in normal pituitary somatotrophs (O'Carroll & Krempels, 1995; Kumar *et al*., 1997).

As shown by the present study, cell exposure to octreotide results in a drastic reduction in cell surface $sst_{2(a)}$ receptor immunofluorescence that, in fact, translocates to the intracellular pool. Previous experiments have revealed that, following agonist stimulation, SRIF receptors are internalized in a peptide- and receptor subtype-specific fashion (see for review Meyerhof, 1998). In particular, the sst_2 receptor rapidly and efficiently internalizes together with its agonist (Hofland *et al*., 1995; Koenig *et al*., 1997; 1998; Nouel *et al*., 1997; Roosterman *et al*., 1997; Roth *et al*., 1997b; Schwartkop *et al*., 1999). The splice variant $sst_{2(a)}$ desensitizes more efficiently than the spliced form $sst_{2(b)}$ (Vanetti *et al*., 1993). As for other G protein-coupled receptors, its efficient internalization may reside in the length of the carboxy terminal segment in which

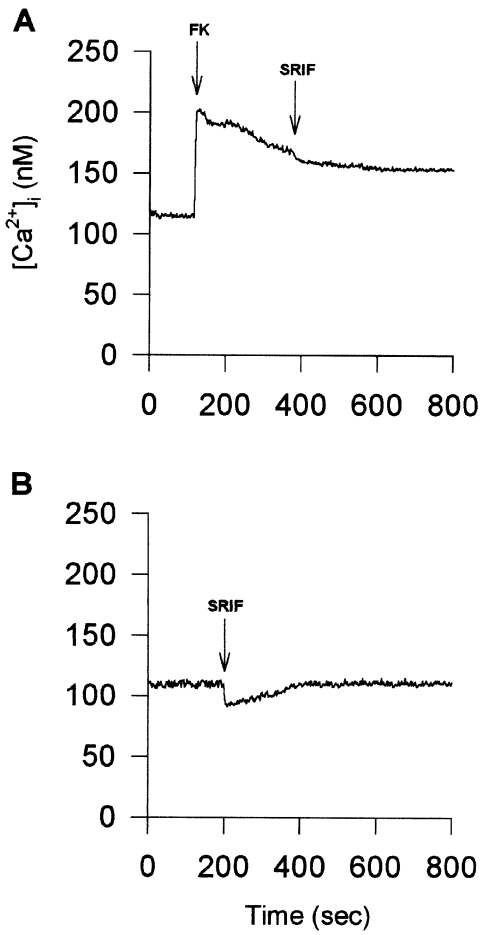

Figure 9 Effects of SRIF (10^{-8} M) on $[Ca^{2+}]_i$. The application of SRIF did not significantly affect the $[Ca^{2+}]_i$ rise in response to forskolin in cells treated with PTX (100 ng ml^{-1}) 18 h before SRIF addition (A). In cells incubated with H-89 (10^{-5} M, 60 min before the addition of 10^{-8} M SRIF), the basal $[Ca^{2+}]_i$ was not significantly different from that of cells incubated in the control medium. In H-89 incubated cells, baseline $[Ca^{2+}]_i$ was still decreased by SRIF application, but the SRIF-induced inhibition was significantly reduced by $56 \pm 2.8\%$. A representative trace is shown in (B). Each trace shown is typical of the recordings made in 4–6 other experiments.

the presence of multiple serine and threonine facilitates the internalization process since these amino acids are potential phosphorilation sites (Roth *et al*., 1997a). As shown by the present results, cell surface sst_1 receptor immunofluorescence does not translocate to the intracellular pool after cell exposure to octreotide. This finding demonstrates the specificity of the effect of octreotide on sst_2 receptor and it is in agreement with previous results showing that sst_1 receptor does not internalize or desensitize (for reference see Meyerhof, 1998).

At the functional level, SRIF does not influence $[Ca^{2+}]_i$ when cells are pretreated with high concentrations of octreotide, thus suggesting that sst_2 receptor signalling desensitizes in response to prolonged ligand stimulation. There is now good evidence that desensitization of G protein-coupled receptor-mediated responses can arise from a number of different mechanisms. At the receptor level, for instance, desensitization can be due to receptor phosphorylation mediated either by specific G protein-coupled receptor kinases or by second messenger-dependent kinases (for reference see Beaumont *et al*., 1998). As shown by our results, internalized $sst_{2(a)}$ receptor fades and is recycled to the cell surface after agonist wash-out indicating complete structural and functional recovery of surface receptor. Indeed, in our experiments, SRIF applications after octreotide wash-out inhibit $[Ca^{2+}]_i$ as in cells without octreotide treatment.

Signal transduction pathways

SRIF receptors are generally coupled to specific G proteins sensitive to PTX. For instance, PTX-sensitive G proteins mediate the SRIF-induced inhibition of Ca^{2+} influx in rat sympathetic ganglion cells, ovine somatotrophs, GH_3 cells (Ikeda & Schofield 1989; Kleuss *et al*., 1991; Chen & Clarke 1996), rat cortical neurons (Wang *et al*., 1990) and PC12 cells (Traina & Bagnoli, 1999). As shown for other cells, also in GC cells, the SRIF-induced inhibition of $[Ca^{2+}]_i$ is mediated by PTX-sensitive G proteins. The activation of sst_2 receptors, with an involvement of PTX-sensitive G proteins, would presumably lead to inhibition of AC activity. Indeed the SRIF- or octreotide-induced decrease of cyclic AMP intracellular accumulation is completely abolished by blockade of the sst_2 receptor, indicating that this receptor mediates the SRIF inhibition of cyclic AMP intracellular levels. SRIF application has been reported to decrease the cyclic AMP accumulation in rat somatotrophs (Epelbaum *et al*., 1987), while other studies of the same cells have failed to demonstrate inhibition of AC activity following SRIF application (Narayanan *et al*., 1989). In GC cells, SRIF as well as its analogue octreotide have been recently shown to inhibit AC activity (Traina *et al*., 1998).

Our results indicate that AC inhibition may mediate SRIF effects on $[Ca^{2+}]_i$. However, the intracellular cyclic AMP content, although reduced by SRIF or octreotide, still remains elevated if compared to the cyclic AMP baseline. On the other hand, both SRIF and octreotide are able to almost recover to baseline the $[Ca^{2+}]_i$ rise induced by FSK. The fact that SRIF is more efficient in inhibiting $[Ca^{2+}]_i$ than in decreasing cyclic AMP concentration suggests that a component of the SRIF-induced $[Ca^{2+}]_i$ decrease may depend on transduction mechanisms other than AC inhibition. This possibility is supported by the observed persisting SRIF effects after PKA blockade. In addition, there is experimental evidence that sst_2 receptor may be functionally coupled to different signal transduction pathways. Indeed, although sst_2 receptor has been generally reported to inhibit AC activity, its efficacy in coupling to AC inhibition appears to depend on the type of the cell where the receptor is expressed. For instance, sst_2 receptor is efficiently coupled to AC activity in F_4C_1 cells, whereas it does not mediate an efficient inhibition of AC in GH_12C_1cells (Chen *et al*., 1997).

Many neurotransmitters that are coupled to cyclic AMP pathways are also known to inhibit or to activate the hydrolysis of phosphatidil inositol (PI), depending on either their specific receptor or the type of G-protein to which the receptor is coupled (Abdel-Latif, 1991). The sst_2 receptor, for instance, has been reported to couple promiscuously to different G proteins (see for reference Meyerhof, 1998). In different cell types, sst_2 receptor activation not only inhibits AC activity but also stimulates phospholipase C (PLC) and Ca^{2+} mobilization (Tomura *et al*., 1994; Chen *et al*., 1997; for review see Meyerhof, 1998). In the case that in GC cells sst_2 receptor activation might involve, at least in part, PLC stimulation, the consequent $[Ca^{2+}]_i$ increase due to release from intracellular stores, could be possibly minimized by other systems, such as PKG-dependent pathways. Indeed, in chick ciliary ganglion cells, the SRIF-induced inhibition of $[Ca^{2+}]_i$ has been shown to involve cyclic GMP-dependent PK (Meriney *et al*., 1994). In addition, biochemical and

functional interactions between IP_3-Ca^{2+} systems and cyclic AMP pathways have been reported in various tissues and cylic AMP levels have been shown to influence IP_3 production (Abdel-Latif, 1991; Rasmussen *et al*., 1990). In this respect, SRIF inhibition on $[Ca^{2+}]_i$ might result from both a decrease of Ca^{2+} entry through AC inhibition and a $[Ca^{2+}]_i$ increase due to Ca^{2+} mobilization. Therefore, possible interactions between different signal transduction pathways might underly a more complex regulation of $[Ca^{2+}]_i$ in GC cells.

SRIF may also inhibit $[Ca^{2+}]_i$ by acting at a step beyond cyclic AMP, possibly by hyperpolarizing the cells as demonstrated for GH pituitary cells (Lussier *et al*., 1991b). In normal somatotrophs, SRIF lowers $[Ca^{2+}]_i$ by blocking Ca^{2+} entry induced by several GH secretagogues, through an increase of K^+ conductance and, thus, hyperpolarizing the cell. As a consequence of hyperpolarization, L-type voltage-sensitive Ca^{2+} channels close, leading to a decrease in Ca^{2+} influx, with a subsequent drop in $[Ca^{2+}]_i$ (Lussier *et al*., 1991b). However, our experiments using bisoxonol have failed to demonstrate substantial changes of the membrane potential both at the resting condition and during the K^+- or the FSK-induced depolarization.

sst_2 receptors have been also shown to operate through the stimulation of phosphotyrosine phosphatase (PTP) activity which is responsible for the SRIF-induced control of cell proliferation (Buscail *et al*., 1994; 1995). Whether SRIF-induced modulation of Ca^{2+} channels may involve PTP activity is not known.

Another mechanism that, in GC cells, might explain that part of SRIF-induced $[Ca^{2+}]_i$ inhibition which does not depend on cyclic AMP levels is a direct coupling between sst_2 receptors and Ca^{2+} channels. In AtT-20 cells, for instance, the SRIF-induced inhibition of $[Ca^{2+}]_i$ does not involve neither SRIF effects on cyclic AMP intracellular levels nor a SRIF-induced alteration of K^+ conductance, suggesting that SRIF receptors are directly coupled to Ca^{2+} channels *via* G proteins (Reisine, 1990).

In conclusion, a multitude of possible interactions between different signal transduction pathways might contribute to the cordinated action of SRIF on $[Ca^{2+}]_i$ regulation in GC cells.

Conclusion

Of the two receptor subtypes expressed by GC cell membranes, the sst_2 receptor mediates the inhibitory control that SRIF plays on $[Ca^{2+}]_i$. This control may represent part of a mechanism by which SRIF, released from the median eminence into the hypothalamo-hypophyseal portal system, periodically triggers GH release while avoiding large $[Ca^{2+}]_i$ increase. The functional significance of the sst_1 receptor present in these cells and visualized immunohistochemically remains unknown.

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