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Chemokine Stromal Cell-Derived Factor 1α Induces Proliferation and Growth Hormone Release in GH4C1 Rat Pituitary Adenoma Cell Line through Multiple Intracellular Signals

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ABSTRACT

We used GH4C1 cells as a model to study the effects of the chemokine stromal cell-derived factor 1 (SDF1) in pituitary functions. In these cells, SDF1α induced proliferation and growth hormone secretion, suggesting a possible regulatory role for this chemokine at pituitary level. We evaluated the intracellular signaling involved in these effects: SDF1α increased cytosolic [Ca^{2+}] and activated Pyk2, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) channels. To correlate these intracellular effectors with the proliferative and secretory effects, we inhibited their activity using BAPTA-AM (Ca^{2+} chelator), 2′-amino-3′-methoxyflavone (PD98059; a mitogen-activated protein kinase kinase inhibitor), salicylate (Pyk2 inhibitor), and tetraethyl ammonium (K^+ channel blocker). All of these compounds reverted SDF1α-induced proliferation, suggesting the involvement of multiple intracellular pathways. Conversely, only BAPTA-AM reverted growth hormone secretion. To identify a possible cross-talk and a molecular ordering among these pathways, we tested these antagonists on SDF1α-dependent activation of ERK1/2, Pyk2, and BK_{Ca} channels. From these experiments, we observed that the inhibition of $[Ca^{2+}]_i$ increase or BK_{Ca} channel activity did not affect ERK1/2 activation by SDF1α; Pyk2 activation was purely Ca^{2+}-dependent, not involving ERK1/2 or BK_{Ca} channels; and BK_{Ca} channel activity was antagonized by Pyk2 but not by ERK1/2 inhibitors. These data suggest that an SDF1α-dependent increase of $[Ca^{2+}]_i$ activates Pyk2, which in turn regulates BK_{Ca} channel activity. Conversely, ERK1/2 activation is an independent phenomenon. In conclusion, we demonstrate that SDF1α causes both proliferation and growth hormone release from pituitary adenoma cells, suggesting that the activation of CXCR4 may represent a novel regulatory mechanism for growth hormone secretion and pituitary cell proliferation, which may contribute to pituitary adenoma development.

Human pituitary adenomas are benign neoplasms mainly classified according to the characteristic clinical syndromes that accompany the tumor hormone production. In particular, the deregulated increase in growth hormone secretion, resulting in acromegaly or gigantism, represents, after alterations of prolactin release, one of the most common hormone-secreting pituitary adenomatous disease. Otherwise, approximately 25 to 30% of pituitary adenomas are classified as "nonsecreting", although this definition strictly refers to the clinical features of these tumors, because immunohistochemistry studies demonstrate that the majority of these adenomas do synthesize and secrete hormones (mainly α subunit or entire gonadotropines). Therefore, they are presently defined as clinically "nonfunctioning pituitary adenomas" (Gittoes, 1998).

The genesis of pituitary tumors is still controversial. It may involve intrinsic alterations of pituicytes [either onco-

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ABBREVIATIONS: SDF1, stromal cell-derived factor 1; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetracetic acid tetra(acetoxymethyl) ester; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channels; PCR, polymerase chain reaction; RT, reverse transcriptase; RT-PCR, reverse transcriptase-polymerase chain reaction; TEA, tetraethyl ammonium; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; MAP, mitogen-activated protein; PD98059, 2′-amino-3′-methoxyflavone.

genic mutations of different genes (e.g., ras, $G\alpha_s$, protein kinase C) or overexpression of activating genes (e.g., pituitary tumor transforming gene) or loss of tumor suppressor genes), alteration of the hypothalamus-pituitary axis, and overproduction of locally secreted growth factors or cytokines active on the hypophyseal cells (Faglia and Spada, 2001). It was proposed that because the majority of pituitary adenomas seems to derive from the clonal expansion of a single transformed pituicyte, genetic alterations at pituitary level seem to be more likely to occur. The current hypothesis assumes that after an initial mutation, providing the cell with a gain of proliferative function (initiation), secondary mutations, hyperproduction of hypothalamic factors, autocrine/paracrine growth factors (in particular, epidermal growth factor and fibroblast growth factors 2 and 4) (Shimon and Melmed, 1997), or cytokines (mainly interleukin 6) may favor the clonal expansion and tumor progression (promotion) (Faglia and Spada, 2001).

Stromal cell-derived factor 1 (SDF1) is a chemokine of the CXC subfamily, originally characterized as a pre-B-cell stimulatory factor and cloned from bone marrow cell supernatants. SDF1 occurs in three alternative splicing variants—α, β, and γ—of which SDF1α is the most abundant (Bajetto et al., 2001b). In contrast to other chemokines, SDF1α nucleotide and amino acid sequences are highly conserved during the evolution (only 1 amino acid difference between murine and human SDF1α), suggesting that this molecule may play important biological roles. Although similarly to other chemokines, SDF1α recruits cells to sites of inflammation, it was also reported to play different functions. This chemokine is a chemotactic factor for T cell, monocytes, pre-B cells, dendritic cells, and hematopoietic progenitor cells and supports B-cell progenitor and $CD34^+$ cell proliferation. However, its expression is not restricted to the immune and blood cells. SDF1α expression has also been described at the central nervous system level in neuronal, astroglial, and microglial cells (Bajetto et al., 1999; Banisadr et al., 2003; Lazarini et al., 2003). SDF1α exerts its effects by interacting with CXCR4, a member of the seven-transmembrane G-protein coupled receptor superfamily. The interaction between SDF1α and CXCR4 seems to be unique, whereas other chemokines may recognize multiple receptors (Bajetto et al., 2001b). Disruption of the murine genes for CXCR4 or SDF1 causes similar embryological lethal phenotypes, characterized by deficient B-lympho- and myelopoiesis, abnormal cardiac and neuronal development, and defects in vasculogenesis (Tachibana et al., 1998; Zou et al., 1998). CXCR4, as observed for SDF1, is also expressed in a wide range of tissues, including endothelial cells, embryonic germinal neuroepithelium, and mature neurons, glia, and microglia (Bajetto et al., 1999; Banisadr et al., 2000). CXCR4 was also reported to represent one of the coreceptors of CD4 for entry of T-lymphocyte tropic strains of human immunodeficiency virus 1, and it was demonstrated that its fusion to and replication in $CD4^+$ and $CXCR4^+$ cells can be inhibited by SDF1 (Feng et al., 1996). CXCR4 is almost constantly expressed by tumor cells (for example, glioblastomas, breast, and ovary carcinomas), and recent data involved its activation in tumor cell proliferation (Scotton et al., 2002; Barbero et al., 2003; Hall and Korach, 2003), migration, invasion (Scotton et al., 2002), metastasization (Geminder et al., 2001; Helbig et al., 2003), and in the tumoral neoangiogenesis (Tachibana et al., 1998; Salcedo et al., 1999).

To date, no evidence have been provided on the possible role of SDF1/CXCR4 in the anterior pituitary function and, possibly, in the genesis of pituitary adenomas, although it was reported in the expression of CXCR4 in rat pituitary, evaluated by autoradiography of [^{131}I]SDF1 binding on rat brain slices (Banisadr et al., 2000).

In this work, we analyzed the possible participation of SDF1, recently renamed CXCL12 (Bajetto et al., 2001b), in the pituitary function on growth hormone secretion and possibly in the development of pituitary adenomas. In particular, we evaluated the role of this peptide in the hormone secretion and pituitary cell proliferation using the rat pituitary adenoma-derived cell line GH4C1. We characterized the signal transduction mechanisms, activated after the binding of SDF1 to its receptor CXCR4, and correlated these signaling to the biological effects of the peptide.

Materials and Methods

Reagents and Materials. Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were purchased from New England BioLabs (Beverly, MA), anti-phospho-Pyk2 from Biosource Europe S.A. (Nivelles, Belgium), and anti-Pyk2 from BD Biosciences Transduction Laboratories (Lexington KY); PD98059 and BAPTA-AM were from Calbiochem (San Diego, CA); and human-SDF1α was from PeproTech EC Ltd. (London, UK). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell Cultures. GH4C1 cells were obtained from the bank of biological material Interlab Cell Line Collection (Genova, Italy) and cultured in Ham's F-10 medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), as reported previously (Florio et al., 1992). When indicated, pertussis toxin was added to the cell culture 24 h before the SDF1α stimulation at the concentration of 180 ng/ml (Schettini et al., 1989).

Western Blot. GH4C1 cells were lysed in 1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, and 10 mM NaF for 10 min at 4°C. Nuclei were removed by centrifugation in minifuge, and cell lysates were assayed for protein contents using the Bradford protein assay (Bio-Rad, Hercules, CA). Proteins (10 μg) were heath-denatured in 2× reducing sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 0.01% bromphenol blue, 1.43 mM β-mercaptoethanol, and 0.1% glycerol), size-fractioned on 10% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membrane (Bio-Rad), and blotted with the appropriate polyclonal antibodies. The detection of immunocomplexes was performed by enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

[^{3}H]Thymidine Incorporation Assay. DNA synthesis activity was measured by means of the [^{3}H]thymidine uptake assay (Florio et al., 1992). Cells were plated at 5×10^4/well in 24-well plates and serum-starved for 48 h before being treated with SDF1α for 16 h; in the last 4 h, cells were pulsed with 1 μCi/ml [^{3}H]thymidine (GE Healthcare). At the end of the incubation, cells were trypsinized (15 min at 37°C), extracted in 10% trichloroacetic acid, and filtered under vacuum through fiberglass filters (GF/A; Whatman, Maidstone, Kent, UK). The filters were then washed sequentially under vacuum with 10 and 5% trichloroacetic acid and 95% ethanol. Trichloroacetic acid-insoluble fraction was then counted in a scintillation counter.

Electrophysiology. In all of the experiments, we used the patch-clamp technique in the cell-attached configuration. The patch electrodes were connected to an EPC-7 (List Medical Instruments, Darmstadt, Germany) amplifier. Patch pipettes were manufactured

from borosilicate glass capillaries (TW150-3; World Precision Instruments, Inc., Sarasota, FL) with a programmable Sachs and Flaming puller (model PC-84), and the tips were fired-polished with a microforge (MF-83; Narishige, Greenvale, NY). The holding potential was set to 0 mV in all of the experiments reported.

Ion currents were recorded with Labmaster D/A, A/D converter-driven by pClamp 7 software (Molecular Devices, Sunnyvale, CA). Capacitance transient neutralization and series resistance compensation were optimized. Single-channel currents were amplified and filtered with a low-pass filter (ITHACO, 4382 Dual 24 dB/octave filter) at a cutoff frequency of 1 kHz with a sampling rate of 13.3 kHz. For each cell, the recording time was 2 min before and after SDF1α treatment. Analysis was performed with Sigma Plot (SPSS Inc., Chicago, IL) software and pClamp 6 (Molecular Devices). This program was used to measure channel current amplitudes and to estimate the open probability of single channel. The standard external solution consisted of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 10 mM glucose. The pH was adjusted to 7.4 using NaOH. The pipettes were filled with a corresponding solution in which the K^+ concentration was increased to 140 mM by equimolar replacement of NaCl with KCl. Tested drugs were added to the bath to obtain the final concentrations indicated.

Measurement of $[Ca^{2+}]_i$ at Single Cell Level. Cells were plated on 25-mm clean glass coverslips, previously coated with poly-L-lysine (10 μg/ml), and transferred to 35-mm Petri dishes; after 24 h, cells were serum-starved for a further 24 h. On the day of the experiment, the cells were washed for 10 min with a balanced salt solution containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 5.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM glucose. Then, cells were loaded with Fura-2 penta-acetoxymethyl ester (4 μM) (Calbiochem-Novabiochem, Laufentigen, Switzerland) for 60 min. Fluorescence measurements were performed as reported previously (Florio et al., 2003). In brief, coverslips were mounted in a coverslip chamber, and Fura-2 fluorescence was imaged with an inverted Nikon diaphot microscope using a Nikon 40×/1.3 numerical aperture Fluor DL objective lens (Nikon, Tokyo, Japan). Cells were illuminated with a Xenon lamp with quartz collector lenses. The two excitation filters (340 and 380 nm) were controlled by computer via a monochromator. Emitted light was passed through a 400-nm dichroic mirror, filtered at 490 nm, and collected by a charge-coupled device camera connected with a light intensifier (Visitech, London, UK). Images were digitalized and averaged in an image processor connected to a computer equipped with the Quanticell software (Visitech). For the calibration of fluorescence signals, we used cells loaded with Fura-2; R_{max} and R_{min} are ratios at saturating and zero $[Ca^{2+}]_i$, respectively, and were obtained by perfusing the cells with a salt solution containing $CaCl_2$ (10 mM), digitonin (2.5 μM), and ionomycin (2 μM) and subsequently with a Ca^{2+}-free salt solution containing EGTA (10 mM). The values of obtained R_{max} and R_{min}, expressed as gray level mean, were used to calculate the $[Ca^{2+}]_i$ using the Quanticell software, according to the equation of Grynkiewicz (1985).

Hormone Release. Growth hormone and prolactin release were assayed using an enzyme immunoassay system (GE Healthcare) as reported (Lecchi et al., 2002). In brief, cells were incubated for 2 h, with the test substances and the medium collected and stored at −80°C until the assay was performed. The amount of hormone released was measured by evaluating the competition between the hormone present in the samples and a fixed quantity of biotin-labeled rat growth hormone or prolactin for a limited amount of rat hormone antibody immobilized on precoated microtiter wells. The actual concentration of growth hormone or prolactin in the samples was calculated comparing the results obtained with those derived from a standard curve prepared using known concentrations of rat hormone standards.

Reverse Transcriptase-PCR. Normal rat hypothalami and anterior pituitaries were isolated from adult male Wistar rats (Charles River Italica, Calco, Italy) as reported previously (Schettini et al., 1988, 1989). Total RNA was isolated from rat brain samples and GH4C1 cells using the acid phenol extraction. Before cDNA synthesis, the RNA was treated with 40 U of RNase-free DNase-I (Roche Molecular Biochemicals, Basel, Switzerland) for 45 min at 37°C in 25 mM Tris-HCl, pH 7.2, 20 mM $MgCl_2$, and 0.1 mM EDTA. Total RNA (5 μg) was reverse-transcribed (RT) in a 20-μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 1 mM dNTPs, 22 U RNase inhibitor, 2.5 μM oligo(dT) (16-mer), and 10 U RT (GE Healthcare) for 40 min at 42°C. To control whether contaminating genomic DNA was present, RNA samples not subjected to RT were included in the PCR amplification. The gene-specific primers used for CXCR4 and SDF1 amplification are the following: sense, 5′-ggccctcaagaccacagtca-3′, and antisense, 5′-ttagctggagtgaaaacttgaag-3′ for CXCR4, and sense, 5′-atgaacgccaag-gtcgtggtc-3′, and antisense, 5′-ggtctgttgtgcttacttgttt-3′ for SDF1. PCR amplification was performed in a reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM primers (each), and 1 U TaqDNA Polymerase (Roche Molecular Biochemicals). The PCR program was as follows: one cycle (5 min at 94°C) followed by 30 cycles (94°C for 30 s, 60°C for 30 s, and 72°C at 30 s). Amplification of β-actin was used as a positive control for the PCR reaction using the following primers: sense, 5′-tccggagacggggtca-3′, and antisense, 5′-cctgcttgct-gatcca-3′.

Statistical Analysis. Experiments were performed in quadruplicate and repeated at least three times. Data are expressed as mean ± S.E. values, and statistical significance was assessed by analysis of variance for independent groups. A p value less than or equal to 0.05 was considered statistically significant.

Results

SDF1α Induces a G Protein-Dependent Proliferation and Growth Hormone Secretion In Vitro in the Pituitary Adenoma-Derived Cell Line GH4C1. To characterize the possible involvement of the SDF1/CXCR4 system in pituitary function, we analyzed the effects of CXCR4 activation in the rat pituitary adenoma cell line GH4C1. We identified specific amplification products for CXCR4 and SDF1 mRNAs in both normal rat hypothalamus and anterior pituitary, whereas GH4C1 cells showed only expression of CXCR4 mRNA (Fig. 1A). Thus, we analyzed the ability of SDF1α to induce proliferation of GH4C1 cells, by means of [^{3}H]thymidine incorporation assay. GH4C1 cells were serum-starved for 48 h and treated with increasing concentrations of SDF1α (3.15–200 nM) for 16 h. As shown in Fig. 1C, SDF1α induced a dose-dependent increase of DNA synthesis with a maximum effect at the concentration of 12.5 nM, reaching an increase in DNA synthesis comparable with that induced, in the same experimental conditions, by growth hormone-releasing hormone (300 nM) (data not shown), a compound reported previously to induce GH4C1 cell proliferation and MAP kinase activity (Zeitler and Siriwardana, 2000). Higher concentrations of SDF1α (up to 200 nM) caused an increase in DNA synthesis that was quantitatively lower (although still statistically significant compared with untreated cells), probably caused by desensitization or down-regulation of the receptor (Fig. 1C). The pretreatment with pertussis toxin completely prevented the proliferative effects of SDF1α, demonstrating that these effects were mediated by a pertussis toxin-sensitive G protein activated via CXCR4 (Fig. 1D).

We also tested the effects of SDF1α treatment on hormone secretion from GH4C1 cells. It was reported previously that these cells secrete both growth hormone and prolactin (Wes-

tendorf and Schonbrunn, 1982). Serum-starved GH4C1 cells were treated for 2 h with SDF1α, and both growth hormone and prolactin release were evaluated by enzyme immunoassay. SDF1α increased growth hormone secretion (>100% of the basal secretion) with a maximal effect at 12.5 nM without showing further increases for higher peptide concentrations (Fig. 2A). SDF1α also induced a statistically significant stimulation of prolactin release, although to a lower extent (<40% of the basal secretion, data not shown). We thus focused further characterization on the effects of SDF1 on growth hormone release. As for proliferation, SDF1α was unable to elicit growth hormone release after the pretreatment of GH4C1 cells with pertussis toxin that uncouples CXCR4 from sensitive G proteins (Fig. 2B).

SDF1α Treatment Increases the $[Ca^{2+}]_i$. To evaluate the intracellular pathways mediating the proliferation of GH4C1 cells, we analyzed the effects of SDF1α on the regulation of intracellular $[Ca^{2+}]$ by means of microfluorimetric experiments. Indeed, Ca^{2+} mobilization after chemokine stimulation is a well-characterized intracellular pathway activated by CXCR4 (Bajetto et al., 1999). Our results, reported in Fig. 3A, confirmed that in GH4C1 cells as well, SDF1α treatment induced a significant increase in $[Ca^{2+}]_i$. Moreover, in agreement with previous reports (Bajetto et al., 1999), we show that the increase in $[Ca^{2+}]_i$ was dependent on the release from the intracellular stores, because in experimental conditions in which Ca^{2+} was removed from the external medium, although starting from a lower basal level, the treatment with SDF1α was still able to increase the $[Ca^{2+}]_i$ (Fig. 3B). Moreover, in agreement with previous studies (Banisadr et al., 2000; Liu et al., 2003), SDF1α regulation of $[Ca^{2+}]_i$ was not associated with the induction of $[Ca^{2+}]_i$ transient oscillations (data not shown).

Ca^{2+}-Mediated Intracellular Signaling by SDF1α. The SDF1α-dependent regulation of Ca^{2+} homeostasis may, in turn, modulate a number of intracellular second messengers involved in the control of both cell proliferation and hormone release. In particular, we evaluated the effects of SDF1α on the activation of the Ca^{2+}-dependent cytosolic tyrosine kinase, Pyk2 (also termed RAFTK or Cak-b), known to be activated by G protein-coupled receptors and recently reported to be involved in the CXCR4 signaling (Bajetto et al., 2001a) and the activation of the MAP kinase ERK1/2, which converts extracellular stimuli into intracellular signals that control gene expression, cell proliferation, and differentiation and that was reported previously to be involved in the proliferative effects of SDF1α in glioma cells (Barbero et al., 2003). SDF1α-dependent activation of Pyk2 and ERK1/2 was analyzed in Western blot experiments using phosphospecific antibodies. SDF1α treatment induced Pyk2 phosphorylation/activation that was clearly detectable after 10 min and reached a maximal stimulation after 30 min of

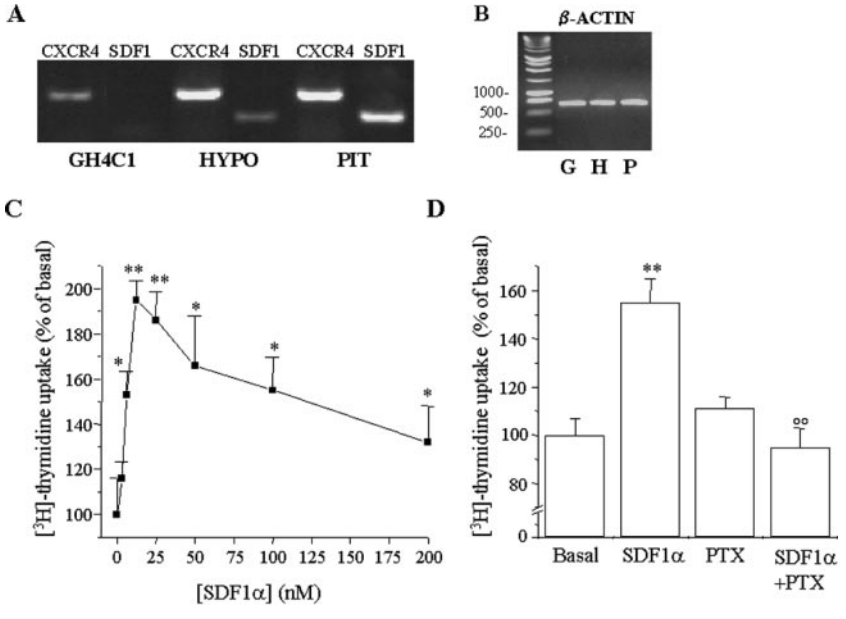

Fig. 1. SDF1α regulation of GH4C1 cell proliferation. A, CXCR4 and SDF1 mRNA expression in rat tissues. GH4C1 rat pituitary adenoma cell line, rat hypothalamus (HYPO), and rat normal pituitary (PIT) were evaluated by RT-PCR analysis. Amplification of RNA before performing the RT reaction demonstrated the lack of genomic DNA contamination in the mRNA samples (data not shown). B, β-actin mRNA expression in rat tissues. GH4C1 rat pituitary adenoma cell line (G), rat hypothalamus (H), and rat normal pituitary (P) were evaluated by RT-PCR analysis for β-actin mRNA expression as a positive control for both the RT and the PCR reactions. C, dose-response effect of SDF1α on the proliferation of GH4C1 cells. GH4C1 cell proliferation was evaluated by means of [^{3}H]thymidine uptake assay. SDF1α induced a dose-dependent increase in DNA synthesis, with a maximal effect at the concentration of 12.5 nM. *, $p < 0.05$, and **, $p < 0.01$ versus basal value. D, involvement of pertussis toxin (PTX)-sensitive G protein in the GH4C1 cell proliferation induced by SDF1α. The pretreatment of GH4C1 cells with PTX (180 ng/ml, 24 h) completely prevented the SDF1α (12.5 nM) stimulation of DNA synthesis evaluated using the [^{3}H]thymidine uptake assay. **, $p < 0.01$ versus basal value; °°, $p < 0.01$ versus. SDF1α stimulation

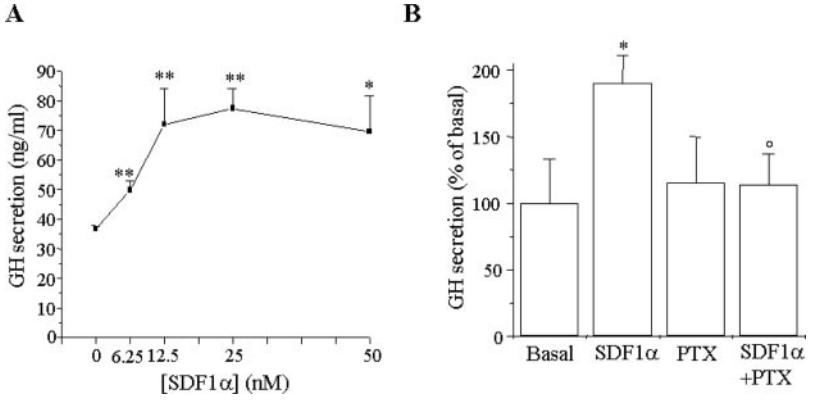

Fig. 2. SDF1α regulation of growth hormone secretion from GH4C1 cells. A, dose-response of the effects of SDF1α on growth hormone secretion. Growth hormone release in the GH4C1 cell culture medium was evaluated by enzyme immunoassay after 2 h of treatment with increasing concentrations of SDF1α (maximal effect at 12.5 nM). *, $p < 0.05$, and **, $p < 0.01$ versus basal value. B, effect of pertussis toxin (PTX) pretreatment (180 ng/ml, 24 h) on SDF1α (12.5 nM) stimulation of growth hormone secretion from GH4C1 cells evaluated by enzyme immunoassay after 2 h of treatment. *, $p < 0.05$ versus basal value; °, $p < 0.05$ versus SDF1α stimulation

treatment (Fig. 4A) at the concentration of 12.5 nM (Fig. 4B). Although a significant amount of phosphorylated ERK1/2 was detected in GH4C1 cells under basal conditions even after 48 h of serum deprivation, the treatment with SDF1α induced a further rapid activation of ERK1/2 (Fig. 4C). This effect was already detectable after 5 min of treatment, lasted up to 10 min, and then decreased after 20 min, thus being much more rapid than the activation of Pyk2. ERK1/2 activation was dose-dependent, with a maximal effect at the concentration of 12.5 nM (Fig. 4D). The analysis of cell lysates for the total expression of Pyk2 and ERK1/2 ensured the equal loading of proteins in the different lanes (Fig. 4).

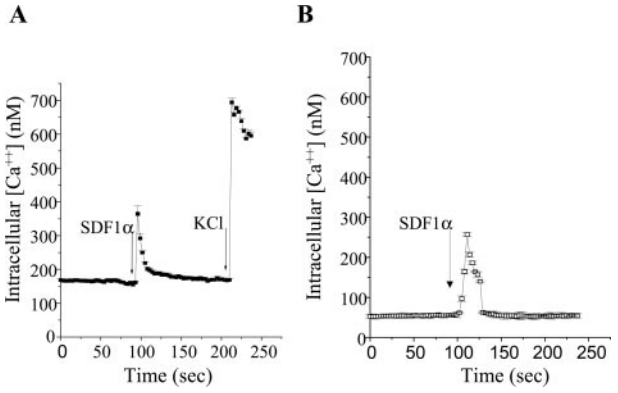

Fig. 3. SDF1α treatment increases $[Ca^{2+}]_i$ in GH4C1 cells. SDF1α effects were evaluated in microfluorimetry at the single-cell level in the presence (A) or absence (B) of Ca^{2+} in the external medium. In both conditions, SDF1α treatment caused a significant spike in $[Ca^{2+}]$ concentration. Data represent the average of at least 15 cells. SDF1α (50 nM) was added as indicated by the arrow.

It was reported that $[Ca^{2+}]_i$ increase induced by CXCR4 stimulation may lead to the activation of the large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) (Liu et al., 2000) known to be involved in the proliferation of different cell types, including GH4C1 and the related cell line GH3 (Huang et al., 2002). Thus, to verify whether the increase in $[Ca^{2+}]_i$ induced by SDF1α may result in an activation of this class of K^+ channels, we tested the activity of BK_{Ca} channels after SDF1α treatment in electrophysiology experiments using the cell-attached patch-clamp configuration. In these conditions, spontaneously active BK_{Ca} channels can be observed at the resting membrane potential (Fig. 5A). No patches with active channel were found when to the electrode-filled solution was added 2 mM tetraethyl ammonium (TEA) (data not shown). At the resting potential, spontaneous activity was observed in 160 of the 200 cell-attached patches examined. The mean conductance of single channel was 148 $\pm$ 14 pS (n = 10). In Fig. 5A, the open probability of the channels is also indicated.

SDF1α (25–100 nM) external application for the whole

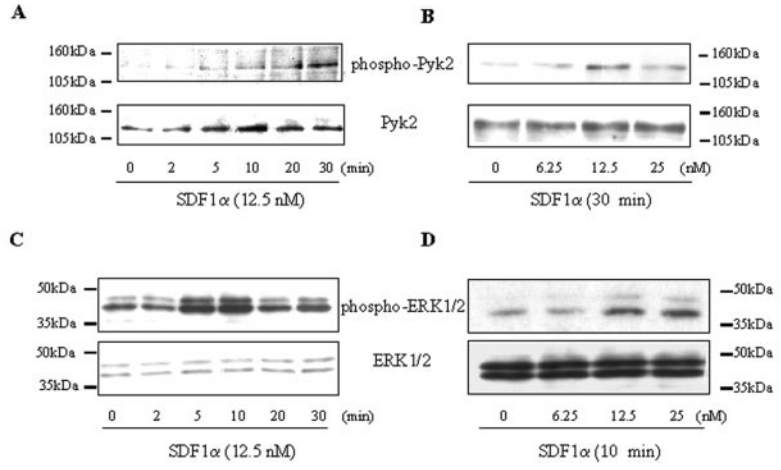

Fig. 4. SDF1α activates the cytosolic tyrosine kinase Pyk2 and the MAP kinase ERK1/2 in GH4C1 cells. Top, time course (A) and dose-response (B) effects of SDF1α on the activation of Pyk2, evaluated in Western blot using a phosphospecific antibody. SDF1α treatment induced a time- and dose-dependent phosphorylation of Pyk2. In both panels, equal protein loading was demonstrated by the Western blot, in a parallel gel, using an antibody that labels the total Pyk2 content. Bottom, time course (C) and dose-response (D) effects of SDF1α on the activation of ERK1/2, evaluated in Western blot using a phosphospecific antibody. SDF1α treatment induced a time- and dose-dependent phosphorylation of ERK1/2. In both panels, equal protein loading was demonstrated by the Western blot, in a parallel gel, using an antibody that labels the total ERK1/2 content.

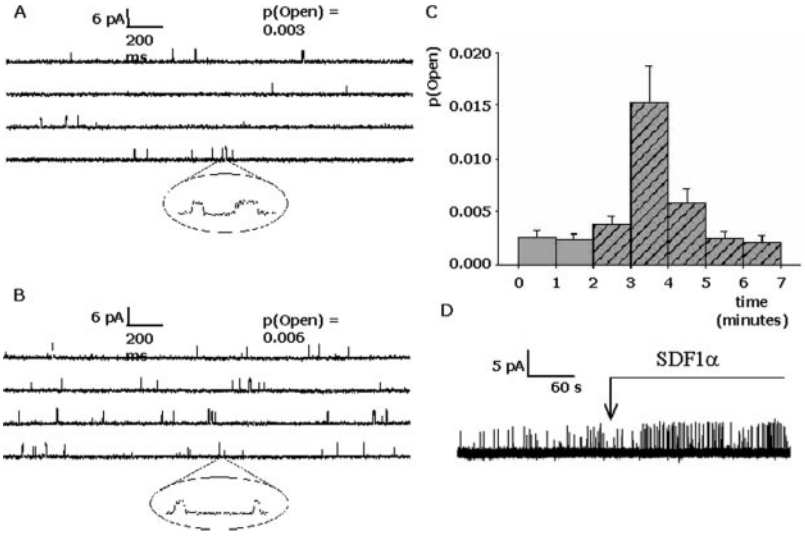

Fig. 5. Enhanced activity of BK_{Ca} channels in a cell-attached patch on a cell stimulated by the addition of SDF1α in the extracellular solution. A, currents from a patch at holding potential of 0 mV under control conditions (the inset shows a 35-ms current trace at an expanded time scale). B, currents from a patch at holding potential of 0 mV in the presence of 25 nM SDF1α. The respective open probabilities of the channel are indicated. C, mean open channel probability before (▤) and after (▨) the addition of SDF1α (100 nM). Each point is expressed as the average $\pm$ S.E. (n = 5). D, current trace recorded in the same patch before and during application. The arrow marks the application of SDF1α (100 nM) that lasted for the whole period of recording. Note that the amplitude of the single channel currents is not statistically different before and after the treatment with SDF1, indicating the same potential across the patch.

period of recording caused a transient increase of the BK_{Ca} channel activity (Fig. 5, B and C). The frequency of the channel openings was increased with an initial period of high channel activity followed by sporadic openings (Fig. 5B). Figure 5D shows a typical recording of single channels before and during exposure to 100 nM SDF1α. The single-channel current slightly increased after SDF1α treatment without reaching a statistically significant difference, whereas the open probability increased more than 4-fold during SDF1α stimulation. This increased activity of the channels was observed in all of the cell-attached patches ($n = 10$) containing spontaneous activity.

Figure 6 shows the mean percentage of opening probability increment recorded in the presence of 25 and 100 nM SDF1α in the external solution. In both cases, the open probability was increased to 150 ± 30% and 340 ± 20%, respectively ($n = 5$).

The effect of SDF1α of BK_{Ca} channels was completely blocked by the treatment of the cells with TEA (2 mM, data not shown) and BAPTA-AM (3 μM; preincubation, 20 min) (Fig. 6), confirming the role of $[Ca^{2+}]_i$ increase in the SDF1α effects.

Effect of the Ca^{2+}-Mediated Intracellular Signaling in the Proliferative and Secretory Effects of SDF1α. To correlate the involvement of ERK1/2 and Pyk2 activation in the GH4C1 cell proliferation induced by SDF1α, we evaluated the [^{3}H]thymidine incorporation into the DNA induced by the chemokine in the presence or absence of drugs able to interfere with the activity of these kinases. GH4C1 proliferation induced by SDF1α was completely inhibited by BAPTA-AM (10 μM), a cell-permeable Ca^{2+} chelator, PD98059 (10 μM), a MAP kinase kinase (MEK) inhibitor, genistein (10 μM), a cytosolic tyrosine kinase inhibitor, and the more specific Pyk2 inhibitor, salicylate (20 mM), all added 20 min before SDF1α (Fig. 7A). Moreover, the specificity of the effects of PD98059 and BAPTA-AM pretreatments on SDF1α-dependent ERK1/2 and Pyk2 activation was analyzed in Western blot. In agreement with recent data (Wang and Brecher, 2001), salicylate (20 mM) completely abolished Pyk2 phosphorylation induced by SDF1α (Fig. 7C). Similar to what observed in the [^{3}H]thymidine incorporation experiments, PD98059 reduced SDF1α-stimulated ERK1/2 phosphorylation (Fig. 7E), although it was unable to reduce SDF1α-stimulated Pyk2 phosphorylation (Fig. 7D). Furthermore, BAPTA-AM did not affect phosphorylation of ERK1/2 under SDF1α-stimulated conditions (Fig. 7E), whereas it reduced SDF1α-stimulated Pyk2 phosphorylation (Fig. 7D). Thus, these data show that ERK1/2 and Pyk2 pathways are independently involved in the intracellular signaling from SDF1α stimulation of GH4C1 cell proliferation. The [^{3}H]thymidine incorporation induced by SDF1α was also strongly reduced by treatment with the BK_{Ca} channel blocker TEA (2 mM) (Fig. 7A), clearly confirming that BK_{Ca} channels are also involved in the proliferative signal mediated by SDF1α.

To correlate the activation of these channels with the regulation of the kinases involved in the proliferation induced by SDF1α, we examined the effects of TEA treatment on the activity of ERK1/2 and Pyk2 by Western blot analysis. Our

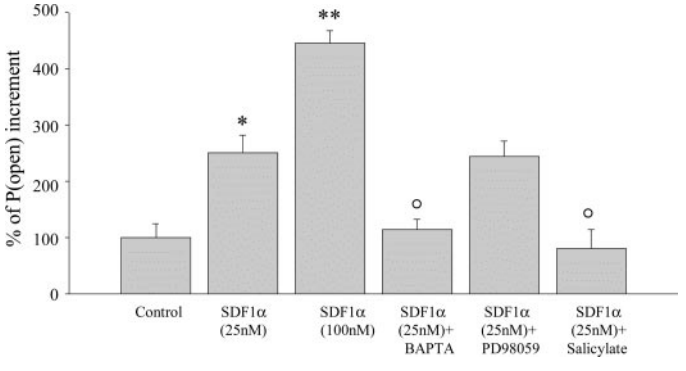

Fig. 6. Signal transduction of the SDF1α regulation BK_{Ca} channel activity. Summary of data showing the effect of SDF1α (25 and 100 nM) on BK_{Ca} channel activity and the effects of BAPTA-AM, PD98059, and salicylate on SDF1α (25 nM)-induced channel activity. Each point is expressed as a percentage ± S.E. compared with the controls (100%) ($n = 5$). BAPTA-AM and salicylate completely prevented SDF1α activation of BK_{Ca} channels, whereas PD98059 was ineffective. *, $p < 0.05$, and **, $p < 0.01$ versus control value; °, $p < 0.05$ versus SDF1α 25 nM value.

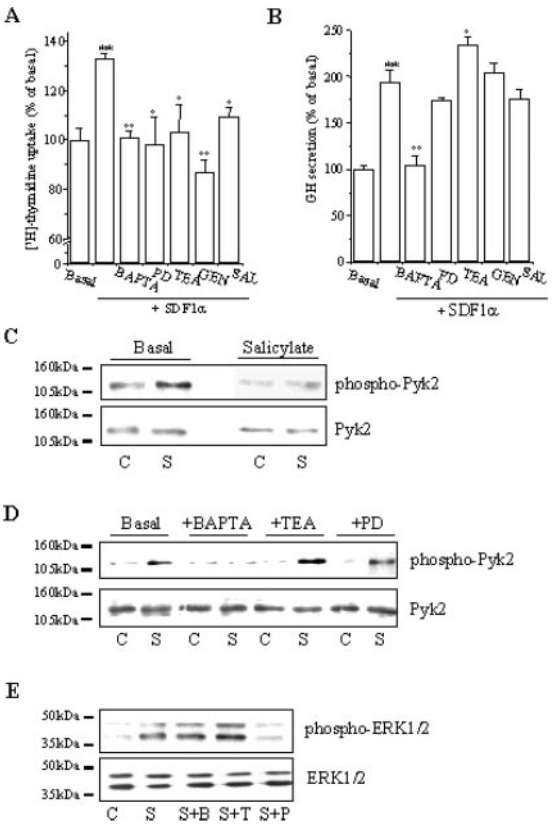

Fig. 7. Intracellular mechanisms involved in SDF1α-induced GH4C1 proliferation and growth hormone secretion and cross-talk between the intracellular pathways activated by SDF1α in GH4C1 cells. A and B, different signal transduction inhibitors were used to dissect the intracellular pathways mediating SDF1α (12.5 nM)-dependent GH4C1 cell proliferation (A) and growth hormone release (B). The compounds used were BAPTA-AM (BAPTA, 10 μM), PD98059 (PD, 10 μM), TEA (2 mM), genistein (GEN, 10 μM), and salicylate (SAL, 20 mM). All of the antagonists were added to the cell culture medium 20 min before SDF1α. **, $p < 0.01$ versus basal value; °, $p < 0.05$, and °°, $p < 0.01$ versus respective SDF1α stimulations. C, effect of salicylate (20 mM) on SDF1α-mediated Pyk2 activation, evaluated in Western blot using a phosphospecific antibody. Equal protein loading was demonstrated by Western blot in a parallel gel using an antibody that labels the total Pyk2 content. D and E, effect of BAPTA-AM (10 μM), TEA (2 mM), and PD98059 (10 μM) pretreatment on SDF1α activation of Pyk2 (D) and ERK1/2 (E), evaluated in Western blot using phosphospecific antibodies. All of the antagonists were added to the cell culture medium 20 min before SDF1α. Total Pyk2 and ERK1/2 expression was evaluated to demonstrate equal protein loading in the gels. C, control; S, SDF1α; B, BAPTA-AM; T, TEA; P, PD98059.

results show that TEA treatment did not reduce either Pyk2 or ERK1/2 phosphorylation and activation induced by SDF1α (Fig. 7, D and E). Moreover, we evaluated the possible role of ERK1/2 and Pyk2 in the SDF1α regulation of BK_{Ca} channels using the selective inhibitors PD98059 and salicylate. However, also in the presence of PD98059 (10 μM), the addition of 25 nM SDF1α produces a significant increase in the channel activity without altering single-channel conductance, as observed in untreated cells (Fig. 6). On the contrary, the treatment with salicylate (20 mM) completely prevented SDF1α effects (Fig. 6). Thus, these data indicate that ERK1/2 activation by SDF1α is a phenomenon independent of the signaling by which the chemokine controls BK_{Ca} channel activity that, conversely, requires the activation of Pyk2.

Then we characterized the intracellular mechanisms mediating SDF1 effects on growth hormone secretion using a similar approach. We pretreated GH4C1 cells with antagonists of the second messengers that mediate SDF1α effects, as identified above: BAPTA-AM (intracellular Ca^{2+} chelator), TEA (K^+ channels inhibitor), genistein (general cytosolic tyrosine kinase inhibitor), salicylate (a more specific Pyk2 inhibitor), and PD98059 (MEK inhibitor). We found that only the pretreatment with BAPTA-AM was able to completely revert the SDF1α-induced growth hormone secretion, whereas genistein, salicylate, and PD98059 were completely ineffective (Fig. 7B). Thus, SDF1-induced proliferation and regulation of growth hormone secretion have both common and different signaling pathways.

Discussion

In the past years, the application of the general principles of tumorigenesis allowed a substantial advancement in the comprehension of the pathogenesis and progression of pituitary tumors. It is now clear that the genesis of pituitary adenomas also involves a multistep process, including both initiating and promoting events (Faglia and Spada, 2001). There is general consensus that the majority of pituitary adenomas derive from a single, initially transformed cell in which a gain of function as far as proliferative potential allows and, subsequently, its clonal expansion in the presence of promoting factors (Herman et al., 1990; Faglia and Spada, 2001). Among the promoting factors, hypothalamic and locally produced growth factors play a pivotal role (Faglia and Spada, 2001). Here, we identify SDF1α as a possible novel growth factor for pituitary cells. Previous studies identified the presence of binding sites for SDF1 in rat pituitary by means of autoradiographic studies and in hypothalamus using immunohistochemistry techniques (Banisadr et al., 2000, 2003). In the latter study, a coexpression between SDF1 and CXCR4 was observed (Banisadr et al., 2003). We confirmed this observation, showing the presence of both CXCR4 and SDF1 mRNA in rat pituitary and hypothalamus. In consideration of the growing bulk of evidence of the role of SDF1/CXCR4 signaling in the growth of different tumoral histotypes (Scotton et al., 2002; Barbero et al., 2003; Porcile et al., 2005), these data may suggest a possible role for the SDF1/CXCR4 ligand-receptor system also in the regulation of pituitary adenoma proliferation and hormone secretion. To address the issue of the effects of CXCR4 activation in pituitary cells at the cellular and molecular level, we analyzed the effects of SDF1 on the pituitary proliferation and hormone release using the rat pituitary adenoma cell line GH4C1, a well-established in vitro model used to characterize the intracellular mechanisms regulating pituitary adenoma functioning. We show that these cells, which release both growth hormone and prolactin (Westendorf and Schonbrunn, 1982), express CXCR4 mRNA but do not secrete SDF1α, thus representing a good model to assess the biological activity of this chemokine, avoiding possible constitutive receptor desensitization or down-regulation observed in in vitro studies in other cell types (Bajetto et al., 1999; Barbero et al., 2003). Our data clearly demonstrate that SDF1α treatment induces both proliferation and hormone release in GH4C1 cells, suggesting a role for this chemokine in the regulation of pituitary functioning. In this article, we show that low concentrations (maximal effect, 12.5 nM) of SDF1α are able to induce a significant proliferation of GH4C1 cells, involving a pertussis toxin-sensitive G protein. Likewise, the activation of CXCR4 caused a pertussis toxin-sensitive increase in both growth hormone and prolactin secretion, although the latter effect was much less pronounced.

We identified multiple intracellular pathways activated by SDF1α to induce pituitary cell proliferation and hormone secretion. In fact, on one hand, inhibitors of MAP kinase, Pyk2, and K^+ channels and the Ca^{2+} chelator BAPTA all reverted DNA synthesis induced by the chemokine, whereas on the other hand, the treatment with SDF1α induced a significant activation of ERK1/2, an increase of $[Ca^{2+}]_i$, caused a Ca^{2+}-dependent phosphorylation/activation of the cytosolic tyrosine kinase Pyk2, and increased the activity of BK_{Ca} channels. All of these intracellular pathways were reported previously to mediate cell proliferation in many cell types and, more importantly, were reported to play a role in CXCR4-mediated signals in different cell types (Liu et al., 2000; Bajetto et al., 2001a; Roland et al., 2003; Sela et al., 2004). In our experimental model, no cross-modulation was identified on the respective effectors: on one hand, the MEK inhibitor PD98059 affected neither Pyk2 nor BK_{Ca} activation (being both Ca^{2+}-dependent processes); on the other hand, ERK1/2 activity was not inhibited by preventing the $[Ca^{2+}]_i$ increase using the intracellular Ca^{2+} chelator BAPTA-AM. Conversely, the activation of BK_{Ca} channels required the activation of Pyk2, as described already for other K^+ channels (Byron and Lucchesi, 2002). Thus, we propose that in GH4C1 cells, ERK1/2 and Pyk2/BK_{Ca} channels contribute independently to the SDF1α-dependent cell proliferation, but their coordinate activities are necessary for the final biological effect.

Moreover, we tried to correlate the second messengers activated by SDF1α to both its proliferative and secretory activities, because specific cellular functions exerted by SDF1α have not yet been clearly attributed to individual intracellular second messengers. From the data described above, we propose that, although the proliferative activity seems to be dependent on multiple intracellular effectors, the regulation of growth hormone secretion induced by SDF1α seems to represent a pure Ca^{2+}-mediated process. Indeed, also in the presence of blockers of ERK1/2 (PD98059), Pyk2, (salicylate), or K^+ channels (TEA), the chemokine elicited a significant growth hormone release that was reverted only in the presence of BAPTA-AM.

In conclusion, we propose that SDF1α represents a powerful proliferative and secretagogue agent for pituitary cells

that, coming either from the systemic circulation or directly from the hypothalamus, may contribute to the regulation of pituitary function. This hypothesis is also supported by the analysis of normal rat tissues (pituitary and hypothalamus) that express both the chemokine and its receptor. Moreover, the observation that in normal rat pituitary, SDF1 and its receptor CXCR4 are coexpressed, together with their proliferative and secretory effects, may suggest a role for this receptor system in the pituitary adenoma development via an autocrine/paracrine pathway, as described in other tumoral cell types (Barbero et al., 2003). It is conceivable that the activation of this loop in cells with a gain of function in the proliferative pathways (induced, for example, by oncogenic mutations) may contribute to the clonal expansion of the mutated cells to favor the development of the adenomas. Moreover, we identified multiple intracellular pathways involved in the CXCR4-dependent cell proliferation, including ERK1/2 and the Ca^{2+}-dependent tyrosine kinase Pyk2 that, in turn, controls the activity of the Ca^{2+}-regulated K^+ channel, BK_{Ca}. Conversely, the increase in the $[Ca^{2+}]_i$ from the intracellular stores mediated by the chemokine is sufficient to elicit growth hormone secretion.

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