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Somatostatin Inhibits Interleukin 6 Release from Rat Cortical Type I Astrocytes via the Inhibition of Adenylyl Cyclase

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Interleukin 6 is a pleiotropic cytokine produced in the central nervous system (CNS) that has been involved in both direct neurotrophic activities and in the regulation of the production of acute phase proteins both at peripheral and central levels. In rat cortical type I astrocytes, interleukin 6 release is under the control of cAMP-protein kinase A and calcium-phospholipids-protein kinase C systems. Somatostatin is a neuropeptide, acting as a neurotransmitter, highly concentrated within the CNS, where it has been involved in the modulation of learning and memory processes. The aim of this study was to characterize the effects of somatostatin on the release of interleukin 6 from rat cortical type I astrocytes and the intracellular mechanisms involved in this activity. Our results show that somatostatin, in a concentration-dependent manner, inhibited basal and forskolin-stimulated interleukin 6 release from rat cortical type I astrocytes in culture. The EC_{50} of the inhibitory action was calculated to be approximately 10 nM. Furthermore, this effect of somatostatin was completely abolished by pretreating cortical astrocytes with pertussis toxin that, uncoupling, by ADP-rybosylating, the inhibitory GTP-binding protein from the receptors, prevents the activation of the intracellular effectors such as the adenylyl cyclase enzyme. To identify the intracellular mechanism mediating the effects of somatostatin on the interleukin 6 release, we evaluated the peptide modulation of basal and stimulated intracellular accumulation of cAMP. In our experimental conditions somatostatin significantly inhibited both basal and forskolin-stimulated cAMP accumulation. Conversely, somatostatin did not affect the increase of interleukin 6 release induced by dibutyryl-cAMP, a nonhydrolizable cAMP analog that, bypassing the effects of somatostatin on adenylyl cyclase activity, directly activated protein kinase A. These observations support the hypothesis that somatostatin inhibitory activity on interleukin 6 release is mediated by its effects on cAMP production. Somatostatin analog SMS 201-995 did not affect interleukin 6 production either in basal or stimulated conditions. Since, SMS 201-995 was reported to bind with high affinity only to somatostatin receptors type 2, 3 and 5, the lack of effect of this compound on interleukin 6 release suggests that the inhibitory action of somatostatin could be mediated by the activation of either type 1 or type 4 somatostatin receptors. In conclusion, our data demonstrate that the release of interleukin 6 from rat cortical type I astrocytes is inhibited by somatostatin through the activation of a somatostatin receptor coupled to the inhibition of adenylyl cyclase via a G-protein sensitive to pertussis toxin.

Cytokines represent a class of molecules showing neurotropic activities(4,14,25). Interleukin 6 (IL-6) is a pleiotropic cytokine whose role as humoral regulator of the immune system is well-known (1,13,34). Recently, astrocytes have been shown to produce and secrete IL-6 in the central nervous system (CNS) in response to proinflammatory stimuli such as interleukin 1_β (IL-1)(16,3,29,10), tumor necrosis factor (TNF)(16,3), or bacterial lipopolysaccharide (LPS)(16,3). Moreover, the activation of neurotransmitter receptors coupled to cAMP production, such as vasoactive intestinal peptide(9), or the direct activa-

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Abbreviations: SRIH, somatostatin; IL-6, interleukin 6; SSTR, somatostatin receptor; FSK, forskolin; AC, adenylyl cyclase; Bu2-cAMP, dibutyryl-cAMP; CNS, central nervous system.

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tion of adenylate cyclase or protein kinase C (forskolin and phorbol esters, respectively esters)(9,10) greatly increased the release of this cytokine.

The role of IL-6 within the central nervous system is not completely clarified. This cytokine was reported to exert direct neurotrophic effects in vitro (i.e. the induction of the differentiation of PC12 cells into neuronal cells and the induction of survival and neuronal sprouting in catecholaminergic hippocampal neurons (25,14). It has also been suggested that IL-6 could induce an acute phase response within the CNS32, thus leading to the production of the amyloid precursor protein and α-1-antichymotrypsin (31,30,24), that are believed to participate in the etiopathogenesis of neurodegenerative diseases, such as Alzheimer's disease. An imbalance between the reparative effects exerted by IL-6 and the IL-6-dependent production of acute phase proteins, triggered by other incoming situations acting on brain environment, may lead to a shift of the activity of the cytokine toward the production of proteins such as the fragment of the amyloid precursor protein, βA4, and the induction of neurodegenerative effects. Indeed, these effects may be due to the participation of external factors such as IL-1_β, produced during inflammatory conditions, that synergizing with the normal production of IL-6 regulated by neurotransmitter activity, may cause an exaggerate production of this cytokine (10).

Somatostatin (SRIH), is a neuropeptide widely expressed in the brain, where it has been proposed to be involved in the control of cognitive functions (26,28). Somatostatinergic neurotransmission is highly impaired in patients with Alzheimer's disease; immunoreactivity for somatostatin has been revealed in the amyloid plaques (19), and the presence of neurofibrillary tangles (23) has been detected within somatostatinergic internucial neurons. Moreover, it has been shown that somatostatin content is reduced in the cerebral cortex and in the cerebrospinal fluid of patients with Alzheimer's disease (6,2). Recently, five subtypes of the somatostatin receptor (SSTR) have been cloned (22), all of which are coupled to the inhibition of adenylyl cyclase (AC) via GTP-binding proteins (11,20). The five types of somatostatin receptors are distinguished in two major subfamilies according to their structural homology (21) and their sensitivity to SRIH synthetic analogues, such as SMS 201-995, MK 678 or RC160. It was reported that SMS 201-995, MK 678 and RC 160 are able to bind and activate only SSTR 2, 3 and 5 (21), while they are completely ineffective on SSTR1 and SSTR4. Recently it has been reported that astrocytes expressed SSTR type 1, 2, and 4 (8). Conflicting data have been reported on SRIH modulation of AC in astrocytes. Although, it has been reported either no effect (5) or inhibition of cAMP production (7), although SRIH binding sites on astrocytes have been clearly identified (18,5).

Our results demonstrate for the first time in the CNS that IL-6 release can be inhibited by a neurotransmitter throughout the reduction of cAMP generation.

EXPERIMENTAL PROCEDURES

Cell cultures. Cultures of rat cortical type-I astrocytes were obtained as previously described (17), with some modifications (9). Briefly, 2 to 3-day-old Wistar rats (Charles River, Italy) were decapitated, the brains removed under aseptic conditions, and placed in phosphate-buffered saline (PBS) (ICN-Flow) containing 100 IU/ml of penicillin and 100 μg/ml of streptomycin (P/S) (Bio-Whittaker). Under a stereomicroscope the meninges were carefully removed and the cerebral cortex dissected. The tissue was cut into small fragments, exposed to trypsin digestion (ICN-Flow) (0.125% in PBS) for 20 min. at 37°C, and then mechanically dissociated in DMEM containing P/S, 10% fetal bovine serum (FBS) (HyClone) and 2 mM glutamine (ICN-Flow) to obtain single cells. Cell viability was greater than 98%, calculated by the trypan blue exclusion test. Thereafter, the cells were plated (1 brain to 1 75 cm^2 T-flask) in DMEM containing P/S and 10% FBS. The medium was changed after 24h and then twice a week. Once confluent, the cultures were vigorously shaken to remove non-adherent cells and subcultured 1:3. The cells were then mechanically purified and subcultured once again 1:4 before the experiments were performed. This protocol allowed the cultures to be constituted by more than 95% glial fibrillary acidic protein-positive cells (data not shown) (9).

IL-6 bioassay. rhIL-6 was quantified by 7TD1 bioassay, before being used as a standard for the unknown samples (9). 7TD1 cells were grown in Iscove's medium (ICN-Flow) supplemented with L-asparagine (0.24 mM) (Serva), L-arginine (0.55 mM) (Serva), hypoxanthine (0.1 mM) (Serva), thymidine (16 μM) (Serva), 2β-mercaptoethanol (50 μM), P/S and 10% FBS and IL-6 (25ng/ml). The bioassay was set up as previously described33 with modifications (9). Briefly, four standard curves were run for each bioassay with the following IL-6 concentrations: 5.2; 4.55; 3.9; 3.25; 2.6; 1 .95; 1.3; 0.65 pg/ml. Each sample was assayed in duplicate in 96-well plates (Falcon). Undiluted samples (50 ml/well) were placed in the first 12-well line. Hundred μl of Iscove's medium, without IL-6, was added to each well. Subsequently, 50 μl of the content of each of the first 12 wells were serially diluted in the next 7 well-lines, obtaining a 1:3 dilution for each step. Thereafter, 100 μl of a suspension containing 5×104 cells/ml was added to each well. The cells were incubated for 4 days; growth was evaluated by colorimetric determination of intracellular exosaminidase levels (15). The absorbance was then determined at two wave lengths (405 nm for test and 620 nm for reference) using a micro-plate reader (Titertrek Multiscan MCC-Flow) and expressed as arbitrary absorbance units. Within the range of IL-6 concentrations used as standards, the concentration/absorbance ratio was linear. Absorbance values for unknown samples were then compared to those of the standard curve. Finally, the arbitrary units were converted into gravimetric units. Neither of the substances tested as inhibitors or stimulants in the experiments affected in a statistically significant manner, at the dilution used, the proliferation of the cells employed in the bioassay (not shown).

Intracellular cAMP assay. Cells were plated in 24-well dishes at the density of 500,000/well. Before the experiments, the medium was replaced to a FBS-free one supplemented with 500μM IBMX and the testing substances, at stated concentrations. The reaction was stopped after 1h by rapid aspiration of the medium and the addition of a mixture of acidic ethanol (1N HCl/ethanol 1 to 100) to extract intracellular cAMP. Samples were kept at 4°C overnight, neutralized

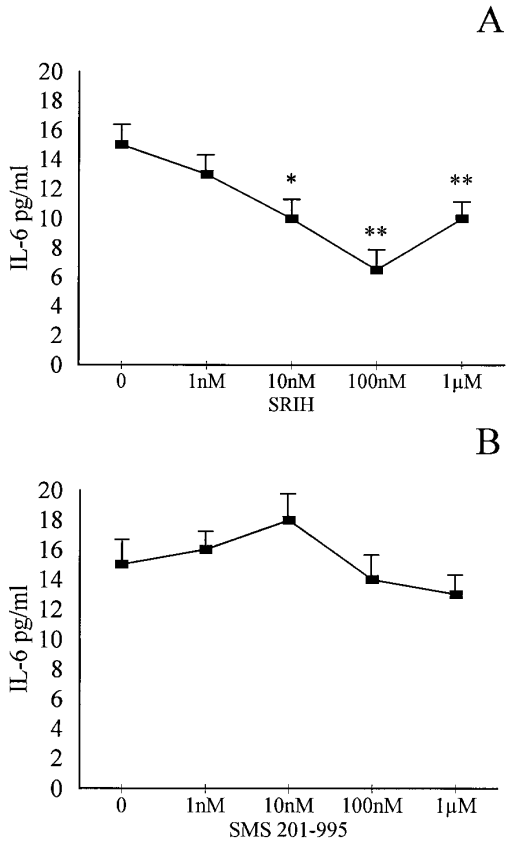

FIG. 1. Effects of somatostatin and SMS201-995 on basal IL-6 release. The treatment of type I astrocytes for 24 h with SRIH caused a concentration-dependent (1nM-1μM) inhibition of the IL-6 release. The effect was statistically significant at the 10 nM concentration and reached the maximal inhibition at the concentration of 100 nM (Panel A). The treatment of type I astrocytes for 24 h with SMS 201-995 (1nM-1μM) was completely ineffective on basal IL-6 release for all the doses tested (panel B). (*=p<0.05 and **=p<0.01 vs. untreated cells)

with 1N NaOH, dried, resuspended in 50ml of a solution of 0.05M Tris, pH 7.5/4mM EDTA) and stored at −80°C. The amount of cAMP produced was determined using the [^{3}H]-cAMP assay system (Amersham, Italy). Intracellular cAMP content was assayed by using a commercial kit purchased from Amersham (Italy). Results were expressed as pg/wells.

Conduction of experiments. In all the experiments astrocytes, grown to confluence in 24-well, plates were used. Experiments were carried out in serum-free DMEM containing 2 mM glutamine, P/S and graded concentrations of test substances. In the case of the concentration response curves with SRIH and SMS 201-995, cells were grown to confluence in six multiwells, to allow an higher basal accumulation of IL-6. After 24 hr of incubation, the medium was collected, under sterile conditions, and stored at −20°C until the bioassays were run. Each experiment was repeated at least three times and each data point was set up in quadruplicate for the release experiments, while each sample was assayed in duplicate in the IL-6 bioassays.

Materials. SRIH was purchased from American Peptides, CA, USA; SMS 201-995 was a kind gift from Sandoz (Milano, Italy). All other reagents were purchased from SIGMA unless otherwise specified.

Statistical analysis. Results were expressed as means ± SEM. The experiments were run in quadruplicate and repeated at least three times. Bioassay results were analyzed by ANOVA followed by Neumann and Keuls test. Differences were considered statistically significant if P value was less than or equal to 0.05.

RESULTS

SRIH modulation of basal IL-6 release. The treatment of cortical type I astrocytes for 24h with SRIH, caused a significant inhibition of IL-6 release. This effect was concentration-dependent (1-1000nM). The inhibitory effect was already significant at 10nM concentration of the peptide. The maximal inhibition was reached at 100nM. Higher concentrations of SRIH did not further reduce the basal secretion of the cytokine (fig.1, panel A). The EC_{50} of the SRIH inhibitory activity was calculated to be about 10 nM (fig. 1, panel A).

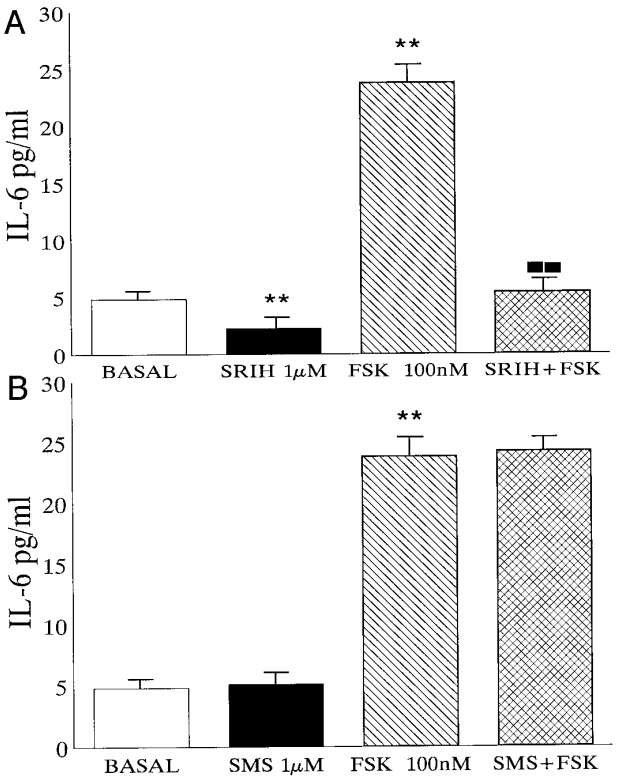

FIG. 2. Effect of somatostatin and SMS 201-995 on forskolin stimulated IL-6 release. The direct activation of adenylyl cyclase with forskolin (FSK) (100nM, for 24 h) caused a significant increase in IL-6 release from type I rat cortical astrocytes. Somatostatin (SRIH) (1μM) significantly reverted this effect, reducing the secretion of cytokine to basal levels (panel A). Similarly to what observed in basal conditions also in conditions of direct activation of adenylyl cyclase with forskolin (FSK) (100nM, for 24 h) SMS 201-995 failed to inhibit IL-6 release from type I rat cortical astrocytes (panel B). (**=p<0.01 vs. basal values; ■ ■=p<0.01 vs. FSK value)

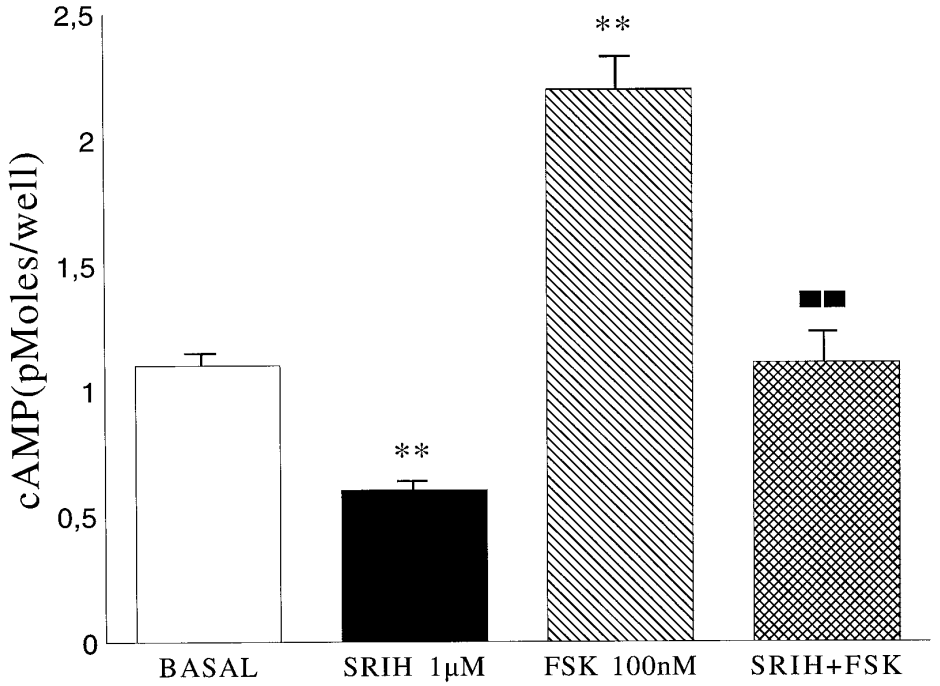

FIG. 3. Effect of SRIH on basal and stimulated intracellular cAMP accumulation. SRIH (1μM, for 1h.) inhibited both basal and FSK (100nM)-stimulated intracellular cAMP accumulation. FSK induced an increase of cAMP accumulation of 250% and this effect was reduced of about 80% by SRIH treatment. (**=$p<0.01$ vs. basal values; ■ ■=$p<0.01$ vs. control value)

On the contrary, the SRIH analogue SMS 210-995 did not affect IL-6 release, in the same experimental conditions at any of the concentrations tested (up to 1 μM) (fig. 1, panel B).

Effect of SRIH on FSK stimulation of IL-6 release. Direct activation of adenylyl cyclase enzyme, in type I cortical astrocytes following FSK (100nM) treatment, significantly increased IL-6 release (about 500%) (fig 2, panel A). Somatostatin (1μM), consistently inhibited the stimulatory activity of FSK (fig. 2, panel A). The inhibitory effect of SRIH was more evident in stimulated than in basal conditions. In fact, SRIH almost completely blocked FSK-induced IL-6 release (fig. 2, panel A). Similarly to the results obtained in basal conditions, SMS 201-995 was completely ineffective in the inhibition of FSK-stimulated IL-6 release, also (fig. 2, panel B).

Effect of SRIH on intracellular cAMP accumulation. To investigate the transducing mechanisms mediating SRIH inhibition of IL-6 release, we assayed the effects of SRIH on basal and FSK-stimulated intracellular cAMP accumulation in rat cortical type I astrocytes. SRIH (1μM) inhibited basal intracellular cAMP accumulation, inducing a reduction of about 50% of the cAMP content. The greatest effectiveness of SRIH was observed when astrocytes were stimulated with FSK (100nM). In these experimental conditions, FSK induced a two-fold increase of cAMP content and SRIH (1μM) caused an almost complete inhibition of intracellular cAMP accumulation induced by the diterpene (fig. 3).

Effect of the nonhydrolyzable cAMP analogue Bu_2-cAMP. To further confirm the involvement of the reduction of cAMP levels induced by SRIH as a mediator of the effect of this peptide on IL-6 release, we tested whether we could abolish the SRIH inhibitory effects by maintaining elevated the intracellular cAMP levels, by adding, an analogue of cAMP, Bu_2-cAMP, permeable to the plasmamembrane. Bu_2-cAMP was previously reported to increase IL-6 release (9), likely acting as a direct PKA activator, thus, by-passing the requirement for adenylyl cyclase activation. In these experimental conditions, Bu_2-cAMP (100μM) significantly increased IL-6 release but SRIH (1μM) was completely unable to revert this effect (fig. 4). These data clearly show that, in the presence of elevated cAMP levels, SRIH is not able to reduce IL-6 secretion.

Effect of pertussis toxin (Ptx) pretreatment on the SRIH inhibitory effects. To better characterize the intracellular mechanism mediating SRIH inhibition of IL-6 release, we investigated the involvement of G-proteins in this effect. We used Ptx, that causes the ADP-rybosylation of G-proteins, incliding Gi, and uncouples the receptors from their intracellular effectors. The pre-

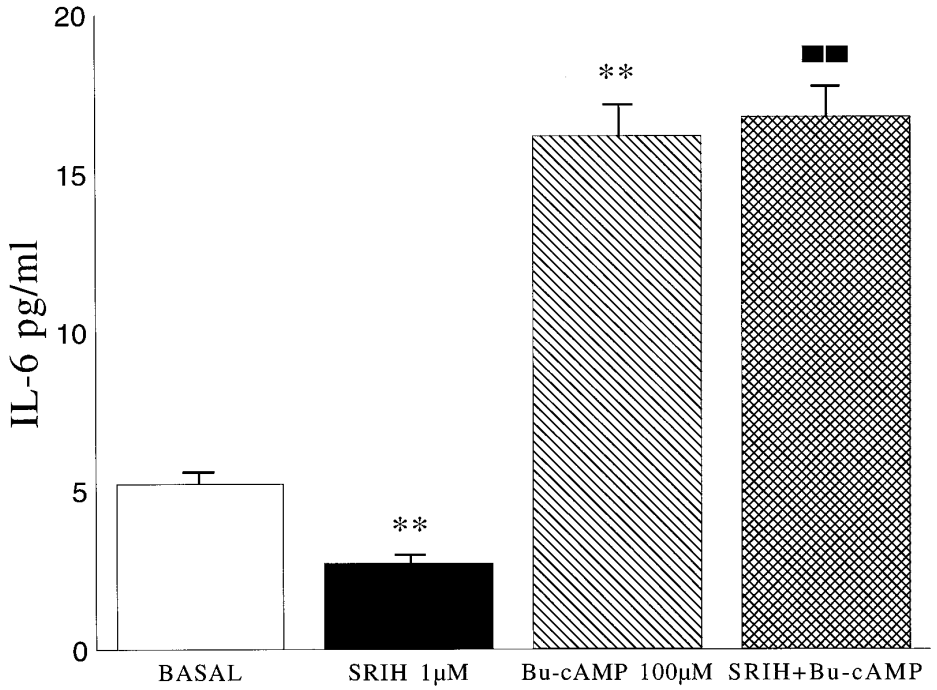

FIG. 4. Effect of the nonhydrolyzable cAMP analog Bu2-cAMP. The treatment of cortical astrocytes with Bu2-cAMP significantly increased IL-6 release. In these experimental conditions, SRIH was completely unable to revert this effect, showing that the SRIH inhibitory effects on IL-6 release are mediated by the SRIH-dependent reduction of intracellular cAMP formation. (**=$p<0.01$ vs. basal value).

treatment for 16 hours of rat cortical type I astrocytes, with Ptx 100 ng/ml, caused a complete abolishment of the SRIH effects on IL-6 release, although it did not modified the ability of FSK to stimulate IL-6 release, thus confirming that the effects of SRIH are dependent on the activation of receptors coupled to G-proteins of the G_i family (fig. 5).

DISCUSSION

It has been reported that SRIH neurotransmission is involved in the learning and memory processes (35,26), and it is known to inhibit adenylyl cyclase activity in different brain areas (27). Somatostatinergic neurons are selectively affected in neurodegenerative diseases, such as Alzheimer's disease (28). In the CNS IL-6 is synthesized and released by type I astrocytes in response to neurotransmitter receptor activation9. This cytokine was reported to play a neurotrophic role within the CNS, but in conditions causing its over-production (i.e. inflammatory conditions in response to IL-1 production), it may induce acute phase proteins and amyloid deposition, leading to neurodegeneration.

In this study we evaluated the effects of somatostatin on the release of IL-6 from rat cortical astrocytes and the transduction mechanisms involved.

In our study, we described for the first time an inhibitory action on basal IL-6 release in rat cortical type I astrocytes. In fact, SRIH induced an inhibition of basal IL-6 release in a concentration dependent manner. This inhibitory effect of SRIH was more pronounced after stimulation of IL-6 release with FSK. From our data, it is conceivable that the antagonistic effects of these two compounds on IL-6 secretion was dependent on their opposing modulation of cAMP production. To verify this hypothesis we evaluated the effects of SRIH on basal and FSK stimulated cAMP production in cortical astrocytes. Although conflicting data have been reported on the capability of SRIH in modulating adenylyl cyclase in astrocytes (7,5), in our experimental conditions SRIH resulted to be a powerful inhibitor of both basal and FSK-stimulated cAMP accumulation, being more effective in the latter conditions. Conversely, when IL-6 production was stimulated by treatment with Bu2-cAMP, thus by-passing the activation of adenylyl cyclase enzyme, providing exogenous cAMP, SRIH was unable to reduce IL-6 production. This observation further confirms that the SRIH effects on the cytokine release are mediated by the inhibitory effects of the peptide on adenylyl cyclase activity. The inhibitory effect of SRIH on IL-6 release was abolished by pertussis toxin pretreatment suggesting that, in astrocytes, a G-protein, likely of the G_i family, couples SRIH-receptors activation to the reduction of IL-

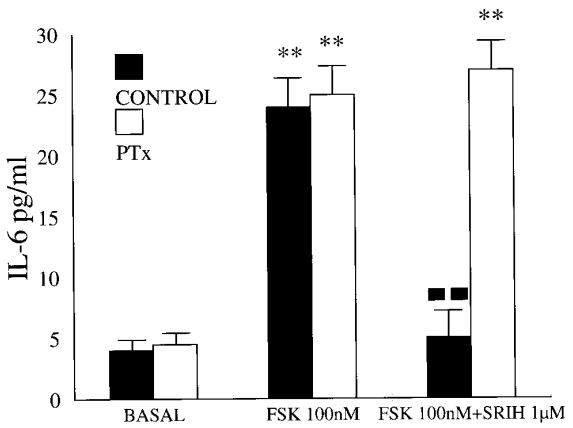

FIG. 5. Effect of pertussis toxin pretreatment on SRIH inhibition of IL-6 production. The pretreatment for 16 hours of rat cortical type I astrocytes, with 100 ng/ml of pertussis toxin (PTx), causing the ADP-rybosylation of the α subunit of the GTP-binding protein G_i, completely prevented SRIH inhibition of IL-6 release, although it did not modified the ability of FSK to stimulate the production of this cytokine. (**=$p<0.01$ vs. basal values; ■ ■=$p<0.01$ vs. control value)

6 release as well as to the reduction of adenylyl cyclase activity.

SMS 201-995, a synthetic SRIH analogue active on SSTR 2, 3 and 5 subtypes (21). was completely unable to inhibit IL-6 release at all the concentrations tested, both in basal or FSK stimulated conditions. Therefore, these SSTR subtypes (2, 3 and 5) do not seem involved in the inhibitory effects of SRIH on IL-6 release.

As far as the physiopathological relevance of this inhibition of IL-6 release, it is noteworthy that in normal conditions a tight regulation of the production of this cytokine could be exerted by different neurotransmitters. In fact, astrocytes express receptors for many neurotransmitters and neuropeptides (12), that, in turn, may modulate in opposite directions the formation of second messengers such as cAMP, keeping the production of IL-6 in a "neurotrophic range". In particular, we previously demonstrated that IL-6 production from cortical astrocytes is stimulated by neurotransmitters that activate PKA, increasing cAMP (such as VIP) (9), or PKC (10). Here we show that agents such as SRIH, can reduce IL-6 release through the inhibition of cAMP production.

Alterations of the somatostatinergic neurotransmission, that were observed in conditions such as Alzheimer's disease (28), may imbalance the control of the production of IL-6. The reduction of the inhibitory activity may result in a net increase of the release of the cytokine. This process together with alteration of neurotransmitter release, such as the modifications of excitatory amino acids observed in some neurodegenerative disease, may further potentiate the release of IL-6, for the synergistic activity with inflammatory mediators such as IL-1_β (10). Thus, these alterations may result in an over-production of IL-6 that may induce an "acute phase response in the brain" with induction of amyloid precursor protein and its metabolites, that may be responsible for some findings of neurodegenerative disease.

In conclusion in this study it is reported for the first time an inhibitory action on basal release of IL-6 in rat cortical type I astrocytes. This inhibitory action is exerted by an inhibitory neurotransmitter, this representing a novel finding, via the reduction of cAMP accumulation and through a mechanisms PTx sensitive involving SSTR other than 2,3, and 5.

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