Somatostatin receptors are expressed by immature cerebellar granule cells: Evidence for a direct inhibitory effect of somatostatin on neuroblast activity

(cerebellar development/cell proliferation/neurotrophic factors/neuromodulation)

B. GONZALEZ*, P. LEROUX*, M. LAMACZ*, C. BODENANT*, R. BALAZS†, AND H. VAUDRY*‡

*European Institute for Peptide Research, Laboratory of Molecular Endocrinology, Centre National de la Recherche Scientifique Unité Recherche Associée 650, Unité Alliée à l'Institut National de la Santé et de la Recherche Médicale, University of Rouen, 76134 Mont-Saint-Aignan, France; and †Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands

ABSTRACT   Somatostatin and somatostatin receptors are transiently expressed in the immature rat cerebellar cortex but virtually undetectable in the cerebellum of adults. Although somatostatin binding sites have been visualized during the postnatal period in the external granule cell layer, the type of cell that expresses somatostatin receptors has never been identified; thus, the potential function of somatostatin in the developing cerebellum remains unknown. In the present study, we have taken advantage of the possibility of obtaining a culture preparation that is greatly enriched in immature cerebellar granule cells to investigate the presence of somatostatin receptors and the effect of somatostatin on intracellular messengers on cerebellar neuroblasts in primary culture. Autoradiographic labeling revealed the occurrence of a high density of binding sites for radioiodinated Tyr-[D-Trp^8]somatostatin-(1–14) on 1-day-old cultured immature granule cells. Saturation and competition studies showed the existence of a single class of high-affinity binding sites ($K_d = 0.133 \pm 0.013$ nM, $B_{max} = 3038 \pm 217$ sites per cell). Somatostatin induced a dose-dependent inhibition of forskolin-evoked cAMP formation ($ED_{50} = 10$ nM), and this effect was prevented by preincubation of cultured immature granule cells with pertussis toxin. Somatostatin also caused a marked reduction of intracellular calcium concentration. These results show the presence of functionally active somatostatin receptors on immature granule cells. Our data suggest the possible involvement of somatostatin in the regulation of proliferation and/or migration of neuroblasts during the development of the cerebellar cortex.

Somatostatin [somatotropin release-inhibiting factor (SRIF)] is a cyclic tetradecapeptide that was first isolated in ovine hypothalamic extracts from its ability to inhibit growth hormone release (1). Subsequent studies have shown that SRIF and SRIF receptors (SRIF-R) are widely distributed in the central nervous system of mammals (2–4), and it is now well documented that SRIF acts as both a neuroendocrine factor inhibiting the secretion of several pituitary hormones and a neurotransmitter and/or neuromodulator controlling a wide spectrum of neuronal activities (5).

In the adult rat, the cerebellum is virtually devoid of SRIF-immunoreactive neurons and SRIF-R (2, 3). Conversely, during development, the occurrence of SRIF and SRIF-R has been demonstrated in the cerebellar cortex (6, 7). In particular, the presence of SRIF-like immunoreactivity has been visualized in Purkinje cells, Golgi cells, and climbing fibers during early postnatal life (6). The existence of SRIF in the primordium of the cerebellum has been detected by radioimmunoassay as early as embryonic day 14 (E14) (8)

and the expression of the SRIF gene has been demonstrated by in situ hybridization between birth and postnatal day 9 (P9) (9). Concurrently, high levels of SRIF-R are expressed in the rat and human cerebellum during development (10, 11). In the rat, SRIF-R are first detectable at E15, reach a maximum between P8 and P13, and gradually vanish from P13 to P23 (12). Autoradiographic labeling revealed the occurrence of a high concentration of SRIF-R in the external granule cell (EGC) layer (12), a proliferative area that generates the majority of the cerebellar interneurons [i.e., granule, stellate, and basket cells] (13), while the disappearance of both SRIF and SRIF-R at the end of the third week (9, 12) coincides with the cessation of neurogenesis (13). However, the cellular localization of SRIF-R and the mode of action of somatostatin in the cerebellum have never been investigated.

The physiological significance of the transient expression of the somatostatinergic system during histogenesis of the rat cerebellum is currently a matter of speculation. The appearance of SRIF and SRIF-R is chronologically associated with the replication and migration of the stem cells of the EGC layer (9, 12, 13). Thus, it has been proposed that SRIF may act as a "trophic factor" involved in the regulation of multiplication of the neuroblasts of the EGC layer (14). However, while SRIF and SRIF agonists have been shown to slow down the growth of various types of human tumors (15–17) and to inhibit proliferation of tumoral cells (18, 19), the function of SRIF in the developing brain remains totally unknown.

In the present study, we have used a culture preparation of 8-day-old rat cerebellum to determine the cell type that expresses SRIF-R and to investigate the mechanism of action of somatostatin. We demonstrate that SRIF-R are borne exclusively by immature granule cells, and we show that these receptor sites are negatively coupled to adenylate cyclase activity and intracellular calcium mobilization. These data suggest that, during postnatal life, SRIF regulates histogenesis in the cerebellar cortex.

MATERIALS AND METHODS

Chemicals. Synthetic SRIF [somatostatin-(1–14)] was provided by G. Chanteclair (Sanofi, Paris). Somatostatin-(1–14) modified by extension at the N-terminal end with a tyrosine residue and replacement of L-Trp-8 with D-Trp-8, Tyr-[D-Trp^8]somatostatin-(1–14), was a gift from D. H. Coy (New

Abbreviations: [Ca^{2+}]$_i$, intracellular calcium concentration; EGC, external granule cell; ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14), radioiodinated Tyr-[D-Trp^8]somatostatin-(1–14) (^{125}I-Tyr-Ala-Gly-Cys-Lys-Asn-Phe-Phe-DTrp-Lys-Thr-Phe-Thr-Ser-Cys-OH); SRIF, somatostatin (somatotropin release-inhibiting factor); SRIF-R, somatostatin receptor(s); P1, etc., postnatal day 1, etc.
‡To whom reprint requests should be addressed.

Orleans). The cAMP radioimmunoassay kit was purchased from Amersham; 3-isobutyl-1-methylxanthine, pertussis toxin, and forskolin were from Sigma. Tyr-[D-Tyr^8]somatostatin-(1–14) was radioiodinated by means of the lactoperoxidase technique as described (20). The radioligand, ^{125}I-Tyr-[D-Tyr^8]somatostatin-(1–14), was purified by reverse-phase HPLC on a Zorbax C$_{18}$ column (25 × 0.4 cm; Merck) by using a gradient of acetonitrile in 0.25 M triethylammonium phosphate buffer at pH 3. The radioiodinated ligand was eluted at 26% acetonitrile. The specific radioactivity was approximately 2000 Ci/mmol (1 Ci = 37 GBq).

Cell Culture. Granule cell suspensions were prepared from cerebella of P8 Wistar rats as described (21). The cells were plated (3 × 10^5 cells per cm^2) onto coverslips coated with poly(L-lysine) at 10 μg/ml. The standard culture medium consisted of 3:1 (vol/vol) Dulbecco's modified Eagle's medium (GIBCO)/Ham's F-12 medium (GIBCO) and the following additions to the mixture: 5 μg of insulin per ml (initially dissolved in 100 μl of 0.1 M NaOH), 100 μg of human transferrin per ml, 20 nM progesterone, 200 μM putrescine, 1 mM sodium pyruvate, 2 mM glutamine, 5 mM KCl, and 30 nM selenium. Mitotic inhibitors were not added because the culture conditions did not favor replication of non-neuronal cells.

Binding Studies. Cells cultured for 1–6 days were used for binding studies. The cells were washed with Tris buffer (pH 5) containing 0.1% bovine serum albumin, 5 mM MgCl$_2$, 0.5 μg of bacitracin per ml, and 100 kallikrein inhibitor units of aprotinin per ml for 10 min. For saturation studies, the cells were incubated for 60 min at 20°C in the same buffer with increasing concentrations of the radiolabeled peptide (70–800 pM). Nonspecific binding was determined by adding 1 μM SRIF. For competition studies, cells were incubated with 300 pM ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) in the presence of various concentrations of unlabeled SRIF (10 pM to 10 μM). At the end of the incubation, the coverslips were removed from the dishes and washed six times with cold Tris buffer, and the radioactivity was counted.

Emulsion-Coated Autoradiographic Studies. Cells were plated at low density (5 × 10^4 cells per cm^2) and incubated with 200 pM radioligand as described above. Then, the coverslips were dipped into Kodak NTB-2 liquid emulsion at 40°C. After 1 week of exposure, the autoradiographic preparations were counterstained with toluidine blue and observed under a light microscope (Leitz; Orthoplan) at high magnification (×1250 under oil immersion).

cAMP Measurement. One-day-old cultured cells were washed and incubated for 5 hr in the absence or presence of pertussis toxin at 100 ng/ml. The cells were then exposed for 30 min to 0.1 mM isobutylmethylxanthine and incubated for another 30 min with 30 μM forskolin in the presence of various concentrations of SRIF. The reaction was stopped by addition of 30% trichloracetic acid at 4°C. Cells and medium were removed and centrifuged at 7000 × g for 3 min. The supernatants were collected and washed three times with diethyl ether and evaporated overnight, and finally the pellet was resuspended with 1 ml of acetate buffer. The cAMP content was assayed by RIA in duplicates as described in the cAMP radioimmunoassay kit.

Calcium Measurement. For microfluorimetric studies, the cells were cultured on coverslips as described above. The cells were incubated in the dark with 5 μM indo-1 acetoxymethyl ester and 0.2% pluronic F 127 in Ringer's solution at 37°C for 10–15 min. At the end of the incubation period, the cells were washed twice with 2 ml of fresh medium. The intracellular calcium concentration ([Ca^{2+}]$_i$) was monitored by a dual-emission microfluorimeter system constructed from a Nikon Diaphot inverted microscope. The fluorescence emission of indo-1, induced by excitation at 355 nm, was recorded at two wavelengths (405 nm and 480 nm) by separate

photometers. The 405/480-nm ratio was determined by using an analogic divider (constructed by B. Dufy, Centre National de la Recherche Scientifique Unité Recherche Associée 1200, Bordeaux, France) after conversion of single photon currents to voltage signals. All three signals (405 nm, 408 nm, and the 405/408-nm ratio) were continuously recorded with a three-channel voltage recorder (BD 100/101, Kipp & Zonen, Delft, The Netherlands). All secretagogues were injected in the vicinity of individual cells by using a pressure ejection system.

RESULTS

The occurrence of binding sites for ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) was visualized on 1-day-old cultured neuroblasts from P8 rat cerebellum by autoradiography (Fig. 1). The culture consisted of more than 90% interneurons, which were easily distinguishable as they are smaller than Purkinje cells and glial cells by a factor of 3–4. Most of the interneurons are immature granule cells; stellate and basket-type cells only account for about 2% (21). Numerous silver grains were
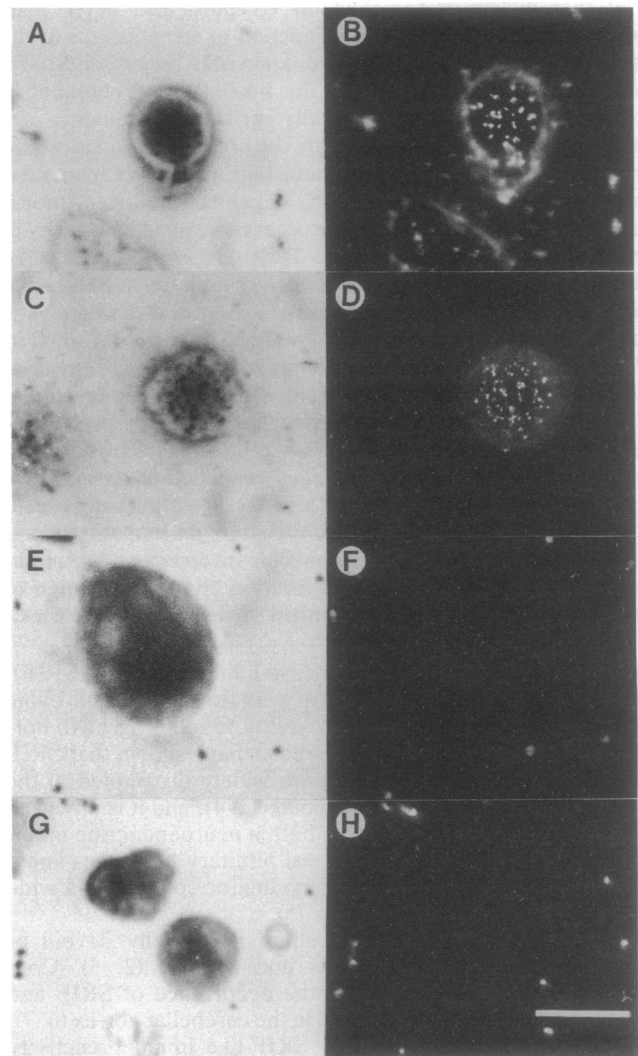

FIG. 1. Visualization by autoradiography of SRIF binding sites on 1-day-old cultured cells from P8 rat cerebellum, incubated with 200 pM ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) in the absence (*A–F*) or presence (*G* and *H*) of 1 μM SRIF. (*A, C, E,* and *G*) Photomicrographs of the cells stained with toluidine blue. (*B, D, F,* and *H*) Dark-field micrographs showing the distribution of silver grains. All microphotographs show immature granule cells except *E* and *F*, which show a much larger (Purkinje or glial) cell. (Bar = 5 μm.)

observed on immature granule cells (Fig. 1 *A–D*), indicating that these cells possess a high density of binding sites. In contrast, the other cell types were not labeled (Fig. 1 *E* and *F*). In the presence of 1 μM SRIF, the labeling of cultured granule cells was totally inhibited (Fig. 1 *G* and *H*).

The evolution of the concentration of SRIF-R on the neuroblasts was studied during 6 days of culture. Three hours after cell dissociation, SRIF-R were almost undetectable in the culture, suggesting the alteration of SRIF-R by the cell dispersion procedure (Fig. 2). The concentration of SRIF binding sites increased rapidly and reached a maximum at 1 day of culture. Then, the level of SRIF-R decreased gradually and disappeared in coincidence with the death of the neuroblasts (Fig. 2).

Saturation studies performed with ^{125}I-Tyr-[D-Trp^8] somatostatin-(1–14) revealed the presence of high-affinity SRIF binding sites on the immature granule cells. Kinetic parameters measured by Scatchard plot analysis indicated that the radioiodinated ligand interacts with a single class of binding sites with a K_d value of 133 $\pm$ 13 pM and a B_{max} of 3038 $\pm$ 217 sites per cell (Fig. 3*A*).

Competition studies using the same radioligand and SRIF as a competitor were carried out with the neuroblasts after 24 hr of culture (Fig. 3*B*). The competition curve was monophasic with an IC$_{50}$ value of 1.91 $\pm$ 0.21 nM and a Hill coefficient of 0.90 $\pm$ 0.01 (Fig. 3*B*).

To determine if these somatostatinergic binding sites represent functional receptors, two different intracellular messengers were studied—i.e., cAMP and calcium. Treatment of immature granule cells with 30 μM forskolin induced a 400–500% stimulation of cAMP levels (Fig. 4*A*). SRIF produced a dose-dependent inhibition of forskolin-evoked cAMP production. Half-maximal inhibition occurred at a dose of 10 μM. In the presence of 100 nM SRIF, the stimulatory effect of forskolin was reduced by 86%. Pretreatment of cultured granule cells with pertussis toxin for 5 hr completely blocked the inhibitory effect of SRIF on forskolin-evoked cAMP production (Fig. 4*B*).

The effect of SRIF on [Ca^{2+}]$_i$ was studied by monitoring the fluorescence signal in a total of 20 neuroblasts. Under the experimental conditions, 90% of the cultured cells responded to SRIF application by a substantial decrease in [Ca^{2+}]$_i$. In two cells, SRIF had no effect. A brief application of SRIF (15 s) by pressure ejection caused an immediate reduction of [Ca^{2+}]$_i$ (Fig. 5*A*). [Ca^{2+}]$_i$ returned to control level within 1.5 min after the end of the administration of SRIF. A 60-s ejection of SRIF caused a sustained decrease of [Ca^{2+}]$_i$, which lasted during the whole exposure of the cell to the neuropeptide (Fig. 5*A*). Repeated pulses of SRIF resulted in sequential inhibition in [Ca^{2+}]$_i$ with gradual attenuation of the response (Fig. 5*B*).
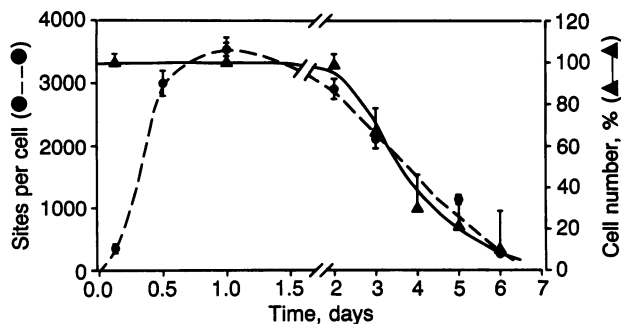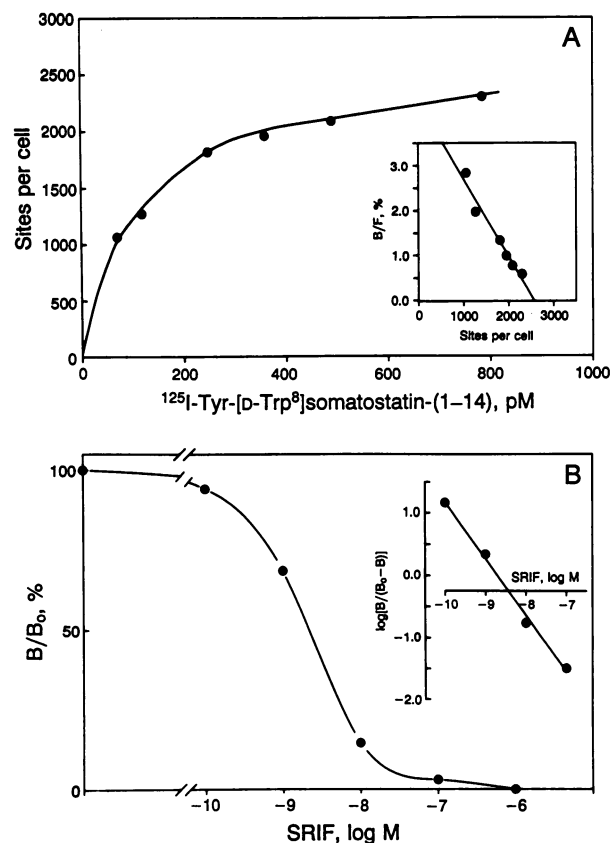

FIG. 3. Characterization of ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) binding sites in 1-day-old cultured immature granule cells. (*A*) Typical saturation curve of ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) binding sites. (*A Inset*) Corresponding Scatchard plot. B, bound; F, free ligand. (*B*) Competition curve of radioligand binding by SRIF. (*B Inset*) Corresponding Hill plot. Each point is the mean of quadruplicates. B$_o$, binding of radioligand in the absence of SRIF; B/B$_o$, fraction of radioligand bound in the presence of increasing concentration of SRIF.

DISCUSSION

Previous studies had shown that SRIF-R are transiently present in the rat cerebellar cortex during the early postnatal period (7, 12). The present data show that SRIF-R are expressed by immature granule cells of the cerebellum in primary culture. The K_d values determined by saturation and competition experiments were similar to those measured by means of membrane binding assay in the adult rat brain (3) and in the immature cerebellum (10). The Hill coefficient was slightly less than 1, suggesting that the radioligand bound to a single population of recognition sites.

To determine whether ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) binding sites actually correspond to authentic SRIF-R, we have examined the effect of SRIF on various second-messenger systems. We first observed that SRIF inhibited in a dose-dependent manner forskolin-induced cAMP biosynthesis by cultured cerebellar neuroblasts. Preincubation of the cells with pertussis toxin totally prevented the inhibitory effect of SRIF on cAMP production, indicating that the action of SRIF on adenylate cyclase is mediated through a pertussis toxin-sensitive guanine nucleotide regulatory binding protein (G$_i$ or G$_o$). We also observed that pressure ejection of SRIF in the vicinity of cultured neuroblasts caused a substantial reduction of [Ca^{2+}]$_i$. Repeated pulses of SRIF resulted in sequential decrease in [Ca^{2+}]$_i$ with gradual attenuation of the response, suggesting the occurrence of a desensitization mechanism. Using the patch-clamp technique, we have recently observed that SRIF causes hyper-

FIG. 2. Effect of the duration of the culture on the density of ^{125}I-Tyr-[D-Trp^8]somatostatin-(1–14) binding sites on immature granule cells (●) and the number of neuroblasts (▲). Each point represents the mean of quadruplicate determinations.
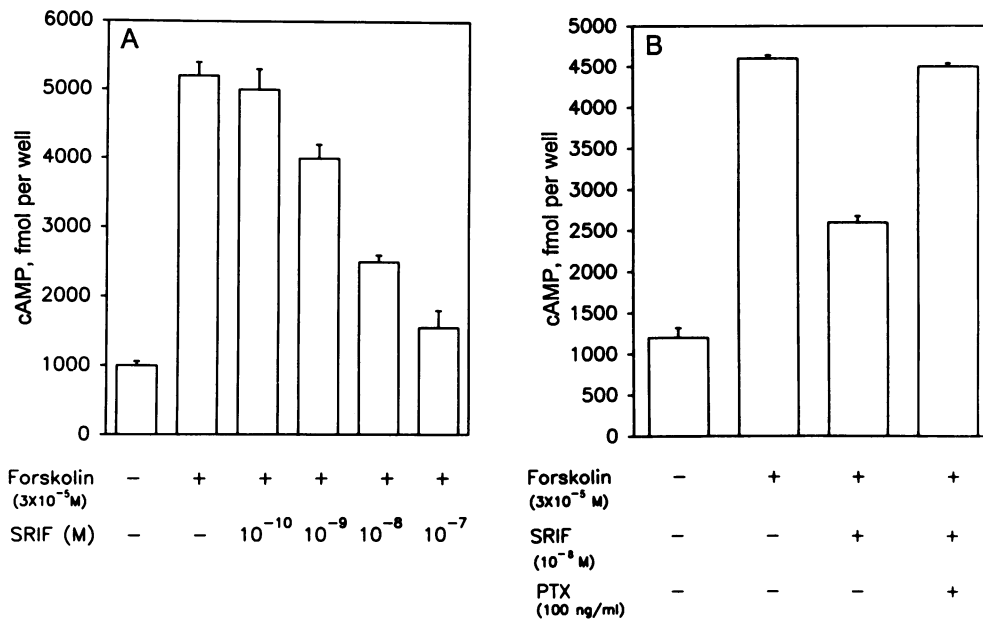
FIG. 4.   Effect of SRIF on forskolin-stimulated cAMP concentrations in 1-day-old cultured immature granule cells. (*A*) Cells were treated with 30 μM forskolin for 30 min in the absence or presence of 0.1 nM to 0.1 μM SRIF. (*B*) Effect of 100 ng of pertussis toxin (PTX) per ml on the inhibitory effect of 10 nM SRIF on forskolin-evoked stimulation of cAMP production. Each value represents the mean ($\pm$SEM) of four independent determinations.

polarization associated with an arrest of spontaneous action potentials in cultured neuroblasts (unpublished data). Previous studies have shown that, in the adult rat brain, SRIF-R are negatively coupled to adenylate cyclase activity (22, 23). In addition, SRIF modulates the conductance of various ionic channels, including potassium (24) and calcium channels (25, 26), through cAMP-independent mechanisms (27). Thus, our data support the view that SRIF-R transiently expressed in immature granule cells of the rat cerebellum exhibit biochemical and functional properties that are similar to those of adult cortical neurons and tumoral cell lines.
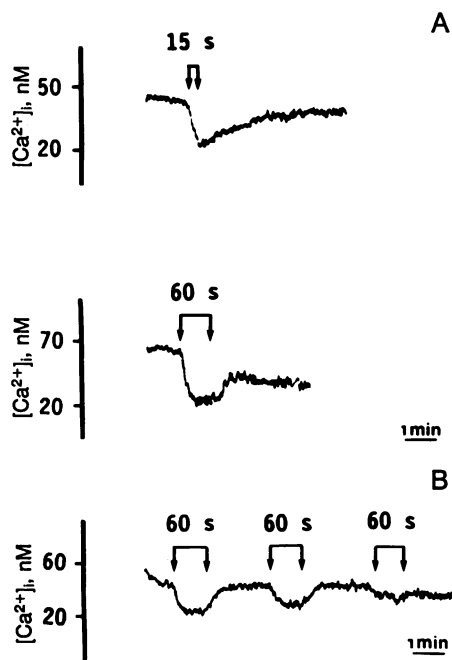

FIG. 5.   Effect of 10 μM (*A*) and 0.1 μM (*B*) SRIF application by pressure ejection on [Ca^{2+}]$_i$ in 1-day-old cultured immature granule cells. The profiles represent the absolute concentration of Ca^{2+} as determined from the ratio of emission fluorescence signal (405 nm/480 nm).

The physiological role of SRIF during development of the central nervous system is currently a matter of speculation. It is now well established that SRIF and its agonists are potent inhibitors of cell proliferation in tumoral cell lines (18, 19) and growth of various human neoplasms (15–17). In the human pancreatic cancer cell line Mia-PaCa-2, SRIF inhibits epidermal growth factor (EGF)-stimulated cell proliferation (17). This effect is mediated through activation of a specific tyrosine phosphatase, independent of the EGF receptor–tyrosine kinase system (28). In the same way, SRIF seems to slow down the development of some glia-derived brain tumors, the presence of high concentrations of SRIF-R being restricted to differentiated tumors with low malignancy (29). Recent studies revealed that SRIF also exerts antiproliferative effects on various types of non-neoplastic somatic cells. In particular, SRIF analogs exert a potent inhibitory effect on multiplication of adrenocortical (30) and vascular smooth muscle cells (31).

We now demonstrate that neuroblasts of the rat cerebellum possess functional SRIF-R. These receptors are only expressed in the EGC layer during the proliferative phase and vanish at the end of neurogenesis (12). These data, together with the presence of transient SRIF-immunoreactive elements during postnatal life, suggest that one of the effects of SRIF is to regulate histogenesis in the cerebellar cortex during development.

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