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Psycholeine, a natural alkaloid extracted from *Psychotria oleoides*, acts as a weak antagonist of somatostatin

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Abstract

The effects of psycholeine, a plant alkaloid, were investigated on binding of radiolabelled somatostatin ([^{125}I]*N*-Tyr-SRIF) and on somatostatin (SRIF)-induced inhibition of adenylate cyclase activity and growth hormone (GH) secretion by rat anterior pituitary cells. Psycholeine was shown to displace specific binding of [^{125}I]*N*-Tyr-SRIF to pituitary membrane preparations, with an IC_{50} of 10^{-5} M. At this concentration, psycholeine was also effective in significantly reducing the SRIF-induced inhibition of adenylate cyclase activity previously stimulated by growth hormone releasing factor (GRF). In parallel, it reduced the SRIF-induced inhibition of GH release stimulated by GRF in primary pituitary cell cultures in a dose-dependent manner. At a moderate concentration, the alkaloid affected neither adenylate cyclase activity nor GH release when applied in the absence of SRIF. These data suggest that psycholeine has antagonistic properties at the SRIF receptor. Quadrigemine C, a precursor of psycholeine, has a similar action.

Keywords: Psycholeine; Quadrigemine C; Somatostatin; Growth hormone; Adenylate cyclase

1. Introduction

We reported previously (Rasolonjanahary et al., 1986) that administration to rats of a crude alkaloid extract obtained from the leaves of *Psychotria oleoides*, a New Caledonian tree, was able to affect plasma levels of growth hormone (GH). When administered to male rats, the organic extract selectively reduced GH plasma levels without affecting other pituitary hormone levels. When the extract was purified, only a few fractions exhibited the effect.

The screening of new active molecules on pituitary hormonal secretion was performed in vivo by using male rats (Rasolonjanahary et al., 1986), a more stable model than female rats for comparing basal and stimulated plasma hormone profiles. In the present work, we provide evidence that two compounds present in purified fractions, psycholeine and quadrigemine C, exhibit antagonistic properties against somatostatin (SRIF) when tested on GH release from dispersed anterior pituitary cells from female rats in vitro. The reason for choosing female pituitaries was that they contain more somatotrophs than male pituitaries and are thus more sensitive to agents affecting GH secretion in vitro.

2. Materials and methods

2.1. Materials

Rat GRF, somatostatin (SRIF) and *N*-Tyr-SRIF were purchased from Peninsula (Belmont, CA, USA). ATP, cAMP, GTP, creatine kinase, creatine phosphate, Dulbecco modified Eagle's medium (DMEM), bovine serum albumin (fraction V), fetal calf serum and glutamine were obtained from Boehringer Mannheim (Indianapolis, IN, USA); Norit-A charcoal

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and Dextran (T70) were from Pharmacia (Piscataway, NH, USA) and culture dishes were from Nunc (Roskild, Denmark). All other compounds were purchased from Sigma (St. Louis, MO, USA). [^{125}I]Na was purchased from Amersham (Les Ulis, France), and [^{3}H]cAMP and [α-^{32}P]ATP from New England Nuclear (Boston, MA, USA).

2.2. Preparation of the alkaloids

Psycholeine and quadrigemine C (Fig. 1) were obtained from a crude mixture of alkaloids extracted from the leaves of *Psychotria oleoides*, a Rubiaceae collected at Montagne des Sources in New Caledonia. Extraction yielded a mixture of very polar alkaloids. These were separated by high performance liquid chromatography (HPLC) on silica gel reverse phase as previously described (Libot et al., 1987; Sévenet et al., 1990; Guéritte-Voegelein et al., 1992). Active fractions were subsequently selected on the basis of their property to bind to pituitary cell membranes, as previously described (Rasolonjanahary et al., 1986). Psycholeine, one of the active derivatives, was purified according to the method of Guéritte-Voegelein et al. (1992).

Quadrigemine C was also isolated from the crude leaf extract. Its structure had formerly been deduced from nuclear magnetic resonance (NMR) experiments (Libot et al., 1987; Sévenet et al., 1990; Guéritte-Voegelein et al., 1992). The easy transformation of quadrigemine C into psycholeine in acidic medium allowed us to determine the precise stereochemistry of psycholeine (Sévenet et al., 1990; Guéritte-Voegelein et al., 1992).

2.3. Anterior pituitary cell culture

Anterior pituitaries were rapidly dissected after decapitation of female rats, Wistar 175–200 g (Charles River, Saint-Aubin-les-Elbeuf, France) and dispersed according to the method of Hopkins and Farquhar (1973). Briefly, they were rinsed, cut into pieces in DMEM 0.3% bovine serum albumin and incubated in DMEM 0.5% trypsin for 15 min at 37°C. Deoxyribonuclease (2 μg/ml) was then added for 2 min to the medium.

After enzymatic digestion, the medium was removed and anterior pituitaries were incubated in DMEM containing a trypsin inhibitor (1 mg/ml) for 5 min at 37°C. After changing the medium, pituitaries were incubated in Ca^{2+},Mg^{2+}-free medium containing EDTA (2 mM) for 5 min at 37°C, followed by a 15-min incubation in the same medium containing only 1 mM EDTA. The cells were rinsed 3 times with Ca^{2+},Mg^{2+}-free medium, before mechanical dispersion in the same medium. After centrifugation ($300 \times g$, 10 min) cells were counted with a Coulter counter ZBI (Coulter Electronics, Hialeah, FL, USA) and plated in Petri dishes (diameter 10 cm) for the adenylate cyclase assay (10×10^6 cells/dish) or in multiwell plates (24 wells) for the study of hormone secretion (0.2×10^6 cells/well). Cells were maintained in DMEM supplemented with 10% fetal calf serum (pre-treated overnight with Norit A charcoal 1% and dextran 0.1%), glutamine 2 mM and antibiotics (penicillin and streptomycin 0.05 mg/ml) for 5 days.

2.4. Hormone secretion

The cells were rinsed and preincubated for 2 h in serum-free medium. Bacitracin (2×10^{-5} M) was added to the incubation medium to prevent peptide degradation. Hormone release was determined after 1 h of incubation in the absence or presence of test substances. At the end of the incubation, the medium was removed and stored at −20°C until assay. Growth hormone and other anterior pituitary hormones were

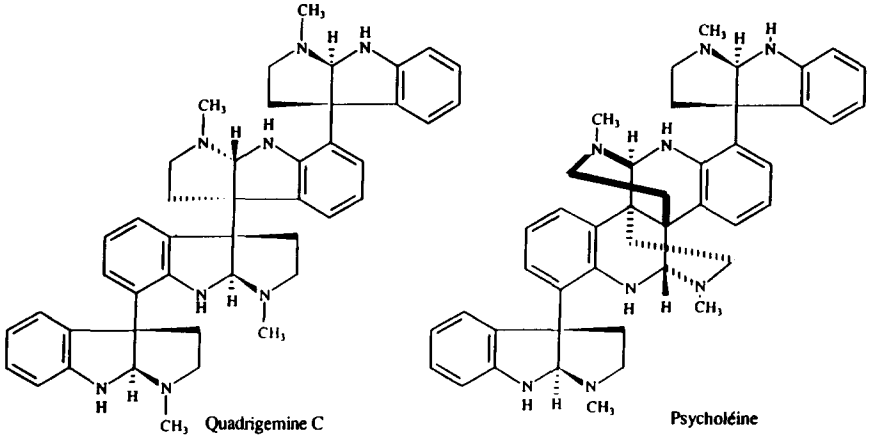

Fig. 1. Chemical structure of psycholeine and quadrigemine C.

assayed in duplicate by radioimmunoassay as previously described (Rieutort, 1972; Niswender et al., 1968,1969; Kieffer et al., 1974; Guillemin et al., 1977).

2.5. [^{125}I]N-Tyr-SRIF binding assay

[^{125}I]*N*-Tyr-SRIF was prepared in the laboratory as previously described by Enjalbert et al. (1982). *N*-Tyr-SRIF was iodinated in the presence of chloramine T. Purification of the labelled tracer was performed on carboxymethyl cellulose CM52 (Whatman, Clifton, NJ, USA). The specific activity of the tracer was 760 ± 17 Ci/mmol.

Anterior pituitary lobes were dissected rapidly at 4°C after decapitation of the rat and homogenized in a glass Teflon (Potter, Elvehjem) homogenizer in 50 mM Tris-HCl buffer at pH 7.5 (10% v/w) and centrifuged for 3 min at 600 × *g*. The supernatant was recentrifuged at 12000 × *g* for 20 min. The resulting pellet was gently resuspended in 50 v/w Tris-HCl buffer, supplemented by 0.1% bovine serum albumin and 0.1% bacitracin to prevent peptide adsorption and enzymatic degradation, respectively. 50 μl of freshly prepared membranes (25–50 μg proteins) were incubated with [^{125}I]*N*-Tyr-SRIF at 20°C for 30 min, in the absence or presence of unlabelled SRIF or the substances to be tested in a total volume of 150 μl. The incubation was stopped by addition of 2 ml ice-cold buffer. Membrane-bound labelled SRIF was separated from the free peptide by filtration on Whatman GF/C filters which were then washed with 10 ml ice-cold buffer.

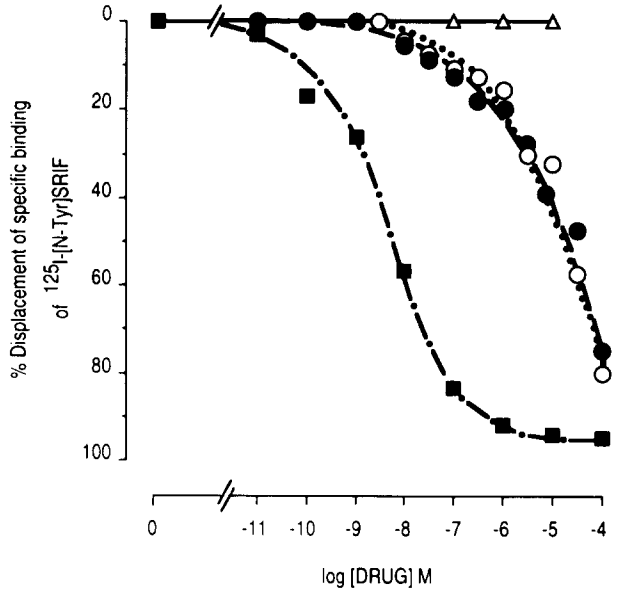

Fig. 2. Displacement of [^{125}I]*N*-Tyr-SRIF-specific binding from pituitary membrane preparations by increasing doses of unlabelled somatostatin (SRIF) (■), quadrigemine (○), psycholeine (●) and fraction 1237 of *Psychotria* (△). Each point represents the mean of three independent determinations performed in triplicate.

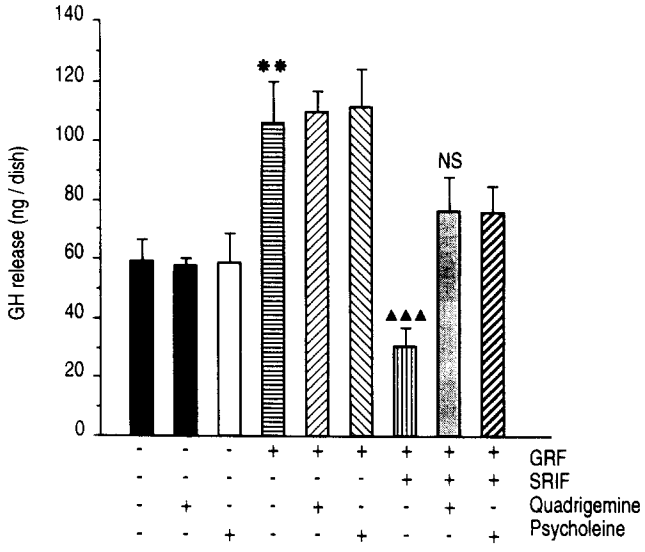

Fig. 3. Effects of quadrigemine C and psycholeine (3×10^{-6} M, 1 h incubation) on growth hormone (GH) release from primary pituitary cell cultures under basal conditions and after GRF stimulation or somatostatin (SRIF) inhibition. Results are the mean ± S.E.M. of three determinations. All experiments were performed at least 3 times. Statistically significant differences are indicated: ** $0.01 < P > 0.001$ vs. control. $^{NS}P > 0.05$; ▲▲▲ $P < 0.001$ vs. GRF stimulation.

Filters were placed in polystyrene tubes and counted in an LKB gamma counter at 70% efficiency. Specific binding was calculated as the difference between total and residual binding observed in the presence of 5×10^{-7} M cold SRIF. This saturable component represented between 70–80% of the total binding. In the absence of membranes, the radioactivity represented less than 0.1% of total.

2.6. Adenylate cyclase assay

Anterior pituitary lobes dissected as under Section 2.5 above were rinsed and homogenized in a glass Teflon (Potter, Elvehjem) homogenizer in 3 mM Tris, 1 mM EGTA buffer (pH 7.2) containing 3% sucrose. The homogenate was centrifuged at 500 × *g* for 3 min and the supernatant was recentrifuged at 12500 × *g* for 15 min. The resulting pellet was gently resuspended in Tris-EGTA buffer containing 10% sucrose. Adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP to [α-^{32}P]cAMP as previously described (Enjalbert and Bockaert, 1983). The final incubation medium (50 μl) contained 50 mM Tris-maleate buffer (pH 7.2), 0.1 mg/ml creatine kinase, 5 mM creatine phosphate, 10 mM theophylline, 1.5 mM $MgSO_4$, 1 mM cAMP, 0.15 mM ATP, 0.01 mM GTP, 0.001 μCi [^{3}H]cAMP (30.5 Ci/mmol), 2 μCi [α-^{32}P]ATP (27 Ci/mmol) and the substances to be tested.

The reaction was initiated by addition of 10 μl membranes (20–50 μg proteins) and lasted for 30 min at 30°C. The incubation was stopped by addition of 1

ml ice-cold buffer composed of 40 mM Tris-maleate buffer (pH 7.2), 4 mM cAMP, 2.5 mM ATP, 10 mM $CaCl_2$ and 0.1 N HCl. Radioactive nucleotides were isolated according to the method of Salomon et al. (1974). Recovery of [^{3}H]cAMP varied from 70–80% and was identical in all experimental groups. Protein content was determined according to the method of Lowry et al. (1951) and results were expressed as picomoles of cAMP formed per mg protein/30 min.

2.7. Statistical analysis

Student's paired *t*-test was performed to determine the statistical significance.

3. Results

3.1. Displacement of [^{125}I]N-Tyr-SRIF binding to pituitary membranes

Both natural and synthetic psycholeine displaced bound [^{125}I]*N*-Tyr-SRIF from pituitary membranes. The IC_{50} was slightly lower than 10^{-5} M, an approximately 1000-fold lower potency than cold SRIF itself (Fig. 2). Other fractions, such as fraction 1237, of *Psychotria*-containing analogs of psycholeine proved inactive at concentrations up to 10^{-5} M (Fig. 2).

3.2. Effect on SRIF inhibition of GH secretion

As expected, SRIF inhibited GRF-stimulated GH release from pituitary cells maintained in primary culture (Fig. 3). The effect of SRIF was dose dependent; a maximal inhibition of 65% was obtained with 10 nM of the peptide (Fig. 4). Concentrations of psycholeine or quadrigemine C up to 3×10^{-6} M were ineffective by themselves in altering GH secretion; a slight stimulation was only observed for concentrations higher than 10^{-5} M (data not shown). In contrast, increasing concentrations of the alkaloid reduced the SRIF inhibition of GH, as shown by a shift of the hormonal response to SRIF, with higher amounts of the peptide being required in the presence of psycholeine in order to induce an equal inhibition of GH (Fig. 4). In parallel, the maximal inhibition induced by SRIF was decreased by about 25% (from 65 to 40%). Other fractions derived from *Psychotria* extracts were ineffective at concentrations up to 10^{-5} M.

3.3. Effect on adenylate cyclase

Pituitary membranes were incubated in the presence of GRF, a potent stimulator of adenylate cyclase in somatotrophs, in order to increase the extent of the

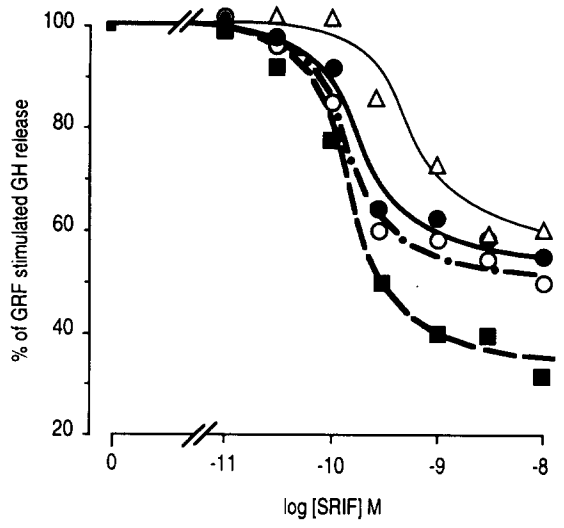

Fig. 4. Effects of psycholeine (10^{-6} M (○), 3×10^{-6} M (●), and 10^{-5} M (△)) on the SRIF (■) inhibition of GRF (10^{-8} M)-stimulated GH release from anterior pituitary cells after a 1-h incubation. Results are expressed in percent of GRF (10^{-8} M) values and they correspond to the mean of three determinations performed in triplicate.

SRIF inhibition of the enzyme. GRF (10^{-6} M) induced a 3-fold increase in adenylate cyclase activity (Fig. 5). SRIF (10^{-6} M) inhibited the GRF-stimulated level of adenylate cyclase by about one-third. Psycholeine (10^{-5} M) by itself did not affect GRF-stimulated levels of adenylate cyclase, but reduced significantly the inhibition induced by SRIF (Fig. 5).

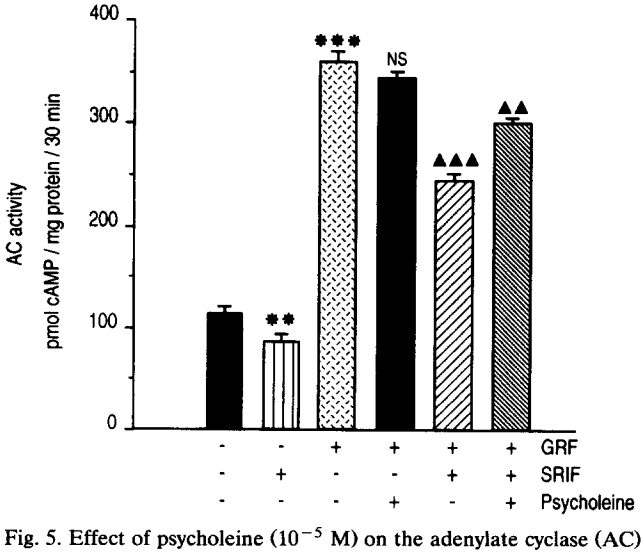

Fig. 5. Effect of psycholeine (10^{-5} M) on the adenylate cyclase (AC) activity in anterior pituitary membrane preparations after a 30-min incubation. Results are the mean ± S.E.M. of three determinations. All experiments were performed at least 3 times. Statistically significant differences are indicated: ** $0.05 < P > 0.01$; *** $P < 0.001$ vs. control. ^{NS} $P > 0.05$; $^{▲▲}$ $0.01 < P > 0.001$; $^{▲▲▲}$ $P < 0.001$ vs. GRF stimulation.

4. Discussion

Using a simple screening test based on radioimmunoassay of pituitary hormones after in vivo administration of unknown moieties, we previously suggested that extracts of *Psychotria oleoides* contained substances capable of affecting selectively GH secretion. Administration to male rats of tartrates of alkaloids prepared from *Psychotria oleoides* reduced GH plasma levels in a dose-dependent manner (Rasolonjanahary et al., 1986; Sévenet et al., 1990). By monitoring the displacement of SRIF bound to pituitary membranes as well as the GH response to SRIF of pituitary cells maintained in primary culture, we were able to characterize an active compound corresponding to a tetrameric alkaloid referred to as psycholeine. Subsequently, psycholeine was shown to reduce the SRIF inhibition of GH release as well as of GRF-stimulated adenylate cyclase activity, without affecting those parameters in the absence of SRIF. Psycholeine thus exhibits antagonistic properties against the SRIF receptor. Quadrigemine C, a precursor of psycholeine, has a similar action.

The effects of psycholeine and quadrigemine C observed in vitro are thus distinct from those of extracts of *Psychotria oleoides*, which contain several moieties, as described in our original observation, since in vivo administration of the extracts resulted in a SRIF agonist-like response.

This apparent discrepancy between the in vivo and in vitro results could partially be explained by the nature and/or the site of action of the tested substances. For in vitro experiments, the active fractions tested were previously purified and they interacted directly with SRIF receptor. In contrast, a crude extract was used for in vivo tests. It contained several alkaloid compounds (about 20 major alkaloids) which may act on the SRIF receptor in diverse ways, but which may also affect GH regulation at other levels, such as the hypothalamus. Under these conditions, comparison of the effect of psycholeine and of fraction 1237 of *Psychotria*, a component with no effect on SRIF binding, is not appropriate.

In the present work we reported the in vitro results obtained from two purified compounds: psycholeine and quadrigemine C. These compounds act as antagonists of the SRIF receptor.

The potencies of antagonists reported here were not very high; reversal of the SRIF effects required a concentration ratio of quadrigemine C or psycholeine to SRIF of approximately 1000. In addition, concentrations of the alkaloid higher than 10 μM could interfere with SRIF non-specific binding as well. This may account for the fact that, under our experimental conditions, psycholeine was not able to completely reverse the SRIF inhibition of GH release or of pituitary membrane adenylate cyclase activity.

Structure-activity studies on psycholeine analogs may open perspectives for synthetizing more potent compounds. At any rate, the antagonistic properties of psycholeine on the SRIF receptor described here are, to our knowledge, the first report concerning an antagonist of the neuropeptide. They may prove of interest for exploring GH dysfunctions and gastrointestinal disturbances, as well as for evaluating the role of endogenous SRIF in hippocampal or cortical brain structures which contain relatively high amounts of the peptide.

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