Effect of the triakontatetraneuropeptide (TTN) on corticosteroid secretion by the frog adrenal gland

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ABSTRACT

Diazepam-binding inhibitor (DBI) was initially isolated from the rat brain as a result of its ability to compete with benzodiazepines for their receptors. Immunohistochemical studies have recently shown the presence of peripheral-type benzodiazepine receptor (PBR)- and DBI-like immunoreactivity in the frog adrenal gland. The aim of the present study was to investigate the effect of two biologically active DBI-derived peptides, the triakontatetraneuropeptide [TTN; DBI(17-50)] and the octadecaneuropeptide [ODN; DBI(33-50)], on corticosteroid secretion by frog adrenocortical cells. Exposure of frog adrenal explants to graded concentrations of TTN (3·16×10⁻⁸ to 3·16×10⁻⁶ M) induced a dose-related increase in corticosterone and aldosterone secretion. In contrast, ODN did not modify corticosteroid output. When repeated pulses of TTN (10⁻⁶ M) were administered at 2-h intervals, the response of the adrenal explants to the second dose of TTN was markedly reduced, suggesting the existence of a desensitization phenomenon. Exposure of dispersed adrenal cells to TTN also induced a marked stimulation of corticosteroid secretion, indicating that TTN acts directly on adrenocortical cells. The central-type benzodiazepine receptor (CBR) agonist, clonazepam, did not stimulate corticosteroid secretion and the CBR antagonist, flumazenil, did not block the stimulatory action of TTN. Similarly, the PBR agonist, Ro5-4864, did not mimic the stimulatory effect of TTN and the PBR antagonist, flunitrazepam, did not affect the stimulatory action of TTN. The present study provides the first evidence for a stimulatory effect of TTN on intact adrenocortical cells. The receptor mediating the corticotrophic action of TTN is not related to central- or peripheral-type benzodiazepine receptors. Our data suggest that TTN, released by chromaffin cells, may act as a paracrine factor regulating the activity of adrenocortical cells.

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INTRODUCTION

Diazepam-binding inhibitor (DBI) is an 86 amino acid polypeptide that was originally isolated and characterized from the rat brain as an endogenous ligand of benzodiazepine receptors (Guidotti et al. 1983). The primary structure of DBI has been subsequently identified from several vertebrate species, including man and ox (Marquardt et al. 1986), pig (Chen et al. 1988), rat (Knudsen et al. 1989), duck (Rose et al. 1994) and frog (Lührmann et al. 1994). The sequence of DBI has been relatively well preserved during evolution, suggesting that this polypeptide has important biological functions. Proteolytic processing of DBI generates several biologically active peptides, including the triakontatetraneuropeptide DBI(17-50) (TTN) and the octadecaneuropeptide DBI(33-50) (ODN), which, like DBI, inhibit benzodiazepine–receptor binding (Ferrero et al. 1986, Slobodyansky et al. 1989, Berkovitch et al. 1990). The term endozepines is generally used to designate all these endogenous ligands of benzodiazepine receptors (Marquardt et al. 1986, Besman et al. 1989).

The DBI gene is widely expressed in the brain and in various peripheral tissues (Alho et al. 1988, Chen et al. 1988, Bovolin et al. 1990, Tonon et al. 1990, Steyaert et al. 1991, Tong et al. 1991, Malagon et al. 1992, 1993). In particular, high concentrations of DBI-related peptides are found in most endocrine glands (Bovolin et al. 1990, Rhéaume et al. 1990, Höög et al. 1991, Johansson
et al. 1991, Rouet-Smih et al. 1992), suggesting that endozepines can act as local regulators of hormone secretion. Consistent with this hypothesis, it has been demonstrated that DBI-related peptides reverse the inhibitory effect of γ-aminobutyric acid (GABA) on pituitary melanotropes (Tonon et al. 1989) and inhibit glucose-induced insulin secretion (Östenson et al. 1994). The mode of action of endozepines on their target cells remains poorly understood. It was initially reported that DBI and its processing products modulate GABA-activated chloride currents through an allosteric regulation of central-type benzodiazepine receptors (CBR) (Bormann et al. 1985). It has also been shown that DBI and TTN exhibit high affinity for the peripheral-type benzodiazepine receptors (PBR) (Slobodyansky et al. 1989, Berkovitch et al. 1990, Bovolin et al. 1990). In addition, DBI acts as an acyl-CoA-binding protein (Knudsen et al. 1989), suggesting that it may exert a physiological role as a lipid-transport protein (Mikkelsen & Knudsen 1987). Recent studies conducted in rat astrocytes indicated that endozepines may also activate a membrane receptor positively coupled to a phospholipase C through a pertussis toxin-sensitive G protein (Patte et al. 1995, Lamacz et al. 1996, Gandolfo et al. 1997).

The occurrence of DBI mRNA and immunoreactive endozepines has been demonstrated in the rat adrenal gland (Bovolin et al. 1990), ovary (Toranzo et al. 1994) and testis (Rhéaume et al. 1990), and in the mouse Y1 adrenocortical cell line (Papadopoulos et al. 1991). In adrenocortical and Leydig cells, endozepines act as intracrine factors that activate steroidogenesis via stimulation of PBR located on the outer mitochondrial membrane (Yanagibashi et al. 1989, Toranzo et al. 1994). In Leydig cells, DBI also acts as a paracrine or autocrine factor, or both, stimulating PBR located on the plasma membrane (Garnier et al. 1993). In contrast, the effect of exogenous endozepines on intact adrenocortical cells has never been investigated.

We have recently shown that the frog adrenal gland expresses the DBI gene. Using an antiserum against human ODN that cross-reacts with DBI and TTN, we have detected the presence of immunoreactive endozepines in chromaffin cells and in a population of mast-like cells called Stilling cells (Lesouhaitier et al. 1996). These observations suggested that DBI-related peptides may be released within the adrenal tissue and may act as local regulators of adrenal cell activity (Lesouhaitier et al. 1995). In the present study, we have investigated the effect of TTN and ODN on corticosteroid secretion by frog adrenocortical cells.

MATERIALS AND METHODS

Animals

Adult male frogs (Rana ridibunda; body weight 40–50 g), were obtained from a commercial supplier (Couétrand, Saint-Hilaire de Riez, France). The animals were housed in a temperature-controlled room (8 °C) under an established photoperiod of 12 h of light/day (lights on, 0600–1800 h) for at least 1 week before use. Animal manipulations were performed according to the recommendations of the French Ethics Committee and under the supervision of authorized investigators.

Chemicals

Synthetic TTN and ODN (human sequences) were obtained from Neosystem (Strasbourg, France). Flunitrazepam (Narcozep) was provided by Roche (Neuilly-sur-Seine, France). Flumazenil and clonazepam were generous gifts from Hoffmann–La Roche (Basel, Switzerland). Ro5-4864 was obtained from Fluka Chimie (St Quentin Fallavier, France). Leibovitz culture medium (L15), collagene (type IA) and protease (from Bacillus polymyxa, type IX) were purchased from Sigma (St Louis, MO, USA). Fetal calf serum, the kanamycin solution (10 000 U/ml) and the antibiotic–antimycotic solution [penicillin G sodium (10 000 U/ml); streptomycin sulfate (10 000 U/ml); amphotericin-B (25 µg/ml)] were from Gibco (Grand Island, NY, USA). Heps [N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)] was from Research Organics (Cleveland, OH, USA). [1,2,6,7-3H]Corticosterone and [1,2,6,7-3H]aldosterone were from Amersham International (Amersham, Buckinghamshire, UK). BSA (fraction V) was from Boehringer Mannheim (Mannheim, Germany).

Perfusion of adrenal explants

The effects of TTN and ODN on corticosteroid secretion were studied using a perifusion system technique for frog adrenal slices as previously described (Delarue et al. 1990a). For each experiment, 48 adrenal glands were dissected free of renal tissue, sliced and preincubated in 5 ml Ringer’s solution (15 mM Hepes buffer, 112 mM NaCl, 15 mM NaHCO3, 2 mM CaCl2 and 2 mM KCl) supplemented with 2 g/l glucose and 0.3 g/l BSA. The Ringer’s solution was gassed with O2–CO2.
(95:5) and the pH was adjusted to 7·4. The tissue slices were rinsed three times with fresh medium and layered between several beds of Bio-Gel P2 (Bio-Rad, Richmond, CA, USA) into perifusion chambers (equivalent of 12 adrenal glands per chamber). The adrenal tissue was continuously perifused with gassed Ringer’s solution at a constant flow rate (200µl/min) and temperature (24°C). The experimental procedure started after a stabilization period of 2 h. The effluent perifusate was collected every 5 min and immediately frozen until required for assay.

**Perifusion of dispersed adrenal cells**

For each experiment, 48 adrenal glands were dissected, sliced and preincubated in 5 ml L15 medium adjusted to Rana ridibunda osmolality (L15 : water = 1 : 0·4), supplemented with 200 mg/l glucose, 63 mg/l CaCl2, 1% kanamycin and 1% antimycotic–antibiotic solutions (f-L15; pH 7·4). The adrenal cells were then enzymatically dispersed at 24°C for 45 min in f-L15 medium containing collagenase (2 mg/ml) and protease (2 mg/ml). After digestion, the tissue was disaggregated by gentle aspiration through a siliconized Pasteur pipette with a flame-polished tip. The suspension was centrifuged (50 x g; 15 min) and the cells were rinsed twice with fresh f-L15 medium and centrifuged. The cells were re-suspended in 5 ml Ringer’s solution, transferred into the perifusion chamber (750 000 cells per chamber) and perifused as described above. The experimental procedure started after a stabilization period of 3 h. The viability of dispersed adrenal cells was assessed by the trypan blue exclusion test.

**Corticosteroid radioimmunoassays**

Corticosterone and aldosterone concentrations were determined by radioimmunoassays (RIA), without prior extraction, in 200–300µl samples of perifusion fractions, as previously described (Leroux et al. 1980, Leboulenge et al. 1982). Direct measurement of corticosterone and aldosterone was validated by RIA quantification of corticosteroids after high performance liquid chromatography analysis of the effluent perifusate. The working ranges of the assays were 20–5000 pg for corticosterone and 5–2000 pg for aldosterone. For both assays, the intra- and interassay coefficients of variation were <3% and 6% respectively. None of the test substances showed any interference in the corticosterone and aldosterone RIAs.

**Statistical analysis**

Each perifusion pattern was established as the mean profile of corticosteroid release (± s.e.m.) calculated over at least three independent experiments. Corticosterone and aldosterone secretion were expressed as percentages of the basal values calculated as the mean of eight samples (40 min) immediately preceding the administration of the test substances. Paired t-test was used after regression analysis for comparison between values. To compare the increase in steroid production induced by TTN and ODN, the areas under the curves (AUC) were calculated using the trapezoidal rule (Contesse et al. 1996).

**RESULTS**

**Effects of endozepines on corticosteroid secretion from perifused adrenal explants**

Administration of graded doses of TTN (3·16 x 10⁻⁸ M to 3·16 x 10⁻⁶ M) over 40 min induced a concentration-related increase in corticosterone (Fig. 1A) and aldosterone secretion (Fig. 1B). The maximum response was observed 40 min after the beginning of the infusion of TTN. At a concentration of 10⁻⁶ M, TTN induced a 130% increase in corticosterone (P<0·001) and a 170% increase in aldosterone output (P<0·01); the EC₅₀ values were, respectively, 2·4 x 10⁻⁷ M and 3·5 x 10⁻⁷ M. In contrast, graded concentrations of ODN (3·16 x 10⁻⁸ M to 3·16 x 10⁻⁶ M) did not modify corticosteroid secretion (Fig. 1A and B).

When consecutive pulses of TTN were administered at 120-min intervals, there was a marked reduction in the secretion of corticosterone (Fig. 2A) and aldosterone (Fig. 2B) in response to the second pulse of TTN. Prolonged exposure of adrenal slices to TTN (10⁻⁶ M) induced an increase in corticosteroid secretion that reached a maximum within 40 min. Thereafter, steroid release gradually decreased, in spite of the continuous infusion of the secretagogue (Fig. 2C and D).

**Effects of endozepines on corticosteroid secretion from perifused dispersed adrenal cells**

We next examined the ability of TTN and ODN to stimulate steroid secretion from enzymatically dissociated adrenal cells. Administration of TTN (10⁻⁶ M) induced a significant increase in corticosterone (P<0·001) (Fig. 3A) and aldosterone secretion (P<0·01) (Fig. 3B). The time-course of the response of dispersed adrenal cortical cells to
TTN was more rapid than that of adrenal explants, the maximum effect being observed 30 min after the onset of TTN administration. Exposure of dispersed adrenal cells to ODN (10^{-6} M) did not significantly affect corticosteroid secretion (Fig. 3C and D).

Receptors involved in the stimulatory effect of TTN

Administration of the CBR agonist, clonazepam (10^{-5} M; 40 min), did not modify corticosterone (Fig. 4A) and aldosterone secretion (Fig. 4B). Prolonged infusion of the CBR antagonist, flumazenil (10^{-5} M; 240 min), induced an inhibition of the basal production of corticosterone (Fig. 4C) and

**Figure 1.** Effects of ODN (open bars) and TTN (black bars) on the secretion of corticosterone (A) and aldosterone (B) by perifused frog adrenal explants. Graded concentrations of each peptide (3·16 × 10^{-8} to 3·16 × 10^{-6} M) were administered as 40-min pulses. The AUC are expressed as percentages of the basal value. The reference values of corticosterone and aldosterone release (100% basal value) were calculated for each experiment as the mean of eight samples just before the administration of ODN and TTN. The data represent the mean (± S.E.M.) of three independent experiments. Each point is the mean corticosteroid secretion of two consecutive fractions collected over 5 min. The reference value of corticosterone or aldosterone release (100% basal value) was calculated for each experiment as the mean of eight samples just before the first pulse of TTN (○). The mean basal values of corticosterone and aldosterone secretion in these experiments were 32·8±1·4 and 29·6±2·9 pg/min per adrenal gland respectively. **P<0·01, ***P<0·001, compared with the reference values (100% basal value).

**Figure 2.** A,B: Effects of repeated pulses of TTN (10^{-6} M; 40 min each) on the secretion of corticosterone (A) and aldosterone (B) by perifused frog interrenal explants. The pulses of TTN (arrows) were given at 120-min intervals. C,D: Effect of a prolonged infusion of TTN (10^{-6} M; 80 min) on the secretion of corticosterone (C) and aldosterone (D) by perifused frog interrenal explants. The TTN solution was renewed after 40 min (arrowheads) to avoid degradation of the diluted peptide. The profiles represent the mean (± S.E.M.) secretion pattern of three independent perifusion experiments. Each point is the mean corticosteroid secretion of two consecutive fractions collected over 5 min. The reference value of corticosterone release (100% basal value) was calculated for each experiment as the mean of eight samples just before the first pulse of TTN (○). The mean basal values of corticosterone and aldosterone secretion in these experiments were 13·1±0·7 and 8·6±1·1 pg/min per adrenal gland respectively.
aldosterone (Fig. 4D). Nevertheless, flumazenil did not block the stimulatory effect of TTN on corticosteroid secretion (Fig. 4C and D).

Administration of the PBR agonist, Ro5-4864 (10^{-5} M; 40 min), had no effect on corticosterone (Fig. 5A) and aldosterone secretion (Fig. 5B). Prolonged administration of the PBR antagonist, flunitrazepam (10^{-5} M; 240 min), did not affect TTN-evoked corticosteroid output (Fig. 5C and D).

**DISCUSSION**

The occurrence of DBI mRNA and DBI-related peptides has been previously reported in rat (Bovolin et al. 1990, Rouet-Smih et al. 1992) and bovine (Besman et al. 1989, Yanagibashi et al. 1989) adrenal glands. However, the effect of endozepines on intact adrenocortical cells has never been investigated in mammals. We have recently shown the presence of endozepine- and PBR-like immunoreactivity in the frog adrenal gland (Lesouhaitier et al. 1996). The present study has demonstrated that the endozepine, TTN, stimulates the secretion of corticosterone and aldosterone in vitro through a non-benzodiazepine receptor.

Adrenochromaffin cells produce various neurotransmitters and neuropeptides that can act as local regulators of adrenocortical cell activity (Bornstein & Ehrhart-Bornstein 1992, Gallo-Payet 1993, Vinson et al. 1994, Lesouhaitier et al. 1995). In contrast to the mammalian adrenal gland, which is organized in cortical and medullary zones, the interrenal gland of amphibians is composed of chromaffin cells intermingled with adrenocortical...
perifused frog adrenal explants. In contrast, the endozepine, ODN, was totally devoid of corticotropic activity. Consistent with these findings, previous studies have shown that ODN and TTN act on distinct receptors (Slobodyansky et al. 1989, Berkovitch et al. 1990). In Leydig cells, both DBI and ODN have been reported to stimulate testosterone biosynthesis (Garnier et al. 1993).

The perifusion technique is particularly well adapted to investigate the temporal changes in steroid secretion and to disclose the occurrence of a desensitization process (Leboulenger et al. 1993, Yon et al. 1993). Using this approach, we observed an attenuation of the response when two consecutive pulses of TTN were administered successively to perifused adrenal slices. We also found that sustained administration of TTN induced a transient stimulation of corticosteroid secretion, which peaked within 40 min and gradually declined toward the baseline value. The decay of the response, observed in spite of the continuous infusion of the peptide, cannot be ascribed to degradation of TTN, as the solution was renewed after 40 min to avoid peptide damage. Taken together, these data indicate that TTN induced desensitization of its receptors. Other corticotropic peptides, such as vasotocin (Larcher et al. 1992), endothelin (Delarue et al. 1990a) and calcitonin gene-related peptide (Esneu et al. 1994), have previously been found to induce homologous desensitization of frog adrenocortical tissue, whereas down-regulation has never been observed with ACTH (Delarue et al. 1990b).

In order to determine whether TTN acts directly on adrenocortical cells to stimulate steroid secretion, we investigated the action of the peptide on enzymatically dispersed frog adrenal cells, a preparation in which the connections between the cells are disrupted. The observation that TTN could stimulate corticosteroid secretion from dispersed cells indicated that the peptide exerts its stimulative effect through a direct action on adrenocortical cells. The fact that substantial concentrations of endozepines are also contained in the rat (Bovolin et al. 1990, Rouet-Smih et al. 1992) and bovine adrenal gland (Besman et al. 1989, Yanagibashi et al. 1989) suggests that, in various vertebrate classes, TTN may act as a paracrine factor stimulating corticosteroid secretion.

Molecular cloning of DBI cDNA has revealed that the polypeptide does not possess the typical signal sequence characteristic of secreted proteins (Webb et al. 1987, Lihrmann et al. 1994). Moreover, in frog adrenochromaffin cells, ODN-like immunoreactivity does not appear to be sequestered in secretory vesicles (Lesouhaitier et al. 1983). This particular histological organization favors cross-talk between the two categories of cells (Yon et al. 1994, Lesouhaitier et al. 1995). For instance, chromaffin cells of the frog adrenal gland contain serotonin (Delarue et al. 1988a), vasotocin (Larcher et al. 1989) and vasoactive intestinal polypeptide (Leboulenger et al. 1983), which all stimulate corticosteroid secretion (Leboulenger et al. 1983, 1984, Delarue et al. 1988b, Larcher et al. 1989, 1992). The occurrence of immunoreactive ODN in frog adrenochromaffin cells (Lesouhaitier et al. 1996) prompted us to investigate the effect of endozepines on adrenocortical cells. The present results show that synthetic TTN exerts a concentration-dependent stimulatory effect on

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**Figure 5.** A,B: Effect of the peripheral-type benzodiazepine receptor agonist, Ro5-4864 (10^{-5} M; 40 min), on the secretion of corticosterone (A) and aldosterone (B) by perifused dispersed frog adrenal cells. C,D: Effect of TTN (10^{-6} M; 40 min) during prolonged infusion of the peripheral-type benzodiazepine receptor antagonist, flunitrazepam (10^{-5} M), on the secretion of corticosterone (C) and aldosterone (D) by perifused dispersed frog adrenal cells. The pulse of TTN was given 90 min after the onset of flunitrazepam administration. The mean basal values of corticosterone and aldosterone secretion in these experiments were 306 ± 123 and 64 ± 18 pg/min per 10^5 cells. See legend to Fig. 2 for other designations.
TTN stimulates adrenal steroidogenesis.

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1996). These observations raise the question as to whether endozepines can be secreted by chromaffin cells. Recent studies, however, have shown that endozepines are actually released from various cell types, including Sertoli and Leydig cells (Garnier et al. 1993) and astrocytes (Lamacz et al. 1996). The fact that other biologically active peptides lacking a recognizable signal sequence, such as interleukins or fibroblast growth factor, are exported through an ATP-driven membrane translocator (MacGrath & Varshavsky 1989, Livi et al. 1991) suggests that the secretion of endozepines from chromaffin cells may also occur via an ATP-binding cassette transport protein.

It has been previously shown that the biological actions of DBI and its processing products are mediated through an interaction with CBR or PBR (Costa & Guidotti 1991, Papadopoulos et al. 1991, Do Rego et al. 1997). In order to explore the type of receptor involved in the action of endozepines on adrenocortical cells, we have investigated the effect of various benzodiazepine receptor agonists and antagonists on spontaneous and TTN-evoked corticosteroid secretion. We found that the CBR agonist, clonazepam, did not mimic the stimulative effect of TTN. In addition, the CBR antagonist, flumazenil, did not block the response of adrenocortical cells to TTN. Similarly, the PBR agonist, Ro5-4864, did not stimulate corticosteroid secretion and the PBR antagonist, flunitrazepam, did not affect the TTN-induced steroid release. Taken together, these data indicate that the stimulative effect of TTN on frog adrenocortical cells is mediated neither through CBR nor through PBR. Consistent with this finding, it has been recently shown that, in rat Leydig cells, endozepines stimulate testosterone secretion via a PBR-independent mechanism (Garnier et al. 1993). It has also been found that, in rat astrocytes, the endozepine ODN activates a membrane receptor positively coupled to a phospholipase C through a pertussis toxin-sensitive G protein (Patte et al. 1995, Lamacz et al. 1996, Gandolfo et al. 1997). Further studies on the mechanism of action of TTN are required to elucidate the type of receptor responsible for the effect of the peptide on adrenocortical cells. Although exogenous TTN does not act through benzodiazepine receptors, it should be borne in mind that PBR and CBR agonists can modulate corticosteroid secretion (Thomson et al. 1992, Whitehouse 1992, Python et al. 1993).

In conclusion, the present study provides the first evidence for a direct stimulatory effect of TTN on intact adrenocortical cells. The receptor mediating the action of TTN is pharmacologically distinct from the central and peripheral benzodiazepine receptor subtypes. Our data suggest that endozepines synthesized within chromaffin cells may act as local regulators of adrenocortical cells through a paracrine mode of communication.

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