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NEUROTENSIN RECEPTORS ON CIRCULAR SMOOTH MUSCLE OF CANINE SMALL INTESTINE: ROLE OF DISULFIDE BRIDGES

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We have studied the effect of sulfhydryl agents on the binding of ^{125}I-tyr^{3}-neurotensin to the purified plasma membranes from circular smooth muscle and on the *in vitro* response of circular muscle strips of canine small intestine to neurotensin. Dithiothreitol (DTT) enhanced the binding by about 80%. Cysteine (a reductant) also enhanced the binding while cystine (an oxidant) reduced the binding to the similar extent. DTT stimulated the tissue in the organ bath and abolished the stimulatory response to low concentrations of neurotensin. The stimulatory response to acetylcholine was not altered by DTT. The implications of the role of disulfide bridges in the neurotensin response is discussed.

A major part of neurotensin-like immunoreactivity (NT-LI) is present in the distal small intestine especially in the endocrine cells (N-cells) of mucosa (1-3). There are, however, some reports describing its presence in enteric nerves (4,5), but not all studies have found NT-LI in neurons (1,6). Neurotensin *in vivo* alters the motility pattern of the gastrointestinal tract (7-10) and *in vitro* either contracts, relaxes or produces a biphasic response depending on the species (see 11). Effects *in vitro* are tetrodotoxin-insensitive. To reinforce the hypothesis of the neurotransmitter role of neurotensin, receptors for this peptide have been characterized on several central as well as peripheral systems (12-15). In the gastrointestinal tract, neurotensin receptors have been demonstrated on the smooth muscle of rats (12) and guinea-pigs (16,13). We have characterized the neurotensin receptors on the circular smooth muscle of canine small intestine (17). In the present study we have studied the role of disulfide bridges in the binding activity and the *in vitro* response to neurotensin using circular smooth muscle of canine small intestine.

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METHODS

The detailed methodology involved in the preparation of the plasma membranes from canine small intestine circular smooth muscle has been published elsewhere (17).

Binding studies using ^{125}I-tyr^3-Neurotensin were performed as described in previous studies (17). In brief, ^{125}I-tyr^3-Neurotensin ($\approx$ 0.1 nM) was incubated at 37°C with the membranes diluted in the incubation buffer (50 mN Tris-HCl, pH 7.4 containing 0.2% BSA) with (for non-specific binding) or without (for total binding) 10^{-6}M unlabelled neurotensin, in a total volume of 250 µL. When preincubation with DTT was studied, membranes were incubated with the required concentration of DTT for the indicated times, then the reaction was started by the addition of labelled neurotensin plus buffer (for total binding) or unlabelled neurotensin (for non-specific binding). The reaction was carried out for 20 minutes after which the reaction was terminated by dilution with 2 mL ice cold incubation buffer and filtration over Gelman GN-6 filters using a Millipore filtration apparatus. This was followed by two additional washes. The filters were recovered and counted for bound radioactivity in a Beckman Gamma 5500 gamma counter. All the experiments were performed in triplicate both for total and non-specific binding.

For the *in vitro* contractility experiments, the circular muscle layer was dissected as described above but in Krebs-Ringer solution; its composition (mM) was: NaCl, 115; KCl, 4.5; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; glucose, 11. Approximately 2-3 mm wide strips were cut in the circular orientation. These strips were tied with fine silk suture to electrodes at one end and to a force transducer on the other end. Initially one gram tension was applied to the strips and the strips were left for about one hour in Krebs solution bubbling with 95% O_2 - 5% CO_2 at 37°C for equilibration before starting the experiment. See reference 9 for a detailed description of these procedures.

RESULTS

Effect of sulfhydryl agents on the binding

Dithiothreitol (DTT) augmented the binding of neurotensin to the smooth muscle plasma membranes. The effect was concentration dependent (fig. 1). A maximum effect of DTT was achieved from 0.5 to 5 mM DTT but at higher concentrations the effect was reduced. The maximum enhancement of the binding was about 80%. No preincubation of the membranes with DTT was required for its effect on the binding. The effect of 1 mM DTT was maximal within 2 in. of preincubation which was the minimum time studied; furthermore, the effect was stable with time for up to 60 minutes (not shown).

The effects of two other sulfhydryl agents having opposing actions were also studied on the binding. Cysteine (a sulfhydryl reducing agent) and cystine (an oxidant) influenced the binding in opposite direction (fig. 2). In a concentration dependent manner, cysteine enhanced the binding while cystine decreased specific binding.

Effect of DTT on the *in vitro* response to neurotensin

Neurotensin *in vitro* increases the frequency and sometimes the amplitude of phasic concentration of circular muscle of canine small intestine at lower concentrations (10^{-12}-10^{-8} M); at concentrations higher than 10^{-8} M, inhibition is observed followed by a tonic contraction with a further increase in frequency of phasic contractions (fig. 3A and ref.

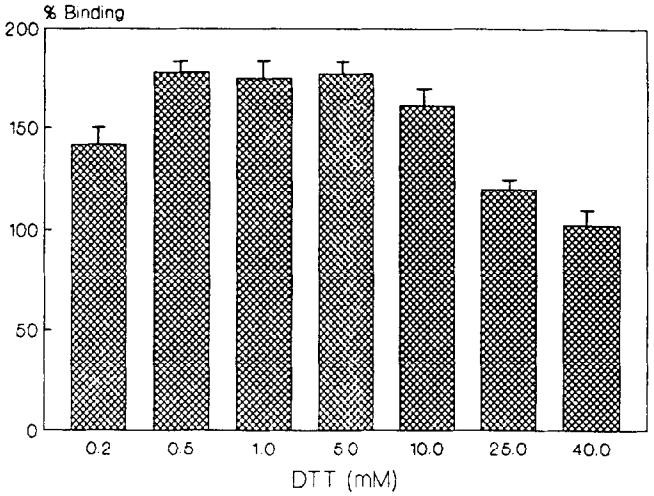

Figure 1. The effect of dithiothreitol (DTT) on the specific binding of [^{125}I]tyr^{3}-neurotensin (70-80 pM) to the circular smooth muscle plasma membranes of canine small intestine. The concentration of [^{125}I]tyr^{3}-neurotensin was chosen to be near the Kd value for the high affinity binding site. The results are plotted as mean percent ± s.e.m. with respect to control (in the absence of DTT) taken as 100%. All values are significantly ($p < 0.05$) different from 100% except at 25 or 40 mM.

9). However, when the tissue was preincubated with 1 mM DTT, the phasic contractile response to any concentration of neurotensin was abolished (n=4 experiments, each with 6 to 8 strips). However, neurotensin could still produce its usual inhibitory response (fig. 3B). The tonic contractile response to higher concentration of neurotensin was still

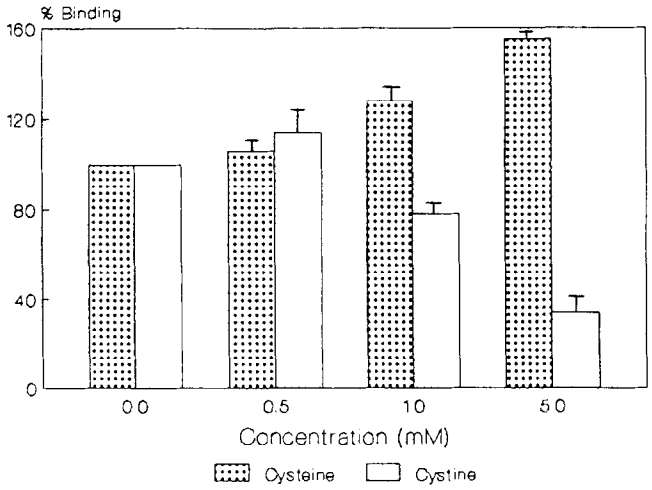

Figure 2. Effect of cysteine and cystine on the specific binding of [^{125}I]tyr^{3}-neurotensin (70-80 pM) to the circular smooth muscle plasma membranes. The reaction was carried out in the absence (control 100%) or in the presence of indicated concentrations of the sulfhydryl agents. Data are expressed the same as in Figure 1.

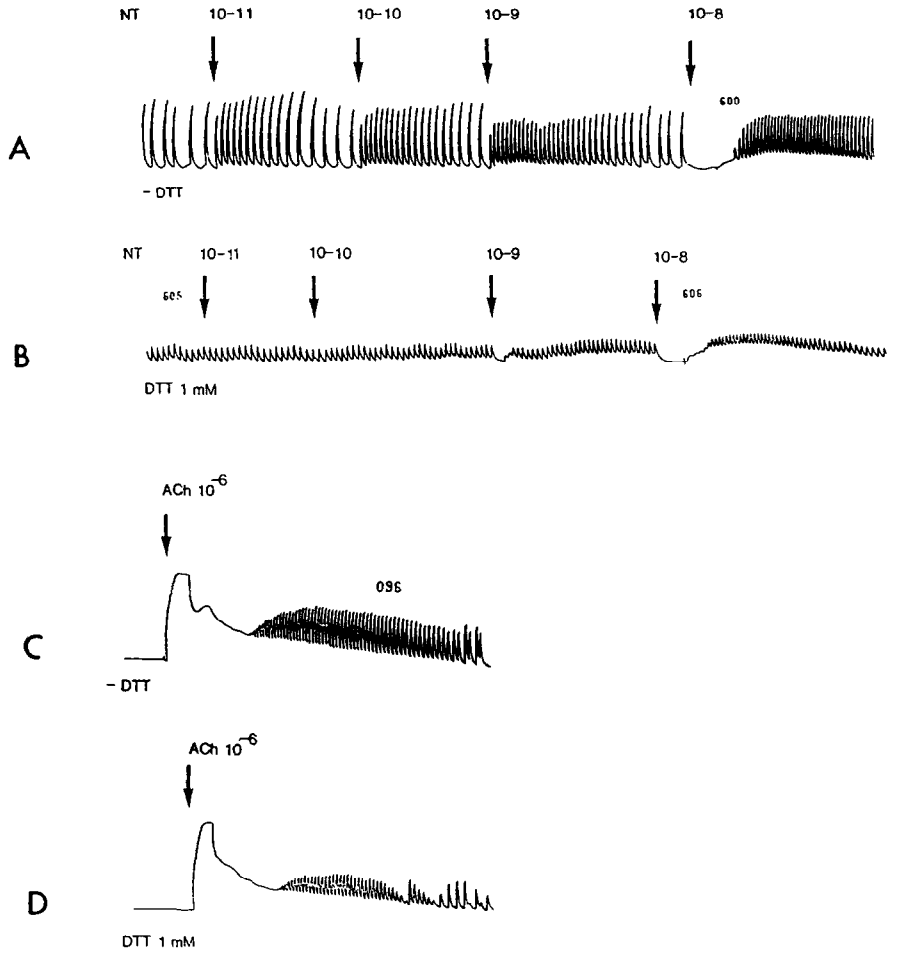

Figure 3. *In vitro* response of the circular smooth muscle of canine small intestine to added neurotensin cumulatively. A, control response; B, response to added neurotensin in the presence of 1 mM DTT; note in A that 10^{-11} M or higher concentrations of neurotensin increase the frequency of phasic actions while 10^{-8} M also causes a preliminry inhibition of phasic contractions and a tonic contraction; C, the control response to acetylcholine and D, the response in the presence of 1 mM DTT.

present. Figures 3C & D further illustrate that the excitatory response to the acetylcholine was minimally affected by DTT pretreatment (compare figs. 3C & 3D). Therefore, the action of DTT on the neurotensin response did not result from a generalized paralysis of the contractile machinery of the tissue, but was, rather selective for the excitatory effects of neurotensin.

DISCUSSION

In this study we showed that sulfhydryl agents, DTT as well as cysteine, enhanced neurotensin binding by as much as 80%. In rat mast cells, no effect of sulfhydryl agents was observed on neurotensin binding (18). Therefore, the receptors in canine small

intestine appear to differ in their molecular organization from other neurotensin receptors. Our unpublished observations also suggest such a difference; the target size, as obtained by the radiation inactivation, of neurotensin receptors from circular smooth muscle is in the range of 200,000 Da. (unpublished) while those from rat fundus and rat brain have a target size of 100,000 Da. (19). Solubilized mouse brain neurotensin receptors also have a molecular weight of 100,000 Da. on SDS-PAGE (20).

The effects of DTT and other thiol modifying agents has been studied in other receptor systems such as nicotinic cholinergic receptors in mouse brain (21), opioid receptors in bovine medulla (22), ß-adrenergic receptors (23) and histamine receptors in guinea pig ileum (24). In many previous studies, the thiol reducing agents decreased the binding and a preincubation with the reducing agents was required; the maximal effect was seen after 60 minutes of the preincubation (22).

In the present studies, however, no preincubation was required for the effect of thiol reducing agent dithiothreitol. Furthermore, these agents increased, rather than decreased the binding. These observations suggest the reduced sulfhydryl groups, rather than the disulfide bridges are required for the neurotensin binding activity. In many previous studies where DTT attenuated the binding, antagonists were used as the ligands (22,23). In the case of histamine receptors, where both antagonists and agonists were used, DTT revealed a high affinity state of agonists without affecting the interactions of antagonists (24). The lack of a proper neurotensin antagonist precludes further studies along this line. However, it is clear that the neurotensin receptors in canine small intestine are different from those on the rat mast cells where DTT failed to influence the binding.

Pretreatment of the tissue with DTT abolished the stimulatory effect of neurotensin on contraction. Reduction of disulfide bridges has been envisaged as a mechanism of receptor activation by ligands (24-26). Sulfhydryl reagents have been reported to inhibit the response to insulin and reducing agents acted as insulinomimetic reagents (25). Furthermore, insulin receptor/kinase activity was shown to be inhibited by N-ethylmaleimide, a sulfhydryl alkylating agent and activated by DTT (25). Beta adrenergic receptor activation in the absence of agonist and even in the presence of antagonist was observed upon exposure to thiol agents capable of cleaving disulfide bridges (see 23 and references therein). DTT caused massive stimulation of the tissue in the present study which subsided with time before neurotensin was added.

One hypothesis to explain these data envisages the neurotensin receptor in intact cells being held partially oxidized and partially reduced by metabolites. Neurotensin interacting with the oxidized receptor would cause its reduction and initiate contraction

by opening Ca-channels. This appears to cause enhanced slow waves and phasic contraction at lower concentrations and tonic contractures as well at higher concentrations (27). The neurotensin-receptor interaction would lead to the formation of more high affinity reduced receptors. DTT would also by acting directly to reduce disulfide bridges cause similar but more massive contractile events as observed. Oxidizing agents, when present together with neurotensin, would hold the receptors in the oxidized low affinity form preventing formation of high affinity receptors by neurotensin and reducing the number already on hand. They should also cause reduced contractions alone. However, they might potentiate contractions by making more oxidized receptors available if rapidly removed just before neurotensin was added. Oxidation or reduction of receptors in membranes would decrease or increase the number of high affinity receptors and these changes would not be affected by metabolites.

Alternatively oxidized (low affinity) receptors may be necessary for the initiation of excitation by neurotensin but are not changed in the process of occupation and response. Some predictions from this theory are similar to those noted above. However, it would be difficult to explain how low concentrations (10^{-11} to 10^{-9} M) of neurotensin cause excitation by occupying receptor which has a Kd of higher than 1 nM.

In contrast the inhibitory effects, which seems to result form opening of Ca^{2+}-activated K^+ channels (27) may be the result of occupation of receptors, presumably low affinity which causes release of intracellular Ca^{2+}. According to both of the hypotheses presented above, these would be in the reduced configuration. It is unclear whether the activation of these receptors requires initial occupation in the oxidized form and subsequent reduction of the receptors.

These proposals do not exhaust the possible explanation. However, they are clearly testable.

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