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Calcitonin modifies ligand binding to muscarinic receptor in CNS membranes

Georgina Rodríguez de Lores Arnaiz*, Patricia G. Schneider

Instituto de Biologia Celular y Neurociencias 'Prof. E. De Robertis', PROBICENE-CONICET, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121, Buenos Aires, Argentina

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Abstract

Calcitonin (CT) is a peptide produced by the thyroid gland, whose best described role is to prevent bone reabsorption, though it also participates in other biological functions through both central and peripheral mechanisms. CT is able to inhibit brain Na^+, K^+-ATPase activity (Rodríguez de Lores Arnaiz, López Ordieres, Peptides 1997;18:613–5) and a relationship between such enzyme activity and cholinergic function has been suggested. Accordingly, we tested CT effect on [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to rat CNS membranes to determine whether the peptide is able to modify the cholinergic muscarinic receptor as well. It was found that 1×10^{-7}–1×10^{-5} M CT decreased 20–70% ligand binding to hippocampal, cerebellar, cortical and striatal membranes. Scatchard analysis of saturation curves showed that 5×10^{-6} M CT significantly modified binding kinetic constants, thus it increased roughly 220% K_d values and decreased 20–36% B_{max} values in cerebral cortical and cerebellar membranes. Since the peptide decreases affinity ligand binding and reduces the number of binding sites, CT may well be acting as a cholinergic modulator through a decrease in muscarinic receptor functionality. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Calcitonin effect; CNS membranes; QNB binding; Cholinergic muscarinic receptor; Cholinergic system; Sodium pump; Na^+, K^+-ATPase

1. Introduction

Calcitonin (CT) is a 32-aminoacid peptide produced by the thyroid gland, whose best described role is to prevent bone reabsorption, though it also participates in other biological functions through both central and peripheral mechanisms. Central administration of CT induces analgesia [1,2] and hyperthermia [2], as well as decreased food intake [3,4], amphetamine-induced locomotor activity [2] and gastric secretion [5], involving both central and peripheral mechanisms.

Previous findings from this laboratory have shown that CT is able to inhibit synaptosomal membrane Na^+, K^+-ATPase activity [6] and evidence pointing to a relationship between Na^+, K^+-ATPase activity (the sodium pump) and cholinergic function is available [7,8]. In the present study, we tested CT effect on [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to rat hippocampal, cerebellar, cortical and striatal membranes to determine whether the peptide is able to modify the cholinergic muscarinic receptor. We observed ligand binding inhibition to CNS membranes with decreased affinity binding and lower binding site number, suggesting that CT may well be acting as a cholinergic modulator through impaired muscarinic receptor functionality.

2. Materials and methods

2.1. Animals and drugs

Male Wistar rats weighing 100–150 g were used. Reagents were analytical grade. Atropine was from Sigma

*Corresponding author. Fax: +54-11-4508-3645.
E-mail address: grodrig@ffyb.uba.ar (G. Rodríguez de Lores Arnaiz)

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Chemical Co., St. Louis, MO, USA; salmon calcitonin (Peptech, Denmark) was provided by Gramón & Co., Argentina; L-[^{3}H]-QNB, with a specific radioactivity of 43.6 Ci/mmol, was purchased from New England Nuclear, DuPont, Boston, MA, USA.

2.2. Membrane preparation

For each experiment, cerebellum, hippocampus, cerebral cortex and striatum from 3 to 4 rats were harvested and separately pooled. Tissues were rapidly homogenized at 10% (w/v), except for cerebral cortex at 4% (w/v), in 0.32 M sucrose neutralized with Tris base solution (0.4 mM Tris final concentration) in a Teflon glass Potter–Elvehjem homogenizer.

Homogenates were centrifuged at $900 \times g$ for 10 min and pellets discarded; resulting supernatants were diluted with 0.16 M sucrose to a final concentration of 0.25 M sucrose, centrifuged at $100\,000 \times g$ for 30 min and membrane pellets stored at -70°C until use.

2.3. [^{3}H]-QNB binding assay

[^{3}H]-QNB binding was determined according to the method described by Yamamura and Snyder [9] with slight modifications, in the presence of variable concentrations of CT. Peptide solutions in 0.006 N HCl were freshly prepared for each experiment and, before adding aliquot membranes suspensions, were neutralized with Tris base solutions. Membrane pellets were resuspended and later diluted in 50 mM sodium–potassium phosphate buffer (pH 7.4) to reach 0.1 mg protein per ml concentration.

Triplicate membrane aliquots were incubated (2 ml final volume) at 30°C for 60 min with 0.5 nM of [^{3}H]-QNB. Non-specific binding was defined as tracer binding in the presence of 5 μM atropine sulfate. After incubation, 3 ml of ice-cold sodium–potassium phosphate buffer were added and samples vacuum-filtered through Whatman GF/B glass disks. Filters were washed twice with 3 ml of ice-cold buffer, placed in plastic vials and dried overnight at 70°C. To each vial, 3 ml of 0.4% PPO in toluene were added and radioactivity quantified in a liquid scintillation counter.

Specific binding was calculated as the difference between the binding in the absence and presence of atropine, and represented 90–95% of total binding.

For saturation studies, membranes were incubated in the presence of [^{3}H]-QNB concentrations ranging from 0.125 to 2.00 nM and processed as described above. Saturation curves were initially analyzed by Scatchard method and fitted by using the nonlinear least square regression analysis program, EBDA (G.A. McPherson 1983 V 2.0).

Protein was assayed according to Lowry et al. [10] using bovine serum albumin as standard.

3. Results

Cholinergic receptor was studied by means of [^{3}H]-QNB binding to CNS membranes in the absence or presence of variable CT concentrations. In the absence of the peptide, ligand binding was 1.55 ± 0.75, 1.49 ± 0.13, 1.29 ± 0.20 and 0.50 ± 0.09 pmoles per mg protein (mean values ± S.E., $n = 3$, 4) for striatum, cerebral cortex, hippocampus and cerebellum membranes, respectively. Since preliminary assays had indicated that CT is able to affect [^{3}H]-QNB binding, as a first step to find an appropriate peptide concentration for further saturation studies, we chose 10^{-7} M, 10^{-6} M and 10^{-5} M CT which proved effective to inhibit synaptosomal membrane Na^+, K^+-ATPase activity [10]. It was found that 1×10^{-7}–1×10^{-5} M CT decreased 55–69 and 37–62% ligand binding in striatal and cerebral cortex membranes, respectively (Table 1). Such CT concentration range exerted a lower effect on hippocampal and cerebellar membranes, since binding inhibition reached 48% (Table 2).

Saturation curves with 0.125, 0.250, 0.500, 0.625, 0.750, 1.00, 1.50 and 2.00 nM [^{3}H]-QNB were performed for cerebral cortex and cerebellum in the presence of 5×10^{-6} M CT and respective Scatchard plots traced (Figs. 1 and 2). It was observed that binding attained maximal values with 0.75–1.00 nM ligand in all cases. Saturation curves as well as Scatchard plots indicated the labelling of an homogeneous population of receptor sites.

Table 1
Calcitonin inhibition of specific L-[^{3}H]-QNB binding to rat striatum and cerebral cortex membranes[a]

Calcitonin	Striatum	Cerebral cortex
1×10^{-7} M	54.9 ± 0.9 (3)[b]	37.0 ± 0.6 (3)[b]
1×10^{-6} M	62.0 ± 0.7 (3)[b]	41.7 ± 0.7 (3)[b]
1×10^{-5} M	68.7 ± 1.9 (3)[b]	62.0 ± 1.0 (3)[b]

[a] L-[^{3}H]-QNB binding to striatum and cerebral cortex membranes was assayed in the absence and presence of CT. Results are expressed as specific binding inhibition taking as 100% values obtained in the absence of the peptide (mean values ± S.E.). Figures between brackets indicate the number of experiments performed in triplicate in the absence or presence of 5×10^{-6} M atropine sulfate to quantify specific binding.
[b] $P < 0.001$ by Student's t test.

Table 2
Calcitonin inhibition of specific L-[^{3}H]-QNB binding to rat hippocampus and cerebellum membranes[a]

Calcitonin	Hippocampus	Cerebellum
1×10^{-7} M	24.2 ± 0.8 (4)[b]	20.4 ± 0.9 (3)[b]
1×10^{-6} M	36.7 ± 0.9 (4)[b]	33.7 ± 0.3 (3)[b]
1×10^{-5} M	48.0 ± 0.7 (4)[b]	47.4 ± 0.7 (3)[b]

[a] L-[^{3}H]-QNB binding to hippocampus and cerebellum membranes was assayed in the absence and presence of CT. Results are expressed as specific binding inhibition taking as 100% values obtained in the absence of the peptide (mean values ± S.E.). Figures between brackets indicate the number of experiments performed in triplicate in the absence or presence of 5×10^{-6} M atropine sulfate to quantify specific binding.
[b] $P < 0.001$ by Student's t test.

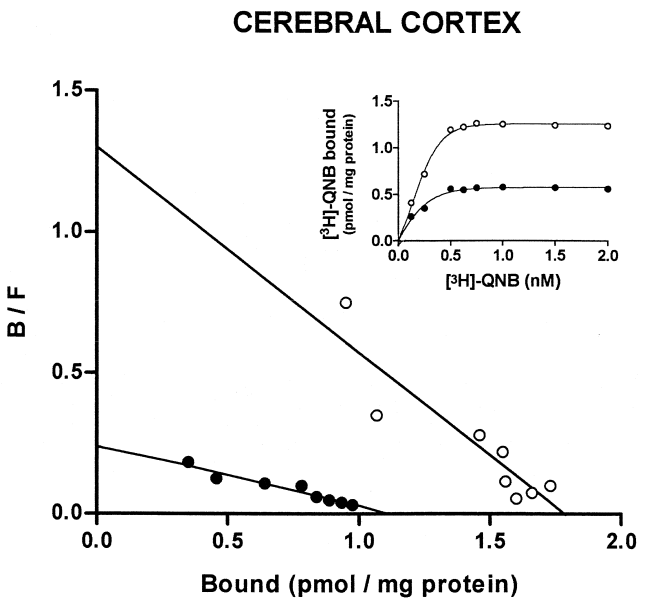

Fig. 1. Scatchard plots for L-[^{3}H]-QNB binding to rat cerebral cortex membranes in the absence (○) or presence (●) of 5×10^{-6} M calcitonin. Results are from a single experiment representative of a set of three, each performed in triplicate. Inset, saturation curves.

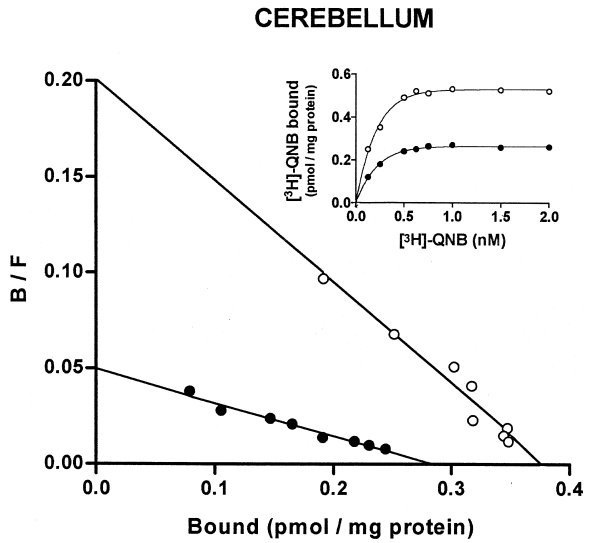

Fig. 2. Scatchard plots for L-[^{3}H]-QNB binding to rat cerebellum membranes in the absence (○) or presence (●) of 5×10^{-6} M calcitonin. Results are from a single experiment representative of a set of three, each performed in triplicate. Inset, saturation curves.

Averaged values from three experiments for cerebral cortex and cerebellum showed that kinetic constants resulting from Scatchard plots were altered by CT. For the former, K_d value increased 225%, indicating a marked decrease in ligand–receptor affinity; concomitantly, a 36% decrease in B_{max} values was found, which indicates a reduction in the number of binding sites. For cerebellum, K_d increased 224% with a 20% decrease in the number of binding sites (B_{max}). Statistical analysis indicated that all differences were highly significant. Hill numbers were close to unity both for cerebral cortex and cerebellum membranes (Table 3).

4. Discussion

Present results showed that CT decreases [^{3}H]-QNB binding to CNS membranes, exerting a greater effect on striatum and cerebral cortex than on hippocampus and cerebellum. Taking into account that the ranking order for ligand binding values in control membranes was striatum = cerebral cortex = hippocampus ≫ cerebellum, it seems that sensitivity to CT inhibition relates poorly to binding site density in assayed CNS areas. In order to determine whether inhibition was due to a change in muscarinic receptor affinity binding and/or in binding site number, saturation curves were performed for cerebral cortex and cerebellum over a wide ligand concentration range. The peptide decreased 3-fold affinity ligand binding and reduced 20–35% the number of binding sites, which showed homogeneous behaviour as the Hill number was invariably close to unity. On the whole, results suggest that CT may act as a cholinergic modulator through a decrease in muscarinic receptor functionality.

The contention that ligand binding inhibition is due to CT complexing with the radioligand, thus reducing its free concentration, is hardly tenable because ligand binding percentage inhibition is remarkably constant when [^{3}H]-QNB concentration is increased from 0.5 to 2.0 nM (see saturation curves in Figs. 1 and 2).

Since percentage ligand binding inhibition by CT to cerebral cortex and cerebellum membranes remained unaltered in saturation curves, interaction can hardly be reversible. Scatchard plots suggest that interaction is neither entirely competitive nor noncompetitive, as there are significant changes in both constants, B_{max} and K_d.

Muscarinic ligand binding inhibition by CT here described may provide a plausible explanation for pain relief by this peptide [1,2], since an analgesic effect has been described for some muscarinic antagonists, thus supporting its anticholinergic action. To illustrate, one of the enantiomers of the atropine racemate, namely R-(+)-hyoscyamine (R-(+)-1), acts as an analgesic and a cognition enhancer in mice [11].

CT modifies gastrointestinal motility and behaves as anticholinergic drugs hexametonium or atropine through a central mechanism which seems independent of peripheral innervation [12]. Our results showing decreased cholinergic receptor functionality by CT may well be related to collateral peptide effects such as those recorded occasionally in patients receiving this peptide [13,14]. Peripherally administered CT inhibits gastrointestinal motility, an effect which seems to occur at CNS level by lowering vagal activity [12], besides exerting antiulcer effect, in which $5HT_3$ appears to be involved [15].

It is known that muscarinic receptors are present both at

Table 3
Effect of calcitonin on L-[^{3}H]-QNB binding constants in cerebral cortex and cerebellum membranes[a]

Area	Condition	K_d (pM)	B_{max} (nmol mg prot^{-1})	N_{Hill}
Cerebral cortex	Control	113.7±17.5	1.78±0.09	0.97±0.04
	5×10^{-4} M CT	369.7±44.4[b]	1.14±0.13[b]	0.92±0.02
Cerebellum	Control	171.7±19.4	0.36±0.02	1.02±0.13
	5×10^{-6} M CT	556.0±55.3[d]	0.29±0.01[c]	1.01±0.02

[a] Cerebral cortex and cerebellum membranes were separated and used to study L-[^{3}H]-QNB binding in the absence or presence of 5×10^{-6} M calcitonin (CT). Data from three independent experiments run with different membrane preparations were processed to calculate kinetic constants. Results presented are mean values (±S.E.).
[b] $P<0.01$
[c] $P<0.02$
[d] $P<0.005$ by Student's *t*-test.

pre- and postsynaptic level, and that, should CT effect on muscarinic receptor occur at postsynaptic level, a decrease in cholinergic neurotransmission would be expected. Alternatively, should receptor blockade occur at presynaptic level, it would presumably modulate acetylcholine release, leading to cholinergic transmission enhancement, as neurotransmitter release is regulated by presynaptic receptors [16–18]. Interestingly, galanin, a peptide similar to CT in size (which contains 29 aminoacids), inhibits K^+-evoked acetylcholine release as well as muscarinic receptor-mediated stimulation of phosphoinositide turnover [19]. Assuming that CT effect takes place at presynaptic level, it is tempting to speculate that CT administration might well compensate neurological deficits exhibited by some patients with cholinergic dysfunction.

CNS [^{3}H]-QNB binding changes have been found to correlate with altered acetylcholinesterase activity following administration of hypotensive Buxaminol [20]; however, administration of acetylcholinesterase inhibitor tacrine is unable to modify cholinergic muscarinic receptor density [21]. Indeed, tacrine proves a potent enzyme inhibitor in whole brain homogenates (IC_{50} 0.083 μmol/l) [22], whereas CT fails to alter synaptosomal membrane acetylcholinesterase activity over a 10^{-6}–10^{-4} M concentration range [6]. Since CT has no effect on acetylcholinesterase activity [6], present findings showing alteration in cholinergic muscarinic receptor seem to rule out any correlation between acetylcholine catabolizing enzyme and receptor molecules.

Efforts have been devoted to characterize novel cholinergic agents which may or may not possess nootropic properties [23]. To exemplify, tacrine has been reported to improve cognitive function and behavioural deficits in Alzheimer's disease [24]. On comparing tacrine and CT affinity for cholinergic muscarinic receptor, very similar IC_{50} values (μmoles/l) were found by assaying [^{3}H]-QNB binding to rat cerebral cortex membranes, i.e. 3.7 [22] and 5.0 (present results) for tacrine and CT, respectively. However, opposite effects for such substances have also been recorded, since a collateral tacrine effect is hypothermia, dose-dependent in nature [22], whereas CT induces hyperthermia [2].

It has been reported that a small proportion of an intravenous CT dose could cross the blood–brain barrier [25], and it is not unlikely that peripheral CT may diffuse in cerebrospinal fluid [26]. CT receptors exist in the circumventricular area, where barrier function is less effective [27,28].

The contention that CT may play a role at CNS level is sustained by the demonstration of binding sites for CT in human and rat brain homogenates [29,30]. Furthermore, salmon CT binding sites have been localized in rat brain by autoradiography [31].

The ability of CT to induce anorexia has been described, an effect proving more marked when injected in regions involved in food intake control. However, the effect poorly correlates with CT binding sites, suggesting that alternative targets may participate in behavioural responses [32]. According to our results, the target may be the sodium pump [6] and/or the muscarinic receptor (present findings). The CT concentration required to evidence such effects is in the micromolar range whereas K_d values for brain CT receptor are in the nanomolar range [33,34]. There is no available information regarding effective CT concentration at the synaptic region, where the peptide would be expected to behave as a Na^+, K^+-ATPase and/or a muscarinic receptor modulator. Herein, we found that [^{3}H]-QNB binding inhibition requires a relatively high CT concentration in the micromolar range. So far, it is hard to assess the actual concentration range in vivo of an active molecule in a given cellular microenvironment.

The functionality of the cholinergic system is essential in learning and memory [35–38]; thus, increased levels of brain acetylcholine are involved in its improvement [39] and some muscarinic receptor antagonists enhance cognitive ability [11]. The decrease in ligand affinity and in the number of binding sites of the cholinergic muscarinic receptor by CT may well lead to cholinergic system changes with potential improvement in cognitive functions.

In a previous article we have shown that CT is able to inhibit the activity of synaptosomal membrane Na^+, K^+-ATPase and K^+-*p*-nitrophenylphosphatase but not of other enzymes such as that of Mg^{2+}- and Ca^{2+}-ATPases or acetylcholinesterase [6]. Present findings point to a de-

crease in cholinergic muscarinic receptor functionality and support the view that sodium pump and cholinergic system responses are not independent, though the mechanism of their putative interaction remains to be elucidated.

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