Invest Clin 40(2): 109-125, 1999

Partial characterization of endogenous modulators of muscarinic acetylcholine receptors in human frontal cortex.

Nelson Enrique Loureiro-Dos Santos[1,2], Carla Silvia Rodríguez-Valenzuela[1], Rafael Armando Bonfante-Cabarcas[1,3], Carmine Pascuzzo-Lima[1,4] y José Antonio Moreno-Yanes[1,3]

[1]Unidad de Bioquímica, Centro de Investigaciones Biomédicas,
[2]Sección de Fisiología. Departamento de Ciencias Funcionales,
[3]Sección de Bioquímica. Departamento de Ciencias Funcionales y
[4]Sección de Farmacología. Departamento de Ciencias Funcionales.
Decanato de Medicina. Universidad Centroccidental "Lisandro Alvarado". Barquisimeto, Venezuela

Key words: Muscarinic acetylcholine receptors homotropic, endogenous modulator, human brain.

Abstract. A soluble fraction from human frontal cortex with molecular weight less than 10 kD was tested for the presence of endogenous substances capable of modulating the [^{3}H]-QNB binding to crude P1 + P2 fractions from the same region. The soluble fraction was able to decrease [^{3}H]-QNB binding in a dose-response manner with an IC_{50} of about 30 µg/ml. The effect appeared to be noncompetitive in nature, since Bmax but not Kd was significantly affected; however, in some specimens a biphasic profile, with an initial inhibition of 88-90% of [^{3}H]-QNB binding and 50-60% ulterior binding recuperation was also found. The modulator appeared to have a molecular weight less than 10,000 Daltons and was heat and trypsin resistant. These results point out the existence of an endogenous factor, which could be heterogeneous in regard to its molecular nature or to its action sites.

Caracterización preliminar de un modulador endógeno del receptor colinérgico muscarínico en corteza cerebral humana. *Invest Clín 1999; 40(2): 109-125.*

Palabras clave: Receptor colinérgico muscarínico, modulador endógeno, cerebro humano, factor endógeno.

Resumen. Se reporta la existencia de un factor soluble endógeno de bajo peso molecular, derivado de la corteza frontal humana, el cual es capaz de modular la unión de [^{3}H]-QNB al receptor colinérgico muscarínico. El efecto de esta sustancia fue de naturaleza no competitiva, ya que afectaba la capacidad máxima de unión, pero no la afinidad; sin embargo, un perfil bifásico, con inhibición hasta un 88-90% de la unión y una ulterior recuperación de hasta 50-60% de la inhibición, también fue observado. La acción de esta sustancia pareció ser reversible, resistente a la acción de la tripsina, termoestable, y presentó un peso molecular no mayor de 10.000 Da. Los resultados sugieren la posible existencia de heterogeneidad molecular en el factor endógeno aislado o la presencia de múltiples sitios de acción sobre los cuales actuaría este factor.

Received: 14-10-98. Accepted: 26-4-99.

INTRODUCTION

Muscarinic acetylcholine receptors belong to a superfamily of coupled G protein receptors with seven transmembrane spanning domains (1, 2). Five distinct subtypes have been identified on the basis of molecular cloning studies, although pharmacological studies have identified only three subtypes (3-5).

Based on classical kinetic theories, muscarinic acetylcholine receptor antagonist binding pattern has been analyzed almost universally in terms of a simple hyperbolic model, assuming non-interacting sites (6, 7). However, recent evidences have demonstrated more complexity in its kinetic, including the presence of heterogeneity and cooperative phenomena. For instance, cooperativity has been found in its two possible ways: homotropic (8-13) and heterotropic (14-18). Many different substances, including nicotinic ligands, calcium channel blockers, dopaminergic agonists, local anesthetics and antiarrhythmic drugs may act on allosteric sites at muscarinic acetylcholine receptor altering the antagonist´s binding in a cooperative fashion (16-18).

The presence of muscarinic acetylcholine receptor allosteric sites suggests the presence of muscarinic acetylcholine receptor endogenous modulators able to interact with these sites and, consequently, modulate muscarinic function. In fact, several studies have supported this hypothesis by reporting the ex-

istence of soluble substances from different tissues and animal species (19-27), those are capable of modifying the binding profile of antagonist. However the results have been diverse and controversial.

Considerable evidence from both animal and human studies, suggests that cholinergic systems are important for learning, memory and cognition (for review see 28). Some exogenous substances such as atropine and scopolamine are able to disrupt the acquisition and performance of learned behaviors, blocking the interaction between acetylcholine and muscarinic acetylcholine receptor (29). Therefore, the existence of endogenous substances able to modulate the interaction between acetylcholine and muscarinic acetylcholine receptor could be involved in learning and memory, either increasing or decreasing these function. An unbalanced production of these endogenous modulators would be traduced in memory and learning disorders. In agreement with this point of view, it has been reported that patients with senile dementia of the Alzheimer type have a serum circulating suppressing factor of muscarinic acetylcholine receptor antagonist binding (26). Moreover, patients that suffer Alzheimer's disease present high activity of muscarinic acetylcholine receptor antagonist binding inhibitor in their brain (23). Then, the knowledge of muscarinic acetylcholine receptor allosteric regulation by endogenous substances, is a necessary step to unveil the dynamic of muscarinic acetylcholine receptor function *in situ* and its relation with brain pathologies that involve the cholinergic system in order to design better therapeutic approaches.

The reported of serum circulating suppressing factor (26) and endogenous brain factor (23, 24, 30) in Alzheimer´s patients are still somewhat controversial, because it has not been fully confirmed. In the present study, the main objective was a further characterization of this endogenous factor derived from a soluble fraction of human frontal cortex that could be able to regulate muscarinic acetylcholine receptor functions in order to enhance the current understanding about this matter. Our results confirm that the soluble fraction from frontal cortex has a [^{3}H]-QNB binding inhibitor but we also found that some brains has another factor that it was able to counteract the inhibitory effect.

MATERIALS AND METHODS

Materials

Five human brains of young males (15-35 years old) were obtained at the Department of Pathology of the "Antonio María Pineda" Hospital. None of the patients had a previous history of diseases including of central nervous system or treatment with any drug. The causes of death include: haemorrhagic shock due to trauma (n = 2) or gun shot (n = 1), mechanical asphyxia (n = 2). The macroscopic integrity of those brains was conserved in all patients without any

sign of brain damage. Delay time between death and sample collection was not longer than 12 hours. Brains were dissected and the frontal cortex (FC) was removed. This procedure was carried out at 4°C.

[^{3}H]-QNB (43.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Ultrafiltration equipment 94353-138 and membranes type Diaflo® (PM10, UK05) were obtained from Amicon Corporation (Lexington, MA, USA).

Preparation of the soluble fraction

Pieces of frontal cortex were suspended 1: 10 w/v in ice cold pentadistilled water (18.2 M of resistance) obtained from a MilliRo distiller equipment (Millipore Corporation, USA) and homogenized three times (4-seg bursts with 1-min interval) using a Polytron homogenizer (Eberbach Corporation, Ann Harbor MI). The homogenate was centrifuged at 43,000 g for 1 hour in a Sorvall RC-5C centrifuge (at 4°C). This procedure gave a supernatant that was called crude soluble fraction and a pellet designated as crude membrane fraction. Crude soluble fraction was filtered under vacuum through glass-fiber filters (Whatman Nº 1) to eliminate the largest particles, followed by Ultrafiltration through a Diaflo membrane PM10 applying 60 PSI Nitrogen pressure. The resulting ultrafiltrates were lyophilized, weighed and resuspended in 25 mM phosphate buffer (pH 7.3) to a final concentration of 2-4 mg dry weight per ml. This material was called purified soluble fraction and it was stored at -70°C until use.

Preparation of the microsomal fraction

The preparation of microsomal fraction and binding assays were done following the protocol standarized by Moreno-Yanez *et al* (12). In brief, crude membrane fraction was resuspended in 25 mM phosphate/1mM EDTA buffer and centrifuged at 43,000 g for 20 minutes. The supernatant was discarded and the pellet was resuspended in 0.32 M Sucrose / 25 mM Phosphate and centrifuged again at 1,000 g (10 minutes). The resulting pellet was resuspended and centrifuged again. Both supernatants were collected and centrifuged at 43,000 g for 45 minutes. Pellets were resuspended and centrifuged (43,000 g for 45 minutes). Resulting pellets, called microsomal fraction, were weighed and resuspended in buffer (1: 20 w/v). Protein content was determined by the method of bicinchioninic acid (31).

^{3}H-QNB binding assays

For inhibition assays, fixed microsomal fraction concentrations (50 µg/2 ml) were preincubated in the absence or presence of the respective soluble fraction (0.5-300 µl) for 60 minutes in 25 mM phosphate buffer (pH 7.3, 37°C). Then, [^{3}H]-QNB (20, 50, 250 or 500 pM) was added and incubated for 120

minutes. The prolonged incubation time has no effect on the activity of microsomal fraction (see Moreno-Yanez *et al.* (12). Saturation assays were performed for each microsomal fraction, in the presence and absence of 1 ID_{50} of each respective soluble fraction. [³H]-QNB (5 - 1000 pM) was incubated in 25 mM phosphate buffer (2 ml) in the presence of microsomal fraction (50 µg of protein) at 37°C for 120 minutes after a 60 min-preincubation with soluble fraction. Nonspecific binding was obtained by adding 1 µM atropine in parallel tubes prepared for each condition. Binding reactions were terminated by filtration under vacuum trough GF/B glass-fiber filters (Whatman Paper Ltd.; Brandell, Inc.) and washed three times with 5 ml ice-cold 10 mM phosphate buffer solution. Filters were dried in vials at 60°C and then, 5 ml of scintillation liquid (PPO / POPOP / Triton X-100 / Toluene) was added. After a 12-hours period, radioactivity on the filters was measured in a liquid scintillation counter with about 40% counting efficiency (Wallac 1410, Pharmacia, Inc., Finland). All assays were carried out at least twice, with 3 replicates for each point.

Stability of soluble fraction

It was determined by heating the soluble fraction in a boiling water bath (91°C, up to 60 minutes) and by treatment with crystallized trypsin 10^{-4} U/ml for 60 minutes at 37°C. Trypsin digestion was terminated by addition of 5 mg/ml trypsin Inhibitor.

Data Analysis

Saturation assays were analyzed by non-linear regression using the following equations:

$$B = \frac{Bmax \times Kd^{nH}}{F + Kd^{nH}} \qquad B = \frac{Bmax \times Kd}{F + Kd}$$

Adjustments were done according to the least square method. The more complex model (Hill model) (32) was accepted when, by comparing the two equations by the F partial test, differences generate a p value ≤ 0.05. Inhibition curves were analyzed by this equation:

$$B = \frac{Bmax}{1 + 10^{\log SF \quad \log IC_{50}}}$$

Other analyses were carried out by ANOVA or Student´s t test. GraphPad Prism® version 2.01 (GraphPad Software, San Diego CA) was used in the analyses.

RESULTS

Characterization of the soluble fraction effect

The effect of soluble fraction at different preincubation times is shown in Fig. 1. As it can be noticed, the maximal effect of the soluble fraction was achieved after about an hour ($t_{½}$ = 11.91 minutes, Fig. 1). This time was used for subsequent experiments.

Initial experiments show that soluble fractions from different brains were able to inhibit [^{3}H]-QNB binding at the same microsomal fraction, but we noted that some soluble fractions increased [^{3}H]-QNB binding after initial inhibition. In or-

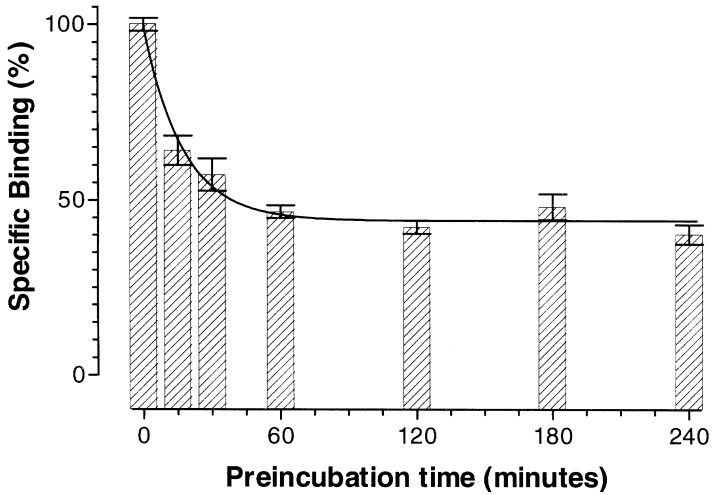

Fig. 1. Effect of the time course of preincubation on soluble fraction action. For the assays, 1 ID_{50} of soluble fraction was added to microsomal fraction and incubated at several times (15 - 240 minutes) and then, [^{3}H]-QNB was added and incubated for 2 hours. Preincubation allowed the consecution of soluble fraction maximal effect in about an hour ($t_{1/2}$ = 11.91 minutes).

der to discard that the observed effect could be a consequence of soluble fractions and microsomal fractions from different brain sources, we matched the soluble fraction with its corresponding microsomal fraction.

Experiments were performed in 5 different brains between the respective soluble fraction-microsomal fraction pairs. In all the brains, soluble fraction was able to inhibit the [^{3}H]-QNB binding in a dose-dependent manner with an IC_{50} broadly around 30 µg dry weight/ml which was independent of the [^{3}H]-QNB concentration (Fig. 2), however, in two brains (40 %) soluble fraction had a biphasic profile. In these later cases (see Fig. 3) there were the initial [^{3}H]-QNB binding inhibition toward 10-12% with a similar potency as observed in the other brains, but unexpected, in these cases there were an almost total recuperation of the specific binding when the soluble fraction concentration was increased above 100 µg/ml, indicating the presence of another factor able to counteract the prior inhibition of [^{3}H]-QNB binding. No correlation was found between the cause of death and the presence of the excitatory effect.

The effect of the inhibitory soluble fraction on the [^{3}H]-QNB binding profile is shown in Table I. Soluble fraction was able to decrease significantly Bmax values ($p < 0.05$; paired t test) while differences of the Kd values were not statistically significant. These results suggest that the inhibitory soluble fraction behave in a noncompetitive

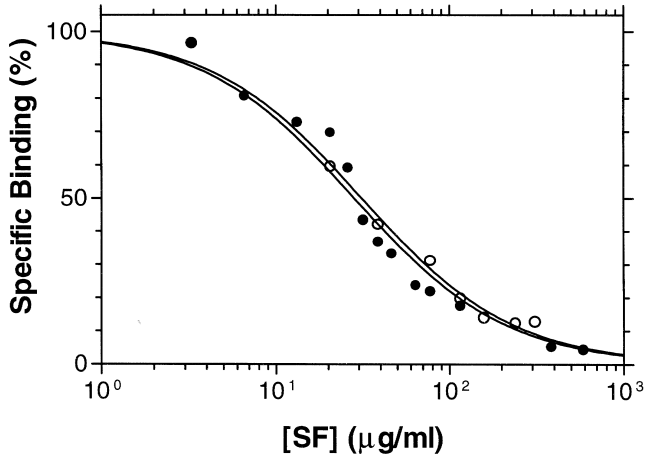

Fig. 2. [^{3}H]-QNB binding Inhibition by soluble fraction. MF (50 µg/2 ml) was incubated with [^{3}H]-QNB in the presence of soluble fraction (0.5-300 µl) for 60 minutes in 25 mM phosphate buffer pH 7,3 at 37°C. Full circles: [^{3}H]-QNB 500pM; Open circles: [^{3}H]-QNB 50 pM. The calculated IC_{50}s were 31.12 (CI 95%= 26.60 to 36.40) and 28.21 (CI 95%= 23.56 to 33.78), respectively.

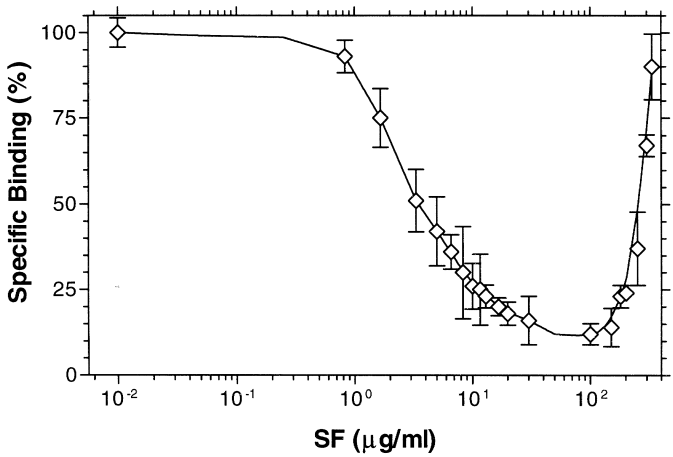

Fig. 3. Biphasic Effect of soluble fraction on [^{3}H]-QNB 500pM binding. The assay was performed in the same conditions described for Fig. 2. In this experiment we can observe an initial inhibition profile toward 10-12 percent of specific binding when the soluble fraction concentration achieve 100 µg/ml; however, when the soluble fraction concentration was increased above 100 µg/ml we can observe an almost total recuperation of the specific binding in a dose-dependent manner instead of more inhibition, indicating the presence of another factor in the soluble fraction able to counteract the inhibition effect. The curve was drawn by a locally weighted (lowest) adjusting.

TABLE I
EFFECT OF SOLUBLE FRACTION ON [^{3}H]-QNB BINDING

MF/SF pair	Bmax		Kd		nH	
	Control	SF	Control	SF	Control	SF
1	577	358	51	87	1.01	1.00
2	435	256	46	46	1.18	1.44
3	352	218	15	8	1.05	0.93
4	350	188	37	49	1.35	1.54
5	414	247	32	25	0.91	0.99
Mean ± SD	425.6±92.6	253.4±64.3	36.2±14.0	43.0±29.7	1.10±0.18	1.18±0.29

Saturation assays were performed in the presence and absence of 1 ID_{50} of the respective SF. [^{3}H]-QNB (5 - 1,000 pM) was incubated in 25 mM Phosphate Buffer (2 ml) in the presence of MF (50 µg of protein) at 37°C for 120 minutes after a 60 minutes-preincubation with SF. Changes on Bmax ($p = 0.0002$; paired T test), but not on Kd ($p = 0.45$; paired T test) or Hill coefficients ($p = 0.30$; paired T test) were statistically significant.

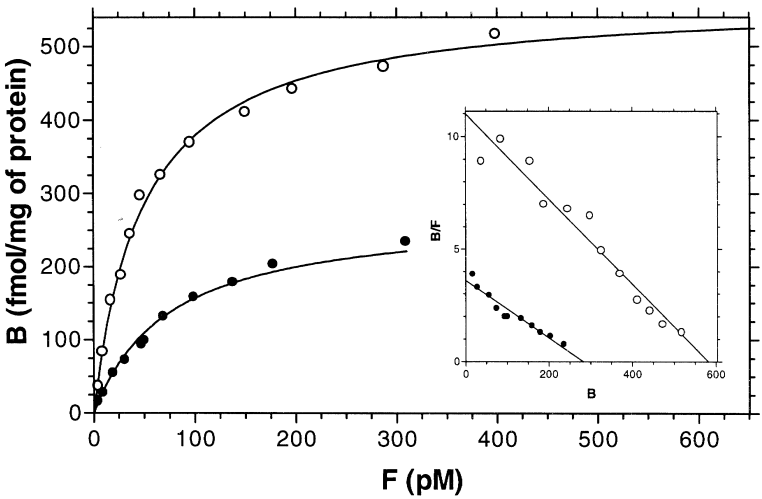

Fig. 4. Effect of soluble fraction on [^{3}H]-QNB Binding. Saturation assays were performed in the presence (full circles) and absence (open circles) of the respective soluble fraction in the conditions described for Table I. The inset displays the respective Scatchard plots.

fashion. Three microsomal fraction displayed a non-cooperative pattern, while the other two showed an apparent positive cooperativity with n_H of 1.18 and 1.35, respectively. Although they affected Bmax, none of the inhibitory soluble fraction induced significant changes on the basal kinetic profile (i.e., there were not significant effects on Hill coefficients, Table I). Fig. 4 displays a typical pair of saturation assays.

Stability of the inhibitory soluble fraction

The effect of heating on the inhibitory soluble fraction actions is shown in Fig. 5. Although there were significant differences between the effect of inhibitory soluble fraction for each time, these differences were considerably low (less than 10% for the 30-60 minutes heating).

Trypsin treatment did not induce significant changes in the inhibitory soluble fraction effect (Fig. 6). A two-fold increase in the trypsin concentration did not alter this result (data not shown).

Molecular weight cut-off activity of inhibitory soluble fraction

In order to determine the size of the putative compound able to modify the muscarinic acetylcholine receptor [^{3}H]-QNB binding profile, 15 ml of soluble fraction were ultrafiltrated through a 500 Dalton (D) cut-off membrane. The ability of the ultrafiltrated and the concentrated non-ultrafiltrated soluble fraction, that remained into the recipient, to inhibit the [^{3}H]-QNB binding was tested and compared against the non treated soluble fraction. The 500 D ultrafiltration effect is shown in Fig. 7. The 500 D

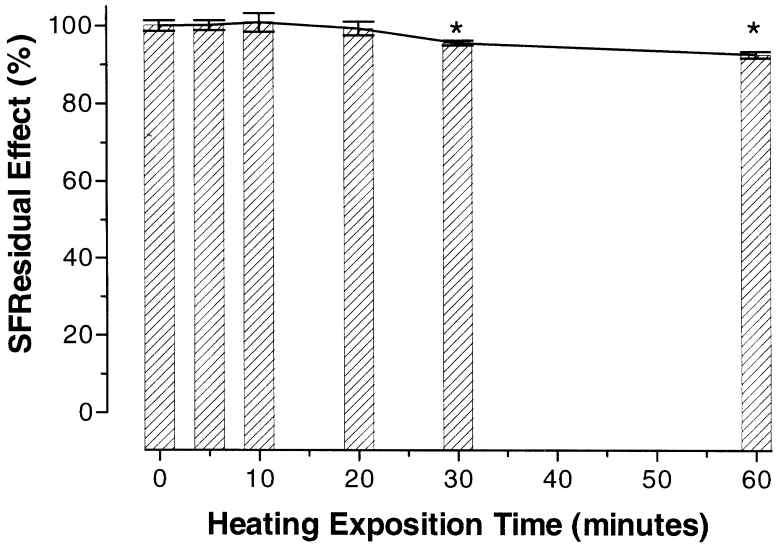

Fig. 5. Effect of Heat on soluble fraction activity. The effect of heating on the action of soluble fraction is shown. soluble fraction was heated in a boiling water bath (91°C) for different periods of time (0 - 60 minutes). Comparisons were done using One-Way ANOVA, yielding significant differences between heating times ($p < 0.0001$). Dunnet's Multiple Comparison Test indicated that both the 30-minutes and 60-minutes heating were able to diminish significantly the soluble fraction effect, however, the differences were numerically negligible (4.6 and 7.6%, respectively).

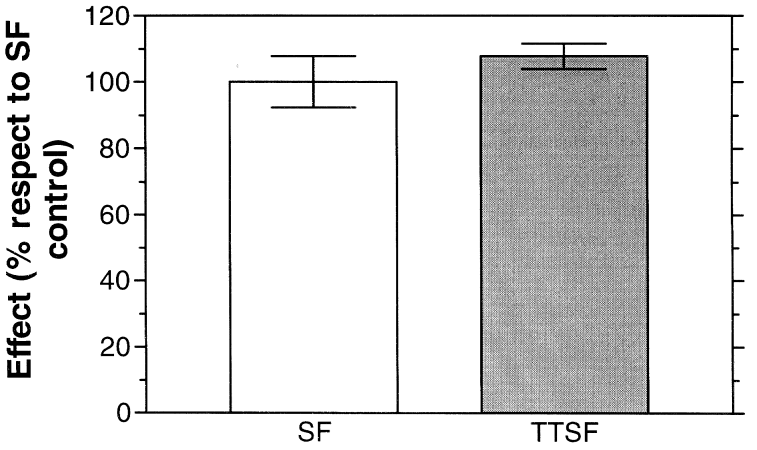

Fig. 6. Effect of trypsin on soluble fraction activity. Soluble fraction was treated with trypsin 10^{-4} U/ml, for 60 minutes at 37°C. Digestion was terminated by addition of 5 mg/ml trypsin Inhibitor. Data are shown as the relative effect respect to untreated soluble fraction. Comparison was performed by using paired T test and no statistically significant differences were found ($p = 0.4216$).

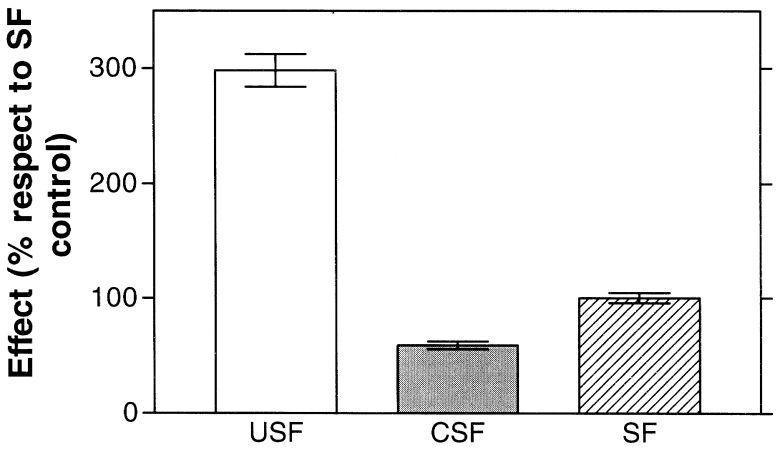

Fig. 7. Effect of Ultrafiltration on soluble fraction activity soluble fraction was ultrafiltrated for 16 hours at 4°C, through UK05 membranes. The action of ultrafiltrated soluble fraction (USF, white bar), concentrate soluble fraction (CSF, gray bar) and non ultrafiltrated soluble fraction (SF, diagonal bar) on [^{3}H]-QNB is shown. Data are shown as the relative effect respect to the soluble fraction control. Comparison was performed using ANOVA and a significant difference was found ($p < 0.0001$). The Dunnett's post-test was used to compare USF and CSF effect with SF one. There were significant differences in both comparisons ($p < 0.01$ and $p < 0.05$, respectively).

ultrafiltrated displayed a three-fold higher effect than non-treated soluble fraction, while the remaining concentrate non 500 D ultrafiltrated soluble fraction exhibited only a 60% of the soluble fraction effect.

DISCUSSION

In the present paper we confirm the existence of an endogenous inhibitor of muscarinic acetylcholine receptor [^{3}H]-QNB binding extracted from apparently healthy human frontal cortex specimens, but interestingly, in contrast to other reports we report the possible existence of another endogenous factor able to counteract the inhibitory effect.

The nature of the inhibitory endogenous factor has been matter of controversy. Results have been variable depending on the animal species, organ and developmental stage (19-25). Similar to other reports from experiments made in human brains (23, 24) the inhibitory soluble factor presented here is heat stable (Fig. 5), trypsin resistant (Fig. 6), is of low molecular weight (Fig. 7) and it has a noncompetitive kinetics, since Kd was not significantly affected (Fig. 4).

It has been suggested that the endogenous inhibitory action is consistent with a free radical mechanism because it has been found that the effect is essentially irreversible, it is enhanced by reduced glutathion and it is blocked by free radical scavengers like Mn^{++}, vitamin E analogous and ascorbate (24). Venters y col (30) have claimed that the inhibitor behave as hemin groups, which are able to generate thiyl radicals (GS•) by nucleophilic attack to reduced gluthation (GSH) or to β-mercaptoethanol. These thiyl radicals may irreversibly inhibit muscarinic acetylcholine receptor via a nucleophilic attack on muscarinic acetylcholine receptor sulphydryl groups. However, this effect is matter of controversy, because it has been shown that agents able to affect these groups at muscarinic acetylcholine receptor, as N-ethylmaleimide (NEM), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and low concentrations of *p*-chloromercuribenzoate (PCMB) are not able to affect significantly muscarinic acetylcholine receptor antagonist binding (7). Moreover, Creazzo and Hartzell (20) failed to augment the endogenous inhibitory effect using another agent similar to glutathion like dithiotreitol (DTT).

Similar kinetic effects as the endogenous inhibitory factor have been reported for Cu^{+1}, Zn^{+2} and Vanadium compounds (Metavanadate, Orthovanadate and Pervanadate) (11, 33). All of them decrease Bmax but do not alter Kd, being the vanadium compound's effect potentiated by glutathion (33). Also Zn^{++} and vanadate (VO_3^{-2}) potentiate the effect of an inhibitory endogenous factor studied by Creazzo and Hartzell (20) and Díaz-Arrastia y col (21), suggesting that the mechanism of inhibition include oxido-reduction chain reactions.

On the other hand, the inhibitory effect shown here was counter-

acted when an endogenous "excitatory" factor is also present in the extract. These fact analyzed together with the kinetic of the inhibitory endogenous factor suggests a binding site on muscarinic acetylcholine receptor where both putative factors are able to interact. However, the counteracting endogenous excitatory factor could be also part of the putative oxido-reduction chain but with only a limited activity, able to appear when the muscarinic acetylcholine receptor attains certain oxido-reduction level. This later aspect of the endogenous modulator could be related to the possibility of reversibility, which has been reported in extract from calf thymus (21). On the other hand, the biphasic profile could suggest heterogeneity not only in the soluble fraction but on the muscarinic receptors binding sites, which could be able to identify the inhibitory and "excitatory" soluble factors with different affinities.

Cholinergic transmission through muscarinic acetylcholine receptor has been involved in the modulation of higher brain functions like learning, memory and cognition (28). Loss of cholinergic synaptic connections may contribute to the pathology of cognitive disorders, being Alzheimer's disease the paradigm of them (34). Some of the neuropathological hallmarks of Alzheimer's disease include early and extensive degeneration of cortically projecting cholinergic neurons in the basal forebrain, decreased levels of brain choline acetyltransferase and reduced number of muscarinic acetylcholine receptor (35, 36). The muscarinic receptors of the M_1 subtype are relatively preserved but their affinities appear increased in the cortex of patients with Alzheimer's disease, whereas the presynaptic receptors, which are of the M_2 subtype are reduced in number, specially at the nucleus basalis of Meynert (35, 37-39). Also, it has been demonstrated that agonist-stimulated GTP binding and subsequent nucleotide hydrolysis is decreased, indicating a failure in muscarinic receptor-G protein coupling (40). The loss of muscarinic receptors has been connected to the neurodegeneration phenomena, which imply a reduced number of neuronal input making synapses at cholinergic neuron (41).

However, an alternative hypothesis related to muscarinic systems involves an endogenous brain factor (23, 24) and a serum circulating suppressing factor (26), both able to inhibit human brain muscarinic acetylcholine receptor antagonist binding. This endogenous brain factor would be expressed physiologically, since it has been found in supernatant fractions of non-demented individuals, but their concentration is three times higher in brains of patients with Alzheimer's disease (23, 24). It has been suggested that the endogenous brain factor is a free heme that could be involved in the generation of superoxide and thiyl radicals, by its interconversion between +2 and +3 oxidation states (30). The possible

identification of the endogenous brain factor as free heme, suggests that free heme compounds cause an oxidative stress in Alzheimer´s brain (30). However, it is unknown whether the concentration of free heme could increases as a result of a neurodegeneration process, or on the other hand, free heme could cause neurodegeneration by itself.

The possible existence of a counteracting endogenous excitatory factor suggest that, in physiological conditions, the action of the endogenous inhibitory factor should be balanced with the excitatory one. An unbalanced production or action of these putative substances would cause disturbance in muscarinic acetylcholine receptor function, traduced in cholinergic hiper or hipoexcitability. It has been reported that the inhibitory factor derived from brains of patients with Alzheimer's disease is more active than the one derived from brains of normal individuals (24). This greater inhibition could be the consequence of a reduced production of the excitatory factor and/or a higher production of the inhibitory one.

The absence of the excitatory effect could be a consequence of an increased activity of the inhibitory factor, since it would be necessary more concentration of the excitatory factor to counteract the action of the inhibitory factor. Indeed our extracts were of limited quantity, therefore, we were not able to discard definitively the presence of the excitatory factor in those brains in which its effect was not discernible. Also, the variability of endogenous soluble fractions' IC_{50} observed here could reflect the presence of different concentration of the excitatory factor in all the brains tested.

The existence of a counteracting endogenous excitatory factor is an exciting possibility that could offer therapeutic hopes in the treatment of disorders like Alzheimer's disease. It has been reported that loss of muscarinic acetylcholine receptor stimulation increases tau phosphorylation and increase amiloid deposition (references in 41), consequently an excitatory endogenous compound could enhance the function of the residual receptors, thus reverting these neurodegenerative triggering processes.

In conclusion, our results confirm the presence of a low molecular weight brain factor that is able to inhibit the antagonist binding to muscarinic acetylcholine receptor in a noncompetitive fashion. Additionally, we suggest an existence of an excitatory factor that would be able to counteract the effect produced by the inhibitory factor either recovering the muscarinic acetylcholine receptor [^{3}H]-QNB binding and/or changing the potency of the soluble factor.

Although the results of this and other reports are encouraging, the nature and real functional significance of endogenous modulators remain unclear. For this reason, further investigation is required, emphasizing on the mechanisms of action and possible role in cerebral physiological and pathological processes.

ACKNOWLEDGMENTS

This work was supported by grants Nº 02 42M 96 and 02 1M 97 from the Consejo de Desarrollo Científico Humanístico y Tecnológico (CDCHT) - Universidad Centroccidental "Lisandro Alvarado"

REFERENCES

1. DOHLMAN H.G., CARON MG, LEFKOWITZ RJ. A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry 1987; 26: 2658-2664.
2. STRADER C.D., FONG T.M., TOTA M.R., UNDERWOOD D. Structure and function of Protein G-coupled receptors. Annu Rev Biochem 1994; 63: 101-132.
3. BONNER T.I, BUCKLEY N.J., YOUNG A.C. The molecular basis of muscarinic receptor diversity. TiNS 1989; 12 (4): 148-151.
4. HULME E.C., BIRDSALL N.J.M., BUCKLEYN J. Muscarinic receptor subtypes. Ann Rev Pharmacol Toxicol 1990; 30: 633-673.
5. MITCHELSON F. Muscarinic Receptor Differentiation. Pharmacol Ther 1988; 37 (3): 357-423.
6. HULME, E.C., BIRDSALL, N.J.M., BURGEN, A.S.V., METHA, P. The binding of antagonist to brain muscarinic receptors. Mol Pharmacol 1978; 14: 737-750.
7. BIRDSALL, N.J.M., HULME E.C. The binding Properties of Muscarinic Receptors. In Joan Heller Ed. The muscarinic Receptors. Clifton, New Jersey, The Humana Press. 1989. p : 31.
8. BOYER J.L., MARTÍNEZ-CARCAMO M., MONROY-SÁNCHEZ J.A., POSADAS C., GARCÍA-SAINZ J.A. Guanine nucleotides-induced positive cooperativity in muscarinic cholinergic antagonist binding. Biochem Biophys Res Commun 1986; 134: 172-177.
9. LIPPO-DE-BÉCEMBERG I., PONTE-SUCRE A., ALFONZO M. Biochemical and pharmacological characterization of octylglucoside-solubilized muscarinic receptors derived from bovine tracheal smooth muscle. Arch Ven Farmacol Ter 1986; 5(4): 244-256.
10. MATTERA R., PITTS B.J., ENTMAN M.L., BIRNBAUER L. Guanine nucleotide regulation of a mammalian myocardial muscarinic receptor system. J Biol Chem 1985; 260: 7410-21.
11. MORENO-YANES J.A., DÍAZ L.E., QUINTERO C.C. Efecto regulador del cobre sobre la función muscarínica del cerebro humano. Arch Ven Farmacol Ter 1995; 14(1): 5-17.
12. MORENO-YANES J.A., PASCUZZO C., BONFANTE-CABARCAS R.A. Inducción de cooperatividad homotrópica positiva en la unión de antagonistas al receptor colinérgico

muscarínico de corteza frontal humana. Ciencia 1995; 3(3): 207-228.
13. WREGGETT K., WELLS J. Co-operativity manifest in the binding of purified cardiac muscarinic receptors. J Biol Chem 1995; 270: 22488-22499.
14. CLARK A.L., MITCHELSON F. The inhibitory effects of gallamine on muscarinic receptors. Br J Pharmacol 1976; 58: 323-331.
15. DUNLAP J., BROWN J.H. Heterogeneity of bindig sites on cardiac muscarinic receptors induced by the neuromuscular blocking agents gallamine and pancuronium. Mol Pharmacol 1983; 24: 15-22.
16. HENIS Y.I., KLOOG Y, SOKOLOVSKY M. Allosteric interactions of muscarinic receptors and their regulation by other membrane proteins In Joan Heller Ed. The muscarinic Receptors. Clifton, New Jersey, The Humana Press. 1989. p 337
17. LEE N., H., EL-FAKAHANY E.E. Allosteric antagonists of the muscarinic acetylcholine receptor. Biochem Pharmacol 1991; 42 (2): 199-205.
18. TUCEK S, PROSKA J. Allosteric modulation of muscarinic acethylcholine receptors. TiPS 1995; 16: 205-212
19. ACTON G., DAILEY J.W., MORRIS S., McNATT L. Evidence for an endogenous factor interfering with antagonist binding at the muscarinic cholinergic receptor. European J Pharmacol 1979; 59: 343-344.
20. CREAZZO T.L., HARTZELL, H.C. Reduction of Muscarinic Acetylcholine Receptor Number and Affinity by an Endogenous Substance. J Neurochem 1985; 45 (3): 710-718.
21. DIAZ-ARRASTIA R, ASHIZAWA T, APPEL S. Endogenous inhibitor of ligand binding to the muscarinic acetylcholine receptor. J Neurochem 1985; 44 (2): 622-628.
22. FANG Y-I., IIJIMA M, OGAWA M, SUSUKI T, MOMOSE, K. Reduction in the numbers of muscarinic receptors by an endogenous protein. Biochem Pharmacol 1993; 46 (4): 637-641.
23. FREY W.H, EMORY C.R., WIEBENGA M.E., SAXENA S., CARDELLI D, ALA T, TOLLEFSON G. Inhibitor of antagonist binding to the muscarinic receptor is elevated in Alzheimer's brain. Brain Res 1994; 655: 153-160.
24. FREY WH 2ND, NAJARIAN M.M., KUMAR K.S., EMORY C.R., MENNING P.M., FRANK J.C., JOHMSON M.N., LA T.A. Endogenous Alzheimer's brain factor and oxidized glutathione inhibit antagonist binding to the muscarinic receptor. Brain Res 1996; 714: 87-94.
25. FRYER, A.D., EL-FAKAHANY E.E. An endogenous factor induces heterogeneity of binding sites of selective muscarinic re-

ceptor antagonists in rat heart. Membrane Biochem. 1989; 8: 127-132.
26. KITANO S., MORIMOTO S., FUKUO K., MIYA K., YASUDA O., KOH E., HIROTANI A., NAKAHASHI T., TAMATANI M., OGIHARA T. Circulating suppressing factor for the muscarinic acetylcholine receptor in patients with senile dementia of the Alzheimer type. Gerontology 1992; 38: 24-28.
27. van KOPPEN, C.J., SOKOLOVSKY, M. Evidence for an endogenous factor involved in maintenence of pirenzepine high-affinity binding in rat brain stem. Biochem Biophys Res Commun 1998; 157 (1): 42-47.
28. FIBIGER, H.C. Cholinergic mechanism in learning, memory and dementia: a review of recent evidence. Trends Neurosci 1991; 14: 220-223.
29. DUNNETT, S. Cholinergic grafts, memory and aging. Trends Neurosci 1991; 14: 371-376.
30. VENTERS HD JR, BONILLA LE, JENSEN T, GARNER HP, BORDAYO EZ, NAJARIAN MM, ALA T.A., MASON R.P., FREY W.H. 2ND. Heme from Alzheimer's brain inhibits muscarinic receptor binding via thiyl radical generation. Brain Res 1997; 764: 93-100
31. SMITH P.K., KROHN R.I., HERMANSON G.T., MALLIA A.K., GARTNER F.H., PROVENZANO M.D., FUJIMOTO E.K., GOEKE N.M., OLSON B.J., KLENK D.C. Measurement of protein using Bicinchoninic Acid. Anal Biochem 1985; 150: 76-85.
32. HILL A.V. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J Physiol 1910; 40: 190-197.
33. VENTERS H JR, ALA T.A., FREY W.H. 2ND . Inhibition of antagonist binding to human brain muscarinic receptor by vanadium compounds. Recept Signal Transduct. 1997; 7: 137-142.
34. LEVEY, Al. Muscarinic acetylcholine receptor expression in memory circuits: implications for treatment of Alzheimer disease. Proc Natl Acad Sci USA 1996; 93(24): 13541-13546.
35. AVERY, E.E, BAKER, L.D., ASTHANA, S. Potential role of muscarinic agonists in Alzheimer's disease. Drugs Aging 1997; 11(6): 450-459.
36. CAULFIELD M.P., STRAUGHAND W., CROSS A.J., CROW T., BIRDSALL N.J.M. Cortical muscarinic receptor subtypes and Alzheimer's Disease. Lancet 1982; ii: 1277.
37. NORDBERG A., ALFUFOFF Y, WINBLAD B. Nicotinic and muscarinic subtypes in the human brain: Changes with aging and dementia. J Neurosci Res 1992; 31: 103-111.
38. NORDBERG A., WINBLAD B. Reduced number of [^{3}H]nicotine and [^{3}H]acetylcholine binding sites in the frontal cortex of

Alzheimer brains. Neurosci Lett 1986; 72: 115-119.
39. SVENSSON AL, ALAFUZOFF I., NORBERG A. Characterization of muscarinic receptor subtypes in Alzheimer and control brains cortices by selective muscarinic antagonists. Brain Res 1992; 596: 142-148.
40. FERRARI-DI LEO, G, MASH DC., FLYNN, DD. Attenuation of muscarinic-G-protein interaction in Alzheimer disease. Mol Chem Neuropathol 1995; 24: 69-91.
41. SELKOE, D.J., Biochemistry of Alzheimer's Disease. In Siegel, G.J., Agranoff, B.W., Albers, R.W., Molinoff, P.B. Eds. Basic Neurochemistry. Fifth edition, New York, Raven Press p: 919-933.