MUSCARINIC RECEPTORS IN THE RAT CEREBELLUM: CHARACTERISTICS AND METHODOLOGY

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1. Introduction

The study of muscarinic receptors has been greatly improved by the introduction of some alkylating derivatives of benzilylcholine [1–3] and also quinuclidinyl benzilate (QNB) [4,5]. QNB has been widely used to study the properties of these receptors in a wide variety of tissues [6–10] including central nervous system (for review see ref. 11).

In the course of a more general study on muscarinic receptors during synaptogenesis in the rat cerebellum, we observed that the measured number of binding sites for QNB is, to a large extent, dependent on the methodology used for the tests. In this paper we report a comparison of different methods used for this purpose and the kinetic properties of QNB binding to rat cerebellar homogenates.

2. Materials and methods

Adults or 20-day-old Wistar albino rats were decapitated after stunning, cerebella were removed and homogenized in 10 vol of ice-cold 50 mM Na-phosphate, (pH 7.4) buffer in a Potter-Elvehjem glass homogenizer with a teflon pestle. Other buffers compared with this are listed in fig.4a. Protein determination on aliquots of homogenates was performed according to Lowry et al. [12]. Specific QNB-binding was measured by the competition between [^{3}H]QNB (16 Ci/mmole; Radiochemical Center, Amersham) and non-radioactive QNB (synthesized in our laboratory according to the method of Whitaker [13]) or atropine (a gift of Juste S. A., Madrid.) and buscapine (a gift of Boehringer Sohn Ingelheim, Barcelona.), specific muscarinic antagonists. The following filtration technique modified from Yamamura and Snyder [6] was used: 25–100 μl of whole cerebellar homogenate were pipetted into 1.5 ml plastic centrifuge tubes, a buffer solution containing [^{3}H]QNB was added and the samples were incubated for 1 h at 37°C with continuous agitation. In each experiment, different concentrations of [^{3}H]QNB ranging from 0.1 to 10 mM were used (fig.1). Six samples were used for each [^{3}H]QNB concentration, three of which contained also a 100-fold excess of unlabelled QNB. At the end of incubation, the samples were placed in ice and then filtered, in a Millipore sintered glass filtration apparatus, through HAWP filters (2.5 cm diameter, 0.45 μm pore size from Millipore, S. A. Molsheim, France). Before use, each filter was preincubated in the buffer for 120 min at +4°C. The material retained on the filter was then washed three times by adding 3 ml, each time, of ice-cold buffer. Comparative experiments in the full range of ^{3}HQNB concentration were carried out with other filters (GA-6 filters, 0.45 μm pore size from Gelman, Ann Arbor, MI.; and GF/F glass fiber filter, from Whatman Ltd., Springfield, Kent) of the same diameter.

In another series of experiments samples were incubated only at a single, saturating [^{3}H]QNB concentration (4 nM). After incubation and in parallel with the filtration method, aliquots of the same homogenate were processed by two other methods. In one procedure, samples were centrifuged (Sorvall

RC-2B) at +4°C for 60 min at 20 000 × *g* and washed with 1.2 ml of ice-cold buffer twice. The tips of each plastic centrifuge tube containing the pellets were cut off and put into a scintillation vial. In the other procedure (equilibrium dialysis) larger samples (200 μl homogenate) were used. 2 ml sample aliquots were dialysed overnight at 4°C in Visking tubes (8 mm width) against 20 ml buffer. Samples containing 100-fold excess of unlabelled QNB were dialysed in parallel.

2.1. *Radioactivity determination*

Millipore and Gelman filters were dissolved in 1 ml dioxane for 1 h at room temperature in scintillation vials. In all other cases the dioxane treatment was omitted. 2 ml of a 5% (w : v) solution of Triton X-100 (scintillation grade, Serva, Heidelberg) in water were added to each vial which was left overnight at room temperature to solubilize proteins. In the equilibrium dialysis method, Triton X-100 (5% final concentration) was added only to the retentate.

In all cases 10 ml Scintigel (Roth, Karlsruhe) were added, the radioactivity was counted in an Intertechnique SL 30 (Plaisir) scintillation spectrometer. Counts were corrected for efficiency and quenching using the method of external standard ratio.

3. Results

3.1. *Kinetic study of the QNB binding*

As shown in fig.1a, the specific binding of QNB is a saturable process (saturation around 3 mM QNB concentration in the medium), while the non-specific binding is linear and characteristic of a non-saturable process. In a 20-day-old rat cerebellum, the number of binding sites calculated from the Scatchard [14] plot was 312×10^{-15} mol QNB/mg protein (fig.1b). The dissociation constant K_d was found to be 5.4×10^{-10} M and only one class of binding sites was detected.

The value of the Hill coefficient [15] calculated from these data was: $n_h = 0.83$. This indicated clearly that no cooperativity occurs. The rate constant for dissociation K_{-1} was determined in separate experiments and found to be 1.25×10^{-2} min^{-1} which is very close to the results of Yamamura and Snyder [6].

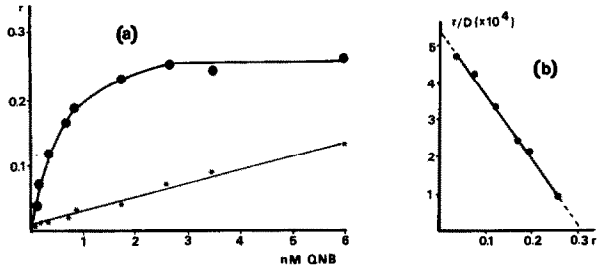

Fig.1. [³H]QNB binding to cerebellar homogenate from 20-day-old rats. Assay carried out (on 25 μl samples in Na-phosphate buffer) by filtration through HAWP Millipore filters. (a) direct plot. Ordinates: *r* = pmol of bound [³H]QNB/mg protein. (•) specific binding; (*) non-specific binding. (b) Scatchard plot of the same experiment. Abscissa: *r* = pmol of specifically bound [³H]QNB/mg protein. Ordinate: *r/D* = ratio of bound to free [³H]QNB.

3.2. *Action of antagonists on the specific binding of QNB*

Figure 2 shows the displacement of the specific binding of radioactive QNB elicited by non-radioactive QNB, atropine and buscapine. 50% inhibition of the specific binding of 1 nM [³H]QNB is achieved at concentrations of 1 nM of non-radioactive QNB, 7.94 nM atropine and 66.1 nM buscapine. The shape of the inhibition curve is characteristic of competitive antagonism, indicating that all binding sites are due to muscarinic receptors.

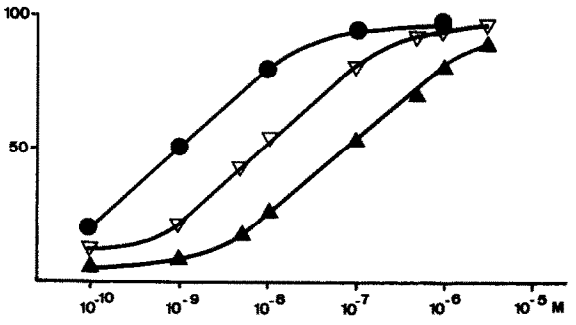

Fig.2. Effect of antagonists on the specific [³H]QNB binding to adult rat cerebellar homogenate. Incubation was in the presence of various concentrations of antagonists, while [³H]QNB was at a constant concentration (1 nM). Other assay conditions as in fig.1. Abscissa: molar concentration of antagonists. Ordinate: percent inhibition of the specific [³H]QNB binding. (•) non radioactive QNB, (▽) atropine, (▲) buscapine.

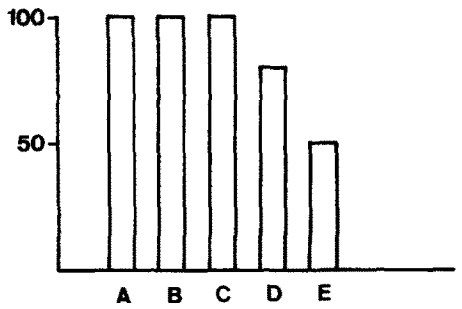

Fig.3. Influence of the assay method on the amount of [^{3}H]QNB specifically bound to adult rat cerebellar homogenates. (A) filtration through Millipore HAWP, (B) centrifugation method, (C) equilibrium dialysis (D) filtration through Whatman GF/F (E) filtration through Gelman GA-6. Ordinate: specific binding as % of the binding measured by filtration through Millipore HAWP. 50 mM sodium phosphate buffer (pH 7.4) was used in all cases.

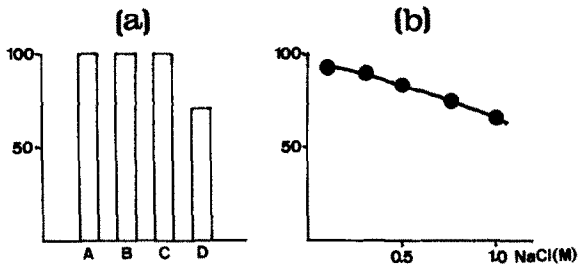

Fig.4. Influence of (a) different buffers and (b) different concentrations of NaCl added to the Na-phosphate buffer, on the specific [^{3}H]QNB binding to cerebellar homogenates. (A) Na-phosphate (B) Na,K-phosphate, (C) Na-acetate, (D) Tris–HCl; all 50 mM (pH 7.4). All other experimental conditions were as in fig.1. Ordinates: specific binding as % of the binding measured in the presence of Na-phosphate buffer.

3.3. *Efficiency of the different methods*

The amount of QNB specifically bound to adult rat cerebellar homogenate, measured by centrifugation, or equilibrium dialysis or by filtration on Millipore HAWP was 25 pmol QNB/g wet weight. This maximal amount was considered as 100%. As shown in fig.3, the pore size of the filter used is important for quantitative estimation of QNB binding, since with GF/F Whatman filters, which do not retain particles smaller than 0.7 μm in diameter, the amount of specific QNB binding is lower (20 pmol/g cerebellum). The type of filter is also important since although both Millipore HAWP and Gelman GA-6 filter have the same pore size, the binding found by using the latter (which is made of cellulose acetate) is only half of that found with the former (which is made of a mixture of cellulose esters). In addition, control experiments without homogenate showed that adsorption of QNB to any of the filters used can be neglected, since it accounts for only 1–2% of the radioactivity bound to the homogenate and thus is smaller than the experimental variation.

Ions and ionic strength can also modify the amount of measured QNB binding. As shown in fig.4a, three of the buffers used had no effect, but Tris–HCl decreased the amount of binding by 30%. Similarly, increasing concentrations of NaCl in the sodium phosphate buffer up to 1 M decreased proportionally the specific binding of QNB (fig.4b). This effect is probably not due to solubilization of muscarinic receptor by NaCl, and hence loss by filtration since, although this solubilization occurs in 2 M NaCl [16–18], 1 M NaCl has either [16] no or small [17] solubilization effect even after a 12 h treatment.

4. Discussion

Our results show that, regardless of the method used and thus of the quantitative results obtained, the measured QNB binding sites have the same characteristics of kinetics and antagonist inhibition. Furthermore, these characteristics are the same as those reported in the literature [6,9,19]. The quantitative results show that filtration on the appropriate filter gives the same results as more time-consuming methods such as centrifugation and equilibrium dialysis and thus is the method of choice. The identity of the results obtained by equilibrium dialysis and the other two methods shows also that all QNB binding sites are membrane bound.

The amount of muscarinic receptor found in cerebella by us, is much higher than that reported by Yamamura and Snyder [6] for cerebellar cortex and by Laduron et al. [20] for whole cerebella. Although Yamamura and Snyder [6] have used only cerebellar cortex, and thus discarded the cholinoceptive cerebellar deep nuclei neurons, we feel that the major reason for these differences is that the Whatman GF/B filters used by these authors do not retain particles

with a diameter smaller than 1 μm, and thus many membranes, including those containing muscarinic receptors, passed through the filter. This indicates that for correct quantitative results with the filtration method, the choice of the filter is crucial.

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