THE MUSCARINIC RECEPTOR OF HEART CELL MEMBRANES

Association with agonists, antagonists and antiarrhythmic agents

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1. Introduction

The adult heart is innervated by the sympathetic and parasympathetic branches of the autonomic nervous system (review [1]). The pharmacological properties of the acetylcholine receptor in the heart are those of a receptor of the muscarinic type [2]. A large body of evidence suggests that acetylcholine shortens the action potential duration of atrial myocardium by increasing the potassium permeability of the membrane [3]. The shortening of the plateau by acetylcholine diminishes calcium influx during the action potential and this has been held responsible for the negative inotropic effect of the neurotransmitter [4].

The availability of receptor specific ligands radiolabelled to a high specific activity makes it now possible to measure directly the association properties of agonists and antagonists to neurotransmitter receptors. 3-Quinuclidinyl benzilate (QNB), a potent muscarinic antagonist in the central [5] and peripheral nervous system [6] has already been shown to bind to guinea pig ileum [7] and rat brain [8] in a very specific way. This paper makes use of [^{3}H] QNB to analyse the molecular properties of the heart muscarinic cholinergic receptor in its interaction with agonists and antagonists.

2. Materials and methods

Heart membranes were prepared according to [9]. The final pellets were kept frozen at −15°C and resuspended in 50 mM Tris at pH 7.4 before use. Binding experiments were carried out as described [8] for membrane fractions isolated from the central nervous system. [^{3}H]QNB (8.4 Ci/mmol) was purchased from Amersham Searle. *Naja mossambica mossambica* neurotoxin I was prepared in this laboratory as described [10]. Protein was determined by the method [11] using bovine serum albumin as a standard.

3. Results

Figure 1 shows that binding of [^{3}H]QNB to heart membranes is a saturable process. Half-maximal saturation ($K_{0.5}$) occurs at 0.12 nM [^{3}H]QNB. The maximal binding capacity is 190 fmol QNB specifically associated/mg whole-heart proteins. The Hill coefficient measured for this binding is 1.0 and indicates no cooperativity. The simplest mechanism of association of QNB to its receptor (R) is therefore to be written:

$$R + QNB \underset{k_d}{\overset{k_a}{\rightleftharpoons}} R - QNB$$

Rates of association (k_a) and dissociation (k_d) were measured at different temperatures. The association process follows bimolecular kinetics (fig.2A). The dissociation process was followed by first incubating heart membranes with [^{3}H]QNB at 25°C for 60 min and then incubating with 10 μM atropine to displace the radioactive label. A parallel evaluation of the rate of receptor degradation was carried out by

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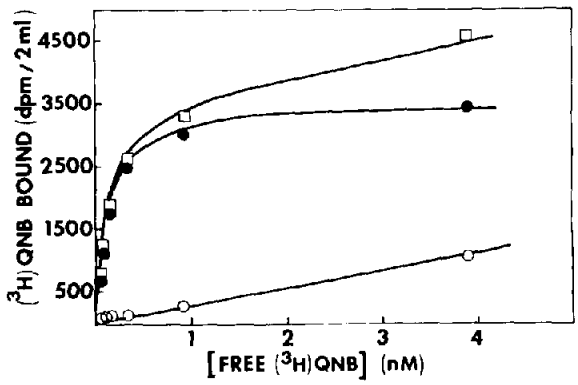

Fig.1. Binding of [^{3}H]QNB to rat heart membranes. Increasing amounts of [^{3}H]QNB (from 4000–250 000 cpm) were incubated 60 min at 25°C with heart membranes (0.25 mg protein/ml) in 2 ml 50 mM phosphate buffer at pH 7.4. The experiment was first carried out in the absence of atropine to measure the total binding capacity (□), then in the presence of 10 μM atropine to measure the non-specific binding not displaceable by the antagonist (○), the specific binding being measured by the difference between the two curves (●). Bound radioactivity was separated from free radioactivity by filtration on GF/B Whatman filters. The filters were placed in vials containing 8 ml picofluor (Packard) and the radioactivity was then measured by liquid scintillation spectrometry (Packard Tri-carb model B 2450) at a counting efficiency of 40–45%.

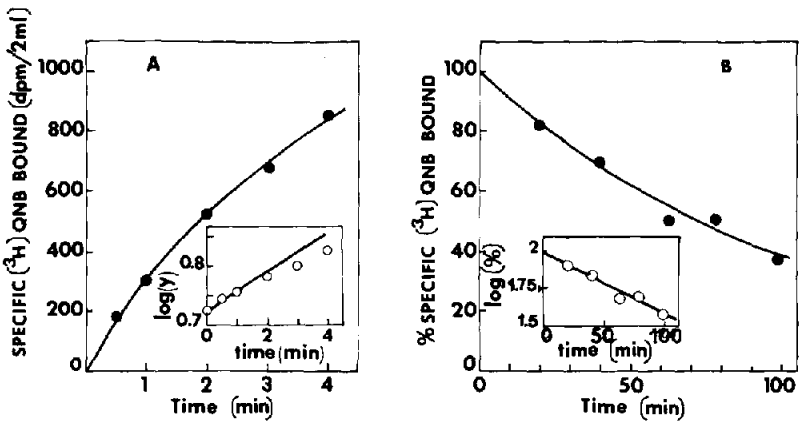

Fig.2. Time course of the [^{3}H]QNB association with rat heart membranes (A) and of [^{3}H]QNB dissociation from the QNB–muscarinic receptor complex (B) at 35°C, pH 7.4.

(A) Specific [^{3}H]QNB binding was measured as described in the legend of Fig.1. Inset: evaluation of the bimolecular rate constant for the QNB–receptor association. The equation of the curve is:

$$\log\left(\frac{[QNB]_0 - x}{[R_0 - x}\right) = \frac{[QNB]_0 - [R_0]}{2.3} k_a t + \log\frac{[QNB]_0}{[R_0]}$$

where $[QNB]_0$ is the concentration of [^{3}H]QNB added at $t = 0$; $[R_0]$, the receptor concentration present in the tissue and x the concentration of specifically-bound [^{3}H]QNB.

$$y = \frac{[QNB]_0 - x}{[R_0] - x}$$

(B) [^{3}H]QNB is first associated with the rat heart membrane at 25°C, pH 7.4 during 60 min. The displacement of the specifically bound [^{3}H]QNB is started with 10 μM atropine. Aliquots are taken at different times to measure the amount of [^{3}H]QNB which remains bound to the membrane. Inset: first-order representation of the [^{3}H]QNB dissociation induced by atropine. log% means log% of specifically-bound [^{3}H]QNB.

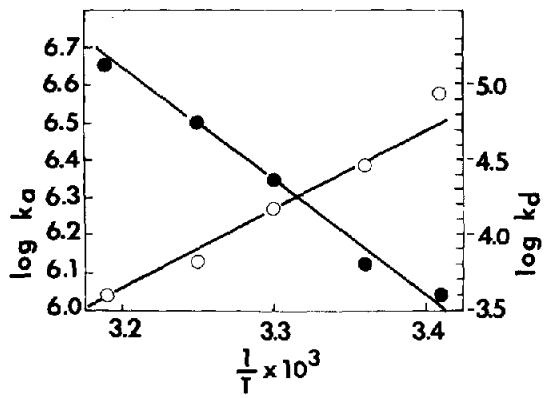

Fig.3. Arrhenius plots of the rate constant of association k_a (•), and of dissociation, k_d (○), corresponding to the formation of the [^{3}H]QNB–receptor complex.

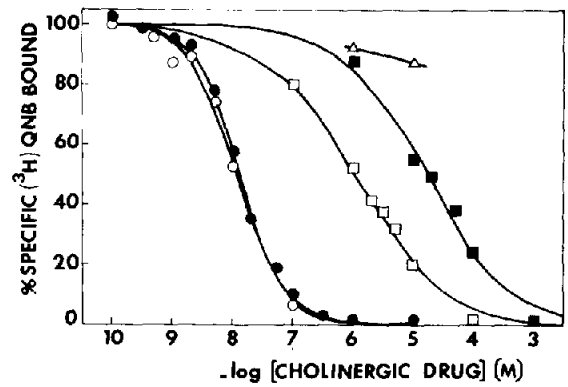

Fig.4. Displacement of specific [^{3}H]QNB binding by various concentrations of scopolamine (○–○), atropine (•–•), acetylcholine (□–□), carbamylcholine (■–■) and *Naja mossambica mossambica* neurotoxin I (Δ–Δ). [^{3}H]QNB (0.6 nM) was incubated 60 min at 25°C with rat heart membranes (0.2 mg protein/ml) in 2 ml 50 mM phosphate buffer at pH 7.4 in the presence of the indicated concentrations of cholinergic drugs. In the particular case of acetylcholine, 10 μM eserine had to be added to the medium to inhibit acetylcholinesterase activity and thus prevent acetylcholine hydrolysis.

looking at the amount of [^{3}H]QNB which remains bound to the membrane in the absence of atropine. No degradation occurred at 20°C. Degradation occurred at higher temperatures: it was 15% initial receptor concentration after 5 h at 25°C, 25% after 150 min at 30°C, 35% after 100 min at 35°C and 50% after 50 min at 40°C. After adequate corrections for receptor degradation, the displacement of [^{3}H]QNB by atropine can be represented as in fig.2B. As expected this displacement follows first order kinetics.

Values of k_a, the second order rate constant of association, and k_d, the first order rate constant of dissociation, are strongly temperature-dependent. Arrhenius plots shown in fig.3 give enthalpies of activation for both processes. ΔH_a^* 13.8 kcal. mol^{-1} and ΔH_d^* 24.3 kcal. mol^{-1}. The enthalpy of binding of QNB to its receptor is $\Delta H^\circ = \Delta H_a^* - \Delta H_d^* =$ −10 kcal. mol^{-1}. At 25°C, ΔG° − 14.5 kcal. mol^{-1} and ΔS° + 13.5 entropy units.

Similarly to atropine, a variety of pharmacologically related muscarinic drugs have the ability to displace [^{3}H]QNB from heart membranes (fig.4). Muscarinic agonists are considerably less potent to displace [^{3}H]QNB than antagonists (fig.4 and table 1). Muscarinic agonists like pilocarpine, acetylcholine, carbamylcholine, or muscarinic antagonists like QNB, scopolamine and atropine show a much better affinity for the acetylcholine receptor of the heart membrane than nicotinic agents such as nicotine, dimethylphenylpiperazinium, phenyltrimethylammonium, decamethonium, *d*-tubocurarine or mecamylamine or the *Naja mossambica mossambica* neurotoxin I (table 1).

Antiarrhythmic drugs like verapamil and D600 were also assayed for their ability to displace [^{3}H]QNB from its receptor site. Verapamil and D600 displaced specific [^{3}H]QNB binding with ED_{50} of 19 μM and 24 μM, respectively.

4. Discussion

The binding characteristics of [^{3}H]QNB to heart membranes ($K_{0.5}$ values, and rate constants k_a and k_d) are very similar to those found for the muscarinic receptor in rat brain or in guinea-pig ileum [7,8]. The apparent dissociation constant of the acetylcholine–receptor complex is of the order of 1 μM and the best antagonist is QNB itself with a dissociation constant of the receptor–QNB complex as low as 0.1 nM. One interesting difference between antagonists and agonists resides in the fact that antagonists bind to the muscarinic receptor with a Hill coefficient of 1, i.e., without any indication of cooperativity, whereas

Table 1
Relative potencies of cholinergic drugs and antiarrhythmic agents in displacing [^{3}H]QNB binding from rat heart membranes

	ED_{50} (nM)	n_H	$K_{0.5}$ (nM)
Muscarinic agonists			
pilocarpine	12,000	0.58	–
acetylcholine	1250	0.50	–
carbamylcholine	20,000	0.56	–
Muscarinic antagonists			
QNB	–	1.0	0.12
scopolamine	11.0	1.0	1.4
atropine	13.0	1.0	2.2
Nicotinic agonists			
nicotine	(a)	–	–
dimethylphenyl-piperazinium	(a)	–	–
phenyltrimethyl-ammonium	(a)	–	–
Nicotinic antagonists			
decamethonium	10,000	–	–
d-tubocurarine	10,000	–	–
mecamylamine	(b)	–	–
Naja mossambica mossambica neurotoxin I	(a)	–	–
Antiarrhythmic drugs			
verapamil	18,600	0.70	–
D600	23,200	0.81	–

[a] 10–20% displacement at 10 μM
[b] No displacement at 10 μM

ED_{50} is the concentration of unlabelled drug which induces half-displacement of specifically-bound [^{3}H]QNB. $K_{0.5}$ is the apparent dissociation constant of the complex formed between heart membranes and the unlabelled drug. When the Hill number is 1.0, $K_{0.5}$ is calculated from the ED_{50} value by the formula:

$$ED_{50} = K_{0.5}\left(1 + \frac{[QNB]}{K_{QNB}}\right),$$

where [QNB] is the concentration of free [^{3}H]QNB at half dissociation and K_{QNB} is the dissociation constant of the QNB–heart membranes complex

agonists display a marked negative cooperativity of binding (table 1) with Hill coefficients of 0.5–0.6.

The dissociation constant measured for QNB binding from kinetic data ($K_{0.5} = k_d/k_a$) is of 0.03 nM at 25°C, very similar to the constant measured through direct binding experiments (fig.1). The rate constant k_a for the association of QNB to its receptor is 1.1×10^6 M^{-1} s^{-1} at 20°C. This rate constant is several orders of magnitude lower than what would be expected for a diffusion controlled process (10^8–10^9 M^{-1} s^{-1}). Therefore it indicates that binding of QNB to the muscarinic receptor involves a conformational rearrangement of the complex [12]. Such an interpretation is confirmed by the fairly large change of entropy (ΔS° = + 13.5 entropy unit) involved in the binding process.

Drugs like verapamil and D600 are well known in antiarrhythmic therapy. Their primary effect is generally believed to be due to a direct interaction of the molecule with the Ca^{2+} entry systems of the cardiac cell membrane [13–15]. It is clear from the results shown in table 1 that drugs like D600 or verapamil bind fairly tightly to the muscarinic receptor (ED_{50} 20–40 μM) and that part of their clinical action may be due to this interaction.

Acknowledgements

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