Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 23, pp. 2801-2806, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00429-5

PROTEIN-LIGAND INTERACTIONS. 6 NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST ACTIVITY OF ISOQUINOLINE ALKALOIDS

C.G. Whiteley* and S. Daya

Department of Biochemistry and Microbiology,
Rhodes University, Grahamstown, South Africa 6140

Abstract. Fluorimetry and spectrophotometry have been used to study the binding of several isoquinoline alkaloids to nicotinic acetylcholine receptor, isolated and purified from *Torpedo fuscomaculata*. Analysis indicates that the ligands are true agonists of the receptor exhibiting positive cooperative binding with more than one class of binding sites. Copyright © 1996 Elsevier Science Ltd

The nicotinic acetylcholine receptor (nAChR) is a ligand gated ion channel protein[1,2]. It contains, in its protein moiety, binding sites for acetylcholine and its agonists and antagonists (receptor function), the calcium channel (response function), and several types of molecular sites (molecular function). The elucidation of its molecular mechanism of function, therefore, requires an intimate understanding of its ligand binding properties.

With the exception of polypeptide neurotoxins all the ligands of acetylcholinesterase (AChE) may also be ligands of the receptor. The ligands of AChE can be subdivided into substrates and competitive inhibitors, while those of the receptor into agonists and antagonists (competitive blockers). It is of interest to investigate the character of protein-ligand interactions in order to reveal if conformational changes that accompany receptor function can be transmitted to a neighbouring subunit(s). The major difficulty that has limited progress in the study of protein-ligand, subunit-ligand, and subunit-subunit interactions is the very nature of these interactions themselves. In many cases the native oligomeric structure of the receptor protein is not easily dissociated, thus making it almost impossible to investigate the monomeric subunit of the receptor. More drastic conditions utilising protein denaturation is unsatisfactory in that extensive subunit unfolding and loss of receptor activity also occur.

In order to answer the questions of the effect of agonist/antagonist on the receptor function it is of interest to investigate the character of subunit-ligand or protein-ligand interactions. The purpose of the present work is to use a ligand with one or more features present that mimic the key parameters of receptor/agonist/antagonist function. Such a molecule must (a) fit the active site of the receptor; (b) have a rigid and fixed conformation; (c) possess a quaternary nitrogen to simulate the natural substrate (acetylcholine); and (d) possess additional functional groups to act as electron donors for specific amino acids on the receptor protein.

As a continuing study on the interaction of biologically active compounds on biochemical processes currently being undertaken in these laboratories[3] we envisaged certain natural products may fulfill our requirements for suitable organic molecules. Indeed, the isoquinoline alkaloids, berberine and papaverine that

exhibit a broad spectrum of biological activity, used as their hydrochloride salts, possess all of the necessary features.

A detailed description of the interaction of these molecules with the receptor might provide insights not only into the molecular mechanism of junctional excitation and permeability change, but also into the principles on which more complex neural functions are based.

With this in mind, and prompted by the availability of highly purified solubilised receptor[4] in a form suitable for biochemical studies, we have undertaken an investigation aimed at both the kinetics of interaction and binding of nicotinic acetylcholine receptor with berberine, papaverine, and isoquinoline (Figure 1).

It should be realised that the analysis reflects changes in optical properties due to the nature of the subunit-ligand binding. Also binding and kinetic studies critically depend on the specificity of the ligands applied and the observed changes in fluorescence.

Fluorescent probes have been valuable tools in the studies involving subunit cooperativity[5], hydrophobic binding sites[6] and the distance between two types of binding sites.[7]

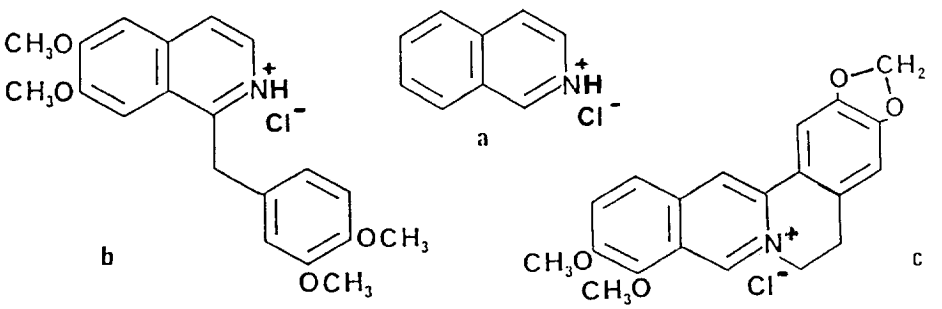

Figure 1 (a) Isoquinoline (b) Papaverine (c) Berberine

Results

Spectrophotometry[8] The binding of the isoquinoline alkaloids to nAChR was also monitored by changes in the ultraviolet absorbance of the protein. The difference in protein absorbance upon binding is sufficiently large to allow evaluation of the titration data. The signal change, measured as the difference between the maxima at a wavelength (230 nm) is considered to be directly proportional to the concentration of alkaloid - nAChR complex and is therefore a reliable parameter to follow such an interaction. The saturation binding curves and the Scatchard plots (Figure 2) for the alkaloids indicated the existence of more than one class of binding site at the receptor. The Kd and B_{max} (Binding Capacity) for both high and low affinity binding sites can be calculated and are all represented (Table 1). As observed the receptor exhibits a high-affinity low capacity, as well as a low affinity high capacity binding sites.

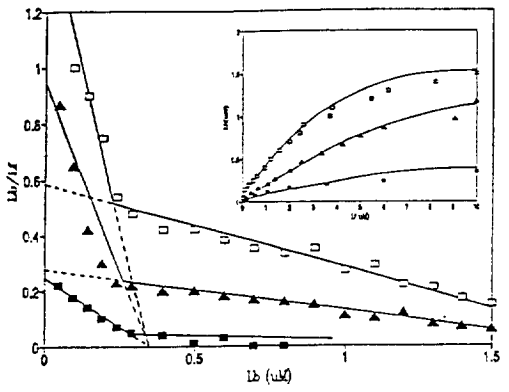

Figure 2 Saturation binding curves (inset) and Scatchard plots for the binding of isoquinoline (■), papaverine (▲) and berberine (□) to nicotinic acetylcholine receptor. L_b is the number of moles of bound alkaloid and L_f is the concentration of free alkaloid.

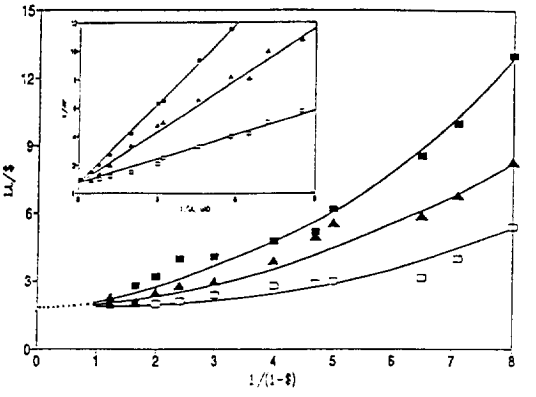

Figure 3 Analysis data and double reciprocal plots of fluorescence change (inset) for the binding of isoquinoline (■), papaverine (▲) and berberine (□) to nicotinic acetylcholine receptor in 100 mM phosphate buffer, pH 7.2.

Fluorimetric Studies[9] Typical Benesi-Hildebrand and analysis plots of 1/(1 - S) versus (Ligand)/$ are shown (Figure 3) for the effect of the alkaloids on nicotinic acetylcholine receptor. The analysis plots were not linear indicating the existence of more than one class of binding site at the receptor. Both acetylcholine and d-tubocurarine also exhibit positive cooperativity and bind to the receptor with two affinities (Table 1). For all of the isoquinoline alkaloids there are two equilibrium dissociation constants - one representing a weak association and the other a more stronger representation - in addition to positive co-operativity. All of these results are consistent with the interpretation that the changes of fluorescence intensity observed upon addition of acetylcholine to receptor-ligand complex are caused by the physical displacement of the alkaloid from the binding site common for acetylcholine.

Figures 4 and 5 show back titrations of tubocurarine fluorescence by alkaloid ligands when the sites are saturated with acetylcholine. The slope of the lines (Figure 4) approximates 2 indicating that the competing ligand bind to a heterogeneous class of dependent sites in causing tubocurarine dissociation.

	Spectrophotometry				Fluorimetry		
Ligand	Kd_1 (μM)	B_{max} (μM)	Kd_2 (μM)	B_{max} (μM)	Kd_1 (μM)	p	Kd_2 (μM)
Tubocurarine	0.03	-	8	-	0.05	1.79	11.4
Acetylcholine	0.18	-	3	-	0.12	2.01	2.3
Isoquinoline	3.3	1.86	49	0.31	3.1	2.22	51.6
Papaverine	2.4	2.07	31	0.27	1.7	1.96	29
Berberine	0.84	2.14	27	0.31	0.77	2.01	34

Table 1: Binding constants obtained by fluorimetry and spectrophotometry of isoquinoline alkaloids on nicotinic acetylcholine receptor. p are the number of binding sites.

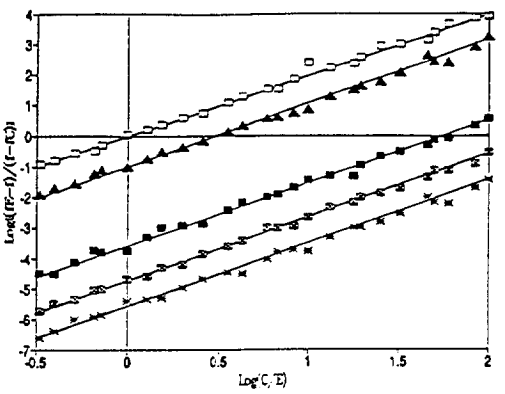

Figure 4 Hill plots of the competitive dissociation of tubocurarine from nicotinic choline receptor membrane in the presence of acetylcholine by isoquinoline (*), papaverine (⊠) and berberine (■). Tubocurarine (□) and acetylcholine (▲) are also represented as standards.

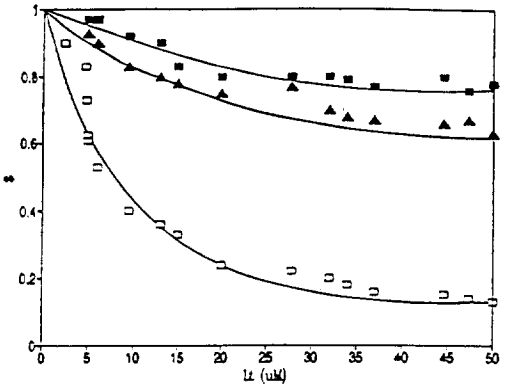

Figure 5 Dissociation of tubocurarine-nicotinic acetylcholine receptor complex in the presence of acetylcholine by isoquinoline (■), papaverine (▲) and berberine (□).

Discussion

Our studies have shown that various isoquinoline alkaloids interact with the agonists binding sites of nicotinic acetylcholine receptor. The potency of a ligand in the interaction with the receptor is determined by the Kd for the receptor-ligand complex. The fit into the binding sites of the protein is largely determined by the size, structure and configuration of the ligand. The capability of such a ligand to bind non-covalently at, or close to, the active site could also influence these values.

Two fundamental properties of the interaction of the alkaloids to the receptor agonist active site are implied. First, there is at least one protonated residue which combines with the ligand within the receptor-complex. Second, the receptor binding site must have a hydrophobic nature. Thus, when the ligands bind to the receptor they pass from a hydrophilic to a hydrophobic environment. This change in environment is a prerequisite for good binding of acetylcholine to the active site and has already been invoked as an important factor.[10] The ease of transition between the hydrophilic and hydrophobic environment coupled with the overall stability of the intermediate formed will be reflected in the value of Kd for the receptor-alkaloid complex.

Detection of the intrinsic fluorescence change in a protein upon binding with the ligands is one of the simplest and most direct methods to study ligand induced conformational change. In the present investigation the quenching of fluorescence of the receptor upon binding with the alkaloids is used to study the protein ligand interactions. The decrease in relative fluorescence on titration with the ligand supports a change in environment of the alkaloid moiety when the binding to the protein takes place. This may arise due to the presence of non-polar regions in or around the tryptophan containing binding site of the receptor.

The intrinsic fluorescence of the receptor is quenched by the alkaloids. Tubocurarine, a specific competitive antagonist of the receptor blocks any specific interaction of the alkaloids with the receptor. This,

and the fact that there is competition at the active site of the receptor when both acetylcholine and alkaloids are present, is clear evidence to support the idea that the alkaloids are potential nicotinic receptor agonists. The curves of the Scatchard plots (Figure 2) indicate a positive co-operative binding and the existence of more than one class of binding site for the isoquinoline alkaloids at the receptor.

Both the low and high affinity binding of the alkaloids are reversible and are displaced by the cholinergic agonists acetylcholine and (antagonist)-d-tubocurarine (Figure 4)[12]. The inhibition of papaverine binding by d-tubocurarine can be explained as a competitive model which takes into account the two binding constants for the alkaloid. Furthermore, the binding constants estimated from fluorimetry agree very well with the values from the Scatchard analysis. The values of Kd estimated by the two methods used are in sufficient agreement to lead us to the conclusion that the decrease in observed fluorescence in the presence of ligand is due to the competitive displacement of alkaloid bound to the agonist receptor site. The effective concentration of papaverine determined by fluorescence and the Kd values from the direct binding are in excellent agreement.

The spectral properties of the ligands bound to the receptor sites appear sensitive to the nature of the ligands bound and it was established that, by analysis of the fluorescence spectra, a 12 nm blue shift occurred when the alkaloids bound to the receptor sites. Extension of information[14,15] suggests that a hipsochromic spectral shift is characteristic of the agonist character of a ligand. The quench in fluorescence intensity caused by the presence of d-tubocurarine/ acetyl-choline can be interpreted as any alkaloid remaining bound to either of the receptor sites. With the one receptor site the emission wavelength is independent of the occupancy of the receptor site while with the other the emission wavelength is sensitive to the nature of the effector (d-tubocurarine or acetylcholine) bound at the receptor site. Ligands bound to the receptor dominate the emission spectrum, even under conditions where a small fraction of the ligand interacts with the protein. When sufficient portion of that population has been displaced, the spectral properties of the molecules bound to the second class of sites become apparent.

The observed competition between the alkaloids and tubocurarine and between acetylcholine and alkaloids can be accounted for by a simple model that assumes that they all bind to a common site and most probably with a 1:1 stoichiometry. This model predicts, and supports, the total number of binding sites for papaverine, berberine, isoquinoline, acetyl-choline and d-tubocurarine should be identical. Indeed, our results conclude that the number of binding sites are 1.96, 2.01, 2.2, 2.01, and 1.79, respectively.

In order to further unravel the mechanism of ligand interaction of the receptor we have used fluorescence titrations and spectrophotometry of various isoquinoline alkaloids with the protein. The employed receptor preparations, monitoring ligands and techniques were particularly suited for this purpose. The receptor, isolated from *Torpedo* exhibited positive cooperativity of acetylcholine binding sites as well as with the alkaloid ligands. A complete analysis in terms of Kd values of binding equilibria of the receptor with a set of agonists and antagonists is presented.

Acknowledgement. We acknowledge the Medical Research Council (South Africa) for financial support.

References and Notes

1. Maelicke, A. In *Molecular Pharmacology*, Burgen, A.S; Roberts, C.G. Eds., Elsevier, North Holland, 1981; pp 1-22.
2. Changeux, J-P.; De Villiers-Thiery, A; Chemouilli, P. *Science*, **1984**, *225*, 1335-1345.
3. Whiteley, C.; Mmutle, T.B. *J. Enz. Inhibition*, **1994**, *8*, 1-16.
4. Whiteley, C.; Kapp, E.A. *Biochim. et Biophys. Acta*, **1990**, *1034*, 29-38.
5. Ivanov, M.V.; Kube, D; Nagradova, N. *Biochem. Biophys. Acta*, **1984**, *789*, 216-223.
6. Roberts, D.D.; Goldstein, I.J. *J. Biol. Chem.*, **1982**, *257*, 11274-11277.
7. Kella, N.K.D.; Roberts, D.D.; Schafer, J.A.; Goldstein, I.J. *J. Biol. Chem.*, **1984**, *259*, 4777-4781.
8. Reaction mixtures (3.0 mL) containing purified receptor (13.33 nM) and ligand (2-50 μM) in potassium phosphate buffer (0.1M, pH 7.4). Absorbances were monitored at respective wavelengths and concentrations of the alkaloids obtained using E_{220} = 6.31 x 10^4 $M^{-1}.cm^{-1}$ (isoquinoline, M_R = 129); E_{240} = 7.94 x 10^4 $M^{-1}.cms^{-1}$ (papaverine, M_R = 376); E_{263} = 5 x 10^4 $M^{-1}.cms^{-1}$ (berberine, M_R = 353). The determination of the number of binding sites and the dissociation constants (Kd) of the receptor-ligand complex were determined according to the method of Scatchard[11] and equations 1 and 2.

$$L_f = L_t - (\Delta A_x/\Delta A_{x.max})A_t \quad \ldots. 1 \qquad \nu/L_f = p - \nu/Kd \quad \ldots. 2$$

where L_t is the total ligand concentration; A_t is the total receptor concentration; ΔA_x is the difference in absorbance observed when ligand is mixed with receptor at wavelength x; $\Delta A_{x.max}$ is the maximum absorbance observed when all of the ligand is bound to the receptor; ν are the moles of ligand bound; p is the number of binding sites; L_f is the concentration of free ligand and Kd is the dissociation constant of ligand-receptor complex.
9. All fluorimetric titrations were carried out at 20 °C (0.1M potassium phosphate buffer; pH 7.2). Increasing concentrations of ligand (5-100 μM) were titrated against a fixed concentration of receptor (100 $\mu g.mL^{-1}$). The mixture was excited at 295 nm and emission measured at 340 nm. The fluorescence data was used to determine the Kd of the alkaloids with the receptor. The results were analysed according to equations 3 and 4 for independent and equivalent binding sites.

$$Lt/\$ = Kd/(1 - \$) + p(A_t) \quad \ldots. 3 \qquad \$ = \Delta F/\Delta F_{max} \quad \ldots. 4$$

\$ is the fractional occupancy of acceptor sites by ligand; ΔF is the change in fluorescence in the presence of an amount of ligand; ΔF_{max} is the change in fluorescence at full saturation with ligand.
10. Jencks, W.P. *Adv. Enzymol.* **1975**, *43*, 219-410.
11. Scatchard, G. *Proc. Natl. Acad.* **1949**, *51*, 660-672.
12. Fluorescence data were plotted according to the logarithmic formulation[13] (equation 5).

$$\log[f_E - f)/(f - f_C)] = p \log K_E/K_C + p \log C/E \quad \ldots. 5$$

Where f_E denotes the initial fluorescence in the absence of competing ligands; f_C denotes the fluorescence when tubocurarine is completely displaced from the nicotinic receptor and f denotes the fluorescence observed at any given concentration of competing ligand, E and C represent free concentrations of tubocurarine and competing ligand, and K_E and K_C are their respective dissociation constants. Dissociation constants for the competing ligand (K_C) were calculated from the intercept on the abscissa. The logarithmic relationship of equation 5 is analogous to the Hill equation and a slope (p) that differs from unity should reflect either heterogeneity in binding sites or co-operativity in binding.
13. Taylor, P.W.; Lappi, S. *Biochemistry* **1975**, *14*, 1989-1997.
14. Herz, J.M.; Johnson, D.A.; Taylor, P.W. *J. Biol. Chem.* **1987**, *262*, 7238-7247.
15. Prinz, H.; Maelicke, A. *J. Biol. Chem.* **1983**, *258*, 10263-10271.

(*Received in USA* 24 *June* 1996; *accepted* 6 *September* 1996)