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Original Article

Binding of Nicotinoids and the Related Compounds to the Insect Nicotinic Acetylcholine Receptor

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In a radio receptor assay on the binding at the [^{3}H]α-bungarotoxin binding site to the nicotinic acetylcholine receptor (nAChR) obtained from housefly and honeybee head membranes, nicotine, nornicotine, anabasine and dihydronicotyrine, all with highly basic nitrogen, had a strong binding affinity, whereas myosmine, nicotyrine and cotinine, with low basic nitrogen, did not. Structure-binding relationships of the above nicotinoids and pyridylmethylamines mostly coincided with the previously studied relationships to the insecticidal activity, the effect on nerve activity and the inhibition of acetylcholinesterase (AChE). Both enantiomers of nicotine had an affinity for nAChR, although the affinity was higher in the *l*-form than in the *d*-form. Imidacloprid interacted at the same site on the nAChR, but oxadiazolone, a potent AChE inhibitor, had no affinity.

INTRODUCTION

Structure-activity relationships of nicotinoids have been studied by various methods. To estimate the intrinsic activity at the site of action, insecticidal activity,[1-3] inhibition of acetylcholinesterase (AChE)[4] as a model of acetylcholine receptor (AChR) and effect on spontaneous nerve activity[4] were used. Quantitative structure-activity relationships were also established by means of substituent constants.[5,6] An overall picture of the mode of action of nicotine in insects was proposed previously.[1,7] In the present study, binding to nicotinic AChR (nAChR) preparations from housefly and honeybee heads was examined as a more direct parameter. Recently, oxadiazolone compounds were found to be potent AChE inhibitors[8] and an imidacloprid type compound was reported to interact with nAChR.[9] These compounds were therefore included in the study for comparison.

MATERIALS AND METHODS

1. *Materials*

N-[Propionyl-^{3}H]propionylated α-bungarotoxin ([^{3}H]α-BGTX; 56 Ci/mmol) was purchased from Amersham. Nicotinoids were synthesized in our laboratory. Imidacloprid was donated by Nihon Bayer Agrochem, and MK 21166 and MK 179 by Mitsubishi Chemical Ind. Ltd. Cholinergic ligands in Table 2 were purchased from Sigma Chemical, except for trimethaphan camsylate which was donated by Dr. M. E. Eldefrawi.

2. *nAChR Preparation*

Honeybee (*Apis mellifera* L.) head membranes were prepared by the method of Sherby *et al.*[10] Housefly (*Musca domestica* L.) heads were homogenized with a Polytron™ setting 7 three times, 30 sec each with a 60 sec rest in-between in 20 mM Na_2HPO_4-HCl buffer of pH 7.4 containing 0.1 mM PMSF and 1 mM EDTA (0.5 g heads/ml). The homogenate filtered through four layers of cheese cloth was centrifuged at 600×*g* for 10 min, and the super-

natant was centrifuged at 20,000×g for 30 min. The pellet was suspended in ice-cold distilled water and incubated at 4°C for 1 hr to remove internal acetylcholine. The suspension was centrifuged at 20,000×g for 30 min, and the pellet was washed by rehomogenizing in the above buffer and recentrifuged as above. Then, the pellet was suspended in the above buffer to the initial volume.

3. Binding Study

A nAChR preparation (0.3–0.6 mg protein/assay) from honeybee or housefly heads was incubated at 25°C for 60 min with 2–3 nM of [^{3}H]α-BGTX and with or without a test chemical at appropriate concentrations in a total volume of 250 μl of 20 mM Na_2HPO_4-HCl buffer of pH 7.4 containing 1 mM EDTA and 0.1 mM PMSF. The reaction was terminated by rapid filtration on a GF/B (Whatman) presoaked in 0.05% polyethyleneimine. The GF/B was rinsed three times with 2.5 ml portion of 0.9% NaCl and transferred into a counting vial. Radioactivity of receptor-radiolabeled ligand complex was counted after overnight incubation in 4 ml of scintillation cocktail (Ready Protein, Beckman). Specific binding was defined by the difference in the radioactivity in the absence and presence of 1 μM unlabeled α-BGTX.

4. Determination of Kinetic Constants

The increase of specific binding ([LR]) of [^{3}H]α-BGTX (L) was plotted *vs.* time (curve 1) until equilibrium (60 min), from which $[LR]_{eq}$ was obtained. Then an excess amount of unlabeled α-BGTX was added, and the decrease of [LR] was plotted *vs.* time (curve 2). k_{-1} was obtained from the slope by plotting $\ln [LR]/[LR]_{eq}$ *vs.* time by transforming curve 2. k_{obs}, defined as $k_1[L]+k_{-1}$, was obtained from the slope when $\ln [LR]_{eq}/[LR]_{eq}-[LR]$ was plotted *vs.* time by transforming curve 1. Then, k_1 was obtained by $k_1=(k_{obs}-k_{-1})/[L]$. $T_{1/2}$ is the half-life of receptor-radiolabeled ligand complex. K_D was calculated from $K_D=k_{-1}/k_1$.

5. Data Analysis

Saturation isotherms were obtained by displacing of [^{3}H]α-BGTX binding with unlabeled α-BGTX at various concentrations and analyzed by iterative nonlinear least-squares regression with the LIGAND program[11] modified by G. A. McPherson for an IBM personal computer. Kinetic constants were analyzed with the KINETIC program. IC_{50} values (molar concentrations necessary for inhibiting 50% of the specific [^{3}H]α-BGTX binding) were determined by iterative nonlinear least-squares regression with the SIGMAPLOT program (Jandel Scientific, CA, USA). The binding affinity of the tested chemicals was evaluated by Ki values obtained by the Cheng and Prusoff equation[12]: $Ki=IC_{50}/(1+[L]/K_D)$, where [L] is a concentration of added radiolabeled ligand. The dissociation constant (K_D) and amount of maximal binding (B_{max}) were estimated by Scatchard analysis of saturation isotherm.

RESULTS AND DISCUSSION

1. Kinetic Constants of Binding of [^{3}H]α-BGTX

Table 1 indicates that α-BGTX had a similar affinity for housefly and honeybee nAChRs, although the k_1, k_{-1} and $T_{1/2}$ were different.

2. Binding Affinity of Cholinergic Ligands for Housefly and Honeybee nAChRs

In Table 2 the order of potency is **1>7>6>2>13>3>9>10>12>8>11>4, 5** for housefly nAChR, and **1>7>6>2>13>3>10>8>9>11>12>5>4** for honeybee nAChR. The

Table 1 Kinetic constants of binding of [^{3}H]α-BGTX.

Insect	K_D (nM)	k_1 (M^{-1} min^{-1})	k_{-1} (min^{-1})	$T_{1/2}$ (min)
Housefly (*Musca domestica*, L)	0.11	$1.26\pm0.16\times10^8$	$1.40\pm0.11\times10^{-2}$	58.6
Honeybee (*Apis mellifera*, L)	0.12	$2.80\pm0.41\times10^8$	$3.35\pm0.27\times10^{-2}$	21.1

The values are mean±standard error, $n=3$.

Table 2 Binding affinity of cholinergic drugs for the [^{3}H]α-BGTX binding site.

No.	Drug	*Ki* (μM)	
		Housefly	Honeybee
1	Cytisine	1.23	0.65
2	Lobeline	12.8	9.13
3	DMPP	86.3	49.5
4	Mecamylamine	>1000	453
5	Coniine	>1000	262
6	Trimethaphan	2.75	5.79
7	Tubocurarine	1.88	3.57
8	Carbamylcholine	212	175
9	Decamethonium	103	224
10	Pentolinium	108	72.2
11	Arecoline	516	240
12	Nereistoxin	187	254
13	Atropine	32.5	26.3

profiles were essentially the same between the two species, with some discrepancy, indicating that the target receptor in the crude preparations was the nAChR compared with those obtained for other organisms.[13]

3. Binding Affinity of Nicotinoids for nAChR

Table 3 summarizes the results on binding as well as p*Ka'*, calculated ionized % at pH 7, relative insecticidal activity, neuro-activity and AChE inhibitory activity examined in the previous studies. The binding profiles were almost the same between the two species. Nornicotine, anabasine, nicotine and dihydronicotyrine are known to have high insecticidal activity and nitrogen basicity,[1] and also they had high binding affinity for nAChR. Myosmine, nicotyrine, and cotinine, which have low insecticidal activity and nitrogen basicity,[1] had no effect on nAChR. As previously reported,[4] the AChE inhibitory activity somewhat reflected the insecticidal activity, but the IC_{50} value of nicotyrine was too low as compared with other biological parameters. Toxicity of *d*- and *l*-isomer of nicotine to houseflies was the same.[14] To AChE, the *d*-isomer was more inhibitory than the *l*-isomer, but in the binding study both enantiomers of nicotine showed an affinity for nAChR, although it was far higher in the natural or *l*-isomer. The previous statement derived from the study on their toxicity to many organisms that the both enantiomers of nicotine behave similarly at the site of action[14] should be modified.

As shown in Table 3-2, among the three isomers of pyridylmethyl-*N*, *N*-diethylamines, only the 3-isomer having a side chain like nicotine showed higher insecticidal activity.[2,8] In the binding study on pyridylmethyl-*N*, *N*-dimethylamines, too, only the 3-isomer showed higher binding affinity. These results indicated that the previously proposed essential moiety for insecticidal activity[1] is also essential for binding.

Most of 3-pyridylmethylamines, as shown in Table 3-3, had a sizable affinity for nAChR. The order of potency was **30>31>26>28>24>22>27>21>23**, **25**, **29**, **32** for housefly nAChR, and **30>31>24>27>29>28>26>22>21>25>23**, **32** for honeybee nAChR. The profiles were essentially the same for the two species, although there was some discrepancy. The results shown in Tables 2 and 3 justify the use of commercially available honeybees for this kind of study. When the amino nitrogen is a part of heterocycles, the potency became higher as seen with **30** and **31**. However, **32**, not provided with an essential moiety because of the lower basicity of morphoryl nitrogen, was not insecticidal[2,3] and had no binding affinity.

4. Binding Affinity of Chemicals Supposed to Interact with nAChR

Acetylcholine is both a ligand for nAChR and a substrate for AChE. Nicotine, although cited as a non-AChE inhibitor, dose inhibit AChE at higher concentrations.[4,14] Oxadiazolone compounds such as MK 179 are potent insecticides and AChE inhibitors,[8] and there is a reason to suspect that they interact with nAChR. However, the results shown in Table 4 indicate that a potent insecticide, MK 179, has no binding affinity. MK 21166 is neither insecticidal, nor AChE-inhibitory, nor has any binding affinity. On the other hand, imidacloprid has a strong binding affinity for nAChR, which confirms the results by Bai *et al.*,[9] while the simplified structures had no affinity.

5. Mode of Action

Scatchard analysis of saturation isotherms

Table 3 Structure-bioactivity relationship of nicotinoids.

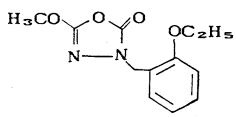
Compounds	(No.)	Basicity pK_a' 25°C (37°C)	Ionized % (pH 7)	Relative toxicity (H. fly)	Neuro-activity[4] EC_{50} (mM)	AChE inhibition[4] IC_{50} (mM)	Binding to nAChR *Ki* (μM) (H. fly)	(H. bee)
3-1.								
Nornicotine	(**14**)	9.0	99.0	*l*		4.2		
		(8.76)		*dl* 0.3		9.7	8.77	7.61
Anabasine	(**15**)	8.7	98.0	*l*		3.9	3.71	3.91
		(8.48)		*dl* 0.6				
Nicotine	(**16**)	7.9	88.8	*l* 1.0	0.20	2.6	2.48	1.48
		(7.66)		*dl* 1.0		0.62		
				d 1.0		0.23	59.9	43.7
Dihydronicotyrine	(**17**)	7.4	71.5	*dl* 2.6	0.08	0.35	1.92	1.27
		(7.21)						
Myosmine	(**18**)	5.5	3.1	0.36		6.8	>1000	>1000
		(5.23)						
Nicotyrine	(**19**)	4.7	0.5	0.18		2.3	>1000	>1000
		(4.51)						
Cotinine	(**20**)	4.5	0.1	0.03		260	>1000	>1000
		(—)						
3-2.								
Pyridylmethyl-*N*,*N*-dimethylamines								
2-Isomer							>1000	>1000
3-Isomer	(**26**)	(7.76)		0.45	0.06	5.1	201	208
4-Isomer							>1000	>1000
Pyridylmethyl-*N*,*N*-diethylamines								
2-Isomer		(8.42)		0.1		3.0		
3-Isomer	(**27**)	(8.12)		0.46	0.14	0.89	450	155
4-Isomer		(7.96)		0.1		7.7		
3-3.								
3-Pyridylmethylamines (R_1, R_2: substituents on amino nitrogen)								
R_1 R_2								
H Me	(**21**)	(8.30)		0.30		31	600	377
H Et	(**22**)	(8.96)		0.34		7.1	380	289
H *n*-Pr	(**23**)	(8.38)		0.15		17	>1000	>1000
H *i*-Pr	(**24**)	(8.46)		0.46		3.9	226	103
H *n*-Bu	(**25**)	(8.66)		0.16		23	>1000	406
Me Me	(**26**)	(7.76)		0.45	0.06	5.1	201	208
Et Et	(**27**)	(8.12)		0.46	0.14	0.89	450	155
n-Pr *n*-Pr	(**28**)	(8.13)		0.16		1.7	220	200
n-Bu *n*-Bu	(**29**)	(8.08)		0.28		2.8	>1000	175
Pyrrolidyl	(**30**)	(8.11)		0.43	0.54	1.4	16.4	14.3
Piperidyl	(**31**)	(8.16)		0.79	0.17	1.5	67.6	48.7
Morphoryl	(**32**)	(5.76)		0.04	14.0	[a]	>1000	>1000

[a] Too high to calculate.

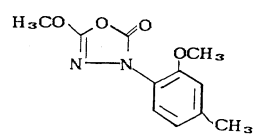
on binding of [^{3}H]α-BGTX with or without nicotine and imidacloprid showed the convergence of the lines to a point (Table 5), indicating that all the chemicals interact with the same α-BGTX binding site. Yamamoto[1,7] has reported that all the insecticidal nicotinoids are provided with a 3-pyridylmethylamine moiety in which the amino nitrogen is highly basic, while compounds not provided with this moiety are of low toxicity; protonated nicoti-

Table 4 Binding affinity of tested chemicals for the [^{3}H]α-BGTX binding site.

Compounds	*Ki* (μM)	
	Housefly	Honeybee
MK 21166	>100	>100
MK 179	>1000	>1000
Imidacloprid	7.47	2.53
Compound **A**	>500	>500
Compound **B**	>500	>500
Compound **C**	>1000	>1000



MK 21166



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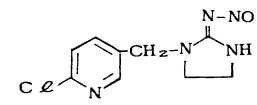
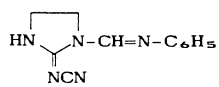
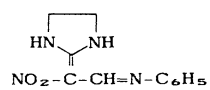


IMIDACLOPRID



COMPOUND A



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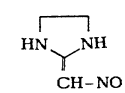

COMPOUND C

Table 5 Effect of *l*-nicotine and imidacloprid on K_D and B_{max} values of [^{3}H]α-BGTX binding to honeybee nAChR.

Drugs	K_D (nM)	B_{max} (fmol/mg protein)
Control	7.45±0.89	451±21.9
With		
1 μM *l*-nicotine	23.8±15.9	463±51.4
2 μM imidacloprid	25.8±10.1	425±83.5

The values are means±standard error, $n=3$.

noids, provided with such essential moiety, resemble acetylcholine in the conformation and electronic makeup. The results of the present binding study support this proposal. It is interesting to note that imidacloprid is provided with the above moiety.

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要　　約

ニコチノイドおよび関連化合物の昆虫ニコチン性アセチルコリンレセプターへの結合

富澤元博，山本　出

イエバエおよびミツバチ頭部のニコチン性アセチルコリンレセプター (nAChR) 画分の α–ブンガロトキシン (α-BGTX) 結合部位への薬物の結合を，ラジオレセプターアッセイにより検討した．塩基性の高いニコチン，ノルニコチン，アナバシン，ジヒドロニコチリンでは親和性が強く，塩基性の低いミオスミン，ニコチリン，コチニンでは弱かった．ピリジルメチルアミン類の3位異性体で塩基性の高いものの親和性が強かった．ニコチンの光学異性体では，*d* 体より *l* 体のほうが強かった．以上の結果はこれらニコチノイドの殺虫活性と対応していた．アセチルコリンエステラーゼの強力な阻害剤であるオキサジアゾロン化合物の nAChR への親和性は認められなかった．ニコチンと同じ構造部分をもつイミダクロプリドは強い親和性を示し，α-BGTX やニコチンと同一部位に相互作用することが示された．