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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

Species-Specific Differences in the Substrate–Inhibitory Specificity of Cholinesterases from Optical Ganglia of Squids of the Gonatidae Family

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Abstract—A comparative study was carried out of the substrate and inhibitory specificity of cholinesterase preparations from squids, representatives of 3 genes and 5 species of the Gonatidae family: *Berryteuthis* (*B. magister* and *B. anonichos*), *Gonatus* (*G. kamtschaticus* and *G. tinro*), and *Gonatipsis* (*G. borealis*), that have overlapping habitation areals in the Bering Sea. As substrates, there were used bromides of acetylthiocholine, propionylthiocholine, and butyrylthiocholine, as organophosphorus inhibitors, diisopropylfluorophosphate, a cation-containing inhibitor, and 4 hydrophobic compounds. The homogeneity of the cholinesterase activity in these preparations has been shown, the intergenus and interspecies differences in the enzyme properties are revealed, and also the peculiarity of properties of enzymes from Gonatidae squids is emphasized in comparison with cholinesterase from the Pacific squid *Todarodes pacificus* and "standard" mammalian enzymes (from human erythrocytes and horse blood serum). The revealed interspecies differences are discussed in terms of evolutionary development of the Gonatidae family.

INTRODUCTION

More than 30 years ago we started a systematic study of the substrate–inhibitor specificity of cholinesterases (ChE) from optical ganglia of squids [1, 2]. This comparative enzymologic study of many species of this suborder of cephalopod molluscs [3] was carried out using a large set of ChE effectors: substrates, onium reversible inhibitors and irreversible organophosphorus inhibitors (OPI) [4]. The ChE substrate–inhibitor specificity was studied in the greatest detail in the Pacific squid *Todarodes pacificus* [5, 6] and in the Commodore squid *Berryteuthis magister* [7, 8]. Our studies [4] have revealed both interspecies and intraspecies differences in ChE reactivity, which has allowed us to use parameters of the ChE specificity as a tool for specification of taxonomic position of squids [9].

Recently [10] we have got an opportunity to study ChE properties in squids, representatives of one family, Gonatidae, that have a limited habitation area in the Bering Sea aquatorium, some of the studied squid species of this family being considered more ancient, while others, younger and more changeable in the evolutionary aspect [11]. Such comparative study of the substrate–inhibitor specificity will allow more substantiated conclusions to be made about intergenus and interspecies differences.

MATERIALS AND METHODS

The study was performed on 5 species of squids of the Gonatidae family, which belong to 3 genes: *Berryteuthis* (*B. magister* and *B. anonichos*), *Gonatus* (*G. kamtschaticus* and *G. tinro*) and *Gonatipsis* (*G. borealis*). Freshly caught squids (5 and more individuals of each species without differentiation by sex) collected on the research ship "Profes-

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Table 1. The substrate–inhibitory analysis of homogeneity of cholinesterase preparations from optical ganglia of squids of the family Gonatidae

Enzyme source	OPI	k_{II}, M^{-1} min^{-1}		
		Substrates		
		AThCh	PThCh	BThCh
Gonatopsis borealis	I	8.9×10^2	6.1×10^2	1.9×10^3
	II	8.4×10^4	1.2×10^4	2.4×10^4
	III	3.9×10^2	2.3×10^2	3.9×10^2
Berryteuthis magister	I	2.7×10^6	2.8×10^6	3.1×10^6
	II	2.1×10^7	1.3×10^7	1.3×10^7
	III	6.1×10^5	4.6×10^5	7.1×10^5
Berryteuthis anonychos	I	8.1×10^5	9.3×10^5	1.3×10^6
	II	1.4×10^7	1.5×10^7	9.6×10^6
	III	4.2×10^5	6.8×10^5	8.0×10^5
Gonatus kamtschaticus	I	4.0×10^5	4.5×10^5	4.9×10^5
	II	1.4×10^6	1.6×10^6	2.0×10^6
	III	2.7×10^4	6.0×10^4	5.7×10^4
Gonatus tinro	I	2.8×10^5	2.5×10^5	2.9×10^5
	II	1.6×10^6	2.0×10^6	2.2×10^6
	III	3.5×10^4	3.4×10^4	4.0×10^4

Note: AThCh–acetylthiocholine bromide; PThCh–propionylthiocholine bromide; BThCh–butyrylthiocholine bromide; OPI–organophosphorus inhibitors (see their formulas in "Materials and Methods"); k_{II}–bimolecular rate constant of the OPI interaction with enzyme (see "Results and Discussion"). The same is in Tables 2 and 3.

sor Kiziwetter" in different zones of the Bering Sea aquatorium (the Olyuter Bay, East Kamchatka, Northern Kuril Islands), were totally frozen at −18°C and delivered to TINRO (Vladivostok), where their taxonomic position was determined. After that, their optical ganglia were isolated and stored at −18°C [10]. The source of enzyme was supernatant (800 g, 15 min) of water homogenate (3 mg/ml) of the ganglion tissue. The cholinesterase activity was determined by colorimetric Ellman's method [12] using bromides of acetylthiocholine (AThCh , propionylthiocholine (PThCh), and butyrylthiocholine (BThCh) (Chemapol) as substrates. The following OPI were studied: $[(CH_3)_2CHO]_2P(O)F$ (I) (Merck), $C_2H_5O(CH_3)P(O)SC_2H_4S^+(CH_3)C_2H_5 \cdot CH_3SO_4^-$ (II), $C_2H_5O(CH_3)P(O)SC_2H_4SC(CH_3)_3$ (III), $C_2H_5O(CH_3)P(O)SC_5H_{11}$ (IV), $C_2H_5O(CH_3)P(O)SC_6H_{13}$ (V), $C_2H_5O(CH_3)P(O)SC_7H_{15}$ (VI) (the OPI II–VI were synthesized at the Laboratory of N.N. Godovikov of Nesmeyanov Institute of Organoelement Compounds of the Russian Academy of Sciences [4]).

RESULTS AND DISCUSSION

First of all it was necessary to check whether the studied enzyme preparation contains only one ChE or a mixture of several ChE (like the nervous tissue of some animals [4]).

To determine homogeneity of the studied cholinesterase preparations, the most adequate is the method of substrate–inhibitor analysis, using different specific effectors [4, 13]. Thus, the specific OPI (Table 1) were used in this study: diisopropylfluorophosphate (I) specific for butyrylcholinesterase (BuChE) [4], cation-containing OPI (II) specific for AChE [4], and the hydrophobic OPI, containing *tert*-butyl group (III) specific for ChE from the Pacific squid *Todarodes pacificus* [6], as well as 3 thiocholine substrates, including butyrylthiocholine (BThCh), specific substrate of BuChE [4]. If the anticholinesterase potency of OPI (the bimolecular constant of the inhibition rate $k_{II} = 1/([I]\,t) \ln (E_0/E_t)$ [4]) does not depend on nature of substrate used for measurements of the initial (E_0) and residual (E_t) ChE after t min of incubation with OPI (I), it may be concluded that the tissue contains only one ChE [13]. As seen from Table 1, the k_{II} values for each of the tested OPI, determined with the aid of three substrates, are close to each other. This allows the conclusion that ChE preparations from the studied Gonatidae family representatives are homogeneous, i.e., they contain only one enzyme.

The studied ChE preparations differed essentially by values of the specific activity measured from the rate of hydrolysis of different substrates (Table 2). Thus, the highest specific activity while using AthCh, was observed in *B. magister* and *B. anonichos*; it was 5 times lower in *G. kamtschati-*

Table 2. Parameters of the substrate specificity of ChE of optical ganglia of squids of the family Gonatidae

Parameters	Substrates	*B. magister*	*B. anonichos*	*G. kamtschaticus*	*G. tinro*	*G. borealis*
Specific activity, μmol mg^{-1} min^{-1}	AThCh	0.355	0.357	0.067		
	PThCh		0.267		0.084	0.286
	BThCh			0.117	0.041	0.385
V_{max}, mM min^{-1}	AThCh	5.33	5.36	1.01	2.41	4.65
	PThCh	2.17	4.00	2.11	1.26	4.29
	BThCh	2.17	4.06	1.75	0.61	5.78
V_{max}, rel. %	AThCh	100	100	100		100
	PThCh	40	75		50	
	BThCh			175	25	
K_M, mM	AThCh	2.90	0.20	0.82	1.09	0.27
	PThCh	1.19	0.12	1.81	1.69	0.49
	BThCh	0.71	1.82	0.41	1.34	2.16
V_{max}/K_M, min^{-1}	AThCh	2	25		2	
	PThCh					10
	BThCh					

Note: V_{max}—maximal rate of enzymatic hydrolysis; K_M—Michaelis constant [4].

cus. At the same time, the PThCh and BThCh hydrolyses were catalyzed at the highest rate by ChE of *G. borealis* and *B. anonichos*, and at the lowest (3–10 times lower), by the enzyme of *G. tinro*. There were very significant interspecies differences in K_M values for each of the substrates. Thus, for enzymes of *B. magister* and *B. anonichos*, which belong to one genus *Berryteuthis*, these differences were 10–15-fold in the cases of AThCh and PThCh. The K_M values for BThCh were 50 times lower in ChE of *G. kamtschaticus*, than that of *G. borealis*.

When comparing substrate specificity of cholinesterases, usually two indexes are considered: qualitative, the relative rate of substrate hydrolysis in a row of several substrates, and quantitative, the V_{max}/K_M ratio value that reflects, to a degree, the substrate affinity to enzyme [4]. Using the both indexes, essential interspecies differences have been revealed. Thus, ChE of *B. magister* and *B. anonichos* hydrolyze PThCh and BThCh at equal rate, while AThCh 1.5–2 times faster, which distinguishes the enzyme of these species not only from mammalian ChE (the ratio of AThCh : PThCh : BThCh hydrolysis rates is 100 : 55 : 5 for erythrocyte AChE, and 100 : 140 : 230 for serum BuChE [4]), but also from the Pacific squid *Todarodes pacificus* ChE (100 : 100 : 50) [5]. The properties of ChE from other Gonatidae species also are different from each other and from "standard" enzymes, and ChE of *G. kamtschaticus* should be classified by the accepted criterion [4] as PChE, while ChE of *G. borealis*, as BuChE (with the reservation that this is hydrolysis of thiocholine substrates). The interspecies differences in the substrate "affinity" to the studied enzymes (by the V_{max}/K_M ratio values) are found. Thus, in the *B. anonichos* and *G. borealis* ChE the "affinity" to BThCh is lower, and in the *G. kamtschaticus* ChE, on the contrary, higher, than to AThCh and PThCh. Besides, it is interesting to note that the "affinity" to PThCh in the *B. anonichos* ChE was 70 times higher, than in the *G. tinro* ChE.

Table 3. Inhibitory specificity (log k_{II}) of ChE in optical ganglia of squids of the Gonatidae family

Enzyme source	Substrate	Organophosphorus inhibitors						Ratio of k_{II} values	
		I	II	III	IV	V	VI	II/III	III/V
Gonatopsis borealis	AThCh	2.95	4.38	2.59	2.00	2.12	3.00	60	3
	PThCh	2.78	4.08	2.36	1.78	2.17	3.11	50	2
	BThCh	3.28	4.38	2.59	2.18	2.04	3.23	60	3
Berryteuthis magister	AThCh	6.43	7.32	5.79	3.52	3.64	3.76	35	140
	PThCh	6.45	7.12	5.66	3.64	3.74	3.95	30	85
	BThCh	6.49	7.11	5.85	3.21	3.96	3.84	45	80
Berryteuthis anonychos	AThCh	5.91	7.15	5.62	3.36	3.76	3.88	35	75
	PThCh	5.97	7.16	5.83	3.25	3.86	3.89	25	100
	BThCh	6.11	6.98	5.91	3.41	4.06	3.96	15	70
Gonatus kamtschaticus	AThCh	5.60	6.14	4.43	3.41	3.36	3.03	50	10
	PThCh	5.65	6.21	4.78	3.56	3.16	3.25	30	40
	BThCh	5.69	6.30	4.76	3.61	3.57	3.54	30	15
Gonatus tinro	AThCh	5.45	6.19	4.55	3.79	3.74	4.00	45	7
	PThCh	5.40	6.30	4.53	3.64	3.83	3.78	60	5
	BThCh	5.46	6.34	4.60	4.00	4.00	3.84	55	4
Todarodes pacificus [6]	ACh	6.84	7.64	6.41	5.03	5.38	4.90	15	10
	BCh	6.86	7.56	6.30	–	5.44	–	20	7
Human erythrocyte AChE [4]	ACh	4.98	8.12	3.20	3.42	4.20	4.33	10^5	0.1
Horse serum BuChE [4]	ACh	7.18	6.57	3.68	3.42	3.58	3.52	10^3	1

Note: ACh—acetylcholine chloride, BCh—butyrylcholine iodide, AChE—acetylcholinesterase, BuChE—butyrylcholinesterase.

For analysis of inhibitor specificity of the studied ChE preparations (Table 3), the OPI differing in their structure were tested: the effective phosphorylating agent diisopropylfluorophosphate (I), the cation-containing OPI (II), and a group of hydrophobic OPI (III–VI). The intergenus and interspecies differences were revealed in sensitivity of ChE to some of the studied OPI. Thus, reaction capability of the *G. borealis* ChE to most OPI was several orders lower, than of other ChE. It is for the first time that ChE of squids show such a low sensitivity to the OPI that are as efficient as diisopropylfluorophosphate (I) and cation-containing II. Among the mollusc enzymes, this is rather similarity perhaps with ChE from hemolymph of the Pacific mussel [14]. The degree of inhibition by specific OPI (I–III) (see above) of ChE from the *Berryteuthis* genus was somewhat higher, than from the *Gonatus* genus. To analyze effects of the OPI structure, using a small set of inhibitors, we compared two pairs: the cation-containing OPI (II) with the structurally similar hydrophobic (the influences of cationic grouping) OPI (III), as well as the OPI containing a *tert*-butyl grouping (III) with its isomer of a normal structure (V) (the influence of the "neo-hexyl" structure specific of the squid ChE [6]). As a whole, "the effect of cationic grouping" was practically identical for ChE of the Gonatidae family representatives, but it was somewhat higher than for ChE of *T. pacificus* (probably, due to the high specificity of III to this ChE

[6]). As to the so-called "isomeric effect" (advantage of the "neo-hexyl" structure over the *n*-hexyl one [6]), it was the most expressed in ChE of the *Berryteuthis* genus squids, the value of the k_{II} ratio of III to V being much higher in the *Gonatidae* ChE, than in the *T. pacificus* ChE. The comparison of the squids ChE with the "standard" mammalian ChE has confirmed the peculiarity of properties of squid enzymes [4, 6].

Thus, the performed study of properties of ChE preparations from squids, representatives of three genes and five species of the Gonatidae family, which inhabit the overlapping Bering Sea areas, has shown homogeneity of the cholinesterase activity of these preparations, has revealed intergenus and interspecies differences in their substrate and inhibitory specificity, and also has emphasized a peculiarity of properties of ChE from Gonatidae squids in comparison with ChE from the Pacific squid *T. pacificus*, the human erythrocyte AchE, and the horse blood serum BuChE. There is a suggestion that evolution of Gonatidae proceeds from the pelagic to benthic mode of life, and the studied squid species differ in the degree of "evolutionary changeability:" *B. anonichos, G. kamtschaticus,* and *G. tinro* are considered to be more ancient species of this family, while *B. magister* and *G. borealis,* younger and more changeable in the evolutionary aspect [3, 11]. The revealed interspecies differences in the substrate–inhibitor specificity of enzymes seem to be due to that "the Gonatidae are in the process of evolution on their pathway of specialization and development of the life form «the squid, a predator of depths»" [3].

REFERENCES

1. Rozengart, E.V., Inhibitory Specificity of Cholinesterase from Squid Optical Ganglia. Anticholinesterase Efficiency of O-ethyl-*n*-alkylmethylthiophosphonates, *Reakts. Sposob. Org. Soed.,* 1967, vol. 4, pp. 954–962.
2. Turpaev, T.M., Brestkin A.P., Rosengart, E.V., *et al.*, Cholinesterase of Squid Optical Ganglia, *Eur. J. Biochem.,* 1968, vol. 6, pp. 55–59.
3. Nesis, K.N., *Okeanicheskie golovonogie mollyuski* (Oceanic Cephalopod Molluscs), Moscow, 1985.
4. Brestkin, A.P., Kuznetsova, L.P., Moralev, S.N., *et al., Kholinesterazy nazemnykh zhivotnykh i gidrobiontov* (Cholinesterases of Terrestrial Animals and Hydrobionts), Vladivostok, 1997.
5. Rozengart, E.V., Basova, N.E., Khovanskikh, A.E., and Epshtein, L.M., Various Aspects of Substrate Specificity of Cholinesterase from Optical Ganglia of the Pacific Squid *Todarodes pacificus, Zh. Evol. Biokhim. Fiziol.,* 1996, vol. 32, pp. 384–392.
6. Rozengart, E.V., Khovanskikh, A.E., and Epshtein, L.M., Peculiarities of Inhibitory Specificity of Cholinesterase of the Pacific Squid *Todarodes pacificus, Zh. Evol. Biokhim. Fiziol.,* 1993, vol. 29, pp. 475–481.
7. Rozengart, E.V., Reactivity of Cholinesterase of the Commodore Squid *Berryteuthis magister.* Substrates and Organophosphorus Inhibitors, *Zh. Evol. Biokhim. Fiziol.,* 1996, vol. 32, pp. 576–583.
8. Rozengart, E.V., Basova, N.E., Khovanskikh, A.E., *et al.*, Interaction of Cholinesterase of Commodore Squid *Berryteuthis magister* Individuals from Different Zones of the Habitation Areal with Onium Reversible Inhibitors, *Zh. Evol. Biokhim. Fiziol.,* 1997, vol. 33, pp. 371–382.
9. Epshtein, L.M., Shevtsova, S.P., Rozengart, E.V., *et al.*, A Method for Determination of the Species Specificity of Aquatic Organisms, USSR Inventor's Certificate no. 1001898 from 09.11.1982, *Byull. Izobret.,* 1983, no. 9 from 07.03.1983.
10. Rozengart, E.V., Derzhavin, D.K., Kovalev, N.N., *et al.*, Specific Differences in Substrate Specificity of Cholinesterases of Far East Squids of the Gonatidae Family, *Doklady RAN,* 1995, vol. 342, pp. 703–704.
11. Epshtein, L.M., Comparative Studies of Serine Hydrolases in Hydrobionts, *Doctorate Sci. Diss.,* St. Petersburg, 1992.
12. Ellman, G.H., Courtney, R.D., Andres, V., and Featherstone, K.M., A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, *Biochem. Pharmacol.,* 1961, vol. 7, pp. 88–95.
13. Rozengart, E.V., Khovanskikh, A.E., and Epshtein, L.M., Study of Homogeneity of Cholinesterases in the Nervous Tissue of Squids by a Method of the Substrate–Inhibitor Analysis, *Zh. Evol. Biokhim. Fiziol.,* 1994, vol. 30, pp. 15–22.
14. Grigor'eva, G.M., Rozengart, E.V., and Turpaev, T.M., Characteristics of Specificity of Cholinesterases in the Heart and Hemolymph of Molluscs, *Fiziologiya i biokhimiya bespozvonochnykh* (Physiology and Biochemistry of Invertebrates), Kreps, E.M., Ed., Leningrad, 1968, pp. 166–175.