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Onchidal: A Naturally Occurring Irreversible Inhibitor of Acetylcholinesterase with a Novel Mechanism of Action

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SUMMARY

Onchidal has been identified as the major lipid-soluble component of the defensive secretion of the mollusc *Onchidella binneyi*, and it has been proposed as the compound responsible for the chemical protection of *Onchidella* [*Bioorg. Chem.* 7:125–131 (1978)]. In support of this hypothesis, we now report that onchidal can be found in several different species of *Onchidella* and that it is toxic to fish. Because onchidal is an acetate ester similar to acetylcholine, its ability to interact with nicotinic acetylcholine receptors and acetylcholinesterase was investigated. Although onchidal did not prevent the binding of ^{125}I-α-bungarotoxin to nicotinic acetylcholine receptors, it inhibited acetylcholinesterase in a progressive, apparently irreversible, manner. The apparent affinity of onchidal for the initial reversible binding to acetylcholinesterase (K_d) was approximately 300 μM, and the apparent rate constant for the subsequent irreversible inhibition of enzyme activity (k_{inact}) was approximately 0.1 min^{-1}. Onchidal was a substrate for acetylcholinesterase, and approximately 3250 mol of onchidal were hydrolyzed/mol of enzyme irreversibly inhibited. The calculated k_{cat} for onchidal was 325 min^{-1}. Irreversible inhibition resulted from either onchidal itself or a reactive intermediate in the enzyme-catalyzed hydrolysis of onchidal, rather than from the hydrolysis products of onchidal. Irreversible inhibition of enzyme activity was prevented by coincubation with reversible agents that either sterically block (edrophonium and decamethonium) or allosterically modify (propidium) the acetylcholine binding site. Enzyme activity was not regenerated by incubation with oxime reactivators; therefore, the mechanism of irreversible inhibition does not appear to involve acylation of the active site serine. Because onchidal contains a potentially reactive α,β-unsaturated aldehyde, irreversible inhibition of acetylcholinesterase may result from formation of a novel covalent bond between the toxin and the enzyme. Thus, this novel toxin could potentially be exploited in the design of a new class of anticholinesterase insecticides and in the identification of amino acids that contribute to the binding and hydrolysis of acetylcholine.

Naturally occurring toxins are often selective inhibitors of protein function, and this property can often be exploited for a variety of purposes. For instance, physostigmine, a naturally occurring inhibitor of acetylcholinesterase, has proven to be useful in the treatment of glaucoma as well as in understanding the kinetic mechanism of acetylcholinesterase (1, 2). Naturally occurring inhibitors of nicotinic acetylcholine receptors, such as the snake α-toxins, *d*-tubocurarine, and lophotoxin, have also proven to be useful either clinically or in elucidating structural details of the nicotinic acetylcholine receptor (3, 4).

The Onchidiacea family of molluscs do not have the protection of a hard external shell as do most molluscs. Instead, when molested they secrete a viscous fluid from specialized glands. In two species of Onchidella (*Onchidella floridanum* and *Onchidella borealis*), this defensive secretion has been shown to act as a deterrent to potential predators (5, 6). Onchidal has been identified as the major lipid-soluble component of the defensive secretion of *Onchidella binneyi*, and it was therefore proposed as the compound responsible for the chemical protection of *Onchidella* (7). However, the distribution of onchidal in different species of *Onchidella* was not reported and, apart from inhibiting the growth of *Staphylococcus aureus*, no biological activity of onchidal was reported (7). We have extended these initial studies by demonstrating that onchidal is contained in several different species of *Onchidella* and by demonstrating that purified onchidal can be toxic to fish.

Because onchidal is an acetate ester similar to acetylcholine and because cholinergic neurotransmission is often the site of action of natural products involved in chemical defense, we investigated the ability of onchidal to inhibit acetylcholinesterase and the nicotinic acetylcholine receptor. Although onchidal did not prevent the binding of ^{125}I-α-bungarotoxin to nicotinic acetylcholine receptors, it was shown to be an active site-directed irreversible inhibitor of acetylcholinesterase. The

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ABBREVIATIONS: TMB-4, 1,1′-(trimethylene)bis-[4-(hydroxyimino)methyl]pyridinium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HI-6, 1,1′-[oxybis(methylene)]-[4-carbamoyl-2′-(hydroxyimino)methyl]pyridinium.

structure and pharmacology of onchidal suggest that the irreversible inhibition of acetylcholinesterase results from a novel mechanism involving covalent reaction between onchidal and an amino acid within the acetylcholine binding site.

Materials and Methods

Chemicals. ^{125}I-α-Bungarotoxin (approximately 17 Ci/g) was obtained from New England Nuclear. 5′,5′-Dithiobis-(1-nitrobenzoic acid), TMB-4, acetylthiocholine, malate, malate dehydrogenase (2700 units/mg), and citrate synthetase (150 units/mg) were purchased from Sigma. Coenzyme A, ATP, NAD, acetate kinase (200 units/mg), and phosphotransacetylase (1000 units/mg) were purchased from Boehringer Mannheim. HI-6 was kindly provided by Dr. B. P. Doctor (Walter Reed Army Research Institute) and 3,3-dimethylbutyl methylphosphonofluoridate was kindly provided by Dr. H. A. Berman (State University of New York at Buffalo).

Purification of onchidal. Intact specimens were placed in acetone at the site of collection. An ethyl acetate extract was then purified by flash chromatography on a silica gel column (Kieselgel 60) or by high pressure liquid chromatography on a silica column (Partisil M9), using 25:75 ether/hexane as the eluant. Onchidal was identified by thin layer chromatography on silica gel G using 50:50 ethyl acetate/hexane (R_f = 0.3), and its structure was confirmed by proton NMR. The proton NMR of onchidal contains a signal at δ 9.40 (d, J = 1 Hz) due to the aldehyde proton, three methyl singlets at δ 0.89, 0.99, and 2.05, signals for the exocyclic methylene protons at δ 4.50 and 4.81, signals at δ 6.10 (dd, J = 14, 1 Hz) and 8.26 (d, J = 14 Hz) due to the *trans*-enol protons, and a signal at δ 6.41 (t, J = 7 Hz) due to the β-proton at the α,β-unsaturated aldehyde. No other secondary metabolites were present in the acetone extract. Purified onchidal was brought up to a concentration of 10 mM in ethyl acetate or dimethyl sulfoxide and stored at −20°. Purity was checked by thin layer chromatography periodically, and onchidal was stable for several months.

Fish toxicity bioassay. Onchidal was dissolved in solvent (acetone, ethanol, or methanol), and either onchidal or solvent alone was added to 40 ml of water. A single goldfish was added to the water and observed for 1 hr. Two or three similar sized goldfish were utilized for control and experimental conditions. At the end of the hour, the number of dead fish was recorded and the remaining live fish were placed in a separate tank for further observation.

Inhibition of acetylcholinesterase and the nicotinic acetylcholine receptor. The 11 S form of acetylcholinesterase was purified from *Torpedo californica* (8). Onchidal was incubated at 23° with acetylcholinesterase in 100 μl of 100 mM sodium phosphate buffer (pH 7.0) containing 1% dimethyl sulfoxide. Aliquots (5 μl) were removed and the enzyme was diluted at least 100-fold into 1.0 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM acetylthiocholine and 0.5 mM 5′,5′-dithiobis-(1-nitrobenzoic acid). The initial rate of color formation resulting from hydrolysis of the acetylthiocholine was determined in a Gilford spectrophotometer at 412 nm. For protection experiments, acetylcholinesterase was incubated with the reversible inhibitors edrophonium, decamethonium, propidium, or *N*-methylacridinium for 15 min before the addition of onchidal. Aliquots (5 μl) were removed every 15 min for the first 2 hr and every 30 min for the next 4 hr, and the enzyme was diluted 1000-fold for determination of enzyme activity. Inhibition of enzyme activity due to the presence of the reversible inhibitors was negligible after the 1000-fold dilution.

Membranes containing nicotinic acetylcholine receptors (1–2 nmol of α-toxin sites/mg of protein) were purified from *T. californica* (4). Onchidal (up to 1.0 mM) was incubated at 23° for 2 hr with the receptor (0.22 pmol of α-toxin sites) in 125 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 1.0 mM EDTA, 1.0 mM EGTA, and 1% dimethyl sulfoxide. The ability of the receptor to bind ^{125}I-α-bungarotoxin was then determined as described previously (4).

Hydrolysis of onchidal. The acetate produced by hydrolysis of onchidal was assayed using a sensitive coupled enzyme assay (9). Onchidal and 11 S acetylcholinesterase were allowed to react in 200 μl of 100 mM sodium phosphate buffer (pH 7.0) containing 1% dimethyl sulfoxide at 23° for 3 to 4 hr, until greater than 99% of the enzyme activity was irreversibly inhibited. The acetate produced was then determined by the addition of 1.0 ml of the coupled enzyme assay mix containing 150 mM triethanolamine buffer (pH 8.0), 10 mM malate, 3 mM $MgCl_2$, 1.0 mM NAD, 170 μM coenzyme A, 2.7 mM ATP, 5 units of acetate kinase, 7 units of phosphotransacetylase, 4 units of citrate synthetase, and 8 units of malate dehydrogenase. The conversion of acetate to citrate and the resultant reduction of NAD to NADH reached equilibrium within 60 min at 23°. The concentration of NADH was then determined in a Gilford spectrophotometer at 340 nm. A standard curve of absorbance at 340 nm versus known concentrations of acetate was utilized to determine the concentration of acetate produced by hydrolysis of onchidal.

Regeneration of enzyme activity by oxime reactivators. Acetylcholinesterase was incubated at 23° in 100 mM sodium phosphate buffer (pH 7.0) containing 1% dimethyl sulfoxide and either 3,3-dimethylbutyl methylphosphonofluoridate or onchidal, until greater than 99% of the enzyme activity was inhibited (approximately 5 min and 4 hr, respectively). The inhibited enzyme was then diluted 100-fold into 100 mM sodium phosphate buffer (pH 7.0) containing an oxime reactivator (HI-6 or TMB-4) and was allowed to incubate at 23°. Aliquots (50 μl) were removed and the enzyme was diluted 20-fold into 1.0 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM acetylthiocholine and 0.5 mM 5′,5′-dithiobis-(1-nitrobenzoic acid), for determination of the initial rate of enzyme activity. The oxime reactivators HI-6 and TMB-4 did not inhibit enzyme activity when kept below 0.1 mM.

Results

Onchidal was initially isolated from the defensive secretion of *O. binneyi*, collected from Baja California, Mexico (7). However, onchidal could also be obtained from several other species of *Onchidella*, collected from different countries (Table 1). The only reported biological activity of onchidal is inhibition of the growth of *S. aureus* (7). Because inhibition of bacterial growth presumably does not provide a deterrent to predation, the biological activity of onchidal was investigated in simple bioassays as part of a general screening program directed towards identification of biologically active marine natural products (10). Concentrations of onchidal greater than 36 μM were toxic to 100% of the goldfish tested (Table 2).

Onchidal is a relatively small molecule that contains an acetoxy group similar to that found in acetylcholine (Fig. 1). The structural similarity between onchidal and acetylcholine suggests that the toxicity of onchidal might result from inhibition of proteins involved in cholinergic neurotransmission. Onchidal was, therefore, investigated *in vitro* for its ability to inhibit acetylcholinesterase or the nicotinic acetylcholine receptor purified from the electric organ of *T. californica*. Membranes enriched in the nicotinic acetylcholine receptor were allowed to equilibrate with onchidal for 2 hr, and the receptors were then assayed for their ability to bind ^{125}I-α-bungarotoxin.

TABLE 1
Concentration of onchidal in different species of *Onchidella*

Organism	Collection site	Onchidal concentration
		μg/animal
O. binneyi	Baja California, Mexico	230
O. borealis	Central California, USA	33
O. nigricans	New Zealand	18
O. patelloides	Australia	42

TABLE 2
Concentration-dependent toxicity of onchidal in goldfish

Onchidal concentration	Survival after 1 hr
μM	%
3.62	100
18.1	50
36.2	0
90.5	0
181	0
362	0

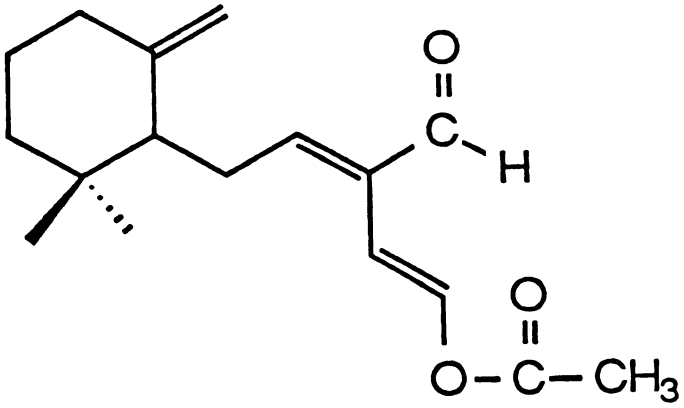

Fig. 1. Structure of onchidal.

Concentrations of onchidal up to 1.0 mM had no effect on the ability of nicotinic acetylcholine receptors to bind ^{125}I-α-bungarotoxin.

Onchidal was incubated with purified acetylcholinesterase in the absence of other substrates or inhibitors, and the enzyme was then diluted 200-fold for determination of enzyme activity. Onchidal inhibited acetylcholinesterase in a progressive, time-dependent, irreversible manner (Fig. 2A). The apparent first-order inhibition rate constant appeared to saturate at high concentrations of onchidal, presumably reflecting saturation of an initial reversible complex between onchidal and the enzyme (Fig. 2B). These results can be described by the kinetic scheme:

$$I + E \underset{}{\overset{K_d}{\rightleftharpoons}} IE \xrightarrow{k_{inact}} I\text{-}E^* \qquad (1)$$

where K_d represents the dissociation constant of an initial reversible bimolecular complex (IE) between onchidal (I) and acetylcholinesterase (E), and k_{inact} represents a subsequent irreversible unimolecular rate constant resulting in the formation of an inactive enzyme complex (I-E^*). Evaluation of a double-reciprocal plot revealed that the K_d for onchidal was approximately 3×10^{-4} M and that the k_{inact} was approximately 0.1 min^{-1} (Fig. 2C).

Because onchidal is an acetate ester, the ability of acetylcholinesterase to utilize onchidal as a substrate was investigated. A sensitive coupled enzyme assay was employed to determine the acetate released upon hydrolysis of onchidal (9). Acetylcholinesterase was incubated with an excess of onchidal under conditions that resulted in complete irreversible inhibition of enzyme activity. Analysis of the amount of acetate produced versus the amount of enzyme inhibited revealed a linear relationship, and approximately 3250 mol of onchidal were hydrolyzed/mol of enzyme irreversibly inhibited (Fig. 3). Thus, the

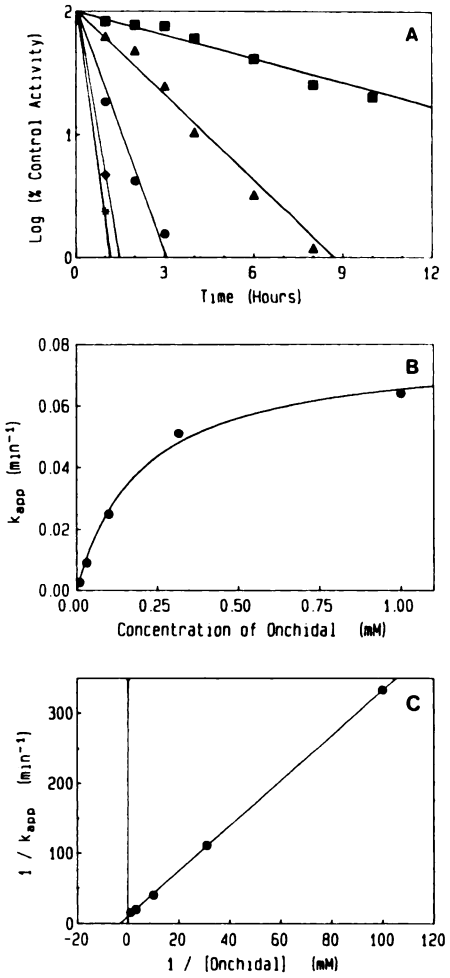

Fig. 2. Progressive irreversible inhibition of acetylcholinesterase by onchidal. Acetylcholinesterase (9.3×10^{-8} M) was allowed to react with 1.0×10^{-3} M (•), 3.3×10^{-4} M (◆), 1.0×10^{-4} M (●), 3.3×10^{-5} M (▲), or 1.0×10^{-5} M (■) onchidal. At the indicated times, 5-μl aliquots were removed and the enzyme was diluted 200-fold for determination of the initial rate of acetylcholinesterase activity. A, The $\log_{10}$ of the percentage of control enzyme activity is shown versus time. B, The apparent first-order rate constant (k_{app}) for inhibition of enzyme activity (obtained from linear regression of the data in A) is shown versus the concentration of onchidal. C, A double-reciprocal plot of the dependence of k_{app} (obtained from B) on the concentration of onchidal.

interaction of onchidal with acetylcholinesterase is more appropriately described by the kinetic scheme:

$$I + E \underset{}{\overset{K_d}{\rightleftharpoons}} IE \begin{matrix} \xrightarrow{k_{inact}} I\text{-}E^* \\ \xrightarrow{k_{cat}} E + P \end{matrix} \qquad (2)$$

where k_{cat} represents the rate of catalysis of onchidal and product (P) represents the hydrolysis products of onchidal. Knowledge of k_{inact} (0.1 min^{-1}) and the partition ratio (3250) allows calculation of k_{cat} (325 min^{-1}) for onchidal.

Because onchidal was hydrolyzed by acetylcholinesterase, it was of interest to determine whether onchidal itself or hydrolysis products of onchidal resulted in irreversible inhibition of enzyme activity. Incubation of onchidal with increasing concentrations of acetylcholinesterase revealed that onchidal was unable to completely inhibit higher concentrations of the enzyme, even when the onchidal remained in molar excess over enzyme (Fig. 4). Separate experiments under similar conditions

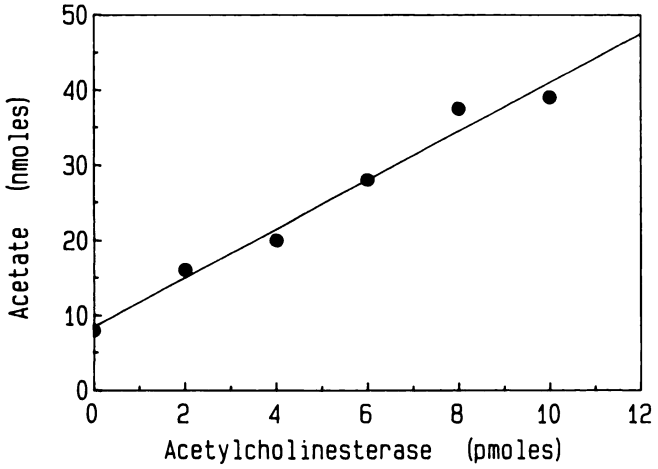

Fig. 3. Hydrolysis of onchidal by acetylcholinesterase. Onchidal (1×10^{-3} M) was incubated with acetylcholinesterase (0–5 × 10^{-8} M) until the enzyme activity was completely inhibited. The acetate produced by hydrolysis of onchidal was then determined with a sensitive coupled enzyme assay, as described in Materials and Methods. The amount (nmol) of acetate produced is presented versus the amount (pmol) of acetylcholinesterase irreversibly inhibited.

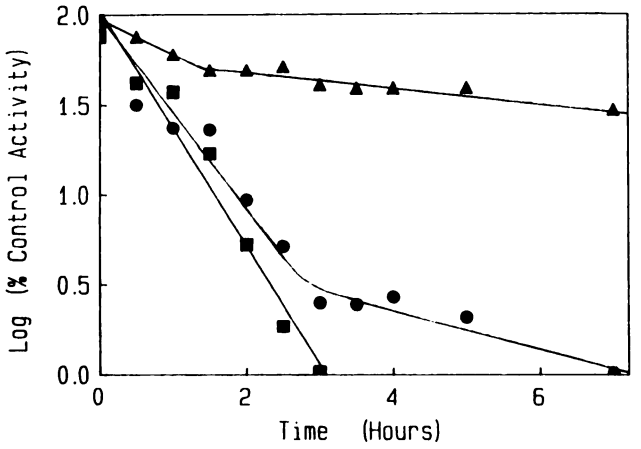

Fig. 4. Inhibition of increasing concentrations of acetylcholinesterase by onchidal. Onchidal (1×10^{-4} M) was allowed to react with 9.3×10^{-9} M (■), 9.3×10^{-8} M (●), or 9.3×10^{-7} M (▲) acetylcholinesterase. At the indicated times, 5-μl aliquots were removed and the enzyme was diluted 100-, 1,000-, and 10,000-fold, respectively, for determination of the initial rate of acetylcholinesterase activity.

revealed that the increased concentrations of acetylcholinesterase resulted in the complete hydrolysis of onchidal (data not shown).

The structural similarity between onchidal and acetylcholine, the ability of onchidal to inhibit enzyme activity, and the hydrolysis of onchidal by acetylcholinesterase suggest that onchidal binds to the acetylcholine recognition site. Therefore, several reversible enzyme inhibitors were investigated for their ability to prevent the irreversible inhibition of enzyme activity produced by onchidal. Edrophonium, decamethonium, and propidium protected the enzyme from irreversible inhibition by onchidal, whereas *N*-methylacridinium was unable to protect the enzyme (Fig. 5).

Esterification of the active site serine is the common mechanism of action of most, if not all, irreversible inhibitors of acetylcholinesterase (1, 2). Deesterification of the active site serine can be effected by active site-directed oximes such as

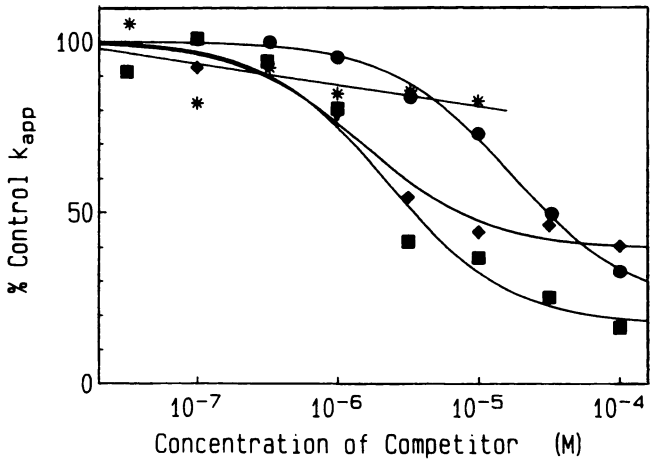

Fig. 5. Protection of onchidal-mediated inhibition of acetylcholinesterase by reversible enzyme inhibitors. Acetylcholinesterase (9.3×10^{-8} M) was incubated with onchidal (3.3×10^{-4} M) in the presence or absence of the reversible inhibitors edrophonium (■), decamethonium (●), propidium (◆), or *N*-methylacridinium (*). Aliquots (5 μl) were removed every 15 min for the first 2 hr, followed by every 30 min for the next 4 hr, and the enzyme was diluted 1000-fold for determination of the initial rate of enzyme activity. The apparent rate constant (k_{app}) for the onchidal-mediated irreversible inhibition of enzyme activity was determined for each concentration of reversible inhibitor (as described in Fig. 2), and the percentage of control k_{app} is shown versus the concentration of reversible inhibitor.

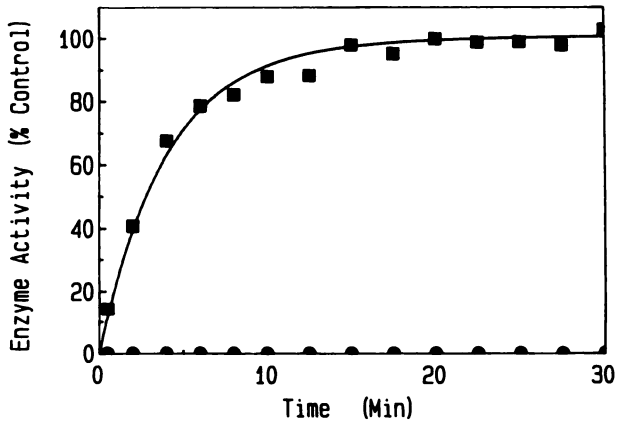

Fig. 6. Effects of HI-6 on enzyme activity inhibited by an organophosphonate or onchidal. Acetylcholinesterase (1×10^{-6} M) was incubated with either 5×10^{-5} M 3,3-dimethylbutyl methylphosphonofluoridate (■) or 2×10^{-3} M onchidal (●), until greater than 99% of the enzyme activity was inhibited. The inhibited enzyme was then diluted 100-fold into buffer containing HI-6 (1×10^{-4} M). At the indicated times, 50-μl aliquots were removed and the enzyme was diluted 20-fold for determination of the initial rate of enzyme activity.

the bis-quaternary bis-oxime TMB-4 and the bis-quaternary mono-oxime HI-6 (1, 11). Although HI-6 (0.1 mM) rapidly regenerated enzyme activity inhibited by the organophosphonate 3,3-dimethylbutyl methylphosphonofluoridate, enzyme inhibited by onchidal could not be regenerated (Fig. 6). Similar results were obtained with 1.0 mM HI-6 and 0.1 mM or 1.0 mM TMB-4 (data not shown).

Discussion

Onchidella secrete a viscous fluid from specialized glands when they are molested, and this defensive secretion has been shown to act as a deterrent to potential predators (5, 6).

Onchidal is the major lipid-soluble component of the defensive secretion of *O. binneyi* and was, therefore, proposed as the compound responsible for the protection of *Onchidella* (7). However, the distribution of onchidal in different species of *Onchidella* was not investigated, and a biological activity consistent with the proposed role of onchidal was not reported.

The results presented herein demonstrate that onchidal can be found in four of the eight known species of *Onchidella*. In addition, onchidal was found to be toxic to goldfish. Although goldfish are not potential predators of *Onchidella*, these results demonstrate that onchidal has a distribution and a biological activity consistent with its proposed role in the chemical defense of *Onchidella*.

The structural similarity between onchidal and acetylcholine suggested that the toxicity of onchidal could result from inhibition of either nicotinic acetylcholine receptors or acetylcholinesterase. Because *in vitro* assays require substantially less onchidal than *in vivo* studies, the ability of onchidal to inhibit either the nicotinic acetylcholine receptor or acetylcholinesterase was investigated *in vitro*.

Onchidal (1.0 mM) did not inhibit the ability of nicotinic acetylcholine receptors to bind ^{125}I-α-bungarotoxin. However, onchidal produced a progressive irreversible inhibition of acetylcholinesterase. The apparent first-order inhibition of enzyme activity appeared to saturate with increasing concentrations of onchidal, consistent with the formation of a reversible complex between onchidal and the enzyme, followed by the subsequent irreversible inhibition of enzyme activity. Although the apparent affinity of the initial reversible complex was relatively low (K_d = 300 μM), the subsequent irreversible step proceeded sufficiently rapidly (k_{inact} = 0.1 min^{-1}) to allow for substantial inhibition of enzyme activity even at relatively low concentrations of onchidal.

Incubation of onchidal with acetylcholinesterase resulted in the production of acetate, demonstrating that onchidal is a substrate for acetylcholinesterase. Analysis of the extent of acetate production after complete inhibition of different concentrations of acetylcholinesterase revealed that approximately 3250 mol of onchidal were hydrolyzed/mol of enzyme inhibited. Organophosphate and carbamate inhibitors of acetylcholinesterase have partition ratios (mol of toxin hydrolyzed/mol of enzyme irreversibly inhibited) that approach unity. Therefore, the relatively high partition ratio for onchidal suggests that the mechanism of inhibition utilized by onchidal may be distinctly different from other irreversible inhibitors (12). The rate of hydrolysis of onchidal (k_{cat}) can be calculated from the k_{inact} and the partition ratio (k_{cat} = k_{inact} × the partition ratio). The calculated k_{cat} of 325 min^{-1} for onchidal is relatively slow (k_{cat} for acetylcholine is greater than 10^5 min^{-1}), suggesting that onchidal is not a very good substrate (13).

The ability of acetylcholinesterase to hydrolyze onchidal raised the question of whether inhibition of enzyme activity resulted from onchidal itself or from a product of the enzymatic hydrolysis of onchidal. Titration of onchidal with increasing concentrations of enzyme revealed that onchidal was unable to completely inhibit higher concentrations of acetylcholinesterase. In these experiments onchidal was in molar excess and was completely hydrolyzed. Therefore, the hydrolysis products of onchidal were not responsible for irreversible inhibition of enzyme activity. Thus, irreversible inhibition of enzyme activity resulted either from onchidal itself or from a reactive intermediate produced during hydrolysis of onchidal. In the latter case, onchidal would be considered a mechanism-based inhibitor of acetylcholinesterase (12).

The ability of acetylcholinesterase to hydrolyze onchidal demonstrated that onchidal is capable of interacting with the esteratic subsite of the enzyme. However, protection experiments with reversible inhibitors were employed to define the onchidal binding site involved in irreversible inhibition of enzyme activity. *N*-Methylacridinium is thought to interact selectively with the quaternary ammonium binding subsite involved in binding the quaternary ammonium group of acetylcholine (2, 14). Thus *N*-methylacridinium effectively inhibits the hydrolysis of substrates containing a quaternary ammonium group, but it does not affect or even enhances the hydrolysis of neutral substrates (14). Because *N*-methylacridinium was unable to protect the enzyme from irreversible inhibition, onchidal apparently does not interact with the quaternary ammonium binding subsite. This observation is perhaps not unexpected, because onchidal does not contain a positively charged nitrogen and it presumably behaves like other neutral esters.

In contrast to *N*-methylacridinium, several other reversible inhibitors of enzyme activity were able to protect the enzyme from irreversible inhibition by onchidal. These included decamethonium, edrophonium, and propidium. Decamethonium is thought to interact with the same quaternary ammonium binding subsite as acetylcholine, as well as with an additional quaternary ammonium binding site located approximately 14 Å away (15), whereas edrophonium is thought to interact with both the esteratic and quaternary ammonium binding subsites (1, 2). In contrast, propidium is thought to interact selectively with a peripheral anionic site and thereby allosterically alter the esteratic subsite (15, 16). Taken together, the protection experiments suggest that interaction between onchidal and the esteratic subsite of acetylcholinesterase results in irreversible inhibition of enzyme activity.

All known irreversible inhibitors of acetylcholinesterase that are substrate analogs inhibit enzyme activity by covalent modification of the active site serine (1, 2). This covalent modification can be reversed, and enzyme activity thereby regenerated, by active site-directed nucleophiles such as the oxime reactivators HI-6 and TMB-4 (1, 2, 11). Although HI-6 and TMB-4 were able to regenerate enzyme activity inhibited by a representative organophosphonate, enzyme activity inhibited by onchidal could not be regenerated. These results suggest that the mechanism of irreversible inhibition utilized by onchidal is substantially different from that utilized by all other known irreversible inhibitors.

Several possible reaction mechanisms could be utilized by onchidal. For instance, the aldehyde of onchidal could form a Schiff base with a reactive lysine in the enzyme. In addition, the α,β-unsaturated double bond in conjugation with the aldehyde is potentially reactive, and nucleophilic attack of a cysteine, lysine, histidine, or tyrosine could occur at the electron-deficient β-unsaturated carbon. Alternatively, hydrolysis of onchidal could result in the formation of a reactive intermediate that is capable of irreversible inhibition of enzyme activity. For instance, hydrolysis of onchidal presumably results in an enol that could tautomerize to form a second aldehyde. The 1,4-dialdehyde could then react with a lysine to form a pyrrol. Isolation and characterization of the covalent reaction product

will ultimately be required to determine whether onchidal is a mechanism-based suicide substrate or simply a covalently reactive affinity reagent that can be utilized by the enzyme as a substrate (12).

The structure and pharmacology of onchidal suggest that inhibition of acetylcholinesterase results from a novel covalent reaction between onchidal and an amino acid within the acetylcholine binding site. Thus, onchidal could potentially be exploited in the design of a new class of acetylcholinesterase insecticides and in the identification of amino acids that contribute to the binding and hydrolysis of acetylcholine. Further investigation of the toxicity of onchidal in higher organisms may help clarify the role of onchidal in the chemical protection of *Onchidella*, whereas investigation of structural analogs, and in particular radiolabeled analogs, may elucidate the covalently modified amino acid and the mechanism of action of this novel toxin.

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