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Cartap Hydrolysis Relative to Its Action at the Insect Nicotinic Channel

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The insecticide cartap is the bis(thiocarbamate) derivative of 2-(dimethylamino)propane-1,3-dithiol, which on oxidation forms the natural toxicant nereistoxin (NTX) [4-(dimethylamino)-1,2-dithiolane]. Both cartap and NTX are ion channel blockers of the nicotinic acetylcholine receptor (nAChR). Cartap was originally proposed to act only after metabolic conversion to NTX and later suggested to block directly without activation. The present study uses a new approach to differentiate these hypotheses, that is, pH effects on channel-blocking activity and hydrolysis rates. As controls, mecamylamine (the classic channel blocker) and NTX are stable and similar in channel-blocking potency ([^{3}H]-thienylcyclohexylpiperidine binding assay, honeybee nAChR) at pH 6.1−8.4. In contrast, cartap is >200-fold more effective at pH 7.4 than at pH 6.1, indicating that it undergoes hydrolytic activation. Cartap slowly hydrolyzes to cartap monothiol at pH 6.1 but quickly forms the dithiol and some NTX at pH 7.4. The relationship between potency and hydrolysis products at various pH ranges suggests that cartap dithiol is the most plausible blocking agent.

KEYWORDS: Bensultap; cartap; ion channel blocker; nereistoxin; nicotinic acetylcholine receptor

INTRODUCTION

The insect nicotinic acetylcholine receptor (nAChR)/ion channel complex is an important target for the action of insecticides. Nicotinoids and neonicotinoids act as agonists, inducing excitatory neurotoxicity (*1*−*3*). On the other hand, cartap and bensultap (two commercial insecticides) (*4*−*8*) and nereistoxin (NTX) (their natural product prototype) (*5*, *9*) (**Figure 1**) have the opposite action; that is, they are blockers causing inhibitory neurotoxicity (*5*, *7*, *10*−*18*). Interestingly, the different mechanisms of action (nicotinic agonists versus blockers) should circumvent possible cross-resistance at the nAChR. We recently concluded that cartap, without the requirement of metabolic activation, acts directly and selectively at the noncompetitive blocker site of the insect nAChR/channel (*19*). This topic is revisited here on considering the rapid hydrolysis of cartap with a $t_{1/2}$ of 10 min at pH 7 (*20*). On this basis cartap may not act directly but instead undergo activation with one of the hydrolytic intermediates or NTX serving as the actual channel blocker. This possibility is examined by considering the effect of pH on cartap hydrolysis and the action of cartap and NTX at the noncompetitive blocker site of the honeybee (*Apis mellifera*) nicotinic channel. We finally discuss the active form of cartap or its hydrolysis products relative to channel-blocking activity and toxicity.

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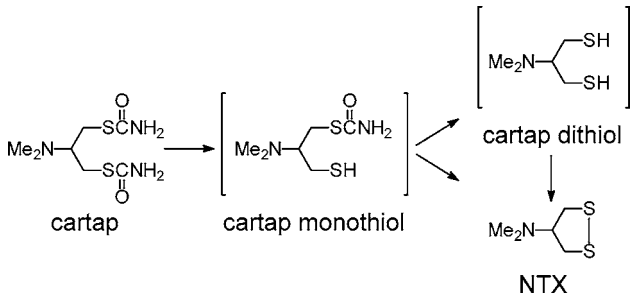

Figure 1. Structures of cartap, its mono- and dithiol, and nereistoxin (NTX). Bensultap is the bis(benzenethiosulfonate) derivative of cartap dithiol.

MATERIALS AND METHODS

Chemicals. Sources were as follows: [^{3}H]-*N*-[1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP, 42 Ci/mmol) from NEN Life Science Products (Boston, MA); cartap hydrochloride (>98% purity) from Takeda Chemical Industries (Tokyo, Japan); NTX oxalate from Wako Pure Chemical (Osaka, Japan); mecamylamine hydrochloride from Sigma (St. Louis, MO). Cartap dithiol was prepared by treatment of NTX in water [10% (w/v), 100 μL] with 3 equiv of sodium borohydride for 3 h at 25 °C.

Hydrolysis Products of Cartap Analyzed by Liquid Chromatography−Mass Spectrometry (LC-MS). A Finnigan-MAT model TSQ-700 triple-quadrupole mass spectrometer was used with an electrospray ionization (ESI) source. The mobile phase of acetonitrile (15%) and aqueous 0.1% trifluoroacetic acid (TFA) (85%) was pumped with a Perkin-Elmer model 250 HPLC (Norwalk, CT) to a Rheodyne model 7125 injector (Cotati, CA) with a 10 μL sample loop. Samples

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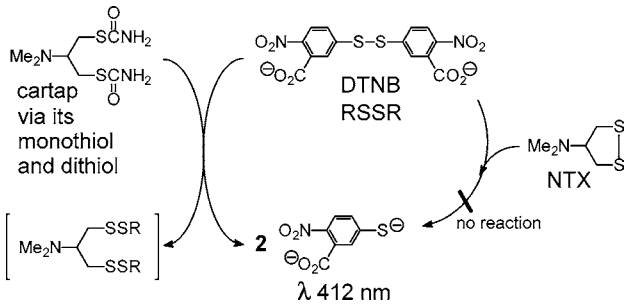

Figure 2. Reaction of cartap hydrolysis products with DTNB (Ellman's reagent). R = 3-carboxy-4-nitrophenyl.

were analyzed on an Alltech ODS2 column (150 mm × 4.6 mm i.d., 3 μm) (Deerfield, IL) at 0.2 mL/min with UV detection (HP 1050 series) at 205 nm prior to entering the source of the TSQ-700 operated at 4.0 kV potential. The heated capillary temperature was set at 250 °C, and nitrogen gas was used at a sheath gas pressure of 40 psi. The hydrolysis products of cartap were studied in water and 100 mM pH 6.0, 7.4, and 9.0 phosphate buffers after incubation for 30 min at 25 °C. Standards used for comparison were cartap, cartap dithiol, and NTX described above.

Hydrolysis of Cartap Analyzed According to the 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) Procedure. Liberation of free thiol group(s) from cartap in the presence of DTNB results in stoichiometric formation of the yellow 3-carboxy-4-nitrophenylthiolate anion (*21*) (**Figure 2**). NTX, which lacks a free thiol group, was used as a control. The test compound (50 μM) and DTNB (330 μM) in 100 mM phosphate buffer at pH 6.0, 7.4, or 9.0 (3 mL) were incubated at 25 °C, and the absorbance at 412 nm was determined at 0, 5, 10, 20, 30, 60, 90, and 120 min. Standards for stoichiometric determinations were GSH and dithiothreitol at 50 μM (1 and 2 free thiol equiv, respectively) giving appropriate absorbance values that did not vary over the pH range examined.

Radioligand Binding Assays with *Apis* Head Membranes. Using the procedure of Lee et al. (*19*), heads from bees frozen at −80 °C were homogenized with a Polytron in 9 volumes of 20 mM Tris−hydrochloride buffer (pH 7.4) containing 0.02% sodium azide and 0.1 mM diisopropyl fluorophosphate (to inhibit acetylcholinesterase) for 30 s and, following a 60 s pause, the homogenization and pause cycle was repeated twice. The homogenate was passed through four layers of cheesecloth and the filtrate centrifuged at 1000*g* for 10 min and the supernatant at 40000*g* for 45 min. The pellet was washed with distilled water by centrifugation at 40000*g* for 45 min and then prepared at 2 mg of protein/mL in distilled water.

[^{3}H]TCP was used as the reporter molecule for the noncompetitive blocker site in the *Apis* nAChR/channel. The method was first developed with [^{3}H]phencyclidine (*18*) and then adapted for use with [^{3}H]TCP (*19*). The standard assay involved *Apis* head membranes (0.2 mg of protein), 5 nM [^{3}H]TCP, a candidate inhibitor or none (control), and unlabeled mecamylamine (0 or 1 mM) in 250 μL of 100 mM phosphate buffer at the specified pH. The binding reaction of 60 s at 25 °C was terminated by rapid filtration on a GF/B filter (presoaked in 0.1% polyethyleneimine) and rinsing three times with 2.5 mL of ice-cold 0.9% sodium chloride. Total, specific, and nonspecific binding were determined at each pH. Specific binding was considered to be the difference between total and nonspecific binding in the absence and presence, respectively, of mecamylamine at 1 mM. The IC_{50} value for a test chemical, the molar concentration necessary for 50% displacement of radioligand specific binding, was determined by iterative nonlinear least-squares regression using the SigmaPlot program (Jandel Scientific Software, San Rafael, CA).

Special attention was given to the effect of pH on [^{3}H]TCP binding and inhibition thereof. The assay tubes initially contained 100 mM phosphate buffer at various pH values (125 μL) and mecamylamine (0 mM for total binding or 1 mM for nonspecific binding) in distilled water (25 μL). The candidate inhibitor was added in 100 mM pH 6.0 phosphate (25 μL) followed by [^{3}H]TCP in distilled water (25 μL). The membrane protein (0.2 mg) in distilled water (100 μL) was added last with incubation for 60 s before filtration, washing, and determination of bound [^{3}H]TCP. The initial pH values for the phosphate buffer of 5.0, 6.0, 7.0, 7.4, 8.0, and 9.0 were finally 5.4, 6.1, 6.9, 7.2, 7.5, and 8.4, respectively, after the various additions before the 60 s binding assay.

Table 1. LC-MS Characteristics of Cartap, Cartap Monothiol, Cartap Dithiol, and Nereistoxin (NTX)

compound	t_R[a] (min)	ESI/LC-MS, *m/z* (amu) (% relative abundance)
cartap	11.23	195 $[M + H - CONH]^+$ (100), 238 $[M + H]^+$ (62), 152 $[M + H - (CONH)_2]^+$ (33), 105 $[(CH_3)_2NCHCH_2SH]^+$ (10)
cartap monothiol	12.36	195 $[M + H]^+$ (100), 152 $[M + H - CONH]^+$ (31)
cartap dithiol	15.22	152 $[M + H]^+$ (100), 193 $[M + H + CH_3CN]^+$ (47)
NTX	16.25	191 $[M + H + CH_3CN]^+$ (100), 150 $[M + H]^+$ (40)

[a] See Materials and Methods for conditions.

Table 2. Hydrolysis Products of Cartap in Water and at pH 6.0, 7.4, and 9.0 Analyzed by LC-MS

	relative area for $[M + H]^+$ at 30 min and indicated pH[a]			
compound	water	pH 6.0	pH 7.4	pH 9.0
cartap	267	190	79	14
cartap monothiol[b]	81	52	420	173
cartap dithiol[b]	0	0	78	149
NTX[b]	1	1	43	72

[a] Cartap is the only compound detected at 0 min. [b] Data are not corrected for sensitivity factors and are therefore not appropriate for quantitative comparisons between compounds.

RESULTS

Hydrolysis Products of Cartap Analyzed by LC-MS. Cartap, cartap monothiol, cartap dithiol, and NTX are well separated by LC, and they give characteristic ESI fragments for $[M + H]^+$ (all compounds), $[M + H - (CONH)_2]^+$ and/or $[M + H - CONH]^+$ (thiocarbamates), and $[M + H + CH_3CN]^+$ (cartap dithiol and NTX) (**Table 1**).

Cartap undergoes extensive conversion to the monothiol, dithiol, and NTX at pH 7.4 and 9.0 within 30 min (**Table 2**). In contrast, cartap monothiol is the only major product detected in water or at pH 6.0 after 30 min.

Stoichiometry and Hydrolysis Rate of Cartap To Liberate Free Thiol Group(s). Hydrolysis of cartap to liberate free thiol substituents in the presence of DTNB yields the carboxynitrophenylthiolate anion, allowing monitoring of the stoichiometry and overall rates. As expected, the reaction rate increases with pH, that is, 6.0 < 7.4 < 9.0. Cartap generates 1.8 equiv of free thiol at pH 7.4 or 9.0, and the reaction appears to be essentially complete in 120 min (**Figure 3A**). Assuming the reaction at pH 9.0 is complete in 120 min, the loss of bound thiol equivalent follows apparent first-order kinetics at pH 6.0, 7.4, and 9.0 (**Figure 3B**). The free thiol equivalent liberated in 5 min is 5, 16, and 33% of that at 120 min with the $t_{1/2}$ for total free thiol liberation of 116, 24, and 12 min, respectively (**Figure 3**).

As a control, NTX does not react with DTNB at pH 6.0, 7.4, or 9.0 but instead gives the same absorbance curve over 120 min as DTNB alone (see Supporting Information).

Effect of pH on [^{3}H]TCP Binding (Figure 4). Specific binding of [^{3}H]TCP at pH 6.1 is 26% and at pH 8.4 is 84% of optimal at pH 7.5 (100%), allowing the determination of IC_{50} values for cartap and its derivatives in the range of pH 6.1−8.4. Very little binding is evident at pH 5.4.

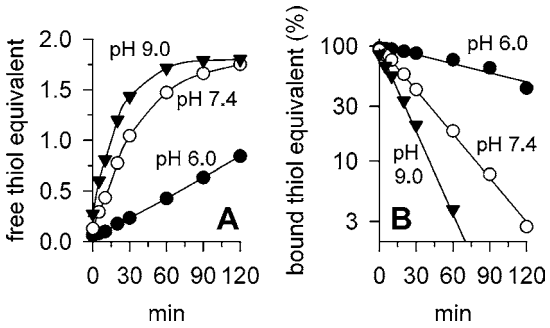

Figure 3. Stoichiometry (**A**) and hydrolysis rates (**B**) of cartap at pH 6.0, 7.4, and 9.0 determined according to the DTNB procedure. A free thiol equivalent of 1.0 gives absorbance 0.85 independent of the pH range examined based on GSH and dithiothreitol (one and two free thiol equivalents, respectively) as the standards. Rate curves (**B**) assume the reaction at pH 9.0 is complete in 120 min. Cartap has two bound thiol equivalents (100%). Symbols are means of three separate determinations with <10% SD. See **Figure 2** for reactions involved.

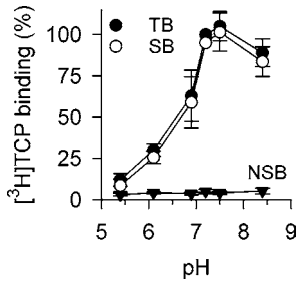

Figure 4. Effect of pH on [^{3}H]TCP binding. Specific binding (SB) is the difference between total binding (TB) and nonspecific binding (NSB). NSB is a minor component of TB and is not greatly affected by pH, for example, 14 ± 2% at pH 6.1 and 6.0 ± 1.5% at pH 7.2. TB of 100% at pH 7.5 was 14500 ± 2100 dpm.

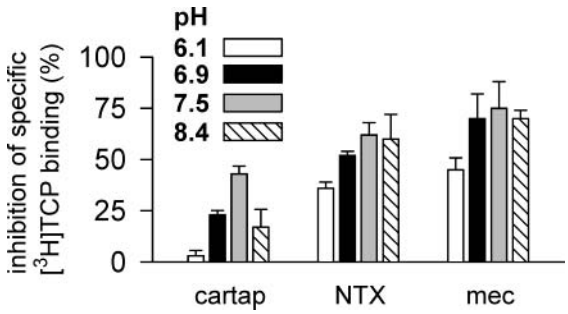

Figure 5. Effect of pH on the potency of cartap, NTX, and mecamylamine (mec) as inhibitors (5 μM) of [^{3}H]TCP binding.

Effect of pH on the Potency of Cartap, NTX, and Mecamylamine as Inhibitors of [^{3}H]TCP Binding. The inhibitors at discriminating concentrations were assayed in pH 6.1, 6.9, 7.5, and 8.4 buffers to determine possible pH effects on potency (**Figure 5**). Cartap gives the largest pH effect with much lower inhibition at pH 6.1 compared with 6.9 and above. The other compounds give smaller or no pH effects.

The results of another experiment are given in **Table 3** as IC_{50} values, which again emphasize the much greater pH effect on the potency of cartap (i.e., >200-fold at pH 7.4 versus 6.1) than of the other compounds (2.9- and 1.2-fold for NTX and mecamylamine, respectively).

On the basis of our earlier study (*19*), cartap and mecamylamine are poor inhibitors of [^{3}H]IMI binding with IC_{50} values of >1000 μM, whereas NTX is more potent with an IC_{50} value of 48 μM (**Table 3**).

Table 3. Effect of pH on the Potency of Cartap, NTX, and Mecamylamine as Inhibitors of [^{3}H]TCP and [^{3}H]IMI Binding

	IC_{50} (μM) ± SD (n = 3)		
	channel blocker site [^{3}H]TCP binding		agonist site [^{3}H]IMI binding
compound	pH 6.1	pH 7.4	pH 7.4
cartap	≥1000 (46%)[a]	4.3 ± 0.2[b]	≥1000 (47%)[a,b]
NTX	13 ± 2	4.5 ± 0.5[b]	48 ± 6[b]
mecamylamine	1.4 ± 0.1	1.2 ± 0.3[b]	>1000 (31%)[a,b]

[a] Percent inhibition at indicated concentration. [b] Data from our earlier investigation (*19*) reconfirmed in the present study.

DISCUSSION

Direct Action of NTX as a Nicotinic Channel Blocker. NTX directly blocks the nicotinic channel of insects (*18*, *19*). This is further supported in the present investigation, because there is no thiol liberation based on reactivity with DTNB and little pH effect on potency as an inhibitor of [^{3}H]TCP binding.

Hydrolysis of Cartap (Figure 1). Cartap hydrolyzes readily at pH 7 with a $t_{1/2}$ of 10 min at 25 °C based on a titrimetric analysis (*20*). The present LC-MS analyses of cartap-derived products in water and pH 6.0 phosphate buffer after 30 min reveal cartap monothiol with almost no cartap dithiol and NTX; at pH 7.4 and 9.0 there is more extensive loss of cartap and formation of the mono- and dithiol and NTX. The detection of NTX indicates some oxidation of cartap dithiol. No evidence was observed for $Me_2NCH(CH_2S)_2C{=}O$ as an intermediate on hydrolysis. These LC-MS studies serve to identify the products but not to quantify their ratios. Accordingly, the hydrolysis of cartap was monitored at pH 6.0, 7.4, and 9.0 in the presence of DTNB as free thiol equivalent based on carboxynitrophenylthiolate absorbance. At pH 7.4 and 9.0 cartap generates 1.8 equiv of carboxynitrophenylthiolate in 90 min, suggesting the formation of largely 2-(dimethylamino)propane-1,3-dithiol in derivatized form via cartap monothiol and dithiol (**Figure 2**). Some or all of the remaining 0.2 equiv not detected as free thiol and therefore "apparently lost" may in fact be an artifact resulting from hydrolysis of DTNB or mixed disulfides at pH 9.0 (*21*).

Activation of Cartap as a Nicotinic Channel Blocker. The [^{3}H]TCP binding assay with *Apis* brain membranes is the most direct available for determining insect nAChR channel blocker activity. Cartap gives a monophasic interaction with the noncompetitive blocker site at pH 7.4 (*19*), indicating that only one type of inhibitor is involved in this assay. The active form of cartap was initially considered by determining the relative potencies of cartap and its derivatives at pH 7.4. Cartap is >10-fold more active than its mono- and didemethylation products and its sulfoxide and sulfone derivatives (*19*), indicating that demethylation and sulfoxidation are detoxification rather than activation reactions. The relationship between cartap and NTX is less clear because they are equipotent and cartap is converted in part to cartap dithiol and NTX at pH 7.4. However, the product composition is dependent on the incubation time (data not shown) and pH. The inhibitor solutions are prepared and used in no more than 5 min, and the binding reaction is terminated at 60 s. Cartap undergoes very little hydrolysis within 5 min at pH 6.0 compared with pH 7.4 and 9.0 and is a very poor blocker at pH 6.1 compared with pH 6.9−8.4. The actual blocking agent is therefore not cartap or the monothiol present at pH 6.1 but instead cartap dithiol and/or NTX formed at higher pH conditions. Studies below on the agonist site rule against significant conversion of cartap to NTX under the assay

conditions, leading to the conclusion that cartap dithiol is the most plausible channel-blocking agent. In support of this conclusion, the neuroblocking activity of cartap dithiol on the American cockroach nerve cord is similar to that of NTX (*6*), consistent with the present channel-blocking data using [^{3}H]-TCP binding.

Action at the Nicotinic Agonist Site. NTX acts at the channel blocker and agonist sites with high and low affinities, respectively (*18*, *19*). Cartap and mecamylamine have little or no activity at the agonist site. The very low potency of cartap at pH 7.4 on the agonist site indicates that NTX is not formed as a major product within the 60 min assay period.

Active Form of Cartap as an Insecticide. The primary use of cartap is as a systemic insecticide. It is ultimately converted to NTX in plants and many other biological systems (*22*). In conclusion, although cartap dithiol is probably a major contributor to the channel-blocking action of cartap in the rapid binding experiments described here, in the long term the insecticidal activity as a systemic is more likely due to NTX formed within the plant (*22*) or insect (*13*).

Relationship between Cartap and Bensultap Activation. Both insecticides metabolically give NTX (*13*), but we cannot confirm NTX as a principal product of bensultap in aqueous medium at pH 6.0−8.4. In our earlier radioligand binding experiments (*19*), bensultap was tested as an emulsion. The solubility limitation affects both the binding assay and the reaction kinetics. Thus, the possible relationship of bensultap action to cartap-type activation remains to be established (see Supporting Information).

ABBREVIATIONS USED

DTNB, 5,5-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); ESI, electrospray ionization; IC_{50}, molar concentration of inhibitor necessary for 50% displacement of specific radioligand binding; IMI or [^{3}H]IMI, imidacloprid or its tritiated form; LC-MS, liquid chromatography−mass spectrometry; nAChR, nicotinic acetylcholine receptor; [^{3}H]TCP, [^{3}H]-*N*-[1-(2-thienyl)-cyclohexyl]piperidine; TFA, trifluoroacetic acid.

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Supporting Information Available: Hydrolysis of bensultap and action as nicotinic channel blocker. This material is available free of charge via the Internet at http://pubs.acs.org.

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