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Blocking effects of promethazine, triprolidine and their analogues on the excitation caused by the peptide, achatin-I

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Abstract

An *Achatina* endogenous tetrapeptide, achatin-I (Gly-D-Phe-Ala-Asp), applied by brief pressure, produced an inward current (I_{in}) on an *Achatina* giant neurone type, PON (periodically oscillating neurone). Promethazine, triprolidine and their analogues tested, applied by perfusion, showed a tendency to inhibit the I_{in}, suggesting that the effective structures vary to a wide extent. With respect to promethazine and its analogues, the presence of 2-bromo, 5-oxo, 3-dimethylsulfamido and 2-methoxy weakened the effects. 10-(2-methylamino-2-methylethyl) instead of 10-(2-dimethylamino-2-methylethyl) of promethazine and the azepine ring instead of phenothiazine ring potentiated the effects. From the dose (pressure duration)-response study of achatin-I, the two promethazine analogues, RP 6497 and RP 6549 (the structures are shown in Fig. 1), inhibited the I_{in} in partly competitive and partly noncompetitive manners. Regarding triprolidine and its analogues, the compounds in *Z*-configuration seemed to be more effective than those in *E*-configuration. The presence of 4-methyl in 1-phenyl, and 1-(4-pyridyl) instead of 1-(2-pyridyl) potentiated the effects. 3-Dimethylamino instead of 3-pyrrolidino weakened the effects. The two triprolidine analogues, Trip Der 3 and Trip Der 6 (the structures in Fig. 2), inhibited the I_{in} in an uncompetitive manner.

Keywords: Achatin-I (Gly-D-Phe-Ala-Asp); Excitatory neuropeptide; Promethazine; Triprolidine; Histamine H_1 receptor antagonist; Blocking effect; Neuron; (Snail)

1. Introduction

Achatin-I (Gly-D-Phe-Ala-Asp), isolated from the ganglia of an African giant snail (*Achatina fulica* Férussac), was proposed as an excitatory neurotransmitter for *Achatina* neurones (Kamatani et al., 1989; Kim et al., 1991a,b). The inward current (I_{in}) produced by achatin-I on an identifiable *Achatina* giant neurone type, PON (periodically oscillating neurone), measured under voltage clamp, was mainly due to an increase in the neuromembrane permeability to Na^+ (Na^+-dependent) (Kim et al., 1991a), whereas the I_{in} on another neurone type, v-RCDN (ventral-right cerebral distinct neurone), was partly (approximately half) Na^+-dependent (Emaduddin, Zhang and Takeuchi, unpublished data).

It was demonstrated that an inhibitor of the adenosine-3′,5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA), H-89 (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide) (Chijiwa et al., 1990), and a calmodulin inhibitor, W-7 (*N*-6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) (Hidaka et al., 1978), inhibited the I_{in} produced by achatin-I on PON, whereas an inhibitor of the guanosine-3′,5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase (PKG), KT5823 (8*R*,9*S*,11*S*)-9-methoxy-9-methoxycarbonyl-2*N*,8-dimethyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[a,g]cycloocta[c,d,e]-trinden-1-one) (Ito and Karachot, 1990), inhibited the I_{in} on v-RCDN. These findings suggested that the I_{in} on PON was mediated by the cyclic AMP – PKA and Ca^{2+} – calmodulin systems, whereas the I_{in} on v-RCDN was mediated by cyclic GMP – PKG system (Emaduddin et al., 1996).

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Besides, achatin-I at low concentrations modulated the responses of *Achatina* neurones to the putative neurotransmitters, for example, 5-hydroxytryptamine and oxytocin, indicating that this peptide also acts as a neuromodulator of these neurones (Liu and Takeuchi, 1993a,b). Some neuroactive peptides including oxytocin modulated the I_{in} produced by achatin-I (Liu and Takeuchi, 1995).

It was recently evidenced that some histamine H_1 receptor antagonists, promethazine, triprolidine, chlorcyclizine and homochlorcyclizine, inhibited the I_{in} caused by achatin-I on PON (Santos et al., 1995).

In the present study, which was designed as one of the serial works on the effects of achatin-I, the blocking effects of promethazine, triprolidine and their analogues on the I_{in} produced by achatin-I on PON were examined, to elucidate their structure-activity relationships. Achatin-I was ejected locally onto the neurone by pneumatic brief pressure, to prevent the transsynaptic events as much as possible. The two histamine H_1 receptor antagonists and their analogues were applied by perfusion around the dissected ganglia containing the neurone to be tested.

2. Materials and methods

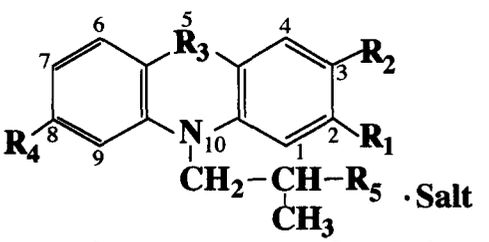
2.1. Preparations and electrophysiological arrangements

African giant snails (*Achatina fulica* Férussac) were flown from Cebu, Philippines, and kept in our laboratory within 2 weeks. An *Achatina* giant neurone type, PON (periodically oscillating neurone), identified in the suboesophageal ganglia, was used throughout the experiments. Localization in the ganglia and sensitivity to the putative neurotransmitters of this neurone type were reported previously (Takeuchi et al., 1985, Takeuchi et al., 1987; Liu et al., 1991a; Araki et al., 1995). The suboesophageal ganglia were dissected from the animal, and incubated with 0.33% trypsin (Type III, Sigma Chemical Co., USA) for 5–10 min at room temperature (21 ± 1 C°). The connective tissue covering the ganglia was removed with fine forceps, to expose the neurone to be tested. The ganglia were fixed on a Sylgard layer in the experimental chamber of approximately 0.2 ml in volume, with a suction pipette and fine tungsten wires.

Conventional voltage clamp technique using the two microelectrodes implanted into a neurone soma (Okamoto et al., 1976) was employed. The membrane voltage was kept at −60 mV (holding voltage, V_h), near the resting potential level of this neurone type. The membrane voltage and current were recorded with a pen-writing galvanometer and stored with a video recorder through an A-D converter.

2.2. Compounds used and application methods

Synthetic achatin-I (Gly-D-Phe-Ala-Asp) was donated by E. Munekata of Tsukuba University (Japan). Chemical structures of promethazine hydrochloride, triprolidine hy-

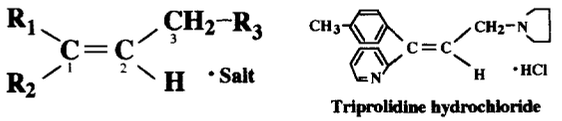


Promethazine hydrochloride

No.	Compound	R_1	R_2	R_3	R_4	R_5	Salt
1.	Promethazine	-H	-H	-S-	-H	$-N(CH_3)_2$	hydrochloride
2.	RP 4692	-Br	-H	-S-	-H	$-N(CH_3)_2$	hydrochloride
3.	RP 4726	$-OCH_3$	-H	-S-	-H	$-N(CH_3)_2$	hydrochloride
4.	RP 12323	$-NO_2$	-H	-S-	-H	$-N(CH_3)_2$	maleate
5.	RP 6581	$-CH_2-CH_3$	-H	-S-	-H	$-N(CH_3)_2$	hydrochloride
6.	RP 5257	$-OC_6H_5$	-H	-S-	-H	$-N(CH_3)_2$	methanesulfonate
7.	RP 20642	-H	$-SO_2-N(CH_3)_2$	-S-	-H	$-N(CH_3)_2$	maleate
8.	RP 6497	-H	-H	-S-	-H	$-NHCH_3$	methanesulfonate
9.	RP 6778	-H	-H	-S-	-H	$-CH_2-N(CH_3)_2$	maleate
10.	RP 6743	-H	-H	-S-	-H	$-CH_2-N(CH_3)_{2*}$	maleate
11.	RP 6549	-H	-H	-S-	-H	$-CH_2-N(CH_3)_2$	tartrate
12.	RP 6457	$-OCH_3$	-H	-S-	-H	$-CH_2-N(CH_3)_2$	
13.	RP 7185	$-OCH_3$	-H	-S-	-H	$-CH_2-N(CH_3)_{2*}$	tartrate
14.	RP 14357	$-OCH_3$	-H	-S-	$-OCH_3$	$-CH_2-N(CH_3)_2$	
15.	RP 6732	-H	-H	-S(O)-	-H	$-CH_2-N(CH_3)_2$	
16.	RP 7162	-H	-H	$-CH_2-CH_2-$	-H	$-CH_2-N(CH_3)_2$	maleate
17.	RP 5103	-H	-H	-S-	-H	$-N(-CH_2-CH_2-)(-CH_2-CH_2-)$	hydrochloride

(*, dextrorotatory form)

Fig. 1. Chemical structures of promethazine and its analogues tested in the present study. Numbering of carbon, sulfur and nitrogen in phenothiazine ring is shown.



No.	Compond	R_1	R_2	E / Z	R_3	Salt
1.	Triprolidine	CH_3		E		hydrochloride
2.	Trip Der 1			E		lactate
3.	Trip Der 2	Br		E		lactate
4.	Trip Der 3	CH_3–CH_2		E		lactate
5.	Trip Der 4			E		lactate
6.	Trip Der 5			Z		lactate
7.	Trip Der 6		Cl	Z		lactate
8.	Trip Der 7			Z		lactate
9.	Trip Der 8			Z	$-N(CH_3)_2$	lactate

Fig. 2. Chemical structures of triprolidine and its analogues tested. Carbon numbering in structure is shown.

drochloride and their analogues are illustrated in Figs. 1 and 2. Promethazine was donated by Shionogi (Japan), and its analogues were donated by G. Jolles of Rhone-Poulenc Rorer (France). Triprolidine was commercially obtained from Sigma Chemical Co., and its analogues were synthesized in our laboratories.

The *Achatina* physiological solution was formulated according to the amounts of main inorganic ions in the hemolymph of this animal (Takeuchi et al., 1973). Achatin-I was dissolved at 10^{-3} M in this solution, filled into a glass micropipette together with 0.5% Fast Green (Sigma Chemical Co.), and applied locally to the neurone tested by brief pneumatic pressure ejection (2×10^5 Pa, mostly 400 ms, 10^{-3} M, at 5–10 min interval). The application method of achatin-I was described in detail in a previous report (Santos et al., 1995).

Promethazine, triprolidine and their derivatives were also dissolved in the physiological solution, and perfused into the experimental chamber around the ganglia at a constant flow (2.0 ml/min). Among these compounds, RP 4726, RP 5257, RP 20642, RP 6457 and RP 14357 were dissolved in the physiological solution containing 0.25% dimethyl sulfoxide (DMSO).

To measure the dose (pressure duration)-response curves of achatin-I, this peptide at 10^{-3} M was ejected by varying the duration of the pressure ejection from 35 to 500 ms.

2.3. Statistics

The data were presented as the mean ± standard error of the mean (S.E.M.) for n trials. The data repeatedly obtained from one neurone were compared by the analysis of variance (ANOVA) for repeated measurements and Bonferroni's t test (Glantz, 1987). The two data sets were compared by the two-tailed Student's t test for paired or unpaired data. A datum was considered to be significantly different from another at $P < 0.05$ (*).

The dose (pressure duration)-response curves were analyzed by the probit method (Litchfield and Wilcoxon, 1949) using a computer program. Through this, ED_{50} (the confidence limit at 95%), ideal sigmoidal curve (r value) and Hill coefficient (r value) were obtained. Lineweaver-Burk plot was performed from the mean of the reciprocal of each data of the dose (pressure duration)-response curves. The straight lines through these points were drawn by linear regression.

Table 1
Blocking effects of promethazine and its analogues, perfused at 10^{-4} M, on the inward current (I_{in}) produced by achatin-I, applied by brief pressure ejection (2×10^5 Pa, 400 ms, 10^{-3} M, at 10 min interval), on PON (periodically oscillating neurone)

No.	Compound	Control (nA)	30 min after perfusion		n
			(nA)	(%)	
1	Promethazine	2.19 ± 0.24	0.81 ± 0.05 [c]	40.46 ± 4.66	7
2	RP 4692	2.39 ± 0.43	1.35 ± 0.15	63.55 ± 11.61	4
3	RP 4726	2.75 ± 0.79	1.30 ± 0.38 [a]	48.53 ± 4.02	4
4	RP 12323	2.54 ± 0.42	1.05 ± 0.11 [a]	46.68 ± 8.37	4
5	RP 6581	3.38 ± 0.41	1.48 ± 0.11 [b]	45.63 ± 4.95	4
6	RP 5257	2.20 ± 0.17	1.00 ± 0.10 [c]	45.21 ± 2.07	4
7	RP 20642	2.27 ± 0.31	1.16 ± 0.18 [c]	50.41 ± 5.61	5
8	RP 6497	2.75 ± 0.34	0.85 ± 0.11 [c]	30.84 ± 1.13	4
9	RP 6778	2.16 ± 0.36	0.76 ± 0.09 [c]	37.79 ± 5.27	5
10	RP 6743	1.73 ± 0.21	1.02 ± 0.16 [b]	58.06 ± 2.38	6
11	RP 6549	1.47 ± 0.24	0.60 ± 0.16 [c]	40.27 ± 7.12	5
12	RP 6457	2.04 ± 0.24	1.14 ± 0.20 [c]	55.78 ± 7.58	8
13	RP 7185	1.89 ± 0.15	0.80 ± 0.11 [c]	41.94 ± 3.87	5
14	RP 14357	2.15 ± 0.31	0.83 ± 0.07 [b]	40.32 ± 3.97	4
15	RP 6732	1.85 ± 0.31	1.10 ± 0.09 [a]	62.96 ± 5.19	5
16	RP 7162	2.79 ± 0.43	0.88 ± 0.12 [c]	32.00 ± 2.53	4
17	RP 5103	1.71 ± 0.31	0.80 ± 0.23 [a]	43.91 ± 4.49	4

The I_{in} value (nA) measured 30 min after perfusion of each drug was compared with the mean of the values obtained before the drug perfusion (control) by ANOVA for repeated measurements and Bonferroni's t test ([a] $P < 0.05$; [b] $P < 0.01$; and [c] $P < 0.001$). The ratio (%) of the former value to the latter (control) was also described.

3. Results

3.1. Stability of the inward current (I_{in}) induced by achatin-I

Achatin-I, applied by brief pressure ejection (2×10^5 Pa, 400 ms, 10^{-3} M, at 5–10 min interval), produced an inward current (I_{in}) on an *Achatina* giant neurone type, PON (periodically oscillating neurone). The I_{in} values (mean ± S.E.M.) were 2.01 ± 0.05 nA ($n = 230$) in physiological solution. These were stable for at least 75 min. The relation between time course (abscissa) and the I_{in} caused by achatin-I (5 min interval) (ordinate) ($n = 4$) was y (nA) = $1.8801 - 0.0060853x$ (min).

The I_{in} values caused by achatin-I in physiological solution containing 0.25% dimethyl sulfoxide (DMSO) were 2.24 ± 0.13 nA ($n = 50$), which were not significantly different from those in physiological solution by Student's t test for paired data. These I_{in} values were stable in the presence of DMSO for more than 70 min.

3.2. Blocking effects of promethazine and its analogues on the I_{in} induced by achatin-I

The chemical structures of promethazine and its analogues, tested in the present study, are illustrated in Fig. 1. The results obtained are summarized in Table 1.

Two promethazine derivatives, RP 6497 and RP 6549,

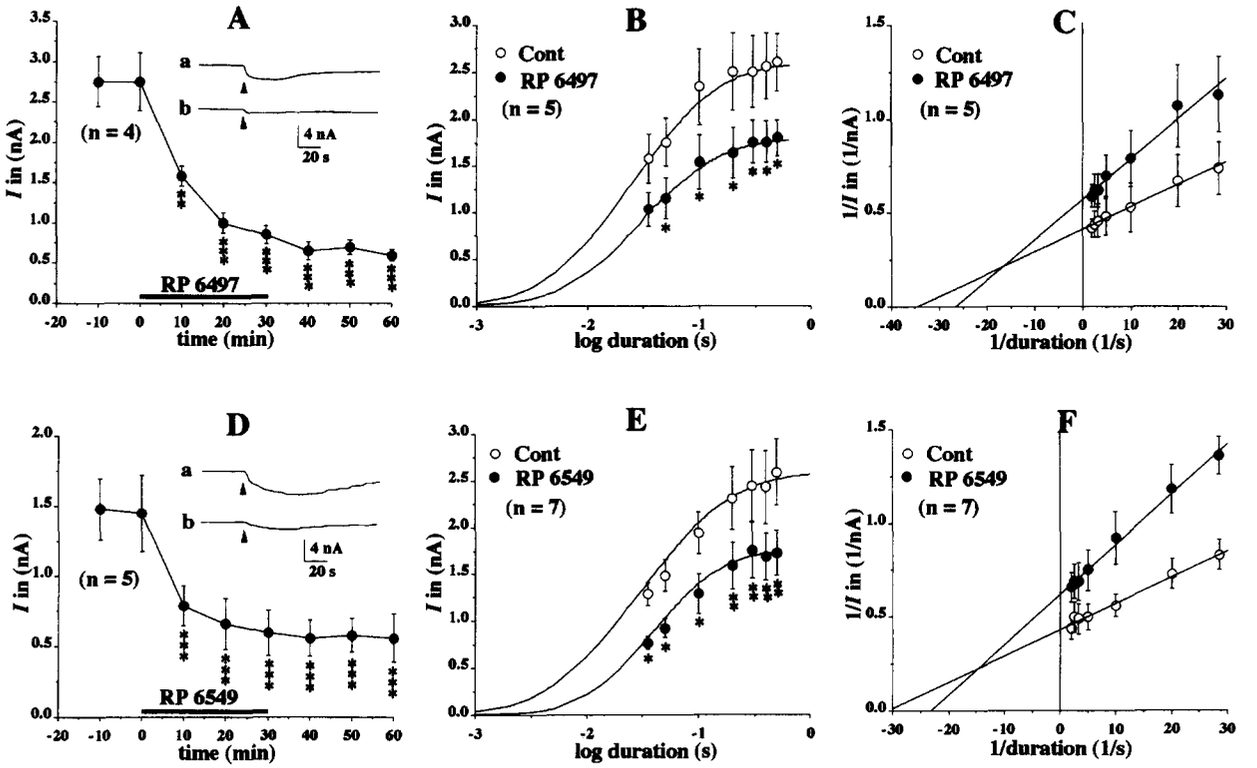


Fig. 3. Blocking effects of promethazine derivatives, RP 6497 and RP 6549, applied by perfusion, on the inward current (I_{in}) caused by achatin-I, ejected by brief pressure (2×10^5 Pa, 10^{-3} M, at 5–10 min interval), on PON (periodically oscillating neurone). Number of observations are indicated in parentheses. A: time course of the effects of RP 6497 at 10^{-4} M on the I_{in} (pressure duration: 400 ms). Inset of A: recordings of the I_{in}. B: effects of RP 6497 at 3×10^{-7} M on the dose (pressure duration)-response curves of achatin-I (pressure duration: varied). C: Lineweaver-Burk plot of the data shown in B. D: time course of the effects of RP 6549 at 10^{-4} M on the I_{in} (pressure duration: 400 ms). Inset of D: recordings of the I_{in}. E: effects of RP 6549 at 3×10^{-7} M on the dose (pressure duration)-response curves of achatin-I. F: Lineweaver-Burk plot of the data shown in E. In A and D: abscissa, time course (min) (horizontal bar: perfusion of RP 6497 (A) and RP 6549 (D)); and ordinate, I_{in} (nA) (small bar: S.E.M.). The I_{in} values measured under drugs and after washout were compared with the mean of the values before the drug perfusion (control) by ANOVA for repeated measurements and Bonferroni's t test (** $P < 0.01$; *** $P < 0.001$). Inset of A and D: (a) in physiological solution; and (b) 30 min after perfusion of each drug at 10^{-4} M. Arrows indicate the achatin-I ejection. In B and E: abscissa, pressure duration of the achatin-I ejection in logarithmic scale (s); and ordinate, I_{in} (nA) (small bar: S.E.M.). The values measured under drugs (drug curve) (●) were compared with those of physiological solution (control curve) (○) in the corresponding pressure duration by Student's t test for paired data (* $P < 0.05$). The curves were drawn by fitting the ideal sigmoidal curves calculated by the computer program (r value: 0.989576 for control curve and 0.984036 for drug curve in B, and 0.966493 for control curve and 0.975142 for drug curve in E). In C and F: abscissa, the reciprocal of the pressure duration (1/s); and ordinate, the reciprocal of the I_{in} (1/nA) (small bar: S.E.M.). The line measured in physiological solution (control line) (○) and the line under drugs (drug line) (●) were drawn by linear regression (r value: 0.990 for control line and 0.969 for drug line in C, and 0.984 for control line and 0.994 for drug line in F).

perfused at 10^{-4} M, markedly inhibited the I_{in} induced by achatin-I, ejected by brief pressure (10 min interval). The effects of the two compounds were saturated approximately 20 min after the perfusion, and there was no recovery up to 30 min after washout. With respect to RP 6497, the I_{in} caused by achatin-I (mean ± S.E.M.) ($n = 4$) were 2.75 ± 0.34 nA for the mean of the values before perfusion of this drug (control), 0.85 ± 0.11 nA ($P < 0.001$, compared with the mean of control by ANOVA for repeated measurements and Bonferroni's t test) 30 min after the perfusion, and 0.60 ± 0.07 nA ($P < 0.001$) 30 min after washout (Fig. 3A). Regarding RP 6549, the I_{in} ($n = 5$) were 1.47 ± 0.24 nA for the mean of control, 0.60 ± 0.16 nA ($P < 0.001$) 30 min after drug perfusion, and 0.56 ± 0.17 nA ($P < 0.001$) 30 min after washout (Fig. 3D). The I_{in} recordings are shown in insets of Fig. 3A and Fig. 3D.

The I_{in} obtained in physiological solution (control) and the I_{in} measured 30 min after perfusion of each drug at 10^{-4} M are described in Table 1. All of the compounds tested at this concentration had a tendency to inhibit the I_{in}. RP 4692 having 2-bromo of the promethazine structure, RP 6732 having 5-oxo, RP 20642 having 3-dimethylsulfamide and RP4726 having 2-methoxy were less effective than promethazine. In contrast, RP 6497 having 10-(2-methylamino-2-methylethyl) instead of 10-(2-dimethylamino-2-methylethyl) of promethazine and RP 7162 having azepine ring instead of phenothiazine ring were more effective than promethazine. In addition, the time course for the blocking effects of promethazine and its analogues, listed in Table 1, was similar to that of RP 6497 and RP 6549, shown in Fig. 3A and Fig. 3D.

3.3. Effects of promethazine analogues on the dose (pressure duration)-response curve of achatin-I

The dose (pressure duration)-response curves of achatin-I, ejected by varying the pressure duration (5 min interval) were measured on PON in physiological solution (control curve) and under either RP 6497 or RP 6549 at 3×10^{-7} M (drug curve) from one neurone. The first (control) curve was measured in physiological solution, then the measurement of the second (drug) curve started 20 min after drug perfusion.

With respect to RP 6497 ($n = 5$), ED_{50} (confidence limit at 95%), E_{max} and Hill coefficient (r value) were 24.3 ms (18.3–32.4 ms), 2.60 ± 0.30 nA and 1.33946 (0.987113), respectively, for control curve, and 30.5 ms (24.0–38.9 ms), 1.80 ± 0.19 nA ($P < 0.05$, compared with E_{max} of control curve by Student's t test for paired data) and 1.42646 (0.984036), respectively, for drug curve. ED_{50} of drug curve was somewhat larger than that of control curve, and E_{max} of drug curve was significantly smaller than that of the former (Fig. 3B).

From the Lineweaver-Burk plot of these data, the relation between the reciprocal of the pressure duration (abscissa) and the reciprocal of the I_{in} (ordinate), obtained by linear regression, was $y\ (1/\text{nA}) = 0.41263 + 0.011927x$ (1/s) in physiological solution (control line), and $y = 0.57028 + 0.021401x$ under RP 6497 (drug line). The cross point of the two lines was $x = -16.519$ and $y = 0.214165$ (Fig. 3C).

Regarding RP 6549 ($n = 7$), ED_{50} (confidence limit at 95%), E_{max} and Hill coefficient (r value) were 29.2 ms (18.1–47.0 ms), 2.59 ± 0.35 nA and 1.23075 (0.947374), respectively, for control curve, and 38.4 ms (26.9–54.7), 1.76 ± 0.30 nA ($P < 0.01$) and 1.64306 (0.964878), respectively, for drug curve. ED_{50} of drug curve was larger than that of control curve, and E_{max} of drug curve was significantly smaller than that of the former (Fig. 3E).

From the Lineweaver-Burk plot of these data, the relation of the reciprocal of the pressure duration and the reciprocal of the I_{in} caused by achatin-I was $y\ (1/\text{nA}) = 0.43602 + 0.013976x$ (1/s) for control line, and $y = 0.61952 + 0.026798x$ for drug line. The cross point of the two lines was $x = -14.311$ and $y = 0.236$ (Fig. 3F).

Table 2
Blocking effects of triprolidine and its analogues, perfused at 10^{-4} M, on the I_{in} produced by achatin-I, ejected by brief pressure (2×10^5 Pa, 400 ms, 10^{-3} M, at 10 min interval), on PON

No.	Compound	Control (nA)	30 min after perfusion		n
			(nA)	(%)	
1	Triprolidine	2.11 ± 0.26	0.99 ± 0.16 [c]	48.76 ± 6.08	7
2	Trip Der 1	1.97 ± 0.15	1.12 ± 0.12 [b]	57.59 ± 6.27	5
3	Trip Der 2	1.63 ± 0.22	1.07 ± 0.20 [a]	63.44 ± 4.97	5
4	Trip Der 3	1.61 ± 0.06	0.67 ± 0.08 [c]	42.21 ± 5.11	7
5	Trip Der 4	2.38 ± 0.23	0.90 ± 0.15 [c]	37.61 ± 4.01	4
6	Trip Der 5	1.71 ± 0.21	0.74 ± 0.09 [c]	44.38 ± 3.98	5
7	Trip Der 6	1.84 ± 0.31	0.68 ± 0.17 [c]	35.78 ± 4.78	6
8	Trip Der 7	1.70 ± 0.29	0.98 ± 0.29	53.45 ± 8.90	3
9	Trip Der 8	1.83 ± 0.18	1.15 ± 0.11 [b]	64.80 ± 8.36	4

The I_{in} value (nA) measured 30 min after perfusion of each drug was compared with the mean of the values obtained before the drug perfusion (control) by ANOVA for repeated measurements and Bonferroni's t test ([a] $P < 0.05$; [b] $P < 0.01$; and [c] $P < 0.001$).

3.4. Blocking effects of triprolidine and its analogues on the I_{in} induced by achatin-I

The chemical structures of triprolidine and its analogues are illustrated in Fig. 2. The results obtained are summarized in Table 2.

Two triprolidine analogues, Trip Der 3 and Trip Der 6, perfused at 10^{-4} M, inhibited the I_{in} induced by achatin-I, ejected by brief pressure (10 min interval). The effects of these drugs were saturated about 20 min after the perfusion, and there was no recovery up to 30 min after washout. With respect to Trip Der 3, the I_{in} ($n = 7$) were 1.61 ± 0.06 nA for the mean of control, 0.67 ± 0.08 nA ($P < 0.001$, compared with the mean of control) 30 min after drug perfusion, and 0.43 ± 0.09 nA ($P < 0.001$) 30 min after washout (Fig. 4A). Regarding Trip Der 6, the I_{in} ($n = 6$) were 1.84 ± 0.31 nA for the mean of control, 0.68 ± 0.17 nA ($P < 0.001$) 30 min after drug perfusion, and 0.57 ± 0.16 nA ($P < 0.001$) 30 min after washout (Fig. 4D). The recordings of the I_{in} were illustrated in insets of Fig. 4A and Fig. 4D.

All of the compounds, listed in Fig. 2, at 10^{-4} M showed a tendency to inhibit the I_{in}. Trip Der 1 having 1-phenyl and 1-(2-pyridyl) in *E*-configuration were slightly less effective than Trip Der 5 having the similar structure in *Z*-configuration. Triprolidine having 4-methyl in 1-phenyl of Trip Der 1 was more effective than Trip Der 1. Trip Der 4 having 1-(4-pyridyl) instead of 1-(2-pyridyl) of Trip Der 1 was more effective than Trip Der 1. Trip Der 8 having 3-dimethylamino instead of 3-pyrrolidino of Trip Der 5 was less effective than Trip Der 5.

3.5. Effects of triprolidine analogues on the dose (pressure duration)-response curve of achatin-I

The dose (pressure duration)-response curves of achatin-I, ejected by varying the pressure duration (5 min

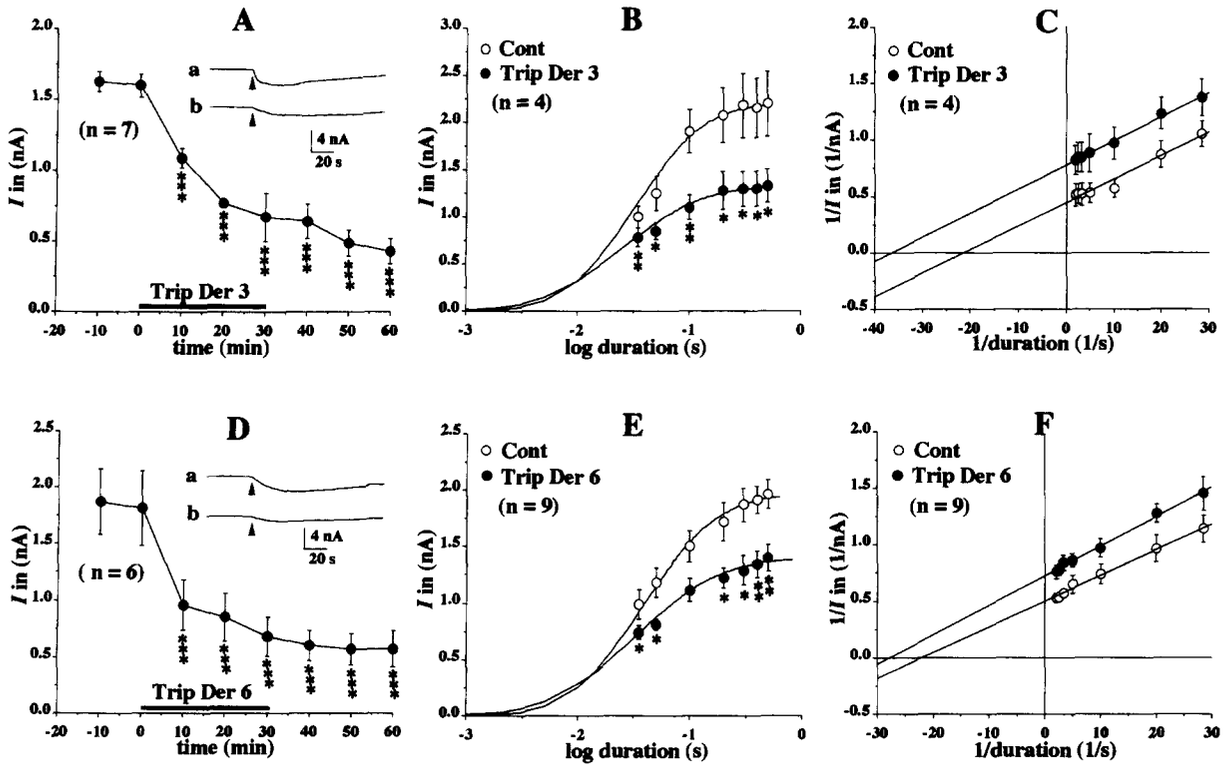


Fig. 4. Blocking effects of triprolidine derivatives, Trip Der 3 and Trip Der 6, applied by perfusion, on the I_{in} caused by achatin-I, ejected by brief pressure (2×10^5 Pa, 10^{-3} M, at 5–10 min interval), on PON. Number of observations are indicated in parentheses. A: time course of the effects of Trip Der 3 at 10^{-4} M on the I_{in} (pressure duration: 400 ms). Inset of A, Recordings of the I_{in}. B: effects of Trip Der 3 at 10^{-6} M on the dose (pressure duration)-response curves of achatin-I (pressure duration: varied). C: Lineweaver-Burk plot of the data shown in B. D: time course of the effects of Trip Der 6 at 10^{-4} M on the I_{in} (pressure duration: 400 ms). Inset of D: recordings of the I_{in}. E: effects of Trip Der 6 at 10^{-6} M on the dose (pressure duration)-response curves of achatin-I. F: Lineweaver-Burk plot of the data shown in E. In A and D: abscissa, time course (min) (horizontal bar: perfusion of Trip Der 3 (A) and Trip Der 6 (D); and ordinate, I_{in} (nA) (small bar: S.E.M.). The I_{in} values measured under drugs and after washout were compared with the mean of control by ANOVA for repeated measurements and Bonferroni's t test. Inset of A and D: (a) in physiological solution; and (b) 30 min after perfusion of each drug. In B and E: abscissa, pressure duration of the achatin-I ejection in logarithmic scale (s); and ordinate, I_{in} (nA) (small bar: S.E.M.). The values of drug curve (●) were compared with those of control curve (○) in the corresponding pressure duration by Student's t test for paired data. The curves were drawn by fitting the ideal sigmoidal curves calculated by the computer program (r value: 0.98110 for control curve and 0.992471 for drug curve in B, and 0.990844 for control curve and 0.981566 for drug curve in E). C and F: abscissa, the reciprocal of pressure duration (1/s); and ordinate, the reciprocal of the I_{in} (1/nA) (small bar: S.E.M.). Control line (○) and drug line (●) were drawn by linear regression (r value: 0.970 for control line and 0.995 for drug line in C, and 0.993 for control line and 0.993 for drug line in F).

interval), on PON were measured in physiological solution (control curve) and under either Trip Der 3 or Trip Der 6 at 10^{-6} M (drug curve) from one neurone. The measurement of the second (drug) curve started 20 min after drug perfusion. With respect to Trip Der 3 ($n = 4$), ED_{50}, E_{max} and Hill coefficient (r value) were 32.8 ms (23.5–45.8 ms), 2.20 ± 0.34 nA and 1.71589 (0.985963), respectively, for control curve, and 25.1 ms (19.7–31.8 ms), 1.33 ± 0.18 nA ($P < 0.05$, compared with E_{max} of control curve) and 1.51099 (0.991407), respectively, for drug curve. ED_{50} and E_{max} of drug curve were smaller than those of control curve (Fig. 4B).

From the Lineweaver-Burk plot of these data, the relation of the reciprocal of the pressure duration and the reciprocal of the I_{in} induced by achatin-I was $y\ (1/nA) = 0.44082 + 0.020788x\ (1/s)$ for control line, and $y = 0.77596 + 0.021264x$ for drug line. The two lines were almost parallel (Fig. 4C).

Regarding Trip Der 6 ($n = 9$), ED_{50} (confidence limit at 95%), E_{max} and Hill coefficient (r value) were 38.4 ms (31.1–47.5 ms), 1.96 ± 0.13 nA and 1.5728 (0.980699), respectively, for control curve, and 31.9 ms (22.8–44.5 ms), 1.40 ± 0.12 nA ($P < 0.05$, compared E_{max} of control curve) and 1.34225 (0.967545), respectively, for drug curve. Both ED_{50} and E_{max} of drug curve were smaller than those of control curve (Fig. 4E).

From the Lineweaver-Burk plot of these data, the relation between the reciprocal of the pressure duration and the reciprocal of the I_{in} caused by achatin-I was $y\ (1/nA) = 0.50127 + 0.022705x\ (1/s)$ for control line, and $y = 0.72601 + 0.026022x$ for drug line. The two lines were nearly parallel, as in the case of Trip Der 3 (Fig. 4F).

4. Discussion

It was demonstrated previously that some mammalian histamine H_1 receptor antagonists, including promethazine and triprolidine, inhibited the inward current (I_{in}) produced by achatin-I (Gly-D-Phe-Ala-Asp) on an *Achatina* giant neurone type, PON (periodically oscillating neurone). However, other histamine H_1 receptor antagonists, such as diphenhydramine, pyrilamine, (+)-chlorphenylamine, cyclizine and cyproheptadine, did not block the I_{in}, suggesting that the effective compounds mentioned above did not act as histamine receptor antagonists to inhibit the I_{in} induced by achatin-I (Santos et al., 1995). Besides, this neurone type, PON, was slightly sensitive (excited) or insensitive to histamine (Takeuchi et al., 1985, 1987).

In addition, histamine H_2 receptor antagonists, acetylcholine (nicotinic and muscarinic) receptor antagonists, GABA ($GABA_A$) receptor antagonists, L-glutamate (NMDA) receptor antagonists, dopamine (D_2) receptor antagonists, α- (nonselective and α_2) and β- (non-selective and β_1) adrenoceptor antagonists and 5-hydroxytryptamine ($5\text{-}HT_{1C}$ and $5\text{-}HT_2$) receptor antagonists did not antagonize the I_{in} caused by achatin-I on this neurone type (Santos et al., 1995).

The present study was designed as one of the serial investigations on the effects of achatin-I using *Achatina* giant neurones. All of the compounds tested, promethazine, triprolidine and their analogues illustrated in Figs. 1 and 2, showed a tendency to inhibit the I_{in} produced by achatin-I on PON, suggesting that the effective structures vary to a wide extent.

Among promethazine and its analogues tested, promethazine was one of the most effective compounds for blocking the I_{in} induced by achatin-I on PON. As to their structure-activity relationships, the presence of 2-bromo, 5-oxo, 3-dimethylsulfamide and 2-methoxy in the promethazine structure weakened the effects. On the other hand, 10-(2-methylamino-2-methylethyl) instead of 10-(2-dimethylamino-2-methylethyl) of promethazine and the azepine ring instead of phenothiazine ring potentiated the effects.

From the dose (pressure duration)-response curves of achatin-I on PON under the two promethazine analogues, RP 6497 and RP 6549, and the Lineweaver-Burk plot of these data, these two compounds inhibited the I_{in} in partly competitive and partly noncompetitive manners. It was suggested previously that the I_{in} produced by achatin-I on this neurone type was mediated by the cyclic AMP-cyclic AMP-dependent protein kinase (PKA) and Ca^{2+}-calmodulin systems (Emaduddin et al., 1996). In addition, the intracellular injection of cyclic AMP and cyclic GMP into an *Achatina* giant neurone, including PON, produced a depolarization (Liu and Takeuchi, 1993c). Therefore, we propose that the inhibition of the I_{in} by these promethazine analogues is partly (for competitive inhibition) the event in the achatin-I receptor sites, and is partly (for noncompetitive inhibition) caused by affecting the activity of these intracellular signal transduction systems mentioned above or the Na^+ channels linked with achatin-I receptors on this neurone type.

As to the structure-activity relationships of triprolidine and its analogues for blocking the I_{in} produced by achatin-I on PON, these compounds in *Z*-configuration seemed to be more effective than those in *E*-configuration. The presence of 4-methyl in 1-phenyl of triprolidine, and 1-(4-pyridyl) instead of 1-(2-pyridyl), potentiated the effects. Further, 3-dimethylamino instead of 3-pyrrolidino weakened the effects.

From the dose (pressure duration)-response curves of achatin-I under the two triprolidine analogues, Trip Der 3 and Trip Der 6, and on the Lineweaver-Burk plot of these data, these two compounds inhibited noncompetitively the I_{in} caused by achatin-I. Therefore, we also propose that the inhibition caused by these compounds is not the event in the achatin-I receptor sites, but is caused by affecting the activity of the cyclic AMP – PKA and/or Ca^{2+} – calmodulin systems or the Na^+ channels.

It was demonstrated by the binding assay of triprolidine and its analogues using the histamine H_1 receptors in the guinea pig that the ratio of the affinity constant between triprolidine in *E*-configuration and its isomer in *Z*-configuration was 1 : 100–600, indicating that the compound in *E*-configuration is much more effective than that in *Z*-configuration. Triprolidine analogues having 1-(3-pyridyl) or 1-(4-pyridyl) were less effective for binding to the histamine H_1 receptors than triprolidine which has 1-(2-pyridyl) (Casy et al., 1992). These results on the mammalian histamine H_1 receptors were completely different from their inhibitory effects on the I_{in} caused by achatin-I.

For the next phase of our series of investigations on the effects of achatin-I, we shall examine whether or not the guanosine 5′-triphosphate (GTP)-binding protein is linked with the achatin-I receptors to produce the I_{in}. Besides, we shall examine the effects of promethazine, triprolidine and their analogues on the I_{in} caused by achatin-I on another neurone type, v-RCDN (ventral-right cerebral distinct neurone), which was suggested to be due to the cyclic GMP – cyclic GMP-dependent protein kinase (PKG) system. We shall also examine the effects of these compounds on the outward currents (I_{out}) induced by other neuroactive peptides, APGW-amide (Ala-Pro-Gly-Trp-NH_2) and fulicin (Phe-D-Asn-Glu-Phe-Val-NH_2), which were both isolated from the *Achatina* ganglia (Liu et al., 1991b; Ohta et al., 1991).

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