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Multiple intracellular signal transduction pathways mediating inward current produced by the neuropeptide, achatin-I

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Abstract

The effects of intracellular signal transduction system inhibitors on the inward current (I_{in}) caused by achatin-I (Gly-D-Phe-Ala-Asp), an *Achatina* endogenous tetrapeptide having a D-phenylalanine residue, applied locally onto the neurone tested, were examined under voltage clamp using two identifiable *Achatina* giant neurone types, v-RCDN (ventral-right cerebral distinct neurone) and PON (periodically oscillating neurone). H-89 (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide) (adenosine-3′,5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase inhibitor) markedly suppressed the achatin-I-induced I_{in} on PON, whereas this drug was ineffective on the I_{in} of v-RCDN. Dose (pressure duration)-response study of achatin-I on PON in a physiological solution and in the presence of H-89, and Lineweaver-Burk plot of these data, indicated that H-89 inhibited the I_{in} in a noncompetitive manner. KT5823 (*N*-methyl-(8*R**,9*S**,11*S**)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-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) (guanosine-3′,5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase inhibitor) suppressed the achatin-I-induced I_{in} of v-RCDN in mainly noncompetitive and partly uncompetitive manners, but this drug had no effect on the I_{in} of PON. W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide) (calmodulin inhibitor) suppressed noncompetitively the I_{in} of PON, but this drug had no effect on the I_{in} of v-RCDN. IBMX (3-isobutyl-1-methylxanthine) (cyclic nucleotide phosphodiesterase inhibitor) enhanced the achatin-I-induced I_{in} of v-RCDN, but this drug was ineffective on the I_{in} of PON. However, IBMX might have effects on the achatin-I receptor sites on v-RCDN. These findings suggest multiple intracellular signal transduction pathways mediating the achatin-I-induced I_{in}: the I_{in} of PON is via cyclic AMP-dependent and probably Ca^{2+}/calmodulin-dependent protein kinases, and that of v-RCDN via cyclic GMP-dependent protein kinase. Other signal transduction system inhibitors including calphostin C (2-[12-[2-(benzyloxy)-propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-peryleny]-1-methylethyl carbonic acid 4-hydroxyphenyl ester) (protein kinase C inhibitor) did not significantly affect the I_{in} of both v-RCDN and PON.

Keywords: Achatin-I (Gly-D-Phe-Ala-Asp); Peptide neurotransmitter; Intracellular signal transduction system; H-89; KT5823; W-7; IBMX (3-isobutyl-1-methylxanthine); Neurone; (Snail)

1. Introduction

Achatin-I (Gly-D-Phe-Ala-Asp), isolated from an African giant snail (*Achatina fulica* Férussac), was the first of the nervous tissue-derived neuroactive peptide containing a D-amino acid residue to be discovered. Of achatin-I and its seven possible stereoisomers, only achatin-I showed marked excitatory (depolarizing) effects on some identifiable *Achatina* giant neurone types, indicating that the effects of this peptide are stereo-specific (Kamatani et al., 1989). Achatin-I markedly produced excitatory effects on nearly half of the *Achatina* giant neurone types tested including v-RCDN (ventral-right cerebral distinct neurone) and PON (periodically oscillating neurone). This peptide showed no inhibitory effect. With these findings, we proposed that achatin-I is an excitatory neurotransmitter of the *Achatina* neurones. The inward current (I_{in}) produced by achatin-I, measured under voltage clamp, was mainly due to an increase in the neuromembrane permeability to Na^+ (Na^+-dependent) on PON (Kim et al., 1991a) but partly (approximately half) Na^+-dependent on v-RCDN (Emaduddin and Takeuchi, unpublished data). Among achatin-I and its 19 derivatives,

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only achatin-I markedly showed excitatory effects on the *Achatina* neurones, indicating that the effects of this peptide are also structure-specific (Kim et al., 1991b).

Further, we demonstrated on *Achatina* neurones (Liu and Takeuchi, 1993a, b) that achatin-I at a low concentration enhanced the I_{in} caused by 5-hydroxytryptamine and the outward current (I_{out}) caused by FMRFamide (Phe-Met-Arg-Phe-NH_2), a neuroactive peptide isolated from a clam (*Macrocallista nimbosa*) (Price and Greenberg, 1977), and suppressed the I_{in} caused by oxytocin and the I_{out} caused by acetylcholine, suggesting that achatin-I is also acting as a neuromodulator of *Achatina* neurones. In addition, we demonstrated (Liu and Takeuchi, 1995) that FMRFamide and oxytocin at low concentrations modulated the I_{in} caused by achatin-I.

We showed (Santos et al., 1995) that some histamine H_1 receptor antagonists, chlorcyclizine, promethazine and triprolidine, inhibited the I_{in} caused by achatin-I on PON. These compounds suppressed the I_{in} not as the histamine H_1 receptor antagonists, since among the 17 H_1 receptor antagonists tested only a few suppressed the I_{in} in a noncompetitive manner. These findings suggest that these antagonists act on the intracellular signal transduction systems or the ionic channels linked with achatin-I receptors on this neurone type.

It was aimed in the present study to examine the effects of inhibitors for intracellular signal transduction systems on the achatin-I-induced I_{in} of v-RCDN and PON under voltage clamp. To prevent the transsynaptic events as much as possible, achatin-I was applied locally onto the neurone tested by a brief pneumatic pressure ejection. Effects of the following inhibitors, applied by perfusion, were examined: H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) (adenosine-3′,5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase inhibitor) (Chijiwa et al., 1990; Geilen et al., 1992), KT5823 (*N*-methyl-(8*R**,9*S**,11*S**)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-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) (guanosine-3′,5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase inhibitor) (Ito and Karachot, 1990, 1992), calphostin C (UCN-1028C; 2-[12-[2-(benzyloxy)propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-peryleny]-1-methylethyl carbonic acid 4-hydroxyphenyl ester) (protein kinase C inhibitor) (Kobayashi et al., 1989; Bruns et al., 1991), ML-9 (1-(5-chloro-naphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine) (myosin light-chain kinase inhibitor) (Nagatsu et al., 1987), genistein (5,7-dihydroxy-3-(4-hydroxy-phenyl)-4*H*-1-benzopyran-4-one) (tyrosine protein kinase inhibitor) (Akiyama et al., 1987), W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide) (calmodulin inhibitor) (Hidaka et al., 1978; Nishikawa et al., 1980), IBMX (3-isobutyl-1-methylxanthine) (cyclic nucleotide phosphodiesterase inhibitor) (Beavo et al., 1970; Snyder et al., 1981), fluphenazine nitrogen-mustard ((2-chloroethyl)-4-[3-(2-trifluoromethyl-10-phenothiazinyl)-propyl]piperazine) (calmodulin-dependent phosphodiesterase inhibitor) (Hait et al., 1987), indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid) (prostaglandin cyclooxygenase inhibitor) (Humes et al., 1981), okadaic acid (9,10-deepithio-9,10-didehydro-acanthifolicin) (type 1, 2A and 2B protein phosphatase inhibitor) (Bialojan and Takai, 1988) and calyculin A (type 1 protein phosphatase inhibitor) (Kato et al., 1986).

2. Materials and methods

2.1. Preparations and electrophysiological arrangements

Preparations and electrophysiological arrangements adopted in the present study were essentially similar to those of our previous reports (Santos et al., 1995; Emaduddin et al., 1995). In brief, the two giant neurone types, v-RCDN (ventral-right cerebral distinct neurone) and PON (periodically oscillating neurone), identified in the ganglia of an African giant snail (*Achatina fulica* Férussac), were used. Their localizations in the ganglia and sensitivities to the putative neurotransmitters were previously reported (Takeuchi et al., 1985a, b, 1987; Liu et al., 1991a, b; Araki et al., 1995). The ganglia containing the neurone to be tested were dissected out, incubated with 0.67% trypsin (Type III, Sigma Chemical Co., USA) for 3–5 min at room temperature (21 ± 1°C) to soften the covering connective tissue, and fixed on a Sylgard layer in the experimental chamber (about 0.2 ml in volume) with a suction pipette. The connective tissue was carefully removed with fine tweezers under a binocular microscope, to expose the neurone to be tested.

The experiments were carried out under voltage clamp using the two microelectrodes implanted into a neurone soma (Okamoto et al., 1976). Holding voltage (V_h) was kept at −50 mV, close to the resting potential level of these neurones.

2.2. Compounds used and application methods

Achatin-I (Gly-D-Phe-Ala-Asp) was synthesized in our laboratories. KT5823 (*N*-methyl-(8*R**,9*S**,11*S**)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-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) was donated by Dr M. Inoue of Kyowa Hakko Kogyo Co. (Japan). The inhibitors for the intracellular signal transduction systems were obtained commercially as follows: H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide dihydrochloride) and W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) from Seikagaku Kogyo Co. (Japan); calphostin C (UCN-1028C; 2-[12-[2-

(benzyloxy)-propyl]-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-peryleny]-1-methylethyl carbonic acid 4-hydroxyphenyl ester) and IBMX (3-isobutyl-1-methylxanthine) from Sigma Chemical Co.; ML-9 (1-(5-chloro-naphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride) from Biomol Research Laboratories (USA); and genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one), fluphenazine nitrogen-mustard (2-chloroethyl)-4-[3-(2-trifluoromethyl-10-phenothiazinyl)-propyl]piperazine dihydrochloride), indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid), okadaic acid (9,10-deepithio-9,10-didehydro-acanthifolicin) and calyculin A from Research Biochemicals International (USA).

The *Achatina* physiological solution was formulated according to the amounts of main inorganic ions in its hemolymph as follows (Takeuchi et al., 1973): NaCl (65.6 mM), KCl (3.3), $CaCl_2$ (10.7), $MgCl_2$ (13.0), Tris-HCl (10.0) and Tris base (1.0) (pH = 7.5). 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 onto the neurone tested by a brief pneumatic pressure ejection (2×10^5 Pa, mostly 400 ms and 5–10 min intervals) under a constant flow (2–3 ml/min) of the physiological solution. The application method of achatin-I was described in detail in a previous report (Santos et al., 1995).

Inhibitors for the intracellular signal transduction sys-

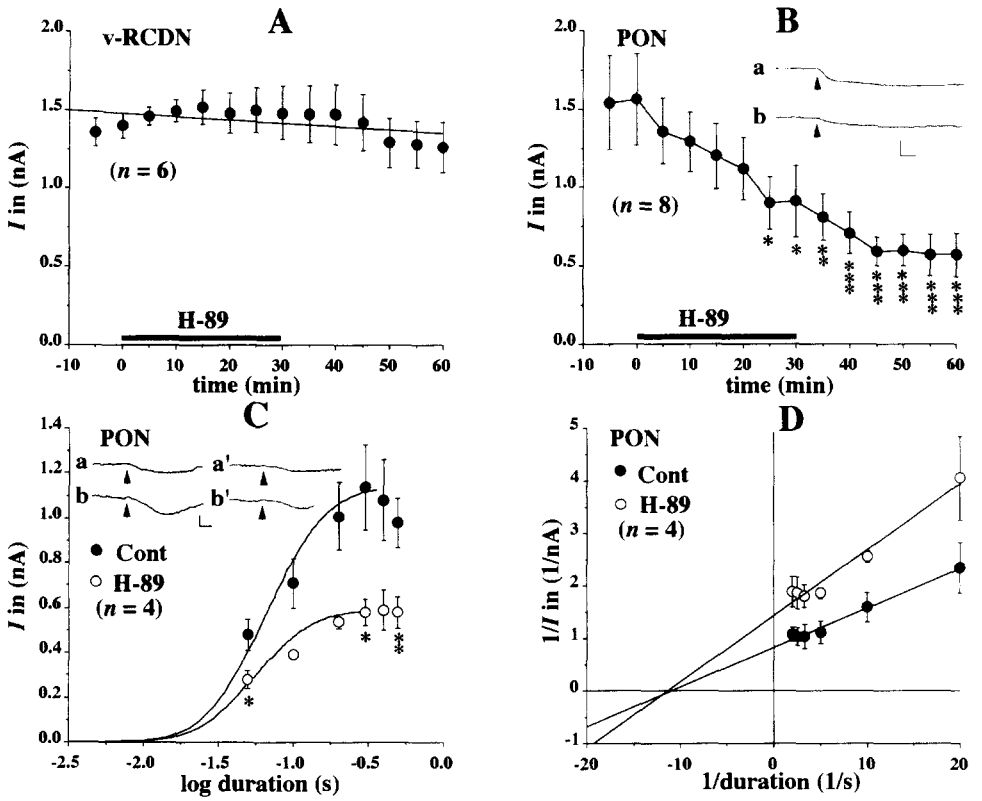

Fig. 1. Effects of H-89 (adenosine-3′,5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase inhibitor) on the inward current (I_{in}) caused by achatin-I (Gly-D-Phe-Ala-Asp) on v-RCDN (ventral-right cerebral distinct neurone) and PON (periodically oscillating neurone). Numbers of observations are indicated in parentheses. (A) Effects of H-89 perfused at 2×10^{-5} M on the I_{in} caused by achatin-I, applied by a pneumatic brief pressure ejection (2×10^5 Pa, 400 ms, 10^{-3} M and 5 min intervals) on v-RCDN. (B) Effects of H-89 perfused at 2×10^{-5} M on the I_{in} caused by achatin-I, applied in the same manner, on PON. Inset of (B): achatin-I-induced I_{in} on PON. (C) Dose (pressure duration)-response curves of achatin-I (2×10^5 Pa, varied durations, 10^{-3} M and 5 min intervals) on PON. Inset of (C): I_{in} caused by achatin-I in different pressure durations on PON. (D) Lineweaver-Burk plot of the data shown in C on PON. In (A) and (B), abscissa, time course (min) (horizontal bar: drug perfusion); and ordinate, I_{in} (nA) (small bar: S.E.M.). The values obtained during the drug perfusion and washout were compared with the mean of the values obtained before the drug perfusion (control) by analysis of variance (ANOVA) for repeated measurements and Bonferroni's *t*-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Inset of (B): a, control; b, 30 min after the drug perfusion. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); vertical bar, calibration (1 nA). (C) Abscissa, pressure duration in logarithmic scale (s); ordinate, I_{in} (nA) (small bar: S.E.M.). The dose (pressure duration)-response curves of achatin-I were measured in physiological solution (control curve) (●) and in the presence of H-89 at 10^{-5} M (drug curve) (○) from one neurone. The values of the drug curve were compared with the corresponding values of the control curve by Student's *t*-test for paired data. The curves were drawn by fitting the ideal sigmoidal curves calculated by the computer program ($r = 0.95424$ for the control curve, and 0.98340 for the drug curve). Inset of (C): a, I_{in} obtained by 100 ms pressure duration of the achatin-I ejection in physiological solution (control); b, 300 ms in control; a′, 100 ms in the presence of the drug at 10^{-5} M; b′, 300 ms in the presence of the drug. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); and vertical bar, calibration (1 nA). (D) Abscissa, reciprocal of pressure duration (1/s); ordinate, reciprocal of I_{in} (1/nA). The lines in the physiological solution (control line) (●) and in the presence of the drug (drug line) (○) were drawn by linear regression ($r = 0.98336$ for the control line, and 0.99196 for the drug line).

tems were dissolved at relatively low concentrations in the physiological solution containing 0.1–1.0% DMSO (dimethyl sulphoxide), and perfused into the experimental chamber at the same constant speed as mentioned above.

To obtain the dose (pressure duration)-response curves of achatin-I, the first (control) curve of this peptide, ejected with 5 min intervals at varied (50–600 ms) durations, was measured; then, 20–30 min after drug perfusion the measurement of the second (drug) curve was performed under the drug in the same way from the same neurone.

2.3. Statistics

Statistical calculations of the data were performed in the same manner as those of our previous reports (Santos et al., 1995; Emaduddin et al., 1995). In brief, the data were expressed as means ± standard error of the mean (S.E.M.). The data repeatedly obtained from one neurone were compared by the analysis of variance (ANOVA) for repeated measurements and Bonferroni's t-test (significantly different at * $P < 0.05$) (Glantz, 1987). The two data obtained from one neurone were compared by the two-tailed Student's t-test for paired data.

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

3. Results

3.1. Stability of inward current caused by achatin-I

Achatin-I, applied by a brief pneumatic pressure ejection (2×10^5 Pa, 400 ms, 10^{-3} M and 5 min intervals), produced the inward current (I_{in}) on the two *Achatina* giant neurone types, v-RCDN (ventral-right cerebral distinct neurone) and PON (periodically oscillating neurone), under voltage clamp. The I_{in} values, obtained by the first and second ejections of achatin-I on each neurone, in physiological solution (mean ± S.E.M.) were 1.04 ± 0.03 nA ($n = 188$) for v-RCDN and 0.93 ± 0.03 nA ($n = 168$) for PON. The I_{in} values of the two neurone types were stable at least for 70 min in physiological solution. The relations between the time course (abscissa) and the I_{in} (ordinate), obtained by linear regression, were Y (nA) = 0.95033 − 0.0017429X (min) ($n = 5$) for v-RCDN and $Y = 0.71750 - 0.00021429X$ ($n = 5$) for PON.

Intracellular signalling system inhibitors were dissolved in physiological solution containing DMSO (dimethyl sulphoxide) at 0.1–1.0%. The mean I_{in} values caused by the first and second ejections of achatin-I on each neurone in physiological solution containing 1.0% DMSO were 0.95 ± 0.16 nA ($n = 8$) for v-RCDN and 0.89 ± 0.09 nA ($n = 12$) for PON, indicating that the I_{in} in the presence of 1.0% DMSO were comparable to those measured in normal physiological solution. The I_{in} values of the two neurone types under 1.0% DMSO were also stable for at least 60 min. The relations between the time course and the I_{in} were Y (nA) = 0.99165 − 0.0016703X (min) ($n = 4$) for v-RCDN and $Y = 0.89793 - 0.0027560X$ ($n = 6$) for PON.

3.2. Effects of H-89

H-89 (cyclic AMP-dependent protein kinase inhibitor) was dissolved at 2×10^{-5} M in physiological solution containing 0.1% DMSO. This drug, perfused at this concentration for 30 min, did not affect the I_{in} caused by achatin-I, ejected by a brief pressure in the manner mentioned above, on v-RCDN ($n = 6$). The I_{in} values were 1.38 ± 0.09 nA for the mean of the values measured before the drug perfusion (control) and 1.48 ± 0.17 nA 30 min after the perfusion (NS, not significantly different; compared with the mean of the controls by analysis of variance (ANOVA) for repeated measurements and Bonferroni's t-test) (Fig. 1A). In contrast, H-89 at 2×10^{-5} M inhibited markedly the I_{in} caused by achatin-I on PON, and the inhibition of the I_{in} was irreversible ($n = 8$); the I_{in} was 1.55 ± 0.29 nA for the mean of control, 0.91 ± 0.23 nA 30 min after the drug perfusion (* $P < 0.05$; compared with the mean of control), and 0.57 ± 0.14 nA 30 min after the washout (*** $P < 0.001$) (Fig. 1B).

The two dose (pressure duration)-response curves of achatin-I (2×10^5 Pa, 10^{-3} M and 5 min intervals) on PON were measured from one neurone by varying the pressure duration of achatin-I ejection, in physiological solution (control curve) and in the presence of H-89 at 10^{-5} M (drug curve). The curves ($n = 4$) were analysed by the probit method. ED_{50} (95% confidence limit), E_{max} as mean ± S.E.M. and Hill coefficient (r value) were 65.5 ms (16.2–107.2 ms), 1.14 ± 0.19 nA and 2.55179 (0.95424), respectively, for the control curve and 55.3 ms (26.0–79.3 ms), 0.59 ± 0.09 nA (*, compared with the E_{max} of the control curve by Student's t-test for paired data) and 2.7511 (0.983397), respectively, for the drug curve. ED_{50} became slightly smaller, and E_{max} was significantly smaller in the presence of H-89 on PON, when compared to those of the control curve (Fig. 1C).

From the Lineweaver-Burk plot of the data of these dose (pressure duration)-response curves, the relations between reciprocal of the pressure duration (abscissa) and reciprocal of the I_{in} (ordinate) ($n = 4$), obtained by linear regression, were Y (1/nA) = 0.82950 + 0.075250X (1/min) for the control (control line) and $Y = 1.4424 + 0.12551X$ for the drug (drug line). The cross point of the two lines was $X = -12.195$ and $Y = -0.882$, suggesting that H-89 inhibited the achatin-I-induced I_{in} in a noncompetitive manner (Fig. 1D).

3.3. Effects of KT5823

KT5823 (cyclic GMP-dependent protein kinase inhibitor) was dissolved at 2×10^{-5} M in physiological solution containing 0.5% DMSO. This drug, perfused at this concentration, inhibited the I_{in} caused by achatin-I, ejected by a brief pressure, on v-RCDN, and this inhibition was semi-reversible ($n = 5$); the I_{in} was 1.04 ± 0.10 nA for the mean of control, 0.72 ± 0.09 nA 25 min after drug perfusion (*, compared with the mean of control), 0.61 ± 0.03 nA 15 min after washout (***), and 0.71 ± 0.08 nA 30 min after (*) (Fig. 2A). In contrast, this drug at the same concentration did not affect the I_{in} caused by achatin-I, applied in the same manner, on PON ($n = 4$); the I_{in} was 1.07 ± 0.12 nA for the mean of the controls and 0.85 ± 0.10 nA 30 min after the drug perfusion (NS) (Fig. 2B).

The dose (pressure duration)-response curves of achatin-I on v-RCDN were measured from one neurone, in physiological solution (control curve) and in the presence of KT5823 at 10^{-5} M (drug curve). ED_{50} (95% confidence limit), E_{max} and Hill coefficient (r value) of these curves ($n = 4$) were 60.1 ms (51.7–70.0 ms), 1.20 ± 0.09 nA and 2.57599 (0.995829), respectively, for the control curve, and 53.7 ms (not measurable – 117.0 ms), 0.75 ± 0.08 nA (** $P < 0.01$; compared with the E_{max} of the control curve) and 2.10995 (0.923654), respectively, for the drug curve. ED_{50} became slightly smaller, and E_{max} was significantly smaller in the presence of KT5823 than those of the control curve (Fig. 2C).

From Lineweaver-Burk plot of these data, the relations of reciprocal of the pressure duration and reciprocal of the I_{in} were Y (1/nA) = 0.64208 + 0.075357X (1/s) for the control line and $Y = 1.0832 + 0.10976X$ for the drug line.

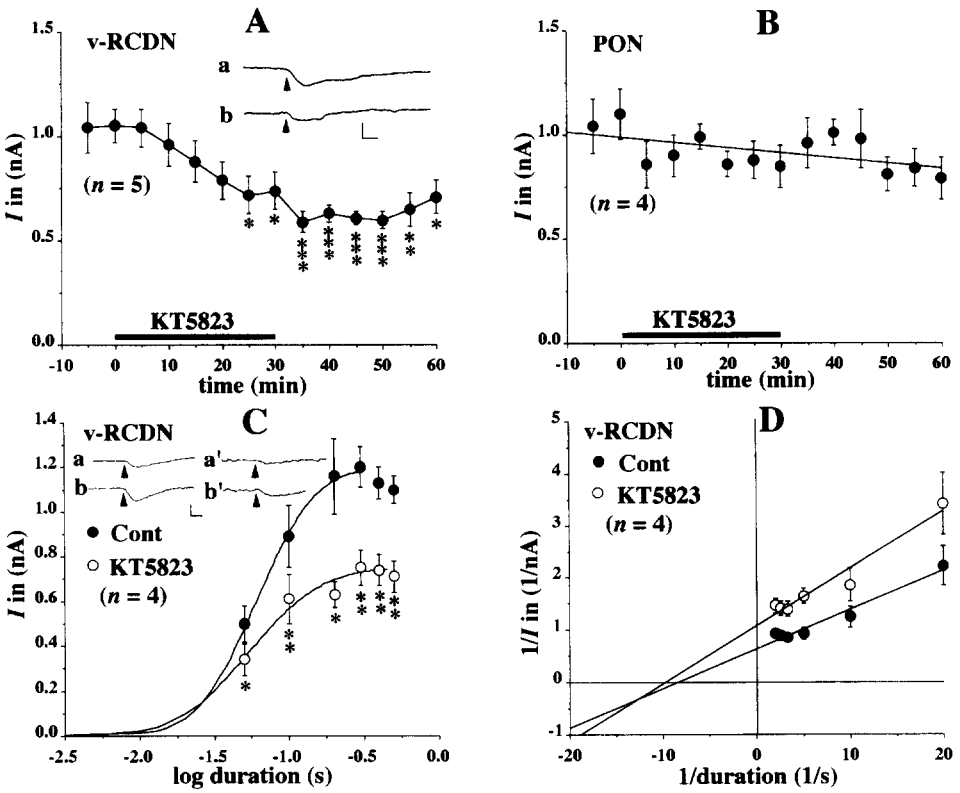

Fig. 2. Effects of KT5823 (guanosine-3′,5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase inhibitor) on the I_{in} caused by achatin-I. Numbers of observations are indicated in parentheses. (A) Effects of KT5823 perfused at 2×10^{-5} M on the I_{in} caused by achatin-I, ejected by a brief pressure (2×10^{5} Pa, 400 ms, 10^{-3} M and 5 min intervals), on v-RCDN. Inset of (A): achatin-I-induced I_{in} on v-RCDN. (B) Effects of KT5823 perfused at 2×10^{-5} M on the I_{in} caused by achatin-I, applied in the same manner, on PON. (C) Dose (pressure duration)-response curves of achatin-I (2×10^{5} Pa, varied durations, 10^{-3} M and 5 min intervals) on v-RCDN. Inset of (C): I_{in} caused by achatin-I in different pressure durations on v-RCDN. (D) Lineweaver-Burk plot of the data shown in (C) on v-RCDN. (A,B) Abscissa, time course (min) (horizontal bar: drug perfusion); ordinate, I_{in} (nA) (small bar: S.E.M.). The values obtained during the drug perfusion and washout were compared with the mean of the controls by ANOVA for repeated measurements and Bonferroni's t-test. Inset of (A): a, control; b, 25 min after the drug perfusion. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); vertical bar, calibration (1 nA). (C) Abscissa, pressure duration in logarithmic scale (s); ordinate, I_{in} (nA) (small bar: S.E.M.). The dose (pressure duration)-response curves of achatin-I were measured in physiological solution (control curve) (●) and in the presence of KT5823 at 10^{-5} M (drug curve) (○) from one neurone. The values of the drug curve were compared with the corresponding values of the control curve by Student's t-test for paired data. The curves were drawn by fitting the ideal sigmoidal curves calculated by the computer program ($r = 0.99828$ for the control curve, and 0.93916 for the drug curve). Inset of C: a, I_{in} obtained by 50 ms pressure duration of the achatin-I ejection in control; b, 300 ms in control; a′, 50 ms in the presence of the drug at 10^{-5} M; b′, 300 ms in the presence of the drug. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); vertical bar, calibration (1 nA). (D) Abscissa, reciprocal of pressure duration (1/s); ordinate, reciprocal of I_{in} (1/nA). The control line (●) and the drug line (○) were drawn by linear regression ($r = 0.97929$ for the control line, and 0.97468 for the drug line).

The cross point of the two lines was calculated to be $X = -12.82214$ and $Y = -0.32416$. This suggests that KT5823 inhibited the I_{in} caused by achatin-I in mainly noncompetitive and partly uncompetitive manners (Fig. 2D).

3.4. Effects of calphostin C

Calphostin C (protein kinase C inhibitor) was dissolved at 10^{-5} M in physiological solution containing 1.0% DMSO. This drug at this concentration did not affect the I_{in} caused by achatin-I, ejected by a brief pressure, on both neurone types tested. The I_{in} of v-RCDN ($n = 4$) was 0.88 ± 0.04 nA for the mean of the controls and 0.83 ± 0.04 nA 30 min after the drug perfusion (NS). The I_{in} of PON ($n = 4$) was 1.22 ± 0.20 nA for the mean of the controls and 1.39 ± 0.18 nA 30 min after the drug perfusion (NS) (data in detail are not shown).

3.5. Effects of W-7

W-7 (calmodulin inhibitor) was dissolved at 5×10^{-5} M in physiological solution containing 0.25% DMSO. This drug, perfused at this concentration, did not affect the I_{in} caused by achatin-I, ejected by the same brief pressure, on v-RCDN ($n = 8$); the I_{in} values were 1.45 ± 0.22 nA for the mean of the controls and 1.44 ± 0.19 nA 30 min after the drug perfusion (NS, compared with the mean of the controls) (Fig. 3A). In contrast, this drug at the same concentration markedly inhibited the I_{in} caused by achatin-I on PON, and this inhibition was irreversible ($n = 7$); the I_{in} was 1.04 ± 0.10 nA for the mean of the controls,

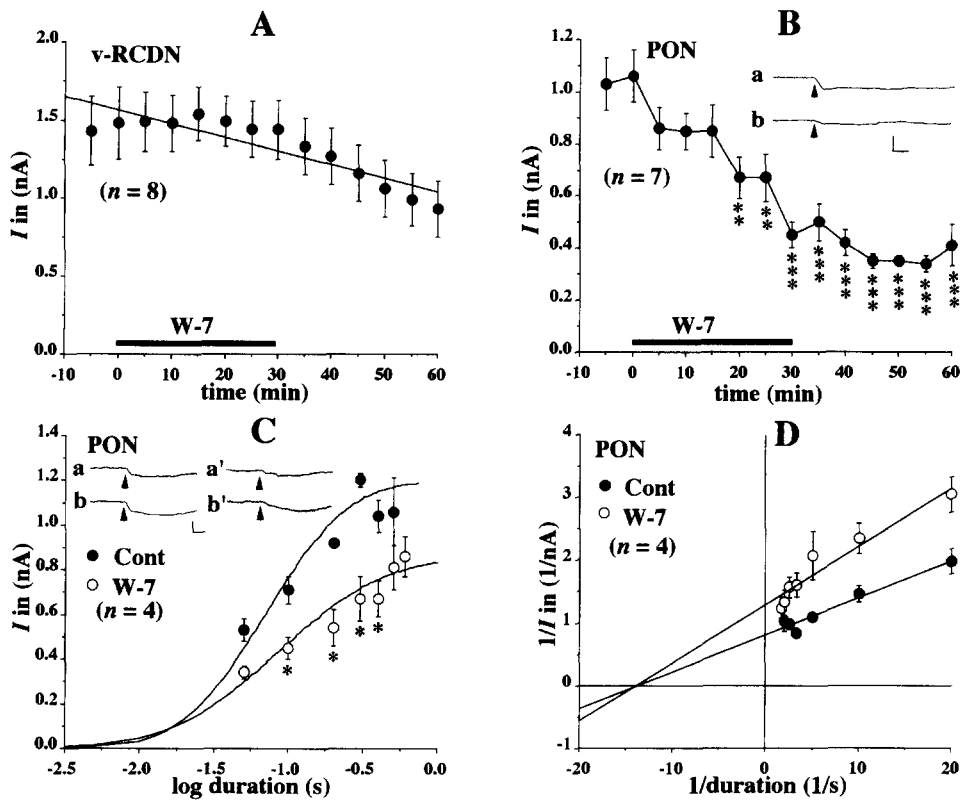

Fig. 3. Effects of W-7 (calmodulin inhibitor) on the I_{in} caused by achatin-I. Numbers of observations are indicated in parentheses. (A) Effects of W-7 perfused at 5×10^{-5} M on the I_{in} caused by achatin-I, ejected by a brief pressure (2×10^5 Pa, 400 ms, 10^{-3} M and 5 min intervals), on v-RCDN. (B) Effects of W-7 perfused at 5×10^{-5} M on the I_{in} caused by achatin-I, applied in the same manner, on PON. Inset of (B): achatin-I-induced I_{in} on PON. (C) Dose (pressure duration)-response curves of achatin-I (2×10^5 Pa, varied durations, 10^{-3} M and 5 min intervals) on PON. Inset of (C): I_{in} caused by achatin-I in different pressure durations on PON. (D) Lineweaver-Burk plot of the data shown in C on PON. (A,B) Abscissa, time course (min) (horizontal bar: drug perfusion); ordinate, I_{in} (nA) (small bar: S.E.M.). The values obtained during the drug perfusion and washout were compared with the mean of the controls by ANOVA for repeated measurements and Bonferroni's t-test. Inset of (B): a, control; b, 30 min after drug perfusion. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); and vertical bar, calibration (1 nA). (C) Abscissa, pressure duration in logarithmic scale (s); ordinate, I_{in} (nA) (small bar: S.E.M.). The dose (pressure duration)-response curves of achatin-I were measured in physiological solution (control curve) (●) and in the presence of W-7 at 10^{-5} M (drug curve) (○) from one neurone. The values of the drug curve were compared with the corresponding values of the control curve by Student's t-test for paired data. The curves were drawn by fitting the ideal sigmoidal curves calculated by the computer program ($r = 0.92294$ for the control curve, and 0.90242 for the drug curve). Inset of (C): a, I_{in} obtained by 100 ms pressure duration of the achatin-I ejection in control; b, 400 ms in control; a', 100 ms in the presence of the drug at 10^{-5} M; b', 400 ms in the presence of the drug. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); and vertical bar, calibration (1 nA). (D) Abscissa, reciprocal of pressure duration (1/s); ordinate, reciprocal of I_{in} (1/nA). The control line (●) and the drug line (○) were drawn by linear regression ($r = 0.97571$ for the control line, and 0.96125 for the drug line).

0.45 ± 0.05 nA 30 min after the drug perfusion (***), and 0.41 ± 0.08 nA 30 min after the washout (***) (Fig. 3B).

The dose (pressure duration)-response curves of achatin-I on PON were measured from one neurone, in physiological solution (control curve) and in the presence of W-7 at 10^{-5} M (drug curve). ED_{50} (95% confidence limit), E_{max} and Hill coefficient (r value) of these curves ($n = 4$) were 69.2 ms (not measurable – 204.2 ms), 1.20 ± 0.03 nA and 1.75284 (0.922938), respectively, for the control curve, and 83.9 ms (34.7–153.7 ms), 0.86 – 0.10 nA (*, compared with the E_{max} of the control curve) and 1.34070 (0.874301), respectively, for the drug curve. ED_{50} became slightly larger, and E_{max} was significantly smaller in the presence of W-7 than those of the control curve (Fig. 3C).

From the Lineweaver-Burk plot of these data, the relations of the reciprocal of the pressure duration and the reciprocal of the I_{in} were Y (1/nA) = 0.80645 + 0.059335X (1/min) for the control line and $Y = 1.29310 + 0.093224X$ for the drug line. The cross point of the two lines was calculated to be $X = -14.360116$ and $Y = -0.045607$, suggesting that W-7 inhibited the I_{in} caused by achatin-I in a noncompetitive manner (Fig. 3D).

3.6. Effects of IBMX

IBMX (cyclic nucleotide phosphodiesterase inhibitor) was dissolved at 10^{-4} M and 4×10^{-4} M in physiological solution containing 0.5% DMSO. This drug, perfused at 10^{-4} M, enhanced reversibly the I_{in} caused by achatin-I, ejected by a brief pressure, on v-RCDN ($n = 7$); the I_{in} was 1.11 ± 0.09 nA for the mean of the controls, 1.53 ±

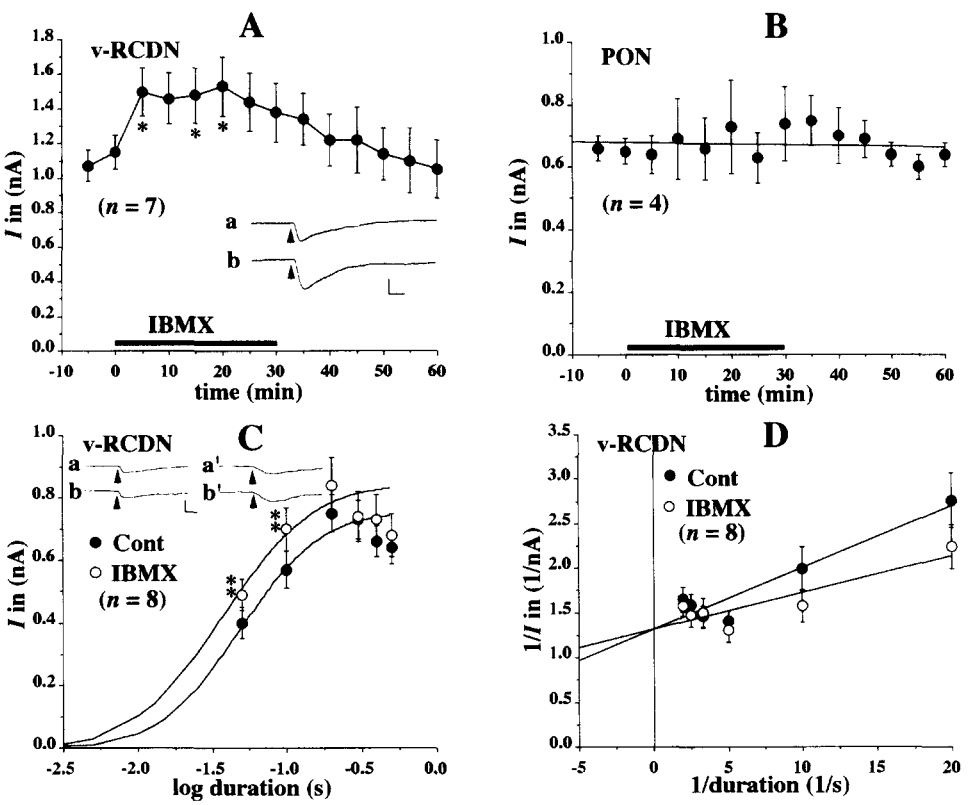

Fig. 4. Effects of IBMX (cyclic nucleotide phosphodiesterase inhibitor) on the I_{in} caused by achatin-I. Numbers of observations are indicated in parentheses. (A) Effects of IBMX perfused at 10^{-4} M on the I_{in} caused by achatin-I, ejected by a brief pressure (2×10^5 Pa, 400 ms, 10^{-3} M and 5 min intervals), on v-RCDN. Inset of (A): achatin-I-induced I_{in} on v-RCDN. (B) Effects of IBMX perfused at 4×10^{-4} M on the I_{in} caused by achatin-I, applied in the same manner, on PON. (C) Dose (pressure duration)-response curves of achatin-I (2×10^5 Pa, varied durations, 10^{-3} M and 5 min intervals) on v-RCDN. Inset of (C): I_{in} caused by achatin-I in different pressure durations on v-RCDN. (D) Lineweaver-Burk plot of the data shown in (C) on v-RCDN. (A,B) Abscissa, time course (min) (horizontal bar: drug perfusion); ordinate, I_{in} (nA) (small bar: S.E.M.). The values obtained during drug perfusion and washout were compared with the mean of the controls by ANOVA for repeated measurements and Bonferroni's t-test. Inset of (A): a, control; b, 20 min after drug perfusion. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); vertical bar, calibration (1 nA). (C) Abscissa, pressure duration in logarithmic scale (s); ordinate, I_{in} (nA) (small bar: S.E.M.). The dose (pressure duration)-response curves of achatin-I were measured in physiological solution (control curve) (●) and in the presence of IBMX at 10^{-4} M (drug curve) (○) from one neurone. The values of the drug curve were compared with the corresponding values of the control curve by Student's t-test for paired data. The curves were drawn by fitting the ideal sigmoidal curves calculated by the computer program ($r = 0.992712$ for the control curve, and 0.994516 for the drug curve). Inset of (C): a, I_{in} obtained by 50 ms pressure duration of the achatin-I ejection in control; b, 300 ms in control; a', 50 ms in the presence of the drug at 10^{-4} M; b', 300 ms in the presence of the drug. Arrows indicate the achatin-I application. Horizontal bar, time course (10 s); and vertical bar, calibration (1 nA). (D) Abscissa, reciprocal of pressure duration (1/s); ordinate, reciprocal of I_{in} (1/nA). The control line (●) and the drug line (○) were drawn by linear regression ($r = 0.95131$ for the control line, and 0.87693 for the drug line).

0.17 nA 20 min after the drug perfusion (*), and 1.05 ± 0.17 nA 30 min after the washout (NS) (Fig. 4A). On the other hand, this drug, even at 4×10^{-4} M, did not affect the I_{in} caused by achatin-I, applied in the same manner, on PON ($n = 4$); the I_{in} was 0.66 ± 0.03 nA for the mean of the controls and 0.74 ± 0.12 nA 30 min after the drug perfusion (NS) (Fig. 4B).

The dose (pressure duration)-response curves of achatin-I on v-RCDN were measured from one neurone, in physiological solution (control curve) and in the presence of IBMX at 10^{-4} M (drug curve). ED_{50} (95% confidence limit), E_{max} and Hill coefficient (r value) of these curves ($n = 8$) were 47.8 ms (not measurable – 119.0 ms), 0.75 ± 0.06 nA and 1.75837 (0.988753), respectively, for the control curve, and 37.0 ms (not measurable – 82.2 ms), 0.84 ± 0.09 nA (NS, compared with the E_{max} of the control curve) and 1.59769 (0.997234), respectively, for the drug curve. ED_{50} was smaller, but E_{max} was almost unchanged in the presence of IBMX on v-RCDN, when compared to those of the control curve (Fig. 4C).

From the Lineweaver-Burk plot of these data, the relations of reciprocal of the pressure duration and reciprocal of the I_{in} were $Y\,(1/\text{nA}) = 1.3171 + 0.041037X\,(1/\text{s})$ for the control line and $Y = 1.3126 + 0.069677X$ for the drug line. The cross point of the two lines was calculated to be $X = 0.15712$ and $Y = 1.323548$, almost on the ordinate. From these findings, we consider that IBMX would act on the receptor sites of achatin-I to enhance the I_{in} caused by this peptide (Fig. 4D).

3.7. Effects of other inhibitors

ML-9 (myosin light-chain kinase inhibitor) at 2×10^{-5} M (dissolved in physiological solution containing 0.5% DMSO), genistein (tyrosine kinase inhibitor) at 2×10^{-5} M (0.25% DMSO), fluphenazine nitrogen-mustard (calmodulin-dependent phosphodiesterase inhibitor) at 10^{-4} M (0.5% DMSO), indomethacin (prostaglandin cyclooxygenase inhibitor) at 5×10^{-5} M (0.1% DMSO), okadaic acid (type 1, 2A and 2B protein phosphatase inhibitor) at 10^{-6} M (1.0% DMSO) and calyculin A (type 1 protein phosphatase inhibitor) at 10^{-6} M (1.0% DMSO), all did not significantly affect the I_{in} caused by achatin-I, ejected by a brief pressure, on both v-RCDN and PON ($n \geq 4$) (data not shown).

4. Discussion

It was proposed that achatin-I (Gly-D-Phe-Ala-Asp) is an excitatory neurotransmitter for the *Achatina* neurones, since this peptide was isolated from the *Achatina* ganglia and excited nearly half of the identifiable *Achatina* giant neurone types tested (Kamatani et al., 1989; Kim et al., 1991a).

In the present experiments, achatin-I was applied locally to the neurone tested by a brief pressure ejection. The excitatory effects of this peptide were considered to be the postsynaptic (not transsynaptic) events of the neurone tested.

We demonstrated in the present study that H-89 (cyclic AMP-dependent protein kinase inhibitor) inhibited noncompetitively the achatin-I-induced I_{in} on an *Achatina* giant neurone type, PON (periodically oscillating neurone), suggesting that this current is produced via intracellular cyclic AMP system on this neurone type. In addition, W-7 (calmodulin inhibitor) inhibited noncompetitively the I_{in}, suggesting that calmodulin plays roles in producing this current probably through Ca^{2+}/calmodulin-sensitive adenylate cyclase and/or Ca^{2+}/calmodulin-dependent protein kinase. It was reported that the achatin-I-induced I_{in} of this neurone type was mainly Na^+-dependent (Kim et al., 1991a). Together with the present results, it is considered that the above signal transduction pathways finally activate the Na^+ channels in this neurone type by phosphorylation.

On the other hand, KT5823 (cyclic GMP-dependent protein kinase inhibitor) inhibited the I_{in} in mainly noncompetitive and partly uncompetitive manners on another *Achatina* giant neurone type, v-RCDN (ventral-right cerebral distinct neurone), suggesting that this I_{in} is produced via the cyclic GMP system. However, our ongoing work on ionic dependence revealed that this current was partly (about half) Na^+-dependent. Further studies are needed to elucidate the linkages between the signal transduction pathways and ionic channels finally activated by achatin-I on this neurone type.

It was reported (Takeuchi et al., 1985a, b, 1987) that small molecule putative neurotransmitters, such as dopamine, 5-hydroxytryptamine, γ-aminobutyric acid (GABA) and acetylcholine, showed either excitatory or inhibitory effects on *Achatina* giant neurone types, suggesting the presence of their receptor subtypes. Based on the pharmacological features, GABA and dopamine receptors of *Achatina* neurones were classified into their subtypes (Kim and Takeuchi, 1990; Emaduddin et al., 1995). In contrast, neuroactive peptides including achatin-I produced only excitatory or inhibitory effects on *Achatina* neurone types, suggesting that the receptor features of a peptide would be homogenous (Liu et al., 1991a, b; Araki et al., 1995). Unexpectedly, the present study revealed that the different signalling pathways mediated the effects of achatin-I on different *Achatina* neurone types, and all of these pathways produced only the excitatory effects.

In addition to v-RCDN and PON, five *Achatina* neurone types were markedly excited by achatin-I (Araki et al., 1995). The pharmacological features and the signal transduction system involvement of the achatin-I receptors in these *Achatina* neurone types will be studied in the future, to classify the receptors into their subtypes. These investigations will be carried out based on the following evidence: some histamine H_1 receptor antagonists, chlorcyclizine, promethazine and triprolidine, inhibited non-

competitively the I_{in} caused by achatin-I on PON (Santos et al., 1995); and the ionic mechanism of the I_{in} caused by achatin-I was partly different in v-RCDN and PON as mentioned above.

It was evidenced in the present study that IBMX (cyclic nucleotide phosphodiesterase inhibitor) enhanced the achatin-I-induced I_{in} on v-RCDN. However, the dose (pressure duration)-response study and the Lineweaver-Burk plot of these data indicated that this drug might act on achatin-I receptor sites, probably by increasing the binding activity of achatin-I to its receptors. We found (Emaduddin and Takeuchi, unpublished data) that IBMX inhibited competitively the outward current (I_{out}) caused by fulicin (Phe-D-Asn-Glu-Phe-Val-NH_2), a neuroactive peptide originally isolated from the *Achatina* ganglia (Ohta et al., 1991), on an *Achatina* neurone type, d-LPeLN (dorsal-left pedal large neurone). It was reported that IBMX acted to be an adenosine receptor antagonist in rat diaphragm (Sebastiao and Ribeiro, 1988). In addition, we consider that this compound modulates the binding activity of other neurotransmitters to their receptors, by either enhancing or blocking.

On the other hand, unexpectedly IBMX was ineffective on the achatin-I-induced I_{in} of PON, which was mediated by the intracellular cyclic AMP system. Further studies are needed to elucidate the features of phosphodiesterase related to the achatin-I receptors in this neurone type.

We demonstrated previously (Liu and Takeuchi, 1993c) that the intracellular injections of cyclic AMP and cyclic GMP showed the excitatory (depolarizing) effects concordantly on several *Achatina* giant neurone types tested, including PON (v-RCDN was not tested), and the same injection of inositol 1,4,5-triphosphate (IP_3) inhibited (hyperpolarized) these neurones. These findings supported the results obtained in the present experiments.

With respect to the excitatory neurotransmitters other than achatin-I, Kirk et al. (1988) demonstrated that egg-laying hormone (ELH) (Kirk and Scheller, 1986) induced an I_{in} on a neurone type of a marine mollusc, *Aplysia californica*. 8-Bromo-cyclic AMP (cyclic AMP agonist) and 8-bromo-cyclic GMP (cyclic GMP agonist) produced a similar current on the same neurone type, and the prior application of 8-bromo-cyclic AMP prevented the ELH-induced I_{in}, suggesting that the excitatory effects of ELH were mediated by either cyclic AMP or cyclic GMP.

Matsumoto et al. (1988) reported that dopamine produced the Na^+-dependent I_{in} on the *Aplysia* neurones. The intracellular injection of cyclic AMP produced the same current, and IBMX enhanced the currents induced by both dopamine and cyclic AMP, suggesting that the effects of dopamine were mediated by the cyclic AMP system.

Sawada et al. (1995) described that hydroxylamine and sodium nitroprusside (nitric oxide generators) produced the Na^+-dependent I_{in} on the *Aplysia* neurones. The intracellular injection of cyclic GMP also induced the same current. These currents were enhanced by IBMX and suppressed by methylene blue (guanylate cyclase inhibitor), suggesting that the I_{in} induced by nitric oxide was mediated by the cyclic GMP system.

The findings on the intracellular signalling systems in the reports cited above were in concordance with the present results on achatin-I, except for those of IBMX.

In contrast, Kudo et al. (1991) reported that 5-hydroxytryptamine produced the Na^+-dependent I_{in} on *Aplysia* neurones, and this current was inhibited by GDPβS (guanosine-5′-*O*-(2-thio-diphosphate)) (guanosine-5′-monophosphate (GTP)-binding protein (G_s) inhibitor) and cholera toxin (G_s activator). However, cordycepin (3′-deoxyadenosine) (adenylate cyclase inhibitor) and H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide) (cyclic AMP- and cyclic-GMP-dependent protein kinase inhibitor) did not affect this current, suggesting that this I_{in} was mediated by G_s without the activation of the cyclic AMP system.

Chiba et al. (1992) reported that the effects of GDPβS, cholera toxin, cordycepin and H-8 on the I_{in} caused by FMRFamide on *Aplysia* neurones were similar to those on the 5-hydroxytryptamine-induced I_{in} studied by Kudo et al. (1991), suggesting that the FMRFamide-induced I_{in} was also mediated by G_s without cyclic AMP system activation.

Sudlow et al. (1993) reported that the intracellular injection of cyclic AMP produced the Na^+-dependent I_{in} on the neurones of a marine snail, *Pleurobranchaea californica*. This current was not affected by protein kinase inhibitor protein (Cheng et al., 1986), suggesting that this current was caused by the direct activation of Na^+ channels by cyclic AMP.

The findings described in the last three reports were different from those of the achatin-I-induced I_{in} on the *Achatina* neurones. The involvement of the GTP-binding protein to produce the excitatory effects for achatin-I as well as the classification of the achatin-I receptors into their subtypes will be studied in our serial investigations on the achatin-I effects.

In summary, the present study demonstrated the existence of the multiple intracellular signal transduction pathways mediating the achatin-I-induced current in different neurone types even in the simple *Achatina* nervous system, although the ultimate response of these neurone types seemed to be similar.

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