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Dopamine stimulates snail albumen gland glycoprotein secretion through the activation of a D1-like receptor

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Summary

The catecholamine dopamine is present in both the central nervous system and in the peripheral tissues of molluscs, where it is involved in regulating reproduction. Application of exogenous dopamine to the isolated albumen gland of the freshwater pulmonate snail *Helisoma duryi* (Wetherby) induces the secretion (release) of perivitelline fluid. The major protein component of the perivitelline fluid of *Helisoma duryi* is a native 288 kDa glycoprotein that is secreted around individual eggs and serves as an important source of nutrients for the developing embryos. The secretion of glycoprotein by the albumen gland is a highly regulated event that must be coordinated with the arrival of the fertilized ovum at the carrefour (the region where the eggs receive albumen gland secretory products). In order to elucidate the intracellular signalling pathway(s) mediating dopamine-induced glycoprotein secretion, albumen gland cAMP production and glycoprotein secretion were measured in the presence/absence of selected dopamine receptor agonists and antagonists. Dopamine D1-selective agonists dihydrexidine, 6,7-ADTN and SKF81297 stimulated cAMP production and glycoprotein secretion from isolated albumen glands whereas D1-selective antagonists SCH23390 and SKF83566 suppressed dopamine-stimulated cAMP production. Dopamine D2-selective agonists and antagonists generally had no effect on cAMP production or protein secretion. Based on the effects of these compounds, a pharmacological profile was obtained that strongly suggests the presence of a dopamine D1-like receptor in the albumen gland of *Helisoma duryi*. In addition, secretion of albumen gland glycoprotein was not inhibited by protein kinase A inhibitors, suggesting that dopamine-stimulated protein secretion might occur through a protein kinase A-independent pathway.

Key words: dopamine, dopamine receptor, albumen gland, perivitelline fluid, glycoprotein, secretion, cAMP, snail, exocrine.

Introduction

The catecholamine dopamine has a widespread distribution in the molluscan central nervous system (CNS; Hetherington et al., 1994; Trimble et al., 1984; Audesirk, 1985; Muneoka et al., 1991), where it is a neurotransmitter regulating numerous physiological processes including learning and memory (Lukowiak and Syed, 1999), the regulation of neurite outgrowth and synaptic connectivity (Spencer et al., 1998), embryonic neural development (Croll, 2000) and the regulation of neural circuits involved in feeding (Elliott and Susswein, 2002) and respiration (Taylor and Lukowiak, 2000). Dopamine is also found in peripheral tissues of molluscs, where it controls physiological processes such as smooth muscle contraction (Gies, 1986), motility of the gastrointestinal tract (Hernadi et al., 1998) and salivary secretion (Kiss et al., 2003).

Catecholaminergic axons have been shown to innervate the reproductive organs of various molluscs (Hartwig et al., 1980; Smith et al., 1998; Croll et al., 1999; Croll, 2001; Kiehn et al., 2001), and histochemical analyses have revealed that catecholaminergic cell bodies and axon processes are concentrated in the region of the exocrine albumen gland (AG) of the freshwater snails *Bulinus truncatus* (Brisson and Collin, 1980; Brisson, 1983) and *Helisoma duryi* (Kiehn et al., 2001). The AG is a female accessory reproductive gland that secretes a viscous substance known as the perivitelline fluid (PVF) around the individual eggs as they enter the carrefour, the area where the main duct of the AG empties. The PVF consists mainly of glycoproteins and galactogen (a highly branched galactose polymer), which provide the main source of nutrients to the developing embryos (Duncan, 1975; Geraerts and Joosse, 1984). Therefore, the timely release (secretion) of PVF is a key regulatory process governing egg production in freshwater snails.

Although the neuroendocrine control of polysaccharide (galactogen) synthesis in the AG has been studied extensively in freshwater pulmonate molluscs (de Jong-Brink et al., 1982; Wijdenes et al., 1983; Miksys and Saleuddin, 1985, 1988; Mukai et al., 2001b), little is known with respect to the regulation of PVF release. We have identified the major protein produced by the AG of the freshwater snail *H. duryi* as a 288 kDa glycoprotein, which is composed of several 66 kDa

subunits (Morishita et al., 1998). The gene encoding for the 66 kDa glycoprotein subunit has recently been cloned (Mukai et al., 2004) and the corresponding protein was given the name *Helisoma duryi* albumen gland protein (HdAGP). The release of HdAGP is known to be stimulated by a novel brain peptide (Morishita et al., 1998) through a cAMP signalling pathway (Mukai et al., 2001a).

The secretion of HdAGP coincides with the arrival of the eggs at the carrefour, and the existence of a control mechanism over AG secretory activity has been postulated (Mukai et al., 2004). Kiehn et al. (2001) showed that the AG and carrefour region of *H. duryi* is innervated by dopaminergic nerve fibres. Moreover, dopamine has also been shown to induce the secretion of total protein from isolated AGs of *H. duryi* (Mukai, 1998; Saleuddin et al., 2000) and *Biomphalaria glabrata* (Santhanagopalan and Yoshino, 2000). Subsequent studies by Boyle and Yoshino (2002) showed that *B. glabrata* AG dopamine levels increased during the initial stage of egg mass production (the period during which the AG is secreting PVF), whereas protein levels in the AG decreased during the latter stages of egg mass production. Collectively, these results suggest that the secretion of protein by the AG of freshwater pulmonate snails is regulated by dopamine and, by inference, specific dopamine receptors in the AG.

Dopamine receptors were originally classified into two categories, D1 and D2, based on their ability when activated to either stimulate or inhibit, respectively, adenylate cyclase activity (reviewed by Civelli et al., 1993). The genes for five distinct dopamine receptor subtypes (D1, D2, D3, D4, D5) have been cloned in mammals and placed into one of the two dopamine receptor groups based on their gene structure and pharmacology (O'Dowd, 1993). The dopamine D1-like receptors, which stimulate cAMP formation, comprise the D1 and D5 subtypes, whereas the dopamine D2-like receptors, comprising the D2, D3 and D4 subtypes, either inhibit or have no effect on adenylate cyclase (Missale et al., 1998). Using a number of dopamine receptor agonists and antagonists, Saleuddin et al. (2000) suggested that a dopamine D1-like receptor mediates total protein secretion by AGs of *H. duryi*. Here, we extend our previous study and examine the intracellular signalling pathway activated after AGs were treated with dopamine. Addition of exogenous dopamine to AG explants stimulates secretion of HdAGP *via* the activation of the cAMP signalling pathway. A number of D1-selective and D2-selective agonists and antagonists were used to obtain a pharmacological profile of the AG dopamine receptor. It is concluded that the AG dopamine receptor regulating protein secretion is distinct but functionally similar to vertebrate D1-like receptors and mediates secretion of HdAGP through an elevation in glandular cAMP, possibly through a protein kinase A (PKA)-independent pathway.

Materials and methods

Animals

Helisoma duryi were reared in 2-litre plastic containers with dechlorinated tap water at 22°C and maintained under a 16 h:8 h L:D photoperiod. The snails were fed a diet of boiled lettuce every 2–3 days, occasionally supplemented with fish pellets. The water was changed at least once per week. Two weeks prior to experimentation, adult snails (15 mm shell diameter) were selected and then individually placed in plastic cups to monitor their egg-laying activity. Only snails that had not laid eggs within the previous 12 h were used for experiments.

Chemicals

Dopamine (3-hydroxytyramine), serotonin, acetylcholine, γ-aminobutyric acid, norepinephrine, histamine, octopamine, glutamate, forskolin (7β-acetoxy-8,13-epoxy-1α, 6β, 9α-trihydroxylabd-14-en-11-one), IBMX (3-isobutyl-1-methylxanthine), apomorphine hydrochloride, bromocriptine (2-bromo-α-ergocryptine methanesulfonate salt), (R)(+)-SCH23390 hydrochloride, (R)(±)-SKF-38393 hydrochloride, R(+)-SKF81297 hydrobromide, (±)-SKF83566 hydrochloride, dihydrexidine hydrochloride {(±)-trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine}, 6,7-ADTN [(±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronapthalene], haloperidol {4-(4-[4-chlorophenyl]-4-hydroxy-1-piperidinyl)-1-(4-fluorophenyl)-1-butanone}, (–)-butaclamol hydrochloride, *cis*-flupenthixol, chlorpromazine, eticlopride, Rp-cAMP and H-89 were purchased from Sigma-Aldrich Canada, Oakville, Ontario, Canada.

Bioassay

Albumen glands were dissected free from surrounding tissue under *Helisoma* saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes, 1 mmol l^{-1} glucose, pH 7.4, 120 mOsm H_2O), cut into halves and then washed in several changes of saline. One half served as a control gland while the other was treated with test compound. Each AG piece (~0.5–1.5 mg) was placed in a separate well of a 96-well culture plate (Becton-Dickinson and Co., Lincoln Park, NJ, USA) containing 100 μl of saline. The saline surrounding the AG was removed and replaced with another 100 μl of fresh saline every 20 min. The collected saline was placed in 1.7 ml polypropylene microtubes (Brinkmann Instruments Inc., Westbury, NY, USA) and centrifuged at 2000 ***g*** for 1 min. An 80 μl sample was removed and added directly to a 1.5 ml polystyrene cuvette containing 420 μl of Triton X-100 (0.0095% in water). Total protein was determined by adding 125 μl Bio-Rad Protein Dye Reagent Concentrate (Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada) and measuring the absorbance at 595 nm with a Zeiss PM2DL spectrophotometer (Carl Zeiss, Oberkochen, Germany). Basal HdAGP secretion was measured for the first 60 min. A stock solution of dopamine (10 mmol l^{-1}) was prepared in deoxygenated-demineralized water and diluted in saline to a final concentration of 10 μmol l^{-1} immediately before use. All the test compounds were dissolved in *Helisoma* saline (with or without dopamine) to their final concentrations and applied to the AGs at 60 min. The test agents were removed at 80 min and replaced with

normal saline for another 60 min. Dopamine receptor antagonists (D1-selective or D2-selective) or PKA inhibitors (Rp-cAMP and H-89) were first preincubated with AGs at 40 min, then removed and replaced with antagonist plus dopamine at 60 min. The amount of HdAGP secreted between 40 and 60 min (control) was compared with the amount secreted between 60 and 80 min (treated).

Electrophoresis

To qualitatively determine the proteins secreted by the AG *in vitro* after dopamine stimulation, the saline surrounding the AG was collected and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The collected saline was first evaporated to dryness using a Savant SVC 100 Speed-Vac (Instruments Inc., Farmingdale, NY, USA), then resuspended in SDS-PAGE sample buffer and separated on a 9% mini-gel apparatus (Bio-Rad) according to Laemmli (1970). Following electrophoresis, the gel was stained overnight with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol/10% acetic acid. The gel was destained the next day and dried onto Whatman 3MM filter paper using a slab gel dryer (Hoefer Scientific Instruments, San Francisco, CA, USA).

cAMP determinations

Albumen glands were dissected under snail saline, then quartered and rinsed in normal saline. Prior to treatment, each AG piece was preincubated for 20 min with 1 mmol l^{-1} IBMX in saline to allow the phosphodiesterase inhibitor to penetrate the AG cells. All test compounds were dissolved in IBMX saline and applied to the AG for 10 min unless indicated otherwise. For the time course experiment, one AG piece served as a control, the second piece was treated with dopamine (10 μmol l^{-1}), the third piece was treated with forskolin (10 μmol l^{-1}) and the fourth piece was treated with both dopamine and forskolin. At the end of each time point, the AGs were immediately plunged into liquid nitrogen, then stored in 1.7 ml microtubes at –80°C. To extract cAMP, AGs were homogenized in 3% ice-cold perchloric acid using a motor-driven Teflon pestle and then centrifuged at 10 000 ***g*** (10 min at 4°C). The precipitates were kept for subsequent protein determinations and resuspended in 0.1 mol l^{-1} NaOH (60°C for 1 h) prior to use. The acidic supernatant was transferred to a new tube and neutralized to pH 6 with 2.6 mol 1^{-1} potassium bicarbonate. The resultant potassium perchlorate precipitate was discarded and the recovered supernatant was evaporated to dryness. The dried residue was resuspended in cAMP assay buffer and the concentration of AG cAMP was measured with a commercial [3H]cAMP assay kit (Diagnostic Products Corp., Los Angeles, CA, USA). For the pharmacological characterization of the AG dopamine receptor, AGs were quartered: one AG piece served as a control; the second piece was treated with 10 μmol l^{-1} dopamine (positive control); the third piece was treated with dopamine receptor agonist or antagonist (10 μmol l^{-1}); and the fourth piece was treated with dopamine plus agonist or antagonist. The AGs were extracted for cAMP as described above. Data were expressed as pmol cAMP mg^{-1} AG protein.

Results

Effect of dopamine on HdAGP secretion

Dopamine was previously demonstrated to induce the release of total protein from *H. duryi* AGs maintained *in vitro*; however, the identity of proteins released was not confirmed. To this end, we collected the saline surrounding the AG and identified the major protein secreted *in vitro* as the 66 kDa subunit of HdAGP (Fig. 1). The time course of basal protein secretion was measured for 60 min, followed by the addition of 10 μmol l^{-1} dopamine for 20 min. Dopamine induced a rapid increase in the amount of protein released *in vitro*, as quantified by protein assay. The SDS-PAGE separation of the secreted material confirmed that the major protein secreted was the 66 kDa subunit of HdAGP (Fig. 1). After removal of the dopamine stimulus at 80 min, HdAGP secretion showed a precipitous decline and then maintained a secretory rate comparable with that observed before stimulation. The time course of HdAGP secretion as identified by SDS-PAGE was identical to the amount of total protein released as quantified by protein assay.

The specificity of the AG response to dopamine was tested by applying other known neurotransmitters found in molluscan

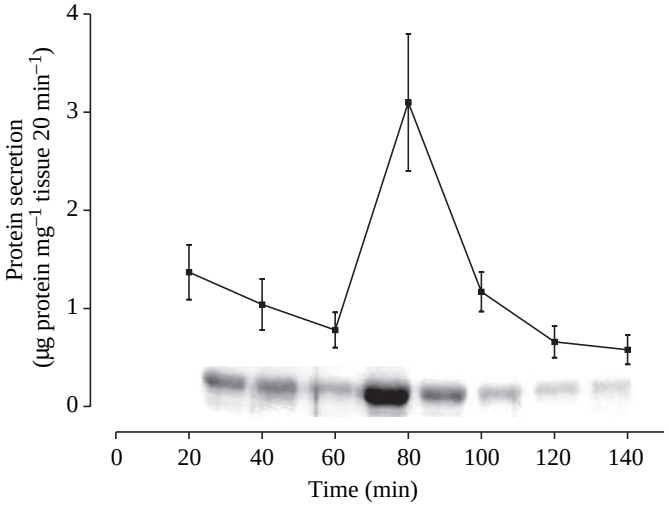

Fig. 1. Effect of dopamine on the *in vitro* secretion of protein by the albumen gland. Basal protein secretion was quantified using a protein detection kit (line graph) or by collecting the medium around the albumen glands and analyzing secreted proteins by SDS-PAGE. Basal protein secretion was monitored for 60 min, then 10 μmol l^{-1} dopamine was added for an additional 20 min. The major protein secreted by the albumen gland of *Helisoma duryi* is the 66 kDa glycoprotein subunit of *Helisoma duryi* albumen gland protein (HdAGP), which appears as a heavily stained band. Addition of dopamine induced a rapid increase in the amount of HdAGP released. Removal of the stimulus at 80 min caused HdAGP secretory rate to rapidly return to basal levels. Each point on the line graph represents the mean ± S.E.M. of 6–8 samples.

nervous tissue to the AG and then measuring *in vitro* secretion of HdAGP. Serotonin, acetylcholine, γ-aminobutyric acid (GABA), norepinephrine, histamine, octopamine and L-glutamate were tested at 0.1, 1 and 10 μmol l^{-1}. With the exception of dopamine, none of the other neurotransmitters were capable of inducing the secretion of HdAGP from the AG (Table 1). A significant stimulation of HdAGP secretion was induced at a concentration of 1 and 10 μmol l^{-1} dopamine.

The effect of dopamine on cAMP production in the AG

Since the existence of a dopamine D1-like receptor on the AG was indicated by previous protein secretion bioassays (Saleuddin et al., 2000) and since forskolin (an adenylate cyclase activator) is known to be a potent stimulator of HdAGP secretion (Morishita et al., 1998), we tested for the effect of dopamine on intracellular cAMP production. The time course of AG cAMP production was measured over a 20 min period after application of 10 μmol l^{-1} dopamine, 10 μmol l^{-1} forskolin or both of these compounds together. Both dopamine and forskolin caused a significant increase in cAMP production by the AG between 5 and 20 min after their application. Furthermore, dopamine-stimulated cAMP levels were augmented in the presence of forskolin and increased linearly for at least 20 min (Fig. 2). Subsequent cAMP determinations were performed using a 10 min treatment unless otherwise indicated. The addition of dopamine (Fig. 3A) or forskolin (Fig. 3B) to AGs increased cAMP production in a dose-dependent manner. Significant elevation of cAMP levels were observed at 10 μmol l^{-1} and 100 μmol l^{-1} for both compounds; however, forskolin was a more potent activator of cAMP production than dopamine.

Effects of dopamine receptor agonists on AG cAMP production

To assess the effect of various dopamine receptor agonists on AG cAMP production, D1-selective agonists (dihydrexidine, SKF81297 and 6,7-ADTN) and D2-selective agonists (bromocriptine and apomorphine) were tested. Treatment of AGs with dopamine (10 μmol l^{-1}) increased AG cAMP levels 3–6-fold above basal AG cAMP levels (Fig. 4). The D1-selective agonists dihydrexidine (Fig. 4A) and 6,7-ADTN (Fig. 4B) stimulated AG cAMP production significantly (3-fold and 5-fold, respectively), whereas SKF81297 had a modest (2-fold) non-significant stimulatory effect on AG cAMP production (Fig. 4C). No augmentation of cAMP production was detected when both dopamine and the

Table 1. *The effect of various neurotransmitters on glycoprotein secretion in the albumen glands of* H. duryi

		Protein secretion (μg protein mg^{-1} tissue 20 min^{-1})		
Neurotransmitter	Concentration (μmol)	Basal	Treated	Ratio
Serotonin	0.1	1.20±0.28	1.09±0.36	0.91
	1	1.37±0.26	1.21±0.27	0.88
	10	1.32±0.79	1.03±0.27	0.78
Acetylcholine	0.1	1.28±0.16	1.11±0.11	0.86
	1	1.15±0.18	1.02±0.18	0.89
	10	0.93±0.12	0.79±0.08	0.85
GABA	0.1	1.09±0.22	0.83±0.20	0.76
	1	1.08±0.26	0.78±0.18	0.72
	10	0.69±0.13	0.55±0.13	0.80
Norepinephrine	0.1	1.82±0.37	1.27±0.21	0.70
	1	1.66±0.27	1.26±0.19	0.76
	10	1.49±0.37	1.43±0.30	0.96
Histamine	0.1	1.38±0.25	1.11±0.21	0.80
	1	1.19±0.24	0.74±0.15	0.62
	10	1.22±0.27	1.14±0.28	0.93
Octopamine	0.1	1.36±0.54	1.13±0.46	0.83
	1	0.85±0.15	0.81±0.16	0.95
	10	0.91±0.15	0.84±0.32	0.92
Glutamate	0.1	1.10±0.18	0.89±0.20	0.81
	1	1.15±0.25	1.10±0.24	0.95
	10	1.24±0.13	1.23±0.15	0.99
Dopamine	0.1	1.61±0.75	1.30±0.52	0.81
	1	0.93±0.20	1.66±0.29*	1.78
	10	0.87±0.23	3.61±0.74**	4.15

Serotonin, acetylcholine, γ-aminobutyric acid (GABA), norepinephrine, histamine, octopamine, glutamate and dopamine were tested at three different concentrations (0.1, 1 and 10 μmol l^{-1}). Dopamine was the only neurotransmitter capable of inducing glycoprotein secretion by isolated albumen glands. A statistically significant increase in glycoprotein secretion was observed at a concentration of 1 and 10 μmol l^{-1} dopamine. Values represent the mean ± S.E.M. for 5–6 samples. *P=0.0042; **P=0.012, paired t-test.

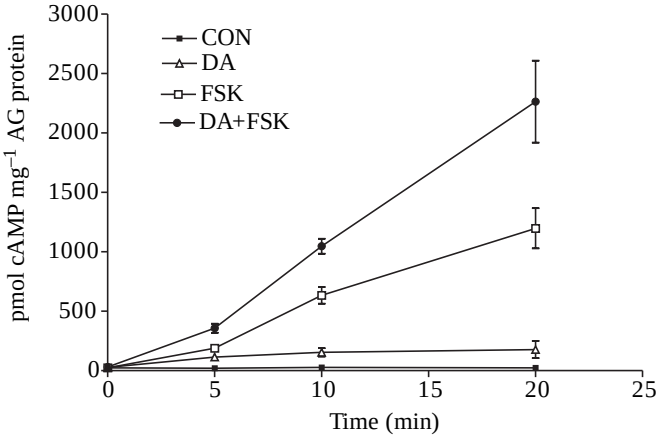

Fig. 2. The time course of cAMP production by the albumen gland of *H. duryi* after application of dopamine (DA) or the adenylate cyclase activator forskolin (FSK). The application of dopamine (10 μmol l^{-1}) or forskolin (10 μmol l^{-1}) significantly increased albumen gland cAMP production between 5 and 20 min. Note, in the presence of forskolin, dopamine-stimulated cAMP production was potentiated. Each point represents the mean ± S.E.M. of 4–6 samples. CON, control.

D1-selective agonists were added to the AGs. By contrast, neither of the two D2-selective agonists apomorphine (Fig. 5A) nor bromocriptine (Fig. 5B) had a statistically significant effect on basal cAMP production. Bromocriptine had no effect on dopamine-stimulated cAMP production (Fig. 5B) but apomorphine unexpectedly caused a significant inhibition of dopamine-stimulated cAMP production (Fig. 5A).

Effects of dopamine receptor antagonists on dopamine-stimulated cAMP levels in the AG

Various D1-selective antagonists were tested for their ability to inhibit dopamine-stimulated cAMP production. At the concentrations (10 μmol l^{-1}) tested in this study, the benzazepines SCH23390 (Fig. 6A) and SKF83566 (Fig. 6B) suppressed dopamine-stimulated cAMP production by 62% and 48%, respectively. The compound flupenthixol had a slight inhibitory effect (22%) on dopamine-stimulated cAMP production but it was not statistically significant. None of the D1-selective antagonists affected basal cAMP levels significantly. The D2-selective antagonists chlorpromazine (Fig. 7A), eticlopride (Fig. 7B) or haloperidol (Fig. 7C) and the mixed D1/D2 antagonist butaclamol (Fig. 7D) had no effect on either basal or dopamine-stimulated cAMP production.

Effects of D1 and D2 agonists and D1 antagonists on HdAGP secretion

To confirm our previous results on the presence of a dopamine D1-like receptor, which mediates protein secretion, in the AG of *H. duryi*, we extended the number of D1-like receptor agonists and antagonists used. The D1-selective agonists SKF81297 and dihyrexidine were tested for their

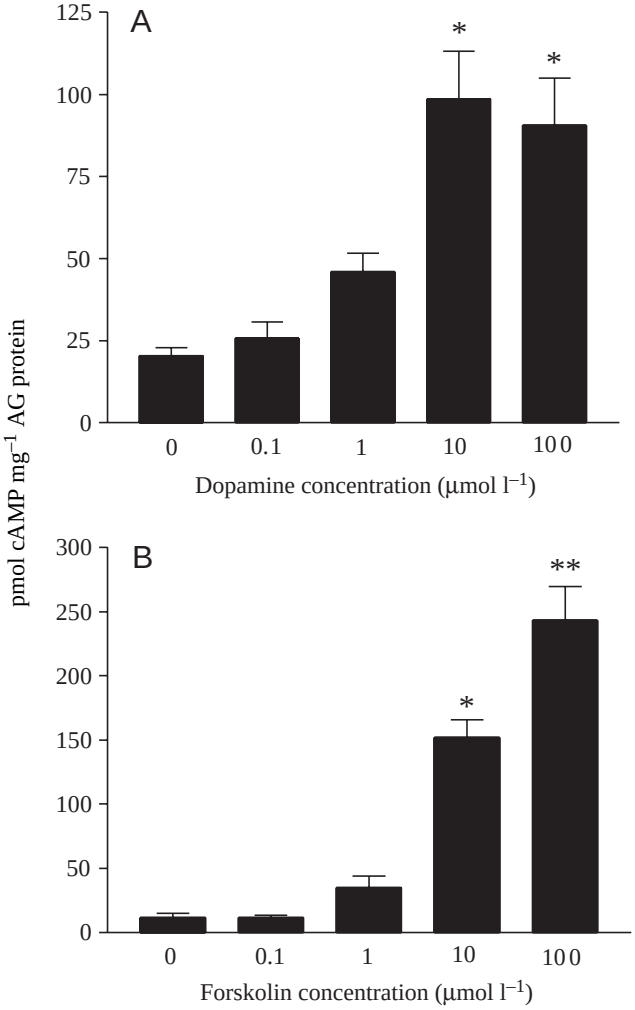

Fig. 3. The effect of different concentrations of (A) dopamine and (B) forskolin on albumen gland cAMP production during a 10 min incubation. Note that dopamine caused a dose-dependent elevation in albumen gland cAMP levels between 0.1 and 10 μmol l^{-1}. Dopamine treatment induced a statistically significant elevation of albumen gland cAMP levels at a concentration of 10 and 100 μmol l^{-1} when compared with untreated control glands (**P*<0.001, Tukey test). Forskolin caused a dose-dependent increase in albumen gland cAMP production between 0.1 and 100 μmol l^{-1} (**P*<0.05 and ***P*<0.001, Tukey test). Note that forskolin is a more potent stimulant than dopamine on albumen gland cAMP production. Bars represent the mean ± S.E.M. of 6 samples.

ability to induce protein secretion from the AGs whereas the D1-selective antagonists SKF83566 and flupenthixol were tested for their ability to inhibit dopamine-induced protein secretion. Both SKF81297 (Fig. 8A) and dihyrexidine (Fig. 8B) were capable of inducing HdAGP secretion by AG explants, although dihydrexidine was the more potent stimulator. A concentration of 10 μmol l^{-1} dihyrexidine stimulated HdAGP secretion 5-fold above control levels whereas 100 μmol l^{-1} of SKF81297 was required to increase protein secretion 3.9-fold. By contrast, the D2-selective agonists apomorphine and bromocriptine had no effect on

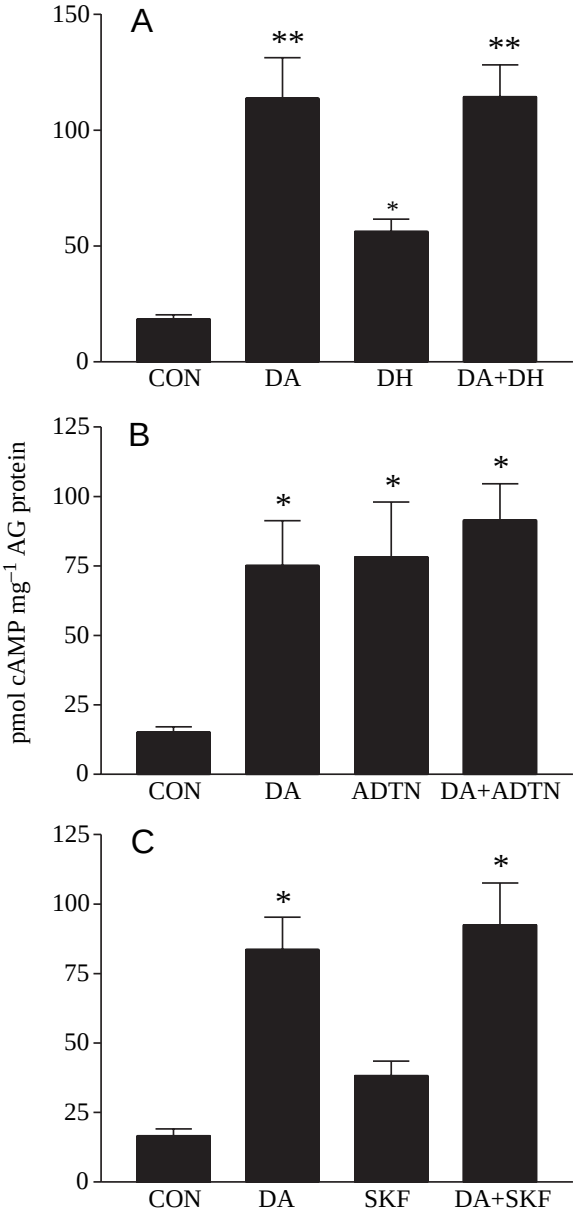

Fig. 4. The effect of dopamine and various D1-selective agonists on albumen gland cAMP production. Albumen glands were quartered and treated with either dopamine, a D1-selective agonist or dopamine + agonist. (A) Dopamine (DA) caused a significant elevation of albumen gland cAMP levels when compared with untreated control glands (CON), whereas dihydrexidine (DH) caused a modest but statistically significant elevation of albumen gland cAMP levels. Application of dopamine and dihydrexidine together was unable to elevate cAMP levels above those of dopamine-treated glands (**P*<0.05, ***P*<0.001, Tukey test). (B) The non-selective dopamine receptor agonist 6,7-ADTN was equally as potent as dopamine on albumen gland cAMP production (**P*<0.05, Tukey test). (C) The partial D1-selective agonist SKF81297 (SKF) induced a modest elevation above basal cAMP production in the albumen gland but it was not statistically significant when compared with control glands (**P*<0.001, Tukey test). All agents were used at a concentration of 10 μmol l^{-1}. Bars represent the mean ± S.E.M. of 5–6 samples.

protein secretion (Fig. 9). Previous studies showed that SCH23390 (100 μmol l^{-1}), a D1-selective antagonist, was a potent inhibitor of dopamine-stimulated protein secretion (Saleuddin et al., 2000). In the present study, protein secretion was also inhibited by two D1-selective antagonists, SKF83566 (50 μmol l^{-1}) and flupenthixol (10 μmol l^{-1}). Both antagonists inhibited dopamine-stimulated HdAGP secretion by 50% (Fig. 10A) and 37% (Fig. 10B), respectively.

Effect of PKA antagonists on HdAGP secretion

The involvement of PKA in mediating AG protein secretion was determined using the PKA antagonists Rp-cAMP (1 mmol l^{-1}) and H-89 (10 μmol l^{-1}). Both Rp-cAMP (Fig. 11A) and H-89 (Fig. 11B) did not significantly inhibit dopamine-stimulated protein secretion and neither inhibitor affected basal protein secretion.

Discussion

The results of the present study support the existence of a specific dopamine D1-like receptor in the AG of the freshwater

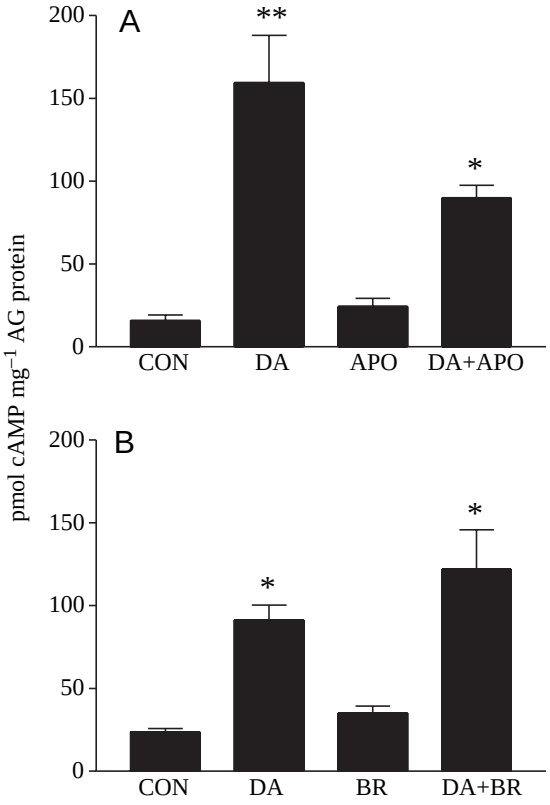

Fig. 5. The effect of the D2-selective agonists apomorphine and bromocriptine on albumen gland cAMP production. All agents were applied to albumen glands at a concentration of 10 μmol l^{-1} for 10 min. (A) Apomorphine (APO) had no effect on basal cAMP production when compared with control glands (CON) but, surprisingly, it suppressed dopamine-stimulated cAMP production (**P*<0.05, ***P*<0.001, Tukey test). (B) Bromocriptine (BR) had no effect on either basal or dopamine-stimulated cAMP production. Bars represent the mean ± S.E.M. of 5–6 samples.

snail *H. duryi* that controls glycoprotein secretion. Addition of exogenous dopamine induced the secretion of the 66 kDa subunit of HdAGP, the major protein produced by the AG of *H. duryi*. Although other minor proteins are released when the AG is stimulated with dopamine, HdAGP is clearly the most prominent. Similar results were obtained when AGs were stimulated with either forskolin (Morishita et al., 1998) or brain peptide (Mukai et al., 2001a). Furthermore, the time course and profile of HdAGP secretion mirrors that of total protein secretion. Hence, total protein secretion by the AG consists primarily of HdAGP. Other neurotransmitters commonly found in the CNS of molluscs, including serotonin, acetylcholine, norepinephrine, GABA, histamine, octopamine and glutamate (Audesirk, 1985; Hetherington et al., 1994), had no effect on AG protein secretion, suggesting that the effect of dopamine on the AG was specific. Indeed, dopamine was the only neurotransmitter detected in significant quantity in the AG of *H. duryi* by HPLC (Kiehn et al., 2001).

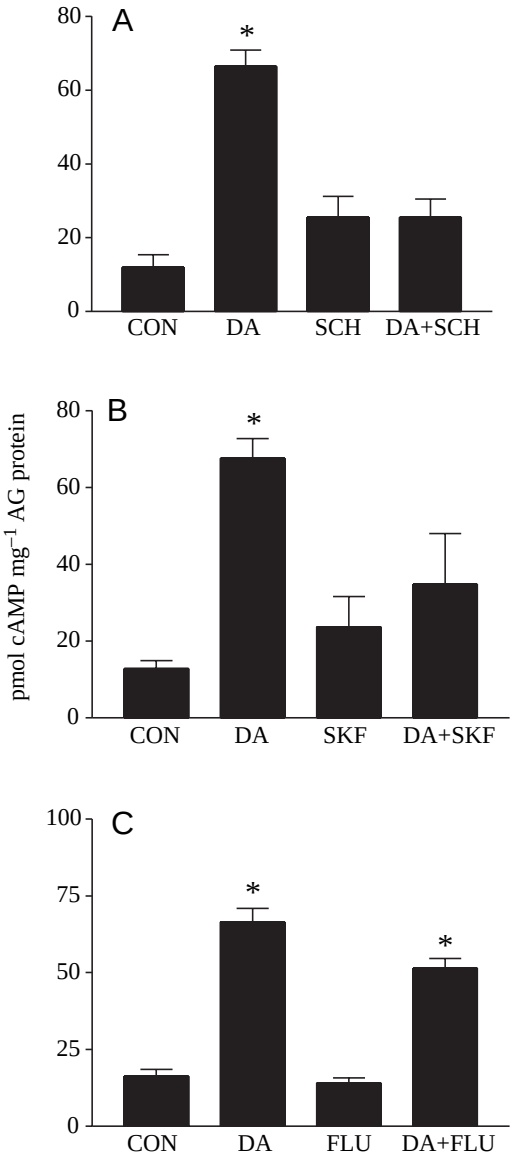

Fig. 6. The effect of D1-selective antagonists on dopamine-stimulated cAMP production in the albumen gland of *H. duryi*. All agents were used at a concentration of 10 μmol l^{-1} and applied to the glands for 10 min. For the dopamine plus antagonist combinations, the glands were first pre-incubated in antagonist for 20 min. Dopamine (DA) significantly increased cAMP levels in the albumen glands compared with untreated control glands (CON). (A) The D1-selective antagonist SCH23390 had no effect on basal cAMP levels but significantly inhibited dopamine-stimulated cAMP production (*P<0.001, Tukey test). (B) The D1-selective antagonist SKF83566 significantly suppressed dopamine-stimulated cAMP production and had no effect on basal cAMP levels (*P<0.001, Tukey test). (C) By contrast, flupenthixol (FLU), another D1-selective antagonist, did not cause a statistically significant suppression of dopamine-stimulated cAMP production (*P<0.05, Tukey test). All agents were used at a concentration of 10 μmol l^{-1}. Bars represent the mean ± S.E.M. of 5–6 samples.

Despite the widespread occurrence of dopamine in the peripheral tissues of molluscs, little information is available about the pharmacological properties of peripheral dopamine receptors, particularly in reproductive organs. In the salivary duct muscle of *Helix pomatia*, dopamine-induced contraction was inhibited by the D1-selective antagonists flupenthixol and fluphenazine, whereas the D1-selective agonist SKF38393 mimicked the effect of dopamine (Kiss et al., 2003). In the AG of *B. glabrata*, the D2-selective antagonist chlorpromazine inhibited dopamine-induced protein secretion (Santhanagopalan and Yoshino, 2000) but it was without effect in *H. duryi*, as shown in an earlier report (Saleuddin et al., 2000) and in the present study. Previous studies have indicated the presence of a D1-like receptor that mediated AG protein secretion in *H. duryi* (Saleuddin et al., 2000). Since D1-like receptors are known to stimulate adenylate cyclase activity (Missale et al., 1998) and since forskolin is a potent stimulator of protein secretion (Morishita et al., 1998), we tested for the effect of dopamine on AG cAMP production. Addition of dopamine or forskolin to AG explants increased cAMP production in a time- and concentration-dependent manner. In the presence of forskolin, dopamine-stimulated cAMP production was enhanced because forskolin bypasses the receptor and directly activates the catalytic subunit of adenylate cyclase (Insel and Ostrom, 2003).

Using a number of different dopamine receptor agonists and antagonists it was possible to obtain a pharmacological characterization for the dopamine receptor in the AG of *H. duryi*. The D1-selective benzazepine agonist SKF81297 showed a slight stimulatory effect on AG cAMP production at a concentration of 10 μmol l^{-1}, indicating that it is a weak agonist at the AG dopamine receptor. This result is consistent with its effect on the secretion of HdAGP, where it was only half as potent as dopamine. In vertebrates, SKF81297 is considered to be a partial D1 agonist, i.e. it modestly activates adenylate cyclase when compared with dopamine (Andersen and Jansen, 1990). Saleuddin et al. (2000) found that SKF38393, another vertebrate D1-selective agonist, also had little effect on AG protein secretion, suggesting that benzazepines are relatively weak agonists on the AG dopamine receptor. In locust salivary glands, SKF38393 was also inactive

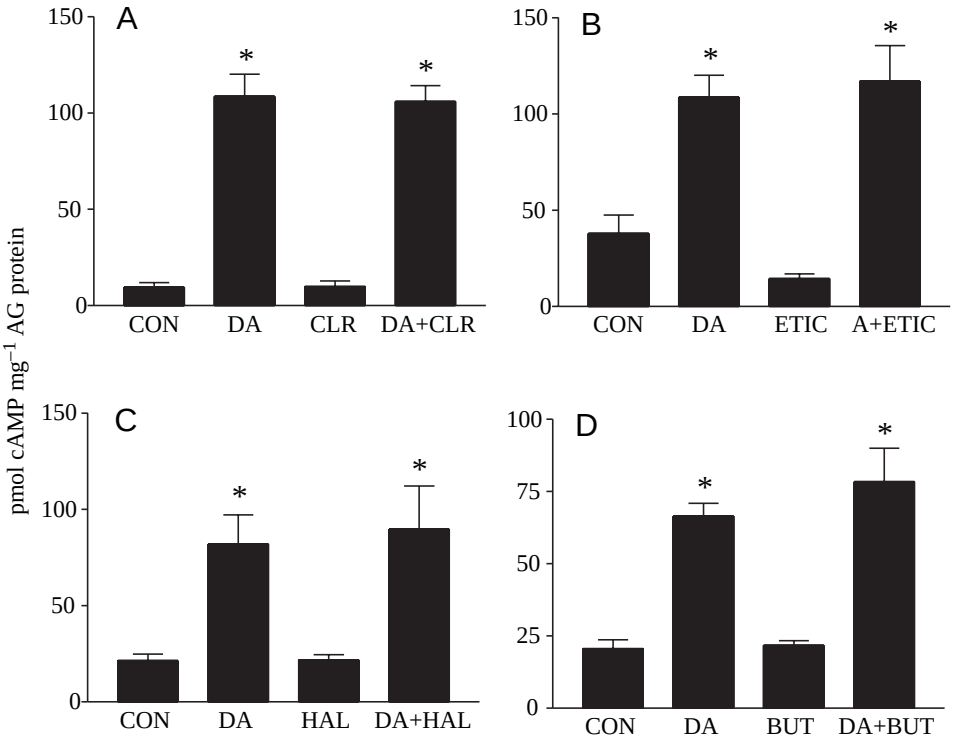

Fig. 7. The effect of various D2-selective antagonists on dopamine-stimulated cAMP production in isolated albumen glands of *H. duryi*. The D2-selective inhibitors chlorpromazine (A), eticlopride (B) and haloperidol (C) and the mixed D1/D2 antagonist butaclamol (D) affected neither basal cAMP levels nor dopamine-stimulated cAMP production in the albumen glands. Abbreviations for A: CON, control; DA, dopamine (*P<0.001, Tukey test); CLR, chlorpromazine; DA+CLR, dopamine + chlorpromazine (*P<0.001, Tukey test). Abbreviations for B: CON, control; DA, dopamine (*P<0.01, Tukey test); ETIC, eticlopride; DA+ETIC, dopamine + eticlopride (*P<0.01). Abbreviations for C: CON, control; DA, dopamine (*P<0.05, Tukey test); HAL, haloperidol; DA+HAL, dopamine + haloperidol (*P<0.05, Tukey test). Abbreviations for D: CON, control; DA, dopamine (*P<0.001, Tukey test); BUT, butaclamol; DA+BUT, dopamine + butaclamol (*P<0.001, Tukey test). All agents were used at a concentration of 10 μmol l^{-1}. Bars represent the mean ± S.E.M. of 5–6 samples.

in stimulating cAMP production (Ali and Orchard, 1994) and caused a weak hyperpolarization of the acinar cells as compared with dopamine (Keating and Orchard, 2001). In vertebrates, benzazepine analogues are effective dopamine receptor antagonists but display only limited effectiveness as cAMP agonists (Andersen and Jansen, 1990). Therefore, the *H. duryi* AG dopamine receptor is functionally similar to vertebrate D1-like receptors since its activation leads to cAMP formation but its structure is probably different because of its specificity to various dopamine receptor agonists.

The dopamine receptor agonist that was most effective in elevating AG cAMP levels was 6,7-ADTN. In vertebrates, this tetraline compound does not discriminate between D1-like and D2-like receptors. The activation characteristics of the AG dopamine receptor resembled that of a cloned D1-like receptor in *Drosophila*. This primordial *Drosophila* dopamine receptor also displayed poor affinity for benzazepines and was activated by the tetraline 6,7-ADTN (Sugamori et al., 1995). In the CNS of *Lymnaea stagnalis*, 6,7-ADTN mimicked the effect of dopamine at synapses between right pedal ganglion 1 (RPeD1) and its follower cells; however, this neuronal dopamine receptor was pharmacologically identified as a D2-like receptor (Magoski et al., 1995). The presence of a D2-like receptor that mediates dopamine-induced hyperpolarization in the growth hormone-producing light green cells of *L. stagnalis* has also been shown (de Vlieger et al., 1986; Werkman et al., 1987). Together, these results imply the existence of different dopamine receptor subtypes in molluscan neural and peripheral tissues. The other D1-selective agonist used in the present study was dihydrexidine. Dihydrexidine is a phenanthridine analogue that is considered to be a full agonist at vertebrate D1-like receptors (Brewster et al., 1990; Mottola et al., 1992); however, it was only modestly effective in elevating AG cAMP levels in *H. duryi*. Although dihyrexidine was not as effective as 6,7-ADTN in stimulating AG cAMP production, it was a more potent agonist in inducing HdAGP secretion when compared with 6,7-ADTN (Saleuddin et al., 2000). Dihydrexidine-stimulated HdAGP secretion was dose dependent and its activity was comparable with equivalent concentrations of dopamine. Finally, no additive or synergistic effects on AG cAMP production were observed with any of the agonists used in this study, indicating that these compounds probably act on a single receptor system.

The D2-selective agonists apomorphine and bromocriptine

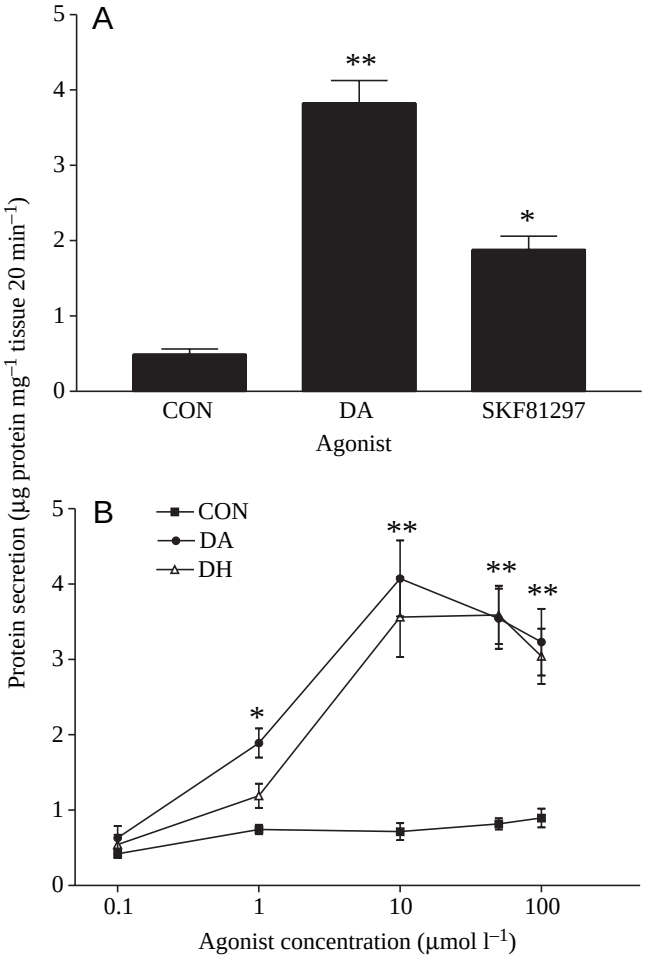

Fig. 8. The effect of the D1-selective agonists SKF81297 and dihydrexidine on the albumen gland glycoprotein secretion. (A) The partial D1 agonist SKF81297 (50 µmol l^{-1}) induced a modest but statistically significant increase in glycoprotein secretion compared with control (CON) glands in saline. However, it was less than half the potency of dopamine (DA) at 50 µmol l^{-1} concentration. Bars represent the mean ± S.E.M. of 16–18 samples (*P<0.05; **P<0.001, Tukey test). (B) Dihydrexidine (DH) caused a dose-dependent increase in albumen gland glycoprotein secretion between 1 and 100 µmol l^{-1}. Note that the shape and magnitude of the dose–response curve for dihydrexidine are similar to those of dopamine. Each point represents the mean ± S.E.M. of 18 samples (*P<0.05; *P<0.001, Tukey test).

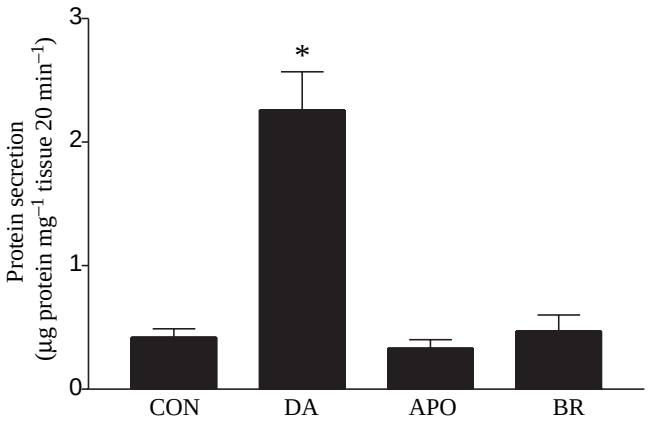

Fig. 9. The effect of the D2-selective agonists apomorphine and bromocriptine on glycoprotein secretion by the albumen gland of *H. duryi*. The application of apomorphine (10 µmol l^{-1}) and bromocriptine (10 µmol l^{-1}) had no significant effect on albumen gland protein secretion compared with control glands incubated in saline. Positive control glands incubated in 10 µmol l^{-1} dopamine showed a fivefold increase in protein secretion compared with saline controls. Abbreviations: CON, control; DA, dopamine; APO, apomorphine; BR, bromocriptine. Bars represent the mean ± S.E.M. of 5–8 samples (*P<0.001, Tukey test).

had no effect on AG protein secretion or basal cAMP levels. However, apomorphine exhibited an unusual effect by attenuating dopamine-stimulated cAMP production. The reason for this is unclear but it is possible that apomorphine occupies the same binding site on the receptor as dopamine, thereby reducing the latter's overall effect on AG cAMP formation.

The effect of various dopamine receptor antagonists was tested on AGs for their ability to inhibit dopamine-stimulated AG cAMP production and dopamine-induced HdAGP secretion. In the present study, the most effective inhibitor of dopamine-stimulated cAMP production was the benzazepine SCH23390, followed by SKF83566. In support of these findings, SCH23390 was also the most effective antagonist inhibiting dopamine-induced protein secretion in the AG of *H. duryi* (Saleuddin et al., 2000). Flupenthixol at a concentration of 10 µmol l^{-1} suppressed dopamine-stimulated AG cAMP production only marginally but significantly inhibited dopamine-induced protein secretion. In the salivary duct muscle of the snail *H. pomatia*, SCH23390 was inactive in suppressing dopamine-induced contractions whereas flupenthixol was shown to be the most potent antagonist (Kiss et al., 2003). This indicates that the peripheral dopamine receptor in the salivary duct muscle of *H. pomatia* displays characteristics of a D1-like receptor and that its pharmacological properties are distinct from the D1-like receptor in the AG of *H. duryi*.

The AG dopamine receptor might represent a member of a novel class of dopamine receptors that is substantially different from those characterized in vertebrates and even other invertebrates. In support of this notion, the dopamine receptor in the corpus allatum of the tobacco hornworm, *Manduca sexta*, exhibits a pharmacological profile distinct from other insect dopamine receptors and has both D1- and D2-like properties (Granger et al., 2000). In the nematode *Caenorhabditis elegans*, a novel dopamine D1-like receptor that did not bind [^{3}H]SCH23390 was recently cloned (Suo et al., 2002). In addition, the D1-like receptor from *C. elegans* also contained introns in the coding region, a feature not present in mammalian or previously characterized invertebrate D1-like receptors, providing further evidence that some

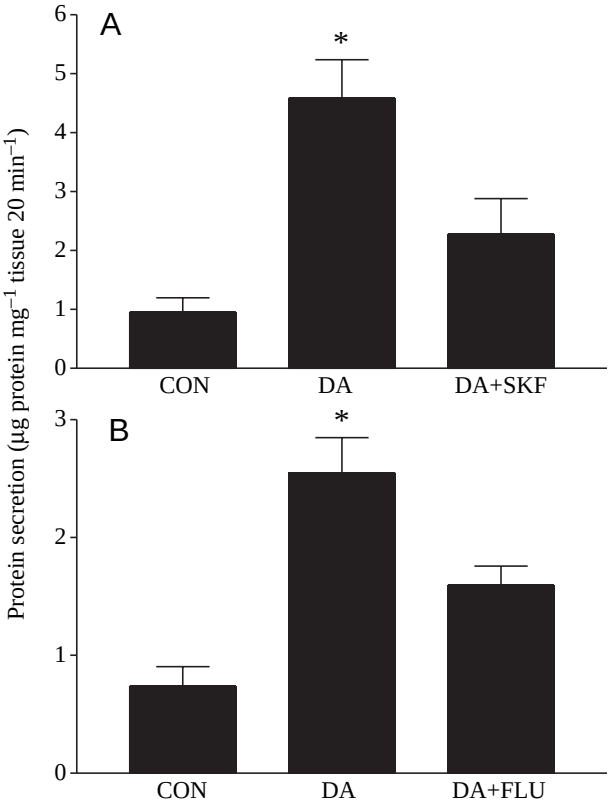

Fig. 10. The effect of the D1-selective antagonists SKF83566 and flupenthixol on dopamine-stimulated glycoprotein secretion by the albumen glands of *H. duryi*. The application of dopamine (50 μmol l^{-1}) stimulated glycoprotein secretion more than fourfold compared with control glands in saline. (A) In the presence of SKF83566 (50 μmol l^{-1}), dopamine-stimulated glycoprotein secretion was significantly suppressed. Abbreviations: CON, control; DA, dopamine; DA+SKF, dopamine + SKF83566. Bars represent the mean ± S.E.M. of 15–20 samples (*P<0.05, Tukey test). (B) The D1-selective antagonist flupenthixol also caused a significant inhibition of dopamine-stimulated protein secretion in isolated albumen glands. Abbreviations: CON, control; DA, dopamine; DA+FLU, dopamine + flupenthixol. Neither antagonist had an effect on basal glycoprotein secretion. Bars represent the mean ± S.E.M. of 15–20 samples (*P<0.05, Tukey test).

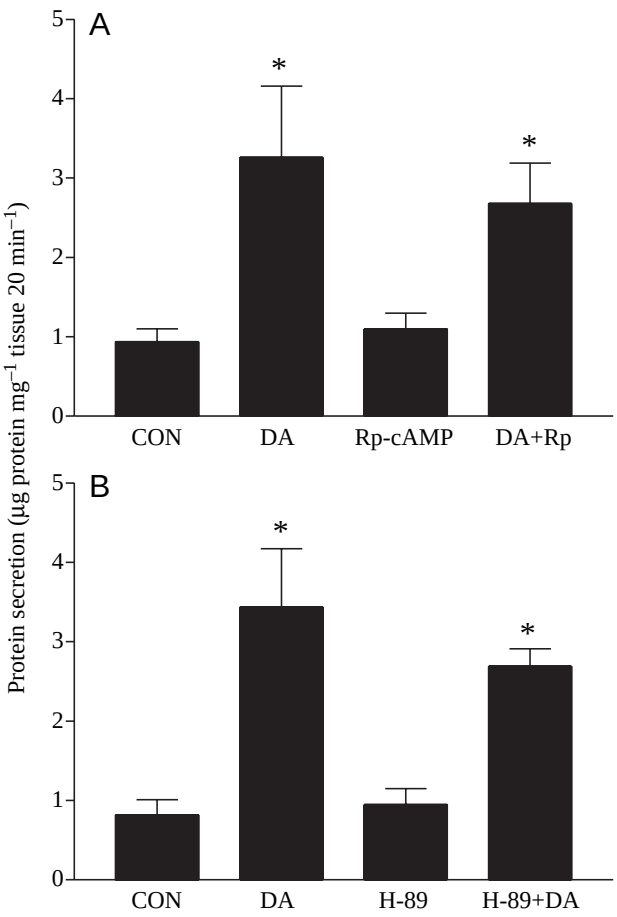

Fig. 11. The effect of protein kinase A (PKA) antagonists on dopamine-induced glycoprotein secretion by the albumen glands of *H. duryi*. Albumen glands were preincubated with either 1 mmol l^{-1} Rp-cAMP (A) or 10 μmol l^{-1} H-89 (B) for 60 min and then dopamine (10 μmol l^{-1}) + Rp-cAMP or dopamine + H-89 were added to the albumen glands for an additional 20 min. Neither PKA antagonist had an affect on basal protein secretion nor did they suppress dopamine-stimulated protein secretion by the albumen glands. Abbreviations: CON, control; DA, dopamine; DA+Rp, dopamine + Rp-cAMP; H-89+DA, dopamine + H-89. Bars represent the mean ± S.E.M. of 6 samples (*P<0.05, Tukey test).

invertebrate dopamine receptors represent a structurally distinct group. However, definitive characterization of the *H. duryi* AG dopamine receptor can only be achieved by molecular cloning and functional expression in a heterologous system.

To investigate intracellular signalling events further downstream of adenylate cyclase and cAMP, we tested for the involvement of PKA. In the vast majority of cAMP-mediated signalling, the primary effector regulated by cAMP is PKA. Cyclic AMP binds to the regulatory subunits of PKA, which in turn releases the catalytic subunits of PKA (Francis and Corbin, 1996). The activated catalytic subunits can then phosphorylate specific intracellular target proteins and mediate the appropriate physiological response. The PKA inhibitors Rp-cAMP and H-89 suppress PKA-mediated signalling in a number of invertebrates, including leeches (Ali et al., 1998), crustaceans (Locatelli et al., 2002), insects (Hazel et al., 2003) and molluscs (Malagoli et al., 2000). Our results demonstrate that dopamine-mediated secretion of HdAGP might be a PKA-independent process because inhibitors of both the regulatory (Rp-cAMP) and catalytic (H-89) subunits of PKA failed to inhibit protein secretion. PKA-independent signalling has recently become a recognized phenomenon due to the identification of novel cAMP-binding proteins, such as guanine exchange factors and cyclic nucleotide-gated channels (reviewed by Dremier et al., 2003; Kopperud et al., 2003), and the establishment of ‘cross-talk’ among other signalling pathways (Cooper et al., 1995; Houslay and Milligan, 1997;

Schwartz, 2001). However, it is possible that these PKA inhibitors were not able to fully penetrate into the glandular cells to exert their effects. Alternative approaches such as microinjection of PKA inhibitors into dissociated glandular cells combined with electrophysiological measurements are required to rule out this possibility.

In addition to dopamine, a putative neuropeptide from the CNS of *H. duryi* also appears to be involved in regulating HdAGP secretion through the cAMP signalling system (Morishita et al., 1998; Mukai et al., 2001). Whether these two molecules are co-released or perhaps affect each other's release is currently unknown. Identifying specific interactions between these two molecules can only be done after the *H. duryi* brain peptide is sequenced. The intracellular target of cAMP action in the AG of *H. duryi* is not known but it is possible that cAMP interacts with other intracellular signalling pathways such as Ca^{2+}. In support of this, we have recently shown that, in addition to cAMP, the influx of Ca^{2+} into the AG cells is an important regulator of protein secretion (Kiehn et al., 2004). Structural elucidation of the brain peptide as well as the identification of downstream targets of cAMP and Ca^{2+} is in progress.

In summary, we have identified a specific receptor to the neurotransmitter dopamine in the AG of *H. duryi* that participates in HdAGP secretion. Based on the pharmacological profile obtained using dopamine receptor agonists and antagonists, it is concluded that the AG dopamine receptor displays functional characteristics of a vertebrate D1-like receptor because of the following: dopamine stimulates cAMP formation and protein secretion in a dose-dependent fashion; D1-selective agonists stimulate basal cAMP formation and induce protein secretion in isolated AGs; D2-selective agonists had no effect on cAMP production or protein secretion; D1-selective antagonists suppress dopamine-stimulated cAMP production and dopamine-induced protein secretion; and D2-selective antagonists had no inhibitory effect on dopamine-stimulated cAMP production. However, the AG dopamine receptor of *H. duryi* also displays some unique pharmacological characteristics in that its activity is stimulated by the non-selective agonist 6,7-ADTN and is attenuated by the D2-selective agonist apomorphine. Finally, dopamine-induced protein secretion might occur through a PKA-independent mechanism involving Ca^{2+} or, perhaps, some unidentified protein that is regulated by cAMP.

Symbols and abbreviations

6,7-ADTN	(±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronapthalene
AG	albumen gland
cAMP	adenosine 3′,5′-cyclic monophosphate
CNS	central nervous system
HdAGP	*Helisoma duryi* albumen gland protein
H-89	N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide
IBMX	3-isobutyl-1-methylxanthine
PKA	protein kinase A
PVF	perivitelline fluid
Rp-cAMP	Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt
SCH23390	R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydo-1H-3-benzazepine hydrochloride
SKF81297	R-(+)-6-chloro-7,8-dihydroxy-1phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide
SKF38393	(±)-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol hydrochloride
SKF83566	(±)-7-bromo-8-hydroxy-3methyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride

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