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Adenosinergic modulation of neuronal activity in the pond snail *Lymnaea stagnalis*

Aqsa Malik[1] and Leslie Thomas Buck[1,2,*]

[1]Department of Cell and Systems Biology, University of Toronto, ON, Canada and [2]Department of Ecology and Evolutionary Biology, University of Toronto, ON, Canada

*Author for correspondence (les.buck@utoronto.ca)

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SUMMARY

Adenosine has been termed a retaliatory metabolite and its neuroprotective effects have been implicated in the hypoxia tolerance of several species; however, its role in the invertebrate CNS remains unclear. To determine if adenosine modulates neuronal activity in invertebrate neurons, we conducted whole-cell recordings from neurons in the central ring ganglia of the anoxia-tolerant pond snail *Lymnaea stagnalis* during exposure to adenosine and pharmacological compounds known to modulate the type I subclass of adenosine receptors (A_1R). Action potential (AP) frequency and membrane potential (V_m) were unchanged under control conditions, and addition of adenosine decreased AP frequency by 47% (from 1.08±0.22 to 0.57±0.14 Hz) and caused significant hyperpolarization of V_m. The A_1R agonist cyclopentyladenosine (CPA) mimicked the results obtained with adenosine whereas antagonism of the A_1R with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) had no effect on AP frequency or V_m but prevented the adenosine and CPA-mediated decreases in neuronal activity. Furthermore, Ca^{2+} measurements with fluo-4 revealed that A_1R activation led to a 12% increase in intracellular Ca^{2+} concentration and this elevation was also antagonized by DPCPX. Our results suggest that adenosine acting via the adenosine receptor (type I subclass) depresses neuronal activity in the adult *L. stagnalis* CNS and this depression is correlated with an increase in cytosolic Ca^{2+} levels.

Key words: adenosine, anoxia, hypoxia, invertebrate, neurons.

INTRODUCTION

Adenosine acts at various parts of the central nervous system to modulate neuronal activity. Chemically classified as a nucleoside, it plays various different roles as an intercellular messenger in the brain, which expresses high concentrations of adenosine receptors and where its role has been implicated in both normal and pathological processes (for a review, see Buck, 2004; Downey et al., 2007; Pagonopoulou et al., 2006). Under conditions of increased demand and reduced supply of ATP, such as hypoxia and prolonged wakefulness, there is an increase in adenosine turnover and adenosine receptor stimulation (Lutz and Kabler, 1997; Lutz and Manuel, 1999; Roman et al., 2008). This is achieved by dramatically increasing adenosine concentrations by the breakdown of high-energy phosphates during episodes of increased nerve activity, hypoxia or ischemia (Schubert et al., 1997). Under these conditions, degradation or transport of adenosine is prevented by adenosinergic transmission-potentiating agents (adenosine deaminase and kinase inhibitors), and elevated adenosine levels offer protection against ischemic or excitotoxic neuronal damage (Wardas, 2002).

The important neuroprotective role of endogenous adenosine in the vertebrate CNS has been well characterized. Increases in extracellular adenosine concentration in response to anoxia have been observed in the striatum of the red-eared turtle, *Trachemys scripta* striatum, leading to the suggestion that the initial energetic shift during hypoxia (90–120 min) is due to the effects of adenosine. Ultimately, the remarkable metabolic rate depression in this species that allows prolonged anoxic survival is achieved through release of inhibitory transmitters [e.g. GABA and glycine (Nilsson and Lutz, 1992)]. The defensive role of adenosine has also been implicated in the anoxia-induced increase in cerebral blood-flow that confers neuroprotection through increased delivery of glycolytic substrates and enhanced removal of acidic anaerobic end products from sensitive brain areas. For example, in the crucian carp, *Carassius carassius*, a 2.2-fold increase in brain blood-flow rate was observed during anoxia with a similar increase following perfusion of the brain with adenosine during normoxia (Nilsson et al., 1994). Although adenosine appears to have a definitive role in mediating anoxia tolerance through different pathways in turtles and the crucian carp, results are variable because it does not have a neuroprotective role during oxygen deprivation in the leopard frog, *Rana pipens* (Soderstrom-Laurtizen et al., 2001) or the shark *Hemicyllium ocellatum* (Soderstrom et al., 1999).

Despite the large number of anoxia-tolerant invertebrate species few studies have utilized invertebrate models to examine a possible neuromodulatory role of adenosine (Table 1). Adenosine is metabolized by many invertebrates (bivalves, gastropods, echinoderms, crustaceans, cephalopods and polychaetes) as indicated by the presence of adenylate-metabolizing enzymes in their tissues (Lazou, 1988). Adenosine was shown to inhibit the acetylcholine-induced depolarization of a specific neuron in the mollusk *Helix aspersa* (Cox and Walker, 1987). Furthermore, the marine invertebrate *Sipunculus nudus* depressed its metabolic rate by 70% during anoxia and neuronal tissue levels of adenosine increased during hypoxia, hypercapnia, and to an even greater degree during anoxic hypercapnia (Reipschlager et al., 1997). Of greater importance, adenosine applied during normoxic conditions depressed oxygen consumption for more than 90 min, whereas application of an adenosine antagonist increased oxygen consumption rate after 30 min of perfusion during hypercapnia (Reipschlager et al., 1997). The finding that adenosine had an excitatory effect on the spontaneous activity and responsiveness of certain interneurons to chemical and electrical stimuli in the spiny lobster *Panulirus argus* (Derby et al., 1987) suggests that invertebrates may have more variable responses.

Table 1. Concentration and effects of adenosine on various invertebrate preparations

Species	Concentration	Physiological actions
Helix aspersa	0.6 μmol l^{-1}	Depression of acetylcholine-mediated depolarization in identified neurons of the isolated subesophageal ganglionic mass[a]
Mytilus edulis	1–10 μmol l^{-1}	Inhibition of neurotransmitter (monoamines) release from the pedal ganglia[b]
Sipunculus nudus	30 μmol l^{-1}	Decrease in oxygen consumption rate of isolated body wall musculature[c]
Panulirus argus	100 μmol l^{-1}	Inhibitory effect on the spontaneous activity of interneurons; certain cells exhibit excitation[d]
Calliphora vicina	10–500 μmol l^{-1}	Decrease in amplitude of evoked EPSCs from the neuromuscular junction
	200–500 μmol l^{-1}	Decrease in frequency of mEPSCs[e]

[a]Cox and Walker, 1987; [b]Barraco and Stefano, 1990; [c]Reipschlager et al., 1997; [d]Derby et al., 1987; [e]Magazanik and Federova, 2003.
mEPSCs, miniature excitatory postsynaptic currents.

Given the dearth of knowledge regarding CNS effects of adenosine in invertebrates, we sought to examine the neuromodulatory role of adenosine in the anoxia-tolerant freshwater mollusk *Lymnaea stagnalis*. *L. stagnalis* can tolerate anoxia for about 40 h in a N_2-bubbled environment at 20°C, with a mortality rate of 10% on day 7 of normoxic recovery (Wijsman et al., 1985). Recent studies have utilized this ability of *L. stagnalis* to examine hypoxia-induced modulation of different protein profiles (Fei and Feng, 2008; Fei et al., 2007). Extensive data on its anaerobic metabolism is also available (Wijsman et al., 1985) along with a detailed morphological and electrophysiological map of the identifiable neurons and neuronal clusters of its paired pedal ganglia (Kyriakides et al., 1989). We used whole-cell patch-clamp techniques and fluorescence microscopy to measure changes in neuronal electrical properties and intracellular calcium levels in response to adenosine receptor modulation (vertebrate A_1R subclass-like) using neurons from cluster F of the left or right pedal dorsal ganglion (LPeDG or RPeDG) within the central ring ganglia of *L. stagnalis*.

MATERIALS AND METHODS

Animals

Laboratory-raised stocks of the freshwater snail *L. stagnalis* L. were maintained in a natural photoperiod for Toronto, ON, Canada. All animals were raised in aquaria filled with well-aerated filtered tap water at 22°C. *L. stagnalis* were fed lettuce *ad libitum* and experiments were performed on snails with a shell length of 10–20 mm (~2 months old).

Dissection and whole-cell patch-clamp recordings

All experiments were conducted at room temperature of 22°C. *L. stagnalis* saline solution included (in mmol l^{-1}): 10 glucose, 51.3 NaCl, 1.7 KCl, 4 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES, set to pH 7.9 using 12 mol l^{-1} HCl. Snails were anesthetized briefly in *L. stagnalis* saline solution containing 30% Listerine, then de-shelled with forceps. Snails were pinned dorsal surface up to the bottom of a Sylgard-filled dissection dish, covered with *L. stagnalis* saline solution, and a medial incision was made from the base of the mantle to the head. Central ring ganglia were removed with a pair of fine surgical scissors, and placed in a modified flow-through perfusion chamber (RC-26 with a P1 platform, Warner Instruments, CT, USA) before being pinned out. The bottom of an RC-26 chamber was modified by replacing the 22 mm×40 mm glass coverslip with 22 mm×40 mm plexiglass plates and a 4 mm layer of Sylgard (total thickness 5 mm). The connective tissue sheath was removed from cluster F neurons within the (LPeDG and RPeDG) with a pair of fine forceps. After being transferred to the microscope stage, the chamber was perfused at a rate of 2–3 ml min^{-1} by gravity flow.

Whole-cell recordings were performed using pipettes with resistance in the range of 2–3 MΩ. Pipettes were pulled from borosilicate glass capillaries (Fisher Scientific, Nepean, ON, Canada) and filled with a filtered intracellular solution (pH 7.4) composed of the following (in mmol l^{-1}): 8 NaCl, 0.0001 $CaCl_2$, 10 NaHEPES, 110 potassium gluconate, 1 $MgCl_2$, 0.3 NaGTP and 2 NaATP. An Axopatch-1D amplifier (Axon Instruments, CA, USA) was used for recordings. The series resistance was monitored throughout each recording and if it varied by >10%, the recording was rejected. No electronic compensation for series resistance was used. After obtaining a gigaohm-seal onto neurons, whole-cell patches were obtained under voltage-clamp mode at a holding membrane potential of –60 mV by applying a brief suction. Spontaneous neuronal activity was recorded after switching to current clamp mode. Recordings were low-pass filtered at 2 kHz using a CV-4 headstage, and a Digidata 1200 (Axon Instruments, CA, USA) and then digitized and stored on a computer using Clampex 7 software (Axon Instruments, CA, USA). Neurons were perfused with *L. stagnalis* saline solution until AP frequency and V_m stabilized (10–20 min). Following this stabilization period, baseline AP frequency and V_m were recorded for 20 min before exposing the preparations to treatment saline for up to 40 min. The preparation was then reperfused with control saline solution to wash out the treatment saline. Each preparation was exposed to only one treatment protocol.

Fluo-4 intracellular Ca^{2+} imaging

The Ca^{2+}-sensitive, membrane-impermeable pentapotassium salt of fluo-4 (Molecular Probes Inc.) was used to determine intracellular Ca^{2+} $[Ca^{2+}]_i$ levels. Fluo-4 was used because of its low background absorbance and increased brightness at lower concentrations (Paredes et al., 2008). Fluo-4 was dissolved in the intracellular recording solution at a final concentration of 100 μmol l^{-1}. Fluo-4 was excited at 488 nm for 0.1 s using a DeltaRam X high-speed random-access monochromator and a LPS220B light source (PTI, NJ, USA). Fluorescent emissions above 510 nm were isolated using an Olympus DM510 dichroic mirror and fluorescence images were acquired (526 nm) at 10-s intervals to limit photobleaching, using an Olympus BX51W1 microscope and a QImaging Rolera MGi EMCCD camera (Roper Scientific Inc., IL, USA). The obtained images were quantitatively analyzed for changes in fluorescence intensities within cells using EasyRatioPro software (PTI). Values of fluo-4 measurements are reported as means ± standard error (s.e.m.), with *N* being the number of cell bodies tested from different animals. The amplitude of the fluo-4 responses was analyzed from the soma and the data expressed as relative fluorescence intensity.

Chemicals

All chemicals were obtained from Sigma Chemical Co. (Oakville, ON, Canada). Adenosine was dissolved in *L. stagnalis* saline solution at a final concentration of 200 μmol l^{-1}. N^6-cyclopentyl-adenosine (CPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)

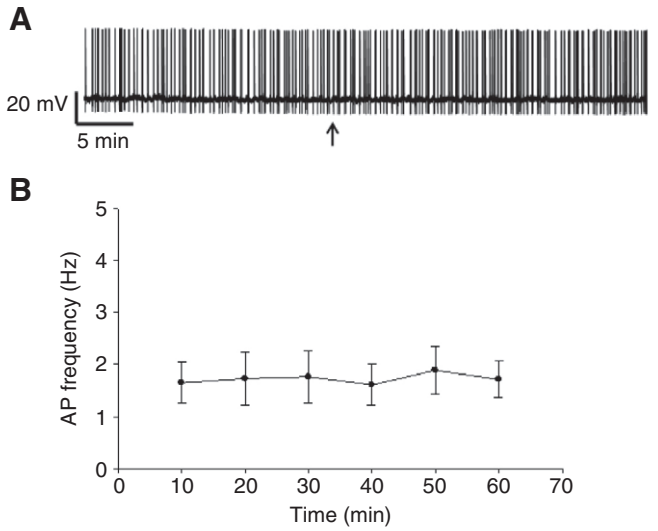

Fig. 1. AP frequency remains stable under control conditions. (A) Raw continuous trace of a recording from a cluster F neuron with a relatively low AP frequency maintained under control conditions. A switch from control to control saline occurred at the arrow, 20 min into the recording. (B) Mean group data (N=8) showing that AP frequency did not change significantly throughout the recording period. AP frequency was assessed every 10 min over a 5 min interval (see Materials and methods).

were initially dissolved in 100% dimethylsulfonic acid (DMSO); and then diluted in *L. stagnalis* saline solution to a final concentration of about 0.3% v/v. Vehicle application alone did not affect the stability of patch-clamp recordings (data not shown). A final concentration of 100 µmol l^{-1} was used for both CPA and DPCPX.

Statistical analysis

AP frequency and V_m were monitored for a 5 min interval in the last 5 min of control (immediately before the switch to treatment perfusion), last 5 min of treatment, and last 5 min of each wash-out perfusion at a sampling rate of 5 kHz. There was significant variation in the AP frequency between neurons (minimum number of APs: 0.02 spikes s^{-1}; maximum number of spikes: 3.2 spikes s^{-1}). Therefore, in order to compare AP frequency between neurons and show the extent of variance within the control values, 5 min of control recordings were used as baseline to normalize data during a single experiment. To assess if neuronal activity changes as a function of time under control conditions without any pharmacological intervention, AP frequency and V_m were determined by analyzing a continuous trace for a 5 min interval at the end of every 10 min, for up to 60 min. For concomitant AP frequency and fluorescence measurements, AP frequency and fluo-4 signals were analyzed for a 1 min interval every 5 min of treatment perfusion for 20 min. Fluo-4 signals were normalized to values obtained immediately prior to treatment perfusion. A dose–response curve was fitted to the Four-Parameter Logistic (4PL) equation. All results are reported as means ± s.e.m. Statistical analysis was performed using SigmaStat software (Point Richmond, CA, USA). Results were analyzed using repeated measures one-way ANOVA with a Tukey's *post-hoc* test or a paired *t*-test, and significance was determined at $P<0.05$ unless otherwise indicated. Values expressed as percentage change were calculated as measured value minus baseline value divided by baseline value multiplied by 100%.

RESULTS

Whole-cell recordings were obtained from visualized neurons in cluster F on the dorsal surface of the RPeDG or LPeDG, which are part of the central ring ganglia in *L. stagnalis* [see figure 1A,B in Cheung et al. (Cheung et al., 2006)]. Recordings were restricted to neurons in cluster F of the PeDG because they have similar electrophysiological properties and their action potential shape has been well characterized (Kyriakides et al., 1989). The majority of neurons tested were spontaneously active, showing APs along with excitatory and inhibitory postsynaptic potentials at their resting membrane potential. Modulation of neuronal activity was

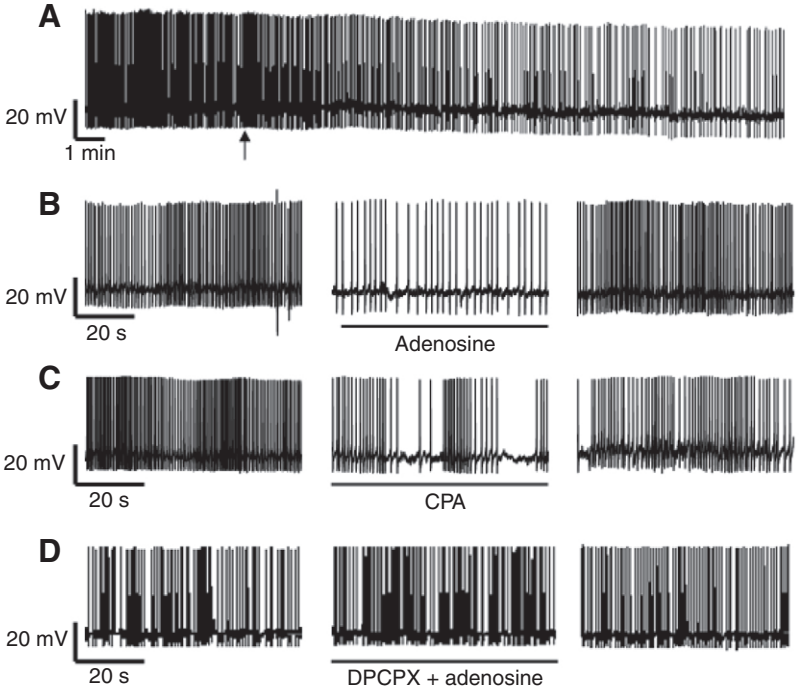

Fig. 2. Effect of A_1 receptor activation or antagonism on AP frequency. (A) Raw continuous trace from an experiment undergoing control to adenosine transition. AP frequency during the last 5 min of control and 15 min of adenosine perfusion is shown; adenosine application began at the arrow. Raw discontinuous traces from individual experiments during control, treatment perfusion with (B) adenosine; (C) CPA; (D) DPCPX + adenosine, and subsequent washout. Discontinuous traces for treatment and recovery parameters are shown for the purposes of clarity and brevity because individual APs were difficult to distinguish in a highly compressed 60 min trace.

Table 2. Effects of adenosine on AP frequency measured in summer animals

No. of cells	Control	Control	100 μmol l^{-1}	200 μmol l^{-1}	500 μmol l^{-1}	1 mmol l^{-1}
8	0.84±0.09	0.85±0.17				
7	1.36±0.18		1.12±0.23			
13	1.38±0.35			1.36±0.37		
10	2.22±0.74				1.95±0.64	
11	0.95±0.25					0.72±0.21

Data are presented as means ± s.e.m. Repeated measures one-way ANOVA with a Tukey's (all pairwise) *post-hoc* test was used to determine significance between treatments (recovery data not shown).
AP frequency is given in Hz.

Table 3. Effects of adenosine, CPA and DPCPX on membrane potential

No. of cells	Control	Control	Adenosine	DPCPX + adenosine	CPA
8	–64.9±3.1	–64.2±3.1			
14	–63.8±3.3		–70.5±4.6*		
6	–56.4±3.8			–60.8±3.9	
6	–72.4±2.6				–76.9±2.1*

Values are means ± s.e.m. Asterisk indicates significant difference from control value (P<0.05).
Membrane potential V_m is given in mV.

determined by changes in AP frequency and V_m. In cells obtained during the summer (May to August), adenosine-mediated decreases in AP frequency were minute and this depression was not statistically significant at any given concentration (Table 2). Significant changes in neuronal activity in response to adenosine treatment were observed during winter months (December to March), and the following results represent data collected during the wintertime.

Under control conditions, there was no significant change in AP frequency for up to 60 min of the recording period. AP frequency remained stable: 1.77±0.56 spikes s^{-1} at 30 min and 1.72±0.39 spikes s^{-1} at 60 min (Fig. 1A,B; N=8). Similarly, no significant change in V_m occurred during the first (–64.9±3.1 mV) and second (–64.2±3.1 mV) halves of the recording period (Table 3; N=8). Application of 200 μmol l^{-1} adenosine caused a significant decrease in AP frequency from 1.08±0.22 to 0.57±0.14 spikes s^{-1}, a 47% reduction (Fig. 2A and Fig. 3; N=14). AP frequency during the recovery period increased to 0.78±0.16 spikes s^{-1} and was not statistically different from the control recording prior to the adenosine perfusion (Fig. 2B and Fig. 3; N=14). Adenosine application also hyperpolarized V_m from –63.8±3.6 to –70.5±4.6 (Table 3; N=14). An adenosine concentration of 200 μmol l^{-1} was chosen because this was the lowest concentration that resulted in changes in V_m and AP frequency. Cells did not respond to 50 and 100 μmol l^{-1} adenosine, but did respond to 200, 500 μmol l^{-1} and 1 mmol l^{-1} adenosine (Fig. 4).

To determine whether the changes in neuronal activity caused by adenosine were A_1R-mediated, AP frequency and V_m were analyzed in the presence of the A_1R agonist CPA. Addition of 100 μmol l^{-1} CPA to the bulk perfusion resulted in a 43% reduction of AP frequency, from 1.11±0.27 to 0.62±0.11 spikes s^{-1} (Fig. 2C and Fig. 3; N=7). As with adenosine application, AP frequency recovered to 0.67±0.11 spikes s^{-1} after CPA addition, and this was not significantly different from the control value prior to treatment onset (Fig. 2C and Fig. 3; N=7). However, an all pair-wise *post-hoc* test revealed that only recovery from the adenosine treatment was significantly different from adenosine treatment alone. This may be the result of tighter binding of the pharmacological modulators and a requirement for a longer washout period. CPA application also hyperpolarized V_m, from –72.4±2.6 to –76.9±2.1 (Table 3; N=7). The A_1R-mediated change was further tested by incubation of the preparation with the A_1R antagonist DPCPX prior to and during adenosine application. Addition of adenosine to DPCPX-treated neurons caused no significant change in AP frequency (from 1.09±0.06 to 0.81±0.03 spikes s^{-1}; Fig. 2D and Fig. 3; N=6) or V_m (from –56.4±2.8 to –60.8±3.9 mV; Table 3; N=6).

The effect of adenosine on Ca^{2+} homeostasis of cluster F neurons was examined using the single wavelength Ca^{2+} indicator fluo-4. Since neurons were not incubated with fluo-4 and the dye was applied through a patch pipette, at least 15 min of baseline recording was required prior to any treatment application to allow maximal loading of cells with the dye and for the fluorescence trace to reach a plateau. The spontaneous activity (both AP frequency and V_m) of these neurons did not change under control conditions and was similar to that observed in previous recordings lacking fluo-4 (data not shown). Thus inclusion of the dye to the intracellular recording

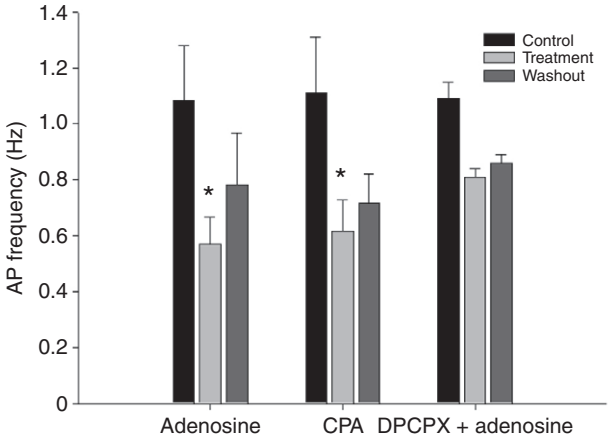

Fig. 3. A_1 receptor-mediated decrease in AP frequency. Summary of changes in AP frequency during control, treatment with adenosine and/or adenosine receptor modulators, and recovery following treatment. Variance within control values was determined by comparison with a 5 min recording period preceding the control interval. Data represent 6–14 replicate experiments and are expressed as means ± s.e.m. Asterisks indicate data significantly different from control values (P<0.05; one-way RM ANOVA).

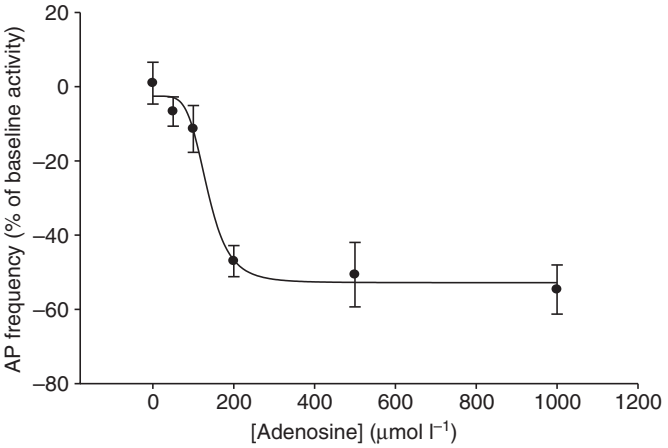

Fig. 4. Concentration–response curve for adenosine effect on AP frequency (normalized values as a percentage of control). 5 min of control recording immediately prior to treatment onset was used to determine changes in AP frequency with adenosine perfusion. Data are presented as means ± s.e.m. Each point represents results of 4–14 separate experiments.

solution did not adversely affect neuronal viability. Application of adenosine provoked a significant 12.5±1.5% increase in the fluo-4 fluorescence; this response had a slow onset and it reached a maxima at ~20 min (Fig. 5A,B; *N*=4). AP frequency and fluo-4 fluorescence were then analyzed at 5 min intervals after treatment onset for 20 min to determine whether the two measurement variables covaried. In response to adenosine perfusion, correlation analysis revealed a linear correlation between the two variables; as Ca^{2+} concentration increased, AP frequency decreased. The correlation between $[Ca^{2+}]_i$ and AP frequency was significant (r^2=0.91, *P*=0.0125) and a linear regression analysis of the data resulted in a slope of –0.11 (Fig. 5C; *N*=4). Conversely, application of adenosine to DPCPX-treated neurons caused no significant change in intracellular calcium levels (Fig. 5A,B; *N*=4).

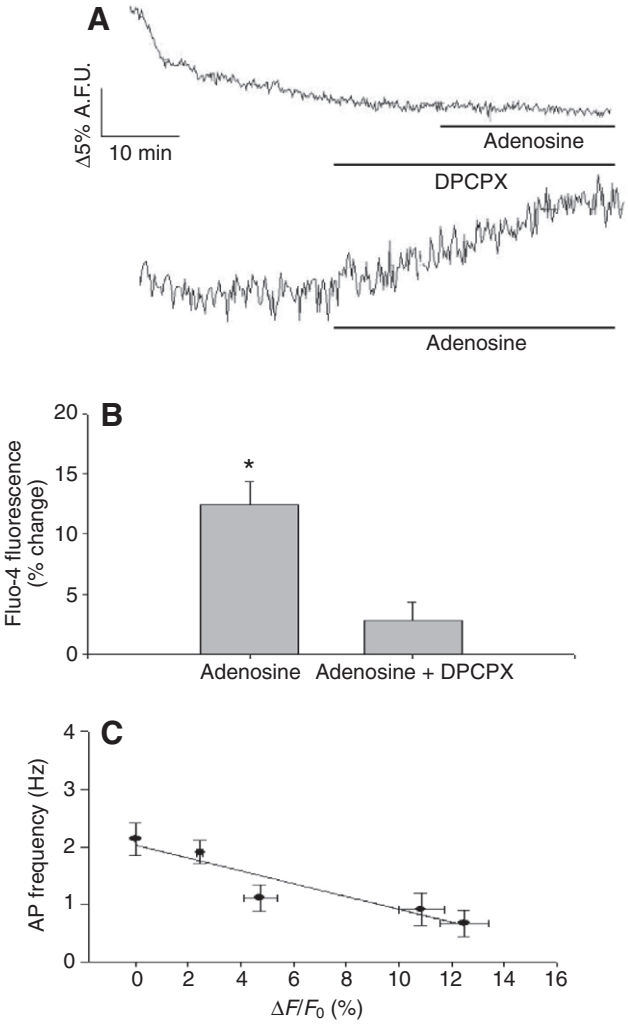

Fig. 5. Effects of adenosine on $[Ca^{2+}]_i$ in cluster F neurons. (A) Raw data traces of fluo-4 fluorescence changes in neurons undergoing treatments as specified by the solid bars under the individual traces. Each trace represents the change in a single neuron. Note that the Ca^{2+} signal does not increase indefinitely, but reaches a plateau towards the end of the recording. (B) Summary graph of normalized changes in fluo-4 fluorescence in neurons undergoing A_1R antagonism with DPCPX (*N*=4) or a control to adenosine transition (*N*=4). Data are expressed as means ± s.e.m. The fluo-4-induced Ca^{2+} signals were quantified as $\Delta F/F_0$ multiplied by 100%. Asterisk indicates significant change from baseline following treatment onset (*P*<0.05; paired *t*-test). (C) Upon adenosine perfusion, AP frequency varies linearly with $[Ca^{2+}]_i$ (*N*=4). Error bars are obscured by symbol in some cases (y=2.45).

DISCUSSION

In this study, we examined the inhibitory effects of adenosine in the central ring ganglia from the pond snail *L. stagnalis*. In response to application of adenosine, cluster F neurons exhibited an 11% hyperpolarization of the resting membrane potential along with a 47% decrease in action potential firing frequency. The reversibility of the depression in neuronal activity upon reperfusion with control saline suggests that this phenomenon may be receptor mediated.

To further explore this possibility, we used a pharmacological A_1R agonist and antagonist to determine changes in neuronal electrical properties. CPA and DPCPX were selected because of their relative A_1R specificity and because the latter has previously proved effective as an A_1R antagonist in invertebrate preparations (Magazanik and Federova, 2003). When A_1-receptor mediated neurotransmission was antagonized with DPCPX, adenosine failed to elicit a robust inhibitory response. We further demonstrated that the inhibitory effects of adenosine are mediated *via* the A_1 receptor, by showing that the response is CPA sensitive. CPA caused a reduction in AP frequency and hyperpolarization of V_m that were comparable to those elicited by adenosine. The inhibitory actions of adenosine are consistent with a previous investigation (Barraco and Stefano, 1990) which showed that adenosine inhibited the release of excitatory neurotransmitters including serotonin and dopamine from the pedal ganglia of the marine bivalve *Mytilus edulis*, whereas an adenosine antagonist blocked the inhibitory effects on neurotransmitter release.

Depression of neurotransmitter release (glutamate in particular) by activation of adenosine receptor is achieved through G-protein-coupled inhibition of Ca^{2+} influx in nerve endings (Fredholm and Dunwiddie, 1988; Wu and Saggau, 1997) along with enhancement of K^+ and Cl^- conductance (Trussel and Jackson, 1985; Mager et al., 1990). Adenosine receptor activation may also cause an increase in cytosolic Ca^{2+} levels *via* an inositol 3-phosphate (Ins(3)*P*)-mediated pathway. All adenosine receptors except the A_{2A} subtype activate phospholipase C (PLC), an enzyme converting phospholipids into diacylglycerol and Ins(3)*P* (Bickler and Buck,

2007; Dunwiddie and Masino, 2001). Ins(3)*P* receptor activation is consistent with the elevation of $[Ca^{2+}]_i$ observed in cluster F neurons upon perfusion with adenosine. Furthermore, a strong correlation was observed between elevated $[Ca^{2+}]_i$ and decreasing AP frequency. The reversibility of this phenomenon could not be tested because of technical limitations (whole cell patches were difficult to maintain beyond 60 min). Despite this shortcoming, it is clear that the Ca^{2+} increase was adenosine dependent because incubation with DPCPX failed to elicit a response in the presence of adenosine.

Unlike the deleterious accumulation that occurs during excitotoxic cell death (ECD) in response to lack of oxygen in hypoxia-sensitive species (Choi, 1994; Choi, 1992), minute increases in cytosolic Ca^{2+} levels can be neuroprotective in certain anoxia-tolerant species (Bickler et al., 2000; Shin et al., 2005). One of the outcomes of elevated Ca^{2+} is inactivation of *N*-methyl-D-aspartate receptors (Ehlers et al., 1996) that are Ca^{2+} permeable glutamate-gated ion channels regulated by $[Ca^{2+}]_i$ and required for fast excitatory neurotransmission in the central nervous system (Albensi, 2007). Thus, the adenosine-mediated decrease in AP frequency and hyperpolarization of V_m, along with a simultaneous increase in Ca^{2+} suggest a possible synergistic mechanism resulting in global neuronal depression.

L. stagnalis is an anoxia-tolerant pulmonate that hibernates during the winter (Jones, 1961). Tolerance of variable oxygen concentrations is often associated with inactivity and hypometabolism induced by cold temperatures. There is a "hemispheric asymmetry" in cold tolerance amongst species that frequently undergo freeze–thaw events (Sinclair et al., 2003; Chown et al., 2004). Because these events occur year-round in high latitudes of the Southern Hemisphere, endemic freeze-tolerant species retain their ability to survive cold temperatures throughout the year without energetically demanding metabolic shifts. By contrast, temperatures in high-latitude areas of the Northern Hemisphere remain sub-zero for several months during winter and rise well above freezing for long periods during the summer. Consequently, native species lose their cold tolerance in the summer, but show exceptional freeze tolerance in winter that requires costly physiological adaptations. These differing strategies amongst freeze-tolerant species in the Southern and Northern hemispheres raise the possibility that anoxia tolerance of *L. stagnalis* may have a seasonal dependence. Furthermore, it might explain our observation of diminished responsiveness to adenosine modulation in neurons obtained during the summer months (May to August). Sensitivity of *L. stagnalis* neurons to seasonal changes has been reported previously. Zapara et al. (Zapara et al., 2004) found that neurons harvested during summertime exhibited a depolarization of the resting membrane potential (anoxic depolarization) in response to ischemia and/or hypoxia, whereas those obtained during the winter showed resistance to anoxic depolarization. More research is required to elucidate the mechanisms that render neurons more sensitive to adenosine regulation during winter (December to March) than during summer.

In conclusion, we demonstrate that adenosine depresses neuronal activity in cluster F neurons of *L. stagnalis via* the A_1R (type I subclass) and this inhibition correlates with changes in $[Ca^{2+}]_i$. Additional experiments are required to ascertain if the adenosine response is mediated entirely through the A_1R, by testing the effects of adenosine type 2 receptor modulators. Furthermore, it remains to be determined whether adenosine confers protection during severe hypoxia and whether this is mediated *via* changes in cytosolic Ca^{2+} levels.

LIST OF ABBREVIATIONS

AP frequency	action potential firing frequency
A_1R	adenosine receptor type I subclass
$[Ca^{2+}]_i$	intracellular calcium concentration
CPA	N^6-cyclopentyladenosine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
PeDG	pedal dorsal ganglia
V_m	membrane potential

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