Retinoic acid affects calcium signaling in adult molluscan neurons

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Submitted 20 June 2014; accepted in final form 9 October 2014

Retinoic acid affects calcium signaling in adult molluscan neurons. J Neurophysiol 113: 172–181, 2015. First published October 15, 2014; doi:10.1152/jn.00458.2014.—Retinoic acid, the active metabolite of vitamin A, is important for nervous system development, regeneration, as well as cognitive functions of the adult central nervous system. These central nervous system functions are all highly dependent on neuronal activity. Retinoic acid has previously been shown to induce changes in the firing properties and action potential waveforms of adult molluscan neurons in a dose- and isomer-dependent manner. In this study, we aimed to determine the cellular pathways by which retinoic acid might exert such effects, by testing the involvement of pathways previously shown to be affected by retinoic acid. We demonstrated that the ability of all-trans retinoic acid (atRA) to induce electrophysiological changes in cultured molluscan neurons was not prevented by inhibitors of protein synthesis, protein kinase A or phospholipase C. However, we showed that atRA was capable of rapidly reducing intracellular calcium levels in the same dose- and isomer-dependent manner as shown previously for changes in neuronal firing. Moreover, we also demonstrated that the transmembrane ion flux through voltage-gated calcium channels was rapidly modulated by retinoic acid. In particular, the peak current density was reduced and the inactivation rate was increased in the presence of atRA, over a similar time course as the changes in cell firing and reductions in intracellular calcium. These studies provide further evidence for the ability of atRA to induce rapid effects in mature neurons.

Lymnaea stagnalis; electrophysiology; retinoid; protein synthesis; second messengers; voltage-gated calcium channels

THE ELECTRICAL FIRING OF NEURONS is important for many developmental and regenerative processes within the nervous system (Al-Majed et al. 2000; Hanson and Landmesser 2004) and can affect neurite outgrowth, growth cone behavior and synaptic plasticity. The vitamin A metabolite, retinoic acid (RA), is also very important for development and regeneration of the nervous system (Maden 2007) and can induce neurite outgrowth (Claggett-Dame et al. 2006; Corcoran et al. 2000), promote growth cone turning (Dmetrichuk et al. 2006; Farrar et al. 2009), as well as modulate hippocampal synaptic plasticity, such as long-term potentiation and depression (Chiang et al. 1998; Misner et al. 2001; Nomoto et al. 2012). We recently investigated whether RA might exert some of its effects by directly modulating the electrical firing of neurons. Using the mollusc Lymnaea stagnalis, it was shown that RA changed the firing properties of adult neurons in a dose- and isomer-dependent manner. Higher concentrations of all-trans RA (atRA), but not its precursor retinol or the isomer 9-cis RA (9-cisRA), induced a transition from tonic firing to bursting activity, increases in action potential duration and the appearance of plateau potentials, followed by cell silencing. Some of these effects, such as cell silencing, were relatively transient (less than 3 h), but the effects on cell firing patterns were longer-lasting (up to 24 h). These longer-lasting effects of RA, but not the transient effects, were blocked by a retinoid receptor antagonist (Vesprini and Spencer 2014). In this study, we aimed to further investigate the underlying mechanisms by which RA, at higher concentrations, might exert its effects on neuronal firing.

The physiological levels of RA in vivo have proven difficult to measure, but it has become clear that either a deficiency or an excess of retinoids can exert detrimental effects on nervous system development (Maden 2007). Classically, RA influences gene expression by binding to nuclear hormone receptors, of which there are two main classes: the RA receptors (RARs) and retinoid X receptors (RXRs). A number of recent studies have, however, shown nonnuclear localization of RARs and RXRs (Carter et al. 2010, 2011; Maghsoudi et al. 2008; Schrage et al. 2006), and others have described nongenomic actions of the retinoid receptors (Aoto et al. 2008; Carter et al. 2010; Liao et al. 2004; Liou et al. 2005; Poon and Chen 2008; Sarti et al. 2012, 2013). Many of these studies described relatively rapid responses to RA, further supporting its role in nongenomic signaling. Our laboratory previously showed that RA rapidly altered the firing properties of cultured molluscan neurons, an effect that also occurred in isolated neurites dissected from the cell body, suggesting a nongenomic mechanism (Vesprini and Spencer 2014). In this study, we determined whether previously identified targets of retinoids and their receptors might play a role in the ability of RA to modify the firing properties of cultured neurons. In particular, we investigated the role of second messengers, such as protein kinase A (PKA) and phospholipase C (PLC), as well as the role of protein synthesis. It has previously been shown that atRA can affect both Ca{sup 2+} channel expression and intracellular calcium concentration ([Ca{sup 2+}]) as a result of its ability to induce cell differentiation. We, therefore, also investigated the effects of RA on [Ca{sup 2+}]{sub i}, as well as transmembrane currents mediated by voltage-gated calcium channels (VGCCs). We found no evidence for the role of protein synthesis, PKA or PLC in the RA-induced changes in neuronal firing, although we found that RA induced rapid reductions in [Ca{sup 2+}]{sub i}, as well as reduced current flow through VGCCs.

MATERIALS AND METHODS

Cell culture. Lymnaea stagnalis were laboratory-reared and housed in dechlorinated water and fed lettuce and Spirulina fish food (Nutrafin Max Spirulina Flakes for Fish). Cell culture techniques were performed as described previously (Dmetrichuk et al. 2006; Vesprini and Spencer 2014). Animals were anesthetized, and the central ring ganglia were removed and bathed in antibiotic saline containing 225 μg/ml gentamycin. Ganglia were exposed to trypsin [2 mg/ml defined

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medium (DM) for 19 min, and pinned out in high osmolarity DM (Gibco Leibovitz’s L-15 medium). After removal of the inner ganglionic sheath, the somata of identified visceral F (VF) neurons were individually removed from the ganglia via a suction pipette. Between 4 and 6 neurons were plated per dish. Culture dishes were coated with poly-L-lysine and contained 3 ml of DM (unless stated otherwise), and cells were incubated at 21°C overnight.

**Intracellular electrophysiological recordings.** Intracellular glass recording electrodes (resistance of 20–40 MΩ) were backfilled with saturated potassium sulfate. Recordings were made from individual neurons after ~18–24 h in culture, using an intracellular recording amplifier (NeuroData IR283A, Cygnus Technology) and a Powerlab 4sp data acquisition system running Chart v4.2 (AD Instruments). Cell activity was recorded in DM for ~10 min prior to the addition of atRA (10^{-7} M final bath concentration) or ethanol (EtOH) (0.1%; vehicle control). The firing activity of the cell was then recorded for a further 60 min in the presence of RA (or EtOH). At 2.5, 15, 35, 50 and 60 min, the membrane potential was manipulated (using depolarizing current injection) to reach firing threshold and to allow the cell to fire up to 10 action potentials at a frequency of ~1 Hz or less. The membrane potential was then briefly depolarized further to induce brief, rapid firing for ~20 s. The membrane potential was then allowed to return to its resting value until the next time point.

**Chemicals.** All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. A stock of atRA or 9-cisRA was made fresh daily and then diluted further using DM. Retinoids were used at a final bath concentration of 1 or 10 µM, and EtOH (0.1%) was used as the vehicle control. In some experiments, neurons were incubated in the presence of antagonists or inhibitors prior to the start of electrophysiological recordings. Solutions with antagonists or inhibitors were added for a final bath concentration of 1 or 10 µM (unless stated otherwise), and the antagonists or inhibitors were added to the bath at least 1 h prior to the start of recordings.

Solutions with antagonists or inhibitors were made fresh daily from 10 mM aliquot stock solutions dissolved in deionized water. The vertebrate RRX pan-antagonist HX531 and the vertebrate RAR pan-antagonist LES40 were obtained from Dr. Kagechika (University of Tokyo, Japan). The antagonists were used at a final bath concentration of 1 µM. Both HX531 and LES40 have previously been shown to effectively block the effects of RA on *Lymnaea* neurons (Carter et al. 2010; Rand 2012). Vehicle controls for the retinoid antagonist experiments used 0.01% DMSO (final bath concentration of 1 µM; H9024). Working solutions of indo-1 AM were made fresh daily from [Ca^{2+}]_i by taking 25 nm fluorescently excitable form of the indo-1 dye is only present once the AM group has been cleaved. Since the fluorescently excitable form of the indo-1 dye is only present once inside neurons, this dye produces minimal background signals in the surrounding culture environment and thus can resolve changes in [Ca^{2+}]_i within cells with a high sensitivity. One day after plating individual neurons into culture dishes, indo-1 AM was loaded into neurons. The culture medium was replaced with saline using a perfusion pump, after which the indo-1 AM ester was added to the dish for a final bath concentration of 1 µM. Cells were then incubated at 21°C for 3 h. When bound to calcium, this dye shifts its emission spectrum, thus providing an opportunity to estimate [Ca^{2+}]_i by taking the ratio of emission at the optimal wavelengths for Ca^{2+}-free and Ca^{2+}-bound dye. This provided ratiometric quantification, and there was no need to control for loading differences across preparations. After dye loading, cells were washed with saline to remove unloaded indo-1 AM dye from the dish.

Once loaded with indo-1, neurons were imaged on an Olympus IX71 inverted microscope (Olympus, Ontario, Canada) equipped with a Tsunami mode-locked Ti:Sapphire laser (model 3960, Spectra Physics) for two photon excitation and an Olympus Fluoview FV300 confocal scanner. Specifically, the Tsunami-mode locked Ti:Sapphire laser was set to a 740-nm excitation wavelength. The laser power at the confocal scanner was set at 13 mW, at which level no autofluorescence was collected from the neurons. Two band-pass filters within the confocal scanner were used to detect both the calcium-free and calcium-bound indo-1 dye (detection wavelengths of 480 ± 25 nm and 405 ± 25 nm, respectively). Fluorescent emission from both wavelengths was simultaneously collected by the confocal scanner, and Fluoview imaging software (version 5.0, Olympus) was used to acquire images on a computer. Fluorescent images of individual cell bodies were taken at 5-min intervals, for 20 min prior to, and 1 h after, application of the retinoid. Images were processed with ImageJ software (version 1.42q) utilizing a custom macro written to calculate the fluorescence from a time-lapse series of two channel images which had been background-subtracted. For each image in a time-lapse series, the perimeter of the cell body was traced, and the ratio of the fluorescence within the neuron was calculated by measuring fluorescence at 405 nm (representing bound dye) and 480 nm (representing free dye). The ratio of free to bound dye during a 20-min period immediately prior to addition of the retinoid was averaged and taken as the preexposure (baseline) level. Changes in [Ca^{2+}]_i, during the 60 min of exposure to retinoid are expressed as a percent change from the baseline value. A mixed-factor ANOVA was used for analysis, where treatment was specified as the fixed factor and time was specified as the repeated factor. The random, repeated factor. A Tukey post hoc test was used for comparisons, which were considered to be significant when P < 0.05. Voltage clamp analysis. The biophysical properties of VGCCs was assessed using whole cell patch-clamp recording. Individual VF neurons from *Lymnaea* were isolated and plated in culture, as described above. Recordings were performed 24 to 48 h later, using a MultiClamp 700A amplifier, a Digidata 1322A digitizer and Clampex 9.2 software (Axon Instruments, Sunnyvale, CA). Calcium channel currents were measured in 10 mM external Ba^{2+} [10 mM BaCl2, 45.7 mM tetraethylammonium chloride (TEA-Cl), 1 mM MgCl2, 10 mM HEPES, 2 mM 4-aminopyridine (4-AP), pH 7.9 with TEA-OH].
electrodes with a resistance between 2 and 4 MΩ were filled with internal solution (29 mM CsCl, 2.3 mM CaCl₂, 2 mM MgATP, 0.1 mM GTP-Tris, 11 mM EGTA, 10 mM HEPES, pH 7.4 with CsOH; Hui and Feng 2008). During all recordings, a test voltage step to 0 mV was used to determine the stability and suitability of the patch. Cells with a leak current greater than 10% of peak current were deemed unsuitable and discarded.

The current-voltage (IV) relationship was determined by holding the cell at −100 mV and then stepping to voltages ranging from −80 to +60 mV, in 10-mV increments for a duration of 400 ms. After an initial control recording, either atRA, DMSO (vehicle control) or saline only were added to the culture dish to produce a final concentration of 10 μM atRA or 0.1% DMSO. Subsequent recordings were made every 5 min after the treatment, for a period of up to 1 h.

For all recordings, leak subtraction was performed offline, and data were filtered using a 500-Hz Gaussian filter in Clampfit 9.2 before further analysis. To produce IV curves, data were exported into Excel (Microsoft), and each Ba²⁺ current was divided by membrane capacitance to calculate current density, which was then normalized to peak density before and at various time points after addition of CdCl₂, and determining the activation and deactivation parameters. The kinetics of deactivation were assessed by dividing the current remaining 300 ms after the peak by peak current at 80 ms, and then normalizing to peak density of the IV curve. Subsequently, the conductance was calculated using the following equation:

\[ G = \frac{1}{V} \frac{dI}{dV} \]  

where \( G \) is conductance, \( V \) is voltage, \( I \) is current, and \( \frac{dI}{dV} \) is the derivative of the IV curve.

Normalized conductance (activation) plots were then exported into Origin 8 software (OriginLab) and curve-fitted with the following Boltzmann equation:

\[ \text{Activation} = \left( \frac{G}{G_{\text{max}}} \right) = \frac{A_1 - A_2}{1 + e^{(V-V_{\text{rev}})/K_e}} \]  

where \( V_{\text{rev}} \) and \( K_e \) represent the half-activation voltage and slope factor of the activation curves, respectively (determined by Origin software). \( A_1 \) and \( A_2 \) equal 1 and 0, respectively, and correspond to the maximum and minimum values allowed in the curve fit. The mean data were plotted, and the IV curve was simulated using the following modified Ohmic-Boltzmann equation:

\[ \frac{I}{I_{\text{max}}} = G_{\text{max}}(V - V_{\text{rev}})(A_2 + (A_1 - A_2)/(1 + e^{(V-V_{\text{rev}})/K_e})) \]  

To quantify calcium channel kinetics, Clampfit 9.2 was employed to determine the time to peak and the inactivation decay 300 ms after the peak at −10, 0 and +10 mV. Current decay (inactivation) was assessed by dividing the current remaining 300 ms after the peak by the peak current. Activation was measured by measuring the time to peak from the start of the voltage step. The kinetics of deactivation were obtained by fitting tail currents with a monoexponential curve and determining the \( \tau \) value. All gating and kinetic data were statistically analyzed using a mixed-factor ANOVA, where treatment was specified as the fixed factor and time as the repeated, random factor. A Tukey post hoc test was used for comparisons (in SigmaStat 3.5 software; Systat Software), which were deemed significant when \( P < 0.05 \).

Cultured VF neurons were also subjected to a voltage ramp protocol to determine the class of VGCCs present. The cells were held at −100 mV, and the voltage increased steadily from −80 mV to +60 mV over the course of 400 ms. Following this, the cells were treated with 100 μM CdCl₂ in an attempt to block high-voltage activated (HVA) channels (Staras et al. 2002) and to also observe the effect on total Ba²⁺ currents elicited by the voltage ramp. The ability of CdCl₂ to block the current was calculated by comparing the peak current density before and at various time points after addition of CdCl₂, and data were analyzed using a one-way ANOVA.

RESULTS

The atRA-induced changes in neuronal firing are independent of protein synthesis, PKA and PLC. It was previously shown that atRA, at micromolar concentrations, induced rapid changes in neuronal firing within 15 min of application (Vesprini and Spencer 2014). As shown previously, application of 10 μM atRA to cultured VF neurons rapidly induced changes in firing properties that included widening of action potentials and atypical firing, such as spike doublets and plateau potentials. Figure 1 shows representative examples of VF neurons firing both before and after the addition of EtOH (vehicle control) and 10 μM atRA. Note that the firing did not change after application of atRA (Fig. 1A), yet cells that were exposed to atRA for between 20 and 30 min showed changes in spike waveform (Fig. 1B) and atypical firing properties, such as plateau potentials (Figs. 1C and 2A). Between 30 and 60 min, a large percentage of neurons became silent (Fig. 2B), and action potentials could not be induced with depolarizing current injection. These effects of atRA on neuronal firing were previously shown to be independent of transcription, because they occurred in isolated neurites (Vesprini and Spencer 2014). However, isolated Lymnaea neurites are capable of local protein synthesis (Spencer et al. 2000; van Minnen et al. 1997), and atRA has been shown to exert translational control in hippocampal dendrites (Poon and Chen 2008). Our first aim was thus to determine whether protein synthesis was required for the atRA-induced changes in neuronal firing. There is also previous evidence that RA modulates the activity of PKA (Kholodenko et al. 2007) and PLC (Liou et al. 2005), and thus we also investigated the role of these second messengers in the effects of atRA on cell firing.

Inhibitors of protein synthesis (anisomycin), PKA (Rp-cAMPS and H-89) and PLC (U-73122) were bath-applied to separate dishes containing VF neurons, 1 h prior to recordings. Intracellular recordings were then made from individual cul-

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**Fig. 1.** All-trans retinoic acid (atRA) alters the firing properties of cultured visceral F (VF) neurons. Representative examples are shown of the firing activity of cultured VF neurons both before (i) and 20–30 min after (ii) the addition of either atRA or ethanol (EtOH, vehicle control). A: a VF cell showed no change in firing properties or spike waveform before addition of the vehicle control, EtOH (0.1%). B and C: different examples of VF cells showing broadening of action potentials (Bii) or atypical firing patterns such as plateau potentials (Ciil) after exposure to atRA (10 μM).
Neurons exposed to atRA and vehicle (n = 28) showed high levels of atypical firing activity (57%), compared with only 4\% of neurons exposed to EtOH (n = 25). Preincubation of cells in anisomycin (n = 14), PKAi (Rp-cAMPs n = 17) and H-89 (n = 6; data not shown), or PLCi (n = 17) had no significant effect on the ability of atRA to induce atypical firing (Fig. 2A). Furthermore, despite 96.4\% of atRA-exposed neurons falling silent by 1 h (compared with 4\% of EtOH controls), the number of neurons that became silent in the presence of the inhibitors was not significantly reduced [Fig. 2B; anisomycin: 77.8\%; Rp-cAMPs (PKAi): 76.5\%; H-89: 83.3\% (data not shown) and U-73122 (PLCi): 82.4\%; P > 0.05]. Together these data suggest that the ability of atRA to induce atypical firing and cell silencing did not require protein synthesis or activation of PKA or PLC.

Analysis of the spike waveforms at both 15 min and 35 min of atRA exposure revealed that atRA produced a significant increase in both half-amplitude duration and decay time compared with application of EtOH alone (Fig. 2C), as shown previously in Vesprini and Spencer (2014). However, we found that the inhibitors of protein synthesis, PKA (Rp-cAMPs) and PLC had no significant effect on these atRA-induced changes in spike waveform parameters. At 15 min, there were no significant differences in either the half-amplitude duration (Fig. 2Ci) or the decay time (Fig. 2Cii) in the inhibitors compared with atRA alone, and all were significantly different from application of EtOH alone. Furthermore, at 35 min, none of the inhibitors showed any significant effect on the ability of atRA to increase half-amplitude duration (Fig. 2Ciii) and decay time (Fig. 2Civ). The inhibitors also had no significant effect on either the peak-to-peak amplitude of the action potential, the rise time, or the input resistance, compared with atRA alone (Table 1).

AtRA significantly reduces \([Ca_{2+}]\), within 15 min of exposure in a dose- and isomer-dependent manner. Changes in \([Ca_{2+}]\), have previously been shown to play a role in modulating bursting activity of neurons (Coulon et al. 2009). Furthermore, alterations in \([Ca_{2+}]\), can cause widespread physiological changes within a cell, potentially leading to changes in the cell silencing, and again, only the application of EtOH alone showed a significant difference from atRA. C: spike analysis of the half-amplitude duration at 15 (i) and 35 min (ii) after application of atRA (or EtOH as a control) in the presence of anisomycin, PKA or PLC inhibitors, shown as a percentage of baseline (set at 0\%). Application of atRA in the presence of the inhibitors exhibited no significant differences from atRA alone at either 15 or 35 min. The decay time of action potentials was also analyzed at 15 (iii) and 35 min (iv) after application of atRA in either the presence or absence of the inhibitors. Once again, application of atRA in the presence of the inhibitors showed no significant differences compared with atRA alone. Note that, in all conditions, the atRA-induced increases in both half amplitude and decay time were larger at the 35-min time point compared with the 15-min time point. Values are means ± SE. Nos. in parentheses, no. of cells. *P values for spike analysis were lower at 35 min, as many of the cells were silent by this time point. **P < 0.05 and ***P < 0.01 for comparisons to atRA, n.s., No significant difference.

### Table 1. Peak-to-peak amplitude, rise time, and input resistance are unaffected by inhibitors of protein synthesis, PKA and PLC

<table>
<thead>
<tr>
<th>10 \umu M RA</th>
<th>Anisomycin + RA</th>
<th>PKAi + RA</th>
<th>PKCi + RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak-to-peak amplitude</td>
<td>89.6 ± 2.7</td>
<td>93.4 ± 4.8</td>
<td>94.0 ± 2.8</td>
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<tr>
<td>Rise time</td>
<td>188.8 ± 40.9</td>
<td>155.9 ± 38.0</td>
<td>130.1 ± 11.7</td>
</tr>
<tr>
<td>Input resistance</td>
<td>113.1 ± 13.1</td>
<td>98.7 ± 10.7</td>
<td>84.1 ± 14.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. All values are expressed as a percentage of baseline (set at 100\%). Action potential waveform analysis was at 35 min following exposure to all-trans retinoic acid (atRA) alone or atRA in the presence of the inhibitors for protein kinase A (PKA; Rp-adenosine 3',5'-cyclic monophosphorothioate), phospholipase C (PLC; U-73122), or protein synthesis (anisomycin). Input resistance was measured 10 min before and 1 h after atRA exposure. In all cases, no significant differences were found.
firing activity through either direct modulation, or downstream modification of ion channels. We next investigated whether acute exposure to atRA could produce changes in $[Ca^{2+}]_{i}$. To this end, we utilized the calcium indicator dye indo-1 AM. Because it was previously demonstrated that the atRA-induced changes in firing properties were both dose dependent and isomer dependent (Vesprini and Spencer 2014), we tested atRA at both 10 $\mu$M and 1 $\mu$M as well as the isomer 9-cisRA at 10 $\mu$M.

VF neurons were individually cultured and then incubated in the presence of indo-1 AM dye for 3 h. Preliminary experiments revealed this incubation time to be sufficient for adequate dye loading of neurons (data not shown). Following incubation, neurons were imaged for 20 min prior to and 60 min after the addition of retinoid or 0.1% EtOH (vehicle control), and changes in $[Ca^{2+}]_{i}$ were expressed as a percent change from the baseline value. A mixed-factor ANOVA revealed a significant interaction between treatment and time ($F_{(36,588)} = 5.22; P < 0.001$). Figure 3A illustrates the significant differences between neurons exposed to atRA (10 $\mu$M; $n = 12$) and those exposed to EtOH (0.1%; $n = 14$). Neurons exposed to 10 $\mu$M atRA showed a significant decrease in $[Ca^{2+}]_{i}$ over time, whereas there was no decline in $[Ca^{2+}]_{i}$, following exposure to EtOH. Compared with the EtOH controls, the atRA-exposed neurons showed a significant decrease in $[Ca^{2+}]_{i}$, as early as 15 min ($P < 0.05$), and the decrease reached a maximum by about 45 min. Interestingly, the time course of the atRA-induced changes in $[Ca^{2+}]_{i}$ closely resembled the atRA-induced changes in firing patterns and spike waveform, which occurred as early as 15 min, but were significantly greater at 35 min than at 15 min (Fig. 2; Vesprini and Spencer 2014).

Figure 3B summarizes data obtained with atRA at both 10 $\mu$M and the lower concentration of 1 $\mu$M, as well as 10 $\mu$M of the isomer 9-cisRA, at specific time points. There were no significant differences between any of the conditions at any time point over the 20-min control period. Interestingly, the neurons exposed to either 1 $\mu$M atRA ($n = 15$) or 10 $\mu$M 9-cisRA ($n = 12$) showed no significant reductions in $[Ca^{2+}]_{i}$, over the 60 min compared with the EtOH controls. Furthermore, the 10 $\mu$M atRA-exposed neurons showed significantly lower $[Ca^{2+}]_{i}$, compared with both 10 $\mu$M 9-cisRA ($P < 0.05$) and 1 $\mu$M atRA-exposed neurons ($P < 0.05$), as early as 10 and 20 min after retinoid exposure, respectively. Taken together, these data suggest that the atRA-induced decreases in $[Ca^{2+}]_{i}$, were both dose and isomer dependent, with only the higher concentration of atRA exerting significant effects. These findings are consistent with the dose and isomer dependency of the RA-induced changes in the electrophysiological properties.

Retinoid receptor antagonists do not block the atRA-induced decrease in $[Ca^{2+}]_{i}$. It was previously shown that the ability of 10 $\mu$M atRA to induce atypical firing, as well as increase the half-amplitude duration and decay time of the action potential, was significantly inhibited in the presence of the retinoid receptor antagonist HX531. Interestingly, however, the more transient effects of atRA-induced cell silencing were not blocked by HX531. The retinoid receptor antagonist LE540 did not block any of the electrophysiological effects of atRA (Vesprini and Spencer 2014).

Our next aim was thus to determine whether either of these retinoid receptor antagonists would block the atRA-induced changes in $[Ca^{2+}]_{i}$. VF neurons were incubated in either the RAR pan-antagonist LE540 (1 $\mu$M; $n = 11$), the RXR pan-antagonist HX531 (1 $\mu$M; $n = 11$) or 0.01% DMSO ($n = 11$ as the vehicle control) for 1 h prior to the start of imaging. atRA (10 $\mu$M) was then applied to all cells (at time $t = 0$ min). A mixed-factor ANOVA revealed no significant interaction between treatment and time ($F_{(24,372)} = 0.771; P > 0.05$), and only a within-group difference reflecting changes across time ($F_{(12,372)} = 18.371; P < 0.001$). As shown in Fig. 4, atRA induced a significant decrease in $[Ca^{2+}]_{i}$, over time ($P < 0.01$ at $t = 60$ min compared with $t = 0$ min), but this also occurred in the presence of either LE540 or HX531, suggesting the effect was independent of receptor activation. These data also suggest that the reduction in $[Ca^{2+}]_{i}$ may occur independently of the changes in spike waveform, as these were previously found to be blocked by HX531. However, the reduction in $[Ca^{2+}]_{i}$ may play a role in the atRA-induced cell silencing, as this effect was also previously found to be independent of receptor activation (Vesprini and Spencer 2014).
Apamin does not mimic the effects of atRA. The observation that 10 μM atRA significantly reduces [Ca^{2+}], suggested that atRA may also indirectly affect membrane ion channels that are dependent on [Ca^{2+}], such as the calcium-dependent potassium channel (K Caf). These K Caf channels can control firing frequency and activity patterns in many neurons. For example, blockade of small-conductance K Caf (SK-like) channels with apamin induces bursting activity in a lobster neuron (Harris-Warrick and Johnson 1987) and silences the firing of snail neurons (Artinian et al. 2010). Both of these effects are similar to those shown previously with atRA (Vespriini and Spencer 2014). Although the presence of these small-conductance K Caf channels in the VF neurons has not previously been demonstrated, we next determined whether apamin might mimic the electrophysiological changes observed with atRA.

Neurons were exposed to either 10 μM atRA (n = 10) or 10 μM apamin (n = 8) for up to 1 h. Neurons exposed to 10 μM RA showed a significant increase in atypical firing (n = 7 of 10; P < 0.01) compared with neurons exposed to 10 μM apamin (n = 0 of 8). Furthermore, 10 μM RA also induced significantly more cell silencing (n = 7 of 10; P < 0.005), compared with 10 μM apamin, which did not induce cell silencing in any of the neurons studied (n = 0 of 8). Analysis of spike waveform at both 15 min (data not shown) and 35 min (Fig. 5), showed significant increases (P < 0.01) in both the half-amplitude duration (Fig. 5A) and decay time (Fig. 5B) in neurons exposed to 10 μM atRA, compared with neurons exposed to apamin. Taken together, these data suggest that impairment of K Caf channel function with 10 μM apamin did not mimic the effects of atRA and thus suggest that small-conductance K Caf (SK) channels do not likely play a role in the electrophysiological effects of atRA in these cells.

AtRA significantly reduces VGCC currents, but does not alter the IV relationship. We next investigated whether the decrease in [Ca^{2+}], might result from a reduction in calcium influx via VGCCs. Although RA has previously been shown to alter the expression of VGCCs during RA-mediated neuronal differentiation, no previous studies have determined whether RA alters VGCC activity in adult neurons.

Whole-cell voltage clamp recordings were used to investigate the changes in Ba^{2+}-current flow through VGCCs. The effect of acute atRA exposure on VGCCs was first assessed by holding the cell at −100 mV and then stepping to voltages ranging from −80 to +60 mV in 10-mV increments while in a Na^{+}-free external bath solution containing 10 mM Ba^{2+} (with TEA and 4-AP to inhibit K^+ channel activity). Patch pipettes were filled with saline containing CsCl to further block K^+ channel activity from the interior of the cell (Hille 2001). Cultured VF neurons were exposed to either 10 μM atRA, or DMSO (0.1%) or saline, as controls.

A mixed-factor ANOVA revealed a significant interaction between treatment and time [F(24,221) = 5.49; P < 0.001]. A post hoc test revealed that the peak current density was significantly reduced within 20 min of application of 10 μM atRA, compared with both saline application (P < 0.001) or DMSO application (P < 0.05) (Fig. 6, A and B). Interestingly, when the IV curves were normalized to peak current to compare the shape of the IV relationship, no significant changes were noted (Fig. 6C). No significant effects on the voltage dependence of activation, half-activation voltage or the slope of the activation curve were observed (data not shown).

The current activation at approximately −40 mV in the VF cells (Fig. 6C) strongly suggested a HVA current. However, both low-voltage activated (LVA) and HVA Ca^{2+} currents have previously been described in other cells of Lymnaea (Staras et al. 2002). To determine which type of current RA might be affecting, we next used a voltage ramp protocol to investigate the possible existence of an LVA component. Previously in the cerebral giant cells of Lymnaea, the LVA component was found to activate at about −65 mV, whereas the HVA component activated at about −40 mV and was selectively blocked by 100 μM CdCl2 (Staras et al. 2002). Our ramp protocol (from −80 mV to +60 mV) failed to indicate
the presence of any LVA current in any of the cells tested (Fig. 6Di; n = 13). Furthermore, the peak current density was significantly reduced by ~84% after 15 min of exposure to 100 μM CdCl₂ (n = 6; P < 0.05; Fig. 6Dii), which was comparable to the percentage of HVA block seen previously in the cerebral giant cells, suggesting a predominantly HVA component. Having shown that both atRA and CdCl₂ reduced the peak current density, we next determined whether 100 μM CdCl₂ would mimic the effects of atRA to produce changes in the firing properties of VF neurons. However, we found that application of 100 μM CdCl₂ did not induce either cell silencing (n = 0 of 8 cells), or atypical firing (n = 0 of 8) in any of the VF neurons tested.

Inactivation of current through the VGCCs occurs more rapidly in the presence of atRA. We next determined whether application of RA induced any changes in Ca²⁺ channel kinetics (Fig. 7). Most notably, at voltages around the peak of the IV curve, VF neurons treated with 10 μM atRA exhibited VGCC currents that were significantly quicker to inactivate. Data are shown for voltage steps to −10 mV (Fig. 7Ai) and +60 mV (Fig. 7Aii) and were analyzed using a mixed-factor ANOVA. A significant interaction between treatment and time was found at −10 mV [F(4,43) = 3.72; P < 0.05]; 0 mV [F(4,46) = 7.85; P < 0.001] and +10 mV [F(4,46) = 21.12; P < 0.001]. Post hoc analysis indicated a significant reduction in the current remaining at 300 ms after peak in 10
μM atRA compared with controls, at both 15 min and 30 min, at 0 mV and +10 mV voltage steps. Representative current traces at 0 mV are shown for each condition at 0 min (Fig. 7Bi), 15 min (Fig. 7Bii) and 30 min (Fig. 7Biii) of treatment. Lastly, no significant changes were found in the kinetics of activation or deactivation, as measured by time to peak and the τ of the tail current, respectively (data not shown). In summary, acute application of 10 μM atRA decreased peak current density (likely through HVA channels) and induced faster current inactivation, resulting in VGCCs passing less current and for a shorter period of time.

**DISCUSSION**

In this study, we have taken steps toward elucidating the mechanisms by which atRA might elicit changes in the firing properties of adult neurons. RA has previously been shown to affect protein synthesis (Poon and Chen 2008), PKA signaling (Khodolenko et al. 2007) and PLC activation (Liou et al. 2005). However, despite our use of pharmacological inhibitors that I) are capable of blocking other effects of RA (Farrar et al. 2009; Liou et al. 2005) and 2) are known effective blockers in *Lymnaea* (Dobson et al. 2006; Farrar et al. 2009; Hamakawa et al. 1999; Lacchini et al. 2006; Marra et al. 2013), there was no evidence to support a role for these particular pathways in the effects of atRA on neuronal firing. We did, however, find that acute exposure to atRA resulted in a significant decline in [Ca^{2+}], as early as 15 min after application. Importantly, the time course and dose and isomer dependency of this reduction in [Ca^{2+}], closely resembled that of the effects of atRA on cell firing (Vesprini and Spencer 2014). We also determined that the peak current density of the VGCCs was significantly reduced by atRA, and that the rate of inactivation of these channels significantly increased. These changes in the properties and kinetics of the VGCCs occurred over the same time period as the changes in firing patterns, yet may not have been the direct cause of such changes. CdCl₂, which also significantly reduced the peak current density through the VGCCs, was unable to mimic any of the effects of RA on cell firing, suggesting that a reduction in Ca^{2+} influx alone was not responsible. However, the reduction in Ca^{2+} influx may have contributed to the reduction in [Ca^{2+}], which also occurred over the same time period, although we currently have no evidence for direct causality. Furthermore, whether there is direct causality between the reductions in [Ca^{2+}], and the changes in firing properties is not yet known. However, our laboratory previously showed that the retinoid receptor antagonist blocked the ability of atRA to induce atypical firing and changes in spike waveform, but not its ability to induce cell silencing (Vesprini and Spencer 2014). In this study, HX531 did not block the atRA-induced reductions in [Ca^{2+}], suggest-
ing either that the reductions in \([\text{Ca}^{2+}]\) do not directly cause the changes in firing patterns, or that these changes in \([\text{Ca}^{2+}]\) are not downstream of receptor activation. However, it is possible that reductions in \([\text{Ca}^{2+}]\) do directly cause cell silencing, as both these effects of atRA were resistant to block by HX531. It seems very likely that RA is capable of inducing multiple and possibly divergent effects on the cells (as shown previously by Liou et al. 2005), which may be either \(\text{Ca}^{2+}\) dependent or independent. It is also well known that changes in \([\text{Ca}^{2+}]\) can trigger numerous changes in various cell-signaling cascades. As just one example, we hypothesized that the reduction in \([\text{Ca}^{2+}]\) may have subsequently reduced activation of \(\text{K}_{\text{Ca}}\) channels. Transcriptome analysis of the \(\text{Lymnaea}\) central nervous system (CNS) has revealed the presence of \(\text{K}_{\text{Ca}}\) channels (Feng et al. 2009), although we did not confirm their presence in the VF neurons. Regardless, we found that application of the small-conductance \(\text{K}_{\text{Ca}}\) (SK) channel blocker, apamin, did not mimic any of the effects of RA, and thus our data did not support any role for this particular channel in the electrophysiological changes induced by RA.

It has previously been shown that atRA affects both \(\text{Ca}^{2+}\) channel expression and \([\text{Ca}^{2+}]\), as a consequence of its genomic role in cell differentiation. RA-induced differentiation of NT2 into NT2N cells results in increased \([\text{Ca}^{2+}]\), and increased L- and N-type \(\text{Ca}^{2+}\) channel expression (Gao et al. 1998). Conversely, store-operated \(\text{Ca}^{2+}\) channels are down-regulated in neuroblastoma cells following 9-cisRA-induced differentiation (Bell et al. 2013). Here, we have now demonstrated that 10 \(\mu\text{M}\) atRA can induce changes in \([\text{Ca}^{2+}]\), as well as directly affect the biophysical properties and kinetics of existing VGCCs of adult CNS neurons. Previous estimates of atRA levels in the \(\text{Lymnaea}\) CNS were in the order of \(\sim 0.7\ \mu\text{M}\) (Dmetrichuk et al. 2008), and, because RA concentrations are rarely uniform, due to the presence of RA gradients, it is thus feasible that micromolar concentrations of RA may exist in various microdomains. Furthermore, although nanomolar concentrations of atRA are often sufficient to elicit genomic effects, most studies demonstrating nongenomic actions of RA have used micromolar concentrations of the retinoid (Aoto et al. 2008; Chen and Napoli 2008; Sarti et al. 2012, 2013), in some cases as high as 30 \(\mu\text{M}\) RA (Liou et al. 2005). Indeed, the effects elicited by 10 \(\mu\text{M}\) atRA on both calcium levels and VGCC currents in \(\text{Lymnaea}\) neurons occurred over a short time period (15–20 min) and are thus consistent with a nongenomic mechanism.

There are a number of recent reports now supporting nongenomic interactions between RA and \(\text{Ca}^{2+}\) signaling in the nervous system. Micromolar concentrations of atRA regulate synaptic strength between neurons (Aoto et al. 2008), and this role in homeostatic plasticity is regulated by postsynaptic \(\text{Ca}^{2+}\) (Wang et al. 2011). It was shown that reductions in postsynaptic \(\text{Ca}^{2+}\) levels activated RA synthesis, and it was concluded that neuronal RA synthesis is tightly regulated by \([\text{Ca}^{2+}]\). Although the mechanism for this interaction has not yet been fully determined, only the ligand- and mRNA-binding domains (but not the DNA-binding domain) of \(\text{RAR}\) were required to mediate the synaptic effect of atRA (Sarti et al. 2012), strongly supporting a nongenomic mechanism. Other nongenomic effects of atRA, such as increased spontaneous transmitter release at a developing neuromuscular synapse, involve second-messenger-induced release of \(\text{Ca}^{2+}\) from intracellular stores (Liou et al. 2005). It is thus becoming increasingly evident that many nongenomc effects of atRA involve changes in \(\text{Ca}^{2+}\) signaling, although the nature of such interactions appear to be both cell and species dependent. It should be noted, however, that changes in neuronal \([\text{Ca}^{2+}]\), or calcium influx, can often induce downstream changes in gene expression, and so atRA may ultimately affect both nongenomic and genomic targets.

In contrast to our study, other studies have shown that atRA often induces increases in \(\text{Ca}^{2+}\) levels, either directly through release from intracellular stores (Liou et al. 2005), or indirectly as a result of increased AMPA receptor function (Wang et al. 2011). However, these previous studies examined either developing synapses or those undergoing activity-induced plasticity. In our study, the neurons were acutely isolated from the CNS and had undergone axotomy. The cells were also cultured in complete isolation from any potential synaptic partners or glial interactions. These isolated, axotomized neurons might thus represent an injury status, where atRA’s primary role might involve regeneration or repair. Indeed, it was previously shown that application of 100 nM atRA to these \(\text{Lymnaea}\) neurons triggers regenerative outgrowth over a period of 24–72 h (Dmetrichuk et al. 2006). Furthermore, it has been suggested that alterations in the firing pattern of injured neurons may act to create a favorable intracellular environment, with reduced \([\text{Ca}^{2+}]\), promoting regeneration of neurons (McClellan et al. 2008). Therefore, in contrast to the role of RA in synaptogenesis and/or synaptic plasticity, our data showing reduced calcium influx and \([\text{Ca}^{2+}]\) levels, together with the changes in firing properties, may reflect the actions of atRA in a regenerative capacity. In support of this, we previously showed that RA induced significant effects on the spike waveform of neurons within the CNS at shorter exposure times following nerve crush injury, than in CNS that had not received the crush injury (Vespri and Spencer 2014).

In summary, we have provided evidence that the effects of RA on electrophysiological properties of adult \(\text{Lymnaea}\) neurons do not appear to require protein synthesis, or the activation of PKA or PLC. However, we have presented evidence to suggest that atRA can induce a rapid reduction in \([\text{Ca}^{2+}]\), in a dose- and isomer-dependent manner, which closely mirrors its effects on cell firing. We have also shown for the first time that atRA can reduce transmembrane ion flux through VGCCs of adult neurons. As calcium signals are thought to play a role in the initiation of neurite outgrowth and to modulate neuronal impulse activity, these findings suggest that atRA, at micromolar concentrations, may have localized effects on neurons in the nervous system.

GRANTS

This research was funded by a Discovery Grant to G. E. Spencer from the Natural Sciences and Engineering Research Council of Canada (NSERC). T. F. Dawson and N. D. Vesprini were supported by NSERC Scholarships. D. Bruce and Y. Yuan also acknowledge support from NSERC Discovery and NSERC Research Tools and Instruments grants.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.D.V., T.F.D., Y.Y., and G.E.S. conception and design of research; N.D.V., T.F.D., Y.Y., and G.E.S. performed experi-
The role of retinoic acid


