Retinoid receptor-based signaling plays a role in voltage-dependent inhibition of invertebrate voltage-gated Ca\(^{2+}\) channels

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The retinoic acid receptor (RAR) and retinoid X receptor (RXR) mediate the cellular effects of retinoids (derivatives of vitamin A). Both RAR and RXR signaling events are implicated in hippocampal synaptic plasticity. Furthermore, retinoids can interact with calcium signaling during homeostatic plasticity. We recently provided evidence that retinoids attenuate calcium current (\(I_{\text{Ca}}\)) through neuronal voltage-gated calcium channels (VGCCs). We now examined the possibility that constitutive activity of neuronal RXR and/or RAR alters calcium influx via the VGCCs. We found that in neurons of the mollusk *Lymnaea stagnalis*, two different RXR antagonists (PA452 and HX531) had independent and opposing effects on \(I_{\text{Ca}}\) that were also time-dependent; whereas the RXR pan-antagonist PA452 enhanced \(I_{\text{Ca}}\), HX531 reduced \(I_{\text{Ca}}\). Interestingly, this effect of HX531 occurred through voltage-dependent inhibition of VGCCs, a phenomenon known to influence neurotransmitter release from neurons. This inhibition appeared to be independent of G proteins and was largely restricted to Ca\(_{2+}\)Ca\(_{2+}\) channels. Of note, an RAR pan-antagonist, LE540, also inhibited \(I_{\text{Ca}}\) but produced G protein-dependent, voltage-dependent inhibition of VGCCs. These findings provide evidence that retinoid receptors interact with G proteins in neurons and suggest mechanisms by which retinoids might affect synaptic calcium signaling.

Voltage-gated Ca\(^{2+}\) channels (VGCCs)\(^2\) play a prominent role in the functioning of both vertebrate and invertebrate nervous systems. High voltage–activated VGCCs include both Ca\(_{1}\) (L-type) and Ca\(_{2}\) (non–L-type) channels. In the nervous system, Ca\(_{1}\) channels are generally involved in the regulation of gene transcription, whereas Ca\(_{2}\) channels play an important role in neurotransmitter release (1). Importantly, high voltage–activated channels are modulated by diverse signaling pathways and can therefore contribute to dynamic changes in cell function.

Retinoic acid, the active metabolite of vitamin A, plays a role in synaptic plasticity (2), as well as learning and memory in many different species (3–5). Interestingly, an interaction between intracellular retinoic acid and calcium has been shown to be important for hippocampal homeostatic plasticity (6). Moreover, we have recently shown in invertebrate neurons that retinoic acid inhibits both Ca\(_{1}\) and Ca\(_{2}\) VGCCs and alters their biophysical properties (7). In particular, retinoic acid reduced voltage-gated Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) density and shifted the voltage dependence of channel activation of both Ca\(_{1}\) and Ca\(_{2}\) channels (7). Some of these effects are also mimicked by retinoid receptor agonists, suggesting a role for retinoid acid receptors (RARs) and retinoid X receptors (RXRs) in the modulation of VGCCs (7). However, the precise signaling mechanisms by which retinoic acid exerts its effects on VGCCs are not yet known.

Classically, retinoic acid exerts its cellular effects by binding to the RAR and/or the RXR (8, 9) to alter the transcription of target genes. However, it has recently been established that retinoic acid can also modulate cell signaling and function in a nongenomic manner, either by activation of retinoid receptors (6) or possibly by direct binding to other molecules (10, 11). Nongenomic retinoid signaling has also been shown to involve the coupling of the RAR and the RXR to G protein subunits. Specifically, the G protein subunits G\(_{\alpha_\text{q}}\) and G\(_{\beta\gamma}\) can associate with the RAR in cancer cells and dopaminergic progenitor cells, respectively (12, 13). Similarly, RXR binds to G\(_{\alpha_\text{q}}\) in platelets and prevents its downstream signaling (14). G protein subunits are also well-known to bind to and modulate VGCCs (15). For example, G\(_{\beta\gamma}\) subunits can bind to Ca\(_{2}\) Ca\(^{2+}\) channels, producing a decrease in \(I_{\text{Ca}}\); a depolarizing shift in the voltage dependence of channel activation and a slowing of activation kinetics. This form of channel inhibition is known as voltage-dependent inhibition, because it is relieved by strong depolarization, which causes the G\(_{\beta\gamma}\) subunits to dissociate from the channel (15–18). It is possible that the modulation of \(I_{\text{Ca}}\) produced by retinoids might include an interaction with G protein subunits.

Recent evidence suggests that the RAR and the RXR may have constitutive activity (19, 20), and we hypothesize that such activity might include regulation of neuronal Ca\(^{2+}\) signaling through interactions with signaling molecules such as G proteins. Receptor antagonists have previously been used to determine the role of constitutive melancortin 4 receptor activity in the modulation of VGCCs (21). In the present study, we used RXR and RAR antagonists to determine whether constitutive...
retinoid receptor activity might affect neuronal calcium influx through VGCCs.

We provide compelling new evidence that RAR and RXR antagonists produce voltage-dependent inhibition of VGCCs through G protein–dependent and possible G protein–independent mechanisms, respectively. These findings strengthen the mounting evidence for an important link between retinoids and calcium signaling in the nervous system.

**Results**

We recently provided evidence that retinoic acid inhibits $I_{Ca}$ and modifies the biophysical and kinetic properties of VGCCs. Retinoic acid shifted the voltage dependence of channel activation to more positive membrane potentials, decreased the rate of both channel activation and recovery from inactivation, and increased the rate of channel inactivation and deactivation. Many of these inhibitory effects were mimicked by retinoid receptor agonists, strongly suggesting a role for the retinoid receptors (RAR and RXR) in mediating this channel inhibition (7). Because of evidence that retinoid receptors might be constitutively active, we aimed to determine whether inhibition of retinoid receptor activity using selective retinoid receptor antagonists would alter VGCC function.

**RXR pan-antagonists PA452 and HX531 exert different effects on $I_{Ca}$**

To characterize the role of the RXR in modulating Ca$^{2+}$ signaling, VF motor neurons were incubated overnight in the RXR pan-antagonists PA452 (1 μM) or HX531 (500 nM or 1 μM) or the highest equivalent concentration of DMSO (0.01%; vehicle control). The cells were voltage-clamped to record $I_{Ca}$, and an $I$–$V$ relationship was established using the voltage-step protocol shown in Fig. 1A. A two-way ANOVA revealed a significant interaction between treatment and voltage ($F_{(6,4,10,73)} = 10.161; p < 0.001$). Raw current recordings in Fig. 1B illustrate that PA452 enhanced $I_{Ca}$ compared with cells incubated in DMSO and post hoc analysis revealed that PA452 significantly enhanced $I_{Ca}$ at membrane potentials between −20 and −5 mV (Fig. 1C). Surprisingly, we discovered that HX531 had the opposite effect to PA452 and instead inhibited $I_{Ca}$ (Fig. 1B). Post hoc analysis revealed that HX531 (1 μM) significantly inhibited $I_{Ca}$ between −5 and +15 mV (Fig. 1C). This inhibition was accompanied by significant slowing of current activation, which was not previously seen in the inhibition produced by retinoic acid and RXR agonists (7).

HX531 is a widely used RXR pan-antagonist and is believed to have differential activity at the RXR compared with PA452 (22). To ensure selective actions of this RXR antagonist, our analysis included a lower concentration of HX531 (500 nM). Raw recordings (Fig. 1B) and post hoc analysis show that 500 nM HX531 also significantly inhibited $I_{Ca}$ between −5 and +25 mV, compared with 0.01% DMSO (Fig. 1C).

The rate at which VGCCs activate helps determine the time course and overall magnitude of Ca$^{2+}$ influx through Ca$^{2+}$ channels. As seen in the raw recordings (Fig. 1B), HX531 (1 μM) appeared to slow the rate of VGCC activation. A Kruskal–Wallis one-way ANOVA on ranks ($H = 30.532; p < 0.001$) and subsequent post hoc analysis of the time constants of channel activation in each condition revealed that, indeed, HX531 (1 μM) significantly increased the time constant of VGCC activation compared with DMSO (Fig. 1D). The time constant of

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**Figure 1. RXR pan-antagonists modulate $I_{Ca}$ in an antagonist-specific manner.** 

**A,** voltage-step protocol used to activate $I_{Ca}$ from cells treated with either 0.01% DMSO, 1 μM PA452, 1 μM HX531, or 500 nM HX531. The arrow indicates slowing of channel activation in cells treated with 1 μM HX531. C, 1 μM PA452 increased peak $I_{Ca}$ density at potentials between −20 and −5 mV compared with 0.01% DMSO-treated cells. In contrast, 1 μM HX531 inhibited $I_{Ca}$ at potentials between −5 and +25 mV. D, 1 μM HX531 significantly increased the time constant of channel activation at +10 mV, compared with DMSO. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with DMSO.
channel activation was not significantly different between cells treated with PA452 or HX531 (500 nM) and DMSO-treated controls (Fig. 1D).

**Differential effects of RXR antagonists on the voltage dependence of channel activation and inactivation**

We next sought to characterize the effects of these RXR antagonists (PA452 and HX531) on the biophysical properties of ICa. We first measured the voltage dependence of channel activation following overnight incubation of VF neurons in either PA452 (1 μM), HX531 (1 μM or 500 nM), or DMSO. This biophysical parameter determines the amount of depolarization required for channel activation and therefore Ca²⁺ influx. A one-way ANOVA (F(3,37) = 47.96; p < 0.001) revealed a significant effect of treatment on the voltage of half-maximal activation. 1 μM HX531 significantly depolarized the voltage of half-maximal activation (p < 0.001), although 500 nM HX531 had no significant effect (p = 0.724) (Fig. 2A, panels i and ii). Conversely, PA452 (1 μM) shifted the voltage dependence of channel activation to more negative potentials (as illustrated by the activation curve; Fig. 2A, panel i) and significantly hyperpolarized the voltage of half-maximal activation (p < 0.001; Fig. 2A, panel ii). These data suggest that PA452 increased the probability of channel opening at more hyperpolarized potentials, whereas HX531 (1 μM) increased the degree of depolarization required to activate VGCCs. The slope factor of channel activation provides information on the voltage sensitivity of a voltage-gated ion channel, and a one-way ANOVA revealed a significant effect of treatment on the slope factor for ICa (F(3,37) = 16.26; p < 0.001). PA452 had no effect on the slope factor, whereas HX531 (at 1 μM and 500 nM) significantly increased the slope factor compared with DMSO (p < 0.001; Fig. 2A, panels iii), suggesting that HX531 decreased the voltage sensitivity of VGCCs.

We next determined whether HX531 also affected the voltage dependence of steady-state channel inactivation, which determines the proportion of VGCCs that are available for activation at a given membrane potential and can thus determine the amount of Ca²⁺ influx through VGCCs upon membrane depolarization. The cells were held at −100 mV, and steady-state inactivation was achieved using 5-s voltage steps, ranging from −100 to +60 mV. ICa was then immediately elicited by a test pulse to +20 mV (Fig. 2B, panel i). The inactivation curve shown in Fig. 2B (panel ii) clearly illustrates that HX531 (1 μM) did not alter the voltage dependence of channel inactivation. Neither the voltage of half-maximal inactivation (t = −1.100; p = 0.286; unpaired t test) nor the slope factor of inactivation (t = −1.889; p = 0.075; unpaired t test) was significantly different between HX531- and DMSO-treated cells (Fig. 2B, panels iii and iv).

**An RAR pan-antagonist, LE540, also inhibits ICa**

Given that we have previously shown that RAR agonists are capable of inhibiting ICa in VF neurons (7), our next aim was to determine whether antagonism of the RAR could also influence ICa. VF motor neurons were treated overnight with the RAR pan-antagonist, LE540 (1 μM), or the equivalent concentration of DMSO (0.01%), and an I–V relationship was established. Raw recordings in Fig. 3A illustrate the inhibition of ICa produced by LE540. A two-way ANOVA revealed a significant interaction between treatment and voltage (F(28,580) = 3.7; p < 0.001); LE540 significantly inhibited ICa between −5 and +45 mV, compared with DMSO (Fig. 3B). However, unlike with 1 μM HX531, 1 μM LE540 did not significantly alter the time constant of channel activation (LE540, 3.95 ± 0.56 ms; DMSO, 5.90 ± 1.24 ms; t = −1.598; p = 0.126; unpaired t test).

We next determined whether the voltage dependence of channel activation was affected by LE540. As shown in the activation curve in Fig. 3C (panel i), LE540 appeared to shift the ICa conductance at more positive potentials (upper portion of activation curve in Fig. 3C, panel i). However, LE540 did not significantly affect the voltage of half-maximal activation (Fig. 3C, panel ii; t = 1.437; p = 0.166; unpaired t test) but did significantly increase the slope factor of activation (Fig. 3C, panel iii; Mann–Whitney U statistic = 102.00; p < 0.01). These data suggest that LE540 inhibits ICa but only moderately affects the voltage dependence of channel activation.

**HX531 and LE540 produce voltage-dependent inhibition of VGCCs**

The slowing of activation kinetics and the depolarizing shift in the voltage dependence of channel activation (with no effect on channel inactivation) produced by HX531 are both characteristic of the voltage-dependent inhibition of VGCCs in vertebrate neurons. Voltage-dependent inhibition can be relieved by a large depolarizing prepulse, known to cause the release of inhibitory Gβγ proteins from the channel (23–27). To determine whether HX531 produced voltage-dependent inhibition in VF neurons, the cells were treated overnight with either 1 μM or 500 nM HX531 or the equivalent concentration of DMSO (0.01%). The cells were then stepped from −100 mV to a test potential of +5 mV for 450 ms to elicit Icontrol. This was again repeated after 5 s to produce Ipost and to confirm that repeated test pulses alone did not facilitate ICa. After 5 s, the cells were then subjected to a large depolarizing prepulse to +150 mV, followed almost immediately by another test pulse to +5 mV for 450 ms to generate Ipost (Fig. 4A).

Raw recordings in Fig. 4B illustrate the inhibition of ICa produced by HX531 (blue traces), compared with DMSO controls (black traces). Analysis of current densities (Icontrol/Iprec and Ipost) for both 1 μM HX531 (Fig. 4B, panel i) and 500 nM HX531 (Fig. 4B, panel ii) with a two-way RM ANOVA revealed a significant interaction between treatment and current density (F(4,54) = 20.971; p < 0.001). Post hoc analysis revealed that Iprec was significantly reduced in both 1 μM (p < 0.01) and 500 nM HX531 (p < 0.05), compared with DMSO. However, strong depolarization of the membrane potential was found to facilitate ICa in HX531-treated cells but not in DMSO-treated cells.
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Ai

Voltage (mV)

g/g_{max}

DMSO (0.01%) (n = 10)
PA452 (1 μM) (n = 8)
HX531 (500 nM) (n = 11)
HX531 (1 μM) (n = 12)

Ai

Voltage of 1/2 Maximal Activation (mV)

DMSO PA452 HX531 HX531
(0.01%) (1 μM) (500 nM) (1 μM)

Ai

Slope Factor

DMSO PA452 HX531 HX531
(0.01%) (1 μM) (500 nM) (1 μM)

Bi

+60 mV
+20 mV

-100 mV

200 ms

5 s

Bii

Voltage (mV)

\[ \frac{I}{I_{\text{max}}} \]

DMSO (0.01%) (n = 9)
HX531 (1 μM) (n = 11)

Biii

Voltage of 1/2 Maximal Inactivation (mV)

DMSO HX531
(0.01%) (1 μM)

Biv

Slope Factor

DMSO HX531
(0.01%) (1 μM)
and analysis of $I_{\text{post}}$ revealed no significant difference in the current density in either 1 μM or 500 nM HX531, compared with DMSO. This suggests that HX531 caused voltage-dependent inhibition, which was completely relieved by the depolarizing prepulse. Indeed, analysis of the prepulse-induced facilitation of $I_{\text{Ca}}$ ($I_{\text{post}}/I_{\text{pre}}$; Fig. 4C) demonstrated significantly greater facilitation of $I_{\text{Ca}}$ in HX531-treated cells compared with DMSO (Kruskal–Wallis one-way ANOVA on ranks; $H = 18.938, p < 0.001$), further confirming that HX531 (at both 500 nM and 1 μM), produced voltage-dependent inhibition of VGCCs.
illustrate that following the depolarizing prepulse, significant facilitation of $I_{\text{Ca}}$ occurred in LE540-treated neurons. A Mann–Whitney rank sum test revealed an effect of treatment on facilitation (Mann–Whitney $U$ statistic $= 145.000; p = 0.001$) and that facilitation of $I_{\text{Ca}}$ occurred only in cells exposed to LE540 but not DMSO (Fig. 4D). However, an analysis of $I_{\text{post}}$ also revealed that the current density in LE540-treated neurons remained significantly lower than DMSO controls ($p < 0.001$), suggesting that LE540 produces inhibition that is only partially relieved by a depolarizing prepulse. These data suggest that the RAR pan-antagonist LE540 inhibited $I_{\text{Ca}}$ only partially through a voltage-dependent mechanism.

In vertebrates, voltage-dependent inhibition of VGCCs can occur rapidly via activation of G protein–coupled receptors (and subsequently G protein $\beta\gamma$ subunits). Our next aim was therefore to determine whether G proteins participate in the voltage-dependent inhibition produced by LE540 or HX531. However, to carry out these experiments, we first needed to confirm that acute addition of the antagonists to the cells would mediate similar effects as overnight treatment. Indeed, both acute perfusion of 1 $\mu$M HX531 or LE540 inhibited $I_{\text{Ca}}$ compared with DMSO (Fig. 5A, panel i), within 10 min of their application. A two-way ANOVA ($F_{(55, 1131)} = 4.183; p < 0.001$) revealed a significant interaction between treatment and voltage. LE540 significantly reduced $I_{\text{Ca}}$ at potentials ranging from $-15$ and $+10$ mV, and HX531 significantly reduced $I_{\text{Ca}}$ at potentials ranging from $-15$ to $+5$ mV, compared with DMSO (Fig. 5A, panel ii). We also found that acute application of LE540 and HX531 shifted the voltage dependence of channel activation to more depolarized potentials (Fig. 5B, panel i). Acutely applied LE540 and HX531 both significantly depolarized the voltage of half-maximal activation compared with DMSO (one-way ANOVA; $F_{(2,39)} = 14.975; p < 0.001$; Fig. 5B, panel ii) and significantly increased the slope factor (one-way ANOVA; $F_{(2,39)} = 14.447; p < 0.001$; Fig. 5B, panel iii). We also tested the effects of acute application of PA452 and, interestingly, discovered that this antagonist did not induce acute effects on $I_{\text{Ca}}$ (Fig. 5C). This highlights the fact that the RXR antagonists HX531 and PA452 likely modulate $I_{\text{Ca}}$ through different, time-dependent mechanisms.

**LE540, but not HX531, produces voltage-dependent inhibition through a G protein–dependent mechanism**

We next determined whether the G protein inhibitor GDP-$\beta$-S (16) could prevent the voltage-dependent inhibition mediated by the retinoid antagonists LE540 and HX531. Cells treated with GDP-$\beta$-S were given 30 min to allow it to diffuse from the recording electrode into the neurons. $I_{\text{pre}}$ and $I_{\text{post}}$ were then measured using the protocol outlined in Fig. 4A to first assess the effects of GDP-$\beta$-S alone. LE540 (1 $\mu$M) or HX531 (1 $\mu$M) was then acutely perfused over the cells until peak inhibition of $I_{\text{Ca}}$ was achieved, at which time $I_{\text{pre}}$ and $I_{\text{post}}$ were reassessed. Vehicle control experiments were first carried out to determine that DMSO alone (0.01%) had no effect on facilitation of $I_{\text{Ca}}$ (before, 0.99 ± 0.06; after, 1.03 ± 0.03; Friedman RM ANOVA on ranks: $\chi^2 = 2.667; p = 0.219$), and separate control experiments also ruled out any change in $I_{\text{Ca}}$ as a function of time alone (data not shown).

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Acutely applied HX531 and LE540 inhibit $I_{Ca}$ and depolarize the voltage dependence of channel activation. A, panel i, raw recordings of $I_{Ca}$ from cells acutely treated with either 0.01% DMSO, 1 μM HX531, or 1 μM LE540. Panel ii, acutely applied 1 μM HX531 significantly inhibited $I_{Ca}$ at potentials ranging from −15 to +5 mV, compared with DMSO. Similarly, acutely applied 1 μM LE540 significantly inhibited $I_{Ca}$ at potentials ranging from −15 to +10 mV, compared with DMSO, and at potentials ranging from −5 to +5 mV, compared with HX531. B, panel i, Boltzmann-fitted activation curve for cells treated with either 0.01% DMSO, 1 μM HX531, or 1 μM LE540. Panel ii, 1 μM HX531 and 1 μM LE540 significantly depolarized the voltage of half-maximal activation compared with DMSO. Panel iii, 1 μM HX531 significantly increased the slope factor of activation compared with DMSO, and 1 μM LE540 significantly increased the slope factor of activation compared with both DMSO and HX531. C, acute treatment of cells with 1 μM PA452 had no effect on $I_{Ca}$ compared with DMSO. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (compared with DMSO); #, $p < 0.05$; ##, $p < 0.01$ (compared with HX531).
A two-way RM ANOVA revealed a significant interaction between LE540 and GDP-β-S treatment on current density ($F_{(3,66)} = 4.848; p < 0.01$). In the absence of GDP-β-S treatment (Fig. 6A, panel i, upper traces), $I_{\text{pre}}$ was significantly reduced by LE540 ($p < 0.001$), with peak inhibition of $I_{\text{Ca}}$ occurring within 10 min. Following the depolarizing prepulse, $I_{\text{post}}$ was signifi-
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cantly increased compared with \( I_{\text{pre}} \) (\( p < 0.001 \)), but the inhibition was again only partially relieved by the prepulse (with overnight incubation in LE540). These data suggest that acute LE540 produces voltage-independent inhibition, in addition to voltage-dependent inhibition of \( I_{\text{ca}} \).

In the presence of GDP-\( \beta \)-S (Fig. 6A, panel i, lower traces), current densities were also significantly reduced following LE540 application (\( I_{\text{post}}/I_{\text{pre}} < 0.001 \); \( I_{\text{post}}/I_{\text{pre}} < 0.01 \)). The effects of LE540 on prepulse-induced facilitation of \( I_{\text{ca}} \) (\( I_{\text{post}}/I_{\text{pre}} \)) in the presence and absence of GDP-\( \beta \)-S (Fig. 6A, panel ii), analyzed using a two-way RM ANOVA, revealed a significant interaction between LE540 treatment and treatment with GDP-\( \beta \)-S (\( F_{(1,22)} = 10.211; p < 0.01 \)). However, post hoc analysis confirmed that significant prepulse-induced facilitation of \( I_{\text{ca}} \) occurred in cells treated with LE540 in the absence of GDP-\( \beta \)-S but did not occur in the presence of GDP-\( \beta \)-S (Fig. 6A, panel ii). That is, following the depolarizing prepulse, \( I_{\text{ca}} \) density in LE540-treated neurons did not significantly increase from \( I_{\text{pre}} \) to \( I_{\text{post}} \). These data strongly suggest that voltage-dependent inhibition of \( I_{\text{ca}} \) mediated by the RAR antagonist, LE540, was indeed G protein–dependent.

We next assessed whether the voltage-dependent inhibition produced by HX531 was also mediated by G proteins. In the same manner as above, HX531 (1 \( \mu \)m) was acutely perfused over the cells (either in the presence or absence of GDP-\( \beta \)-S) until peak inhibition of \( I_{\text{ca}} \) was achieved, at which time \( I_{\text{pre}} \) and \( I_{\text{post}} \) were reassessed. Indeed, in the absence of GDP-\( \beta \)-S, a two-way RM ANOVA (\( F_{(1,36)} = 44.003; p < 0.001 \)) and subsequent post hoc analysis revealed a significant effect of HX531 on \( I_{\text{pre}} \) (\( p < 0.001 \)) but not on \( I_{\text{post}} \) (\( p = 0.293 \)), indicating that the depolarizing prepulse relieved the inhibition produced by HX531. Raw recordings in Fig. 6B (panel i, lower traces) also show voltage-dependent inhibition of \( I_{\text{ca}} \) during acute application of HX531 in the presence of GDP-\( \beta \)-S. Analysis revealed that HX531 continued to significantly reduce \( I_{\text{pre}} \) (\( p < 0.001 \)), but not \( I_{\text{post}} \) (\( p = 0.084 \)) in the presence of GDP-\( \beta \)-S. A two-way RM ANOVA of the current facilitation (\( I_{\text{post}}/I_{\text{pre}} \)) produced in the presence and absence of GDP-\( \beta \)-S revealed a significant effect of HX531 treatment only (\( F_{(1,122)} = 19.177; p < 0.001 \)). Prepulse-induced facilitation of \( I_{\text{ca}} \) seen following HX531 treatment was not blocked by GDP-\( \beta \)-S (Fig. 6B, panel ii).

The data obtained above using GDP-\( \beta \)-S suggested that the voltage-dependent inhibition mediated by HX531 may be independent of G proteins. However, G protein \( \beta \gamma \) subunits can bind to receptors independently of the \( \alpha \) subunit (28), and \( \beta \gamma \) signaling can also be activated independently of Go nucleotide exchange (29). Thus, to further test whether \( \beta \gamma \) subunits might mediate the voltage-dependent inhibition, we next used 1 mM \( \beta \)ARKct, which can sequester G protein \( \beta \gamma \) subunits and inhibit G protein \( \beta \gamma \) signaling at this concentration (30). However, HX531, even in the presence of \( \beta \)ARKct, again induced voltage-dependent inhibition of \( I_{\text{ca}} \) (Fig. 6C, panel i). A two-way RM ANOVA of current facilitation (\( I_{\text{post}}/I_{\text{pre}} \)) indicated a significant effect of HX531 treatment only (\( F_{(1,209)} = 4.813; p < 0.05 \)) and indicated that the prepulse-induced facilitation of \( I_{\text{ca}} \) was not blocked by \( \beta \)ARKct (Fig. 6C, panel ii). Together, these data suggest that the voltage-dependent inhibition produced by HX531 might indeed be mediated through a pathway independent of G proteins, because it was not blocked either by the G protein inhibitor GDP-\( \beta \)-S or by the \( \beta \gamma \) sequestering peptide \( \beta \)ARKct.

HX531 inhibits \( \text{Ca}_{2+} \) channels but enhances \( \text{Ca}_{1} \text{Ca}^{2+} \) currents.

Classical G protein–mediated voltage-dependent inhibition of VGCCs specifically occurs at \( \text{Ca}_{2+} \) channels (15). To determine whether the voltage-dependent inhibition produced by HX531 that appears to be independent of G proteins is also mediated through \( \text{Ca}_{2+} \) VGCCs, we isolated \( \text{Ca}_{2+} \) currents using the dihydropyridine nifedipine (10 \( \mu \)M), which selectively blocks only \( \text{Ca}_{1} \) channels (31). VF neurons were perfused with either 1 \( \mu \)M HX531 or 0.01% DMSO until peak inhibition occurred, and a control \( I_{\text{V}} \) relationship was established. These data indicated the total inhibition of \( I_{\text{ca}} \) produced by HX531. The presence of voltage-dependent inhibition was also established using the depolarizing prepulse-induced current facilitation protocol described previously. The cells were then subsequently perfused with 10 \( \mu \)M nifedipine to inhibit \( \text{Ca}_{1} \text{Ca}^{2+} \) channels. An \( I_{\text{V}} \) relationship was again produced to determine the current carried by \( \text{Ca}_{2+} \) channels (nifedipine-insensitive). Finally, the ability of the depolarizing prepulse protocol to induce current facilitation (mediated by \( \text{Ca}_{2+} \) channels) was again determined.

In the presence of nifedipine, acute application of HX531 inhibited \( I_{\text{ca}} \) compared with DMSO, as shown by the raw recordings in Fig. 7A (panel i). A two-way ANOVA of the effects of HX531 on nifedipine-insensitive currents (\( \text{Ca}_{2+} \)) indicated a significant interaction between treatment and voltage (\( F_{(28,493)} = 9.996; p < 0.001 \)). HX531 treatment significantly inhibited nifedipine-insensitive (\( \text{Ca}_{2+} \)) currents between −5 and +45 mV, compared with DMSO (Fig. 7A, panel iii). HX531 also depolarized the voltage dependence of nifedipine-insensitive (\( \text{Ca}_{2+} \)) channel activation (Fig. 7B, panel i), significantly depolarized the voltage of half-maximal activation (\( t = −4.690; p < 0.001 \)) and subsequently perfused with 10 \( \mu \)M HX531 or 0.01% DMSO until peak inhibition occurred (data not shown). Raw recordings in Fig. 7B (panel ii) show that HX531 also produced voltage-dependent inhibition of nifedipine-insensitive (\( \text{Ca}_{2+} \)) currents, because the depolarizing prepulse again induced current facilitation. A Mann–Whitney rank sum test (Mann–Whitney \( U \) statistic = 80.000; \( p < 0.001 \)) revealed that facilitation of nifedipine-insensitive \( \text{Ca}_{2+} \) currents occurred in HX531-treated cells but not in the DMSO-treated control cells (Fig. 7B, panel iii).

An analysis of possible effects of HX531 on currents carried by \( \text{Ca}_{1} \text{Ca}^{2+} \) channels was obtained by subtracting nifedipine-insensitive (\( \text{Ca}_{2+} \)) currents from the total \( I_{\text{ca}} \). The \( I_{\text{V}} \) relationship of nifedipine-sensitive (\( \text{Ca}_{1} \)) currents was analyzed with a two-way ANOVA and showed a significant interaction between HX531 treatment and voltage (\( F_{(28,493)} = 1.732; p < 0.05 \)). However, HX531 significantly inhibited nifedipine-sensitive (\( \text{Ca}_{1} \)) currents only at −10 mV and instead was found to enhance \( \text{Ca}_{1} \) currents between 0 and +20 mV (Fig. 7C, panel i). HX531 had no effect on the voltage dependence of \( \text{Ca}_{1} \) channel activation (voltage of half-maximal activation: DMSO, −6.898 ± 2.082 mV; HX531, −1.883 ± 2.447 mV; \( t = −1.543; p = 0.133 \)).
Nifedipine-Insensitive (Ca\textsubscript{v}2)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{HX531 produces voltage-dependent inhibition of Ca\textsubscript{v}2 voltage-gated Ca\textsuperscript{2+} channels. \textit{A}, panel \textit{i}, raw recordings of nifedipine-insensitive (Ca\textsubscript{v}2) \textit{I}_{\text{Ca}} at peak current density in cells acutely treated with either 1 \mu M HX531 or 0.01\% DMSO. Panel \textit{ii}, 1 \mu M HX531 significantly inhibited nifedipine-insensitive (Ca\textsubscript{v}2) \textit{I}_{\text{Ca}} density at potentials between −5 and +45 mV, compared with 0.01\% DMSO. \textit{B}, panel \textit{i}, Boltzmann-fitted activation curve of nifedipine-insensitive (Ca\textsubscript{v}2) \textit{I}_{\text{Ca}}, which was absent in cells treated with 0.01\% DMSO. Panel \textit{ii}, Raw recordings of nifedipine-insensitive (Ca\textsubscript{v}2) \textit{I}_{\text{Ca}} before (\textit{I}_{\text{pre}}) and after (\textit{I}_{\text{post}}) a depolarizing prepulse in cells treated with either 1 \mu M HX531 or 0.01\% DMSO. Panel \textit{iii}, 1 \mu M HX531 caused prepulse-induced facilitation of nifedipine-insensitive (Ca\textsubscript{v}2) \textit{I}_{\text{Ca}}, which was absent in cells treated with 0.01\% DMSO. \textit{C}, panel \textit{i}, 1 \mu M HX531 significantly inhibited nifedipine-sensitive (Ca\textsubscript{v}1) \textit{I}_{\text{Ca}} density at −10 mV only but enhanced nifedipine-sensitive (Ca\textsubscript{v}1) \textit{I}_{\text{Ca}} density at potentials between 0 and +20 mV, compared with 0.01\% DMSO. Panel \textit{ii}, 1 \mu M HX531 treatment did not cause prepulse-induced facilitation of nifedipine-sensitive (Ca\textsubscript{v}1) \textit{I}_{\text{Ca}}, compared with 0.01\% DMSO. *, \textit{p} < 0.05; **, \textit{p} < 0.01; ***, \textit{p} < 0.001 compared with DMSO.}
\end{figure}

\begin{align*}
p &= 0.141; \text{ and slope factor: DMSO, } 0.3204 \pm 0.085; \text{ HX531, } 1.118 \pm 0.731; \text{ Mann–Whitney } U \text{ statistic } = 47.000; \text{ } p = 0.903; \text{ data not shown). The absence of depolarizing prepulse-induced current facilitation also indicated that HX531 did not produce voltage-dependent inhibition of the nifedipine-sensitive Ca\textsubscript{v}1 channels (Fig. 7C, panel ii; Mann–Whitney } U \text{ statistic } = 54.000; \text{ } p = 0.230). \text{ Taken together, these data suggest that the G protein–}
\end{align*}
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**Figure 8. PA452 enhances \( I_{\text{Ca}} \) mediated by \( \text{Ca}_{\text{aq}} \) voltage-gated \( \text{Ca}^{2+} \) channels.** A, overnight treatment of cells with 1 \( \mu \text{M} \) PA452 significantly enhanced \( I_{\text{Ca}} \) at \(-15 \text{ mV}\) and potentials ranging from 0 to +10 mV compared with 0.01% DMSO. B, overnight treatment of cells with 1 \( \mu \text{M} \) PA452 also significantly enhanced nifedipine-insensitive \( \text{Ca}_{\text{aq}} \) \( I_{\text{Ca}} \) at \(-15 \text{ mV}\). *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

The effects of overnight application of PA452 on \( I_{\text{Ca}} \). Fig. 8A illustrates the \( I-V \) relationship following overnight application of PA452, again showing the enhanced \( I_{\text{Ca}} \) as indicated by the two-way ANOVA, which revealed a significant effect of treatment \( (F(1,435) = 15.778; p < 0.001) \). Following application of nifedipine, the enhancement of \( I_{\text{Ca}} \) (\( \text{Ca}_{\text{aq}} \) only) persisted \( (F(1,435) = 8.362; p < 0.01; \text{Fig. 8B}) \), and PA452 enhanced \( I_{\text{Ca}} \) of \( \text{Ca}_{\text{aq}} \) at \(-15 \text{ mV}\). However, following subtraction of the \( I-V \) relationship in nifedipine from the total \( I-V \), PA452 was not found to induce any significant enhancement of \( I_{\text{Ca}} \) through \( \text{Ca}_{\text{aq}} \) \( (F(1,435) = 3.596; p = 0.059; \text{data not shown}) \). Taken together, these data indicate that the RXR antagonist HX531 exerts rapid voltage-dependent inhibition of \( I_{\text{Ca}} \) mediated by \( \text{Ca}_{\text{aq}} \), whereas the RXR antagonist PA452 (which fails to exert any rapid effects; Fig. 5) induces an enhancement of \( I_{\text{Ca}} \) mediated mainly by \( \text{Ca}_{\text{aq}} \), following overnight incubation.

The retinoid antagonists exert independent effects on \( I_{\text{Ca}} \)

Given the finding above that the RXR antagonists exert apparently opposing effects on \( I_{\text{Ca}} \) mediated by \( \text{Ca}_{\text{aq}} \), we next determined whether overnight exposure to PA452 (which enhances \( I_{\text{Ca}} \)) could occlude the rapid voltage-dependent inhibitory effects of HX531. The cells were incubated overnight in either 1 \( \mu \text{M} \) PA452 or 0.01% DMSO, and \( I-V \) relationships were obtained. HX531 (1 \( \mu \text{M} \)) was then acutely perfused over cells in each condition until peak inhibition (less than 10 min) was achieved, and a second \( I-V \) relationship was established. A two-way ANOVA \( (F(84,928) = 3.546; p < 0.001) \) revealed a significant interaction between treatment and voltage. As expected, overnight treatment of cells with PA452 significantly enhanced \( I_{\text{Ca}} \) (at potentials ranging from \(-15 \text{ and } +5 \text{ mV}\) (Fig. 9A, panel i)), compared with DMSO (Fig. 9A, panel ii). In both PA452 (Fig. 9A, panel i) and DMSO-treated cells (Fig. 9A, panel ii), HX531 application significantly reduced \( I_{\text{Ca}} \). We next determined the proportion of inhibition produced by HX531 at membrane potentials that elicited peak \( I_{\text{Ca}} \) (obtained prior to HX531 perfusion). A Mann–Whitney rank sum test revealed no significant difference in the inhibition (at peak current) produced by HX531 in cells incubated overnight in PA452, compared with those incubated in DMSO (Fig. 9A, panel iii). Following HX531 perfusion, there was significant depolarization of the voltage dependence of channel activation in both PA452-treated (Fig. 9B, panel i) and DMSO-treated (Fig. 9B, panel ii) cells, but the change in the voltage of half-maximal activation produced by HX531 was not significantly different between groups \((t \text{ test}; t = -0.120; p = 0.906; \text{Fig. 9B, panel iii}) \). Overall, these data suggest that overnight incubation in PA452 was unable to occlude the inhibitory effects of acutely applied HX531.

We next performed co-applications of various antagonists overnight to gain further insights into their possible mechanistic interactions. We co-applied the RXR antagonist HX531 (1 \( \mu \text{M} \)) with either the RXR antagonist PA452 (1 \( \mu \text{M} \)) or with the RAR antagonist LE540 (1 \( \mu \text{M} \)). We then compared the \( I-V \) relationship in each condition, with that obtained with HX531 (1 \( \mu \text{M} \)) alone (or 0.02% DMSO as a control).

The \( I-V \) relationships indicated that \( I_{\text{Ca}} \) was significantly inhibited in all three antagonist treatment conditions, compared with DMSO alone (Fig. 10A). A two-way ANOVA revealed a significant interaction between treatment and voltage \( (F(84,1363) = 8.611; p < 0.001) \). The inhibition produced by co-application of PA452 with HX531 was not significantly different from the inhibitory effects of HX531 alone, further suggesting that PA452 was unable to occlude the effects of HX531 on \( I_{\text{Ca}} \). However, co-application of the RAR antagonist LE540 with HX531 exerted significantly greater inhibitory effects on \( I_{\text{Ca}} \) than HX531 alone, at potentials ranging from \(+15 \text{ to } +60 \text{ mV}\), indicating an additive effect of these two antagonists. This result might be expected considering HX531 likely exerts its effects via the RXR, whereas LE540 exerts its effects via the RAR.

The \( \text{Ca}_{\text{aq}} \) channel inhibitor nifedipine was next used to determine whether the combined inhibitory effects of the antagonists were mainly exerted through \( \text{Ca}_{\text{aq}} \) channels. The \( I-V \) relationship in the presence of nifedipine \((I_{\text{Ca}} \text{ through } \text{Ca}_{\text{aq}}) \) is shown in Fig. 10B. A two-way ANOVA \( (F(84,9860) = 7.664; p < 0.001) \) again revealed a significant interaction between treatment and voltage. In the presence of nifedipine, there was little difference between HX531 and co-application of PA452 and HX531 (Fig. 10B). In contrast, co-application of HX531 and LE540 inhibited \( \text{Ca}_{\text{aq}} \) currents at potentials ranging from \(-15 \text{ to } +60 \text{ mV}\).
from +20 to +60 mV, compared with HX531 alone, suggesting that LE540 also inhibits Ca_2 channels. There was little difference between the inhibition of I_{Ca} either before (Fig. 10A) or after (Fig. 10B) application of nifedipine, indicating that the combined inhibitory effects of the antagonists appear to be largely mediated through Ca_2 (as previously shown with HX531 alone).

In summary, we have demonstrated very different effects of various retinoid receptor antagonists on I_{Ca} in Lymnaea neurons. The effects differed depending on whether the antagonists were applied overnight or were acutely applied, suggesting differences in their mechanisms of action. Importantly, we have provided evidence that both an RXR and RAR antagonist were capable of mediating voltage-dependent inhibition of I_{Ca}, an effect that was, until recently, thought to be restricted to vertebrate neurons. These data further highlight the growing body of evidence linking retinoid signaling with neuronal calcium signaling.

**Discussion**

In this study, we have demonstrated significant effects of both RXR and RAR antagonists on I_{Ca}. Many of these effects were mediated rapidly and largely affected Ca_2 channels, suggesting that these retinoid receptors may mediate fast, non-genomic effects on channels responsible for neurotransmitter release in neurons (in addition to any potential slower transcriptional effects).

An unexpected, but interesting observation from this study was that two RXR antagonists induced opposing effects, with PA452 enhancing I_{Ca} but HX531 inhibiting I_{Ca}. PA452...
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Enhanced $I_{Ca}$ only with overnight incubation and failed to exert any acute effects. This suggests that PA452 might enhance $I_{Ca}$ via a transcriptional or translational mechanism, which may include an increased expression of VGCCs. Indeed, if this was the case, it may explain why PA452 could not occlude the proportional inhibition of $I_{Ca}$ mediated by subsequent addition of HX531. Interestingly, however, HX531 overnight was able to occlude any enhancing effects mediated by PA452. Previous studies in human leukemia cells (22), as well as *Lymnaea* neurons (32), have shown that both 1 μM HX531 and PA452 can inhibit the effects of RXR agonists, supporting their roles as RXR antagonists. However, it should be noted that the antagonistic effects may also extend to actions at heterodimers of RXRs with RAR (or other receptors), at least in vertebrates (22, 33), possibly explaining the differing effects. However, co-application of HX531 with the RAR antagonist LE540 had an additive effect on the inhibition of $I_{Ca}$ in LE540 as might be expected if these antagonists were acting via different receptors. Interestingly, both antagonists appeared to exert their inhibitory effects largely via Ca$_{2,2}$, possibly supporting a role for both RXRs and RARs in the control of VGCCs involved in synaptic transmission.

Taken together, these data indicate that RXR signaling interactions with VGCCs are potentially complex in nature and may involve both rapid voltage-dependent inhibition, as well as an ability to enhance $I_{Ca}$ over longer time periods, possibly via transcriptional/translational mechanisms (and/or potentially as heterodimers with other receptors). Notably, the differing effects of the RXR appeared to largely involve Ca$_{2,2}$ channels (as opposed to Ca$_{2,1}$ channels), which are important for mediating neurotransmitter release during synaptic transmission.

**Modulation of biophysical properties of Ca$^{2+}$ channels are consistent with voltage-dependent inhibition**

In vertebrate neurons, the binding of G protein $\beta$y subunits to Ca$_{2,2}$ VGCCs transitions the channel from the willing (to open) state to a reluctant (to open) state, thus inhibiting the channel and reducing $I_{Ca}$ (23–26). This interaction of $\beta$y subunits with Ca$_{2,2}$ also changes the biophysical properties of the channel, which include slowing of activation kinetics and a depolarizing shift in the voltage dependence of channel activation (16–18, 34). It is thought that these effects result from $\beta$y subunits dissociating from the channel as it opens (while in the reluctant state), temporarily relieving channel inhibition (25). Large depolarizing prepulses (as well as high frequency firing) can rapidly remove $\beta$y subunits from VGCCs and greatly facilitate $I_{Ca}$, which is characteristic of voltage-dependent inhibition.

In this study, HX531 and LE540 reduced $I_{Ca}$, and their inhibitory effects were also relieved by the depolarizing prepulse, resulting in facilitation of $I_{Ca}$. HX531 also produced the typical slowing of activation kinetics and depolarizing shift in channel activation, whereas the voltage dependence of steady-state inactivation was unaffected. These effects are consistent with properties of voltage-dependent inhibition seen in vertebrate neurons (24). Although voltage-dependent inhibition of $I_{Ca}$ occurred with both overnight and acute exposure to LE540, the depolarizing shift in the voltage dependence of channel activation was far more pronounced following acute application. One possible explanation for this is that overnight exposure to LE540 may also have caused down-regulation of Ca$_{2,2}$ channels via a transcriptional or translational mechanism. This might have masked some aspects of voltage-dependent inhibition, perhaps because of Ca$_{2,2}$ channels contributing a smaller proportion of $I_{Ca}$. Whether or not this proves to be the case will, however, require further investigation.

Our data suggest that the retinoid antagonists induce voltage-dependent inhibition of VGCCs, likely through the binding of inhibitory signaling proteins to the channel, which subsequently then dissociate during channel opening. However, it should be noted that the inhibition produced by each antagonist may be biophysically distinct, because of the slowing of activation kinetics in the presence of HX531 but not in the presence of LE540. This effect may relate to differences in the ability of the VGCC to undergo “reluctant openings” in the presence of each antagonist, which might result from involvement of different signaling proteins. The prepulse-induced facilitation of $I_{Ca}$ (characteristic of voltage-dependent inhibition) in LE540 was blocked by GDP-$\beta$S, suggesting that the
voltage-dependent inhibition mediated by LE540 was G protein subunit–dependent (as seen in vertebrate neurons). However, the rapid voltage-dependent inhibition produced by HX531 persisted in the presence of both the G protein blocker GDP-β-S, as well as the βγ sequestering peptide BARKct, suggesting that a novel G protein–independent mechanism might be involved. Interestingly, this possible G protein–independent voltage-dependent inhibition induced by HX531 was also specific to Ca_{2,2} channels. We might speculate that the differences in the biophysical characteristics of the voltage-dependent inhibition, depending on whether it was G protein–mediated (RAR) or not (RXR), may serve to fine-tune the modulation of Ca_{2,2} channels at synapses or perhaps to selectively modulate these channels under different conditions. Indeed, this possible involvement of multiple signaling pathways in mediating voltage-dependent inhibition in invertebrate neurons might highlight the potential importance of this process in regulating synaptic communication and plasticity.

**Invertebrate voltage-gated Ca^{2+} channels are capable of undergoing voltage-dependent inhibition**

Until recently, G protein–mediated voltage-dependent inhibition of Ca_{2,2} channels had been demonstrated only in vertebrate neurons, and only voltage-independent mechanisms had been described in invertebrates. For example, *Lymnaea* Ca_{2,2} channels expressed in HEK cells failed to undergo voltage-dependent inhibition in the presence of purified rat βγ subunits (35), yet *Lymnaea* Ca^{2+} channels can undergo G protein–mediated voltage-independent inhibition (35, 36). Likewise, dopamine, acting at G protein–coupled receptors, produced only voltage-independent inhibition of Ca^{2+} channels in *Aplysia* neurons (37), despite its ability to produce voltage-dependent inhibition of I_{Ca} in vertebrates. More recently, however, evidence has emerged that dopamine mediates inhibition at a *Lymnaea* synapse, at least partially via voltage–dependent inhibition of I_{Ca} (27). Our findings here that invertebrate VGCCs are indeed capable of undergoing G protein–mediated voltage-dependent inhibition provide further compelling evidence that this type of neuronal inhibition is conserved between vertebrates and at least some invertebrates.

**Physiological consequences of voltage-dependent inhibition of I_{Ca}**

Activation of G protein–coupled receptors by neurotransmitters or neuromodulators can produce voltage-dependent inhibition of Ca_{2,2} channels (15, 17, 23, 25), likely via direct binding of the G protein βγ subunit. Because Ca_{2,2} channels play a prominent role in neurotransmitter release during synaptic transmission, voltage-dependent inhibition of these channels results in changes in transmitter release and contributes to synaptic plasticity (34). Indeed, voltage-dependent inhibition allows for dynamic modulation of information processing at synapses, which has new implications for synaptic plasticity in invertebrate nervous systems.

Regulators of G proteins (such as RGS2) have been shown to enhance synaptic output by down-regulating basal G protein–mediated voltage-dependent Ca^{2+} channel inhibition (38). In contrast, activators of G protein signaling (such as AGS1 and Rhes) increase tonic G protein inhibition (39). It is possible that similar mechanisms also exist in invertebrate neurons, further enhancing the complexity of this interaction. Indeed, our data suggest that the RAR might act to inhibit tonic G protein inhibition in invertebrate neurons, possibly by sequestering G proteins and/or AGS proteins.

Retinoid receptors are known to mediate synaptic plasticity, because disruption of RAR and RXR signaling impairs hippocampal LTP and LTD (2, 4). To date, however, the roles of G proteins and retinoid receptors in synaptic plasticity have only been described independently of one another. Indeed, the association of retinoid receptors (RAR and RXR) with G protein subunits (Gq or Gβ) has only previously been described in vertebrate cells such as platelets, cancer cells, and progenitor cells (12–14). We now provide compelling evidence for a link between G proteins and retinoid receptors in adult neurons.

The fact that we have previously shown that retinoids and retinoid receptor agonists can also exert inhibitory effects on I_{Ca} (7) might suggest that VGCCs require an optimal level of retinoid receptor activation for normal functioning, above or below which I_{Ca} becomes inhibited. However, our findings here might also suggest that the RXR and/or RAR are constitutively active. Indeed, there is some evidence for this in their role as nuclear transcription factors (19, 20), where unliganded retinoid receptors are bound to genes that are still undergoing transcription. Traditionally, it was thought that these bound retinoid receptors were acting as repressors of gene transcription, but this view has now been deemed overly simplistic, and more complicated roles of bound retinoid receptors (including silent binding partners) have now been proposed (20).

It is currently unclear whether the RAR/RXR involved in the effects seen here are acting transcriptionally in the nucleus or post-translationally in the cytoplasm and/or membrane. However, the fact that both HX531 and LE540 induced rapid voltage-dependent inhibition of I_{Ca} strongly suggests constitutive binding of the receptors to signaling proteins, either in the cytoplasm or in the membrane. We propose that once these protein–protein interactions are disrupted by the antagonists, the retinoid receptor–interacting proteins are then free to mediate voltage-dependent inhibition of the Ca^{2+} channels.

Evidence for constitutive activity of the retinoid receptors in a nongenomic manner is limited. In cancer cells, RARγ in the cytoplasm binds to the regulatory unit of PI3K (p85α) in the absence of RA, which the authors proposed could be responsible for constitutive activation of the downstream target Akt, known to occur in these cells (40). It is also well-known that some membrane receptors, such as the melanocortin 4 receptor, can display both agonist-evoked and constitutive activity. Constitutive activity of the melanocortin 4 receptor involves inhibition of various Ca^{2+} channel subtypes via G protein–dependent signaling (21).

Although we have not yet identified the class of G protein that might interact with the RAR in *Lymnaea* neurons, it is interesting that the *Lymnaea* G_{q,11} (like the human G_{q}) contains a conserved motif (LXXLL) known to be involved in co-activator binding to retinoid receptors. Vertebrate RAR is also known to localize with G_{q} in lipid rafts in cancer cells (12), suggesting its ability to form microdomain protein complexes.
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with G proteins. Functional cross-talk between RXR and Gproteins has also been demonstrated in human platelets that lack nuclei (14). Interestingly, platelet RXR was found in both cytoplasmic and membrane domains, where it was capable of binding to G proteins, even in the unliganded state (14). LymRXR and LymRAR are also known to localize to both cytoplasmic and membrane domains (32, 41) and can mediate nongenomic effects of retinoids (42) including in Lymnaea VF neurons (43). The next challenge will be to determine which proteins might bind to these retinoid receptors. It will be necessary to determine not only which G proteins (and/or AGS proteins) may bind to the RAR but also which proteins might interact with RXR, to subsequently mediate the possible G protein–independent inhibition of ICa. Furthermore, future down-regulation of each receptor (using siRNA) will hopefully provide more definitive information on possible distinct effects of RAR and RXR on VGCCs.

In summary, we have shown here that various retinoid antagonists exert differential effects on VGCCs and that these effects may also differ over time. Importantly, RAR and RXR antagonists produce voltage-dependent inhibition of Ca2+ channels through G protein–dependent and possible G protein–independent pathways, respectively. These findings link retinoid receptors to G protein signaling in neurons for the first time and provide evidence that multiple signaling pathways might produce voltage-dependent inhibition of ICa in invertebrate neurons. It further adds to the growing body of evidence that retinoid signaling interacts with neuronal calcium signaling (7, 44), possibly to mediate synaptic plasticity (6, 45).

Experimental procedures

Animals

The mollusk Lymnaea stagnalis (pond snail) used in these experiments were bred at Brock University. The animals were raised in a laboratory environment at room temperature and maintained on a fixed 12:12 h light–dark cycle in aerated, dechlorinated water. They were fed a combination of romaine lettuce and Nutrafin Max Spirulina fish food (Hagen) daily. All animals used for cell culture ranged from 18 to 22 mm in shell length.

Cell culture

All dissections were performed under sterile conditions as previously described (46, 47). Briefly, Lymnaea were anesthetized by exposure to saline containing 25% Listerine (containing menthol, 0.042%, w/v), pinned in a dissection dish containing antibiotic saline (normal saline containing 225 μg/ml gentamicin (Sigma–Aldrich)), and dissected to expose the CNS, consisting of the central ring ganglia. The CNS was removed and given three 10-min washes in antibiotic saline. The CNS was then treated with trypsin (Sigma–Aldrich; 2 mg/ml) in defined medium (DM; 50% Leibovitz’s L-15 medium; Gibco) for 18–19 min at 21 °C and was subsequently treated with trypsin inhibitor (Sigma–Aldrich; 2 mg/ml in DM) for 10 min. The CNS was then pinned out in high osmolarity DM, and the outer sheath of connective tissue was removed, followed by removal of the inner sheath to expose the neurons. Individual visceral F (VF) motor neurons were removed from the ganglia using a fire-polished glass pipette coated with Sigmacoat (Sigma–Aldrich) to prevent cell adhesion. Gentle suction was applied via a microsyringe (Gilmont) to remove individual neuronal cell bodies. VF neurons were then plated on poly-L-lysine (Sigma–Aldrich)–coated plastic culture dishes containing DM. The cells were subsequently treated with either the RXR pan-antagonist HX531 (Tocris Bioscience), RAR pan-antagonist PA452 (Tocris Bioscience), or the RAR pan-antagonist LE540 (Tocris Bioscience). Each compound was originally prepared as a 10−2 M stock solution in 100% DMSO and diluted in DM to produce a final concentration of either 500 nM or 1 μM. Control experiments involved treating cells with 0.01% DMSO.

Electrophysiology

The biophysical properties of ICa were assessed using whole-cell voltage clamp electrophysiology. Recordings were performed on cultured VF neurons using a MultiClamp 700A amplifier, a Digidata 1322A digitizer, and Clampex 9.2 software (Axon Instruments, Sunnyvale, CA). ICa was assessed using an external solution containing 10 mM BaCl2, 45.7 mM tetraethylammonium chloride, 1 mM MgCl2, 10 mM HEPES, and 2 mM 4-aminopyridine, with a pH of 7.9 achieved using TEA-Oh. Ba2+ was used as the charge carrier through VGCCs to minimize Ca2+-dependent inactivation (48). Patch electrodes with a resistance between 4 and 8 MΩ were filled with internal solution containing 29 mM CsCl, 2.3 mM CaCl2, 2 mM MgATP, 0.1 mM GTP-Tris, 11 mM EGTA, and 10 mM HEPES, with a pH of 7.4 achieved using HCl (49). The recordings were performed on cells with an access resistance of <15 MΩ, and the series resistance was compensated by 85%. The liquid junction potential was estimated to be +10 mV, which was not adjusted for. Recordings were performed at room temperature.

Current–voltage relationship

To determine the current-voltage (I–V) relationship for ICa, the cells were held at −100 mV and stepped to membrane potentials ranging from −80 to +60 mV in 5-mV increments for 400 ms. The cells were subsequently stepped down to −100 mV for 1 s to remove any inactivation before the next depolarizing pulse was applied (7, 46). Peak ICa was measured for each voltage step using Clampfit 9.2 and normalized to cell capacitance. The I–V relationships were compared across treatment conditions using a two-way ANOVA, followed by a Bonferroni post hoc test.

Activation kinetics

To investigate the kinetics of voltage-gated Ca2+ channel activation, the rising phase of individual traces of ICa were fit in ClampFit 9.2 using the following exponential cumulative probability equation,

\[ f(V) = \sum_{i=1}^{n} p_i (1 - e^{-V/\tau_i}) + C \]  

(Eq. 1)

where \( \tau_i \) represents the time constant. Activation time constants were compared across treatment conditions using either an unpaired t test or a Kruskal–Wallis one-way ANOVA on ranks, as appropriate.
Voltage dependence of channel activation

To determine the voltage dependence of VGCC activation, channel conductance was calculated for each membrane potential used in the $I-V$ relationship protocol described above using the following equation,

$$g = \frac{1}{(V_m - E_{rev})} \quad \text{(Eq. 2)}$$

where $I$ represents current, $V_m$ represents membrane potential, and $E_{rev}$ represents the reversal potential. $E_{rev}$ was calculated by linear extrapolation of the $I-V$ curve at potentials more depolarized than that at which the peak current occurred. Conductance values for each voltage were normalized to the maximal conductance, $g_{\text{max}}$, for each neuron. For each individual neuron, $g/g_{\text{max}}$ values were fit to the following Boltzmann equation,

$$f(V) = \frac{g_{\text{max}}}{1 + e^{(V_m - V)/\nu}} + C \quad \text{(Eq. 3)}$$

where $V_{\text{mid}}$ represents the voltage of half-maximal activation, $V$ represents the membrane potential, $V_m$ represents the slope factor, and $C$ represents a constant. The values for the voltage of half-maximal activation were compared using either an unpaired $t$ test or a one-way ANOVA, as appropriate. Values for the slope were compared using either a one-way ANOVA, an unpaired $t$ test, or a Mann–Whitney rank sum test, as appropriate.

Voltage dependence of channel inactivation

To determine the voltage dependence of VGCC inactivation, the cells were held at $-100$ mV and stepped to $+20$ mV for $200$ ms to generate the control $I_{\text{Ca}}$ ($I_{\text{control}}$). $I_{\text{control}}$ was measured with each sweep of the protocol to account for any rundown that may have occurred over the course of the recording. Voltage-dependent inactivation was achieved by holding cells at $-100$ mV and stepping to membrane potentials between $-100$ and $+60$ mV for $5$ s in $10$-mV increments before immediately applying a test pulse to $+20$ mV for $200$ ms ($I_{\text{test}}$) (50). Because of the pronounced slowing of activation kinetics produced by HX531, the control and test pulses were extended to $400$ ms for HX531. To generate a steady-state inactivation curve, $I_{\text{test}}/I_{\text{control}}$ values for each prepulse potential were then fit in ClampFit using the Boltzmann equation for each individual neuron, as described above. The values for the voltage of half-maximal inactivation were compared using either an unpaired $t$ test, or a Mann–Whitney rank sum test, as appropriate. The slope values were compared using an unpaired $t$ test.

Voltage-dependent inhibition of $I_{\text{Ca}}$

Voltage-dependent inhibition of $I_{\text{Ca}}$ can be relieved through strong depolarization of the membrane potential. To determine whether the inhibition of $I_{\text{Ca}}$ produced by HX531 and LE540 was voltage-dependent, the cells were first stepped from a holding potential of $-100$ mV to $+5$ mV for $450$ ms to generate $I_{\text{control}}$. The cells were then subsequently stepped down to $-100$ mV for $5$ s to remove any inactivation, followed by a second pulse to $+5$ mV for $450$ ms to generate $I_{\text{pre}}$. After being held at $-100$ mV for $5$ s, the cells were given a strong depolarizing prepulse to $+150$ mV for $50$ ms. The cells were then stepped down to $-100$ mV for $25$ ms to remove any inactivation and were subsequently stepped to $+5$ mV for $450$ ms to generate $I_{\text{post}}$. The facilitation produced by the depolarizing prepulse was determined by dividing $I_{\text{post}}$ by $I_{\text{pre}}$. The degree of facilitation produced was compared using either a Kruskal–Wallis one-way ANOVA on ranks, or a Mann–Whitney rank sum test, as appropriate. To ensure that repeated pulses alone did not produce facilitation, as well as to examine differences in $I_{\text{pre}}$ and $I_{\text{post}}$, the current densities at $I_{\text{control}}$, $I_{\text{pre}}$, and $I_{\text{post}}$ were compared using a two-way RM ANOVA.

Voltage-dependent inhibition produced by acute application of HX531 or LE540

To determine whether acute application of HX531 or LE540 produced voltage-dependent inhibition of $I_{\text{Ca}}$, $I_{\text{pre}}$ and $I_{\text{post}}$ were first measured as described above in untreated cells immediately following cell breakthrough. The cells were then perfused with either HX531 or LE540 until peak inhibition was achieved, as determined by $200$-ms pulses to $+20$ mV from a holding potential of $-100$ mV, once every $30$ s. Once peak inhibition was achieved, $I_{\text{pre}}$ and $I_{\text{post}}$ were then reassessed. In control experiments, $0.01\%$ DMSO was acutely applied for a similar duration to that used for LE540 and HX531, and $I_{\text{pre}}$ and $I_{\text{post}}$ were then measured.

To determine whether voltage-dependent inhibition was mediated through a G protein–dependent mechanism, $10$ mM GDP-β-S, was added to the internal solution in the pipette in place of GTP-Tris and allowed to diffuse into the cell for $30$ min following breakthrough. $I_{\text{pre}}$ and $I_{\text{post}}$ were measured, and the cells were then acutely perfused with HX531 or LE540 for the duration of time that was shown to produce peak inhibition. $I_{\text{pre}}$ and $I_{\text{post}}$ were subsequently reassessed in the presence of HX531 or LE540. $I_{\text{post}}/I_{\text{pre}}$ was calculated for each treatment group and compared using a two-way RM ANOVA. A separate series of experiments were also carried out using the same protocol as above, except $1$ mM βARKct, a βγ G protein subunit-sequestering peptide (Tocris) was added to the pipette in place of GDP-β-S.

Voltage-gated Ca$^{2+}$ channel subtype characterization with the use of nifedipine

The selective Ca$^{2+}$ channel blocker nifedipine (Sigma–Aldrich) was used to determine the effects of RXR and RAR antagonists on either Ca$\text{_{1,1}}$ or Ca$\text{_{2,2}}$ VGCCs. A stock solution of nifedipine was prepared at a concentration of $10^{-2}$ M using $100\%$ DMSO and then diluted to $10$ μM in external recording solution. To test the effects of HX531, the cells were acutely perfused with either $1$ μM HX531 or $0.01\%$ DMSO until peak current inhibition occurred. The $I-V$ relationship and the level of prepulse-induced facilitation were then established, as described above. Nifedipine (10 μM in combination with HX531 or DMSO) was then acutely perfused onto the VF motor neurons until peak inhibition occurred. The $I-V$ relationship and the level of prepulse-induced facilitation was again established, now in the presence of nifedipine. The current remaining in the presence of nifedipine was taken to represent $I_{\text{Ca}}$ carried by Ca$\text{_{2,2}}$ VGCCs. Nifedipine-sensitive (Ca$\text{_{1,1}}$ channel)
currents were measured by subtracting the nifedipine-insensitive current from that obtained before nifedipine perfusion. Subtraction was performed offline using Clampfit 9.2. The voltage dependence of channel activation, the voltage of half-maximal activation, and the slope of activation were then determined and analyzed as described above. Similar protocols were used to test the effects of PA452 on Ca\textsubscript{1.2} or Ca\textsubscript{2.3} VCCs, although for this antagonist, the cells were incubated overnight because it did not induce any acute effects. Similarly, to determine the combined effects of HX531 with either PA452 or LE540 on nifedipine-insensitive Ca\textsubscript{2.2} channels, the cells were also incubated overnight in the antagonists.

**Statistical analysis**

All statistical analyses were performed using SigmaStat 3.2, and the graphs were generated using Graph Pad Prism 5.03. The values are presented as the means ± S.E., and differences were deemed significant when \( p < 0.05 \).

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