Direct gating by retinoic acid of retinal electrical synapses

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Retinoic acid (RA), a signaling molecule derived from vitamin A, controls growth and differentiation of a variety of cell types through regulation of gene transcription. In the vertebrate retina, RA also regulates gap junction-mediated physiological coupling of retinal neurons through a nontranscriptional mechanism. Here we report that RA rapidly and specifically modulates synaptic transmission at electrical synapses of cultured retinal horizontal cells through an external $RAR_{\beta/\gamma}$-like binding site, the action of which is independent of second messenger cascades. External application of all-trans retinoic acid (at-RA) reversibly reduced the amplitude of gap junctional conductance in a dose-dependent manner, but failed to affect non-gap-junctional channels, including glutamate receptors. In contrast, internal dialysis with at-RA was ineffective, indicating an external site of action. Selective $RAR_{\beta/\gamma}$ ligands, but not an RAR_{α}-selective agonist, mimicked the action of at-RA, suggesting that gating of gap junctional channels is mediated through an $RAR_{\beta/\gamma}$-like binding site. At-RA did not act on gap junctional conductance by lowering $[pH]_i$ or by increasing $[Ca^{2+}]_i$. A G protein inhibitor and protein kinase inhibitors did not block at-RA uncoupling effects indicating no second messenger systems were involved. Direct action of at-RA on gap junction channels was further supported by its equivalent action on whole-cell hemi-gap-junctional currents and on cell-free excised patch hemichannel currents. At-RA significantly reduced single-channel open probability but did not change unitary conductance. Overall, the results indicate that RA modulates horizontal cell electrical synapses by activation of novel nonnuclear $RAR_{\beta/\gamma}$-like sites either directly on, or intimately associated with, gap junction channels.

Retinoic acid (RA), a retinoid metabolite, is widely distributed in liver, lung, kidney, retina, and brain. It serves as a gene regulator via ligand-activated transcription factors, known as retinoic acid receptors (RAR) and retinoid X receptors (RXR), and plays an important role in regulating the growth and differentiation of a wide variety of cell types. In the vertebrate eye, the RA signaling pathway is involved in early eye and photoreceptor development, through the activation of gene transcription that is mediated by nuclear receptors (1–5). In the mature retina, however, RA biosynthesis has been shown to be regulated by light (6), and RA has also recently been reported to affect the structure and physiological function of retinal horizontal cells through a nontranscriptional mechanism (7, 8).

Electrically coupled networks formed by gap junctions are found between all retinal cell types and are of fundamental importance in transmitting and shaping visual signals. In the outer retina, horizontal cells are second-order interneurons whose electrical synapses are responsible for the lateral spread of inhibitory signals (9). The structure and function of horizontal cell electrical synapses are altered by light adaptation (10, 11), which is mediated by two light-released neuromodulators: dopamine and nitric oxide (10, 12–17). RA may be a third light-neuroactive substance that is involved in modulating gap junctional permeability. Dye or tracer coupling between horizontal cells is inhibited by RA in vertebrate retinas, suggesting that RA is indeed an uncoupling agent (8, 18). However, the underlying mechanisms of RA action on electrical synapses are unclear.

Here we sought to determine the effects of RA on gap junctional channels and the mechanisms of its action in retinal horizontal cells. Although previous reports have shown that RA can modulate dye coupling by regulating connexin expression at the transcription level in other tissues (19–23), our results indicate that RA potently and directly gates electrical coupling in retinal neurons.

Materials and Methods

Cell Culture. Dark-adapted adult striped hybrid bass (*Roccus chrysops* × *Roccus saxitalis*) were killed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. The cell dissociation protocol has been described in detail in ref. 15. Briefly, retinas were enzymatically and mechanically dissociated. Individual cells and cell pairs were studied 2 to 4 days after dissociation.

Solutions and Chemicals. Except for hemi-gap-junctional channel recordings, the composition of pipette solution we used in the experiments contained (in mM) 120 K-gluconate, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 Hepes, pH to 7.5 with KOH. ATP (1 Mg) and GTP (0.1 Na) were added in some experiments indicated in the text. The bath solution contained (in mM) 137 NaCl, 2.5 KCl, 2.25 $MgCl_2$, 2.25 $MgSO_4$, 2 $CaCl_2$, 1 Na-pyruvate, 10 Hepes, 16 glucose, 1 mg/ml BSA (Sigma, Fraction VII), pH to 7.5 with NaOH. For hemi-gap-junctional recordings, K-gluconate in the above pipette solution was totally replaced by CsCl and 10 mM tetraethylammonium chloride (TEA) was added to block potassium channels. $CaCl_2$ in the above bath solution was replaced by 2 mM $MgCl_2$ and 20 mM TEA and 10 mM 4-AP were added in the Ca^{2+}-free bath solution. All-trans retinoic acid (at-RA), all-trans retinaldehyde, all-trans retinol, 9-cis retinoic acid (9-cis RA), GDPγS, H-7, TPA, W-13, and Lucifer yellow were purchased from Sigma. Protein kinase A (PKA) inhibitor 6-22 amide and phosphoglycerate kinase (PKG) inhibitor (peptide) were purchased from Calbiochem. CD336 (Am580), CD666, and CD2314 were synthesized at Galderma R&D (Sophia-Antipolis, France). All retinoids were dissolved as a 100 mM stock in DMSO and then diluted into a working concentration. The maximal final DMSO concentration was less than 0.03%. GDPγS, H-7, W-13, PKA inhibitor 6-22 amide, PKG inhibitor, and Lucifer yellow were directly dissolved in the pipette solution.

Drugs were continuously delivered into the bath from a glass tube near the recorded cells (*ca.* 100–200 μm). The speed of solution superfusion was approximately 1–1.2 ml/min and was increased to 3–4 ml/min in the hemichannel recordings.

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Abbreviations: RA, retinoic acid; at-RA, all-trans retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; NPo, channel open probability.

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Patch-Clamp Recording. Recordings from solitary neurons were performed by using the conventional whole-cell patch clamp configuration, whereas electrical coupling between pairs of horizontal cells was recorded by using the dual whole-cell patch clamp technique. For dual whole cell recordings, a patch pipette was attached to each cell of a pair. Each cell was then voltage-clamped with an independent amplifier. The patch pipettes were pulled from Corning 7052 glass (A-M Systems, Carlsborg, WA) and fire-polished with resistances of 3–4 MΩ. The pipette series resistance and capacitance were compensated by 80%.

Patch currents were recorded from outside-out patches by using a recording pipette of 0.5–1 MΩ and the pipette resistances were 12–20 MΩ in single-channel recording. Single-channel current was filtered at 1 kHz (−3 dB Bessel filter) and digitally sampled at 3–5 kHz. Currents were recorded by using Axopatch 1-D amplifier (Axon Instruments, Foster City, CA) in voltage clamp mode. Voltage commands, data acquisition, and analysis were performed by using PCLAMP 8 software (Axon Instruments).

Data Analysis. The normalized dose–inhibition relationship data were fit by the Hill equation: $I_{retinoid}/I_{control} = 1 - \{A^n/[(IC_{50})^n + A^n]\}$, where $I_{control}$ and $I_{retinoid}$ are currents before and during application of retinoid, respectively. IC_{50} is the concentration giving a half-maximal reduction. A represents a given retinoid concentration and n is the Hill coefficient.

Single-channel currents were analyzed by using PCLAMP 8, FETCHAN, and PSTAT software (Axon Instruments). Single-channel amplitudes were binned and displayed as a histogram fit by the sum of one or more Gaussians. The single-channel open probability was determined from the ratio of the time spent in the open state to the duration of recording T: $NPo = (t_1 + t_2 + \cdots t_n)/T$, where t is the amount of time that N channels are open and N is the number of channels observed in the patch.

Statistics on the data are presented as mean ± SEM. *P* values stated were calculated by using the *t* test or paired *t* test.

Results

To test the hypothesis that RA may affect gap junctional electrical coupling, we specifically examined the effect of RA on macroscopic junctional currents recorded in cultured homologous H1- or H2-type cell pairs by using the dual whole-cell recording configuration. Perfusion with at-RA strikingly decreased the amplitude of macroscopic junctional currents.

Fig. 1*A* illustrates an example of junctional currents (I_j) in an H2-type horizontal cell pair before and during application of at-RA. Generally, both cells of a pair were voltage-clamped to a holding potential of 0 mV, and then alternating steps of 20 mV (V_j) were applied to each cell (Fig. 1*A*d). This procedure minimized the contribution of extrajunctional membrane conductances and voltage-dependent closure of the gap junctions. Before exposure to at-RA, I_j had an amplitude of 355 pA at a V_j of 20 mV (Fig. 1*A*a). After application of 3 μM at-RA for 5 min, the amplitude of I_j had decreased to 125 pA (Fig. 1*A*b). The amplitude of I_j completely recovered to control level on washout (Fig. 1*A*c). Fig. 1*B* shows the time course of an at-RA inhibition on the macroscopic junctional conductance in the same cell pair. The maximal inhibition of junctional conductance was achieved within 5 min after at-RA application. Full recovery of the amplitude of coupling current took 8 min after washout. Similar results were obtained in five other pairs. On average, the amplitude of I_j was reduced by 61% from 238 ± 52 pA to 92 ± 21 pA in the presence of 3 μM at-RA with an onset of 3–5 min ($n = 6$). In 37/37 cell pairs, junctional currents were reduced by at-RA concentrations ranging from 0.3–30 μM. Fig. 1*C* shows a dose–inhibition plot of normalized junctional current vs. at-RA concentrations on a logarithmic scale. The data were fit by a binding equation which yielded a half-maximal inhibitory concentration (IC_{50}) of 2.4 μM and Hill coefficient of 1.6.

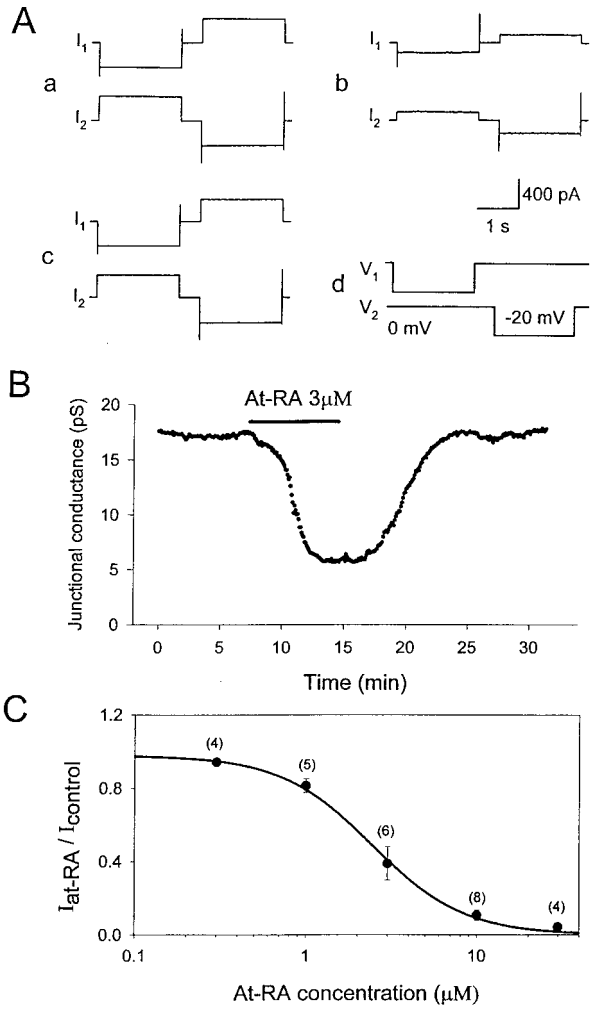

Fig. 1. Effect of retinoic acid on gap junctional conductance in cultured bass horizontal cell pairs. (*A*) Traces of junctional currents before (a), during (b), and after (c) washout of at-RA. Trace d shows stimulation waveform. The amplitude of junctional current was reversibly reduced by 3 μM at-RA. (*B*) A time course of at-RA uncoupling from the same cell pair. (*C*) Normalized dose–response relationship for RA uncoupling effect on junctional currents. Each point shows the mean (± SE) of data from 4–8 cell pairs indicated above point. Smooth curve represents the best fit of the data with the Hill equation.

To determine whether at-RA also reduced non-gap-junctional conductances in retinal neurons, we explored RA's effect on non-junctional currents in solitary cells. Fig. 2*A* shows the current–voltage relationships of non-gap-junctional currents from a solitary H-2 type horizontal cell. However, neither the amplitudes of the peak current nor of the steady-state membrane current was changed in the presence of 10 μM at-RA. Similar results were obtained from five other cells. In addition, we tested the effect of RA on glutamate receptor-mediated currents of horizontal cells. Fig. 2*B* shows that incubation with 10 μM at-RA for 3 min did not change the amplitude of kainate-induced currents. Similar results were obtained from nine other cells. These results suggest that at-RA does not modulate non-junctional channels in bass horizontal cells.

To demonstrate that RA itself, not the products of its chemical degradation (5,6-epoxyretinoic acid and glucuronide), was responsible for the uncoupling effect, we exposed the solution (in which at-RA (10 μM) was dissolved) to a strong light for 5 h (60-W fiber-optic light source). Bleached at-RA had no signif-

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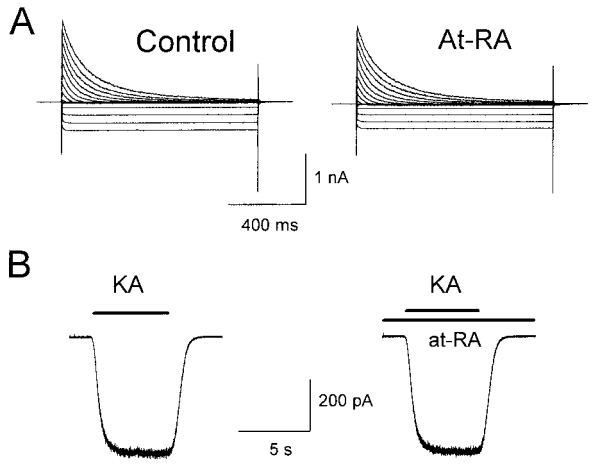

Fig. 2. RA had no effect on non-gap-junctional currents. (*A*) Current–voltage relationships of currents from an individual cell before (*Left*) and during (*Right*) at-RA. The amplitude of either peak or steady-state currents did not change at 10 μM at-RA for 6 min. Voltage-clamp steps were applied in 10 mV increments between −120 mV and 60 mV from a holding potential of −60 mV (duration: 1 second). (*B*) A 50 μM kainate-induced current before (*Left*, 450 pA) and during 10 μM at-RA (*Right*, 443 pA) from an H2-type horizontal cell.

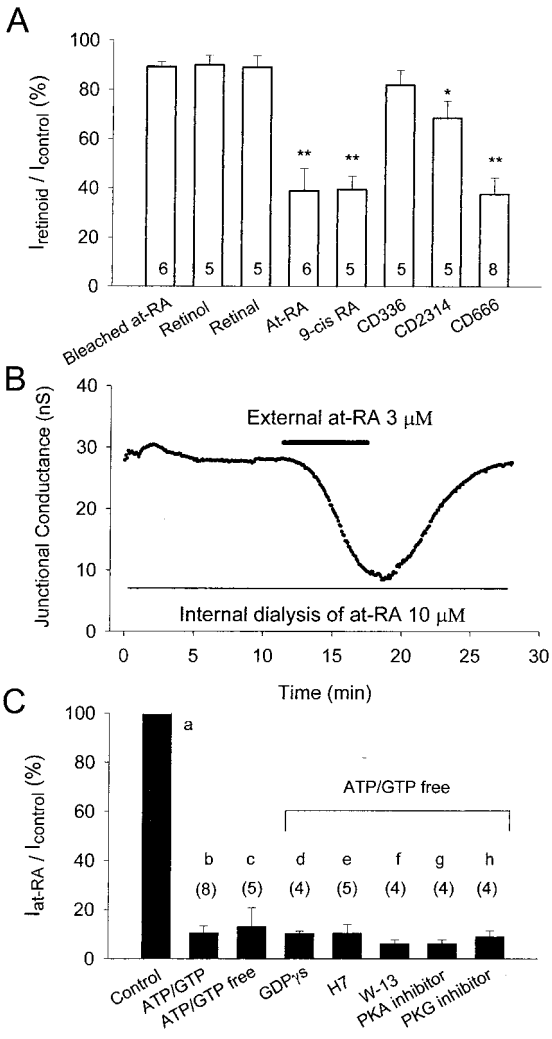

Fig. 3. Mechanisms of at-RA uncoupling effect in horizontal cell pairs. (*A*) Mean reduction of normalized junctional current by a series of retinoids; the name for each is indicated below the bars. Each bar shows the mean (± SE) percentage of control from 5–8 cell pairs indicated inside of the bars. Except for 10 μM bleached at-RA, the concentration of other retinoids is 3 μM. * represents $P < 0.05$ and ** represents $P < 0.01$ compared with the mean value of normalized junctional current by bleached at-RA. (*B*) Time course of junctional conductance from an H2-type horizontal cell pair. Internal dialysis of 10 μM at-RA (pipette solution) did not uncouple gap junctions. In this condition, external application of at-RA reduced gap junctional conductance. (*C*) The action of at-RA is independent of G proteins and phosphorylation. Bars (from bar a to bar h) show amplitude of junctional current before (bar a) and during (from bar b to bar h) 10 μM at-RA under various conditions in changes of pipette solution indicated below each bar as a percentage of control. Error bars show SEM. Compared with bar b, ATP/GTP (bar c) was not necessary for the action of at-RA ($P > 0.05$). Compared with bar c, GDPγS, PKA inhibitor, PKG inhibitor, and W-13 did not block the at-RA uncoupling effect ($P > 0.05$).

icant effect on the amplitude of junctional current (a mean reduction of 11 ± 2%, Fig. 3*A*, $n = 6$). Although RA is a weak acid, both extracellular and pipette solution pH were well-buffered by 10 mM Hepes in the presence of at-RA. To further assure that no pH change was involved in RA's action, we increased the Hepes buffer in the pipette solution from 10 mM to 40 mM. This high concentration of Hepes buffer did not block the at-RA uncoupling effect (data not shown, $n = 8$).

The specific effect on gap junctional currents by at-RA suggested that the lipophilic action of RA that disrupts the lipid bilayer of the plasma membrane may not be involved in the modulation of gap junctions. To further test this idea, we compared the uncoupling effects of retinol and retinaldehyde, the precursors of at-RA, which have the similar lipid solubilities, but different side chain structures than at-RA. We found that these retinoids were much less potent than at-RA in their effects on coupling at 3 μM concentration (a mean decrease of 10 ± 4% for retinol and 11 ± 4% for retinaldehyde; Fig. 3*A*).

We next studied the pharmacological profile of retinoid effects on gap junctional conductance. At-RA and 9-cis RA are equipotent in activating the RAR, but at-RA is 50-fold less potent than 9-cis RA in activating the RXR (24). In Fig. 3*A*, at-RA ($n = 6$) and 9-cis RA ($n = 5$) at 3 μM had similar uncoupling effects (61 ± 9% by at-RA vs. 60 ± 5% by 9-cis RA, $P > 0.05$), suggesting that an RAR-like site, but not an RXR-like site, mediates RA's uncoupling. To further characterize the RAR-like mediator of at-RA uncoupling, we tested selective synthetic retinoid agonists. CD666, an RAR_γ-selective agonist (25, 26), had effects similar to at-RA and 9-cis RA with a mean reduction of 62 ± 6% at 3 μM (Fig. 3*A*, $n = 5, P < 0.01$ compared with the bleached at-RA control). However, CD336 (Fig. 3*A*, $n = 5$), an RAR_α-selective compound (25, 26), had no significant effect on coupling at the same concentration (mean reduction of 18 ± 6%, $P > 0.05$). In addition, CD2314, an RAR_β-selective ligand (25, 26), had a significant, but weaker effect than CD666 with a 31 ± 6% reduction (Fig. 3*A*, $n = 5, P < 0.05$). These results suggest that $RAR_{\beta/\gamma}$-like sites gate gap junction channels.

To determine the location of at-RA's binding site, we compared the effect of internal dialysis of cell pairs with at-RA to external application of at-RA on gap junctional conductance. Fig. 3*B* illustrates junctional currents recorded from an H2-type horizontal cell pair by using a pipette solution containing 10 μM at-RA for internal dialysis. The junctional current was immediately recorded after the whole-cell recording was made. For 12 min of internal dialysis with at-RA, the junctional current remained unchanged. However, when the bath perfusion solution for this cell pair was switched from the normal solution to a 3 μM at-RA solution the junctional current was significantly inhibited. Similar results were obtained in six other cell pairs. Under the same recording conditions, horizontal cells were fully dialyzed with 0.1% Lucifer yellow in 29 ± 3 s ($n = 5$), indicating that this procedure gave at-RA full and quick access to the cell

interior. The results suggest that at-RA acts on an extracellular site to modulate gap junction channels.

Because the patch pipette solution contained a strong Ca^{2+} buffer (11 mM EGTA), it seemed unlikely that RA modulated gap junctional conductance by increasing intracellular Ca^{2+}. In a complementary experiment, we examined the effect of at-RA on intracellular Ca^{2+} by using fura-2 Ca^{2+} imaging studies on solitary horizontal cells. The basal calcium level in horizontal cells was unchanged after application of 10 μM at-RA (data not shown).

Because horizontal cell gap junctions are subject to modulation by kinase-based second messenger systems (16, 27, 28), we tested whether they were involved in the action of RA. To examine whether G proteins were involved in at-RA gating of gap junctional channels, we compared RA action in the presence and absence of ATP/GTP in the pipette solution and found it to be equivalent (Fig. 3*C*, bar b vs. bar c, $P > 0.05$). In addition, GDPγS, a G protein inhibitor, did not block the at-RA uncoupling effect (Fig. 3*C*, bar d, $P > 0.05$ compared with bar c, $n = 4$). We also tested for involvement of kinases. H-7 (30 μM), a nonspecific protein kinase inhibitor, did not block the at-RA uncoupling effect (Fig. 3*C*, bar e, $P > 0.05$ compared with bar c, $n = 5$). Furthermore, the results showed that the at-RA uncoupling was unaffected by the protein kinase A inhibitor 6-22 Amide (Fig. 3*C*, bar g, 100 μM, $P > 0.05$ compared with bar c, $n = 4$), or by protein kinase G inhibitor (Fig. 3*C*, bar h, 150 μM, $P > 0.05$ compared with bar c, $n = 4$). W-13 (150 μM), a specific calmodulin-dependent kinase inhibitor, also failed to block at-RA uncoupling (Fig. 3*C*, bar f, $P > 0.05$ compared with bar c, $n = 4$). In addition, phorbol 12-tetradecanoil 13-acetate (TPA), a protein kinase C (PKC) activator, had no effect on gap junctional currents (185 $\pm$ 24 pA in control vs. 162 $\pm$ 19 pA in the presence of 100 nM TPA, $P > 0.05$, paired *t* test, $n = 5$).

Overall, these experiments suggest the possibility that RA could act directly on gap junctional channels. To further elucidate this mechanism, we sought to study the interaction of RA with hemi-gap-junction channels of horizontal cells. Hemi-gap-junction channels expressed on the plasma membrane of horizontal cells open with lowered extracellular Ca^{2+} and depolarization of the membrane to positive potentials (29, 30). Using the gap junctional uncouplers dopamine (3 μM), a nitric oxide donor (30 μM sodium nitroprusside), heptanol (0.3 mM), and pH (6.5), we confirmed that currents induced by lowered extracellular Ca^{2+} in bass horizontal cells were also mediated by hemi-gap-junctional channels (data not shown). Fig. 4*A* shows the whole-cell hemichannel current induced by Ca^{2+}-free solution at the holding potential of 30 mV in an H2-type horizontal cell. The amplitude of the current was reversibly reduced by external application of 1 μM at-RA. On average, the amplitude of whole-cell hemichannel currents was decreased 67% by 1 μM at-RA ($n = 8$). We also examined effects of other retinoids (retinaldehyde, retinol, CD336, CD2314, and CD666) on hemi-gap-junctional currents. The pharmacological properties of these retinoids from their dose–inhibition relationships are shown in Table 1.

We next tested the effects of at-RA on hemichannel currents in cell-free outside-out patches that were excised from solitary horizontal cells. As shown in Fig. 4*B*, macroscopic patch currents, exhibiting multiple channel openings, were obtained after the extracellular solution around the patch was switched from normal solution to Ca^{2+}-free solution. The amplitude of the current was greatly reduced in the presence of 1 μM at-RA with a 30-s time course. In addition, 1 mM GDPγS in the pipette solution did not block at-RA effects, again indicating a lack of G protein involvement (data not shown). In six excised patches, the amplitude of hemichannel patch currents was reduced 74% on average by 1 μM at-RA. There was no significant difference in the degree of modulation in whole cell vs. cell-free patches

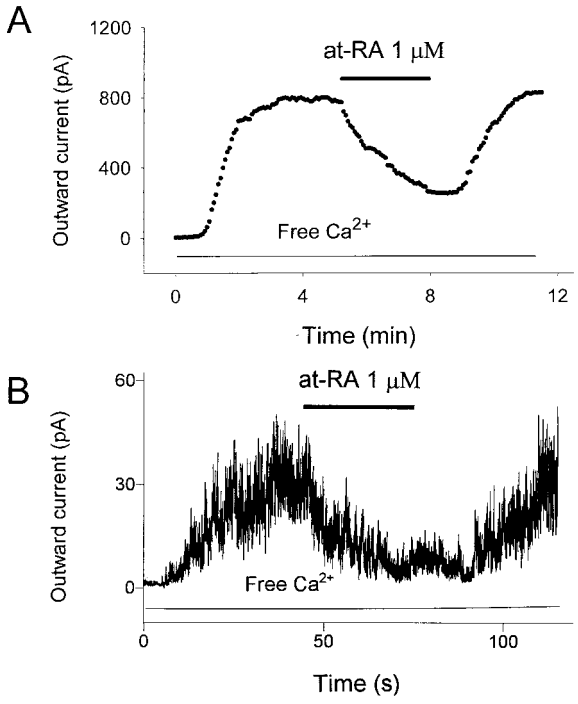

Fig. 4. Effect of at-RA on hemi-gap-junctional channels. (*A*) Hemi-gap-junctional current was induced by Ca^{2+}-free solution at a membrane potential of 30 mV in an H2-type horizontal cell. The current was reduced from 797 pA to 257 pA by 1 μM at-RA. (*B*) Macroscopic patch-current induced by Ca^{2+}-free solution in an outside-out membrane patch excised from an H2-type horizontal cell at the pipette voltage of 30 mV. The amplitude of this current was reduced by 1 μM at-RA. The current was filtered at 1 kHz and sampled at 3 kHz.

($P > 0.05$), indicating that the action of at-RA is independent of cytoplasmic factors.

In 15 excised outside-out patch-recordings in which single hemichannels could be resolved, at-RA significantly decreased the hemi-gap-junctional channel activity measured as channel open probability (NPo). Fig. 5*A* shows traces of single-channel activity recorded from an outside-out patch before and during application of at-RA. Three active channels were apparent in this patch and they opened individually or simultaneously (5*A Upper*). After application of 1 μM at-RA for 30 s, the single-channel activity was clearly reduced (5*A Lower*). The corresponding histogram of NPo vs. recording time shows that NPo was reversibly reduced by 1 μM at-RA (Fig. 5*B*). The amplitude histogram of this recording in the absence of at-RA was fit by Gaussian distributions with three peaks of 41 pS (65%), 87 pS (31%), and 129 pS (4%), as shown in Fig. 5*C Left*. During treatment with at-RA, the conductance-amplitude histogram

Table 1. Pharmacological properties of retinoids on hemichannels of horizontal cells

Compound	IC_{50}, μM	Hill coefficient	I_{max}, %
At-RA	0.4	0.6	95
Retinaldehyde	25.2	1.1	89
Retinol	30.4	1.6	86
CD336	25.8	2.2	26
CD2314	5.7	2.9	95
CD666	1.8	1.3	92

Curves were fit by the Hill equation. IC_{50}, half-maximal inhibitory concentration. I_{max}, maximum inhibition.

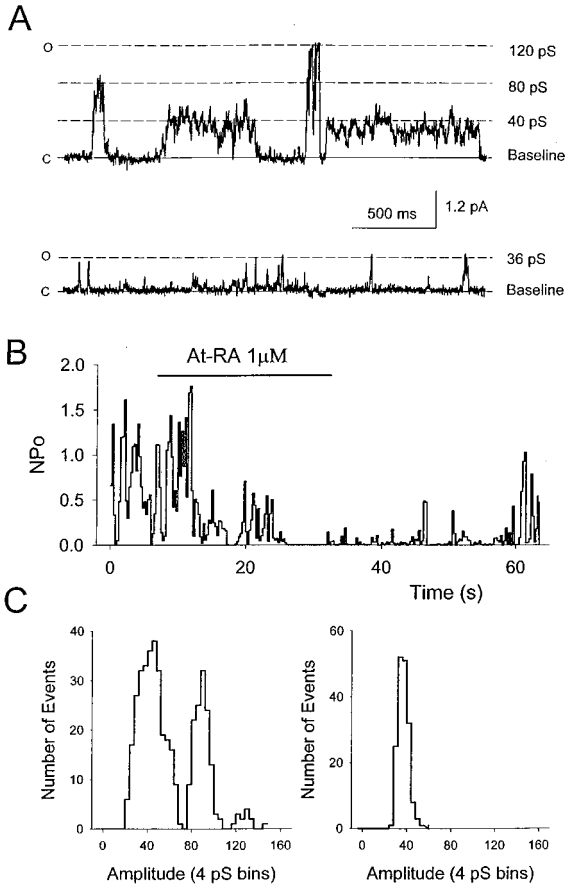

Fig. 5. At-RA reduced single-channel activity but did not alter channel unitary conductance in an outside-out patch. (*A*) The trace in the absence of at-RA (*Upper*) and the trace in the presence of 1 μM at-RA (*Lower*). Channel openings are upward deflections and three channels are present in this patch at the pipette potential of 30 mV. Single-channel current was filtered at 1 kHz and sampled at 5 kHz. o, open; c, closed. (*B*) The histogram of changes in channel activity measured as NPo plotted against time for the activity shown in *A*. Bin width is 0.25 s. (*C*) The corresponding single-channel current amplitude histograms (*Left*) are in the upper trace of *A* with a recording interval of 10 s. (*Right*) Histogram showing the corresponding channel current amplitude at the same patch in the presence of at-RA (recording interval of 30 s).

was fit by a Gaussian with a single-conductance amplitude of 36 pS (Fig. 5*C Right*).

On average in these single-channel experiments, NPo was decreased by 72% from 0.53 $\pm$ 0.11 in the absence of at-RA to 0.15 $\pm$ 0.05 in the presence of 1 μM at-RA ($P < 0.05$, paired t test, $n = 7$), but the mean unitary conductance was similar before and during the application of at-RA (44 $\pm$ 3 pS vs. 40 $\pm$ 2 pS; $P > 0.05$, paired t test, $n = 7$). The 72% reduction in channel open probability accounts for the 74% reduction in macroscopic patch current and the 67% reduction in whole-cell current observed in the presence of 1 μM at-RA.

Discussion

The present study has demonstrated that RA is a potent and selective modulator of electrical coupling through gap junctions in retinal horizontal cells. Unlike many of the previously described actions of RA, this synaptic modulation does not occur through nuclear receptors and transcriptional regulation, but is mediated via an external binding site with $RAR_{\beta/\gamma}$-like agonist specificity which acts independently of cytoplasmic second messenger cascades. The molecular moiety responsible for this novel action may be a previously undescribed type of RAR protein, or an RA binding site on the gap junction channels themselves.

Nontranscriptional Mechanism. Three of our results indicate that the mechanism by which RA reversibly reduced the gap junctional conductance does not involve transcription. First, the time course of RA action on horizontal cell coupling is much more rapid than the previously reported transcriptional effects of RA on cell coupling. RA is known to up-regulate or down-regulate connexin gene expression via RAR/RXR activation in the nucleus, resulting in a change in dye coupling between RA-treated cells in several cell lines (19–23). However, these nonreversible changes take place over the course of hours or days. In contrast, the time course of the onset of the RA uncoupling effect in our experiments is within 3–5 min and the action is readily reversible. Second, internal dialysis of at-RA failed to have an effect on gap junction function providing further evidence that this action is not mediated by nuclear RAR/RXR activation. Third, modulation of gap junction hemichannels in isolated cell-free membrane patches is also inconsistent with transcriptional regulation. This nontranscriptional action is consistent with previous findings for modulation of horizontal cell dye coupling by RA in mammalian and fish retinas (8, 18).

RA Acts by a Specific Receptor-like Mechanism. Although RA is an acidic lipophilic compound that can readily pass across cell membranes, our results clearly show that RA acts by a specific receptor-like mechanism rather than nonspecific pH or lipophilic action. First, although intracellular acidification can uncouple gap junctions, RA at the concentration we were using does not alter the pH of our solutions. In addition, high levels of pH buffer in the intracellular solution do not interfere with uncoupling by RA. Second, if RA, like heptanol, acted within the structure of the phospholipid bilayer of cell membrane, then it might be expected to perturb both gap junctional and non-gap-junctional channels (31). However, RA, at least at concentrations less than 10 μM, specifically acted on gap junction channels of horizontal cells, suggesting that RA action is independent of its lipophilic properties. This postulation is further supported by the evidence that retinoic acid is more potent than either retinaldehyde and retinol in gating gap junctions, even though they are more effective in perturbing phospholipid bilayers than RA (32). In addition, the concentration–inhibition relationships of retinoids on hemi-gap-junctional currents showed that the potency of inhibition by at-RA was 60-fold greater than at-retinaldehyde and 75-fold greater than at-retinol, whereas all compounds had similar efficacy (Table 1). Finally, our single-channel results revealed that RA reduced channel open probability, but did not change the unitary conductance, indicating that RA allosterically modulates channel gating through a specific receptor-like mechanism rather than by simply blocking the channel pore.

An External Receptor Site with $RAR_{\beta/\gamma}$-like Characteristics. Because only external application, not internal dialysis of at-RA, had an effect on junctional conductance, the receptor-like binding site is apparently located on the extracellular side of the plasma membrane. By using selective retinoid receptor ligands, we demonstrated that at-RA and 9-cis RA, which have an equivalent activation on the RAR, had a similar effects on gap junctional conductance, suggesting that RAR-like binding sites might be involved in RA's uncoupling. Furthermore, both a selective RAR_{β} agonist (CD2314) and a selective RAR_{γ} agonist (CD666) had significant effects on gap junctional currents, but a selective RAR_{α} agonist (CD336) did not. Dose–inhibition relationships of these selective RAR agonists on hemi-gap-junctional currents showed that CD666 was more potent than CD2314, whereas they had similar efficacy. However, CD366 was

substantially less potent and efficacious (Table 1). The overall results indicate that at-RA binds to an $RAR_{\beta/\gamma}$-like binding site.

RA Acts Independently of Second Messengers. Unlike NO and dopamine, RA does not modulate horizontal cell gap-junctional channel gating through second messengers. RA also does not act on gap junctions by increasing $[Ca^{2+}]_i$. RA uncoupling effects were equal with or without ATP/GTP in the pipette solution and GDPγS failed to block at-RA uncoupling, indicating that RA regulation of gap junctional currents does not depend on G protein activation. Both cAMP-dependent protein kinase A (PKA) and cGMP-dependent protein kinase G (PKG), which have already been shown to gate gap junctional channels in retinal horizontal cells (14–16, 27, 28), are not involved in the RA uncoupling effect. In addition, the RA uncoupling effect was not mediated by calmodulin kinase or a protein kinase C (PKC) pathway. Importantly, RA gating of hemi-gap-junctional channels occurs in cell-free excised membrane patches, indicating that the normal cytoplasmic factors are not required in the process of RA modulation of gap junction channels. The simplest interpretation of the above results is that at-RA acts directly on gap junction channels to gate channel activity. However, we cannot rigorously exclude the possibility that a separate RA-binding protein closely associated with the gap junction channels is responsible.

Possible Functions of RA in the Retina. Several lines of evidence have shown that RA is a potential endogenous neuroactive substance in the vertebrate retina including the presence of precursors, synthetic enzymes, binding proteins, nuclear receptors, and demonstrated RA release (3, 4, 6, 33–39). Because RA is membrane permeant, it is highly diffusible in tissue. Given that multiple retinal cell types are capable of RA production (6, 33, 38, 39), RA could affect electrical synaptic transmission in other retinal networks as well as in horizontal cells *in vivo*. Synthesis of RA is activated by light adaptation (6), and modulation by RA of morphological and physiological changes in horizontal cells suggest that RA is involved in light adaptation processes (7, 8, 18), perhaps especially during steady light adaptation, which dopamine does not mediate (40–42). In contrast to two other retinal neuromodulators, dopamine and NO, which affect both electrical and glutamatergic synaptic transmission in horizontal cells (14–16, 27, 28, 43, 44), RA acts specifically on electrical synaptic channels. Therefore, RA may play an important role in retinal neuronal networks to maintain high acuity, spatial resolution, and color vision under light-adapting conditions in the vertebrate retina. Although our present results indicate that RA allosterically modulates gap junctional channels in the mature retina, it may be that RA also regulates connexin expression at the transcriptional level over longer time-spans, especially during eye development. Reducing connexin expression during development results in a reduction in eye size and a decrease in the number of dividing retinal cells (45). Our data raise the possibility that both transcriptional and direct regulation of cellular coupling by RA may be involved in eye development (2, 4).

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