(pH 7.0), 70% glycerol, phospholipids from *E. coli* (4 mg/ml) (Avanti Polar Lipids), 1 mM PMSF, and 1 mM EDTA. After incubation at 23°C for 30 min, the solution was diluted with 25 volumes of ice-cold 50 mM sodium phosphate (pH 7.0), 10% glycerol, 1 mM PMSF, 1 mM EDTA. Membrane vesicles were collected by centrifugation at 400,000*g* for 30 min, and the vesicles were washed to remove residual detergent by two cycles of resuspension and centrifugation. The membrane vesicles were resuspended in the same buffer, frozen in liquid nitrogen, and stored at −70°C.

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Intracellular Signaling by 14-Hydroxy-4,14-*Retro*-Retinol

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In mammals, retinol is the precursor for retinoids, which affect various aspects of morphogenesis and development. However, B lymphocytes, although retinol-dependent, do not use retinoic acid as mediator. Retinol is metabolized by B lymphocytes and other cell lines to optically active 14-hydroxy-4,14-*retro*-retinol; it is this compound that mediates the growth control. Thus another second messenger molecule, in addition to retinoic acid and retinal, is derived from retinol.

IN VERTEBRATES, PROCESSES AS DIverse as growth, vision, and reproduction depend on the presence of retinol (vitamin A) (*1*). Retinol serves as a source for a variety of derivatives adapted to specialized functions, such as 11-*cis* retinal, that constitutes the chromophore of the visual pigment rhodopsin (*2*). Furthermore, retinoic acid induces differentiation in many in vivo and in vitro systems (*3*) by binding to a nuclear receptor and subsequently enhancing transcription of specific genes (*4*). Retinol is essential for the growth of a variety of cell types in culture, notably lymphocytes (*5*). However, retinoic acid cannot substitute for retinol in preventing necrotic cell death of activated immune cells (*5–7*). We have identified a retinol derivative on the basis of its growth-promoting properties and have characterized it as 14-hydroxy-4,14-*retro*-retinol (14-HRR).

Cells of the lymphoblastoid line 5/2 were grown in the presence of ^{3}H-labeled retinol–retinol binding protein complex (*8*), and the lipids were extracted from the cell pellet (*9*). High-pressure liquid chromatography (HPLC) on a reversed-phase C_{18} column yielded hitherto undescribed retinoids with retention times of 16 to 18 min, amounting from 4 to 9% of the total cellular retinoids (Fig. 1). These retinoids comprise at least four compounds with similar ultraviolet (UV) absorption spectra, and they are most likely *cis/trans* isomers of the same molecule. The most abundant of these, P3, was purified from lymphoblastoid or HeLa cells by means of a series of reversed-phase columns [a preparative C_{18} column of 250 by 22 mm internal diameter (ID) with water-methanol at 17/83 v/v; a semipreparative C_{18} column of 250 by 10 mm ID with water-acetonitrile at 25/75 v/v; and an analytical C_4 column of 250 by 4.6 mm ID with water-methanol at 78/72 v/v]. From 80 liters of HeLa cells, 30 absorption units at 348 nm (168 μg) of pure P3 were obtained. This compound was unstable on normal-phase columns and in chloroform but tolerated methanol, butanol, benzene, and acetonitrile and remained intact after storage for several months at −80°C.

The characteristic vibronic fine structure of the UV spectrum of P3 (Fig. 2A) suggested a *retro*-retinoid skeleton (*10*). The high-resolution electron ionization (EI) mass spectrum [(matrix perfluoro-kerosine (PFK)] gave an observed value of 302.2265 calculated for $C_{20}H_{30}O_2$ = 302.2246), indicating that P3 has one more oxygen than its precursor retinol, $C_{20}H_{30}O$. The low-resolution EI mass spectrum of P3 is described in (*11*). Proton nuclear magnetic resonance (NMR) measurements (Fig. 3) established that P3 is 14-HRR. The double-bond configuration between positions 6 and 7 was established as E by comparisons with the chemical shifts of 1,1-Me_2 (1.30) and 4-H (5.79) with those reported for 6-E-4,14-*retro*-retinyl acetate (*12, 13*), 1.28 and 5.76 ppm, respectively; the corresponding values for 6-Z-4,14-*retro*-retinyl acetate are 1.11 and 5.63 ppm. Furthermore, the 6-E configuration was confirmed by observation of an approximately 4% nuclear Overhauser effect between 1,1-Me_2 and 8-H. The configuration of the double bond between po-

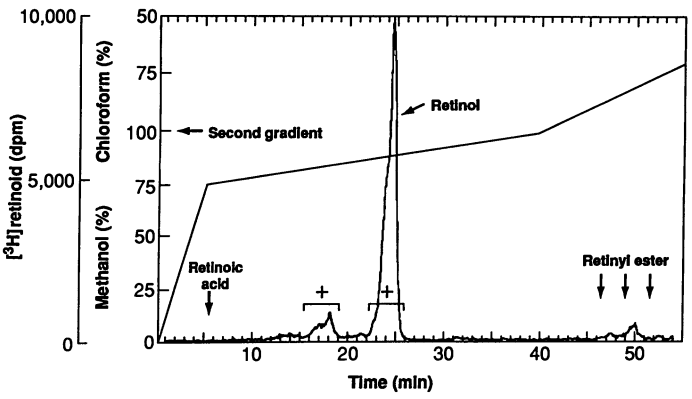

Fig. 1. High-pressure liquid chromatography of retinoids from lymphoblastoid 5/2 cells (Waters, Milford, MA), analytical C_{18} reversed-phase column (Vydac, Hesperia, CA); water-methanol-chloroform gradient; flow rate, 0.5 ml/min; photodiode array detection. Disintegrations per minute were determined with an on-line scintillation counter (Radiomatic, Tampa, FL). Fractions eluting at 16 to 18 min, as well as the original retinol peak, are bioactive in a bioassay performed as described in Fig. 4. The 5/2 cells [1.8 × 10^7 cells in 30 ml of RPMI/0.9% fetal calf serum (FCS) medium] were incubated with 2 × 10^{-6} M holo-retinol–binding protein to which were bound 30 μCi [^{3}H]retinol; specific activity, 49.3 Ci/mmol. Retinol-binding protein was purified from human serum by means of a transthyretin column (*21*). The absorption ratio (280 nm to 324 nm) of the retinol-binding protein–retinol complex used was 0.85. After 24 hours, retinoids were extracted from the washed cell pellet according to the procedure of McLean *et al.* (*9*). Unlabeled reference retinoids (arrows) were used to calibrate the column.

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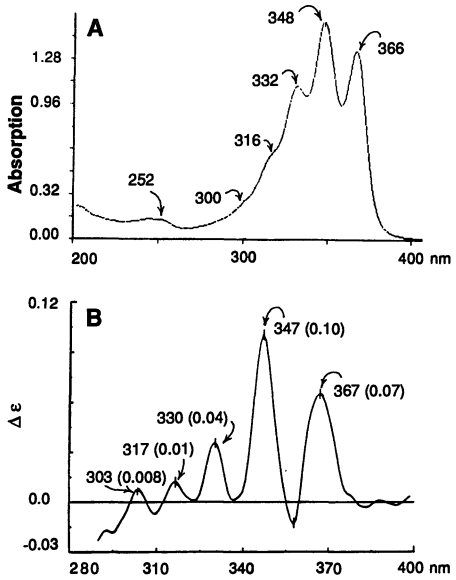

Fig. 2. (**A**) Absorption spectrum of 14-HRR in methanol, Perkin-Elmer model Lambda 4B UV/VIS spectrophotometer. (**B**) Circular dichroism spectrum of 14-HRR in methanol, Jasco J-720 spectropolarimeter.

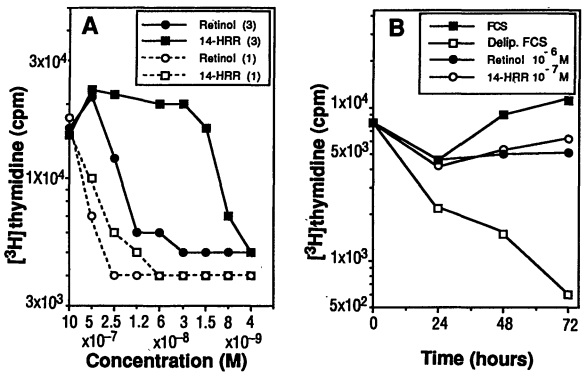

Fig. 4. Growth-supporting activity of 14-HRR compared to that of retinol and serum. (**A**) Dependency of dose response on repeated 14-HRR application. (**B**) Growth kinetics of lymphoblastoid cells. Lymphoblastoid 5/2 cells grown in RPMI/5% FCS were taken from their exponential growth phase, washed twice, and seeded at 30,000 cells/ml in RPMI medium containing in (A) delipidated bovine albumin (1.2 mg/ml), 10^{-6} M linoleic acid, bovine insulin (5 μg/ml), and transferrin (5 μg/ml); in (B) 5% FCS, 5% delipidated FCS (*22*) with or without 10^{-6} M retinol or 10^{-7} M 14-HRR. The assay was done in 96-well microtiter plates in a final volume of 200 μl per well. The cells were cultured for 72 hours, and we determined cell growth by labeling for the last 16 hours (A) or 8 hours (B) with [^{3}H]thymidine (0.8 μCi per well) at the indicated time points. Retinoids at the indicated concentration were added either once at the initiation of culture (A, broken lines) or every 24 hours thereafter as well (A and B, solid lines). Data represent the mean of triplicate measurements and SDs were ≤16%.

sitions 12 and 13 is uncertain.

Because the carbon at position 14 is an asymmetric center, 14-HRR could be optically active. The circular dichroism spectrum of 14-HRR (Fig. 2B) exhibits a weak but distinct positive Cotton effect with fine structure corresponding to its UV spectrum, indicating optical activity. The absolute configuration at C-14 is assigned as R on the basis of the positive "allylic hydroxyl" Cotton effects (*14–16*). However, because this interpretation is dependent on the perturbation of the pentaene absorption at 348 nm by the hydroxyl group which absorbs at ~200 nm (remote from 348 nm) and because an additional 15-OH group is present, the configuration at this center needs to be confirmed by synthesis.

We have demonstrated that most human lymphoblastoid cell lines, normal human, and murine B lymphocytes require retinol at a minimal concentration of 5×10^{-7} M for proliferation. 14-HRR is a biosynthetic product of B cells directly derived from retinol on the basis of radioisotope tracing and optical activity (Fig. 2B); in the absence of retinol, 14-HRR is capable of sustaining the growth of the lymphoblastoid cell line 5/2 (see Fig. 4), as well of T cell lines and fibroblasts (7). Retinoic acid is inactive in this growth assay.

Because a general intracellular mediator should be produced by all cells that show retinol dependency, we have assayed for the presence of 14-HRR in a diverse panel of cell lines, by means of [^{3}H]retinol bound to retinol-binding protein and HPLC analysis. The cell lines tested include fibroblasts, HeLa cells, and leukemias and produced 14-HRR without exception, the relative amounts varying from 0.5 to 9% in reference to total intracellular retinoids.

14-HRR is the first of the *retro*-retinoids to be discovered occurring naturally, although they have been known as a class of synthetic compounds (*10, 13, 17–19*). Natural carotenoids also can exhibit a *retro*-structure skeleton (*20*). 14-HRR occurs in *Drosophila* cells, and other *retro*-retinoids as yet uncharacterized exist in mammalian and insect cells, thus suggesting conservation of a complex family of *retro*-retinoids over a long evolutionary period. 14-HRR, a molecule more potent than its precursor retinol and essential for the growth of a variety of cells in tissue culture, is an intracellular mediator of retinol effects. These results imply the existence of a second pathway of retinol action distinct from the retinoic acid pathway that has been linked to selective gene action and differentiation.

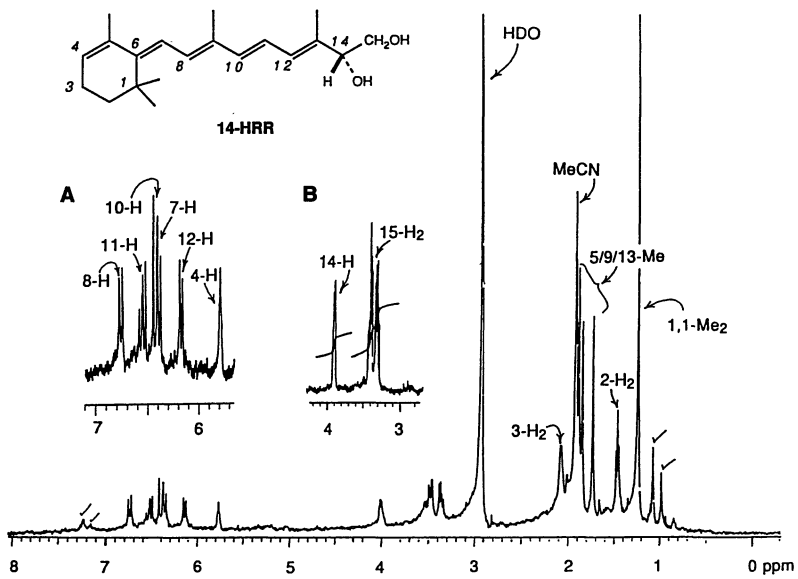

Fig. 3. ^{1}H NMR (CD_3CN, one drop D_2O, Varian VXR-400): 1.30 (s, 6H, 1,1-Me_2), 1.50 (t, J 7.5 Hz, 2H, 2-H_2), 1.76/1.87/1.90 (s, all 3H, 5/9/13-Me), 2.08 (m, 2H, 3-H_2), 3.4 (m, 1H, 15-H), 3.5 (m, 1H, 15-H), 4.02 (m, 1H, 14-H) [see insert (B), in C_6D_6, for better resolution]. Olefinic protons (insert A): 5.79 (t, J 4 Hz, 1H, 4-H), 6.17 (d, J 12 Hz, 1H, 12-H), 6.38 (d, J 12.3, 1H, 7-H), 6.42 (d, J 17, 1H, 10-H), 5.56 (dd, J 17, 12 Hz, 1H, 11-H), 6.76 (d, J 12.3, 1H, 8-H). Checked peaks arise from 14-HRR degradation.

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Participation of Postsynaptic PKC in Cerebellar Long-Term Depression in Culture

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Long-term depression (LTD) in the intact cerebellum is a decrease in the efficacy of the parallel fiber-Purkinje neuron synapse induced by coactivation of climbing fiber and parallel fiber inputs. In cultured Purkinje neurons, a similar depression can be induced by iontophoretic glutamate pulses and Purkinje neuron depolarization. This form of LTD is expressed as a depression of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)–mediated current, and its induction is dependent on activation of metabotropic quisqualate receptors. The effect of inhibitors of protein kinase C (PKC) on LTD induction was studied. Inhibitors of PKC blocked LTD induction, while phorbol-12,13-diacetate (PDA), a PKC activator, mimicked LTD. These results suggest that PKC activation is necessary for the induction of cerebellar LTD.

LTD IS INDUCED AFTER COACTIVATION of parallel fiber (PF) and climbing fiber (CF) inputs to a Purkinje neuron (PN) and is specific to those PF-PN synapses that are active during CF stimulation (*1*). Thus, cerebellar LTD is an anti-Hebbian process: synapses that are active during postsynaptic activation are weakened (*2*). As the PN is the sole output stage of the cerebellar cortex, LTD has been implicated in several forms of motor learning (*3*).

LTD is expressed as a depression of AMPA-mediated current. It is detected with pulses of glutamate, quisqualate, or AMPA but not with pulses of aspartate or NMDA (*4–6*). There is no evidence to suggest a presynaptic function in either the induction or the expression of cerebellar LTD. A quantal analysis has been reported that is consistent with postsynaptic expression (*7*).

Several principles have emerged regarding the induction and expression of cerebellar LTD. The aspect of CF activation that contributes to LTD induction seems to be a prolonged depolarization of the PN (*8*), which results in dendritic Ca^{2+} entry (*9*). As such, induction of LTD is blocked when PNs are electrically inhibited (*1, 6, 10, 11*) or loaded with a Ca^{2+} chelator (*12*), and LTD may be induced when depolarization sufficient to produce Ca^{2+} entry is substituted for CF activation (*5, 6, 10, 11*). Activation of PFs contributes to LTD induction by activating both AMPA (ionotropic quisqualate) receptors and metabotropic quisqualate receptors. Blockade of either of these receptors during conjunctive stimulation blocks induction of LTD (*6, 13*), and activation of both of these receptors substitutes for PF activation during induction of LTD (*5, 6, 14*). NMDA receptors do not seem to contribute to LTD induction (*6, 14*). Thus, three processes are necessary and sufficient for induction of cerebellar LTD, namely, PN depolarization sufficient to produce dendritic Ca^{2+} influx, activation of AMPA receptors, and activation of metabotropic quisqualate receptors.

The metabotropic quisqualate receptor is coupled to phospholipase C, which cleaves the membrane lipid phosphatidylinositol-bisphosphate to yield inositol-1,4,5,-trisphosphate and 1,2-diacylglycerol. The former releases Ca^{2+} from nonmitochondrial internal stores, and the latter is an activator of PKC (*15*). To investigate the possible role of PKC in the induction of cerebellar LTD, we applied PKC inhibitors and activators to PNs in culture to determine their effects on LTD induced by glutamate–depolarization conjunctive stimulation. As this protocol (*16, 17*) does not rely on synaptic stimulation to induce LTD, it allows unambiguous analysis of postsynaptic processes.

Nonpeptide, membrane-permeable PKC inhibitors with greatly improved specificity are now available. Two such compounds are calphostin C, which competes with phorbol esters at the regulatory site of PKC (*18*), and RO-31-8220, which competes with adenosine triphosphate (ATP) at the catalytic site (*19*). Both inhibitors blocked the induction of LTD when applied during glutamate–depolarization conjunction (Fig. 1A). After drug washout for 25 min, a second conjunctive stimulus induced LTD (t = 30 min). Glutamate–depolarization conjunction in the presence of RO-31-8220, but not calphostin C, produced a small potentiation of the glutamate current (RO-31-8220, 118 ± 5.5% of baseline; calphostin C, 104 ± 4.2% at t = 15 min, mean ± SEM, n = 5 per group). This potentiation is similar to that seen when glutamate–depolarization conjunction was applied in the presence of inhibitors of the metabotropic quisqualate receptor or when AMPA–depolarization conjunctive stimulation was applied (*6*). Application of PKC inhibitors after the induction of LTD (t = 10 to 60 min) produced no alteration of the depressed glutamate current (Fig. 1B), suggesting that either after induction, LTD does not require further PKC activity or that PKC activated by glutamate–depolarization conjunction is insensitive to these two PKC inhibitors.

We also applied a PKC inhibitory peptide, PKC(19-36), and a noninhibitory control peptide, [glu^{27}]PKC(19-36), (*20*) to the internal solution of conventional whole-cell patch electrodes (*21*). PNs dialyzed (with Ca^{2+} chelator free vehicle) for either 5 or 10 min before glutamate–depolarization conjunctive stimulation showed LTD (induced four out of four and five out of five times, respectively). However, LTD was

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