The Journal of Experimental Biology 203, 631–640 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2520

DOES MEMBRANE FLUIDITY CONTRIBUTE TO THERMAL COMPENSATION OF β-ADRENERGIC SIGNAL TRANSDUCTION IN ISOLATED TROUT HEPATOCYTES?

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Accepted 3 November 1999; published on WWW 17 January 2000

Summary

The potential role of compensatory adjustments to membrane components in determining the function of the β-adrenergic receptor/adenylyl cyclase (β-AR/AC) signal-transduction system was studied in isolated hepatocytes of 5 °C- and 20 °C-acclimated rainbow trout *Oncorhynchus mykiss*. Rates of epinephrine-stimulated cyclic AMP (cAMP) production, although slowed (by a factor of 1.6- to 2.4-fold) by an acute drop in assay temperature from 20 to 5 °C, were significantly temperature-compensated, being approximately twofold higher in hepatocytes of 5 °C- than of 20 °C-acclimated trout. Membrane order in the bilayer interior of hepatocyte plasma membranes (as assessed by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene) was consistently lower in cold- than in warm-acclimated trout, reflecting an efficacy of homeoviscous adaptation of approximately 50 %. Temperature-induced changes in plasma membrane fatty acid composition (i.e. an increase in the proportions of polyunsaturated fatty acids with acclimation to 5 °C) were consistent with both the observed changes in the order of the bilayer interior and the extent of homeoviscous adaptation. However, isothermal fluidization of the bilayer interior by the addition of benzyl alcohol (30 mmol l^{-1}) decreased rather than increased the rate of cAMP production. Significantly more (1.81-fold) β-adrenergic receptors were present in plasma membranes of hepatocytes from 5 °C-acclimated ($6.23\times10^4\pm4206$ receptors per cell; mean ± S.E.M., N=3) than 20 °C-acclimated fish ($3.44\times10^4\pm4360$ receptors per cell; N=3) when assayed at the acclimation temperature, whereas the equilibrium dissociation constants (K_d) (13.73±4.33 nmol l^{-1} at 5 °C; 9.75±3.29 nmol l^{-1} at 20 °C; N=3) were similar. On the basis of a strong correlation between β-adrenoceptor number and the rate of cAMP production (r^2=0.956), regardless of assay or acclimation temperature, we conclude that modulation of receptor number is the primary acclimatory response of this signal-transduction pathway to temperature change in trout liver.

Key words: temperature, β-adrenergic, liver, hepatocyte, rainbow trout, *Oncorhynchus mykiss*, membrane fluidity.

Introduction

In poikilotherms, endocrine function is frequently compromised by fluctuations in body temperature. For example, the epinephrine-stimulated exchange activity of the Na^+/H^+ antiporter present in trout erythrocytes is highly temperature-dependent: Q_{10} values are 10.8 between 0 and 10 °C, 4 between 6 and 13 °C, and 2.7 between 13 and 20 °C (Cossins and Kilbey, 1990). In addition, the responsiveness of bullfrog hearts to prostaglandins, although evident in 20 °C-acclimated animals, is not seen in animals acclimated to 5 °C (Herman et al., 1986b); similarly, β-adrenergic stimulation of cardiac tissue is evident in 22 °C- but not 12 °C-acclimated bullfrogs (Herman et al., 1986a). Thus, both acute and, in some cases, long-term thermal perturbation can have pronounced effects on endocrine signal-transduction pathways in poikilotherms. However, in animals that remain active over a broad range of temperatures, the ability to maintain cell–cell communication and integrated metabolic function is critical. This implies that the impact of acute changes in temperature on the hormonal activation of cell function may be subject to thermal compensation.

One possible site for thermal compensation of endocrine control mechanisms acting *via* membrane-mediated signal-transduction pathways is the plasma membrane of target tissue cells, because a common mechanism of temperature acclimation is a restructuring of membrane lipid composition (Hazel, 1995). The maintenance of membrane function *in situ* is accomplished, in part, by the restructuring of lipid assemblages, matching lipids of appropriate physical properties to prevailing thermal conditions. Membrane physical properties may influence signal-transduction pathways in at least two fundamentally different ways: (1) by determining the efficiency of diffusion coupling between proteinaceous components anchored in the membrane; and (2) by direct modulation of protein activities or the affinity of proteins for ligands. For example, epinephrine-dependent activation of adenylyl cyclase in turkey erythrocytes both

increases with (Rimon et al., 1978) and parallels (Atlas et al., 1980) a reduction in membrane fluidity, reflecting fluidity-dependent modulation of collision coupling (Hanski et al., 1979). In addition, other studies have established that the activity of adenylyl cyclase itself is dependent on membrane physical properties (Houslay and Gordon, 1982; Calorini et al., 1993).

Isolated hepatocytes constitute an ideal model system for studying the thermal compensation of signal-transduction mechanisms in poikilotherms because they are an easily procurable, homogeneous, differentiated cell type that remains metabolically active after isolation (Berry et al., 1991; Mommsen et al., 1991; Moon et al., 1985). In addition, previous studies have established significant thermal compensation of endocrine mechanisms in trout liver. Both epinephrine-stimulated glucose release and the sensitivity to epinephrine are significantly greater in perfused livers of 5 °C- than 20 °C-acclimated trout (McKinley and Hazel, 1993). These considerations led us to investigate β-adrenergic signaling mechanisms in intact trout hepatocytes in which both the spatial organization of the plasma membrane and the intracellular milieu are conserved.

The present work addresses the potential role of compensatory adjustments in membrane lipid composition in determining the membrane microenvironment and consequent function of the β-adrenergic receptor/adenylyl cyclase (β-AR/AC) signal-transduction pathway in thermally acclimated trout. The specific questions addressed include the following: (i) are rates of epinephrine-stimulated cAMP production temperature-compensated as a consequence of thermal acclimation; (2) do temperature-induced changes in plasma membrane lipid composition produce changes in membrane fluidity; (3) do acclimatory adjustments in membrane physical properties correlate with adrenergic signal-transduction activity and cAMP production; and (4) do changes in receptor number and/or K_d account for acclimatory differences in signal-transduction efficiency?

Materials and methods

All solutions were prepared in glass-distilled water. Na_2HPO_4 was purchased from EM Science. KCl, $MgSO_4$ and $CaCl_2$ were purchased from Baker. NaCl and $NaHCO_3$ were purchased from Fischer Scientific. Hepes, bovine serum albumin (BSA), Trypan Blue, epinephrine bitartrate, heparin, DNAase I, benzamidine and propranolol were purchased from Sigma. Collagenase A was purchased from Boehringer Mannheim. Rainen ^{125}I-labeled cAMP assay kits were purchased from DuPont–New England Nuclear. Methanolic HCl was purchased from Supelco. Bicinchoninic acid protein assay reagents were obtained from Pierce. Reeves Angel papers were purchased from Brandel Scientific.

Animals

Rainbow trout *Oncorhynchus mykiss* (Walbaum) (of both sexes and ranging in body mass from 150 to 400 g) were obtained from the Alchesay National Fish Hatchery in Whiteriver, Arizona, USA. Fish were maintained in 600 gallon (2274 l) cylindrical fiberglass tanks held at 5 °C and 20 °C on a 12 h:12 h light:dark photoperiod and fed Glencoe Mills trout pellets once daily. Water was continuously filtered, and the entire tank volume was replaced with fresh dechlorinated water every 24 h. Animals were acclimated for a minimum of 3 weeks before experimentation.

Hepatocytes

Trout were anesthetized in MS-222 (1/2000 w/v). Hepatocytes were isolated by a slight modification of the perfusion (*via* the hepatic portal vein) conditions of Moon et al. (1985). The initial perfusate was heparinized (20 mg 100 ml^{-1}) Hepes-buffered saline (in mmol l^{-1}: NaCl, 176; KCl, 5.4; $MgSO_4$, 0.81; KH_2PO_4, 0.44; Na_2HPO_4, 0.35; $NaHCO_3$, 8; Hepes, 20; aerated with 98 % O_2/2 % CO_2, pH 7.8; buffer A). Once the liver had cleared of blood, the perfusate was changed to Hepes-buffered saline (buffer A) containing collagenase A (0.2 mg ml^{-1}), but no heparin, until signs of digestion were visible (approximately 15 min). After filtration through 250 and 75 μm nylon mesh, the hepatocytes were washed three times by centrifugation (1000 revs min^{-1} for 5 min, Beckman JA-20 rotor at 4 °C) in the initial perfusion buffer (buffer A) lacking heparin. Cell viability was assessed by Trypan Blue exclusion. Cells were finally resuspended in a Hepes buffer (buffer A) containing 2 % BSA (w/v) and 1 mmol l^{-1} $CaCl_2$ at a density of 2.5×10^6 cells ml^{-1} and allowed to recover for approximately 1 h (while continuously gassed with O_2/CO_2) at the acclimation temperature.

Previous hepatocyte isolations performed in this laboratory failed to isolate cells with intact adrenergic receptors (McKinley and Hazel, 1993). Consequently, in the present experiments, we reduced the exposure to collagenase (0.2 mg ml^{-1} for 15 min compared with 0.6 mg ml^{-1} for 30–45 min) during the isolation procedure. Hepatocytes prepared using the modified method released glucose into the medium in response to β-agonists (epinephrine at 0.5×10^{-6} mol l^{-1}); furthermore, glucose release in the presence of epinephrine was specifically and completely blocked (P<0.003) by the β-specific antagonist propranolol (present at a concentration of 0.5×10^{-6} mol l^{-1}).

Production of cyclic AMP

Hepatocytes (2.5×10^6 cells ml^{-1}) were incubated in Hepes-buffered saline (buffer A), pH 7.8, in a shaking, temperature-controlled water bath, while under a 98 % O_2/2 % CO_2 atmosphere. Cyclic AMP production was stimulated by addition of 0.5×10^{-6} mol l^{-1} epinephrine (in the presence of 4.6 mmol l^{-1} theophylline) (McKinley and Hazel, 1993). The reaction was stopped after 7 min by the addition of 100 μl of 6 % trichloroacetic acid (TCA) and subsequent plunging of samples (600 μl) into liquid nitrogen. After thawing, cell lysates were neutralized with 2 mol l^{-1} $KHCO_3$, centrifuged at 25 000 ***g*** for 15 min at 4 °C, and the supernatant (containing cAMP) was stored at –20 °C until assayed (a period no greater

than 2 days). The quantity of cAMP present was assayed using a Rainen [^{125}I] radioimmunoassay (RIA) kit.

Fluorescence polarization

Plasma membrane order was determined by the measurement of steady-state fluorescence polarization $P=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$, where $\parallel$ is parallel and $\perp$ perpendicular to the beam of incident light, employing 1,6-diphenyl-1,3,5-hexatriene (DPH) to probe the membrane interior and trimethyl ammonium 1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) to probe the interfacial regions. Hepatocytes (2.5×10^6 cells ml^{-1}) were washed free of BSA (in 1 ml of buffer A, pH 7.4) by three sequential centrifugations in a microcentrifuge for 10 s. Cells were diluted to an absorbance of 0.15 at 364 nm (light scattering is negligible under these dilution conditions), and 2 µl of a 2.0 mmol l^{-1} solution (in dimethylformamide) of fluorescent probe was added (probe:lipid molar ratio 0.001–0.002) to 4.0 ml of diluted cells (Williams and Hazel, 1994). Polarization measurements (excitation 360 nm, emission 430 nm) were made using a Perkin Elmer LS 50B luminescence spectrophotometer within 1 min of adding the probe at 5 °C intervals between 5 °C and 20 °C. Cells were incubated at the assay temperature for 5 min prior to the addition of probe. Fresh cells were employed at each assay temperature.

Fluidization of hepatocyte plasma membranes with benzyl alcohol

Benzyl alcohol (30 mmol l^{-1}) reduces membrane order to an extent equivalent to a 6–8 °C rise in temperature in rat adipocytes (Saverheber et al., 1982). To fluidize the plasma membrane of hepatocytes isothermally, cells were incubated (buffer A, pH 7.8) in the presence of varying concentrations (10–40 mmol l^{-1}) of benzyl alcohol. Polarization values for DPH and TMA-DPH were determined for each alcohol concentration as described above.

Membrane purification

Trout liver plasma membranes were isolated by a combination of density gradient and isopycnic centrifugation according to a modification of the method of Armstrong and Newman (1985). Briefly, livers were finely minced and homogenized (with six strokes of a Potter-Elvehjem homogenizer at 500 revs min^{-1}) in four volumes of buffer B (in mol l^{-1}: 0.25 sucrose, 0.02 Tris, pH 7.4 at 20 °C, containing 0.001 mol l^{-1} benzamidine) to which 1 mg ml^{-1} DNAase I was added just prior to tissue disruption. The crude homogenate was filtered through 250 µm nylon mesh and layered over a 15 ml cushion of 41 % (w/v) buffered sucrose (0.02 mol l^{-1} Tris, pH 7.4) prior to centrifugation at 23 000 ***g*** for 30 min (JA-20 rotor). Crude plasma membranes were collected from the interface, diluted in 4 volumes of buffer B, and concentrated by centrifugation at 7000 ***g*** for 15 min (JA-20 rotor). Final purification was accomplished using a Tris-buffered Percoll gradient (0.02 mol l^{-1} Tris, pH 7.4, 10 % 2.5 mol l^{-1} sucrose, 18 % Percoll) and centrifuged at 33 600 ***g*** for 25 min (50.2 Ti Beckman rotor). The plasma membranes were collected from the upper of two distinct bands in the gradient, diluted with 10–20 volumes of buffered saline (0.1 mol l^{-1} NaCl, 0.01 mol l^{-1} Tris, pH 7.4) and concentrated by centrifugation at 185 000 ***g*** for 2 h. Purified plasma membranes were recovered from the top of the Percoll pellet and resuspended in 0.5 ml of 20 mmol l^{-1} Tris (pH 7.4), and samples were stored at –80 °C. The degree of purification was calculated by assaying Na^+/K^+-ATPase (Schwartz et al., 1969) and 5′ nucleotidase (Aronson and Touster, 1974) activities. The enrichment of plasma membrane marker enzymes in the final membrane fraction averaged 20- to 35-fold.

Receptor binding and characterization

Characterization of trout hepatic β-adrenoceptors was investigated by radioreceptor assay techniques using [^{3}H]-labeled CGP 12177 (specific activity 1.26–4.07 TBq mmol^{-1}; Amersham) employing a protocol similar to that of Marttila and Nikinmaa (1988). Washed hepatocytes were diluted to 16×10^6 to 18×10^6 cells ml^{-1}. The time course of [^{3}H]CGP binding was determined using hepatocytes incubated with 10 nmol l^{-1} [^{3}H]CGP for 45, 60, 90 and 120 min. All incubations were in triplicate at an assay temperature corresponding to the acclimation temperature in 10 mm×75 mm borosilicate glass tubes. These studies indicated that an incubation period of 90 min is required to assay [^{3}H]CGP binding.

Isoproterenol competition experiments were performed to determine the concentration of isoproterenol necessary to block completely the binding of [^{3}H]CGP and whether experimental conditions of netting, transport to the laboratory and anesthesia altered receptor binding characteristics. Competition curves were determined by adding 40 µl of suspended hepatocytes (6×10^5 to 7×10^5 cells), isolated from 20 °C-acclimated fish, to 360 µl of Cortland saline (in mmol l^{-1}: 124.1 NaCl, 5.1 KCl, 2.9 Na_2HPO_4, 1.9 $MgSO_4$, 1.4 $CaCl_2$, 11.9 $NaHCO_3$, 5.6 glucose) into which 5 nmol l^{-1} [^{3}H]CGP had been added in combination with 0–200 µmol l^{-1} α-(–) isoproterenol, a β-adrenoceptor agonist. The reaction was allowed to proceed for 90 min at 20 °C and terminated as below.

Saturation binding experiments were initiated by adding 40 µl of suspended hepatocytes (6×10^5 to 7×10^5 cells) to 360 µl of Cortland saline into which 2, 4, 5, 7.5, 10, 15, 20, 25, 30, 40 or 60 nmol l^{-1} [^{3}H]CGP had been added in the presence (nonspecific binding) and absence (total binding) of 200 µmol l^{-1} (–)-isoproterenol.

Incubations were terminated by repeated washings (four) with 5 ml of ice-cold Cortland saline after the hepatocytes had been transferred to borosilicate filters (Reeves Angel, Brandel Scientific) using a Brandel 24R membrane harvester. The filters were then counted (in Readysafe fluor) on a Beckman Rackbeta scintillation counter with counts corrected for quenching using an external standard. The numbers of β-adrenergic receptors and their associated equilibrium dissociation constants (K_d) were determined by non-linear regression employing the computer program Prism.

Analytical procedures

Protein concentration was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985). Phospholipids were extracted from plasma membranes using the procedure of Bligh and Dyer (1959) employing 7.5 mg of butylated hydroxytoluene (BHT) per 100 ml of extraction solvent (2,6-ditert-butyl-p-cresol) as an antioxidant. Fatty acid methyl esters (FAMEs) were prepared by acid-catalyzed transmethylation of total plasma membrane lipids using 3 mol l^{-1} methanolic HCl (Christie, 1982). The FAMEs, dissolved and stored in hexane, were separated on a Hewlett Packard gas chromatograph (model 5840 A) employing a fused silica (film thickness 0.25 μm) Omegawax 320 capillary column (length 30 m; i.d. 0.32 mm; Supelco, Inc. Bellefonte, PA, USA) with nitrogen as the carrier gas under isothermal conditions (190 °C). Fatty acids were identified by comparison with known standards. The cholesterol content of membrane fractions was determined by an enzymatic/fluorometric assay employing cholesterol standards (Crockett and Hazel, 1995). The phospholipid content of membrane fractions was determined colorimetrically by assaying inorganic phosphate levels (Rouser et al., 1970).

Statistical analyses

All statistical differences (at $P<0.05$) were calculated using analysis of variance (ANOVA) employing the computer program Systat. Values are presented as means ± S.E.M.

Results

Production of cyclic AMP by isolated hepatocytes

Epinephrine-stimulated production of cAMP was found to be linear with time for a period of 7 min regardless of assay or acclimation temperature (data not shown); consequently, all assays were conducted for a period of 7 min. Furthermore, epinephrine-stimulated rates of cAMP production were significantly higher ($P<0.024$) than basal rates for both acclimation groups at both 5 °C and 20 °C (Fig. 1). Both epinephrine-stimulated rates of cAMP production and the difference between stimulated and basal rates were consistently higher in hepatocytes assayed at 20 than 5 °C regardless of acclimation temperature; however, hepatocytes from 5 °C-acclimated fish were unusual in that basal rates were actually higher at 5 than 20 °C. Rates of cAMP production (both epinephrine-stimulated and stimulated minus basal) were significantly higher (2.4-fold at 5 °C and 1.7-fold at 20 °C, respectively) in hepatocytes of 5 °C- than of 20 °C-acclimated trout. Thus, β-mediated signal transduction, although slowed by an acute drop in assay temperature, was significantly temperature-compensated. In fact, rates of cAMP production in 5 °C-acclimated trout assayed at 5 °C were significantly ($P<0.05$) greater (1.3-fold for epinephrine-stimulated and 1.6-fold for the difference between stimulated and basal rates) than those in 20 °C-acclimated trout assayed at 20 °C, indicating over-compensation of this signaling pathway.

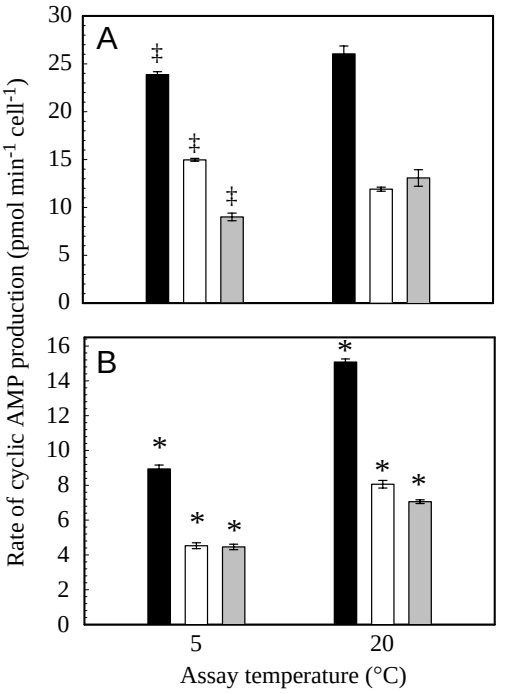

Fig. 1. Basal (open columns) and epinephrine-stimulated (0.5×10^{-6} mol l^{-1}) (filled columns) rates of cyclic AMP production and the difference between stimulated and basal (shaded columns) rates in hepatocytes isolated from 5 °C- and 20 °C-acclimated trout assayed at 5 °C and 20 °C. Values are plotted as means ± S.E.M. for three separate experiments. (A) 5 °C-acclimated trout; (B) 20 °C-acclimated trout (note the difference in ordinate axis dimensions between A and B). In all cases, epinephrine-stimulated rates were significantly ($P<0.05$) higher than basal rates. An asterisk indicates a significant ($P<0.05$) difference between acclimation groups assayed at the same assay temperature. A double dagger indicates a significant difference ($P<0.05$) between acclimation groups when assayed at the acclimation temperature.

Fluorescence polarization measurements

One possible explanation for the observed conservation of endocrine function may be that homeoviscous adaptation (HVA; Sinensky, 1974), by fluidizing the membrane at low temperatures, facilitates collision coupling and the maintenance of transmembrane signal-transduction activity. Therefore, we elected to determine the effect of acclimation temperature on plasma membrane lipid order in whole cells as an index of membrane physical properties. Although an acute reduction in assay temperature ordered the membrane (as reflected in higher polarization values) in both the interfacial (TMA-DPH) and interior (DPH) regions of the bilayer in both acclimation groups, absolute differences in membrane order between acclimation groups were probe-specific (Table 1). In the bilayer interior, lipid order was significantly lower in cold- than in warm-acclimated fish (efficacy of HVA approximately 50 %), whereas the opposite was true in the interfacial region. These data indicate that both the acclimatory and acute effects of temperature on signal-transduction activity are inversely correlated with temperature-dependent changes in membrane

Table 1. *Fluorescence polarization values of intact hepatocytes*

Assay temperature (°C)	5 °C-acclimated	20 °C-acclimated
DPH		
5	0.298±0.003‡	0.316±0.022*
20	0.249±0.006*	0.280±0.054‡
TMA-DPH		
5	0.353±0.013‡	0.335±0.011
20	0.342±0.027	0.325±0.017‡

Polarization values are reported as the mean ± S.E.M. for three separate experiments.

* indicates a significant effect of assay temperature (P<0.036); ‡ indicates a significant effect of acclimation temperature (P<0.006).

DPH, diphenyl hexatriene; TMA-DPH, trimethylammonium diphenyl hexatriene.

order sensed in the interior of the bilayer, i.e. signal-transduction activity is greatest under conditions of lowest order (either at an assay temperature of 20 °C relative to 5 °C in both acclimation groups, or in cold- compared with warm-acclimated trout regardless of assay temperature).

Fatty acid and cholesterol composition of plasma membranes

That membrane order in the bilayer interior is partially conserved as a consequence of temperature acclimation suggests that membrane composition is remodeled in response to a change in growth temperature. Fatty acid analysis of isolated plasma membranes revealed few significant differences in fatty acid composition between acclimation groups, and the small differences in individual fatty acids did not sum to significant differences in the unsaturated fatty acid/saturated fatty acid (UFA/SFA) ratio (2.99±0.39 in 5 °C-acclimated *versus* 2.27±0.103 in 20 °C-acclimated trout) or the unsaturation index (0.92±0.02 *versus* 0.88±0.06); however, polyunsaturated fatty acid (PUFA – those fatty acids with 2 or more double bonds) levels were consistently higher in cold- than warm-acclimated fish (55.6±5.2 % in 5 °C-acclimated fish *versus* 47.5±6.4 % in 20 °C-acclimated fish; Table 2). Elevated levels of PUFAs in 5 °C-acclimated trout arose from a combination of a significant (P<0.054) increase (48 %) in the proportions of eicosapentanaeoic acid (20:5 *n*-3) and the summed contributions of non-significant increases in arachidonic acid (20:4 *n*-6; 52 %), eicosaenoic acid (20:1 *n*-9; 31 %) and eicosatrienoic acid (20:3 *n*-6; 26 %) (Table 2). Conversely, cholesterol levels relative to phospholipid, did not differ significantly between acclimation groups.

Alteration of membrane order and rates of cyclic AMP production by benzyl alcohol

To determine whether the observed conservation of β-adrenergic signal-transduction activity in thermally acclimated trout (Fig. 1) was due to acclimation-temperature-induced changes in membrane order of the magnitude reported in Table 1, we elected to disorder the plasma membrane isothermally (at 5 °C) of 20 °C-acclimated fish (N=3) at 5 °C by the addition

Table 2. *Plasma membrane fatty acids (percentage composition)*

Fatty acid	5 °C-acclimated (mol%)	20 °C-acclimated (mol%)
16:0	19.32±3.71	24.92±5.14
16:1	4.27±0.76	3.46±0.52
18:0	5.83±0.55	7.72±1.05
18:1 *n*-9	12.03±1.66	17.36±5.17
18:2 *n*-6	4.65±0.18	3.89±0.10
20:1 *n*-9	2.96±0.40	2.05±0.32
20:3 *n*-6	4.06±0.06	2.99±0.36
20:4 *n*-6	1.06±0.14	0.49±0.68
20:5 *n*-3	6.52±0.61	3.88±0.28**
22:5 *n*-3	1.09±0.14	1.36±0.01
22:5 *n*-6	2.34±0.64	4.05±1.10**
22:6 *n*-3	35.87±3.39	30.79±3.86
Cholesterol/ phospholipid (mol/mol)	0.085±0.041	0.125±0.046

Values are means ± S.E.M. for three separate experiments.

** indicates significant difference between acclimation temperatures (P<0.05).

of benzyl alcohol until the membrane order was equivalent to that of a 5 °C-acclimated fish assayed at 5 °C and to determine the effect on rates of cAMP production. Thus, in the absence of benzyl alcohol, plasma membranes of hepatocytes from 20 °C-acclimated trout were significantly more ordered at 5 °C (polarization value 0.316) than plasma membranes of hepatocytes from 5 °C-acclimated trout at the same temperature (polarization value 0.298; Table 1) and supported significantly lower rates of cAMP production (Fig. 1). While maintaining the assay temperature at 5 °C, the isothermal addition of benzyl alcohol to hepatocytes from the 20 °C-acclimated trout gradually decreased the membrane order (Fig. 2) until, at a concentration of 30 mmol l^{-1}, membrane order in hepatocytes of the 20 °C-acclimated trout at 5 °C was equivalent to that in 5 °C-acclimated trout at 5 °C in the absence of benzyl alcohol (the dashed line in Fig. 2). If the higher rates of cAMP production at 5 °C by hepatocytes of 5 °C-acclimated (approximately 24 pmol min^{-1} $cell^{-1}$) than of 20 °C-acclimated (approximately 9 pmol min^{-1} $cell^{-1}$) trout are entirely, or in part, due to compensatory adjustments in lipid composition (Table 2) resulting in a less ordered (or more fluid) membrane (as indicated by the data in Table 1), then disordering the plasma membranes of hepatocytes from 20 °C-acclimated trout at 5 °C by the addition of benzyl alcohol would be expected to increase the rate of cAMP production. As illustrated in Fig. 3, a reduction in assay temperature from 20 to 5 °C depressed the rate of cAMP production (by approximately 1.7-fold) in hepatocytes from 20 °C-acclimated trout, consistent with a possible inhibitory effect of increased membrane order at 5 °C on signal-transduction activity. However, even though the addition of 30 mmol l^{-1} benzyl alcohol to hepatocytes of 20 °C-acclimated trout at 5 °C was

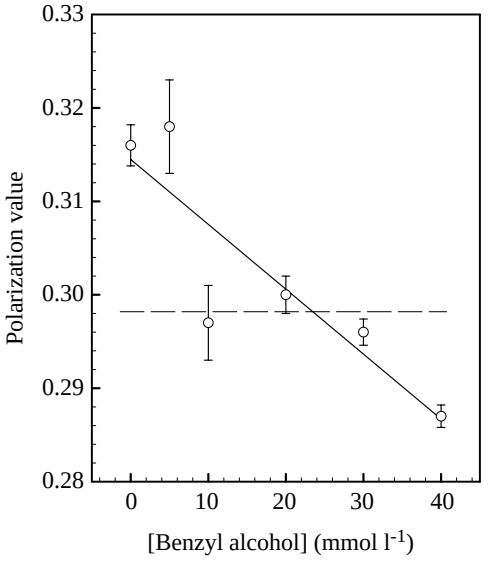

Fig. 2. Effects of benzyl alcohol on plasma membrane order (i.e. DPH polarization values) in hepatocytes isolated from 20 °C-acclimated fish assayed at 5 °C. Values are plotted as means ± S.E.M. for three separate experiments. The dashed reference line indicates the polarization value determined in plasma membranes of hepatocytes from 5 °C-acclimated fish at an assay temperature of 5 °C (refer to Table 1). The solid line is the best-fitting linear regression through the data points (r^2=0.7731, P=0.0209).

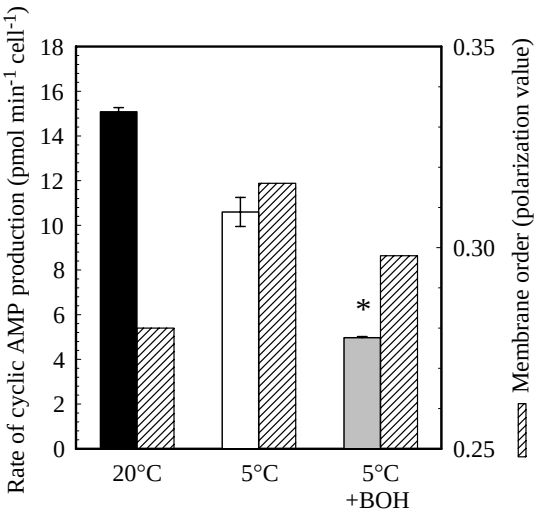

Fig. 3. Effects of assay temperature and the addition of 30 mmol l^{-1} benzyl alcohol (BOH) on epinephrine-stimulated (0.5×10^{-6} mol l^{-1}) rates of cyclic AMP production (filled, open and shaded columns) and membrane order (hatched columns) in hepatocytes isolated from 20 °C-acclimated fish. Values are plotted as means ± S.E.M. for three separate experiments. An asterisk indicates a significant difference (P<0.05) between assays run in the presence and absence of benzyl alcohol at 5 °C.

effective in isothermally fluidizing the plasma membranes to the desired extent (Fig. 2; i.e. to a polarization value of 0.298, identical to that in membranes of hepatocytes from 5 °C-acclimated trout at 5 °C), it not only failed to increase the rate of cAMP production, but actually reduced the rate by an additional twofold (Fig. 3). Thus, rates of cAMP production in this experiment are not consistently correlated with membrane order.

Time course of specific [^{3}H]CGP binding

Since, as indicated by the benzyl alcohol experiments, altered membrane physical properties did not provide an explanation for the elevated signal-transduction activity of cold- compared with warm-acclimated trout, we measured the number and affinity of hepatic β-receptors using whole cells and measuring both the receptor number and the associated K_d. Initial experiments were performed to establish the time required for equilibrium binding. As illustrated in Fig. 4, [^{3}H]CGP binding to adrenoceptors in hepatocytes isolated from 20 °C-acclimated trout assayed at 20 °C required 90 min to reach equilibrium; a similar time course was determined for 5 °C-acclimated fish (data not shown). Consequently, all subsequent binding assays were incubated for 90 min.

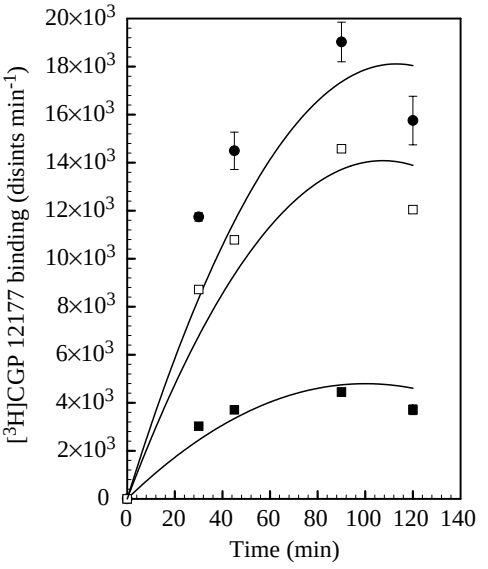

Fig. 4. Representative example of the time course of [^{3}H]CGP 12177 binding. Data are for a 20 °C-acclimated fish assayed at 20 °C. Results are presented as disints min^{-1} for 6×10^5 cells determined with 5 nmol l^{-1} CGP. ●, total binding; □, specific binding; ■, non-specific binding (determined in the presence of 200 μmol l^{-1} isoproterenol).

Isoproterenol competition curve

The objectives of these experiments were (1) to determine the concentration of isoproterenol necessary to displace [^{3}H]CGP fully from binding to the β-adrenergic receptor; (2) to determine whether CGP was binding to a single site or to multiple specific sites; and (3) to assess the effects of netting stress on the binding assay. To this end, the effect of increasing the concentration of isoproterenol (up to 10 mmol l^{-1}) on the binding of the β-agonist [^{3}H]CGP was determined (Fig. 5). Nonlinear least-square regression analysis of these data

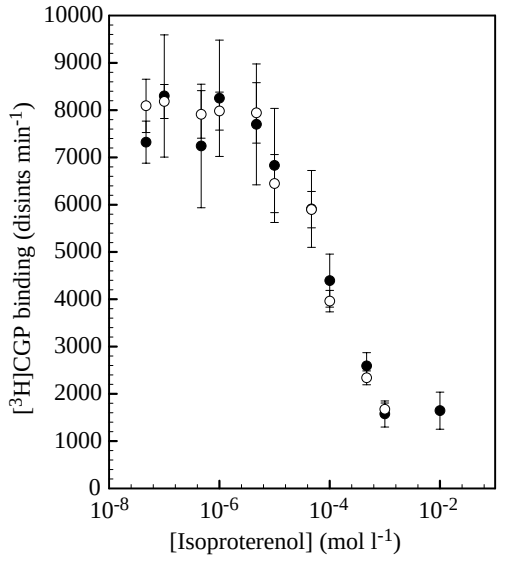

Fig. 5. Isoproterenol competition curve for [^{3}H]CGP 12177 binding to hepatocytes isolated from 20 °C-acclimated fish. ○, anesthesia after transport to the laboratory; ●, anesthesia prior to transport to laboratory. Values are plotted as means ± S.E.M. of triplicate assays for a single experiment.

(employing GraphPad Prism software), in addition to Scatchard analysis (Fig. 6, inset), indicated a significantly better fit to a single-site than to a two-site model. Finally, competition curves for fish transported to the laboratory prior to anesthesia compared with those anesthetized prior to transport were identical, demonstrating the absence of an effect due to handling stress (Fig. 5). In addition, a concentration of 200 μmol l^{-1} isoproterenol was sufficient to displace [^{3}H]CGP binding and was therefore used in all subsequent binding assays to determine nonspecific CGP binding.

Receptor number and K_d

Acclimation temperature significantly influenced the maximal binding of the β-adrenergic agonist CGP to trout hepatocytes (Fig. 6). Most notably, receptor numbers were significantly higher (by 1.81-fold; $P<0.017$) in hepatocytes of cold-acclimated compared with warm-acclimated trout regardless of assay temperature (Table 3). In addition, the number of β-adrenergic receptors was higher in hepatocytes from both acclimation groups when assayed at 20 °C compared with 5 °C. In contrast, K_d values (10–14 nmol l^{-1}) were not significantly affected by either acclimation or assay temperature.

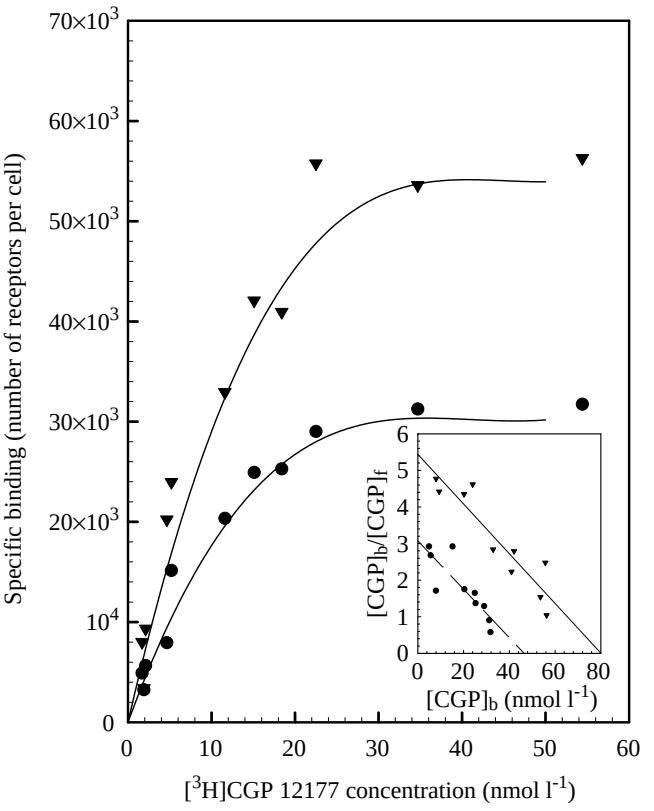

Fig. 6. Representative example of specific binding curves for [^{3}H]CGP 12177 in intact hepatocytes isolated from 5 °C-acclimated fish when assayed at 5 °C (●) and 20 °C (▼). The values plotted are the means of triplicate assays for a single experiment. Inset: Scatchard plots for specific binding data. $[CGP]_b$, concentration of bound CGP; $[CGP]_f$, concentration of free CGP.

Table 3. *[^{3}H]CGP 12177 binding characteristics*

Assay temperature (°C)	5 °C-acclimated	20 °C-acclimated
Receptor number (receptors per cell)		
5	62 320±4 206*,‡	14 560±1 641‡
20	87 969±5 855*	34 366±4 321
K_d (nmol l^{-1})		
5	13.73±4.33	12.16±4.33
20	11.42±3.34	9.75±3.29

Values are means ± S.E.M. for three separate experiments.

* indicates a significant difference ($P<0.017$) between acclimation groups; ‡ indicates a significant difference ($P<0.05$) between assay temperatures within an acclimation group.

Discussion

Although an acute drop in assay temperature from 20 to 5 °C significantly retards epinephrine-stimulated cAMP production in hepatocytes of 20 °C-acclimated trout, long-term exposure to low temperature more than restores the activity of this signal-transduction pathway. In fact, rates of cAMP production were significantly higher (1.6- to 2.4-fold) in hepatocytes isolated from 5 °C- than from 20 °C-acclimated fish regardless of assay temperature (Fig. 1). These results are consistent with previous studies employing perfused liver (McKinley and Hazel, 1993) and reinforce a common theme in the adaptation of vertebrate poikilotherms to low temperature (McKinley and Hazel, 1993; Umminger, 1971a–c): namely, the up-regulation of β-adrenergic regulation of hepatic glucose release and circulating glucose levels secondary to a drop in body

temperature. Although all these studies document, or are suggestive of, elevated rates of hormone-stimulated hepatic glucose release at low temperature, it remains unclear where in the multi-step pathway from the binding of hormone by the receptor to the actual release of glucose from the liver that this compensation occurs. One possibility is that collision coupling between membrane-resident components of the β-AR/AC signal-transduction pathway may be enhanced in cold-acclimated fish because of a decrease in membrane order associated with the well-established temperature-induced restructuring of membrane lipid composition (Hazel, 1995).

Fluorescence polarization measurements from the bilayer interior (reported by DPH), consistent with previous reports (Hazel et al., 1992), indicated significantly less order in plasma membranes of 5 °C- than 20 °C-acclimated trout, and membrane order was partially conserved (homeoviscous efficacy of approximately 50 %) when compared at the acclimation temperature (Table 1). Thus, the higher rates of cAMP production in hepatocytes of 5 °C- than of 20 °C-acclimated trout are in agreement with previous studies on whole cells (Rimon et al., 1978; Atlas et al., 1980; Hanski et al., 1979; Ma et al., 1994), demonstrating a direct correlation between plasma membrane fluidity and the rate of cAMP production. Collectively, these results suggest that acclimatory adjustments to membrane order in poikilotherms may play a role in regulating the activity of the β-adrenergic signaling pathway in response to prolonged thermal challenge.

Furthermore, temperature-induced changes in membrane lipid composition were, for the most part, consistent with the observed adjustments in membrane order. Exposure to cold consistently decreased the content of saturated fatty acids and increased the content of polyunsaturated fatty acids (PUFAs; Table 2). Nevertheless, the reduced rate of cAMP production (Fig. 3) in hepatocytes of 20 °C-acclimated trout isothermally fluidized by the addition of benzyl alcohol to a point matching the fluidity of plasma membranes in hepatocytes of 5 °C-acclimated trout at 5 °C (Fig. 2) indicates that the regulation of membrane physical properties plays no significant role in the thermal compensation of hepatic β-adrenergic signal transduction. However, the failure of changes in membrane order to explain the maintenance of endocrine function in the face of thermal perturbation does not preclude a role for restructured membrane phospholipid composition in the conservation of function that is independent of fluidity regulation. We report here increases in levels of PUFAs such as 20:4 *n*-6, 20:5 *n*-3, 20:1 *n*-9 and 20:3 *n*-6 with acclimation to 5 °C that, while consistent with a reduction in membrane order, may also interact directly with signal-transduction components.

The enzymatic activity of integral membrane proteins is frequently sensitive not only to membrane order but also to the specific chemical composition of their immediate lipid environment. For example, the activity of rat liver adenylyl cyclase increases in the presence of either membrane fluidizing agents (Houslay and Gordon, 1982; Wheeler et al., 1990) or some specific (e.g. *cis*-vaccenic or oleic acids) unsaturated fatty acids. Unsaturation located at the *n*-9 position of 18:1 is most effective in stimulating adenylyl cyclase activity when reconstituted in phosphatidylcholine vesicles (Calorini et al., 1993), while other unsaturated fatty acids (UFAs; i.e. 18:2 *n*-6), which are equally effective in fluidizing the membrane, are significantly less effective in stimulating enzyme activity (Houslay and Gordon, 1982). These data have been interpreted to indicate a requirement for a direct interaction between the cyclase and UFAs with a double bond in the C_9–C_{10} position. Indeed, the putative structure of adenylyl cyclase consists of 12 membrane-spanning helices organized into two transmembrane domains, which anchor and orient two cytoplasmic domains (C_1 and C_2; Hurley, 1999); the determinants of both nucleotide binding and catalysis are shared between the two cytoplasmic domains, and any factor that alters the relative orientation of these domains (e.g. forskolin) can alter the structure of the active site and thus change the substrate affinity, catalytic velocity or both (Hurley, 1999). Two potential regulatory sites that may bind lipid and conceivably alter the orientation of the C_1 and C_2 domains, one facing the cell exterior (Strader et al., 1989) and the other within the hydrophobic region of the bilayer, have been proposed (Levey and Lehotay, 1976). However, since only the levels of 20:5 *n*-3 (which does not possess the appropriate configuration to activate adenylyl cyclase) increased significantly in the plasma membranes of fish acclimated to 5 °C (Table 2) in the present study, this explanation seems unlikely.

One remaining possibility for the increase in β-adrenergic signal-transduction activity of 5 °C-acclimated compared with 20 °C-acclimated trout is an increase in either (or both) the number or affinity of β-adrenoceptors. Although receptor affinity was not significantly influenced by either assay or acclimation temperature (Table 3), receptor numbers were significantly higher (P<0.017) in 5 °C- than 20 °C-acclimated trout at both assay temperatures. When assayed at their respective acclimation temperatures, nearly twice as many β-adrenergic receptors are present in the plasma membranes of 5 °C-acclimated (62 320±4206 receptors per cell) as in 20 °C-acclimated fish (34 367±4321) (Table 3). The numbers of β-adrenergic receptors determined in this study are similar to those previously reported for trout erythrocytes (Reid et al., 1991) and rat liver (Hermsdorf et al., 1991), but are considerably higher than those reported for trout hepatocytes (1600–6000 receptors per cell; Reid et al., 1992). These differences may reflect the longer duration of the incubation with radioligand (90 *versus* 60 min) in the present study and, possibly, differences in acclimation temperatures. In addition, cortisol significantly elevates β-adrenoceptor numbers in trout hepatocytes (Reid et al., 1992; Dugan and Moon, 1998); since fish in the present study were maintained at temperatures (5 and 20 °C) close to the limits for the species, it is likely that chronic stress may have contributed to elevated receptor numbers.

Interestingly, receptor numbers were also significantly higher at 20 °C than at 5 °C in both acclimation groups

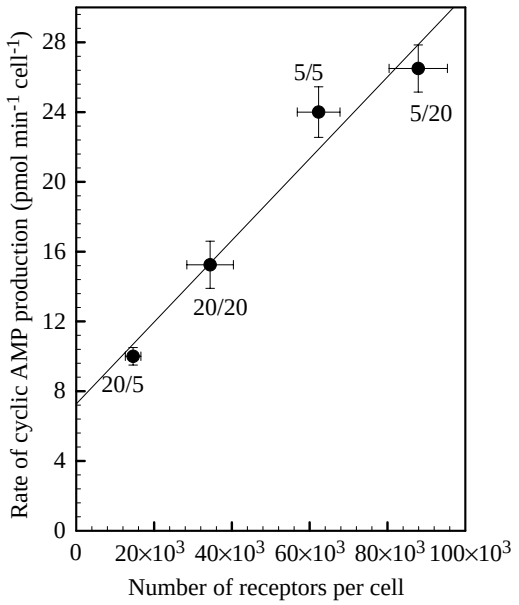

Fig. 7. Relationship between β-adrenergic receptor number and the rate of cyclic AMP production in hepatocytes isolated from 5 °C- and 20 °C-acclimated trout. Values are plotted in all cases as the mean ± S.E.M. for three separate experiments. The solid line represents the best-fitting linear regression of the data (r^2=0.956, P=0.0199). Individual points are identified with respect to acclimation and assay temperature (e.g. '20/5' corresponds to a 20 °C-acclimated fish assayed at 5 °C).

(Table 3). The observations that β-adrenergic receptor numbers vary inversely with acclimation temperature while being positively correlated with assay temperature suggest that independent regulatory mechanisms operate over the short and long term. Mechanisms that regulate the responsiveness of tissues to β-adrenergic stimulation range from receptor desensitization (in response to continuous activation by agonist), resulting from receptor phosphorylation by G-protein-coupled receptor kinases and sequestration/recycling of receptors to/from an intracellular compartment, to alterations in the total complement of receptors (receptor down-regulation) and/or the expression of cellular G-proteins (Jewell-Motz et al., 1997, 1998). Receptor internalization and up-regulation appear to be the most likely candidates for the alterations in receptor numbers observed in the present experiments in response to acute and acclimatory reductions in temperature, respectively. Regardless of mechanism, the correlation between the number of β-adrenergic receptors and the rates of cAMP production (Fig. 7) is strong (r^2=0.956) for all conditions of assay and acclimation temperature, consistent with the report of Marttila and Nikinmaa (1988) that the extent to which fish generate a β-adrenergic response is directly related to the apparent number of receptors. Therefore, we believe that the most likely explanation for the acclimatory response of the β-adrenergic signal-transduction pathway to low temperature in trout liver is a greater number of receptors expressed on the plasma membranes of hepatocytes in fish acclimated to 5 °C compared with 20 °C. This interpretation is supported by the similar observations of Keen et al. (1993) in cardiac tissue of thermally acclimated trout and suggests that up-regulation of receptor numbers is a common adaptational strategy to maintain endocrine signaling *via* the β-adrenergic pathway in organisms that remain active after acclimation to low temperatures.

In summary, the present study documents (1) a 1.6- to 2.4-fold greater β-adrenergic signal-transduction response in hepatocytes of 5 %- than of 20 °C-acclimated trout, (2) a 50 % compensation of membrane physical properties, presumably due to lipid restructuring, (3) a direct correlation between plasma membrane physical state (i.e. membrane order) and cAMP production when physical properties were perturbed isothermally by the addition of benzyl alcohol, and (4) a significantly higher number of β-adrenergic receptors in hepatocytes of 5 °C- than of 20 °C-acclimated trout, with no significant difference in $K_{d.}$ We conclude, on the basis of the strong correlation between β-adrenoceptor number and the rate of cAMP production (Fig. 7), that modulation of receptor number is probably the primary response of the β-adrenergic signal-transduction system to low temperature in trout liver.

This research was supported by NSF grant IBN 9507226 to J.R.H. We are indebted to the personnel of the Alchasay National Fish Hatchery for their assistance and the donation of rainbow trout, to Larry Nienaber for his excellent care and maintenance of the fish, to Paul Brunkow for his statistical expertise and guidance, to Miles Orchinick for use of his laboratory equipment as well as thoughtful guidance and discussions and to two anonymous reviewers for their constructive comments.

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