Immunomodulatory Ionophore Copolymers, T150R1 and T130R2, Induce Corticotropin from Anterior Pituitary Cells (43438)

RIMA HOUSSAMI AND IRENE J. CHECK[1]
Department of Pathology, Emory University, Atlanta, Georgia 30322

Abstract. **T150R1 is a synthetic copolymer with Na^+ ionophore activity. We demonstrated previously that T150R1, when injected into mice, produces rapid thymic involution with depletion of cortical thymocytes. Elevated serum ACTH and corticosterone levels, as well as abrogation of the effects of T150R1 on the thymus by adrenalectomy and hypophysectomy, suggested a pituitary-mediated mechanism. In this work, we investigated the ability of T150R1, and of the related ionophore copolymer T130R2, to stimulate ACTH *in vitro* from the mouse anterior pituitary cell line AtT-20. Copolymer-induced ACTH release was dose-, time-, and temperature-dependent. Hormone induction peaked at 30°C for T150R1 and 37°C for T130R2. The temperature dependence of ACTH release paralleled that of ionophore activity measured in red blood cells, providing evidence that the ability to induce ACTH is related to the ionophore property of the copolymers. Peak ionophore activity and hormonal release occurred at the temperatures when the copolymers form partially soluble complexes which interact optimally with cell membranes. Cotreatment with exogenous phospholipase C inhibited the effects of T150R1, which suggests that the enzyme either blocks the insertion of T150R1 into the cell membrane or that the phospholipase C-induced increase in intracellular calcium inhibits the ionophore activity of T150R1. These data support an ionophore mechanism for copolymer-induced ACTH release in which changes in the physicochemical structure of the copolymers may affect their interaction with cell membranes. The data also suggest that direct stimulation of pituitary ACTH accounts for at least some of the *in vivo* immunomodulatory effects of T150R1.** [P.S.E.B.M. 1992, Vol 200]

T150R1 and T130R2 are amphiphilic compounds composed of a central ethylenediamine and blocks of hydrophilic polyoxyethylene and hydrophobic polyoxypropylene (Fig. 1). They belong to a series of ionophore copolymers (reverse octablock copolymers) that have the ability to mediate the influx of sodium ions into cells by exchange diffusion with intracellular potassium (1).

When administered into mice, copolymer T150R1 produced a dose-dependent thymic involution followed by rebound hyperplasia (2). Elevated serum ACTH (adrenocorticotropic hormone, or corticotropin) and corticosterone levels and diminution of the effects by adrenalectomy indicated that this process was mediated by corticosteroids. T150R1 also caused hyperplasia of the spleen with pronounced hematopoiesis, which resulted in an increased production of granulocytes, erythrocytes, and megakaryocytes. The hematopoietic effects were not explained by corticosteroid action (2).

In vivo evidence suggested that T150R1 stimulates ACTH production by acting directly on the corticotrophs, since hypophysectomized mice injected with T150R1 presented with milder thymic involution and lower levels of serum ACTH than intact mice. T150R1 may also stimulate extrapituitary ACTH-producing cells, since hypophysectomy did not totally abolish ACTH production (2).

In the work reported in this paper, we investigated the effects of T150R1 and T130R2 on the corticotrophs using the mouse anterior pituitary cell line AtT-20. These tumor cells are relatively homogeneous (3) and, like normal corticotrophs, they possess a receptor for the hypothalamic hormone corticotropin-releasing fac-

[1] To whom requests for reprints should be addressed at Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322.

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tor (4). Corticotropin-releasing factor stimulates ACTH from anterior pituitary cells via a cAMP-mediated pathway (5). Other hypothalamic factors, such as arginine-vasopressin and oxytocin, also induce ACTH, but utilize a phosphoinositol-mediated pathway (6).

Since block copolymers have been shown to induce histamine release from mast cells in a calcium-dependent manner which correlated with their ionophore activity (7), we predicted that they might cause ACTH release via a similar mechanism. The experiments in this study were designed to test whether T150R1 and T130R2 could directly stimulate ACTH production from AtT-20 pituitary cells, and to determine whether their ionophore activity and physicochemical structure play a role in this biological effect.

Materials and Methods

Materials. T150R1 and T130R2 were purchased from BASF Wyandotte Corp. (Parsippany, NJ). Phospholipase C was purchased from Sigma Chemical Co. (St. Louis, MO). The copolymers were dissolved in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) for the AtT-20 cell experiments or in 0.9% NaCl for the red blood cell experiments. Because the solubility of these compounds in aqueous conditions decreases with increasing temperature, concentrated solutions were made with cold (4°C) medium (or 0.9% NaCl), then equilibrated to the desired temperature before use. These concentrated solutions were diluted with temperature-adjusted medium at the time of their use. Phospholipase C was dissolved in fresh medium at the desired temperature.

Cell Cultures. Subclones (Passage 44) of AtT-20 mouse pituitary tumor cells were purchased from American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 μg/ml of streptomycin, and 100 units/ml of penicillin. Cultures were maintained at 37°C under 5% CO_2 and 95% air in a humidified atmosphere, and were used in the range of 46th to 64th generations.

ACTH Secretion Experiments. AtT-20 cells (0.5 or 1 ml) were plated in 48-well microtiter plates (Costar, Cambridge, MA) or in culture tubes at a density of 0.5–2 × 10^5 cells/ml and incubated at 37°C for 24 hr. The next day the medium was replaced with serum-free medium, then concentrated reagents were added to the cells. Further incubations were carried out at various temperatures and times as indicated for each experiment. At the end of the incubation period, the medium was removed and centrifuged at 150*g* for 5 min at 4°C to remove residual cells. Aliquots of the supernatant were stored at −20°C for later ACTH determination. Samples treated with serum-free medium alone under similar conditions served as controls. ACTH was measured in centrifuged supernatants for untreated controls,

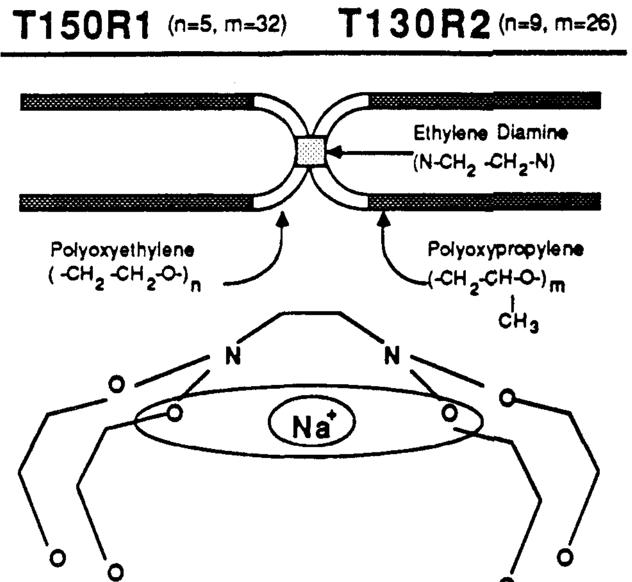

Figure 1. Structure of reverse octablock copolymers. Four blocks of hydrophilic polyoxyethylene flanked by four blocks of hydrophobic polyoxypropylene are attached to a central ethylenediamine. The number of residues in each block is listed for T150R1 and T130R2. A cation-chelating cage is formed by the core ethylenediamine moiety and the proximal hydrophilic polyoxyethylene units. The cage is stabilized by insertion of the hydrophobic peripheral blocks into the lipid bilayers. This structure enables the copolymers to function as ionophores by binding monovalent cations in the hydrophilic center then crossing the cell membrane (1).

and in uncentrifuged, freeze-thawed samples for total release.

ACTH Determination by Radioimmunoassay. ACTH radioassay kits were purchased from ICN Biochemicals Inc. (Carson, CA) and the assay was performed according to instructions in the package insert. Culture supernatant samples were tested either undiluted or diluted, with serum-free culture medium if necessary, to generate results within the linear portion of the standard curve. These kits were designed to measure human ACTH, but they can be used to measure mouse ACTH, since the antibody reacts with sites in residues 1-24 and 1-39. The assay is sensitive to 10 pg/ml of ACTH and is highly specific, showing less than 1% cross-reaction with β-endorphin, α- and β-melanocyte stimulating hormone and α-lipotropin and less than 0.8% with β-lipotropin. The assay is a competitive binding assay in which rabbit anti-ACTH binds to ACTH in the sample, which competes for binding to a radioiodinated synthetic peptide of residues 1-39 of human ACTH. Bound ^{125}I-ACTH was separated from the unbound label by precipitation and radioactivity in the precipitates was counted using a gamma counter (Gamma trac 1191; Tracor Analytic, Elk Grove Village, IL). The concentration of ACTH in the test samples was calculated with reference to a standard curve. The results are expressed in pg/ml.

Ion Flux Measurements in Red Blood Cells. Hu-

man blood anticoagulated with lithium heparin was obtained from healthy normal volunteers. The blood was washed three times with 0.9% NaCl by centrifugation for 10 min at room temperature at 150*g* and diluted with saline to 10% hematocrit. Tubes containing equal volumes of the washed blood suspension were incubated in 4, 20, 25, 30, and 37°C waterbaths for 15 min. Preincubated concentrated solutions of T150R1 and T130R2 copolymers were vortexed thoroughly and added to the red cells for a final copolymer concentration of 30 μg/ml in a total volume of 1 ml/tube. The tubes were incubated in the waterbaths for 30 min, then centrifuged for 10 min at 150*g*. Samples of the supernatant were pipetted and assayed for both potassium and hemoglobin content in order to control for any potassium released due to lysis during handling.

Potassium Measurement. The amount of potassium in the supernatants was determined by flame emission spectroscopy using an IL 943 Flame Photometer (Instrumental Laboratory Inc., Lexington, MA). The results are expressed in mmol/liter. The standards for low and high amounts of plasma potassium (5 and 140 mmol/liter, respectively) were obtained from Instrumental Laboratory.

Hemoglobin Measurement. Hemoglobin content of the supernatants was determined by a spectrophotometric assay in which the absorbance of the sample is measured at three different wavelengths using a Beckman DU-7 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). A mathematical correction is applied in order to minimize interference from bilirubin and lipidemia as explained in the method published by Bradley *et al.* (8). Dilutions of the samples were made when needed using 1.0 *M* phosphate buffer (pH 7.5), which serves as a blank as well. The results are expressed in mg/dl.

Correction of Potassium Values for Hemolysis. The concentration of potassium in samples of copolymer-treated supernatants included potassium released by ionophoric action of the copolymers, as well as trace amounts that may have been released by spontaneous hemolysis during handling. In order to correct for this, we produced a standard curve of potassium concentration versus hemoglobin concentration for washed red cell samples with known percentages of hemolysis ranging from 0 to 5%. The concentration of potassium that could be due to hemolysis was calculated from the standard curve and subtracted from the total potassium in the sample. The concentration of potassium released due to lysis was no more than 0.4% with T150R1 and 0.5% with T130R2.

Assessment of Copolymer Solubility in Aqueous Solutions. In preliminary experiments, T150R1 and T130R2 were solubilized in ice-cold 0.9% NaCl at concentrations ranging from 0.1 to 3 mg/ml and samples of these solutions were incubated in waterbaths of temperatures ranging from 4 to 37°C. After 15 min of equilibration, the absorbance of each sample was read at wavelengths ranging from 300 to 700 nm using a Beckman spectrophotometer. Since the absorbance at 420 nm using copolymer concentrations of 0.37 mg/ml gave a broad range of spectrophotometric readings and agreed with the visual perception of cloudiness in the solutions, these conditions were used in the study of the effects of temperature on copolymer solubility in an aqueous solution. T150R1 and T130R2 were dissolved in 0.9% NaCl at 0.37 mg/ml. Duplicate samples of these solutions were placed in a 5-liter 37°C waterbath, which was allowed to cool to ambient temperature, then was cooled further by adding small amounts of ice with mixing. Samples were taken approximately with every 0.5 to 1°C change in temperature as measured with a thermometer placed in the waterbath. Spectrophotometric measurements were taken at 420 nm using 0.9% NaCl as a blank.

Statistics. Statistical analyses were carried out with Student's two-tailed unpaired t test; $P < 0.05$ was considered significant. Unless otherwise stated, all data are presented as mean ± SE.

Results

Characterization of T150R1 and T130R2-Induced ACTH Release by AtT-20 Cells. *Stimulation of ACTH release by ionophore copolymers T150R1 and T130R2.* Previous studies of the biological activities of reverse octablock copolymers showed that both T150R1 and T130R2 had ionophore activity. T130R2 was a more potent sodium ionophore, but since it was considerably more inflammatory (7), *in vivo* studies of copolymer effects on the immunoendocrine and hematopoietic systems were limited to T150R1 (2). In this study, the effects of both copolymers on ACTH release by AtT-20 pituitary tumor cells were compared. AtT-20 cells were treated with 30 μg/ml of either copolymer or medium for 60 min at 37°C (Fig. 2). Both T150R1 and T130R2 induced significant release of ACTH ($P < 0.001$); however, the ACTH-releasing activity of T130R2 was twice as potent as the activity of T150R1 ($P < 0.001$).

Dose response of ACTH release by T150R1. AtT-20 cells were treated with various concentrations of T150R1 or medium for 60 min at 30 or 37°C. As shown in Figure 3, T150R1 produced dose-dependent ACTH secretion; 10 μg/ml of copolymer were sufficient to induce significant ACTH release ($P = 0.04$) at either temperature. At 30°C, the amount of ACTH released increased with increasing concentration of copolymer. However, at 37°C, higher doses were not significantly more effective than the 10-μg/ml dose. The highest concentration of copolymer showed no detectable effect on the viability of the cells as measured by trypan blue

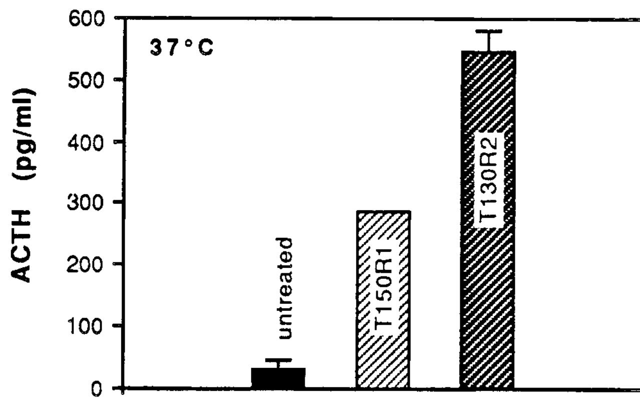

Figure 2. Stimulation of ACTH release by ionophore copolymers T150R1 and T130R2. AtT-20 cells (5×10^4 cells/ml) were preincubated in medium for 24 h at 37°C in a 48-multiwell plate. The next day, the medium in each well was replaced with serum-free medium to which 30 μg/ml of T150R1 or T130R2 equilibrated at 37°C were added. The cells were incubated for an additional 60 min. The supernatants were removed and aliquots of the samples were assayed for ACTH by radioimmunoassay as described in Materials and Methods. Samples from AtT-20 cells treated with serum-free medium alone for 60 min served as untreated controls. This is representative of three experiments, and data points are the mean ± SE of three determinations (T150R1 versus untreated, $P < 0.001$; T130R2 versus untreated, $P < 0.001$; T130R2 versus T150R1, $P < 0.001$).

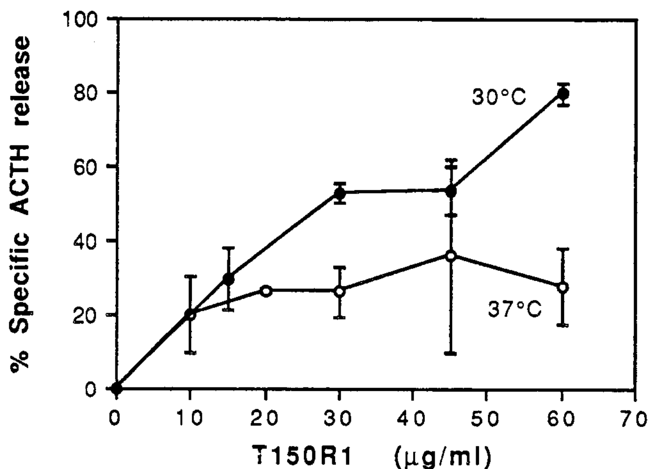

Figure 3. Dose-dependent stimulation of ACTH release from AtT-20 cells by T150R1. AtT-20 cells (5×10^4 cells/ml for the 30°C experiment, and 1.5×10^5 cells/ml for the 37°C experiment) were preincubated in medium for 24 h at 37°C in a 48-multiwell plate. The next day, the medium was replaced with serum-free medium to which different amounts of T150R1 equilibrated at 30°C or 37°C were added, and the cells were incubated at that temperature for 60 min. The supernatants were removed and aliquots of the samples were assayed for ACTH by radioimmunoassay, as described in Materials and Methods. AtT-20 cells treated with serum-free medium alone served as negative controls. Parallel aliquots of cells were freeze-thawed to determine total ACTH content. The percentage of specific ACTH release was calculated as follows: 100 ([total ACTH-sample ACTH]/[total ACTH-untreated control ACTH]). This is representative of three experiments, and data points are the mean ± SE of three determinations (doses 10 μg/ml vs 0 μg/ml, $P = 0.04$ at either temperature).

exclusion, indicating that the ACTH release did not result from lysis of the cells.

Time course of ACTH release by T150R1. AtT-20 cells were treated with 30 μg/ml of T150R1 or medium at 30°C for various times. Within 5 min, T150R1 stimulated approximately a 1.7-fold increase in ACTH as compared with untreated controls ($P = 0.002$). The concentration of ACTH gradually increased throughout the 60-min incubation period (Fig. 4). The data shown are from an experiment in triplicate microtiter wells. Since the cells have a tendency to aggregate, leading to some variability in the number of cells dispensed per well, the experiments were also repeated in test tubes, in which aliquots of supernatant from the same cell population could be sampled over time. Similar time course results were obtained using either configuration at 30 or 37°C (data not shown).

Studies of Mechanism of Copolymer-Induced ACTH Release by AtT-20 Cells. The above experiments showed that at physiologic temperatures, T130R2 released more ACTH than did T150R1. The dose-response and time course experiments with T150R1 showed, however, that T150R1 is more potent at 30°C. Our working hypothesis was that the mechanism of copolymer-induced ACTH release involved their ionophore activity. Since reverse octablock copolymers are soluble in the cold, but their solubility decreases as physiologic temperatures are approached, we wondered whether either biological activity (ACTH release or ionophore activity) was related to tempera-

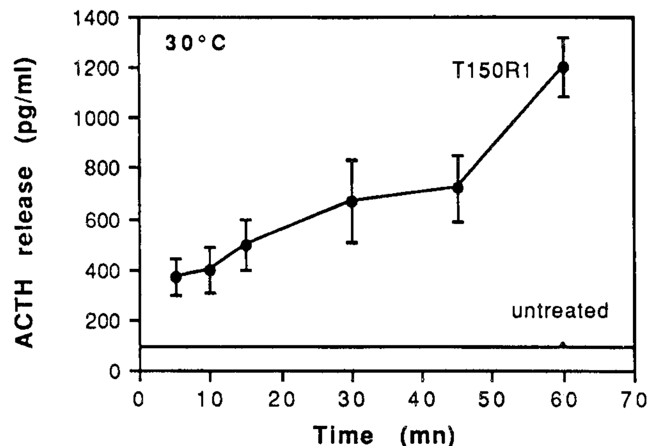

Figure 4. Time course of ACTH release from AtT-20 cells by T150R1. AtT-20 cells (2×10^5 cells/ml) were preincubated in medium for 24 h at 37°C in a 48-multiwell plate. The next day, the medium in each well was replaced with serum-free medium to which 30 μg/ml of T150R1 equilibrated at 30°C were added. The cells were incubated further at that temperature. At the time points indicated, the supernatants were collected and aliquots of the samples were then assayed for ACTH by radioimmunoassay, as described in Materials and Methods. Samples from AtT-20 cells treated with serum-free medium alone for 60 min served as untreated controls as indicated by the dotted line. This is representative of three experiments, and data points are the mean ± SE of three determinations (T150R1 versus untreated, $P = 0.002$ at 5 min).

ture-dependent differences in solubility. The following experiments measured the effects of temperature on ACTH release, ionophore activity, and solubility of copolymers T150R1 and T130R2.

Effect of Temperature on T150R1- and T130R2-Induced ACTH Release by AtT-20 Cells. AtT-20 cells were treated with 30 μg/ml of either copolymer or medium for 60 min at temperatures ranging from 4 to 37°C (Fig. 5a). The two copolymers exhibited different temperature optima in stimulating ACTH secretion. With T150R1, ACTH release was maximal at 30°C and decreased significantly as the temperature was raised or lowered (Fig. 5a, left). The activity at 37°C was only 25% of that at 30°C and there was a small, but statistically significant, amount of ACTH release at 4°C. In contrast, with T130R2, ACTH release peaked at 37°C, decreased to 10% at 30°C, and was minimal at lower temperatures (Fig. 5a, right).

Temperature-Dependent Potassium Release from Human Red Blood Cells by T150R1 and T130R2. In order to assess the effect of temperature on ionophore activity, we measured copolymer-induced potassium release from red blood cells. Atkinson *et al.* (1) have shown that potassium release correlates directly with sodium ion influx and used this system in the original experiments which described the ionophore activity of these compounds. Washed human erythrocytes were treated with 30 μg/ml of T150R1, T130R2, or medium for 60 min at various temperatures, and supernatants were assayed for potassium and hemoglobin content. T150R1 caused essentially no hemolysis, but T130R2 did lyse <0.5% of the cells at 37°C. Therefore, the

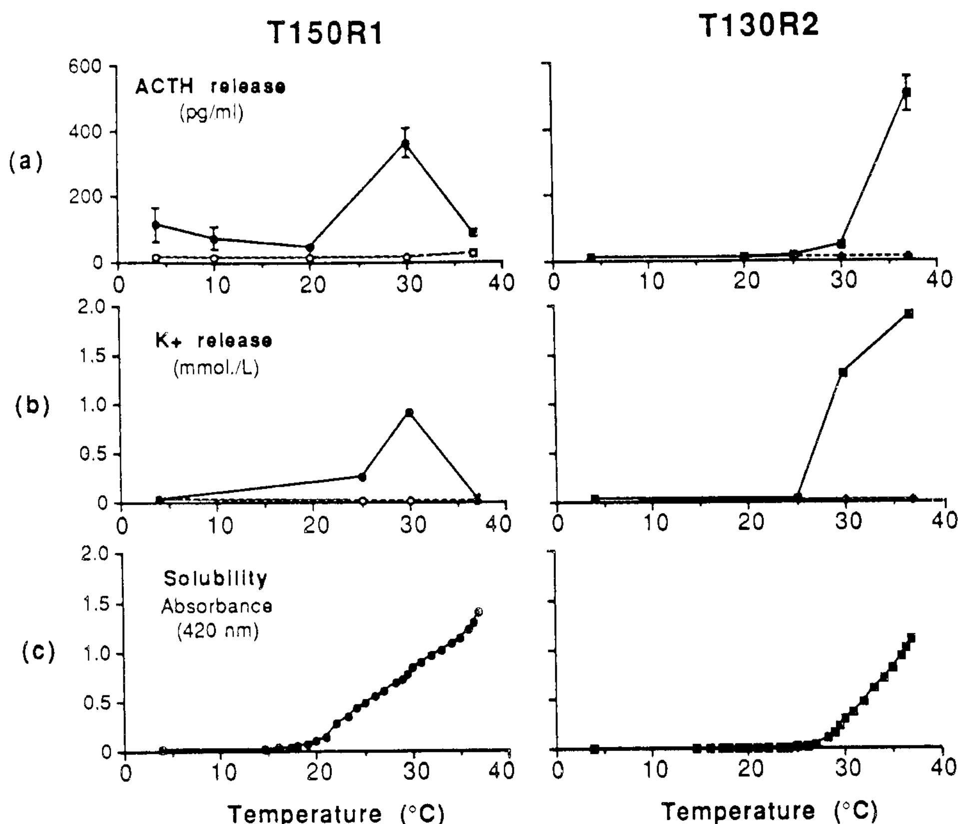

Figure 5. Effects of temperature on ACTH release, potassium release, and solubility of T150R1 and T130R2. (a) *ACTH release.* AtT-20 cells (2×10^5 cells/ml) were preincubated in medium for 24 hr at 37°C in culture tubes. The next day, the medium was replaced with serum-free medium to which 30 μg/ml of T150R1 or T130R2 equilibrated at the indicated temperature were added, and the cells were incubated 60 min in waterbaths at various temperatures. The tubes were centrifuged, supernatants were collected, and ACTH was determined by radioimmunoassay, as described in Materials and Methods. Samples from AtT-20 cells treated with serum-free medium alone for 60 min at different temperatures served as untreated controls. This is representative of three experiments, and data points are the mean ± SE of three determinations (T150R1 versus untreated, $P < 0.001$ at 30°C; $P = 0.02$ at 4°C). (b) *Potassium release.* Tubes containing equal volumes of a 10% suspension of human red blood cells in saline were preincubated in waterbaths at various temperatures. The red cells were treated with temperature-adjusted T150R1 and T130R2 (30 μg/ml), incubated for 30 min, then centrifuged to collect the supernatants. Samples of the supernatants were assayed for potassium and hemoglobin concentration as described in Materials and Methods. This is representative of three experiments that showed similar results. (c) *Solubility.* T150R1 and T130R2 were dissolved in 0.9% NaCl at 0.37 mg/ml and the tubes were placed in a 5-liter 37°C waterbath that was allowed to cool to ambient temperature, then was cooled further by adding small amounts of ice with mixing. Samples were taken approximately with every 0.5–1°C change in temperature as measured with a thermometer placed in the waterbath. Turbidity was measured by absorbance at 420 nm, using 0.9% NaCl as a blank. This is representative of three experiments, and data points represent the means of duplicate values.

potassium data were corrected for cell lysis as described in Materials and Methods. Both copolymers released potassium in a temperature-dependent pattern as shown in Figure 5b. The optimal temperature was 30°C for T150R1 and 37°C for T130R2, similar to the patterns seen for ACTH release by AtT-20 cells. At 30°C, both copolymers showed comparable amounts of ionophore activity. At 37°C, the activity of T130R2 increased while that of T150R1 diminished. Thus, at 37°C, the potency of T130R2 appeared to be 20-fold greater than that of T150R1, as seen previously by Atkinson *et al.* (1).

Effects of Temperature on Copolymer Solubility in Aqueous Solutions. To evaluate the solubility of the ionophore copolymers at different temperatures, we measured light scattering at 420 nm of 0.37-mg/ml solutions of each copolymer (Fig. 5c). Both copolymers were completely soluble at 4°C and their solubility decreased with increasing temperature. At this concentration, slight turbidity of the T150R1 solution was detectable at 15°C and gradually increased with a slope of 0.07 absorbance units/°C (Fig. 5c, left). Turbidity of the T130R2 solution was not detectable until 28°C, but then rapidly increased with a slope of 0.1 absorbance units/°C (Fig. 5c, right). Thus, T150R1 was much less soluble than T130R2 at 30°C, the optimal temperature for the biologic effects of T150R1 in the experiments above. Interestingly, the turbidity of the T150R1 solution at 30°C was nearly comparable to that of the T130R2 solution at 37°C, the temperature optimum for the biological effects of T130R2.

The relationships among copolymer solubility, ionophore activity (potassium efflux from red cells), and the ability to induce ACTH release from AtT-20 are apparent in these temperature curves. With either copolymer, ACTH release was maximal under conditions of maximal ionophore activity. However, at comparable levels of ionophore activity (30°C), T150R1 was more effective at inducing ACTH release.

Effects of cotreatment with T150R1 and phospholipase C on ACTH release by AtT-20 cells. The above experiments suggested that ionophore activity plays a role in T150R1-mediated ACTH release, but other factors related to its interaction with cell membranes must also play a role. Therefore, we examined the effects of exogenous phospholipase C on ACTH release by T150R1. Activation of endogenous phospholipase C within the cell membrane induces hormone release via the phosphoinositol-mediated pathway (5). AtT-20 cells were reacted at 37°C with two concentrations of phospholipase C (0.05 or 0.1 unit/ml), with or without T150R1 (30 μg/ml) (Fig. 6).

Cells treated with phospholipase C (0.05 or 0.1 unit/ml) released over twice as much ACTH as cells treated with medium alone ($P = 0.04$ and 0.05, respectively) (Fig. 6a). T150R1 alone released four times as much ACTH as did phospholipase C ($P < 0.001$) (Fig. 6b), and cotreatment with phospholipase C inhibited this release in a dose-dependent manner ($P = 0.04$ at 0.1 unit/ml) (Fig. 6b).

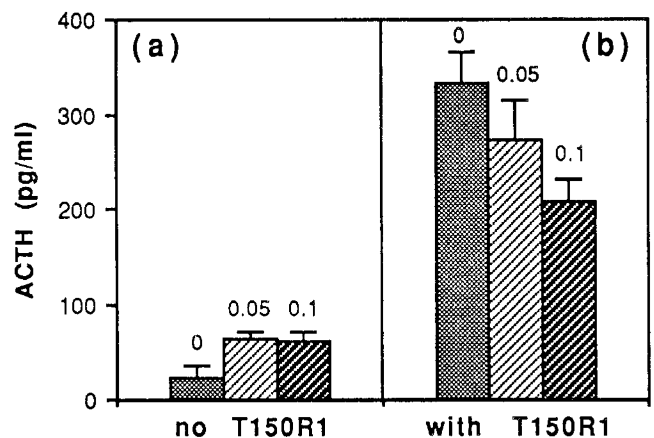

Figure 6. Effects of cotreatment with phospholipase C. AtT-20 cells (5×10^4 cells/ml) were preincubated in medium for 24 hr at 37°C in a 48-multiwell plate. The next day, the medium in each well was replaced with serum-free medium to which was added 0.05 or 0.1 unit/ml of phospholipase C (a) without or with (b) 30 μg/ml of T150R1. The cells were incubated for an additional 90 min at 37°C. The supernatants were collected and aliquots of the samples were assayed for ACTH by radioimmunoassay as described in Materials and Methods. Samples from AtT-20 cells treated with serum-free medium alone or with 30 μg/ml of T150R1 were used as controls. This is representative of two experiments, and data points are the mean ± SE of three determinations (phospholipase C versus untreated, $P = 0.04$ at 0.05 unit/ml and $P = 0.05$ at 0.1 unit/ml; T150R1 versus phospholipase C, $P < 0.001$; T150R1 versus T150R1 + 0.1 unit/ml of phospholipase C, $P = 0.04$).

Discussion

We have shown previously that copolymer T150R1 produces thymic involution followed by rebound hyperplasia when administered to mice. Elevated serum ACTH and corticosterone levels and diminution of the effects by adrenalectomy indicated that this process is mediated by corticosteroids (2). Experiments with hypophysectomized animals suggested that both pituitary and extrapituitary components contributed to ACTH production. In this paper, we examined how T150R1 might stimulate pituitary ACTH *in vivo* by studying the effects of ionophore copolymers T150R1 and the closely related T130R2 on ACTH secretion *in vitro* using the mouse anterior pituitary tumor cell line AtT-20. Since both T150R1 and T130R2 stimulated ACTH secretion from AtT-20 cells, we explored the role of their ionophore activity and physicochemical structure in producing this biological effect.

In the human red cell system described by Atkinson *et al.* (1), T150R1, T130R2, and other reverse octablock copolymers mediated exchange diffusion of Na^+ and K^+ by a carrier mechanism. When tested in excitable cells, these ionophores stimulated histamine release from mast cells and basophils (7, 9). The potency of

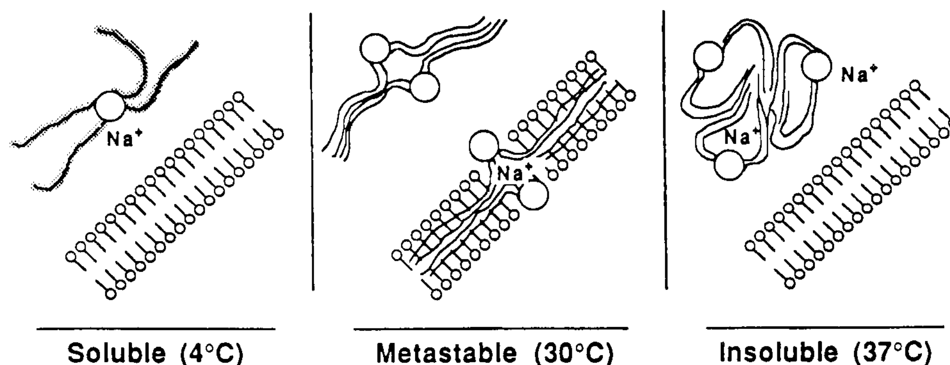

Figure 7. Model for effect of temperature on T150R1 structure and interaction with cell membranes. At 4°C, the copolymer is hydrated via hydrogen bonding with water molecules preventing interaction with cell membranes. At 30°C, the copolymer forms metastable complexes that interact optimally with cell membranes, facilitating sodium transport. At 37°C, large copolymer aggregates form that do not interact well with cell membranes.

their biological effects correlated with their ionophore activity, and, under the physiologic conditions of the assays, T130R2 was a 9-fold stronger ionophore and a 5-fold greater histamine-inducer than T150R1. The authors attributed cell activation to the rise in intracellular Na^+ concentration, which promoted an influx of Ca^{2+} through a Na^+/Ca^{2+} exchanger present in many excitable cells (10). Since ACTH secretion by AtT-20 cells can be triggered by membrane depolarization induced either by electrical stimulation (11, 12) or by agents that activate Na^+ and Ca^{2+} channels and initiate entry of Ca^{2+} (13), we predicted that ionophore copolymers stimulate ACTH release from AtT-20 cells by inducing a Ca^{2+} influx in response to altered intracellular Na^+.

T150R1 and T130R2 both released significant amounts of ACTH from AtT-20 cells and, interestingly, demonstrated distinctly different temperature optima for hormonal stimulation. At physiologic temperatures, T130R2 was 5-fold more potent in inducing ACTH release, consistent with its greater ionophore activity at 37°C. However, at 30°C, T150R1 was 7-fold more potent than T130R2. All of the studies of ionophore activity of T150R1 and T130R2 on red cells and of the induction of histamine release from mast cells reported by Atkinson *et al.* (1, 7) had been done at 37°C. Thus, the relative ionophore and biological activities of various copolymers at other temperatures have not been examined previously. Our studies showed that T150R1 was a more potent ionophore than T130R2 at lower temperatures. The temperature dependence of ionophore activity paralleled that of ACTH release, supporting the concept that ionophore activity had mediated this biological effect. However, under conditions in which T150R1 and T130R2 produced comparable amounts of potassium efflux (30°C), T150R1 released 9-fold more ACTH. If we assume that ionophore activity in AtT-20 cells is comparable to that in red cells, then ionophore activity alone was not sufficient to explain the potency of ACTH induction by T150R1.

We predicted that differences in the partitioning of copolymers into cell membranes might play a role in the biological effects of T150R1 and T130R2. One distinctive feature of reverse octablock copolymers is the decrease in their solubility as they are warmed to physiologic temperatures. This is due to differences in their hydrophile-lipophile balance (HLB), an estimate of surface activity that is related to the combined effects of hydrophobic and hydrophilic domains of amphiphilic compounds (14). Compounds with high HLB values are more water soluble and may not be able to adhere to or penetrate membranes, whereas compounds with low HLB values may be too insoluble to distribute into critical areas of the membrane. The HLB value as measured at 25°C is 1.0 for T150R1 and 2.9 for T130R2 (15). Since partially soluble and completely soluble copolymers might react differently with cell membranes, we studied the relationship between solubility of copolymers and their biological properties.

Interestingly, optimal ionophore activity against red blood cells and pituitary ACTH release occurred not at the temperatures at which the copolymers were completely soluble, but at an intermediate phase of solubility. T130R2 was completely soluble below 28°C and showed no ACTH release at low temperatures. Its optimum biological activity was at 37°C. T150R1 showed both low level turbidity and slight ACTH release at temperatures considerably below 20°C, but its optimum activity was at 30°C. The fact that the absorbance of the T150R1 solution at 30°C was nearly identical to that of the T130R2 solution at 37°C (0.843 vs 1.105 at 0.37 mg/ml) suggests that at this particular phase transition, aqueous dispersibility strongly contributes to biological activity. Under these conditions, the copolymers are neither completely hydrated and soluble, nor do they form stable hydrophobic aggregates. Instead, they presumably form metastable com-

plexes that interact optimally with cell membranes (Fig. 7). The changes in physical structure slightly below physiologic temperature may alter tissue distribution of the copolymer and help to explain both the minimal toxicity and the selectivity of the *in vivo* effects of T150R1.

Interaction of T150R1 with cell membranes was also supported by our data showing that treatment of AtT-20 cells with exogenous phospholipase C together with T150R1 diminished ACTH release by the copolymer. The enzyme by itself actually stimulated small amounts of ACTH secretion, as demonstrated previously (16). Recent evidence has suggested that activation of phospholipase C is the primary event in the mechanism of action of the cAMP-independent ACTH secretagogues (6). Phospholipase C cleaves phosphatidylinositol biphosphate in the plasma membrane to generate two classes of second messengers: inositol triphosphate, which activates the release of Ca^{2+} from intracellular stores, and diacylglycerol, which stimulates protein kinase C (17, 18). Thereafter, protein kinase C phosphorylates cellular proteins, including calcium channels. This induces an increase in Ca^{2+} permeability and ACTH release (19).

The inhibition of T150R1-induced ACTH release by phospholipase C might be of a competitive nature. We propose that phospholipase C reacts with the cell membrane by blocking the insertion of the hydrophobic tails of T150R1 and preventing the central hydrophilic core from shuffling sodium cations. However, blockage of T150R1-induced sodium influx resulting from the increased intracellular calcium induced by phospholipase C cannot be ruled out.

We have shown that T150R1 and T130R2 can directly stimulate ACTH production from AtT-20 pituitary cells. Thus, its immunomodulatory effects *in vivo* may be explained by stimulation of the pituitary adrenal axis with consequent effects on the thymus. Ionophore activity was responsible, at least in part, for copolymer-induced ACTH release from AtT-20 cells. Temperature and solubility studies led us to propose a model to illustrate how changes in the physicochemical structure of the copolymers may affect their interaction with cell membranes.

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