Cytoplasmic Retinoid-Binding Proteins and Retinoid Effects on Insulin Release in RINm5F β-Cells

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Vitamin A (retinol) is required for insulin secretion, and retinoic acid substitutes for retinol in this function. To determine if retinol acts at the β-cell level, we assayed β-cells of the rat insulinoma (RINm5F) line for cytosolic retinol- and retinoic acid–binding proteins (CRBP and CRABP) by radioimmunoassay (RIA) and [^{3}H]retinol and [^{3}H]retinoic acid binding to cytosol extracts. Furthermore, we tested whether insulin release from cells was affected by addition of retinol or retinoic acid to culture medium. RINm5F cells were grown to near confluence before assay of CRBP and CRABP. Scatchard analysis showed the K_d for retinol to be ~6 nM at a level of 4.5 pmol/mg protein or 300,000 sites/cell. Sucrose density-gradient assay showed single discrete peaks migrating at 2S for both retinol and retinoic acid. RIA of whole-cell extracts showed CRBP and CRABP levels of 5.27 ± 0.41 and 2.95 ± 0.75 pmol/mg protein, respectively. Retinol (1.75 μM) and retinoic acid (0.175 and 1.75 μM) increased KCl-induced insulin release. Considered together, the presence of CRBP and CRABP in a β-cell line and the increase in KCl-induced insulin release by retinol and retinoic acid are consistent with the idea that retinol has a functional role in insulin secretion and suggest a potential mechanism of action at the β-cell level similar to that observed in other retinoid-responsive cells. *Diabetes* 38:1544–48, 1989

Vitamin A (retinol) is important for normal growth, reproduction, and vision (1). At the cellular level, retinol deficiency leads to abnormalities in cellular differentiation, glycoprotein synthesis, cell adhesion, and changes in various other membrane functions (2,3). With the exception of its role in vision, the molecular mechanisms of retinoid action have not been defined (4). Retinoid-binding proteins are important in the transport and action of retinoids. Serum retinol-binding protein (RBP) is synthesized in the liver and serves to mobilize retinol from hepatocytes (5). On release, retinol RBP complexes with transthryetin (TTR) and circulates in a 1:1 molar ratio. Intracellularly, retinol and retinoic acid bind to specific cytosolic binding proteins, cellular retinol-binding protein (CRBP), and cellular retinoic acid–binding protein (CRABP), respectively (6). The function of these binding proteins is unknown, but they may serve to transport or shuttle retinoids between subcellular organelles, solubilize intracellular retinoids, serve as a readily available storage pool, or target retinoids to specific metabolic pathways (6,7). Recently, a retinoic acid–binding receptor has been discovered in the nucleus (8); this receptor belongs to a superfamily of nuclear receptors that are gene ligand-transcription factors mediating steroid and thyroid hormone actions through gene expression (9).

Recent studies have demonstrated the presence of relatively high levels of CRBP, CRABP, TTR, and RBP in rat islets by immunohistochemistry and radioimmunoassay (RIA; 10). Furthermore, in a rat model of retinol deficiency, retinol was required for insulin secretion, and retinoic acid substituted for retinol in this function (11). In view of the requirement for retinol in insulin release, we were surprised to find that, immunohistochemically, CRBP was not localized to the β-cell core of the islet (10). We attributed the absence of CRBP from the β-cells to the insensitivity of immunochemical staining.

In an effort to further define the β-cell as a target for retinoid action, we asked whether CRBP and CRABP are present and functional in a β-cell line. Using rat insulinoma (RINm5F) cells, we detected and quantitated CRBP and CRABP by RIA, determined whether [^{3}H]retinoids bind to cytosolic proteins and CRBP binding is saturable and of high affinity similar to that of CRBP in other retinoid-responsive cells

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(6,12–14), and tested for effects of retinoids on insulin release.

RESEARCH DESIGN AND METHDOS

The RINm5F cell line was kindly provided by H.K. Oie and A.F. Gadzar (National Cancer Institute, Bethesda, MD). The β-cell clone 5F was derived from the parent RINm cells of a rat cell islet tumor (15,16). Cells are cultured at 37°C in 5% CO_2/95% air in flasks containing RPMI-1640 with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin.

CRBP and CRABP levels were determined by sensitive and specific RIAs for each protein (10,17). Cells (1–2 million) were washed in phosphate-buffered saline (PBS), pH 7.4, and then rapidly frozen and stored until used for RIA. Cells were thawed and homogenized in 1 ml assay buffer containing 50 mM imidazole buffer (pH 7.4), 0.79% NaCl, 0.03% bovine serum albumin, 0.1% thimerosal, 0.01% leupeptin, and 1% Triton X-100. Values were expressed as picomoles per milligram protein or 10^6 cells. Both assays accurately detect between ~0.065 and 0.65 pmol protein (CRBP and CRABP)/assay tube.

Cytosolic receptor–binding assay. Cytosolic extracts were prepared from RINm5F cells incubated with [^{3}H]retinol (sp act 60 Ci/mmol, Amersham, Arlington Heights, IL) or [^{3}H]-retinoic acid (sp act 60 Ci/mmol, Amersham) and assayed for binding proteins by sucrose density-gradient analyses (13,18). Before assay, cells were grown for 3 days to near confluence in medium containing retinoid-free (UV-irradiated) 10% serum (19). The cells were harvested, and 2 × 10^7 cells/ml were suspended in 50 mM Tris buffer containing 1 mM EDTA, 10 mM KCl, and 1 mM dithiothreitol, pH 7.5, and homogenized for 30 s with a Tekmar homogenizer with a SDT-100EN shaft and generator at a speed of 40. The nuclei were pelleted at 800 × *g*, and the 102,000 × *g* supernatant was used as cytosol. Cytosolic protein concentration was adjusted to at least 1–1.5 mg/ml protein with 260–280 optical density UV absorbance for protein assay for Scatchard analysis or sucrose gradients (20). Protein concentration was more accurately determined later with the Bradford assay (21). To eliminate inhibition of binding of [^{3}H]retinoid by endogenous retinoid or other hydrophobic molecules, the cytosolic extract was treated once with dextran-coated charcoal (Norit A) before addition of label; i.e., cytosol was added to the pellet that resulted from 2000 × *g* centrifugation for 10 min of a 33% volume of buffer (50 mM TRIS, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5) containing 30 mg charcoal and 3 mg dextran/ml, mixed and incubated 15 min with remixing every 5 min. The charcoal was then centrifuged at 2000 × *g* for 10 min. [^{3}H]retinoid was then added to cytosol for a final volume of 250 μl and incubated for 3 h at 0°C in the dark. After incubation, the unbound [^{3}H]retinoid was removed by a second treatment with dextran-coated charcoal. The supernatant, containing bound retinoid, was directly assayed for radioactivity or applied to a sucrose gradient.

For measurement of total binding in sucrose gradients, we used a concentration of 5 × 10^{-8} M [^{3}H]retinoid. For measurement of nonspecific binding, a 200-fold molar excess of unlabeled retinoid was added to the incubation mixture. Final ethanol concentration was 0.1% in sucrose-gradient experiments and 0.08% in Scatchard analyses.

Sucrose gradients were 5–20% sucrose, 5 mM phosphate, 1.5 mM EDTA, 1 mM dithiothreitol, 0.01 M sodium molybdate, and 10% glycerol, pH 7.4. Gradients were run at 378,000 × *g* for 18.5 h with a Beckman SW60Ti rotor. Five- or 10-drop fractions were taken from the bottom of the gradient and were counted in Fisher Scintiverse II liquid scintillant. Myoglobin was used as a 2S sedimentation marker.

Saturation and Scatchard plot analyses. Cytosol was prepared as above. [^{3}H]retinol was added in a final concentration of 2.5–40 × 10^{-9} M in triplicate. Total incubation volume was 250 μl. For measurement of nonspecific binding, a 200-fold molar excess of unlabeled retinol was added to an identical set of tubes, also in triplicate. Tubes were incubated for 20 h at 0°C in the dark. Bound retinol was separated from free retinol with dextran-coated charcoal by adding a 0.125-ml suspension of dextran-coated charcoal and incubating as described above. Supernatant (250 μl) was added to 5 ml of liquid scintillant for counting of bound retinol. Specific binding, the difference between total and nonspecific binding, was calculated for saturation curves and plotting of bound versus bound/free retinol for Scatchard plot analyses (22). Three experiments were performed on separate occasions.

Insulin release. Before study of insulin release, 10^6 cells were seeded into multiwell test plates (Corning) and cultured in medium with or without retinoids for 48 h. Control medium was RPMI-1640 medium supplemented with retinoid-depleted (UV-irradiated) 10% fetal bovine serum, 11 mM glucose, penicillin, and streptomycin. Test medium was the same as control medium, except that retinol or retinoic acid were added back in concentrations of 0.175 or 1.75 μM. To study insulin secretion, culture medium was removed, and cells were washed with Krebs-Ringer bicarbonate buffer (KRBB). Then, 500 μl of control glucose-free KRBB containing 4.7 mM KCl or KRBB supplemented with 25 mM KCl was added to wells, and cells were incubated for 60 min at 37°C. Supernatant was assayed for insulin by RIA. Differences between group means were analyzed by analyses of variance and the multiple-group *t* tests (Student-Newman-Keuls).

RESULTS

Mean ± SE concentrations of CRBP and CRABP in cell homogenates were 4.97 ± 0.39 and 2.74 ± 0.7 pmol/mg protein (n = 6) and 0.86 ± 0.1 and 0.45 ± 0.13 pmol/10^6 cells (n = 6), respectively, based on 15,700 and 15,500 M_r, respectively. CRBP level was almost twofold that of CRABP.

Sucrose density gradient analyses of cytosolic extracts detected single discrete 2S peaks for [^{3}H]retinol (Fig. 1*A*) and [^{3}H]retinoic acid (Fig. 1*B*). A 200-fold molar excess of unlabeled retinol and retinoic acid displaced labeled retinol and retinoic acid from their binding proteins. Thus, typical cytosolic binding proteins similar in size to those described in the cytosol of various cells are also present in RINm5F cells (12–14).

Saturation and Scatchard plot analyses. In the first experiment, total binding increased as the concentration of [^{3}H]retinol was increased in the incubation mixture (Fig. 2). [^{3}H]retinol was not completely displaced by a 200-fold molar

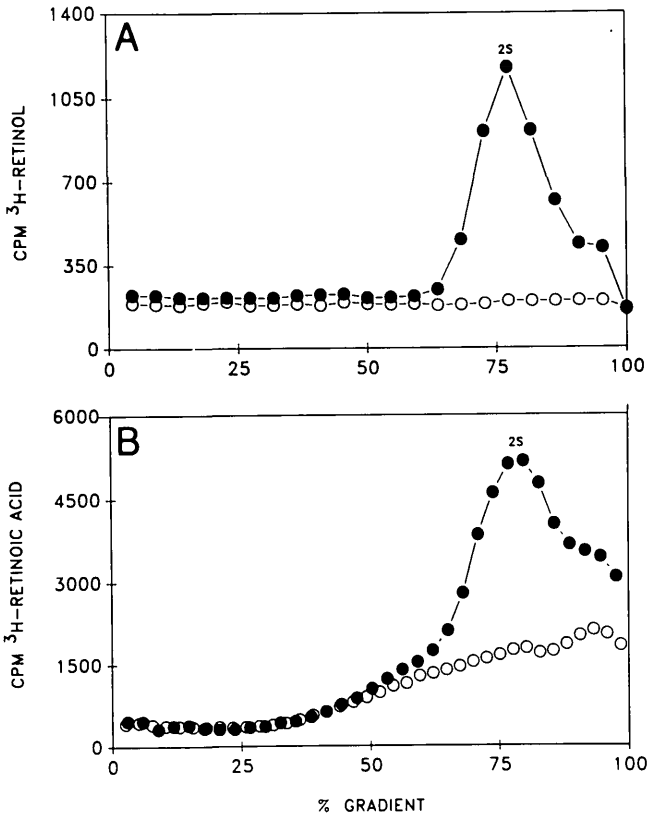

FIG. 1. Sucrose density-gradient analyses of retinol- (*A*) and retinoic acid– (*B*) binding proteins in rat insulinoma (RINm5F) cells. Cytosol (250 μl) containing ~2 mg/ml protein was incubated with 50 nM [^{3}H]retinoic acid or 2.5 nM [^{3}H]retinol alone (●) or with 200-fold excess of unlabeled retinoid (○) for 3 h at 0°C without light. After removal of free retinoid with dextran-coated charcoal, cytosol was analyzed for discrete peaks on 5–20% sucrose gradient. CPM, counts per minute.

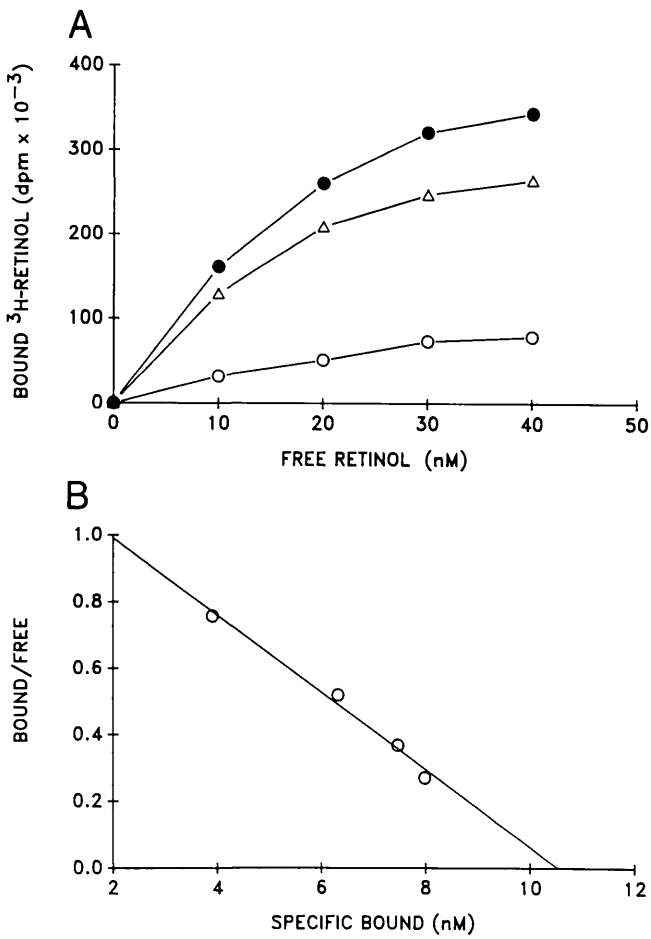

FIG. 2. *A*: saturation plot analysis of retinol binding in rat insulinoma (RINm5F) cytosol. Varying amounts of [^{3}H]retinol were incubated with cytosol at 4°C for 20 h without light. ●, Total binding; ○, nonspecific binding; △, specific binding. *B*: Scatchard plot analysis of retinol binding in RINm5F cytosol. Specific binding was calculated by subtracting nonspecific binding from total binding. K_d = 8.6 nM. Number of sites = 4.21 pmol/mg protein. Protein concentration was 2.5 mg/ml.

excess at each point, indicating the presence of nonspecific binding. Specific binding showed saturation at 40 nM retinol. The findings in our second and third experiments (performed for confirmation, data not shown) were similar. Scatchard analysis showed a high-affinity binding with K_d = 8.6 nM (mean ± SE 5.96 ± 1.32 for 3 experiments), which is in the range of that observed in nonislet cells (12–14). The concentration of receptors extrapolated from the intersection of the curve with the abscissa was ~300,000 sites/cell or 4.21 pmol/mg protein (mean ± SE 4.5 ± 0.33 pmol/mg protein for 3 experiments). These are in reasonable agreement with the values of 5.3 pmol/mg protein and 547,827 sites/cell determined by RIA.

Effects of retinol and retinoic acid on KCl-induced insulin release. Basal release from RINm5F cells incubated in medium containing 4.7 mM KCl without glucose after culture in retinol-depleted medium was not significantly different from that observed from cells cultured in retinol-repleted media (data not shown). KCl (25 mM) stimulated insulin release approximately five- to sevenfold as expected (Table 1). Retinol (1.75 μM, a physiological concentration) added to culture medium increased KCl-induced insulin release, whereas addition of a tenfold lower concentration did not . Retinoic acid (0.175 and 1.75 μM) added to culture medium also increased KCl-induced insulin release. Retinoids increased secretion by ~30–50%.

DISCUSSION

In previous studies with isolated intact islets, we identified cellular retinoid-binding proteins in islets (10) and demonstrated impaired insulin release from islets of retinol-deficient

TABLE 1
KCl-stimulated insulin release in cultured rat insulinoma (RINm5F) cells

Culture condition	KCl (mM)	Insulin (ng · h^{-1} · 10^{-6} cells)
Retinol-free medium	4.7	1.60 ± 0.06
	25	7.60 ± 0.57
Retinol (μM)		
0.175	25	8.20 ± 0.39
1.75	25	10.30 ± 0.39*
Retinoic acid (μM)		
0.175	25	11.90 ± 0.66*
1.75	25	10.10 ± 0.36*

Values are means ± SE; n = 6 for each group. RINm5F cells were cultured for 48 h in medium with or without retinol or retinoic acid. Medium was then changed to contain 4.7 or 25 mM KCl to stimulate insulin release.
*P < .05 vs. 25 mM KCl–stimulated release from control RINm5F cells.

rats (11). Those findings suggested a role for retinoids at the islet level and possibly at the β-cell level. Our studies support the concept that retinoids act at the β-cell level. The identification of cytosolic CRBP by sucrose density-gradient analyses and RIA in a β-cell line, the saturation of CRBP with increasing retinol concentration, and the identification of high-affinity binding sites suggest that these cells are responsive to retinol in their function. The existence of receptors supports the presence of a mechanism of action of retinol in β-cells similar to that observed in other cell lines. Likewise, the presence of CRABP in β-cells demonstrated by sucrose density-gradient analyses and RIA support an action of retinoic acid in β-cells.

CRBP and CRABP differ widely in their tissue distribution (17,23). These binding proteins probably serve different functions in mediating the actions of their respective ligands. In our previous studies with intact isolated rat islets (11), both immunoreactive CRBP and CRABP were present in the islet, and CRBP was present in greater concentrations than CRABP. The presence of both binding proteins, with CRBP occurring in greater concentration, is true of most normal tissues, except for the skin, distal epididymis, vas deferens, and the seminal vesicles (23). In the latter tissues, CRABP is present in greater concentrations than CRBP. RINm5F cells contain both binding proteins, CRBP being present in greater concentrations than CRABP. Most tumor tissues contain both proteins and quantitatively more CRABP than CRBP (6). Cultured cell lines contain CRABP more often than CRBP (6). Comparisons of the relative concentration of CRBP and CRABP among various cell lines may provide insight into cell growth and function, but this information has not been reported yet. Therefore, the significance of the greater concentration of CRBP than CRABP in RINm5F cells and other cells remains to be determined.

Cellular retinoid-binding proteins may have an important role in RIN cell function, growth, and differentiation. Retinol affects gene expression in vivo in rats and cultured cells (6,24). Retinol or retinoic acid could mediate biological processes either directly or indirectly related to hormone release (e.g., transglutaminase production [25–27], protein kinase activity [28]), cell-to-cell adhesion or communication (e.g., gap junction formation [29]), attachment of cells to substratum (32), and phenotypic patterns of differentiation in culture (13,31). Retinoid effects on cellular differentiation may involve the formation of proteins that enable endocrine cells to secrete hormone. These effects of retinoids could be mediated through nuclear cellular binding proteins and cytoplasmic binding proteins, which bind and shuttle retinoids to the nucleus. The cytoplasmic cellular binding proteins may facilitate binding of retinoids to a nuclear receptor, e.g., the newly discovered retinoic acid receptor (8,9), which in turn would bind to DNA and affect gene expression. To date, a nuclear receptor for retinol has not been identified, but receptors remain in the nucleus for which ligands have yet to be identified. If receptors for retinol do not exist, retinol action could occur through the enzymatic conversion of retinol to retinoic acid intracellularly in a similar precursor to product hormone relation as thyroxine to triiodothyronine (32).

In past studies on secretion from perifused isolated rat islets, low concentrations of retinol (10^{-7} M; 33) and high concentrations of 13-*cis*-retinoic acid (10^{-4} M; 29) stimulated secretion, possibly through membrane effects. Retinol at high concentrations (10^{-4} M) inhibited insulin release through effects on glucose oxidation (33,34). It is unclear whether these rapid effects could be mediated through retinoid-binding proteins. By showing that the addition of retinol and retinoic acid to medium increases KCl-induced release from RINm5F cells, our studies support the idea that retinoids have a role in insulin secretion. The RINm5F model should be helpful in studying the cellular mechanisms by which retinoids influence insulin secretion with an effort made to establish dose-response effects on insulin or target proteins. Further studies on the effects of retinoids on insulin secretion and phenotypic growth expression of RINm5F cells and other islet β-cell lines would provide insight into the relation between islet cell differentiation, growth, and insulin secretion. An initial study testing the effects of retinoic acid on RINm5F functions found abnormalities in the incorporation of mannose into glycoproteins without change in insulin secretion (35). More studies are needed comparing the effects of retinoid-free culture medium to medium containing varying concentrations of retinoids, with particular attention to the mode of delivery of retinoids to cells and testing of different concentrations of secretagogues. This knowledge may be applicable to the growth of transplanted islets and cells and defects in growth and replication relevant to the pathogenesis of diabetes.

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