Retinoid-X Receptors and the Effects of 9-*cis*-Retinoic Acid on Insulin Secretion From RINm5F Cells

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Retinoid-X receptors (RXRs) are 9-*cis*-retinoic acid (9CRA)-dependent gene transcription factors, which modulate the action of all-*trans*-retinoic acid (ATRA), fatty acids, thyroid hormone (TH), and vitamin D (VD) by forming dimers with themselves or ATRA, TH, peroxisome proliferator activator receptors (PPARs), or VD receptors (VDRs). To determine if 9CRA and RXRs have a role in secretion, RINm5F cells were assayed for RXR transcripts and effects of 9CRA and ATRA on secretion. A single RXRα transcript and two RXRβ transcripts, but not RXRγ, were evident by Northern blot. Cells were cultured for 48 hours without and with 9CRA 1 to 1,000 nmol/L and then stimulated with glucose 0, 0.5, 2.8, 7, and 11 mmol/L. 9CRA increased secretion at each glucose concentration. 9CRA increased secretion by 50% to 100% (ANOVA, $P < .001$) with consistent concentration-dependent responses (eg, at glucose 2.8 mmol/L, 9CRA: 0 nmol/L, 5.02 ± .20 ng/(10^6 cells · h); 1 nmol/L, 6.97 ± .30; 10 nmol/L, 8.36 ± .18; 100 nmol/L, 9.15 ± .28; 1,000 nmol/L, 10.24 ± .24; n = 6). Although RINm5F cells respond slightly if at all to glucose, 9CRA facilitated glucose-induced insulin release (eg, at 9CRA 100 nmol/L, glucose: 0.5 mmol/L, 7.47 ± .22 ng/(10^6 cells · h); 2.8 mmol/L, 9.15 ± .27; 7 mmol/L, 9.81 ± .19; 11 mmol/L, 11.16 ± .23; n = 6). ATRA increased secretion by 28% to 57% (ANOVA, $P < .001$: at glucose 2.8 mmol/L, ATRA: 0 nmol/L, 6.17 ± .32 ng/(10^6 cells · h); 1 nmol/L, 7.91 ± .29; 10 nmol/L, 9.75 ± .14; 100 nmol/L, 9.66 ± .33; n = 6). 9CRA was more potent than ATRA (eg, at 2.8 mmol/L: baseline, 6.17 ± .32 ng/(10^6 cells · h); ATRA 100 nmol/L, 9.66 ± .33; 9CRA 100 nmol/L, 10.81 ± .15; $P < .05$, n = 6). When 9CRA was combined with ATRA, the combination was not additive or synergistic (eg, at 2.8 mmol/L: ATRA 100 nmol/L, 9.66 ± .33 ng/(10^6 cells · h); 9CRA 100 nmol/L, 10.81 ± .15; ATRA 100 nmol/L + 9CRA 100 nmol/L, 10.79 ± .28; $P < .05$, n = 6). These studies show that (1) 9CRA stimulates insulin secretion from RINm5F cells. This effect appears to be at least equal to if not greater than that observed with ATRA, but additive or synergistic effects with ATRA were not evident; (2) 9CRA may facilitate glucose-induced release; and (3) multiple RXR transcripts are present in insulin-secreting cells, implying specific functions. Our findings support the idea that the effects of 9CRA on insulin secretion are mediated through RXR homodimers or heterodimers with retinoic acid receptors (RARs) or possibly other nuclear receptors. Retinoid deficiency or alterations in retinoid receptor function could lead to abnormalities of cell growth or secretion.

VITAMIN A, or retinol, is an essential dietary nutrient that is required for normal growth, reproduction, and vision.[1] Intracellularly, it is converted to all-*trans*-retinoic acid (ATRA) and a variety of other active metabolites.[1] At the tissue level, ATRA has marked effects on morphogenesis, cell proliferation, and differentiation, and it induces differentiation in a variety of normal and abnormal cell lines.[1,2] These effects are mediated through nuclear retinoic acid receptors (RARs). RARs belong to the superfamily of ligand-dependent transcription factors, which include the vitamin D (VD) receptor (VDR), thyroid hormone (TH) receptor (TR), and peroxisome proliferator activator receptors (PPARs). These receptors are encoded by different genes, and receptor isoforms are generated from the same gene by differential promoter usage or alternative splicing.[1] These receptors form heterodimers and homodimers that bind to DNA response elements, mediating retinoid, VD, TH, and fatty acid actions on growth and secretion through transactivation and regulation of transcription of a set(s) of cell type–specific genes.[1-3]

Intracellularly, ATRA is isomerized to 9-*cis*-retinoic acid (9CRA), another active metabolite that binds not only RARs but also the more recently described retinoid-X receptors (RXRs).[1] Although 9CRA binds to both RARs and RXRs, RXRs specifically bind 9CRA. RXRs modulate the action of RA, TH, VD, and fatty acids by forming dimers with RARs, TRs, VDRs, and PPARs,[1,2] respectively.

Several studies in vivo and in vitro suggest important roles for vitamin A, VD, TH, and fatty acids and their respective receptors in islet growth or secretion or both.[4-19] Vitamin A, VD, and TH deficiency are associated with impairment of insulin secretion.[4-8] Repletion of vitamin A–deficient rats with retinol or ATRA reverses the defect in insulin secretion.[7] RAR transcripts are present in insulin-secreting cells,[10] and ATRA in vitro increases secretion from RINm5F insulin-secreting cells.[11] ATRA also stimulates glucokinase in RINm5F cells.[12] This enzyme is the "glucose sensor" or rate-limiting enzyme for glucose metabolism and insulin release, and is mutated in some families with maturity-onset diabetes of the young.[13] A functional RA/TH-response element was identified upstream of the human insulin gene enhancer, and ATRA increased insulin mRNA.[14] TRs are present in RINm5F cells, and triiodothyronine decreases proinsulin mRNA.[12] In vitro, $1,25(OH)_2D_3$ affects β-cell handling of calcium,[9,15] calcium oscillations,[16] and growth.[17] With the recognition that PPARs are activated by fatty acids and mediate their oxidation,[2,18] others have suggested a potential role for PPARs in modulating the effects of fatty acids on insulin secretion and islet lipotoxicity.[19]

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If RARs, VDRs, TRs, and PPARs interact with RXRs at the islet level as they do in other tissues, then RXRs should be present in insulin-secreting cells. Further, 9CRA and RXRs may affect secretion directly or modulate the effects of RARs, VDRs, TRs, and PPARs on islet growth and secretion. To determine if 9CRA and RXRs have a role in secretion, we tested for the effects of 9CRA on insulin secretion and the presence of RXRs using RINm5F cells. We also compared the effects of 9CRA and ATRA.

MATERIALS AND METHODS

RINm5F Cell Culture and Tissue Preparation

RINm5F cells were kindly provided by H.K. Oie and A.F. Gazdar (National Cancer Institute, Bethesda, MD). RINm5F cells were cultured at 37°C in an atmosphere of 5% CO_2/95% air in RPMI 1640 medium with 100 mL/L fetal bovine serum, 11.1 mmol/L glucose, 100 mg/L streptomycin, and 100,000 U/L penicillin. Under these conditions, cells grow to confluence between day 5 and day 7 and are passed weekly. To study the effects of defined concentrations of retinoids, fetal calf serum is depleted of endogenous vitamin A before addition to media by exposure to UV light (365 nmol/L, 100W bulb, Model B-100A UV lamp; UVP, San Gabriel, CA) for 8 hours,[20] and then defined test concentrations of retinoid are added back to the media. In studies detecting the presence of RXRs and the effect of 9CRA over time on the abundance of transcripts, aliquots of 10^7 cells were seeded into 162-cm^2 polystyrene tissue culture flasks (Corning-Costar, Cambridge, MA) in cell culture medium containing 100 mL/L UV-treated fetal bovine serum. On day 3 of culture, the medium was removed and replaced with fresh vitamin A–deficient medium. On day 5, the medium was removed again and replaced with vitamin A–deficient medium containing a defined concentration of 9CRA 1 to 1,000 nmol/L (kindly provided by Hoffman-LaRoche, Nutley, NJ). After 6, 24, and 48 hours, the medium was removed and 5 mL GTC buffer (5.0 mol/L guanidine isothiocyanate [GIBCO BRL, Gaithersburg, MD], 10 mmol/L Tris, pH 7.5, and 6.7 mmol/L disodium EDTA in diethylpyrocarbonate-treated deionized water) was added to each flask surface. Then, the cells were scraped off the surface, snap-frozen in liquid nitrogen, and stored at −70°C for subsequent isolation of total cellular RNA.

Isolation of Total RNA and Northern Blot Analyses for RXR, α, β, and γ Transcripts

Total RNA was extracted from frozen samples of 10^7 plated cells by the method of Chirgwin et al,[21] denatured in glyoxal and dimethylsulfoxide, fractionated by electrophoresis on 1.2% agarose gel, and transferred to nylon filters. Mouse RXR cDNAs cloned into *Eco*RI sites of pBSSK+ vectors were kindly provided by Ronald Evans (Howard Hughes Medical Institute, Salk Institute for Biological Studies, La Jolla, CA): pSKmRXRα containing a RXRα cDNA 4.8-kb insert, pSKmRXRβ containing a RXRβ cDNA 2.2-kb insert, and pSKmRXRγ containing a RXRγ cDNA 1.6-kb insert. A 1,040-basepair fragment of the RXRα cDNA was obtained for probe synthesis by digestion of the pSKmRXRα construct with *Bam*HI and *Not*I. The pSKmRXRβ construct was digested with *Bam*I and *Eco*RI to obtain a 600-basepair fragment of the RXRβ cDNA for probe synthesis. Full-length RXRγ cDNA was obtained by digestion of the pSKmRXRγ construct with *Eco*RI.

^{32}P-radiolabeled probes for Northern blots were prepared by random oligonucleotide priming of specific gel-purified DNA fragments.[22] Hybridizations with cDNA probes were performed at 42°C overnight. Blots were washed in 2× SSC (1× SSC is 0.15 mol/L NaCl, plus 0.015 mol/L sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 minutes, 0.2× SSC and 0.1% SDS at 50°C for 20 minutes, and 0.2× SSC and 0.1% SDS at 50°C for 60 minutes. Blots were exposed for 5 days at −70°C using an intensifying screen and Kodak XAR-5 film (Eastman Kodak, Rochester, NY). In all Northern blots, rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA[23] was used as an internal standard for RNA quantity and integrity. Commercially available RNA ladders were used as molecular weight markers; these markers were visualized by ethidium bromide staining. Optical densities of RXR and GAPDH bands were determined using a Visage image analyzer (Bio Image; Millipore, Ann Arbor, MI), and OD ratios of RXR to GAPDH were calculated.

RINm5F Cell Insulin Secretion

Aliquots of 5×10^5 cells were seeded into multiwell test plates (Costar-Corning, Cambridge, MA). The incubation medium was the same as the vitamin A–deficient culture medium already described. For secretion studies, a defined concentration of 9CRA was added back to the culture media. Media without retinoids contained the retinoid vehicle, ethanol 2.17 mmol/L. RINm5F cells were cultured for 48 hours in 11.1 mmol/L glucose in the absence or presence of 9CRA at 10, 100, or 1,000 nmol/L. Then, the cells were incubated for 2 hours, the first hour of which served as an equilibration period and the second as a test period during which cells were stimulated with glucose. During the first hour, the media were changed to contain the same concentration of 9CRA and glucose, 0.5 mmol/L, in Krebs-Ringer bicarbonate buffer. During the second hour, media were changed to contain increasing concentrations of glucose: 0.5, 2.8, 7, or 11 mmol/L. At the end of the second hour, the media were removed for assay of insulin by radioimmunoassay.[24] To test for potency differences and additive or synergistic effects between ATRA and 9CRA, a separate set of experiments were performed comparing the effects of 9CRA or ATRA 1.0, 10, and 100 nmol/L or 9CRA 100 nmol/L in combination with ATRA 1.0, 10, or 100 nmol/L.

Statistical Analyses

To analyze differences in insulin responses to glucose and retinoids, statistical analysis was performed with one-way ANOVA followed by the Student-Newman-Keuls procedure for multiple comparisons using a computerized statistical package (SPSS, Chicago, IL). A two-way ANOVA was used to compare interactions at each concentration of 9CRA and the glucose response. An interaction between glucose and 9CRA was found. The increments of insulin for each glucose concentration with increasing 9CRA concentration were further analyzed by one-way ANOVA followed by the Student-Newman-Keuls procedure. To analyze for 9CRA and ATRA effects independent of glucose effects, ANOVA and analysis of covariance was used with glucose as a covariant. P less than .05 was considered significant. Results are expressed as the mean ± SEM.

RESULTS

Northern Blot Analyses for RXR α, β, and γ Transcripts

Separate blots were analyzed for RXRα and RXRβ. Each blot was washed and analyzed for GAPDH transcripts. After exposure for 5 days, blots of RINm5F cell RNA showed one transcript for RXRα and two for RXRβ (Fig 1). RXRα was 4.4 kb. RXRβ transcripts were 2.9 and 2.4 kb, respectively. RXRγ transcripts were not detectable. The GAPDH transcript was 1.4 kb and evident after exposure times as short as 20 minutes.

Effects of 9CRA on Insulin Release From Cultured Cells

Control secretion from cells cultured in vitamin A–deficient media in glucose 0.5 mmol/L was 3.65 ± 0.06 ng/(10^6 cells · h). Compared with glucose 0.5 mmol/L, glucose 2.8 mmol/L stimulated insulin secretion (Fig 2A), after which insulin

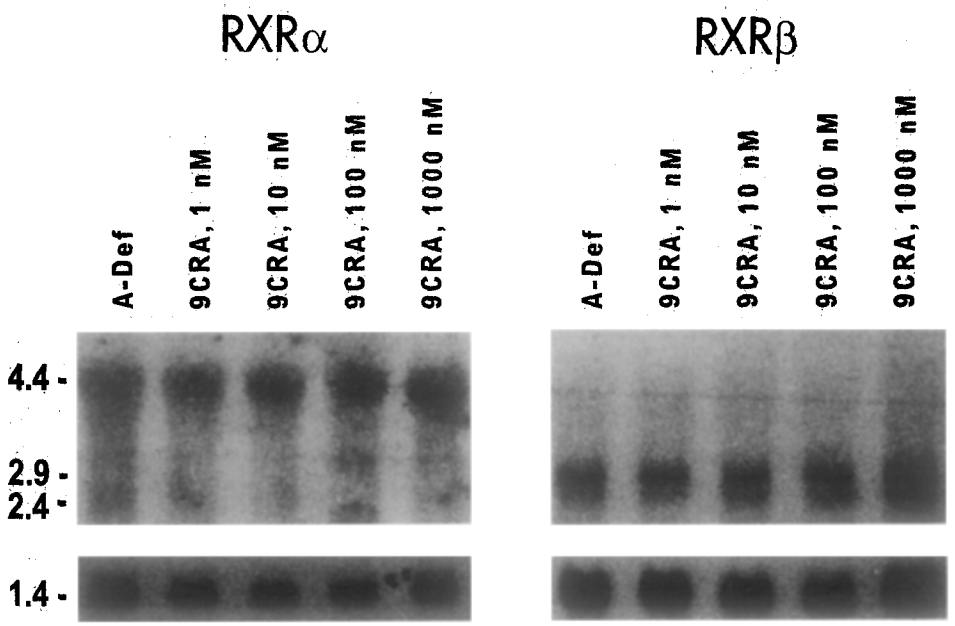

Fig 1. Northern blot analyses of RINm5F cell total RNA shows RXRα and RXRβ transcripts after culture in the absence (A-Def) and presence of varying concentrations of 9CRA. Blots for analysis of RXRs are shown with respective GAPDH analyses. One RXRα at 4.4 kb and 2 RXRβ bands at 2.9 and 2.4 kb are evident. GAPDH bands migrating at 1.4 kb are at the bottom of each lane.

secretion plateaued. The increments in response to glucose 2.8 mmol/L were similar at different concentrations of 9CRA. A further increment in secretion was evident when glucose was increased from 7 to 11.0 mmol/L at both 9CRA 100 and 1,000 nmol/L ($P < .05$), but not at 0, 1, and 10 nmol/L. At each glucose concentration, 9CRA stimulated release to a larger extent than the vitamin A–deficient control (n = 6, ANOVA, $P < .001$) with consistent concentration dependence (Fig 2B). At the highest concentrations of 9CRA, secretion was increased by 75% to 100%.

Comparison Between Effects of 9CRA and ATRA on Insulin Secretion

Insulin responses in the presence and absence of retinoids at glucose 0.5 and 2.8 mmol/L are shown in Table 1. Compared with secretion in the absence of retinoids, both ATRA and 9CRA increased insulin secretion at each retinoid concentration at both glucose concentrations. ATRA alone or in combination with 9CRA 100 nmol/L increased the insulin response further ($P < .05$), although the difference between 10 and 100 nmol/L was not significant. At glucose 2.8 mmol/L, 9CRA alone from 0 to 100 nmol/L increased insulin secretion in a concentration-dependent manner ($P < .05$), similar to that described in the experiment earlier that included 9CRA 1,000 nmol/L. ATRA or 9CRA at 10 or 100 nmol/L increased the insulin response by 10% to 25% greater at glucose 2.8 mmol/L than at 0.5 mmol/L, suggesting that retinoids facilitated the response to glucose, albeit slightly.

Maximal effects overall were observed with 9CRA at 100 nmol/L in combination with ATRA. When retinoid effects on secretion were analyzed independently of glucose concentration, the combination of 9CRA 100 nmol/L plus ATRA 1, 10, or 100 nmol/L increased insulin secretion to a greater extent than either 9CRA or ATRA alone at 1, 10, or 100 nmol/L, respectively (ANOVA, $P < .01$). However, this combination effect was not synergistic and was less than additive, and at 2.8 mmol/L glucose, 9CRA 100 nmol/L alone was equally effective in stimulating secretion as the combination of 9CRA 100 nmol/L plus ATRA 100 nmol/L (Table 1).

DISCUSSION

These studies show that (1) multiple RXR transcripts are present in insulin-secreting cells, implying specific functions; (2) 9CRA stimulates insulin secretion from RINm5F cells. This effect is concentration-dependent and appears to be at least equal to if not greater than that observed with ATRA; (3) 9CRA combined with ATRA has a greater effect than either has alone; and (4) 9CRA and ATRA at concentrations of 10 and 100 nmol/L may facilitate glucose-induced release slightly. Our previous studies show a requirement for vitamin A in insulin secretion in the rat and that vitamin A deficiency causes defects in secretion of insulin.[7,11] In studies using insulin-secreting cell lines, ATRA increased potassium-induced release by 30% to 50%, and RARs were present. We have proposed that vitamin A may affect insulin secretion by inducing either glucokinase or transglutaminase.[10] Our findings here showing that both ATRA and 9CRA have direct effects indicate that 9CRA and ATRA may have a role in insulin secretion. Further, the presence of multiple RXRs suggests specific actions for each RXR and a diversity of functions. Since these cells are tumoral cells, our findings require confirmation in cell lines that are physiologically responsive to glucose. Further, detectable RXR mRNA does not indicate that detectable RXR protein is present. However, since message is present and the effects of 9CRA are mediated through RARS and RXRs (the latter specific for 9CRA), RXR protein must be present with a high degree of certainty.

The effect of 9CRA was greater than that of RA. Although this effect was not additive or synergistic, the concentration dependence and greater effects of 9CRA suggest a dominant effect of 9CRA. The greater response to 9CRA in combination with ATRA observed at glucose 0.5 mmol/L suggests an interaction between RARs and RXRs, which could modulate insulin secretion. In the nucleus, ATRA binds to RAR α, β, γ,

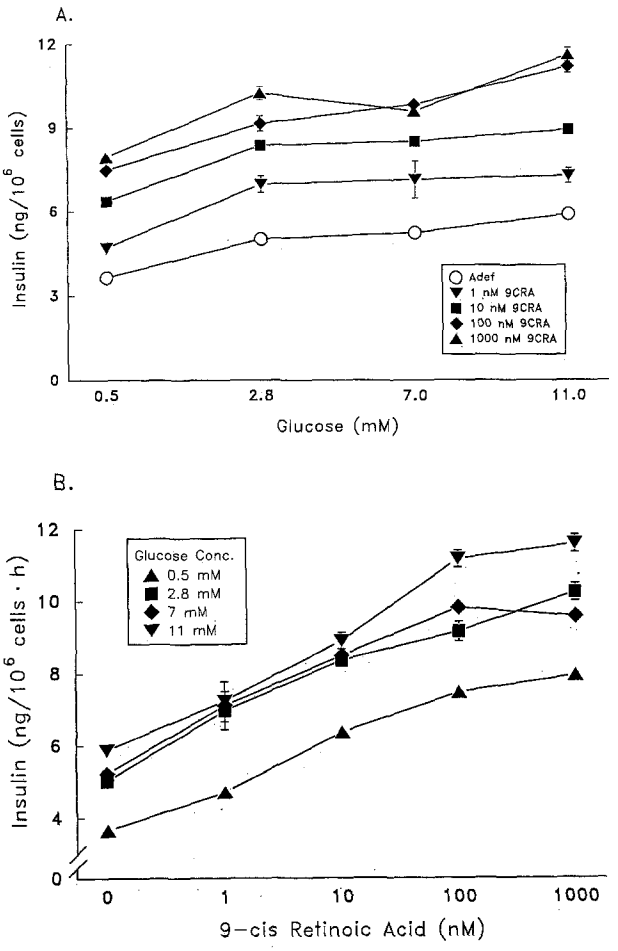

Fig 2. Effects of 9CRA 0 to 1,000 nmol/L on insulin secretion from RINm5F insulin-secreting cells at different glucose concentrations. Cells were cultured in 9CRA for 48 hours and then tested for response to glucose. (A) At each glucose concentration, insulin release from the vitamin A–deficient (Adef) group was significantly less than for 9CRA at 1, 10, 100, or 1,000 nmol/L ($P < .01$). Increments in insulin secretion at 2.8 mmol/L glucose are similar at each 9CRA concentration. The further increment evident when glucose was increased from 7 to 11.0 mmol/L was significant at both 9CRA 100 and 1,000 nmol/L ($P < .05$), but not at 0, 1, and 10 nmol/L. (B) Data from A are plotted as a function of 9CRA concentration. 9CRA stimulated insulin secretion in a retinoid concentration–dependent manner at each glucose concentration ($P < .01$ by ANOVA). $P < .01$ for differences between Adef *v* 9CRA at 1, 10, 100, and 1,000 nmol/L. Differences in insulin release at different 9CRA concentrations independent of glucose concentrations were significant at $P < .05$ for 9CRA 1 nmol/L > Adef, 9CRA 10 nmol/L > 1 nmol/L, 9CRA 100 nmol/L > 10 nmol/L, 9CRA 1,000 nmol/L > 100 nmol/L. The stimulatory effect of 9CRA at 100 and 1,000 nmol/L on 11-mmol/L glucose–induced release is evident (top line). (n = 6 for each point; brackets are the SEM, and where not shown, are within the symbol.)

while 9CRA binds to RAR α, β, γ and RXR α, β, γ. Furthermore, 9CRA binds to RARs with high affinity. Since 9CRA is present in lower concentrations than ATRA in tissues, its effect is more likely mediated through RXRs than RARs. However, 9CRA and RXRs may modulate ATRA and RAR action by promoting RXR heterodimerization with RARs and increasing the affinity of RARs for ATRA or RA response elements (RAREs).[1] Notwithstanding this possibility, the lack of a synergistic or additive effect suggests that (1) 9CRA and ATRA share RARs to some extent, (2) 9CRA binding to RXRs and dimerization to RARs does not enhance the effects of RARs, or (3) 9CRA binding to RXRs and RXR dimerization is not important for secretion. Studies using RAR and RXR receptor-selective retinoids should help determine which receptors play the major role in mediating secretion.

Since RXRs may form heterodimers with other transcription factors (RXR-VDR, RXR-TR, and RXR-PPAR) in islet cells, we can propose a central role for 9CRA and RXRs in hormone signaling and regulation of islet secretion. 9CRA may have direct effects or modulate the effect of other receptors that affect secretion. VD, TH, and fatty acids have been shown to influence insulin secretion. Their effects may be mediated through their specific nuclear receptors whose dimerization with RXRs, binding to DNA response elements, or both are modulated by RXRs and its ligand, 9CRA. With regard to metabolism, a link between fatty acids, 9CRA, and insulin secretion may occur through PPARs.[2,18,19] Fatty acids stimulate insulin secretion and are natural ligands for PPARs. PPAR-RXR heterodimers may be activated by and mediate fatty acid oxidation. Excessive fatty acids may have a role in inducing β-cell abnormalities and glucose intolerance.[19] Abnormalities in fatty acid or retinoid metabolism could alter dimerization and, in turn, affect fatty acid oxidation and islet function adversely.

Aside from secretion, 9CRA may affect gene expression of specific proteins that mediate the growth and differentiation of fetal or adult islet cells. In most normal tissues, one or more RXRs and RARs are present in various combinations.[1,2] In embryonic development, receptor appearance is temporal and spatial, ie, receptors are expressed in a tissue-specific and time-dependent manner, indicating a role in tissue specification and organogenesis. Receptors are expressed in specific loca-

Table 1. Comparison Between the Effects of ATRA and 9CRA Alone and in Combination on Insulin Secretion From RINm5F Cells at Glucose 0.5 and 2.8 mol/L

Glucose (mmol/L)	Retinoid (nmol/L)	ATRA	9CRA	9CRA 100 nmol/L + ATRA
0.5	0		6.15 ± 0.35	
	1	8.31 ± .16	8.90 ± .11	9.48 ± .26
	10	8.85 ± .20	9.00 ± .38	10.32 ± .23
	100	8.65 ± .36	8.59 ± .30	10.41 ± .13
2.8	0		6.17 ± 0.32	
	1	7.91 ± .29	9.34 ± .28	10.64 ± .20
	10	9.75 ± .14*	9.49 ± .15	10.79 ± .16
	100	9.66 ± .33*	10.81 ± .15*	10.79 ± .28

NOTE. Cells were cultured for 48 hours in 9CRA or ATRA alone at 1, 10, and 100 nmol/L or in 9CRA 100 nmol/L in combination with 1, 10, and 100 nmol/L ATRA. Then, cells were tested for secretion in response to glucose 0.5 and 2.8 mmol/L. Mean baseline secretion rates in the absence of retinoids at glucose 0.5 and 2.8 mmol/L are shown in the row above the results for the effects of retinoids. ATRA and 9CRA increased insulin secretion at all concentrations ($P < .05$ for difference between vitamin A–deficient *v* 9CRA and ATRA at 1, 10, and 100 nmol/L. At glucose 2.8 mmol/L, ATRA and 9CRA showed a dose-response with greater effects at higher concentrations. Values are the mean ± SEM in ng (10^6 cells · h); n = 6 for each value.

*$P < .05$ for ATRA 10 or 100 *v* 1.0 nmol/L, and for 9CRA 100 *v* 10 nmol/L.

tions in the late stages of embryogenesis.[25,26] Gene knockout experiments have been successful in revealing the phenotypic effects of RXRs. Of the three RXRs, only RXRα homozygous mutants show an abnormal phenotype, ie, ventricular hypoplasia. Double mutations of RXRα and RXRγ show the same defect, ie, ventricular hypoplasia. However, the RXRα mutation can occur with two different phenotypes, one associated with eye defects observed also in vitamin A deficiency, connoting a function requiring RXR-RAR dimerization, and another with liver defects, not associated with retinoid signaling and possibly related to a function requiring heterodimer formation of RXR with PPAR.[26,27] Further studies are needed to determine the roles and interactions of these receptors in the hormone secretion and growth of insulin-secreting cells, as well as possible exocrine functions and interactions between the exocrine and endocrine pancreas.

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