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CYPROHEPTADINE-INDUCED ALTERATIONS IN CLONAL INSULIN-PRODUCING CELL LINES

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Abstract—Cyproheptadine (CPH) inhibits glucose-stimulated insulin synthesis and secretion, and reversibly depletes pancreatic insulin content in the rat. To examine whether the inhibitory actions of CPH on insulin cell function are linked to the ability of glucose to stimulate insulin synthesis and secretion, studies were performed in two different insulin-producing cell lines. CPH effects were compared in HIT-T15 cells, which respond to glucose with increased insulin synthesis and secretion, and in glucose-unresponsive RINm5F cells. CPH produced similar alterations in both cells lines. After a 48-hr culture period in the presence of 0, 0.1, 1.0 or 10.0 μM CPH, cellular insulin stores and media insulin levels were decreased in a concentration-dependent manner. At 10.0 μM CPH, RIN and HIT cell insulin content declined to 34 and 33% of controls respectively. Cellular insulin returned to control levels 48 hr after removal of CPH. In experiments designed to test a direct inhibitory effect on stimulated insulin secretion, 1 and 10.0 μM concentrations of CPH were found to inhibit glucose-stimulated insulin release from HIT cells, and K^+, alanine and glyceraldehyde-stimulated release from RIN cells. CPH was also shown to inhibit insulin biosynthesis in both cell lines at concentrations that did not alter the synthesis of total cellular proteins. All of these alterations in cellular function were shown to occur at CPH concentrations that did not affect cell growth or viability. The results show that the actions of CPH do not appear to be dependent upon the existence of operational glucose signalling mechanisms for insulin synthesis and secretion.

The use of clonal insulin-producing cell lines has advanced our understanding of the normal regulation of insulin synthesis and secretion. Two such cell lines, RINm5F and HIT-T15, are currently being used in this laboratory to investigate mechanisms by which xenobiotic substances can perturb insulin synthesis and secretion.

The HIT cell line was established in culture from SV40-transformed hamster pancreatic islet cells [1], and the RIN cell line from a radiation-induced transplantable islet cell tumor [2, 3]. HIT cells have been shown to secrete and synthesize insulin in response to stimulation by glucose [4–8]. RIN cells, while known to be essentially unresponsive to glucose stimulation [9, 10], will secrete and synthesize insulin in response to stimulation by other physiological and pharmacological stimuli [9–11].

Cyproheptadine (CPH), a diabetogenic drug, directly inhibits glucose-stimulated proinsulin synthesis and insulin secretion in isolated islets from rats [12–14], and reversibly depletes pancreatic proinsulin and insulin content after administration to rats [12, 15]. The mechanisms by which CPH inhibits insulin synthesis and secretion are not clear, but these actions are unrelated to the antihistaminic and antiserotonergic actions of the drug [16]. Interference with calcium movement across the β-cell plasma membrane has been implicated in the inhibition of insulin secretion [13, 17], but no mechanistic information exists on the CPH-induced block in proinsulin synthesis. The effects of CPH on the function of clonal insulin-producing cell lines have not been studied, and this report provides the first information on the sensitivity of RINm5F and HIT-T15 cells to the diabetogenic actions of CPH.

Extracellular glucose concentration is the major controlling factor in the synthesis and secretion of insulin [18]. In the present study, we examined whether the differences in glucose-responsiveness of RINm5F and HIT-T15 cells are manifested in differences in the susceptibility of the cell lines to CPH-induced alterations in the synthesis and secretion of the hormone. If similar effects could be demonstrated in glucose-responsive and -unresponsive cells, it would indicate that CPH actions are not dependent upon the existence of functional glucose signalling mechanisms for insulin synthesis and secretion.

MATERIALS AND METHODS

Materials

Cell culture. RPMI 1640 culture medium (180 mg/dL glucose), Ham's F-12 Nutrient Mixture (200 mg/dL glucose), fetal bovine serum, dialyzed horse serum, penicillin/streptomycin and trypsin were obtained from the Grand Island Biological Co. (Grand Island, NY). RINm5F cells were the gift of Dr. Paolo Meda (Geneva, Switzerland), and HIT-T15 cells were provided by Dr. Robert Santerre (Eli Lilly, Indianapolis, IN).

Chemicals. Cyproheptadine, as the hydrochloride monohydrate, was obtained from the Merck Institute for Therapeutic Research (West Point, PA). The purity was checked by HPLC as described by Chow and Fischer [19]. DL-Glyceraldehyde, potassium

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chloride, DL-alanine, D-glucose, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), bovine serum albumin, bisbenzimide trihydrochloride (Hoescht No. 33258) and EDTA were obtained from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]Leucine (NET-135H) and ^{125}I-labeled porcine insulin (NEX-196) were purchased from the New England Nuclear DuPont Co. (Wilmington, DE). Radiochemical purity of [^{3}H]leucine and ^{125}I-labeled insulin was checked by thin-layer chromatography [20] and high performance liquid chromatography [21] respectively. Protein A-Sepharose was obtained from Pharmacia LKB (Piscataway, NJ). Guinea pig anti-insulin serum was obtained from the Department of Pharmacology, University of Indiana (Indianapolis, IN). Highly purified rat standard insulin was purchased from Novo Biolabs (Danbury, CT). All other reagents were of the highest quality commercially available.

Methods

Cell culture. HIT-T15 cells were maintained in culture according to the specifications of Santerre *et al.* [1]. Cells were grown at 37° in an atmosphere of 5% CO_2 and 95% O_2 in Ham's F-12 Nutrient Mixture supplemented with 15% dialyzed horse serum, 2.5% fetal bovine serum, 200 μg/mL penicillin and 200 milliunits/mL streptomycin. HIT-T15 cells were plated (2×10^5 cells/dish) in 35-mm Falcon tissue culture dishes. HIT-T15 cells used for these experiments were from passage number 57–68. During this interval, cells remained responsive to glucose stimulation of insulin release. Later passage numbers (>70) were not responsive to glucose stimulation and were not used for these experiments.

RINm5F cells were cultured as described by Gazdar *et al.* [3]. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 μg/mL penicllin and 200 milliunits/mL streptomycin. RINm5F cells were plated (2×10^4 cells/dish) in 35-mm Falcon tissue culture dishes. The RINm5F cells used for these experiments had undergone from 10 to 40 passages in our laboratory. At passage number 40, the insulin secretory response to glyceraldehyde, potassium and alanine was similar in magnitude to the response at passage number 10. For both cell lines, stock cultures were passaged weekly, and received fresh media every 2 days. The cells did not reach confluence during the experimental period.

Measurement of cellular insulin content. Experiments were first conducted to determine whether CPH would deplete cellular insulin content in RINm5F and HIT-T15 cells. Cells were plated as described above, and then cultured for 3 days prior to starting the experiments. After this acclimation period, the media were aspirated and replaced by test media containing 0, 0.1, 1.0 or 10.0 μM CPH. CPH was added to culture media as 100-fold concentrated stock solutions in sterile water. Control dishes received the same volume (20 μL) of sterile water. Cells were cultured in test media for 48 hr; then media were collected and cells were processed for analysis of insulin. Culture media were removed, centrifuged (500 *g*, 10 min), and frozen until insulin radioimmunoassay (RIA) [22]. Cells were harvested by gentle trypsinization, then washed two times and resuspended in 1 mL of cold Hanks' balanced salt solution. Cells in 450-μL aliquots of this suspension were pelleted (150 *g*, 5 min), and resuspended either in 450 μL of 1 N acetic acid or 450 μL of DNA assay buffer (2 M NaCl, 50 mM $NaPO_4$, 2 mM EDTA, pH 7.4) [23]. Cells that were resuspended in 1 N acetic acid were heated for 5 min at 95°, stored at 4° for 12–15 hr to fully extract insulin and proinsulin, and centrifuged (10,000 *g*, 10 min, 4°). The supernatant fraction was frozen until insulin radioimmunoassay. Cells resuspended in DNA assay buffer were sonicated (three times, 10 sec) with a Virsonic 300 sonicator (Virtis Co., Gardiner, NY) at setting 4 (microprobe) and then stored at 4° (12–24 hr) until assayed for DNA [23].

Since CPH-induced depletion of pancreatic insulin content in rat β-cells is reversible upon removal of the drug [15], we determined whether the CPH-induced depletion of cellular insulin content seen after 48-hr exposures of RINm5F and HIT-T15 cells was also reversible. For these experiments, cells were cultured in test media (0 or 10 μM CPH) for 48 hr (with media change at 24 hr), then washed (3 × 2 mL) and cultured in CPH-free media for an additional 48-hr period. At the end of the exposure period and after 24 and 48 hr of recovery, media were collected for insulin RIA, and cells were harvested as above for estimation of cellular insulin and DNA.

For both cell lines, it has been reported previously that there is passage to passage variability in cellular insulin content and secretion [3, 5, 24, 25]. In our laboratory, we also have observed some passage-dependent variability in these parameters. To normalize for this variability, results are expressed as percent of control for each experiment, where all dishes for a given experiment were from the same passage. Over the course of these experiments, control RINm5F cells contained 15.4 ± 2.6 ng insulin per μg DNA (mean ± SE, N = 13) and released 1148 ± 167 ng insulin per culture dish during the 48-hr exposure interval. Control HIT-T15 cells contained 37.4 ± 6.9 ng insulin per μg DNA (N = 19), and released 301 ± 48 ng insulin per culture dish during the 48-hr exposure interval.

Measurement of secretagogue-stimulated insulin release. The ability of CPH to directly inhibit the stimulated secretion of insulin from the cells was examined. Cells received fresh media on day 3 after plating and were used for secretion tests on the following day. Culture media were aspirated and the cells were washed (3 × 2 mL) and preincubated for 30 min at 37° in a modified Krebs–Ringer bicarbonate (KRB) buffer containing 10 mM HEPES, bovine serum albumin (5 mg/mL for RINm5F cells and 1 mg/mL for HIT-T15 cells), and 2.8 mM glucose (RINm5F cells only). After the preincubation, cells were incubated for 45 or 60 min in 1 mL of KRB/HEPES containing CPH (0, 0.1, 1.0 or 10.0 μM) and selected secretagogues (HIT-T15 cells: 50 mg/mL D-glucose; RINm5F cells: 10 mM DL-alanine, 10 mM DL-glyceraldehyde, or 20 mM potassium chloride). Concentrations of secretogogues were selected based on preliminary concentration–response experiments. The test concentrations elicited 80–90% of maximum stimulated release. After the test incubations, the buffer was removed, centrifuged to remove any cells

(500 *g*, 10 min) and frozen until analyzed for immunoreactive insulin.

Measurement of insulin biosynthesis. Media from cells grown in culture for 3 days were aspirated and replaced with 1 mL of fresh culture media containing 0, 0.1, 1.0, 5.0 or 10.0 μM CPH and 50 μCi/mL [^{3}H]Leucine (added in 50 μL of 0.01 N NaOH). After 24 hr of labeling under culture conditions, cells were washed (3 × 2 mL) with KRB/HEPES; then 200 μL of 1 N acetic acid was added to each culture dish. Dishes were kept at 4° for 12–15 hr to extract insulin and proinsulin; then the acetic acid was removed and centrifuged (500 *g*, 10 min, 4°), and the supernatant fraction was frozen at −20° until analysis for labeled insulin-like proteins. Incorporation of [^{3}H]leucine into insulin immunoreactive proteins and total cellular proteins was quantitated by Protein A-Sepharose immunoprecipitation [26] and trichloroacetic acid precipitation [27], respectively, followed by liquid scintillation counting. Control RINm5F cells and HIT-T15 cells typically incorporated 3–4 × 10^5 dpm of [^{3}H]leucine into insulin and proinsulin per 10^6 cells during the labeling period.

Measurement of cytotoxicity. To determine whether CPH effects were due to general cytotoxicity, cells were exposed to CPH under similar conditions as for the insulin depletion experiments, then harvested by gentle trypsinization and examined for their ability to exclude trypan blue. Additionally, since both RINm5F and HIT-T15 cells rapidly divide and proliferate in culture, comparison of growth rates between treated and control cells served as a confirmatory index of cell viability. Growth rates for the cells were estimated by measuring DNA content per culture dish (see above) at various time points following plating.

Statistical analysis. Where appropriate, data were analyzed by a randomized block design analysis of variance [28] and then expressed as a percentage of controls (except where noted). Statistical significance was evaluated at the $P = 0.05$ level. Typically, experiments consisted of 3–5 culture dishes per treatment and were repeated on 3–5 separate occasions. For statistical analyses, the averaged value from replicate dishes in a single experiment represented an N of 1.

RESULTS

The ability of CPH to alter the cellular insulin content of RINm5F and HIT-T15 cells is shown in Fig. 1. Culture of cells for 48 hr with various concentrations of CPH depleted cellular insulin content in both cell lines. This depletion was concentration dependent, and reached approximately 30% of controls using 10 μM CPH. There was no apparent difference between the cell lines in their sensitivity to CPH-induced depletion of cellular insulin.

Media insulin levels after 48 hr of exposure of cells to CPH are shown in Fig. 2. CPH reduced media insulin levels during culture of both cell lines. The reduction was also concentration dependent, and maximum using 10 μM CPH. As for cellular insulin depletion caused by CPH, both cell lines appeared equally sensitive to reductions in insulin released into media.

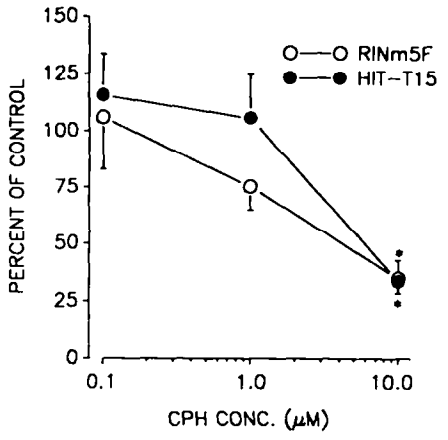

Fig. 1. Cellular immunoreactive insulin content of RINm5F and HIT-T15 cells after culture for 48 hr in the presence of CPH. Values were calculated as ng of cellular insulin per μg of DNA and are expressed as a percent of control (no CPH) for each experiment. See Materials and Methods for information on control values. Each point is the mean value (± SE) of three or four separate experiments (five culture dishes per treatment per experiment). Asterisks denote values significantly different from controls for each cell line ($P < 0.05$).

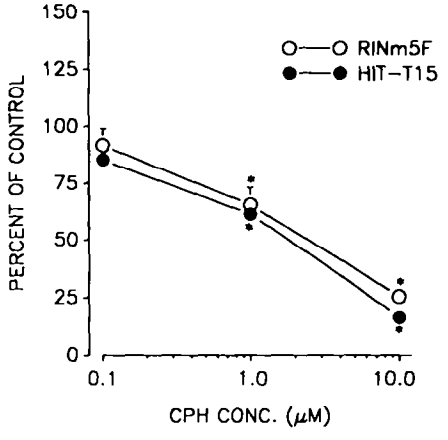

Fig. 2. Immunoreactive insulin levels in media after culture of RINm5F and HIT-T15 cells for 48 hr in the presence of CPH. Values were calculated as total ng insulin in the media, and are expressed as a percent of control (no CPH) for each experiment. See Materials and Methods for information on control values. Each point is the mean value (±SE) of four separate experiments (five culture dishes per treatment per experiment). These values varied between passages and according to the cell density during the experiment. For some data points the SE is within the dimensions of the symbol. Asterisks denote values significantly different from controls for each cell line ($P < 0.05$).

Experiments were conducted to investigate whether the depletion of cellular insulin content caused by CPH was reversible. Results shown in Fig.

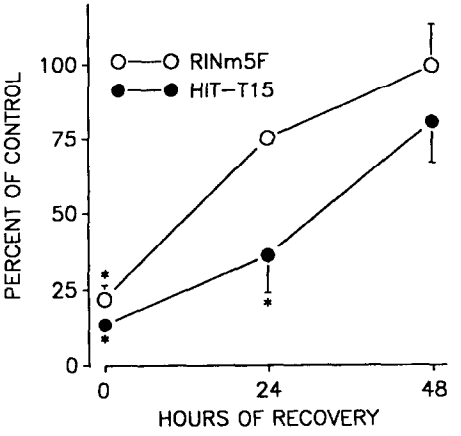

Fig. 3. Recovery of cellular immunoreactive insulin content in RINm5F and HIT-T15 cells after removal of 10 μM CPH (48-hr exposure). Values were calculated as ng of cellular insulin per μg of DNA, and are expressed as a percent of control for each experiment (no CPH during the exposure period; see Materials and Methods for information on control values). Each point is the mean value (± SE) of three separate experiments (three culture dishes per treatment per time point per experiment). For some data points the SE is within the dimensions of the symbol. Asterisks denote values significantly different from controls for each cell line at each time point ($P < 0.05$).

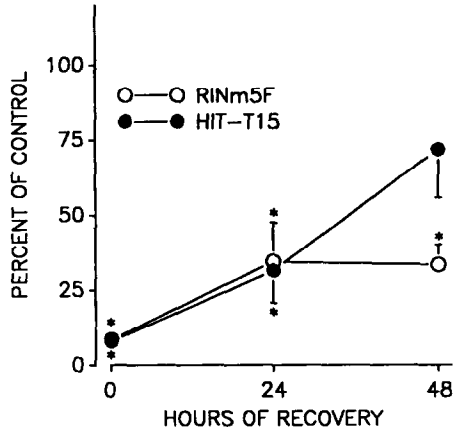

Fig. 4. Recovery of media insulin during culture of RINm5F and HIT-T15 cells after removal of 10 μM CPH (48-hr exposure). Values were calculated as ng insulin in media from each 24-hr interval during the recovery period, and are expressed as a percent of control values (no CPH exposure) at each time point. See Materials and Methods for information on control values. Each point is the mean value (± SE) of three separate experiments (three culture dishes per treatment per time point per experiment). For some data points the SE is within the dimensions of the symbol. Asterisks denote values significantly different from controls for each cell line at each time point ($P < 0.05$).

3 demonstrate that during a 48-hr recovery period following exposure for 48 hr to 10 μM CPH, cellular insulin content in both RINm5F and HIT-T15 cells returned to control levels. Media levels of insulin during recovery of cells are shown in Fig. 4. Media insulin did not return to control as readily as cellular insulin content. Media insulin levels from HIT-T15 cells recovered to a greater extent than from RINm5F cells, but were still less than control at the end of the 48-hr recovery period.

To determine if the CPH-induced effects were associated with cytotoxicity, experiments were conducted to characterize the effects of CPH on cell viability and division. Cells exposed to 10 μM CPH showed no reduction in the ability to exclude trypan blue relative to control cells after 24 and 48 hr of exposure (>95% of control cells excluded trypan blue, data not shown). Effects of CPH on cell division were estimated by comparison of DNA content per culture dish after 48 hr of exposure to 0 or 10.0 μM CPH. Dishes containing cells exposed to 10.0 μM CPH showed no reduction of DNA content after 48 hr of exposure (Fig. 5). Dishes containing cells exposed to 10.0 μM CPH for 48 hr and then cultured for 24 or 48 hr in CPH-free media (Fig. 5) also showed no reduction of DNA content relative to untreated controls. These results suggest that 10 μM CPH does not alter viability or inhibit cell division for either cell line.

Experiments were conducted to determine the ability of CPH to inhibit insulin biosynthesis in RINm5F and HIT-T15 cells. Results shown in Fig. 6 illustrate that CPH selectively inhibited insulin synthesis in both cell lines. Using CPH concentrations as high as 10.0 μM, incorporation of [^{3}H]leucine into total cellular proteins was not inhibited, whereas incorporation of radioactivity into insulin immunoreactive proteins was reduced to about 25% of that observed in controls. The inhibition of insulin biosynthesis was concentration dependent in each cell line.

The ability of CPH to directly inhibit secretagogue-stimulated insulin release from RINm5F and HIT-T15 cells is shown in Fig. 7 (A–D). Inhibition of glucose-stimulated insulin release from HIT-T15 cells is shown in Fig. 7A. CPH inhibited glucose (50 mg/dL) stimulated insulin release from HIT-T15 cells in a concentration-dependent manner with 1 and 10 μM CPH producing significant inhibition. Basal release of insulin (no glucose present) was not affected by CPH exposure (data not shown). The ability of CPH to inhibit insulin release from RINm5F cells when stimulated by 10 mM DL-glyceraldehyde, 10 mM DL-alanine or 20 mM potassium chloride is shown in Fig. 7 (B, C and D). The CPH concentration–responses for inhibition of release varied somewhat with the secretogogue used. Generally, significant inhibition of release occurred at CPH concentrations between 1.0 and 10.0 μM. As for HIT-T15 cells, basal release in RINm5F cells (no stimulus present) was not altered by CPH (data not shown).

DISCUSSION

Both RINm5F and HIT-T15 cells responsed to CPH exposure with reversible losses of cellular insulin content, and diminished insulin biosynthetic and secretory capacities. CPH-induced responses were concentration dependent, and occurred at non-cytotoxic concentrations. These results are consistent with those observed following CPH administration

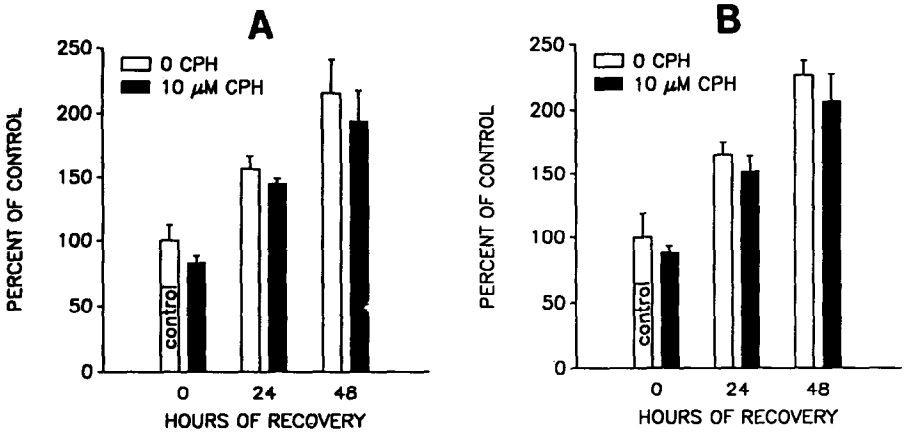

Fig. 5. Lack of CPH effects on RINm5F (A) and HIT-T15 (B) cell growth during recovery from exposure. Values were calculated as μg of DNA per culture dish and are expressed as a percent of the amount in control dishes (no CPH) at the beginning of the recovery period. Control dishes of RINm5F and HIT-T15 cells contained 52–101 and 25–44 μg of DNA respectively. Each bar represents the mean value (± SE) of three separate experiments (three culture dishes per treatment per time point per experiment).

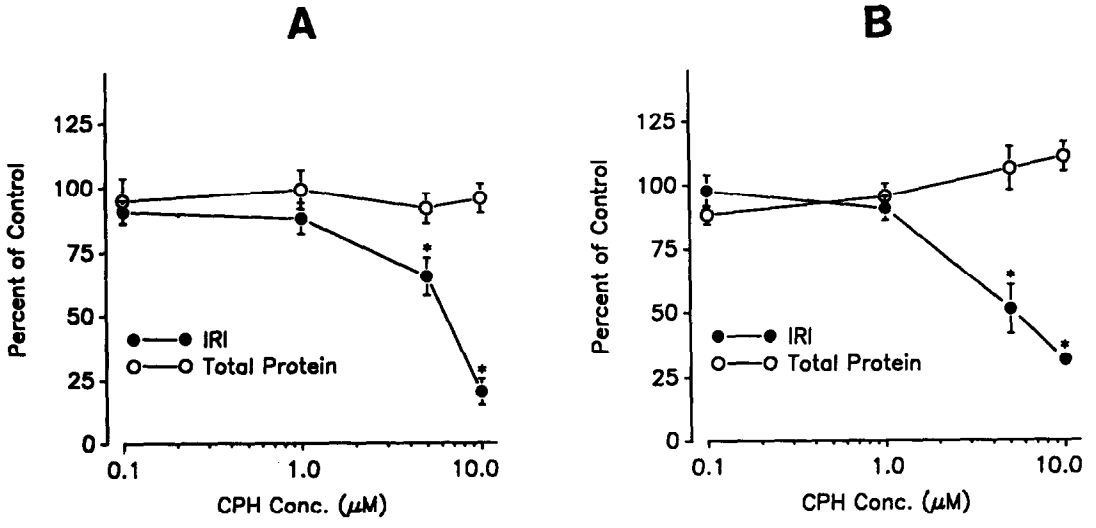

Fig. 6. CPH-induced inhibition of immunoreactive insulin and total protein biosynthesis in RINm5F (A) and HIT-T15 (B) cells during a 24-hr [^{3}H]leucine labeling period in the presence of CPH. Values were calculated as radioactivity incorporated into immunoreactive (IRI) and total proteins (see Materials and Methods), and expressed as a percent of control (no CPH) for each experiment. See Materials and Methods for information on control values. Each point is the mean value (± SE) of three to five separate experiments. For some data points the SE is within the dimensions of the symbol. Asterisks denote values significantly different from controls for each cell line ($P < 0.05$).

to rats [15], and those occurring in isolated rat pancreatic islets exposed to CPH *in vitro* [12, 14]. On the basis of these findings, RINm5F and HIT-T15 cells appear to be adequate models for CPH actions in rat islet β-cells.

The depletion of cellular insulin content in the clonal cells appears to be due to the ability of CPH to inhibit biosynthesis, because the drug reduced incorporation of [^{3}H]leucine into immunoprecipitable protein. It is likely that in RINm5F and HIT-T15 cells, as has been shown for freshly isolated rat islets [12], CPH inhibits the production of insulin by inhibiting the synthesis of its precursor, proinsulin. We have not provided direct evidence for this in these studies because the antibody used in the immunoprecipitation binds both insulin and proinsulin. The biochemical mechanisms by which CPH inhibits insulin synthesis are currently the subject of investigations in this laboratory, and the RIN and HIT cell lines will be of use in these studies.

The reduction of media insulin levels observed after 48-hr exposures of the cells to CPH is probably

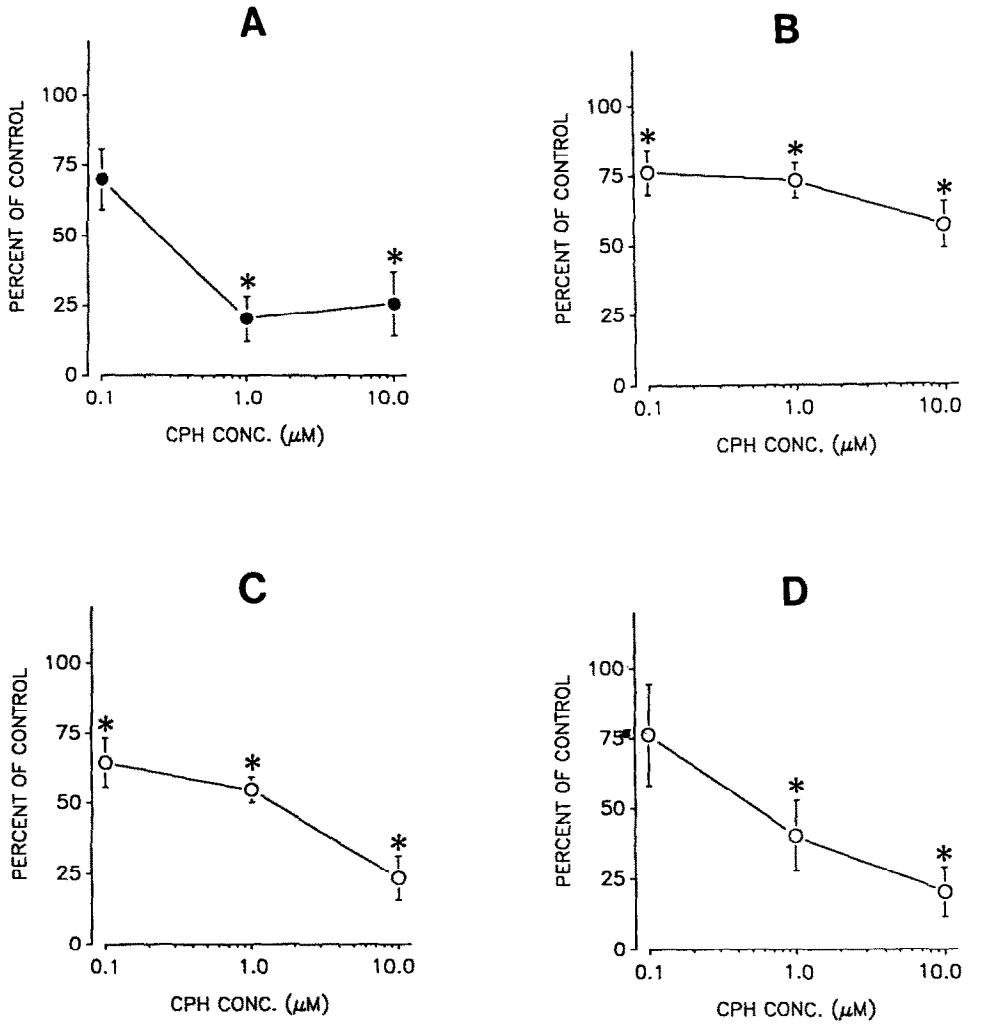

Fig. 7. CPH-induced inhibition of D-glucose (50 mg/dL)-stimulated immunoreactive insulin release from HIT-T15 cells (panel A) and of DL-glyceraldehyde (10 mM, panel B), DL-alanine (10 mM, panel C) or potassium (20 mM KCl, panel D)-stimulated insulin release from RINm5F cells. Stimulated secretion was calculated from increases above basal release (secretogogue absent, CPH absent) and are expressed as a percent of maximal secretion (secretogogue present, CPH absent). Glucose alone produced a 2.5-fold stimulation of insulin release from HIT-T15 cells. Glyceraldehyde, alanine and potassium, each alone, produced 2-, 4-, and 10-fold stimulations, respectively, of insulin release from RINm5F cells. Each point is the mean value (± SE) from three to ten separate experiments (three culture dishes per treatment per experiment). Asterisks denote values significantly different from controls for each cell line ($P < 0.05$).

due to direct inhibition by the drug of insulin secretion, because as shown in this study, CPH inhibits secretogogue-stimulated insulin release. The lower concentration of insulin in the media may also be due to the depleted cellular insulin content, resulting in reduced amounts of releasable insulin in the cells.

CPH-induced depletion of cellular insulin content in RINm5F and HIT-T15 cells was completely reversible upon removal of the drug, and this is consistent with recovery of pancreatic insulin seen after administration of CPH to rats [15]. A complete recovery of media insulin levels was not observed, but this result may not accurately reflect the status of insulin secretion by the cells at the end of the 48-hr recovery period. The media insulin values represent cumulative hormone release over a 24-hr period, and full recovery attained during the 24- to 48-hr period would not have been detected.

The fact that CPH inhibited insulin secretion in response to stimulation by glyceraldehyde, alanine or potassium depolarization in RINm5F cells is consistent with current knowledge of CPH actions in β-cells. The insulin secretory responses of RINm5F cells to these stimulants have been shown to be calcium dependent [11], and it is known that CPH can interfere with calcium movement into islet β-cells [13, 17]. CPH inhibition of glucose-stimulated insulin release from HIT-T15 cells is also consistent with the proposed calcium antagonist properties of

CPH, as glucose-stimulated insulin release from HIT-T15 cells is known to be calcium dependent [5, 29].

Steady-state cellular insulin content and biosynthesis rates are parameters associated with insulin gene expression. Regulation of insulin gene expression in HIT-T15 cells is under glucose control [6, 7], whereas in RINm5F cells it appears not to be [10, 30]. Sensitivity to alterations of insulin synthesis and content caused by CPH might be expected to differ in cells that regulate insulin gene expression differently. The observation that CPH inhibited insulin synthesis and depleted insulin content in both cell lines suggests that its mechanism of action may not be dependent upon the existence of operational glucose signalling mechanisms for insulin synthesis and secretion.

The biochemical basis for the glucose-unresponsiveness of RINm5F cells is not well understood. It has been suggested that abnormalities of glucose transport [31–33] and/or metabolism [34–36] may account for the failure of RIN cells to secrete insulin in response to simulation by glucose. The reasons for the glucose insensitivity of insulin biosynthesis in RINm5F cells have not been elucidated. It is reasonable to suggest that CPH acts at a site that is distal to the abnormality that causes RINm5F cells to be unresponsive to glucose.

RIN cells have been used to investigate a possible link between glucose handling and the selective toxicity of streptozotocin to insulin-secreting cells. Unlike CPH, the actions of streptozotocin appear to be dependent upon the existence of normal glucose signalling mechanisms because RIN cells were found to be less sensitive to the actions of streptozotocin when compared to normal islet β-cells [37, 38]. This lower sensitivity may be due to a reduced ability of RIN cells to transport glucose and the glucose-containing streptozotocin molecule into cells. When compared to published results obtained for isolated rat islets [12, 14], RINm5F cells appear to be as sensitive as isolated pancreatic islets to the insulin-inhibitory actions of CPH. Since RINm5F cells respond to CPH in a manner similar to that of normal islet β-cells, and to HIT-T15 cells, CPH actions do not appear to be dependent upon the existence of normal glucose signalling mechanisms.

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