Insulin Secretion and cAMP Metabolism in HIT Cells

Reciprocal and Serial Passage-Dependent Relationships

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The HIT cell is a variably glucose-responsive clonal line of pancreatic islet β-cells. To ascertain whether insulin responsiveness to glucose, arginine, isoproterenol, forskolin, and K^+ varied in a predictable fashion, full concentration-response curves with these agonists were examined with cells from a span of 25 passages. Basal and stimulated cAMP metabolism were also examined. The findings indicate that insulin responses to glucose diminish progressively with increasing passage number and that studies of glucose-induced insulin secretion should be limited to passages 81 and earlier. This defect in insulin secretion is a general rather than a glucose-specific phenomenon in that insulin responses to the other nonglucose secretagogues also diminished with increasing passage number. All changes in glucose-stimulated responses were limited to diminutions in maximal responses; no alterations in apparent half-maximal effective concentrations (EC_{50}s) were observed. In contrast to the continually diminishing insulin responsiveness observed, hormone inhibition by somatostatin of insulin secretion and basal cAMP metabolism remained intact throughout the passages examined. Interestingly, a reciprocal relationship between insulin responsiveness and cAMP responsiveness was observed. Dramatic cAMP responses to isoproterenol and forskolin were observed with the later passages. We conclude that loss of insulin responsivity in HIT cells is a passage-dependent process that is serial rather than sporadic and global rather than glucose specific. Dramatic reciprocal changes in cAMP metabolism occur in the later passages. *Diabetes* 38:44–48, 1989

The HIT cell is a clonal line of pancreatic islet β-cells that resulted from simian virus 40 transformation of Syrian hamster pancreatic islets. Since the initial report on the HIT cell in 1981 by Santerre et al. (1), many investigators have recognized the value of this cell line and have used it to study β-cell function (2–20). The HIT cell has the important features of secreting insulin in response to glucose (1,5,7–9,18) and of responding to hormonal inhibitors of insulin secretion (1,3,11). However, one limitation is that insulin secretion from the HIT cell has been observed to vary with passage number (1,9).

These studies were designed to answer four specific questions. *1*) Is the decrease in insulin secretion from HIT cells glucose specific? *2*) Is this decrease serially passage dependent or sporadic? *3*) Is the diminished insulin response due to decreased HIT cell sensitivity to secretagogues or to decreased insulin content? *4*) Are there associated changes in cAMP metabolism? To answer these questions, we used cells from a span of 25 passages to examine insulin-response curves to incremental doses of five agonists and to examine inhibition and stimulation of cAMP metabolism.

MATERIALS AND METHODS

HIT cell cultures. The original culture from which the cells used in these studies were derived was provided by A.E. Boyd III (Baylor University, Waco, TX). Studies were performed with culture passages 70–94. HIT cells were grown in 5% CO_2/95% air at 37°C and maintained in RPMI-1640 medium containing 10% fetal calf serum and 11.1 mM glucose. Cells from later passages grew more rapidly; consequently, cells were split for subculture at 1:3, 1:4, 1:5, and 1:6 for passages 70–75, 76–80, 81–85, and 86–90, respectively. Before each study was conducted, cells were subcultured in the absence of experimental drugs for 2 days by plating ~10^6 cells per well in 12-well plates. Cells were monitored for bacterial contamination, and none (specifically

Insulin 1 pM = 0.139 μU/ml

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mycoplasma) was found in earlier or later passages. No antibiotics were used.

Insulin secretion, insulin content, and cAMP accumulation. At the beginning of the 3rd day of culture, the plates were incubated twice for 30 min at 37°C in Krebs-Ringer buffer (KRB) containing 119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM HEPES at pH 7.4 and 0.1% albumin. After each incubation, the cells were washed twice with KRB. Static incubations were then performed for 30 min with varying concentrations of pharmacologic agents in KRB containing 0.8 mM glucose. The entire 1-ml volume was taken at time 0 or after a 30-min incubation for determination of insulin, which was measured as reported (21). Insulin content in acid extracts of HIT cells was determined by the method of Santerre et al. (1). Cyclic AMP accumulation was measured by modification of the method of Barber et al. (22) in separate studies with KRB containing 0.8 mM glucose and 1 or 5 μCi/well of [^{3}H]adenine. The cells were preincubated 90 min at 37°C, washed twice with KRB, then incubated 30 min without and with isoproterenol or forskolin. The cells were then treated with 2.8% trichloroacetic acid for 20 min. Supernatants were used for isolating [^{3}H]cAMP by Dowex and alumina columns for subsequent counting (22).

The pharmacologic agents used in these experiments included incremental concentrations of glucose (Sigma, St. Louis, MO) at 0.4, 0.8, 1.7, 5.6, and 16.7 mM; arginine (Sigma) at 0.25, 0.50, 1.0, 2.0, and 10 mM; isoproterenol (Sigma) at 0.001, 0.01, 0.10, 1.0, and 10 μM; forskolin (Calbiochem, La Jolla, CA) at 0.001, 0.01, 0.10, 1.0, and 10 μM; potassium (Mallinckrodt, Paris, KY) at 5, 10, 20, 40, 80, and 160 mM; and somatostatin (Bachem, Torrance, CA) at 0.1 μM.

Statistics. Statistical comparisons were performed by Student's *t* test for unpaired intergroup comparisons.

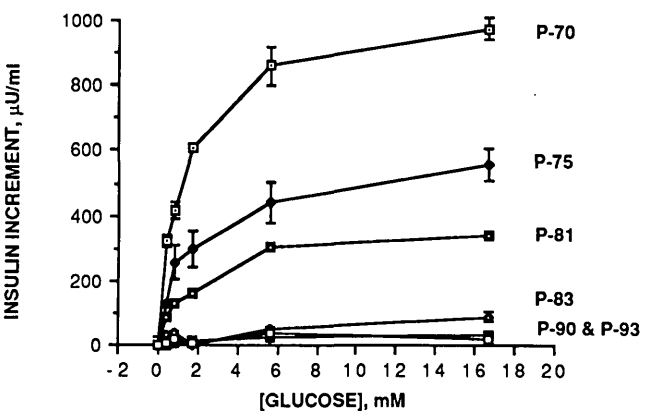

FIG. 1. Insulin responses to glucose in various passages of HIT cells. Basal insulin levels in absence of glucose after 30 min for passages 70, 75, 81, 83, 90, and 93 were 580 ± 14, 459 ± 24, 387 ± 8, 180 ± 4, 50 ± 0, and 116 ± 13, respectively. Insulin increments calculated by subtracting basal insulin levels from the level observed at end of the 30-min incubation with the designated concentration of glucose. All experiments were performed using triplicate wells of HIT cells for each glucose concentration in each passage number; insulin samples were measured in duplicate. Passage-dependent diminution in glucose-stimulated insulin responses was observed, but no change in apparent EC_{50} was observed (1.7 mM in passages P-70, P-75, and P-81).

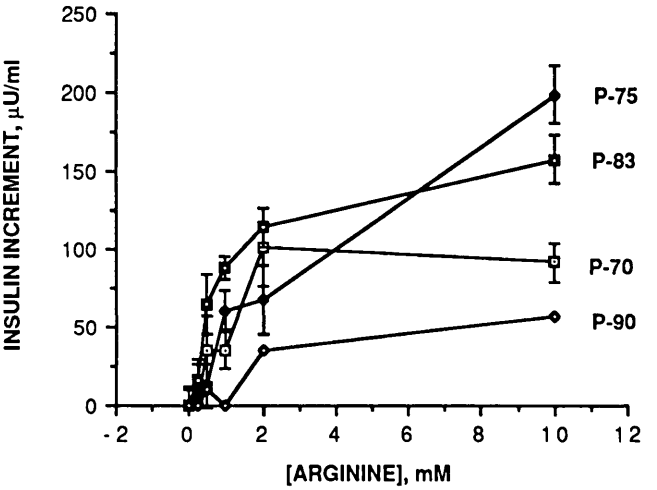

FIG. 2. Insulin responses to increasing concentrations of arginine in various passages of HIT cells in presence of 0.8 mM glucose. Experimental conditions as described in Fig. 1.

RESULTS

Insulin response curves to glucose, arginine, isoproterenol, forskolin, and potassium and insulin content. Insulin responses to increasing concentrations of glucose were examined via six different passages from passages 70–93 (Fig. 1). Total protein concentration per culture well was 0.236 ± 0.038 mg/10^6 cells (mean ± SD). Basal insulin levels in the absence of glucose ranged from 50 to 580 μU/ml and generally decreased as passage number increased. The incremental insulin response to increasing concentrations of glucose was greater in the earlier passages and progressively smaller in later passages. The maximal response observed in passage 70 was approximately threefold; the maximum response observed in passage 94 was barely detectable. The concentration of glucose that provided the apparent half-maximum response (apparent EC_{50}) did not change as passage numbers increased.

The general trend for decreasing maximal insulin response with increasing passage number was also seen with arginine, isoproterenol, forskolin, and potassium as secretagogues with certain differences (Figs. 2–5). For example,

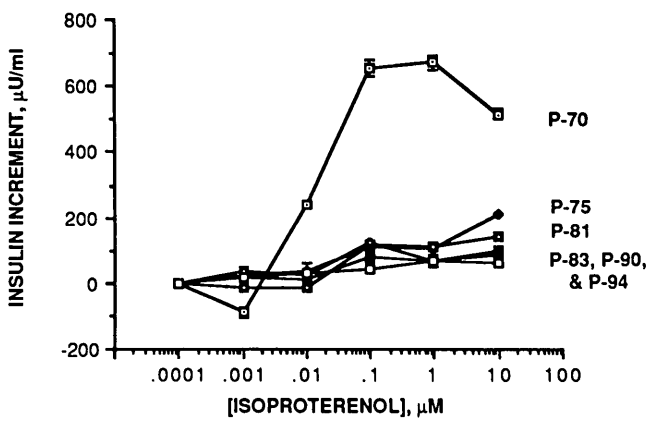

FIG. 3. Insulin responses to increasing concentrations of isoproterenol in various passages of HIT cells in presence of 0.8 mM glucose. Experimental conditions as in Fig. 1.

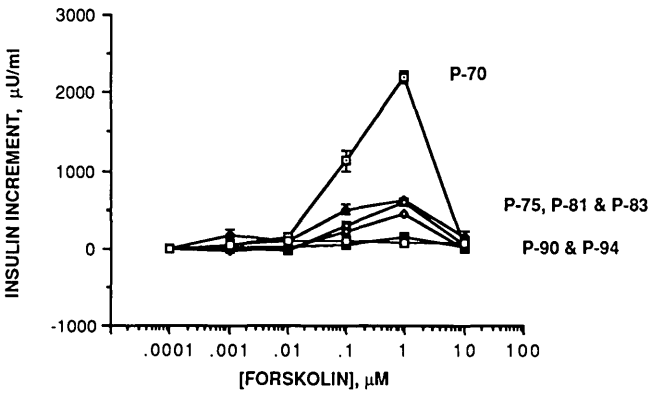

FIG. 4. Insulin responses to increasing concentrations of forskolin in various passages of HIT cells in presence of 0.8 mM glucose. Experimental conditions as in Fig. 1.

insulin responses to isoproterenol and forskolin in passage 70 were widely separated from the other passages. Passage 90 was relatively more responsive to arginine and potassium than to glucose, isoproterenol, and forskolin. Potassium elicited convincing insulin responses from the latest passages that were essentially unresponsive to glucose, isoproterenol, and forskolin. Total acid-extractable insulin contents for passages 70, 75, 82, and 94 were 5931 ± 698, 2629 ± 190, 2322 ± 480, and 706 ± 294 μU/mg protein, respectively.

Inhibition and stimulation of cAMP metabolism. Six passages were selected to ascertain whether relationships existed between basal, inhibited, and stimulated cAMP metabolism and insulin secretion. Basal cAMP levels tended to increase (Table 1) and basal insulin levels decreased (Fig. 6) with increase in passage number. Somatostatin retained its ability to inhibit cAMP production and insulin secretion as passage number increased (Fig. 6). Somatostatin inhibited basal cAMP generation by 27, 23, and 55% in passages 72, 85, and 93, respectively. The corresponding figures for inhibition of insulin secretion by somatostatin were 33, 52, and 34%. Isoproterenol and forskolin were used to determine whether stimulated levels of cAMP changed with increasing passage number. At 10-μM concentrations, forskolin was up to 100 times more effective than isoproterenol. Cyclic AMP responses to each agonist were similar for passages 70, 75, and 81 but were dramatically increased in passages 92–94 (Fig. 7; Table 1). The fold responses over basal cAMP levels for isoproterenol were 1.2, 1.4, 1.2, 26, and 56 for passages 70, 75, 81, 92, and 94, respectively. The fold responses for forskolin were 137, 127, 128, 540, and 673 for passages 70, 75, 81, 93, and 94 (Fig. 7).

TABLE 1
cAMP concentrations from HIT cell incubations

Passage	Basal	Isoproterenol (10 μM)	Forskolin (10 μM)
70	275 ± 12	334 ± 23	37,706 ± 650
75	234 ± 15	324 ± 25	29,793 ± 703
81	278 ± 31	327 ± 53	35,443 ± 232
92	535 ± 66	13,705 ± 1456*	
93	371 ± 35		200,234 ± 7158*
94	330 ± 31	18,556 ± 3162†	222,223 ± 4419*

cAMP concentrations (dpm/ml) in presence of 0.8 mM glucose with or without isoproterenol or forskolin for 30 min with 1 μCi [^{3}H]adenine/well. Values are means ± SE.
*P < .001, †P < .01 compared with passage 70.

DISCUSSION

Our data indicate that the decline in HIT cell responsiveness to glucose was progressive and occurred serially throughout the 25 passages examined. Therefore, it appears that studies of glucose-induced insulin secretion should be limited to passages 81 and earlier. The experiments with arginine, isoproterenol, forskolin, and potassium as secretagogues also demonstrated a general decline in insulin responsiveness with increasing passage number. Interestingly, forskolin paradoxically inhibited insulin secretion in all passages at the highest concentration we used (10 μM). We determined in control experiments that this inhibition was not due to forskolin's solvent (0.1% ethanol). The passage-related decreases in total extractable insulin suggest a defect in some aspect of insulin biosynthesis leads to the decline in insulin responsivity. This conclusion is supported by our finding that the apparent EC_{50} for glucose remained the same regardless of passage number, with changes in insulin secretion limited to diminutions in maximal responses.

In contrast to the decline in stimulated insulin levels, hormonal inhibition by somatostatin of cAMP generation and insulin secretion remained intact throughout the passages

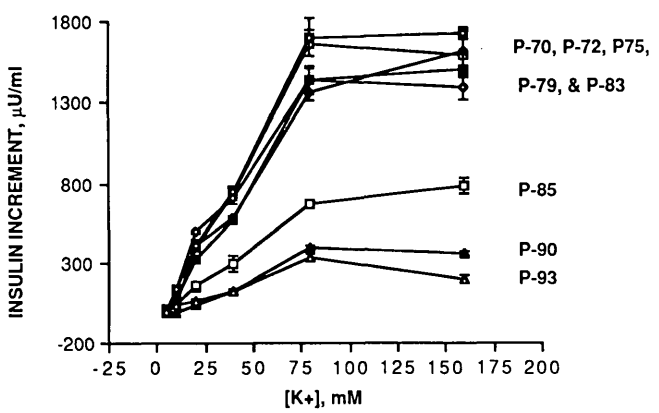

FIG. 5. Insulin responses to increasing concentrations of potassium in various passages of HIT cells in presence of 0.8 mM glucose. Experimental conditions as in Fig. 1.

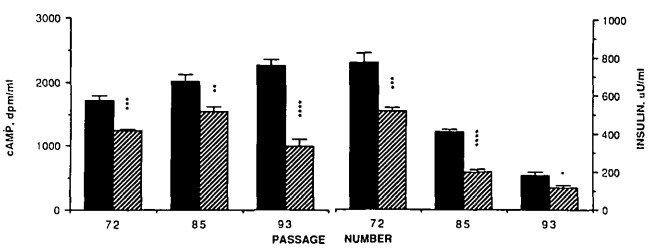

FIG. 6. Basal cAMP (*left*) and glucose-stimulated (5.6 mM) insulin (*right*) levels in various passages of HIT cells with (*hatched bars*, 0.1 μM) and without (*solid bars*) simultaneous incubation with somatostatin. Basal cAMP levels increased and insulin levels decreased with increasing passage number. Somatostatin inhibited both cAMP and insulin levels in all passages. All experiments were performed with triplicate wells of HIT cells for each passage number and 5 μCi [^{3}H]adenine/well. Values are means ± SE. *P = .05, **P < .02, ***P < .01, ****P < .001, somatostatin vs. no somatostatin.

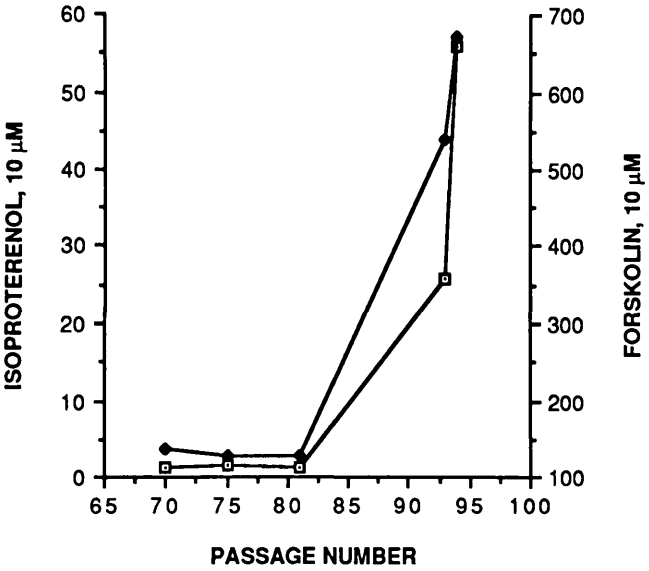

FIG. 7. Expression of data in Table 1 as fold responses of cAMP in response to isoproterenol (♦) or forskolin (⊡) in various passages of HIT cells. A striking increase was observed in response to each secretagogue in the later passages. Forskolin scale is 10 times isoproterenol scale.

we examined. The increase in basal cAMP and its response to isoproterenol and forskolin as passage number increased demonstrates a reciprocal relationship between insulin secretion and cAMP generation in the HIT cell. The dramatic cAMP responses to isoproterenol and forskolin in the later passages indicate functional β-adrenergic receptors and catalytic subunits of adenylate cyclase, respectively. Thus, these do not play roles in the failure of the later passages to release insulin after stimulation by isoproterenol and forskolin.

Although it is generally accepted that later passages of HIT cells lose glucose responsiveness, very little data have been published substantiating this contention. Santerre et al. (1) reported only that passage 41 released 30 times as much insulin as passage 88. However, it is not clear what glucose concentration was used during their experiments, and no glucose-concentration–insulin-response curves were reported. Ashcroft et al. (9) reported more extensive data by use of passages 70–77 to examine insulin content and basal and stimulated insulin responses to 10 mM glucose. They observed decreasing insulin secretion with increasing passage number but could not assess whether later passages developed decreasing sensitivity to glucose because glucose-concentration–insulin-response curves were not determined. Our data provide complete concentration-response curves for six passages ranging from 70 through 94 and demonstrate that the insulin secretory defect was limited to the maximal efficacy of glucose, whereas the potency (EC_{50}) of glucose remained the same in passages where an insulin response was observed. Our HIT cells were exquisitely sensitive to glucose as reflected by an EC_{50} of ~1.7 mM. Although other investigators have examined different doses of glucose, most did not report a sufficiently wide range of glucose concentrations to assess minimal and maximal responses. Where such data exist, there appear to be differences in EC_{50} in different laboratories. For example, in contrast with our value of 1.7 mM, data from Ashcroft et al. (9) and Hammonds et al. (16) indicate a value in the range of 7 mM. It can only be assumed that these differences reflect differences in handling the cells or in culture conditions before glucose-concentration–insulin-response experiments.

These experiments have also provided concentration-response data for four other agonists (arginine, isoproterenol, forskolin, and potassium) and indicated that insulin secretory loss was not glucose specific, i.e., it was not limited to glucose signals and was a shared characteristic of all five secretagogues examined. Interestingly, decreasing insulin responsivity was not found to occur uniformly at specific passage numbers for the different agonists. The meaning of this variation is not readily apparent. Very little information has been published about cAMP production by HIT cells. In 1986, Ashcroft et al. (9) reported that both glucose and forskolin increased HIT cell cAMP levels. Hill et al. (13) demonstrated that forskolin stimulates cAMP levels in a concentration-dependent fashion but did not observe increases in cAMP with glucose. Robertson et al. (11) reported inhibition of cAMP levels by prostaglandin E_2 and, based on pertussis toxin ADP-ribosylation experiments, postulated mediation of this event by negative guanine nucleotide regulatory components of adenylate cyclase.

We conclude that the loss of insulin responsivity of HIT cells is passage dependent in a serial rather than sporadic manner and that this loss is global rather than specific for glucose. Cyclic AMP production is reciprocally related to insulin secretion, and inhibition of cAMP production and insulin secretion by somatostatin is unaffected by passage number. The HIT cell appears to be a valuable investigative tool for studying questions on the relationships between and changes in insulin secretion and cAMP metabolism during the aging process in β-cells.

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