Characteristics of the Interaction of the Glucagon Receptor, cAMP, and Insulin Secretion in Parent Cells and Clone 5F of a Cultured Rat Insulinoma

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SUMMARY

Rat insulinoma cells, which grow in culture and secrete insulin, were used to study the mechanism of stimulation of insulin release by glucagon. The parent cell line (RIN-m) and a clone that secretes high levels of insulin (5F) had been shown to possess specific receptors for glucagon. Glucagon (1 μM) stimulated a rapid increase in cyclic adenosine 3′:5′-monophosphate (cAMP) that was followed by an increase in insulin secretion in both cell lines. The concentration of glucagon necessary for half-maximal stimulation of cAMP was 50 nM in parent and approximately 0.5 μM in 5F, whereas the concentration required to inhibit binding by 50% was 0.5 nM and 30 nM, respectively. In 5F, the dose-response relationships for cAMP and insulin secretion were superimposable. The glucagon effects on insulin secretion and cAMP did not require either glucose or amino acids in the incubation media. No refractoriness to glucagon stimulation of cAMP or insulin was noted.

It may be concluded that (1) there are significant differences between glucagon binding and glucagon responses in parent cells and clone 5F, (2) there are glucagon receptors that are not coupled to adenylate cyclase, and (3) cAMP mediates glucagon-stimulated insulin release. DIABETES 1985; 34:717–22.

We have had the opportunity to investigate cells from a rat islet cell tumor that have been grown in continuous culture for over 4 yr.[1] This tumor cell line (RIN-m-parent) and one of its clones (5F) secrete insulin in response to the addition of glucagon.[2,3] We have also demonstrated that these cultured cells have specific receptors for glucagon[4] and that the interaction of glucagon with these receptors is not a simple bimolecular reaction.[5] Furthermore, the characteristics of the Scatchard analyses and the affinity curves reveal significant differences between the parent and clone 5F.[4]

Glucagon-stimulated insulin secretion from the islets of normal rats is in some way mediated by an increase in cellular cAMP.[6] However, cAMP per se is rarely the primary signal for insulin secretion. With glucose and other secretagogues, cAMP provides a *facilitating* action in insulin secretion thought to involve the redistribution of intracellular calcium.[7,8] We therefore attempted to characterize more precisely the relationship between glucagon binding and insulin secretion by studying the kinetics and stoichiometry of the cAMP and insulin responses to glucagon in the insulinoma parent cell line and clone 5F.

MATERIALS AND METHODS

Cell culture. RIN-m, a continuous, clonable rat cell line was established from a nude mouse heterotransplant of a rat islet cell tumor.[1] For this study, the parent cell line RIN-m and an insulin-secreting clone derived from it, 5F, were grown to confluence in 24-well Falcon plates in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum without antibiotics. The culture media were changed every third day and on the day before use. Immediately preceding the experiment, the media were removed by gentle aspiration and replaced with 1 ml of RPMI 1640 containing 1% heat-inactivated fetal bovine serum and various concentrations of glucagon. In addition to a full complement of amino acids, RPMI media contain 200 mg/dl of glucose. Incubations were carried out at 37°C in 95% air:5% CO_2. Experiments were performed on cells between passages 5 and 25 because the insulin content and the magnitude of glucagon-stimulated insulin release declined with later passages. Cell counts were performed on several wells of each plate ($6.9 \pm 1.6 \times 10^5$ cells/well, N = 25). These cells were also examined for viability by measurement of trypan blue exclusion. Cells in the wells were in a tightly adherent monolayer and did not float up into the supernatant during incubation. Sporadic exami-

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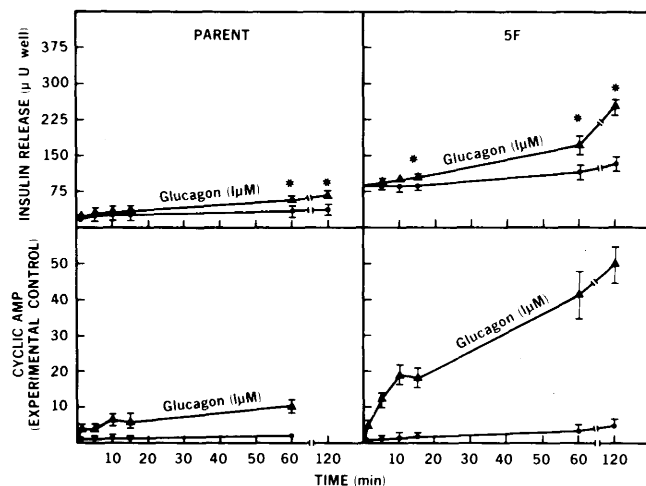

FIGURE 1. Time course of glucagon action on insulin release and cAMP production in parent and 5F. In each experiment, each value was determined in three separate wells and results shown are means ± SEM from three separate experiments. Basal insulin values were significantly higher in 5F, while cAMP values were not different (20.7 in parent versus 29.3 pmol/10⁶ cells in 5F). Because of interexperiment variability, results for cAMP were expressed as the ratio of the value obtained at the time indicated (experimental) divided by the value measured at time 0 (control), with (▲) and without (●) 1 μM glucagon. *P < 0.05 by Student's paired t-test. All values for glucagon-stimulated cAMP were significantly different than control at P < 0.01.

nation of cells after the 1-h incubations revealed no loss of viability as evidenced by: (1) capacity to generate cAMP and release insulin in the second hour of incubation, and (2) trypan blue exclusion.

To determine time-course responses to glucagon, 400 μl of incubation media were removed at varying incubation times and assayed for immunoreactive insulin (IRI). Iced (4°C) absolute ethanol was then added to the remaining volumes (cells plus 600 μl media) and cAMP measured by radioimmunoassay as detailed below. All results are expressed as IRI secretion and cAMP generation per well or per 10^6 cells. To measure extracellular cAMP, the entire medium (1 ml) was removed and added to an equal volume of iced (4°C) absolute ethanol. The remaining cells that *adhered* to the bottom of the plastic well were then extracted with iced (4°C) absolute ethanol to measure intracellular cAMP.

In experiments designed to assess the effect of glucagon in the absence of glucose or amino acids, cells were washed 2–3 times by changing media from RPMI to Krebs-Henseleit bicarbonate buffer containing 1% bovine serum albumin (Sigma, St. Louis, Missouri). Incubations were then performed with and without glucagon (1 μM) in the absence of added substrate, or in the presence of either 5 mM glucose or 20 mM 2-deoxyglucose. At the end of 1 h, 400 μl of incubation buffer was assayed for IRI and remaining cells and buffer extracted for cAMP. In each experiment, each condition was carried out in triplicate and the entire experiment repeated three times.

Insulin secretion. Insulin (IRI) was measured by the double-antibody method of Morgan and Lazarow.[9] Aliquots of incubation medium were stored at −20°C until assayed for IRI.

cAMP formation. cAMP was determined using the radioimmunoassay procedure developed by Brooker et al.[10] The cAMP extraction procedure was modified as previously reported.[11] Samples were kept at 4°C for 15 min then frozen and stored at −70°C. For measurement of cell cAMP, the previously frozen, alcohol-treated well contents were scraped and then centrifuged at 10,000 × g for 5 min. An aliquot of supernatant was removed and diluted in 5 mM sodium acetate (pH 4.75) then acetylated at 4°C by adding 10 μl of triethylamine followed by 5 μl of acetic anhyride. Duplicates of each acetylated sample (50 μl) were added to 12 × 75 glass tubes and incubated with 50 μl of (^{125}I)-cyclic AMP (8000 cpm) and 200 μl of goat anti-cyclic AMP antibody at 1:200,000 dilution for 16 h at 4°C. At the end of the incubation, 1 ml of charcoal suspension (2 mg/ml Norit-A charcoal in 100 mM potassium phosphate buffer, pH 6.3, containing 2.5 mg/ml bovine serum albumin) was added to each assay tube, allowed to stand at 4°C for 15 min, and centrifuged at 1500 × g (4°C) for 10 min. The supernatant was removed and measured for radioactivity in a Beckman Gamma Counter 5500 (Beckman Instruments, Irvine, California). Statistical analyses were performed using the Student t-test, and two-way analysis of variance (ANOVA).

Materials. RPMI 1640 with glutamine and fetal bovine serum was obtained from Grand Island Biological, Grand Island, New York; bovine plasma albumin (fraction V) was from Armour Pharmaceutical, Phoenix, Arizona; all other chemicals were from Sigma Chemical; (^{125}I)2′-O-succinyl (tyrosine methyl ester)-cyclic AMP was from Meloy Laboratories, Springfield, Virginia; and goat anti-cyclic AMP antibody was a gift from Dr. Gary Brooker, Georgetown University School of Medicine, Washington, D.C. Glucagon was a gift from Dr. William Bromer, Lilly Research Laboratories, Indianapolis, Indiana.

RESULTS

Time-course glucagon action: insulin and cAMP. The time course of glucagon action on insulin secretion and cAMP

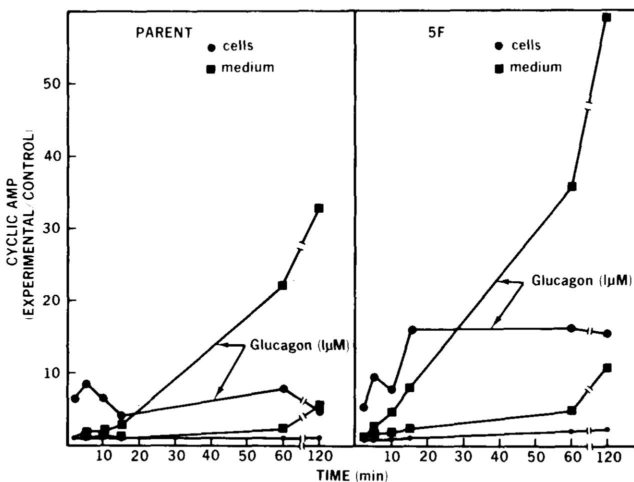

FIGURE 2. Time course of glucagon action on intracellular and extracellular cAMP. In each experiment, each value was determined in three separate wells and results shown are means from three separate experiments. Results are calculated as the intracellular or extracellular value with or without 1 μM glucagon (experimental) divided by the corresponding intracellular or extracellular value measured at time 0 (control). At time 0, intracellular cAMP equaled extracellular cAMP. For parent and 5F, total cAMP was 4.8 pmol/10⁶ cells.

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TABLE 1
Absence of refractoriness of 5F to glucagon-stimulated cAMP and insulin release

Additions	Second Incubation None		Glucagon (1 μM)	
	cAMP (pmol)	IRI (μU/well)	cAMP (pmol)	IRI (μU/well)
First incubation				
None	0.30 ± 0.04	70.5 ± 3.6	4.57 ± 0.36	378.0 ± 37.7
Glucagon (1 μM)	0.33 ± 0.07	63.4 ± 3.2	4.36 ± 0.28	378.8 ± 44.6

In the first incubation, cells were divided into two groups and incubated with and without 1 μM glucagon. At the end of 30 min the medium was removed, cells were washed twice with RPMI, and then resuspended in fresh RPMI medium. These cells were then incubated with and without 1 μM glucagon (second incubation). At the end of 120 min IRI and cAMP were measured. In each experiment, each point was determined in three separate wells and the results (mean ± SEM) shown are representative of four separate experiments. The data for cells whose first incubation in glucagon was 3 h revealed similar findings to those shown.

production was examined in parent and its insulin-secreting clone 5F (Figure 1). The data shown represent a mean of three experiments with clone 5F and with parent cells. In the absence of glucagon, insulin secretion from parent increased from a basal value of 18 μU/well to 36 μU/well at 1 h and 42 μU/well at 2 h. Addition of 1 μM glucagon resulted in no significant change in insulin secretion over the initial 15 min, but there was a significantly increased secretion to 57 μU/well and 67 μU/well at 1 and 2 h, respectively. Clone 5F showed basal insulin secretion of 85 μU/ml, namely a fourfold increment over parent. In the absence of glucagon, insulin secretion gradually increased to 120 μU/well at 1 h and 135 μU/well at 2 h. In this instance the addition of glucagon significantly increased insulin secretion to 105 μU/well by 15 min, and substantially greater secretion of 175 μU/well and 255 μU/well at 1 and 2 h when compared with parent.

Adding 1 μM glucagon to parent stimulated a threefold increase in cAMP (media plus cells) after 1 min, which increased to approximately fivefold after 10 and 15 min and increased further to 10-fold after 60 min. Similarly, adding 1 μM glucagon to 5F stimulated a fourfold increase in cAMP by 1 min, which increased to approximately 18-fold after 10–15 min and increased further to 42-fold after 60 min. In the absence of additions, after 60 min, cAMP increased less than onefold in parent and twofold in 5F.

For parent cells and clone 5F, the basal cAMP was not significantly different: 20.7 ± 5.1 (N = 10) in parent and 29.3 ± 8.6 (N = 9) pmol/10^6 cells in clone 5F. After 120 min of incubation with 1 μM glucagon, cAMP was 102.6 ± 18.3 for parent cells and 217.7 ± 51.9 pmol/10^6 cells for clone 5F ($P < 0.05$).

Intra- and extracellular cAMP. Because the initial rapid increase in glucagon-stimulated cAMP was not accompanied by a concomitant increase in insulin secretion and the timecourse for cAMP appeared multiphasic, we decided to examine the action of glucagon on the intracellular and extracellular distribution of cyclic AMP (Figure 2).

In both cell lines, 1 μM glucagon caused an immediate (1 min) increase in cellular cAMP, which was characterized thereafter by an early peak. In clone 5F, this was at 5 min followed by a second higher peak at 15 min and subsequently a plateau level was seen in both cell lines. The extracellular medium, which contained little cAMP early, rapidly rose after 15 min and continued to increase throughout the experiment. This increase in extracellular cAMP was especially marked in the 5F incubation. Basal values (unstimulated) of parent and 5F were similar in these experiments so that 5F cells generated significantly more cAMP. It appears that significant insulin responses to glucagon occur subsequent to the intracellular peaks of cAMP, and are concomitant with the increasing extracellular cAMP.

Lack of refractory response to glucagon. Since there was a continuous accumulation of cAMP over the 2-h period of exposure to glucagon, it seemed likely that the cells were continuing to generate cAMP and that no refractoriness to the hormone had developed.[12] To examine this question, 5F cells were first incubated for 30 min or 3 h with and without 1 μM glucagon. The cells were then washed twice to remove glucagon from the medium and resuspended in fresh medium with and without 1 μM glucagon. Table 1 illustrates that (1) washing the cells after the first incubation reduces basal cAMP levels, (2) the continued presence of glucagon is required for cAMP response, and (3) that a response to glucagon (cAMP and IRI) persists despite prior exposure to the hormone. Similar results were obtained for the 3-h incubation experiment (data not shown).

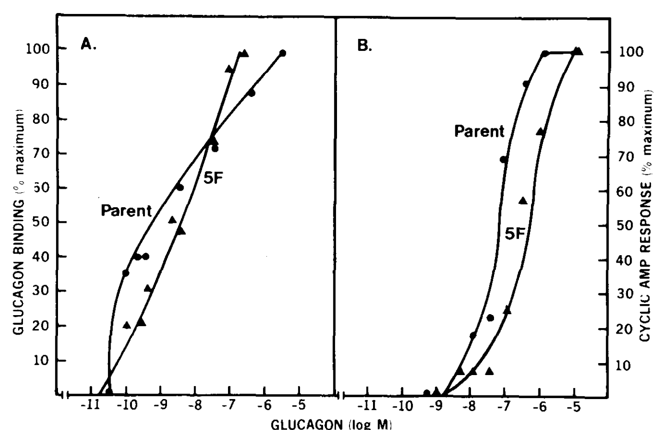

FIGURE 3. Comparison of ^{125}I-glucagon binding and glucagon-stimulated cAMP in parent and 5F. (A) Glucagon binding values are obtained from prior studies[4] and expressed as the percentage of maximum binding. Scraped cells were incubated in Tris buffer at 22°C. (B) Results for cAMP are expressed as the percentage of the increase caused by 10 μM glucagon. Cells were incubated in RPMI 1640 at 37°C. Results given for cAMP and glucagon binding are means of three separate experiments and each value was determined in three separate wells (cAMP) or three separate collections of cells (binding).

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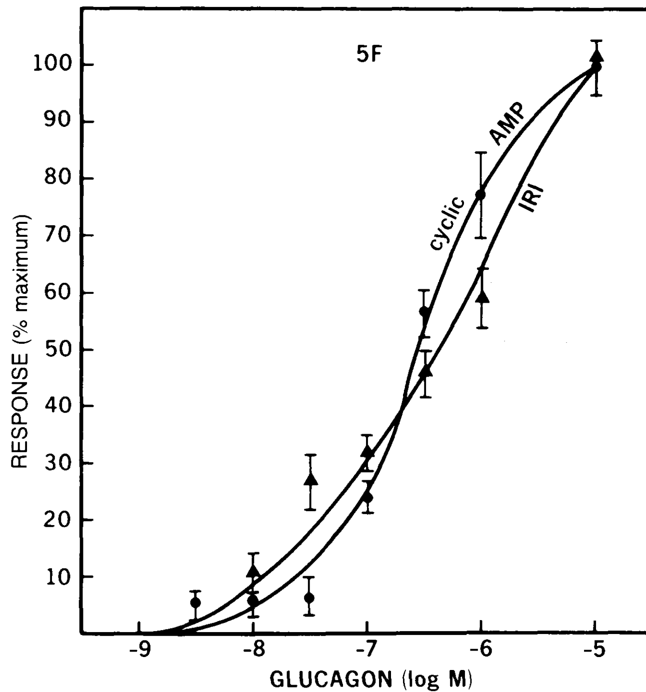

FIGURE 4. Effect of glucagon on cAMP and insulin release in 5F. Values are expressed as the percentage of the increase caused by 10 μM glucagon. In each experiment, each value was determined in three separate wells and results shown are means ± SEM from three separate experiments.

Glucagon binding and cAMP. Previous studies from this laboratory demonstrated that both parent and 5F possess high-affinity glucagon receptors (Figure 3A), that (^{125}I)-glucagon binding cannot be accounted for by a simple bimolecular reaction, and that the concentration of native glucagon necessary to inhibit binding by 50% is 0.5 nM and 30 nM for parent and 5F, respectively.[4] To further explore the relationship between glucagon binding and the increase in glucagon-stimulated cAMP, we examined the effect of increasing concentrations of glucagon on cAMP in both cell lines. In parent, 3 nM glucagon caused a significant increase in cAMP and the response was maximal at 1 μM, with a half-maximal response occurring at 50 nM (Figure 3B). In 5F, the cAMP response to the lowest concentration of glucagon was evident at the same concentration as in parent; however, a maximal response may not have been achieved at the highest glucagon concentration tested (10 μM). (It was not possible to obtain a higher concentration for testing due to the solubility characteristics of glucagon.) Despite this, it is clear that the dose-response curve for cAMP in 5F compared with parent is shifted to the right. Furthermore, in both 5F and parent, the cAMP response curves compared with the respective glucagon-binding curves are also shifted to the right (1 log). (Single glucagon-binding curves were carried out with parent and 5F cells indicating that no significant changes had occurred when compared with our previous published data[4].)

Because of the difference in time-course curves between early and late cAMP responses to glucagon, we examined the glucagon dose-response curves after a 5-min incubation period. Reducing the duration of the incubation did not alter the glucagon-stimulated cAMP dose-response relationships for either parent or 5F (data not shown). Furthermore, adding the phosphodiesterase inhibitor IBMX (1 μM) did not alter the concentration of glucagon required for half-maximal cAMP response in either parent or 5F (data not shown). Thus, the concentration of glucagon that inhibits binding by 50% is an order of magnitude less than the concentration of glucagon required for half-maximal stimulation of cAMP.

Since these data suggested the presence of glucagon receptors that are not coupled to a cAMP response, we examined the dose-response relationship between glucagon-stimulated cyclic AMP and insulin secretion in clone 5F, in which insulin secretion is high. Figure 4 illustrates the dose-response curves for glucagon-stimulated cyclic AMP and insulin secretion and shows that they are superimposable. These findings support the role of cAMP as an intracellular mediator of insulin release.

Exogenous substrates and glucagon action. To assess the contribution of glucose and amino acids in glucagon-stimulated insulin release (both substrates are components of RPMI-1640 media), 5F cells were studied in glucose- and amino acid-free Krebs-Henseleit buffer. As noted in the MATERIALS AND METHODS section, cells were washed twice, and then incubated with and without 1 μM glucagon in the Krebs buffer for 60 min (Table 2).

In the absence of additions, glucagon increased insulin secretion by 70.8 μU/well. The presence of 5 mM glucose, 20 mM 2-deoxyglucose, or glucose plus 2-deoxyglucose did

TABLE 2
Glucagon stimulates IRI release and cAMP in the absence of glucose or amino acids

Additions	IRI secretion (μU/well)		cAMP formation (pmol)	
	None	Glucagon (1 μM)	None	Glucagon (1 μM)
None	89.2 ± 11.4	160.0 ± 6.8*	9.4 ± 0.8	19.7 ± 1.8*
Glucose (5 mM)	120.2 ± 9.3	179.7 ± 15.1*	11.4 ± 2.4	39.6 ± 3.7*†
2-Deoxyglucose (20 mM)	99.8 ± 5.6	147.5 ± 20.0*	7.5 ± 0.7	16.1 ± 2.2*
Glucose (5 mM) + 2-Deoxyglucose (20 mM)	97.2 ± 11.1	143.8 ± 10.4*	8.6 ± 2.5	21.6 ± 4.0*

Clone 5F cells were washed twice with Krebs-Henseleit buffer without glucose and subsequently incubated in that buffer for 60 min with and without glucagon. In each experiment, each point was determined in three separate wells. Results shown represent the mean ± SEM of three separate experiments.
*$P < 0.01$ by Student's paired *t*-test for effect of glucagon.
†$P < 0.05$ comparing glucose plus glucagon effect on cAMP versus other incubations by two-way ANOVA and Duncans Multiple Range test.

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not affect the increment in insulin secretion produced by 1 μM glucagon. Glucagon stimulated a mean increase of 9.5 pmol of cAMP in the absence of substrate or in the presence of 2-deoxyglucose alone, while in the presence of 5 μM glucose, glucagon stimulated an increase of 28.2 pmol cAMP ($P < 0.05$ comparing glucagon stimulated cAMP levels with each other).

DISCUSSION

Most studies have focused on the role of cAMP in glucose-induced insulin release and have concluded that cAMP is a positive modulator of insulin release rather than the primary trigger.[6,7] It has been assumed that because glucagon activates adenylate cyclase and also stimulates insulin release, that glucagon-stimulated insulin secretion is mediated by cAMP, although whether this is a primary or facilitating action is not clear. The availability of insulin-secreting tumor cell lines with functional glucagon receptors has provided an opportunity to define more precisely the relationship between glucagon binding, cAMP generation, and insulin secretion. These studies were performed using two established cell lines, a parent cell (RIN-m), which secretes small quantities of insulin and somatostatin, and an insulin-secreting clone (5F) derived from the parent cell line, which secretes large quantities of insulin and essentially no somatostatin.[1,2] The possibility that the secretion of somatostatin by RIN-m cells might decrease insulin secretion by those cells seems unlikely, since the concentration of exogenous somatostatin required to inhibit insulin release by 5F was extremely high.[2]

Glucagon binding in the two cell lines differs.[4] The concentration of glucagon required for 50% inhibition of binding is greater for 5F (30 nM) than for parent (0.5 nM) (Figure 3A). Furthermore, the concentration of glucagon required for half-maximal cAMP stimulation is also greater for 5F than for parent (50 nM versus 0.3 μM) (Figure 3B). Although the relationship between binding characteristics and cAMP generation is maintained in the two cell lines, even under disparate incubation conditions, comparison of these two sets of experiments suggests that glucagon binding occurs without any cAMP response. It would have been desirable to compare binding and cAMP response curves under identical conditions of incubation.

Unfortunately, glucagon binding studies (cells scraped and suspended in Tris-Hepes buffer) could not be performed in the 24-well plates in RPMI 1640 medium, which was optimal for studies of cAMP and IRI response to glucagon. It was, however, reassuring that binding curves carried out at the time of the cAMP studies were essentially unchanged from those previously reported. Further, since there was no significant degradation of glucagon in either buffer,[12] destruction of glucagon in RPMI 1640 could not account for the shift of the cAMP response curves to the right compared with the binding curves. We interpreted these data to indicate that a significant number of glucagon binding sites are not coupled to cAMP production. Such uncoupling may be a manifestation of cellular transformation, although a somewhat similar observation has been made with diabetic hepatocytes.[13] Other possible manifestations of the "transformation" process were seen. In contrast to most studies of stimulation of cAMP in intact cells by glucagon[14] and β-adrenergic agents,[15,16] the insulinoma cells did not exhibit refractoriness to glucagon (Table 1). This finding may be due to the deletion of a regulatory component responsible for receptor-mediated desensitization or perhaps islet cells differ from liver and other cells in this regard.

It is clear from the time course in Figures 1 and 2 that glucagon stimulates a rapid cAMP response, whereas the insulin response appears after a short (15-min) latent period in 5F. The failure to detect early increases in insulin secetion in parent may reflect either (1) the decreased magnitude of the cAMP response to glucagon, or (2) our inability to detect significant differences in insulin secretion at very low insulin levels. We also noted that in the presence of 1 μM glucagon, total cAMP continues to accumulate. The intracellular cAMP rises rapidly to an early peak, and then reaches a plateau (in 5F) for 2 h, whereas the extracellular cAMP continues to increase. This increase in extracellular cAMP appears to be due to movement of intracellular cAMP into the extracellular space. Whether this is due to cell leakage or to a specific transport process is not known.

Since 5F releases large quantities of insulin, it was possible to demonstrate that the glucagon-stimulated cAMP and insulin dose-response curves were superimposable (Figure 4). It was also of interest that clone 5F generated more cAMP as well as more insulin in response to glucagon when compared with parent cells. These data suggest that cAMP mediates glucagon-induced insulin release. Further support for this concept may be found in the data of Table 2. It is evident that neither glucose nor amino acids are required in the incubation medium for glucagon-stimulated insulin release or cAMP generation. Of course, one can argue that there are endogenous substrates within the cells that may serve a similar purpose to the exogenous substrates. However, in contrast with fetal pancreas, where addition of glucose to the medium increased glucagon-stimulated insulin release markedly,[17] in clone 5F there was no enhancement of the glucagon-induced insulin response in the presence of 5 mM glucose. Praz et al.[3] reported in clone 5F that glucose utilization increased but insulin secretion did not increase with increasing concentrations of glucose (2.8–33.4 mM). This was also observed by our group.[2] However, we had not looked at the difference between no glucose and low glucose concentrations. Our very preliminary data (Table 2) tend to confirm the observation of Praz et al.[3] that low glucose levels compared with no glucose stimulates insulin release, and that glucose is transported into the cell and metabolized. The possible role of glucose in potentiating glucagon-stimulated cAMP levels is an interesting finding that requires future elucidation.

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