Expression and signal transduction of the glucagon receptor in βTC3 cells

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Abstract

The expression and signal transduction of the glucagon receptor (GR) have been studied in βTC3 cells. Northern blot and RT-PCR analysis indicated the expression of the GR gene in βTC3 cells. One-5 nM glucagon stimulated a 2.5-fold increase in the IP_s production. At glucagon concentrations higher than 5 nM, the production of IP_s was blunted but not abolished. The accumulation of intracellular cAMP was observed following the stimulation with 5 nM of glucagon. A maximal 4.5-fold increase in cAMP was observed using 250 nM glucagon and higher. Comparative studies using a glucagon anatogonist, des-His^1[Glu]^{9}glucagon, showed no effect on intracellular cAMP and IPs in βTC3 cells. Our data shows that the GR gene is expressed in βTC3 cells. The GR in βTC3 cells transmits its intracellular signal by causing the accumulation of both IP_s and cAMP.

1. Introduction

Glucagon is a twenty nine amino acid hormone that plays an important role in the regulation of glucose homeostasis and the pathogenesis of diabetes [1]. The main target organ of glucagon action is the liver [1]. Glucagon functions to maintain basal glucose level by activating two enzymatic pathways in the liver, glycogenlysis and gluconeogenesis [2]. In pancreatic islets, glucagon acts as an insulin secretagogue [3]. It has been shown using rat hepatocytes that glucagon can activate adenylate cyclase by a Gs-mediated process [4,5]. In addition, glucagon can also elicit a rapid, transient increase in hepatic protein kinase C and phospholipase C activity [4–8]. Two populations of hepatic glucagon receptors have been proposed based upon both binding and functional studies [9–12].

The glucagon receptor cDNA has been cloned from rat, human and mouse liver [13–15]. The cDNA is approximately 1.9kb and it encodes a polypeptide of 485 amino acids. The glucagon receptor is approximately 55kDa and contains four potential N-linked glycosylation sites [13]. Sequence comparisons have shown that the glucagon receptor belongs to a family of hormone receptors specific for intermediate-sized peptides such as glucagon like peptide-1, calcitonin, parathyroid hormone and parathyroid hormone-related peptide [13]. All members of this receptor subfamily are predicted to have seven transmembrane domains [13]. Transfection of the rat hepatic glucagon

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receptor cDNA into BHK cells demonstrated that glucagon can stimulate the intracellular accumulation of cAMP and a transient increase in intracellular calcium levels [13].

The existence of functional glucagon receptors on β cells is supported by early studies demonstrating ^{125}I-glucagon binding and activation of adenylate cyclase in immortal Syrian hamster β-cells [16]. To date the expression and molecular mechanism(s) of the glucagon receptor signal transduction in β cells have not been completely elucidated. The cloning of the glucagon receptor cDNA made it possible to study glucagon receptor expression in β-cells using a molecular approach. The mouse pancreatic β cell line-βTC3 is derived from insulinomas that developed in transgenic mice expressing the SV40 large T antigen under the control of the insulin promoter [17]. βTC3 cells have low level constitutive insulin release and regulated insulin secretion in response to a number of secretagogues [18]. In this report we demonstrate, using Northern blot and RT-PCR analysis, that the glucagon receptor mRNA is expressed in βTC3 cells. Additionally, the dose-response curves to glucagon for the accumulation of intracellular cAMP and inositol phosphates in βTC3 cells were characterized. Comparative studies using a glucagon antagonist, des-His[1][Glu][9]glucagon amide, show no effect on intracellular cAMP and inositol phosphates. Results of these studies clearly demonstrate that the glucagon receptors expressed in βTC3 cells transmit intracellular signals via adenylate cyclase and phospholipase C system.

2. Materials and methods

2.1. Materials

Glucagon was a kind gift from the Eli Lilly Corp. (Indianapolis, IN) and des-His[1][Glu[9]]glucagon amide was a kind gift from Drs. C. Unson and B. Merrifield. [^{3}H]inositol and ^{3}H-cyclic AMP was purchased from NEN-Dupont (Boston, MA). ^{32}P-dCTP and the random prime DNA labelling kit were from Amersham Corp. (Arlington Heights, IL). Accell QMA anion exchange SEP-PAK cartridges were obtained from Waters Assoc. (Milford, MA). Taq polymerase, reverse transcriptase and all cell culture reagents were purchased from Gibco Life Technology (Gaithersburg, MD). Forskolin was purchased from Calbiochem-Behring (La Jolla, CA). Methylisobutylxanthine (IBMX), cyclic AMP dependent protein kinase and other chemical reagents were purchased from Sigma Chemical Corp. (St. Louis., MO).

2.2. βTC3 cell culture

The βTC3 cell line was a kind gift from Dr. S. Efrat. The cells were cultured according to Efrat et al. [17]. Briefly, cells were grown on 30 mm^2 plates in Dulbecco's Modified Eagle's Medium supplemented with 15% horse serum, 2.5% fetal bovine serum and 1% penicillin-streptomycin in 5% CO_2–95% O_2 at 37°C. Cells were harvested by trypsinization with 0.05% trypsin and 0.53 mM EDTA. Most studies reported used βTC3 cells from passages 35–45.

2.3. Northern blot analysis

Total RNA from βTC3 cells, mouse liver and skeletal muscle was extracted using Tri-reagent (Molecular Research Center, Cinicinnati, OH) according to the manufacturer's instructions. Approximately 30 μg of the total RNA was loaded onto an 1.2% agarose/formaldehyde gel. After electrophoresis the gel was transferred to a nylon membrane and hybridized to a random prime ^{32}P-labeled probe derived from the rat glucagon receptor cDNA using high strigency conditions (50% formamide, 42°C). The filter was washed using high strigency conditions (0.2XSSC, 0.1% SDS, 42°C, 40 min) and subjected to phosphoimage analysis for visualization and quantitation of the results.

2.4. RT-PCR analysis

Oligonucleotide primers used in the RT-PCR reactions were derived from the mouse glucagon receptor cDNA sequence [15]. Primer 1 was from basepair +926 to +946 (5′GATTCTGGTGGATCCTGCGT-3′) and primer 2 from basepair +583 to +605 (5′TCTATTGGCTGCTGAAGACACG3′). Reverse transcription reactions were carried out at 42°C for 50 min using 10 μg of total RNA. Thirty cycles of PCR reactions were carried out at 94°C for 45 s, 56°C for 45 s, and 72°C for s. A 296 bp product was expected

from the PCR reaction. To control for the integrity of the RNA, β-actin RT-PCR was conducted on the same RNA samples. The oligonucleotide primers and RT-PCR reaction conditions were as previously described [19]. The PCR products were electrophoresed on agarose gels and identified following Southern blot analysis and high stringency hybridization to a randon prime ^{32}P-labeled DNA probe specific for either the mouse glucagon receptor or β-actin. Following high strigency washing the membranes were subjected to phosphoimage analysis for visualization and quantitation of the results.

2.5. Measurement of intracellular cAMP

Near-confluent βTC3 cells were cultured overnight without serum before each experiment. In the morning cells were washed with D-PBS and incubated in modified Krebs-Ringer buffer containing 10 mM HEPES, 2.54 mM $CaCl_2$, 119 mM NaCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$ and 0.1% BSA (pH 7.4) with 0.5 mM IBMX for 45 min. Different concentrations of glucagon were added to the cells and incubated for 15 min. Cells were then washed twice with ice cold PBS and scraped from the plates using ice-cold 0.1 N HCl. The lysed cells were subjected to sonication and the nuclei and unbroken cells were spun down at $1000 \times g$ for 5 min at 4°C. Supernatants were harvested and lyophilized overnight. The cAMP content of the lyophilized supernatant was assayed using a modified Gilman protein binding assay [20].

2.6. Measurement of intracellular inositol phosphates

Near-confluent βTC3 cells were cultured overnight in DMEM without inositol. In the morning the cells were labeled with ^{3}H-inositol (5 μCi/ml) for 3 h in modified Krebs-Ringer buffer. 10 mM LiCl was added 15 min before glucagon and then incubated for an additional 30 min. The cells were washed twice with ice cold PBS, scraped into 15% cold TCA and centrifuged for 5 min at $1000 \times g$ at 4°C. Supernatants were then extracted four times with an equal volume of ether. Separation of the [^{3}H]inositol phosphates was according to the modifications of Larocca et al. [21].

3. Results

3.1. Glucagon receptor mRNA expression in βTC3 cells

Expression of the glucagon receptor in βTC3 cells was studied by Norhtern blot and RT-PCR analyses. Nothern blot analysis was performed using total RNA from βTC3 cells and mouse liver to verify glucagon receptor expression in these cells. A 1.9 kb mRNA was detected in both βTC3 and liver using a rat glucagon receptor cDNA probe (Fig. 1A). The size of the glucagon receptor mRNA detected in βTC3 cells is approximately the same as that detected in mouse and rat liver [13,15]. Densitometric analysis revealed that the steady state levels of glucagon receptor mRNA in βTC3 cells is approximately 3-fold less than that detected in mouse liver. To be certain that the mRNA detected in βTC3 cells is in fact specifically the glucagon receptor mRNA, reverse transcription (RT)-PCR was performed on βTC3 total RNA using glucagon receptor-specific primers [15]. Oligonucleotide primers derived from the mouse glucagon receptor cDNA sequence were used in both the reverse transcription and PCR reactions. Mouse liver and muscle total RNA were used as positive and negative controls, respectively. The RT-PCR products were electrophoresed and subjected to Southern blot analysis and high stringency hybridization with a murine glucagon receptor cDNA probe. A 296 bp glucagon receptor-specific RT-PCR product was verified in liver and βTC3 cells but not in muscle (Fig. 1B). The quality of the RNAs used in this study were verified by performing RT-PCR analysis using oligonucleotide primers specific for β-actin on the same RNA used to study glucagon receptor expression (Fig. 1B). The results of Northern blot and RT-PCR analyses clearly demonstrate that the glucagon receptor is expressed in βTC3 cells.

3.2. Intracellular cAMP production in response to glucagon

To examine the action of glucagon on intracellular cAMP accumulation in βTC3 cells, we incubated the cells with varying concentrations of glucagon (500 pM to 250 nM) in the presence of 0.5 mM of IBMX and in the absence of glucose. Fig. 2 diagrams the

changes in intracellular cAMP levels over the range of glucagon doses. Glucagon induces a dose-dependent increase in intracellular cAMP accumulation in β TC3 cells. Increased intracellular cAMP accumulation can be observed in cells incubated with as little as 5 nM glucagon, however, a 4.3-fold increase in intracellular cAMP levels is noted in cells treated with 100 nM glucagon. Half maximal accumulation of intracellular cAMP is observed in cells incubated with approximately 20 nM glucagon.
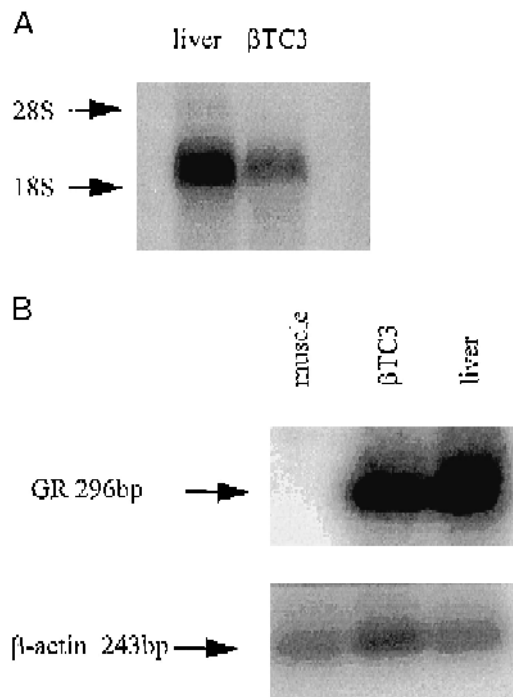

Fig. 1. Glucagon receptor expression in β TC3 cells. (A): Northern Blot analysis of Glucagon receptor mRNA in β TC3 cells. Approx. 30 μg of the total RNA prepared from β TC3 cells and mouse liver was loaded onto an agarose/formaldehyde gel and subjected to Northern blot analysis. The glucagon receptor mRNA was detected following high stringency hybridization to the insert from the rat glucagon receptor cDNA. The integrity of the RNA used was determined by ethidium bromide staining of the gel. (B). RT-PCR analysis of GR mRNA in β TC3 cells. Reverse transcription-PCR (RT-PCR) was performed on 5 μg of total RNA extracted from β TC3 cells, mouse liver and mouse skeletal muscle using oligonucleotide primers specific for glucagon receptor and β-actin. The RT-PCR products were identified by Southern blot analysis using mouse glucagon receptor and β-actin specific ^{32}P-labeled probes. The glucagon receptor (296 bp) and β-actin specific (243 bp) products were identified following phosphoimage analysis. Skeletal muscle is included as a negative control tissue that deos not express glucagon receptor mRNA.
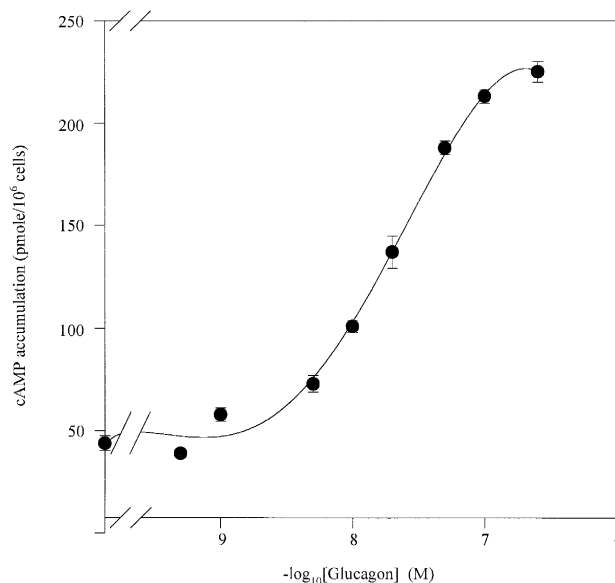

Fig. 2. Dose response curve of the effect of glucagon on intracellular accumulation of cAMP in β TC3 cells. β TC3 cells were incubated with 0.5 mM IBMX for 30 min at 37°C in the presence of the indicated glucagon concentrations. The accumulation of cAMP was determined as described. Values are the mean $\pm$ S.E.M. for three replicate samples from one typical experiment using an average of 3×10^{-6} cells per plate. When the error bar is not shown, it is so small that it falls into the symbol. The data shown are representative of three separate experiments.

The influence of the glucagon analogue des-His[1][Glu[9]]glucagon amide on intracellular cAMP accumulation in β TC3 cells was also examined. Des-His[1][Glu[9]]glucagon amide is a well established antagonist of glucagon which has been shown to bind to rat hepatocytes as well as native glucagon but has no glucagon-associated biological effect on these cells [22]. Similar to that noted previously in hepatocytes, no increase in intracellular cAMP levels is observed in β TC3 cells treated with 100 pM, 1 nM or 100 nM des-His[1][Glu[9]]glucagon amide (Fig. 3).

3.3. Intracellular inositol phosphates production in response to glucagon

The treatment of β TC3 cells with 1 nM glucagon results an intracellular increase in the inositol phosphates. The chromatographic separation of the [3H]inositol phosphates (IPs) revealed that glucagon treatment results principally in an increase in the accumulation of [3H]inositol 1-phosphate (IP). How-
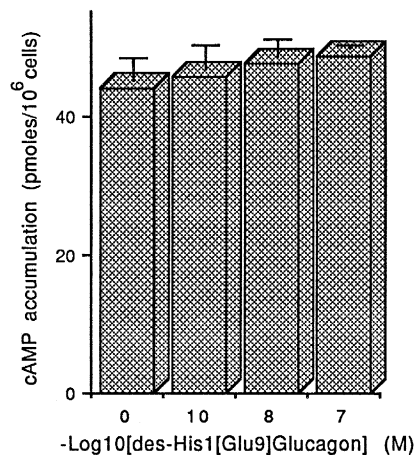
Fig. 3. Effect of the glucagon analogue des-His[1][Glu[9]]glucagon amide on the accumulation of intracellular cAMP in βTC3 cells. The experiments were carried out as described in Fig. 2 using 0, 100 pM, 10 nM or 100 nM of des-His[1][Glu[9]]glucagon amide. Values are the mean $\pm$ S.E.M. for triplicate samples from one typical experiment using an average of 3×10^{-6} cells per plate. The data shown are representative of three separate experiments. Differences between groups were determined by two tailed Student's t test.

ever, a glucagon-dependent increase in levels of [³H]inositol 1,4-bisphosphate, [³H]inositol 1,4,5-triphosphate (IP3) was also observed. In addition, it was observed a glucagon-dependent increase in a mixture of IPs that contained [³H]inositol 1,3,4,6-tetrakisphosphate (IP$_4$), [³H]inositol 1,3,4,5,6-pento-

Fig. 4. The effect of glucagon on the intracellular accumulation of inositol phosphates in βTC3 cells. (A) Elution pattern of inositol phosphates in a typical run following the glucagon stimulation of βTC3 cells prelabeled with [³H]inositol. βTC3 cells were labelled with [³H]inositol (5 μCi/ml) for 3 h, washed, and stimulsated with 10^{-9} M glucagon for 30 min in the presence of 10 mM Li$^+$. Main graph: Labeled inositol phosphates were chromatographed on Accell QMA Sep-Pak cartridge, with a discontinuous gradient of ammonium formate/formic acid in 5 mM sodium borate. The inset shows in greater detail the distribution of radioactivity in fraction 11–21. O-O: control, ●-●: glucagon. (B) Dose response curve of the effect of glucagon on the intracellular accumulation of inositol phosphates in βTC3 cells. βTC3 cells were labeled with ³H-inositol (5μCi/ml) for 3 h at 37°C followed by the addition of the indicated concentrations of glucagon and incubated for 30 min. The accumulation of intracellular IPs was determined as described above. Values are the mean $\pm$ S.E.M. from four replicate samples from one typical experiment using an average of 8×10^{-5} cells per plate. The same experiment was repeated three times with similar results.

kisphosphate (IP$_5$) and [³H]inositol hexakisphosphate (IP$_6$) (Fig. 4A).

The intracellular accumulation of inositol phosphates in response to varying concentrations of glucagon was examined. The accumulation of intracellular IPs in the presence of 10 mM LiCl is clearly biphasic in the function of glucagon (Fig. 4B). The stimulatory phase of the response begins at 100 pM glucagon and reaches a maximal response level in cells treated with 1–5 nM glucagon. An approx. 2-fold increase in the accumulation of inositol phosphates is observed in the presence of 1–5 nM
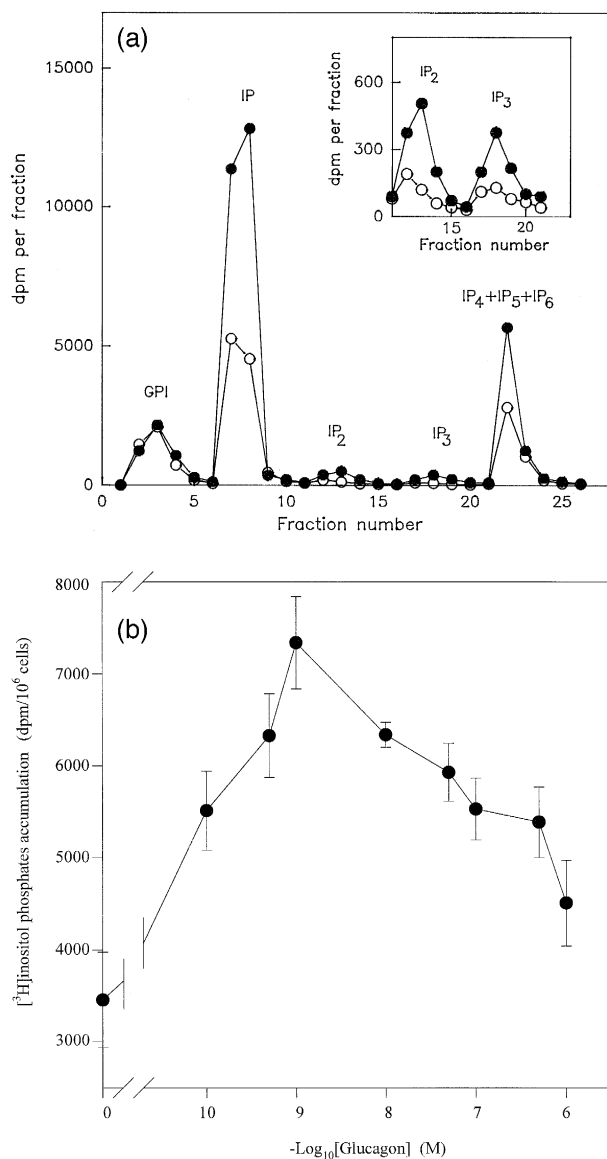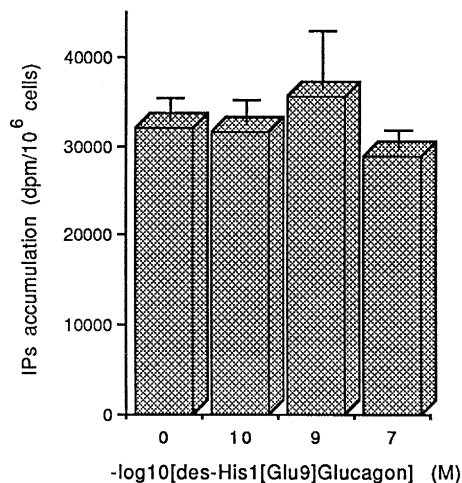
Fig. 5. Effect of the glucagon analogue des-His[1][Glu[9]]Glucagon amide on the intracellular accumulation of inositol phosphates in β TC3 cells. The experiments were carried out as described in Fig. 4B using 0, 100 pM, 10 nM and 100 nM of des-His[1][Glu[9]]glucagon amide. The data shown are the average $\pm$ S.E.M. of three replicates from one experiment using an average of 3×10^6 cells/plate. The same experiment was repeated three times with similar results. Differences between groups were determined by two tailed Student's t test.

glucagon. The EC_{50} of the stimulatory phase is approximately 100 pM. Inhibition of the accumulation of inositol phosphates is noted in cells incubated with glucagon concentrations above 5 nM. In β TC3 cells incubated with $1 \mu M$ glucagon, the accumulation of intracellular IPs decreased to approx. 30% that of the untreated cells. The EC_{50} for the inhibitory phase of the glucagon response is approx. 50 nM.

We also studied the accumulation of intracellular inositol phosphates using the glucagon analogue des-His[1][Glu[9]]glucagon amide. IPs accumulation is not modified in β TC3 cells treated with 100 pM, 1 nM or 100 nM of des-His[1][Glu[9]]glucagon amide (Fig. 5).

4. Discussion

Glucagon has been shown to be an insulin secretagogue [3]. We therefore set out to study expression of the glucagon receptor and glucagon-stimulated signalling pathways in pancreatic β cells using the β TC3 cell line. β TC3 cells have been used as a model β cell system to study glucose sensing and insulin secretion in response to a number of stimuli

[18]. In this study we demonstrate that the glucagon receptor mRNA is expressed in β TC3 cells and it is functional in transmitting intracellular signals through both the adenylate cyclase and phospholipase C second messenger systems. These results shed new light on the intracellular mechanisms of glucagon-stimulated insulin secretion.

Results of both Northern blot and RT-PCR analyses indicated the presence of only one type of glucagon receptor in β TC3 cells. The glucagon receptor mRNA detected in β TC3 cells is the same size as that observed in the mouse liver but it is of relatively lower abundance. These results cannot exclude the possibility that two species of glucagon receptor mRNA are expressed in β TC3 cells. An alternatively spliced glucagon receptor mRNA approximately the same size as the known glucagon receptor mRNA would not have been distinguished by Northern blot or RT-PCR analysis if the oligonucleotide primers chosen could not distinguish the difference between the two receptor mRNAs. Additionally, the glucagon receptor protein may undergo post-translational modifications which result in two populations of receptors that couple to different G proteins. Ligand binding affinity analysis and chromatographic purification of glucagon receptors from rat hepatocytes have suggested that there may be two populations of glucagon receptors [9–12].

Early studies of Birnbaumer and co-workers examined the glucagon receptors expressed in Syrian hamster pancreatic β cell tumors [16]. Crude membrane fractions were shown to contain specific ^{125}I-glucagon binding sites [16]. Glucagon-stimulated cAMP production displayed a half maximal activation at 3 nM. An approximate 2-fold increase in cAMP was detected under the above conditions. In the present study, we demonstrated that β TC3 cells exhibit a 4.3-fold increase in the accumulation of intracellular cAMP upon glucagon stimulation. The EC_{50} for glucagon-dependent cAMP formation is approximately 20 nM. Further characterization of the glucagon receptor in β TC3 cells using the glucagon analogue-des-His[1][Glu[9]]glucagon amide were performed. This analogue has been shown to have a binding affinity similar to native glucagon in rat hepatocyte membranes [22]. β TC3 cells incubated with des-His[1][Glu[9]]glucagon amide exhibited no change in intracellular cAMP accumulation. These

results demonstrate that the glucagon receptor expressed in βTC3 cells couples with adenylate cyclase.

Additionally, our studies demonstrate that the glucagon receptor expressed in βTC3 cells also couples to phospholipase C. The glucagon-stimulated accumulation of IPs is biphasic. The maximal stimulation of accumulation of IPs was observed at lower concentrations than the maximal cAMP response detected. Our results showed that the increase in the accumulation of [^{3}H]IPs were primarily caused by the accumulation of IP. In addition, a glucagon dependent increase in IP_3 was also observed. The increase in the level of IP_3 indicated that glucagon stimulation resulted in an increase in the hydrolysis of PIP_2. However, we cannot rule out the possibility that glucagon treatment also elicited the hydrolysis of PIP and PI. Similar pattern of glucagon-stimulated intracellular inositol phosphates and cAMP accumulations have been observed in rat hepatocytes [4–8]. To further characterize the phospholipase C mediated glucagon response, the βTC3 cells were incubated with des-His^1[Glu^9]glucagon amide. Earlier studies showed that this analogue does not induce the accumulation of inositol phosphates or cAMP in rat hepatocytes [22]. Similarly, no significant change in the accumulation of intracellular inositol phosphates was observed in des-His^1[Glu^9]glucagon amide-treated cells.

The molecular mechanism underlying the induction of two different signal transduction pathways by glucagon is currently unknown. Nevertheless, the glucagon receptor is not the only receptor that has been shown to induce the accumulation of both cAMP and IPs in response to ligand binding. To date, the mammalian PTH/PTHrP and LH receptors are other seven transmembrane domain receptors able to couple to both phosphoinositol breakdown and cAMP accumulation [23,24]. However, the biphasic nature of inositol phosphate accumulation in response to glucagon is unique. Earlier studies on rat hepatocyte membranes suggested that the activation of phospholipase C by glucagon may result in the inhibition of glucagon-stimulated adenylate cyclase activity [4,5]. Two functionally different subpopulations of glucagon receptors was postulated based upon the two different signalling features noted in rat hepatocytes [25]. When the rat liver glucagon receptor cDNA was transfected

into BHK cells, an increase in intracellular cAMP accumulation and calcium concentration was observed [13]. Although the accumulation of intracellular inositol phosphates in BHK cells transfected with the glucagon receptor cDNA was not measured it was concluded that the glucagon-induced $[Ca^{2+}]_i$ increase was due to the activation of the phopholipase C second messenger system [13].

The glucagon receptor in βTC3 cells activates similar intracellular signalling pathways to those observed in hepatocytes. Glucagon stimulates hepatic glucose production by inducing glycogen breakdown and gluconeogenesis [2]. Since glucagon has very different biologic effects on hepatocytes and pancreatic β-cells, it is important to explore the regulatory features of each. The physiologic importance of the glucagon-stimulated intracellular accumulation of inositol phosphates and cAMP in βTC3 cells remains to be explained. Future studies will examine the above signal transduction features of the glucagon receptor(s) in βTC3 cells in the presence of glucose and the insulin secretory response to different concentrations of glucagon.

Acknowledgements

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