Glucagon-mediated Ca\(^{2+}\) signaling in BHK cells expressing cloned human glucagon receptors

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Hansen, Lars H., Jesper Gromada, Pierre Bouchelouche, Ted Whitmore, Laura Jelinek, Wayne Kindsvogel, and Erica Nishimura. Glucagon-mediated Ca\(^{2+}\) signaling in BHK cells expressing cloned human glucagon receptors. Am. J. Physiol. 274 (Cell Physiol. 43): C1552–C1562, 1998.—From video imaging of fura 2-loaded baby hamster kidney (BHK) cells stably expressing the cloned human glucagon receptor, we found the Ca\(^{2+}\) response to glucagon to be specific, dose dependent, synchronous, sensitive to pertussis toxin, and independent of Ca\(^{2+}\) influx. Forskolin did not elicit a Ca\(^{2+}\) response, but treatment with a protein kinase A inhibitor, the Rp diastereomer of 8-bromoadenosine-3',5'-cyclic monophosphothioate, resulted in a reduced glucagon-mediated Ca\(^{2+}\) response as well as Ca\(^{2+}\) oscillations. The specific phospholipase C inhibitor U-73122 abolished the Ca\(^{2+}\) response to glucagon, and a modest twofold increase in inositol trisphosphate (IP\(_3\)) production could be observed after stimulation with glucagon. In BHK cells coexpressing glucagon and muscarinic (M\(_3\)) acetylcholine receptors, carbachol blocked the rise in intracellular free Ca\(^{2+}\) concentrations in response to glucagon, whereas glucagon did not affect the carbachol-induced increase in Ca\(^{2+}\). Furthermore, carbachol, but not glucagon, could block thapsigargin-activated increases in intracellular free Ca\(^{2+}\) concentrations. These results indicate that, in BHK cells, glucagon receptors can activate not only adenylyl cyclase but also a second independent G protein-coupled pathway that leads to the stimulation of phospholipase C and the release of Ca\(^{2+}\) from IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores. Finally, we provide evidence to suggest that cAMP potentiates the IP\(_3\)-mediated effects on intracellular Ca\(^{2+}\) handling.

The glucagon receptor belongs to the superfamily of seven transmembrane-spanning receptors that couple to heterotrimeric guanine nucleotide-binding proteins (G proteins). Furthermore, on the basis of structural homology, glucagon receptors, together with those for glucagon-like peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP), secretin, vasoactive intestinal polypeptide (VIP), growth hormone-releasing factor, corticotropin-releasing factor, pituitary adenylate cyclase-activating polypeptide, parathyroid hormone, and calcitonin (5, 18), form a subfamily of closely related receptors that is now emerging as a group of G protein-coupled receptors able to activate multiple signaling pathways. All these receptors are able to stimulate adenylyl cyclase, but, in addition, many have been found to activate alternative intracellular second messengers. For example, stimulation of the parathyroid hormone receptor leads to intracellular accumulation of cAMP, inositol trisphosphates (IP\(_3\)), and Ca\(^{2+}\) (1), and splice variants of the pituitary adenylyl cyclase-activating polypeptide receptor are able to differentially couple to adenylyl cyclase and phospholipase C (23). The cloned receptors for calcitonin (7), GLP-1 (9, 28), GIP (29), and glucagon (16) have been shown to stimulate cAMP production and a rise in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)).

It had long been postulated that the cellular effects of glucagon are mediated not only by cAMP but also by [Ca\(^{2+}\)], since it has been reported that, in perfused rat liver or cultured hepatocytes, glucagon is able to increase the [Ca\(^{2+}\)] by inducing a Ca\(^{2+}\) influx as well as by stimulating the release of intracellular Ca\(^{2+}\) stores (3, 6, 21). However, the mediator(s) of this rise in [Ca\(^{2+}\)] remains to be determined, particularly since there appears to be some controversy as to whether this Ca\(^{2+}\) response to glucagon is a cAMP-mediated effect or a result of a separate signaling pathway. Experiments demonstrating that the effects of glucagon on Ca\(^{2+}\) mobilization in rat liver cells can be reproduced by cAMP analogs have led some investigators to conclude that the glucagon-mediated rise in [Ca\(^{2+}\)] is mediated by cAMP (8, 24, 25). In contrast, others have reported that cAMP-independent effects of glucagon on intracellular Ca\(^{2+}\) mobilization (22, 27). On the basis of their studies demonstrating that TH-glucagon, a biologically active glucagon analog that is unable to activate adenylyl cyclase, could stimulate inositol phosphate production in rat hepatocytes, Wakelam et al. (27) had proposed the possible existence of two distinct hepatic glucagon receptors: one that couples to adenylyl cyclase and the other that couples to other signaling pathways.

GLUCAGON, a pancreatic hormone that plays an important role in maintaining glucose homeostasis, is secreted from the α-cells in response to low blood glucose levels and stimulates hepatic glucose output by increasing glycogenolysis and gluconeogenesis while at the same time inhibiting glycolysis (5). In addition to its metabolic actions in the liver, glucagon is also involved in the regulation of adipose, cardiac, renal, gastrointestinal, and pancreatic functions, including the potentiation of glucose-induced insulin secretion (5). These effects are mediated by specific glucagon receptors, which have been shown to have a wide tissue distribution corresponding to the observed multiple functions of glucagon (12).
other to the breakdown of inositol phospholipids. However, we have since demonstrated that the cloned rat hepatic glucagon receptor is capable of mediating both a cAMP and a Ca\(^{2+}\) response (16).

In the current study we have used baby hamster kidney (BHK) cells expressing the cloned human glucagon receptor (19) to further characterize our original observation that the glucagon receptor is able to stimulate an increase in [Ca\(^{2+}\)]. By video imaging of fura 2-loaded cells, we have monitored the Ca\(^{2+}\) response to glucagon and have addressed the questions as to the source of the glucagon-mediated rise in [Ca\(^{2+}\)] and the possible molecular mechanisms involved. In view of glucagon's central role in regulating glucose metabolism and our previous findings that a mutated glucagon receptor may in some manner contribute to the development of non-insulin-dependent diabetes (10, 11), the characterization of the molecular signaling pathways activated by glucagon is not only important for understanding the normal physiological mechanisms involved in glucagon action in target tissues such as the liver, fat, or pancreas, but it may also lead to the identification of defects in glucagon signal transduction that may occur in diabetes.

### MATERIALS AND METHODS

Cell culture and transfections. BHK cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 10 µg/ml streptomycin, and 2 mM l-glutamine at 37°C in 5% CO\(_2\)-95% air. The transfection of BHK cells with the human glucagon receptor cDNA plasmid (16) was carried out using the Lipofectamine reagent, as previously outlined in detail (11). The clones analyzed in the present study have been characterized previously for the level of glucagon receptor expression (~3 x 10\(^6\) receptors/cell) and for their ability to bind 125\(^I\)-labeled glucagon (dissociation constant ~10 nM) as well as stimulate cAMP production in response to glucagon (EC\(_{50}\) ~2 pM) (11). For the BHK cells coexpressing the glucagon and muscarinic (M\(_1\)) receptors, the glucagon receptor-expressing BHK cells transfected with the human glucagon receptor plasmid pM1-R by use of the calcium-phosphate method (20). [Ca\(^{2+}\)] measurements. BHK cells (~3 x 10\(^6\), seeded onto thin, circular coverslips (22 mm) that had been precoated with poly-d-lysine (10 µg/ml), were cultured for 3 days. Before Ca\(^{2+}\) measurements, the cells were loaded with 7 µM fura 2-AM in the presence of the nonionic detergent Pluronic F-127 (25%) at 37°C for 30 min, and then an equal volume of HEPES-buffered RPMI culture medium was added. The cells were then incubated for a further 30 min to allow complete deesterification of fura 2. Subsequently, the cells were washed three times in NaCl-HEPES buffer (in mM: 145 NaCl, 5 KCl, 1 Na\(_2\)HPO\(_4\), 0.5 MgSO\(_4\), 20 HEPES, 5 glucose, 1 CaCl\(_2\), pH 7.5). Finally, the coverslips were mounted in a recording chamber and placed on an inverted microscope (model 135 TU, Zeiss axiophot, Oberkochen, Germany). The recording chamber was continuously perfused with NaCl-HEPES buffer supplemented with 1% BSA at 1.5–2 ml/min. All test compounds were applied through the perfusate to give the concentrations indicated. For experiments that required Ca\(^{2+}\)-free medium, the CaCl\(_2\) in the NaCl-HEPES buffer was replaced with 1 mM EGTA.

The [Ca\(^{2+}\)] measurements were performed essentially as outlined previously (4, 9, 26). Briefly, the cells were illuminated with alternating wavelengths (340 and 380 nm) by using interference filters mounted on a filter wheel and observed through a 510-nm emission filter. The images were captured in real time with a low-light-level intensified charge-coupled devise videocamera system. The system was calibrated for free Ca\(^{2+}\) concentrations by use of the formula

\[
[K_{d}] = \frac{b \cdot (R - R_{340/380, \text{min}})/(R_{340/380, \text{max}} - R)}{d}
\]

where \(K_d\) is the dissociation constant of the dye (225 nM for fura 2), \(b\) is the proportionality coefficient (4), and \(R\) is the wavelength ratio. Calibrations of the fura 2 fluorescence signal were performed by dialyzing single cells with 100 µM fura 2 pentapotassium salt and Ca\(^{2+}\)-EGTA buffers with free Ca\(^{2+}\) concentrations ranging from 0 to 39.8 µM. Thus, R\(_{340/380, \text{max}}\) was determined under saturating Ca\(^{2+}\) concentrations, whereas R\(_{340/380, \text{min}}\) was measured for the same field under Ca\(^{2+}\)-free conditions. The ratio images (R\(_{340/380}\)) were obtained by dividing the 340-nm images by the 380-nm image on a pixel-by-pixel basis, whereas the numeric R\(_{340/380}\) values were calculated after the pixel gray values obtained for each wavelength were averaged. Ratio values were then converted to Ca\(^{2+}\) concentrations by use of the calibration curve, and the background (field without cells) was subtracted.

For the experiments shown in Table 1 and Fig. 1, the recordings were made using the Tardis imaging software (Applied Imaging, Tyne & Wear, UK). For the toxin sensitivity studies, the cells were pretreated with 10 µg/ml pertussis or cholera toxin for 24 h at 37°C in the culture medium. The recordings in Figs. 3, 4, and 6–8 were made using a fluorescence imaging system (IonOptix, Milton, MA).

IP\(_3\) determinations. BHK cells transfected with the human glucagon receptor or the human M\(_1\) acetylcholine receptor were seeded (5 x 10\(^4\) cells) into 60-mm tissue culture dishes and allowed to grow for 3–4 days to ~90% confluency. For an estimate of cell number, the cells from two dishes were counted for each cell line. The cells were then incubated for 1 h in DMEM culture medium containing 10 mM myo-inositol and for an additional 30 min in the presence of 10 mM myo-inositol and 10 mM LiCl in DMEM. Cells were subsequently stimulated for 30 s with various concentrations of glucagon or carbachol prepared in DMEM containing 1% BSA. The reactions were terminated by the addition of 2 ml of ice-cold 15% TCA. The cells were then scraped off and transferred onto ice for 20 min, and then they were centr-
fuged for 15 min at 250 g at 4°C. The supernatants were then transferred to polypropylene tubes containing 10 µl of 5 mM EDTA. To the remaining pellets 0.5 ml of 15% TCA was added, and the pellets were incubated for an additional 20 min on ice. The resulting supernatant was combined with the first and extracted four times with one volume of diethyl ether (H2O saturated). The aqueous phase was freeze-dried, resuspended in 2 ml of H2O, and adjusted to pH 7.5 with 1 M NaHCO3.

Amersham’s D-myoinositol 1,4,5-trisphosphate (IP3) 3H assay system was used to measure IP3 in the BHK cell extracts according to the manufacturer’s instructions. For each cell extract, duplicate determinations of a 1:10 dilution were assayed. The samples were counted for 4 min in a beta scintillation counter, and the amount of IP3 in each sample was determined by interpolation from the standard curve.

cAMP measurements. Cells were seeded out in six-well plates (3 × 105 cells/well) and cultured overnight. Before stimulation the cells were washed once in Hanks’ balanced salt solution and again in RPMI 1640 medium, and finally in 1 ml of RPMI medium supplemented with 0.5% fetal bovine serum and 0.45 mM IBMX was added to each well. The cells were stimulated with glucagon and carbachol, and after 20 min of incubation at 37°C, 1 ml of 65% ethanol was added to each well. The cells were scraped off and transferred to Eppendorf tubes, which were then centrifuged at 300 g for 15 min. The resulting supernatants were dried down overnight in a Speed-Vac and stored at −20°C until they were assayed. The cAMP concentrations were determined using the cAMP 125I scintillation proximity assay (Amersham). The dried cell extracts were resuspended in 1 ml of assay buffer and diluted 1:150. For the assay the acetylation protocol described by the manufacturer was followed, and the cAMP determinations were normalized to cell number.

Reagents. All tissue culture flasks and dishes were from Nunc (Roskilde, Denmark). Thin (0.2-mm), circular (22-mm-diameter) glass coverslips were purchased from Struers Kebo Lab (Albertslund, Denmark). Cell culture medium and components, FCS, and Lipofectamine reagent were from Gibco BRL (Paisley, Scotland). Poly-d-lysine, forskolin, pertussis toxin, cholera toxin, thimerosal, EGTA, LaCl3, carbachol, and myo-inositol were purchased from Sigma Chemical (St. Louis, MO). Glucagon was obtained from Novo Nordisk (Bagsvaerd, Denmark); GLP-1-(7—36) amide and VIP were from Peninsula Laboratories (Belmont, CA). The cAMP antagonist, an Rp diastereomer of 8-bromoadenosine 3'5'-cyclic monophosphothioate (Rp-8-BrcAMPS), and the cAMP agonistic Sp diastereomer of adenosine 3'5'-cyclic monophosphothioate (Sp-8-BrcAMPS) were obtained from BIOLOG Life Science Institute (Bremen, Germany). Ryanodine was from Alomone Labs (Jerusalem, Israel). The phospholipase C inhibitor U-73122 and its inactive analog U-73343 were obtained from Calbiochem (Bad Soden, Germany). Fura 2-AM and fura 2 pentapotassium salt were from Molecular Probes (Eugene, OR). The IP3 3H assay system and cAMP 125I scintillation proximity assay system (dual range) from Amersham (Little Chalfont, England) were used for IP3 and cAMP measurements.

RESULTS

Specific, dose-dependent rise in [Ca2+]i mediated by glucagon. In BHK cells stably expressing the cloned human glucagon receptor, we found that stimulation by glucagon results in a transient, synchronized, and dose-dependent rise in [Ca2+]i (Figs. 1 and 2). The response is rapid and recovers to prestimulus levels within 2–3 min. As can be noted in Fig. 1, it is not only the amplitude of the response to glucagon that increases in a dose-dependent manner but also the...
number of responding cells rises with increasing concentrations of glucagon, such that at high levels (≥25 nM) all cells respond. Although the amplitude of the Ca2+ response to glucagon varies from cell to cell, it appears that all responding cells do so in a synchronous manner. There is a delay in the response that becomes progressively less when higher concentrations of glucagon are used; there is a 75 ± 6 s lapse from the time 5 nM glucagon is introduced to the time the rise in [Ca2+]i peaks, whereas the delay is only 53 ± 1, 38 ± 1, and 31 ± 2 s for 10, 25, and 50 nM glucagon, respectively (n = 6). The observed effects of glucagon on the increase in [Ca2+]i appear to be specific, mediated by the binding and activation of specific glucagon receptors, since the related hormones, GLP-1-(7–36) amide and VIP, were not able to elicit a rise in [Ca2+]i in these cells (Table 1).

Finally, in nontransfected BHK cells, no Ca2+ response to glucagon could be observed.

Toxin sensitivity of the glucagon-mediated rise in [Ca2+]i. The glucagon-induced rise in [Ca2+]i was found to be mediated by the activation of a pertussis toxin-sensitive G protein, since pretreatment of the human glucagon receptor expressing BHK cells with pertussis toxin resulted in the near ablation of the Ca2+ response to glucagon (Table 1). Under identical conditions, pertussis toxin had no effect on the carbachol-stimulated Ca2+ response in BHK cells expressing the M1 cholinoergic receptors. In contrast, a glucagon-mediated rise in [Ca2+]i was still observed in cells pretreated with cholera toxin, although the amplitude of the response was decreased in comparison to that observed in nontreated cells (Table 1).

Source of the glucagon-mediated rise in [Ca2+]i. To determine the source of the observed increase in [Ca2+]i, we examined the Ca2+ response to glucagon in the absence of extracellular free Ca2+, which was achieved by using Ca2+-free medium to which was added a further 1 mM EGTA. Under these conditions, glucagon was still able to elicit a robust rise in [Ca2+]i of the same magnitude as obtained in the presence of extracellular Ca2+ (Fig. 3, A and B), suggesting that this transient rise in [Ca2+]i is a result of the release of intracellular Ca2+ stores. This is also confirmed by our finding that LaCl3, a general Ca2+ channel blocker, did not affect the Ca2+ response to glucagon (Fig. 3C).
Mechanisms involved in the glucagon-mediated rise in [Ca^{2+}]i. Binding of glucagon to its receptors results in activation of heterotrimeric G proteins (G_s subtype), stimulating the production of cAMP, which ultimately functions as the second messenger mediating many of glucagon's cellular effects. However, because it has also been reported that stimulation of hepatocytes with glucagon leads to activation of phospholipase C and production of IP_3, we have examined the putative role of this second messenger in glucagon receptor signaling. As evident from Fig. 4A, incubation of the cells with the phospholipase C inhibitor U-73122 completely abolished the ability of glucagon to mobilize intracellular Ca^{2+}. The inactive isoform of the inhibitor U-73343 was without effect (Fig. 4B), indicating that activation of phospholipase C is indeed involved in the glucagon-induced Ca^{2+} response. Furthermore, incubation of the cells with 50 µM ryanodine had no effect on the Ca^{2+} response to subsequent stimulation with glucagon (Fig. 4C). These findings are consistent with our observation that glucagon can stimulate a twofold increase in IP_3 production (Fig. 5A). This response, however, appears to be relatively modest compared with the 100-fold increase obtained when carbachol is used to stimulate BHK cells expressing M_1 cholinergic receptors, which are known to activate phospholipase C, resulting in the formation of IP_3 and release of IP_3-sensitive intracellular Ca^{2+} stores. Figure 5B also shows that stimulation with carbachol does not lead to any concomitant increase in cAMP production, indicating that signaling by the M_1 receptors, when transfected into BHK cells, is essentially normal. The effectiveness of the phospholipase C inhibitor U-73122 is illustrated in Fig. 5C, where treatment of the cells with this agent (15 µM) decreases the carbachol-mediated IP_3 response by 67.5 ± 7.8% (n = 4) compared with untreated cells. In addition, the specificity of U-73122 can be seen in Fig. 5D, where this inhibitor was found to have no effect on adenylate cyclase activity as determined by the accumulation of cAMP in response to glucagon.

Further characterization of the Ca^{2+} response to glucagon was carried out in BHK cells coexpressing glucagon and M_1 receptors. As expected, repeated stimulation of these cells with the same agonist, glucagon (Fig. 6A) or carbachol (Fig. 6B), results in desensitization of the Ca^{2+} response. Interestingly, we found that although prior stimulation with carbachol could prevent the Ca^{2+} response to a subsequent glucagon treatment (Fig. 6D), glucagon pretreatment was not able to prevent the rise in [Ca^{2+}]i elicited by a subsequent carbachol stimulation (Fig. 6C). These results indicate that glucagon releases Ca^{2+} from the IP_3-sensitive intracellular stores but that, unlike carbachol, glucagon is not capable of emptying these stores completely. This is further substantiated by our finding that, after depletion of thapsigargin-sensitive intracellular Ca^{2+} stores, glucagon is unable to induce a rise in [Ca^{2+}]i (data not shown). In addition, unlike carbachol, which can deplete thapsigargin-sensitive intracellular Ca^{2+} stores (Fig. 6F), glucagon only partially empties the thapsigargin-releasable intracellular Ca^{2+} stores, since treatment with thapsigargin immediately after glucagon stimulation can still elicit a rise in [Ca^{2+}]i (Fig. 6E).

To investigate whether cAMP may be involved in the glucagon-induced rise in [Ca^{2+}]i, we examined the effects of forskolin, which stimulates the accumulation of intracellular cAMP by directly activating adenylate
As can be seen in Fig. 7A, forskolin was not in itself able to elicit a Ca\(^{2+}\) response in these cells, whereas the subsequent addition of glucagon was still able to stimulate a rise in [Ca\(^{2+}\)]. Consistent with these results, the activator of protein kinase A (PKA), Sp-8-BrcAMPS, was also unable to stimulate an increase in [Ca\(^{2+}\)] (Fig. 7C). In addition, we examined the effects of Rp-8-BrcAMPS, a cAMP analog that specifically inhib-
its PKA (8), the effectiveness of which has been demonstrated in BHK cells by its ability to inhibit the translocation of the catalytic subunit of PKA, as determined by video-imaging techniques (O. Thastrup, personal communication). Here we found that Rp-8-BrcAMPS inhibits the Ca\textsuperscript{2+} response to glucagon, as can be seen by the diminished [Ca\textsuperscript{2+}] transients (Fig. 7B). Furthermore, in some of the Rp-8-BrcAMPS-treated cells, glucagon-evoked Ca\textsuperscript{2+} oscillations could be observed; the amplitude of the repetitive spikes appeared to be somewhat lower than that of the initial response (Fig. 7B). These data suggest that the rise in [Ca\textsuperscript{2+}] after glucagon stimulation is most likely not mediated by cAMP itself but that it may in some way modulate or potentiate the Ca\textsuperscript{2+} response. Further support of this suggestion is the finding that, together with forskolin, the IP\textsubscript{3} receptor-sensitizing agent thimerosal is able to elicit a Ca\textsuperscript{2+} response (Fig. 8A), whereas on its own, thimerosal does not affect the [Ca\textsuperscript{2+}] (data not shown). A Ca\textsuperscript{2+} response of normal magnitude is observed after stimulation with glucagon, which is then followed by a number of Ca\textsuperscript{2+} oscillations (Fig. 8A). To further test this hypothesis that cAMP potentiates the Ca\textsuperscript{2+} response to IP\textsubscript{3}, we examined the effect of cAMP on the carbachol-stimulated rise in [Ca\textsuperscript{2+}] in BHK cells expressing M\textsubscript{1} receptors. We found that subthreshold concentrations of carbachol could induce a Ca\textsuperscript{2+} response in the presence of forskolin (Fig. 8B and C). Finally, in BHK cells transfected with a mutant (Gly40Ser) glucagon receptor that had previously been identified in some diabetic patients (10), we observed a reduction in the Ca\textsuperscript{2+} response to glucagon (Fig. 7D), and, interestingly in some of these cells, glucagon-induced Ca\textsuperscript{2+} oscillations occurred that were similar to those seen in cells treated with Rp-8-BrcAMPS or thimerosal (Figs. 7B and 8A, respectively).
DISCUSSION

We previously demonstrated that glucagon stimulates a concentration-dependent rise in intracellular cAMP production in BHK cells expressing human glucagon receptors (11, 19). Here we provide evidence to indicate that activation of the cloned human glucagon receptor expressed in BHK cells leads to a rise in [Ca\(^{2+}\)]i that does not appear to be due to cAMP-mediated activation of Ca\(^{2+}\) influx. This suggests that the glucagon receptor, like many others of the secretin receptor family, can stimulate two independent signaling pathways: one mediated by cAMP and the other by Ca\(^{2+}\).

From studies aimed at characterizing the rise in [Ca\(^{2+}\)]i stimulated by glucagon, we found it to be independent of extracellular Ca\(^{2+}\), since it was not affected by blocking of membrane Ca\(^{2+}\) channels or by removal of extracellular Ca\(^{2+}\). These findings indicate that the source of the glucagon-stimulated rise in [Ca\(^{2+}\)]i is intracellular Ca\(^{2+}\) stores. Thus the general features of the glucagon-mediated rise in [Ca\(^{2+}\)]i appear to differ from those described in response to activation of GLP-1 and GIP receptors, the two most closely related to the glucagon receptor. In contrast to the Ca\(^{2+}\) response to glucagon, the Ca\(^{2+}\)-mobilizing effects of GLP-1 in pancreatic β-cells have been demonstrated to be dependent on extracellular Ca\(^{2+}\), and mimicked by stimulation with cAMP. This response has been proposed to involve influx of extracellular Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels as well as a release of Ca\(^{2+}\) from intracellular stores (15). From further studies on β-cells, it has been proposed that the mobilization of Ca\(^{2+}\) from intracellular stores in response to GLP-1 reflects Ca\(^{2+}\)-induced Ca\(^{2+}\) release, since it was blocked by ryanodine (9). On the other hand, the rise in [Ca\(^{2+}\)]i elicited by GIP through the cloned receptor expressed in COS-7 cells has been shown to be biphasic, where the first transient rise in [Ca\(^{2+}\)]i has been attributed to the mobilization of intracellular thapsigargin-sensitive Ca\(^{2+}\) stores and the second sustained rise in Ca\(^{2+}\) has been found to be a result of Ca\(^{2+}\) influx through a cation channel (29). Despite these differences among the Ca\(^{2+}\) signaling mediated by the various related receptor types, the characteristics of the Ca\(^{2+}\) response that we observe after activation of the cloned human glucagon receptor nonetheless are supported by previous studies in which endogenous hepatic glucagon receptors were found to have Ca\(^{2+}\)-mobilizing effects that could not be reproduced by cAMP (22, 27). We do recognize, however, that by overexpressing the glucagon receptor in BHK cells we run the risk of “forcing” the coupling to effectors that may not necessarily occur in cells expressing the endogenous receptors. Nevertheless, previous studies using cultured primary hepatocytes have demonstrated that glucagon is able to activate cAMP- and Ca\(^{2+}\)-mediated signaling pathways, which supports our findings with use of the BHK cells transfected with the cloned glucagon receptor. In addition, signaling by the cloned M1 receptors in BHK cells overexpressed to the same extent as the glucagon receptor were stimulated with 200 nM glucagon. Each trace represents response in a single cell and is representative of at least 20 single-cell recordings from 3 different experiments.

Fig. 7. Role of protein kinase A in glucagon-mediated Ca\(^{2+}\) response. BHK cells expressing wild-type human glucagon receptor were stimulated with 200 nM glucagon after a previous stimulation with 10 µM forskolin (A), 100 µM Rp diastereomer of 8-bromoadenosine 3',5'-cyclic monophosphothioate (Rp-8-BrCAMPs, B), or 100 µM Sp diastereomer of 8-bromoadenosine 3',5'-cyclic monophosphothioate (Sp-8-BrCAMPs, C) for time period indicated by horizontal bar. D: BHK cells expressing mutant (Gly40Ser) glucagon receptors were stimulated with 200 nM glucagon. Each trace represents response in a single cell and is representative of at least 20 single-cell recordings from 3 different experiments.
pared with the response observed with carbachol (Fig. 5A). This difference in the magnitude of IP₃ formation provides an explanation for our heterologous desensitization experiments, from which it is apparent that, even at a maximal concentration, glucagon only partially empties the Ca²⁺ stores mobilized by IP₃ after activation of M₁ cholinergic receptors. The fact that stimulation with carbachol could desensitize the Ca²⁺ response to a subsequent stimulation with glucagon whereas the opposite is not the case suggests that glucagon and carbachol mobilize Ca²⁺ from the same IP₃-sensitive intracellular stores but to varying degrees. This is further exemplified by the finding that thapsigargin is still able to release Ca²⁺ stores after stimulation by glucagon but not by carbachol (Fig. 6, E and F). Finally, Hofer et al. (14) found that the intragranellar sequestration of Ca²⁺ in BHK cells is not affected by ryanodine, which supports our finding that this agent has no effect on glucagon's Ca²⁺ response (Fig. 4C). Although it is thus apparent that phospholipase C-catalyzed IP₃ production is involved in the Ca²⁺ response to M₁ and glucagon receptor activation, the upstream signaling mechanisms leading to this phospholipase C activation are clearly different, since we find that pertussis toxin blocks the glucagon-mediated rise in [Ca²⁺] (Table 1). Because M₁ cholinergic receptors couple to phospholipase C by a pertussis toxin-resistant G protein (Gq) (2), the glucagon-induced Ca²⁺ signal must involve a G protein distinct from that used by the M₁ receptors. It has been shown that the βγ-subunit of the pertussis toxin-sensitive Gi is able to activate the β₂-isofrom of phospholipase C (17), and since it has been recently shown that the luteinizing hormone receptor is able to couple to Gαs and Gαi to activate adenylate cyclase and phospholipase C, with the βγ-subunits released from either G protein contributing to the stimulation of phospholipase C β-isofroms (13), it is possible that this mechanism may also be involved in the glucagon-mediated Ca²⁺ response.

Our results also indicate that Gs- and Gi-activated pathways are involved in the Ca²⁺ response to glucagon, since Rp-8-BrcAMPS, which acts as an inhibitor of PKA, was found to reduce the glucagon-stimulated increase in [Ca²⁺] (Fig. 7B), whereas neither forskolin nor the cAMP agonist Sp-8-BrcAMPS was able to elicit a Ca²⁺ response on its own. Furthermore, the IP₃-sensitizing agent thimerosal, although unable to elicit a Ca²⁺ response itself, leads to an increase in IP₃ if the cells have previously been exposed to forskolin (Fig. 8A). Further substantiation of the possible interaction between cAMP and IP₃ is given by our finding that a subthreshold concentration of carbachol (1 nM) is also able to evoke a Ca²⁺ response after prior treatment with forskolin (Fig. 8, B and C). This suggests that although cAMP is not responsible for the rise in [Ca²⁺] stimulated by glucagon, cAMP can potentiate the Ca²⁺ response. It is interesting to note that the induction of Ca²⁺ oscillations that occurred after thimerosal (Fig. 8A) or Rp-8-BrcAMPS (Fig. 7B) treatment was similar to our observation in BHK cells expressing the Gly40Ser mutant receptor (Fig. 7D), which we have reported to be present in a subset of diabetic patients (10). We previously demonstrated that this mutation results in a decreased sensitivity of the receptor to glucagon in terms of binding and cAMP production (11). However, the mechanisms underlying the induction of this oscil-
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latory response to glucagon under certain conditions and the significance of these oscillations remain to be determined.

Thus, taking into consideration all our findings, we suggest the following model for glucagon-mediated Ca\(^{2+}\) signaling in which two distinct signaling pathways are involved. On the basis of sensitivity to pertussis and cholera toxins, it appears that the glucagon receptor can couple to multiple G proteins: G\(_s\) and possibly G\(_i\). In addition, the glucagon-stimulated cAMP production appears to play a permissive role in the rise in [Ca\(^{2+}\)], such that cAMP, although itself unable to elicit a response, in concert with the small incremental release of IP\(_3\) is able to mediate the observed rise in [Ca\(^{2+}\)] in response to glucagon. Previously, we proposed that a similar mechanism underlies the GLP-1-stimulated release of Ca\(^{2+}\) from intracellular stores in pancreatic \(\beta\)-cells, such that a cAMP-induced phosphorylation of the IP\(_3\) receptor (mediated by PKA) may enhance the Ca\(^{2+}\) mobilization in response to the low levels of IP\(_3\) arising from activation of phospholipase C by localized Ca\(^{2+}\) influx (9). Therefore, although we did not see an increase in Ca\(^{2+}\) in response to cAMP stimulation alone (via forskolin), the cAMP produced in response to activation of glucagon receptors in these cells may potentiate the effects of the rather small increase in IP\(_3\) we observed after glucagon stimulation. This model is supported by our observation that thimerosal or a subthreshold concentration of carbachol is able to induce a Ca\(^{2+}\) response in the presence of forskolin. In summary, we have analyzed the general characteristics of the glucagon-mediated rise in [Ca\(^{2+}\)], and have provided evidence to suggest that, contrary to earlier proposals of two glucagon receptor subtypes in the liver (27), the cloned human glucagon receptor is able to couple to multiple G proteins, thereby activating two distinct signaling pathways. It remains a challenge, however, to elucidate the physiological role of these cAMP-Ca\(^{2+}\) signaling pathways stimulated by glucagon in its target tissues.

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