Glucagon release dependent on and independent of changes in cytosolic Ca^{2+} : Studies using Ca^{2+} -clamped rat pancreatic islets

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ABSTRACT

The role of calcium ion (Ca^{2+}), and of cyclic AMP-dependent and Ca^{2+} -activated, phospholipid-dependent protein kinases (protein kinases A and C, respectively) in glucagon release was examined using isolated rat pancreatic islets permeabilized with digitonin whose cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was clamped with Ca-EGTA buffer. Ca^{2+} (1–100 μM) stimulated glucagon release from the Ca^{2+} -clamped islets in a concentration-dependent manner. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, increased glucagon release at substimulatory (0.1 μM) and stimulatory (10 μM) concentrations of Ca^{2+} . Forskolin, an activator of adenylate cyclase, also stimulated glucagon release at 0.1 μM Ca^{2+} but not at 10 μM Ca^{2+} . These findings suggest that all three messenger systems have a stimulatory role in glucagon release, and the activation of protein kinase A or C regulates the hormone release, at least in part, without changing $[\text{Ca}^{2+}]_i$.

Ca^{2+} is an important intracellular messenger in a wide variety of cellular functions, including hormone release (18). However, the role of Ca^{2+} in glucagon release is still a matter of debate (for review, see reference 13), which may result from a difficulty in directly estimating the effect of cytosolic Ca^{2+} concentration on glucagon release. One of the methods to overcome the difficulty is to clamp $[\text{Ca}^{2+}]_i$ at arbitrary levels by permeabilizing the plasma membrane with digitonin followed by incubating the cells in Ca-EGTA buffer. We have recently reported that the Ca^{2+} -clamped islets are useful to analyze the role of internal signaling systems in insulin release (23). Using these islets, one can elucidate the role of $[\text{Ca}^{2+}]_i$ directly and also discriminate phenomena independent of changes in $[\text{Ca}^{2+}]_i$ from those dependent on its changes. In the present study, we examined the effect of various concentrations of Ca^{2+} in the media as

well as effects of forskolin, an activator of adenylate cyclase, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, on glucagon release from the Ca^{2+} -clamped islets.

Islets were isolated from pancreases of fed male Wistar rats by the collagenase digestion method. The media used during the isolation was Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and gassed with 5% CO_2 /95% O_2 . The isolated islets were preincubated for 1 h at 37°C in Krebs-Ringer bicarbonate buffer containing 3 mM glucose and 5 mg/ml bovine serum albumin. Then, they were treated with digitonin (25 $\mu\text{g}/\text{ml}$) (Sigma, St Louis, MO) for 5 min (23). After washed 3 times, groups of 10 islets were incubated for 30 min at 37°C in the media at various Ca^{2+} concentrations with or without forskolin (5 μM) or TPA (162 nM). The composition of the media used for the digitonin treatment and the incu-

Fig. 1 Glucagon release from digitonin-treated pancreatic islets at increasing concentrations of Ca^{2+} . Isolated rat pancreatic islets were treated with digitonin ($25 \mu\text{g/ml}$) for 5 min. Groups of 10 islets were incubated for 30 min at 37°C in the Ca -EGTA buffer containing various concentrations of Ca^{2+} . Values are expressed as means $\pm$ SE, for the numbers of observations shown in parentheses. * $P < 0.05$ and ** $P < 0.01$, relative to the value at $0.1 \mu\text{M}$ Ca^{2+} .

bation was as follows (mM): K-glutamate, 100; Na-glutamate, 42; Mg-ATP, 3; HEPES, 16; EGTA, 1; and bovine serum albumin, 5 mg/ml. No glucose was added. CaCl_2 was supplemented appropriately according to stability constants of EGTA (23). The pH of the media was adjusted to 7.0 with NaOH, and the media was saturated with pure O_2 . The stock solution of forskolin (Sigma) was prepared in 95% ethanol, and that of TPA (Calbiochem, La Jolla, CA) in dimethyl sulfoxide, respectively. Samples, taken after 30 min of incubation, were immediately frozen at -20°C and stored until assayed.

Glucagon was measured by radioimmunoassay using Glucagon Kit 'Daiichi' (Daiichi Radioisotope Laboratories, Tokyo). Bovine glucagon was used as standard. The C-terminal region-specific anti-glucagon antibody was used for the assay. Destruction of glucagon during the incubation of islets and the assay procedure was estimated by the method reported by Tahara *et al.* (22); the destruction

did not occur to a significant extent. None of the media used and the compounds added interfered with the assay. Statistical significance was assessed by Student's *t* test for unpaired data (Fig. 1) or for paired data (Table 1).

Conflicting observations have been reported as to the role of Ca^{2+} in the control of glucagon release. Enhancement of glucagon release by acute Ca^{2+} deprivation was first reported using incubated pieces of duct-ligated rat pancreas (14), and thereafter in various experimental systems (13) including isolated islets of rat pancreas (1, 3). The reintroduction of Ca^{2+} following a period of Ca^{2+} deprivation (15) or stepwise increase in the extracellular Ca^{2+} concentration in the range of 0.5–3 mEq/l (17) was reported to inhibit glucagon release. On the contrary, stimulation of glucagon release by increasing the extracellular Ca^{2+} concentration was observed in the perfused rat pancreas (17) and in monolayer cultures of newborn rat pancreas (30). A Ca^{2+} ionophore, A23187, has been found to stimulate glucagon release from perfused rat islets (1) and from monolayer cultures of rat pancreas (30). The stimulatory role of Ca^{2+} in glucagon release is also supported by the observation that Ca^{2+} uptake in A cell-rich islets was increased with a low concentration of glucose, which is known to increase glucagon release (2). However, the studies with verapamil, a voltage-dependent Ca^{2+} channel blocker, revealed complex effects of the antagonist on glucagon release (16). There has been little direct evidence for the role of intracellular Ca^{2+} on glucagon release.

Fig. 1 shows glucagon release from digitonin-treated pancreatic islets at increasing concentrations of Ca^{2+} (0.01–100 μM). Raising the Ca^{2+} concentration resulted in stimulation of glucagon release from the treated islets in a concentration-dependent manner with the threshold of Ca^{2+} for the hormone release being between 0.1 and 1 μM , like in insulin release (23). These results suggest that Ca^{2+} has a positive role in glucagon release. Such an interpretation is also in agreement with the recent finding, using the pyroantimonate precipitation technique, that the cytoplasmic calcium content was increased in A cells stimulated by glucose at low concentrations (19).

The role of cyclic AMP in the regulation of glucagon release has also been a matter of controversy (25). Exogenous cyclic AMP or dibutyryl cyclic AMP was reported to stimulate (9,

Table 1 *Effects of Forskolin and TPA on Glucagon Release from Digitonin-treated Pancreatic Islets at Low (0.1 μM) and High (10 μM) Concentrations of Ca^{2+}*

Ca^{2+} (μM)	Control output (pg/islet/30 min)	Additions	Increment (pg/islet/30 min)	<i>P</i> (versus control)
0.1	6.21 $\pm$ 1.06 (18)	Forskolin (5 μM)	+8.29 $\pm$ 2.15 (18)	<0.01
	8.83 $\pm$ 1.93 (7)	TPA (162 nM)	+4.89 $\pm$ 1.18 (7)	<0.01
10	20.45 $\pm$ 1.99 (7)	Forskolin (5 μM)	-8.14 $\pm$ 0.99 (7)	<0.001
	19.90 $\pm$ 3.07 (15)	TPA (162 nM)	+8.66 $\pm$ 2.68 (15)	<0.01

Groups of 10 islets permeabilized with digitonin were incubated for 30 min in the Ca-EGTA buffer containing 0.1 μM or 10 μM Ca^{2+} with or without forskolin or TPA. Values are expressed as means $\pm$ SE, for the numbers of observations shown in parentheses.

10, 27, 29) or inhibit glucagon release (24, 29). Conflicting results have also been reported using theophylline, an inhibitor of phosphodiesterase (4-6, 9, 24, 26, 29). Forskolin raises an intracellular level of cyclic AMP by activating adenylate cyclase, and enhanced glucagon release from perfused dog pancreas (7).

The effect of forskolin on glucagon release from the Ca^{2+} -clamped islets is shown in Table 1. Forskolin (5 μM) markedly stimulated glucagon release in the presence of 0.1 μM Ca^{2+} , suggesting that cyclic AMP has a stimulatory effect on glucagon release independently of changes in $[\text{Ca}^{2+}]_i$. In contrast, an inhibitory effect of forskolin was observed at a higher concentration (10 μM) of Ca^{2+} . A similar observation was previously reported by Wollheim *et al.* (29), that exogenous cyclic AMP stimulated glucagon release from monolayer cultures of rat pancreas incubated at a low concentration (1 mM) of Ca^{2+} , whereas it inhibited the hormone release at a higher concentration (2.5 mM) of Ca^{2+} . Such a discrepancy could be partly explained by the influence of other pancreatic hormones on glucagon release. As previously reported (23), forskolin stimulated insulin release from the Ca^{2+} -clamped islets at a higher concentration (10 μM) of Ca^{2+} , but not at a low concentration (0.1 μM) of Ca^{2+} . Under the former condition, glucagon release may be inhibited by insulin concomitantly released.

Although available data concerning the participation of protein kinase C in glucagon release are still limited at present, TPA, an activator of protein kinase C, was found to stimulate glucagon release from isolated rat pancreatic islets (8). In the present experiment, TPA (162 nM) significantly increased glucagon release from Ca^{2+} -clamped islets at both 0.1 and 10 μM of Ca^{2+} (Table 1), again

suggesting that the activation of protein kinase C results in an increase in the hormone release without changing $[\text{Ca}^{2+}]_i$. The stimulatory effects of TPA on several physiological phenomena without elevating $[\text{Ca}^{2+}]_i$ have also been documented in other types of cells or tissues (12, 20), including the pancreatic B cell (11, 23). It has been recently shown, however, that changes in $[\text{Ca}^{2+}]_i$ under stimulation differ in different regions of the cell (21, 28). Although the medium used in the present study contains a sufficient amount of EGTA to buffer Ca^{2+} mobilized from intracellular organelles (23), further experiments are required to elucidate whether the spatial and temporal changes in $[\text{Ca}^{2+}]_i$ can be uniformly canceled by Ca-EGTA buffer.

The present findings suggest that three major internal signaling systems have a stimulatory role in glucagon release, as has been suggested for insulin release.

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