Insulin release independent of a rise in cytosolic free Ca^{2+} by forskolin and phorbol ester

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Received 1 March 1985

The role of cytosolic free Ca^{2+} in insulin release was evaluated using isolated rat pancreatic islets permeabilized with digitonin and incubated in Ca-EGTA buffers to fix free Ca^{2+} concentration at arbitrary levels. Ca^{2+} induced insulin release in a concentration-dependent manner with the threshold being between 0.1 and 1 μM. The hormone release was increased by forskolin and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a potent activator of adenylate cyclase and that of protein kinase C, respectively. The findings suggest that activation of both protein kinase A and protein kinase C modulate insulin release without a concomitant increase in cytosolic free Ca^{2+}.

Insulin release *Digitonin* *Ca-EGTA* *Forskolin* *Phorbol ester* *Cytosolic free Ca^{2+}*

1. INTRODUCTION

It has been widely accepted, mainly based on $^{45}Ca^{2+}$ flux studies, that all initiators or potentiators for insulin release exert their effects through a common mechanism, i.e., increasing cytosolic free calcium concentration ($[Ca^{2+}]_i$) [1]. The view was also supported by the data obtained with the recent introduction of fluorescent Ca^{2+} indicators into pancreatic B cells [2–4]. However, the latest measurements of $[Ca^{2+}]_i$ with the indicator showed that forskolin, a potent activator of adenylate cyclase, evoked insulin release without a rise in $[Ca^{2+}]_i$ [5]. Thus, the role of cytosolic free Ca^{2+} as an intracellular messenger for insulin release is still a matter of controversy. Taking another experimental approach, we have attempted to clarify whether insulin release can be evoked independently of a rise in $[Ca^{2+}]_i$. To avoid changes of $[Ca^{2+}]_i$ on stimulation, we permeabilized rat pancreatic islets with digitonin and incubated them in Ca-EGTA buffers. Under these conditions, insulin release was increased by forskolin and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a potent activator of protein kinase C.

2. EXPERIMENTAL

Islets were isolated from pancreases of fed male Wistar rats by collagenase digestion, and preincubated for 1 h in Krebs-Ringer bicarbonate buffer containing 3 mM glucose and 5 mg/ml bovine serum albumin. Then, they were treated with digitonin (25 μg/ml) (Sigma, St Louis, MO) for 5 min according to the method used in adrenal medullary cells [6]. After washing 3 times, groups of 5 islets were incubated at 37°C for 30 min in the media with various concentrations of Ca^{2+} and test substances. The composition of the media for digitonin treatment and incubation (mM): K-glutamate, 100; Na-glutamate, 42; Mg-ATP, 1; Hepes, 16; glucose, 3; EGTA, 1; and bovine serum albumin (5 mg/ml). $CaCl_2$ was added appropriately and the pH was adjusted to 7.0 with NaOH. Using stability constants of EGTA [7], concentrations of free Ca^{2+} in the presence of the chelator were kindly computed by Dr A. Takai, Department of Physiology, University of Nagoya, Japan. Forskolin (Calbiochem, La Jolla, CA) was added to the incubation media from stock solution (20 mM) prepared in 95% ethanol, and TPA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

(Sigma) was from that (162 μM) prepared in dimethyl sulfoxide, the final concentrations being 5 μM and 162 nM, respectively. Insulin release in the media was determined by radioimmunoassay using rat insulin as standard. Statistical significance was assessed by Student's t-test for unpaired data.

3. RESULTS AND DISCUSSION

Digitonin is known to form pores which make the membrane freely permeable to inorganic ions by interacting with cholesterol in the cholesterol-rich plasma membrane, while it does not affect membranes which contain negligible amounts of cholesterol such as those of intracellular organelles [8]. The islets thus permeabilized with digitonin were incubated with Ca-EGTA buffers to give arbitrary concentrations of free Ca^{2+}. Since the total calcium content of an islet was reported to be 4.7 pmol on average [1], even the maximal contribution of mobilized Ca^{2+} from the intracellular organelles to $[Ca^{2+}]_i$ should be nullified with a sufficient amount of EGTA (1 mM) in the buffer.

Insulin release from digitonin-treated islets was stimulated by Ca^{2+} in a concentration-dependent manner, and the threshold was between 0.1 and 1 μM (fig.1), which is similar to the results obtained from digitonin-treated chromaffin cells [9], or those from permeabilized islets by electric discharge [10]. Colchicine (1 mM) inhibited insulin release induced by 10 μM Ca^{2+} by 36%, suggesting that the microtubular system is operating in the treated islets.

A rise in the cyclic AMP content of islets is known to enhance insulin release when the islets are exposed to suitable secretagogues. The mechanism of the enhancement has been considered to mobilize intracellular stored calcium, leading to an increase in $[Ca^{2+}]_i$, since cyclic AMP or agents that raise intracellular cyclic AMP levels increased $^{45}Ca^{2+}$ efflux from pancreatic islets preloaded with the isotope [1]. However, a recent finding using a fluorescent Ca^{2+} indicator revealed that cyclic AMP stimulation of insulin release was not accompanied by increased $[Ca^{2+}]_i$ [5]. Thus, the interpretation of the results derived from $^{45}Ca^{2+}$ flux studies is still a matter for debate. Our present data, in support of the results using the Ca^{2+} indicator, showed that 5 μM forskolin

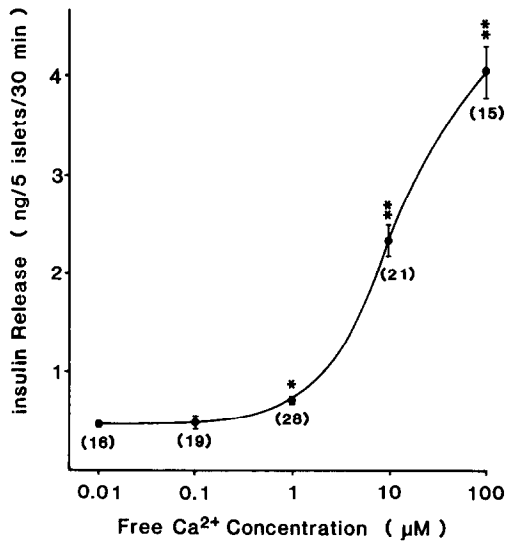

Fig.1. Dependence of insulin release from digitonin-treated pancreatic islets upon Ca^{2+}. Values are means ± SE (represented by bars), for the numbers of experiments shown in parentheses. Significance levels: * $p < 0.01$, ** $p < 0.001$, vs the value at 0.01 μM Ca^{2+}.

Table 1

Effects of forskolin and TPA on insulin release from digitonin-treated pancreatic islets at various concentrations of Ca^{2+}

$[Ca^{2+}]$ (μM)	Additions	Insulin release (ng/5 islets per 30 min)
0.01	none	0.48 ± 0.03 (14)
	forskolin	0.58 ± 0.04 (15)
	TPA	1.34 ± 0.13[b] (16)
0.1	none	0.72 ± 0.15 (9)
	forskolin	1.03 ± 0.12 (8)
	TPA	2.00 ± 0.15[b] (9)
1	none	1.09 ± 0.09 (7)
	forskolin	1.54 ± 0.11[a] (7)
	TPA	4.53 ± 0.41[b] (7)
10	none	3.60 ± 0.25 (13)
	forskolin	4.89 ± 0.29[a] (7)
	TPA	9.94 ± 0.49[b] (16)

Values are means ± SE for the numbers of experiments shown in parentheses. Significance levels: [a] $p < 0.01$, [b] $p < 0.001$, vs control without addition

significantly augmented insulin release at 1 or 10 μM Ca^{2+}, as shown in table 1. The findings that forskolin did not significantly stimulate insulin release at lower concentrations of Ca^{2+}, 0.01 and 0.1 μM, are reminiscent of those in intact islets that the effects of cyclic AMP are usually small at low, non-stimulatory glucose concentrations [1]. Although the underlying mechanism(s) of the potentiation is not yet clear, one of the possibilities may be that cyclic AMP sensitizes the secretory machinery of pancreatic B cells to Ca^{2+}, in analogy with the view on the role of cyclic nucleotides in platelet secretion [11].

Growing attention has been paid to the involvement of protein kinase C in a wide variety of cellular functions, including insulin release. TPA is known to substitute for diacylglycerol and increase the affinity of protein kinase C for Ca^{2+} resulting in its activation without elevating $[Ca^{2+}]_i$ [12]. As shown in table 1, addition of 162 nM TPA caused a significant insulin release at 0.01 and 0.1 μM Ca^{2+}, and augmented that induced by 1 or 10 μM Ca^{2+}. These data indicate that the phorbol ester modulates insulin release by a mechanism independent of a rise in $[Ca^{2+}]_i$. It was reported that TPA stimulated catecholamine release from adrenal medullary cells made leaky by exposure to an intense electric field [13]. TPA was also shown in human platelets to evoke secretion and aggregation without elevating $[Ca^{2+}]_i$ using a fluorescent Ca^{2+} indicator [14]. Taken together, it seems likely that activation of protein kinase C can modulate insulin release without a concomitant rise in $[Ca^{2+}]_i$. Two points should be noted. First, the results obtained under the present conditions do not rule out the possible involvement of mobilization of stored calcium in insulin release stimulated by TPA, as was suggested in [15] based on the data derived from $^{45}Ca^{2+}$ efflux study, although $^{45}Ca^{2+}$ efflux may not necessarily reflect $[Ca^{2+}]_i$, as mentioned above. Second, the effect of TPA on insulin release observed at 0.01 μM Ca^{2+} may not be mediated via activation of protein kinase C, but may rather be attributable to some other action(s) of TPA, since protein kinase C is reported to be hardly activated at Ca^{2+} concentrations below 0.1 μM [12].

Our data lend support to the view that cyclic AMP modulates insulin release independently of changes of $[Ca^{2+}]_i$ and also provide the first evidence that TPA, probably via protein kinase C, can stimulate insulin release without a rise in $[Ca^{2+}]_i$.

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