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Minireview

Protein kinase C in insulin releasing cells

Putative role in stimulus secretion coupling

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The evidence for the involvement of protein kinase C (PKC) in insulin secretion stimulated by glucose and Ca^{2+}-mobilizing receptor agonists has been reviewed. Results of phorbol ester binding to intact cells and the measurements of the proportion of PKC associated with the membrane after cell fractionation are presented. Glucose stimulation leads to increased phorbol ester binding without causing membrane insertion of the enzyme which, however, occurs with receptor agonists. It is suggested that the rise in cytosolic Ca^{2+} in response to glucose favours the apposition of PKC to the membrane whereas intercalation of the enzyme requires phospholipase C-mediated generation of diacylglycerol. It is possible that this effect of glucose on PKC, although not involved in the initiation of secretion, could explain the potentiation of insulin release observed in the presence of the receptor agonists.

Protein kinase C; Insulin secretion; Glucose; Carbachol; Ca^{2+}

1. INTRODUCTION

The secretion of insulin is regulated by fuel stimuli, hormones and neurotransmitters to obtain precise control of blood glucose homeostasis. The mode of action of the different secretagogues spans from the depolarization of the membrane (e.g. glucose), to Ca^{2+} mobilization (e.g. acetylcholine) and cyclic AMP generation (e.g. glucagon) [1,2]. Therefore, the β-cell offers a good experimental model in which the various signalling pathways of importance for cell activation in general can be reviewed. In the present short overview the actions of fuel stimuli and those of Ca^{2+}-mobilizing receptor agonists will be discussed, because of particular persistent controversy regarding the role of protein kinase C (PKC). The cyclic AMP pathway, less controversial in nature, will not be treated here and we refer the reader to a recent review [2].

2. Ca^{2+}-MOBILIZING RECEPTOR AGONISTS

The neurotransmitter acetylcholine and its analogue carbachol bind to muscarinic receptors which, in insulin secreting cells, leads to the activation of phospholipase C (PLC) via GTP-binding protein not sensitive to pertussis toxin [1]. In this manner, as in other cells, the hydrolysis of phosphatidylinositol-4,5-bisphospate $(PIP)_2$ generates the second messengers diacylglycerol (DAG) and inositol-1,4,5-tripsphosphate $(IP)_3$ [3]. The former activates PKC [4] while the latter promotes Ca^{2+} mobilization from intracellular stores [1,3,5]. In insulin secreting cells the same pathway was also demonstrated for cholecystokinin [6], ATP acting as purinergic transmitter [7,8], bombesin [9] and, in the cell line RINm5F, vasopressin [10]. These substances also increase Ca^{2+} influx into the cell, perhaps via the IP_3 receptor as recently proposed [11]. Furthermore, some of the agonists depolarize insulin secreting cells by closing K^+ channels which is believed to lead to gated Ca^{2+} influx [12].

3. GLUCOSE AND OTHER FUEL STIMULI

Glucose, in contrast to receptor agonists, enters the β-cell by facilitated diffusion via low affinity high capacity glucose transporters [13,14] and is then metabolized by glycolysis and oxydised in the mitochondria [13]. This leads to the production of ATP, reduced pyridine nucleotides and other putative coupling factors thought to link metabolism to ion fluxes across the plasma membrane [2]. It is now well established that glucose and other nutrient stimuli depolarize the membrane potential of insulin secreting cells by closing ATP-sensitive K^+ channels [15]. This, in turn, causes Ca^{2+} influx via

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voltage-dependent Ca^{2+} channels mainly of the L-type [16]. Although there is general agreement on the intitial sequence of events in response to glucose, the exact nature of the coupling of metabolism to the membrane events is still a matter of debate. An increase of the ATP concentration and/or of the ATP/ADP ratio has retained most attention [15], but other mechanisms including the activation of PKC have been put forward [17]. In fact, activation of PKC by phorbol esters and synthetic DAG stimulates insulin secretion [17–19]. These agents have been shown to depolarize insulin secreting RINm5F cells by closing ATP-sensitive K^+ channels therein mimicking the effect of the triose glyceraldehyde [17]. The depolarizing action of carbohydrates was therefore proposed to be mediated, at least in part, by the activation of PKC. In the following, work analysing the role of PKC in glucose stimulated insulin release will be discussed.

4. GLUCOSE ACTIVATION OF PHOSPHOLIPASE C

Many papers have demonstrated an effect of glucose on the turnover of inositol-containing phospholipids and an increased production of IP_3 [2,20]. However, in contrast to the Ca^{2+}-mobilizing agonists, the effect of glucose was small and clearly dependent on extracellular Ca^{2+} [20]. Therefore PLC is not directly activated by glucose but rather stimulated by the increase in cytosolic Ca^{2+} secondary to Ca^{2+} influx. Indeed, in permeabilized RINm5F cells, as in other cell types, Ca^{2+} was found to enhance PLC activity as demonstrated by measuring both IP_3 [21] and DAG (22) production.

5. DE NOVO SYNTHESIS OF DAG AND ACTIVATION OF PKC

The incubation of insulin secreting cells with stimulatory concentrations of glucose generates glycolytic intermediates which can be acylated to form phosphatidic acid and, in turn, DAG (17,23–25). Since the de novo synthetized DAG could stimulate PKC, the possible involvement of this enzyme was examined in the presence of glucose concentrations that enhance insulin secretion. Different methods have been applied. In a first attempt the effects on insulin secretion of various inhibitors of the enzyme were tested. Most of them did not interfere with glucose stimulation [26], while some of them were found to inhibit the glucose response [27,28]. Therefore, probably because of lack of specificity of PKC inhibitors, this approach gave no unequivocal answer to the question.

PKC is a specific receptor for phorbol esters that bind to and activate the enzyme [4]. Since prolonged pretreatment of the cells with these agents has been shown to down-regulate PKC [29], this property was used in several studies to establish the role of the enzyme in the mechanism of glucose response. In such pretreated islets glucose still caused insulin secretion [26,30–33] and in one case the effect of the carbohydrate was even potentiated [32]. These experiments are, however, not clear cut: first, a relevant amount of PKC activity that could account for the glucose stimulated insulin secretion was still detectable after prolonged phorbol ester treatment [31,33,34]; second, it is noteworthy that phorbol ester-pretreated islets not only preserve glucose stimulated insulin release but also that induced by carbachol, an agonist believed to act mainly via PKC activation [31,32]. The latter observation suggests that the down-regulation manoeuvre is not easy to interpret in intact pancreatic islets. A role for PKC has been suggested from protein phosphorylation experiments in intact islet cells and after cell fractionation, since some of the protein substrates were tentatively identified to be common to glucose and phorbol esters [35] or glucose and carbachol [33]. However, the effect of glucose was much less pronounced than that of carbachol and could not be unambigously attributed to PKC, since the activation of another Ca^{2+}-dependent protein kinase following Ca^{2+} influx was not ruled out [33,35].

An alternative approach was to measure the fraction of PKC activity associated in a chelator stable manner with membranes. An increase in the amount of enzyme bound to the membrane has been reported in many different cell types [36,37], including insulin secreting cells [31,38], in response to Ca^{2+} mobilizing receptor agonists. As shown in Table I, incubation of RINm5F cells for 2 min in the presence of carbachol leads to an increase in the PKC activity recovered in the membrane

Table I

Effect of carbachol, oleyl-acetyl-glycerol (OAG) and KCl on membrane-associated protein kinase C activity in RINm5F cells

(A)	
control	100 ± 3 (5)
carbachol 100 μM	179 ± 10 (5)
(B)	
control	100 ± 7 (4)
OAG 5 μg/ml	110 ± 17 (4)
OAG 50 μg/ml	144 (2)
KCl 48 mM	103 ± 7 (4)
OAG 5 μg/ml + KCl 48 mM	222 ± 38 (4)
OAG 50 μg/ml + KCl 48 mM	220 (2)

RINm5F cells were cultured in suspension for 3 h and then washed twice with modified Krebs-Ringer bicarbonate buffer [45]. The cells were then incubated for 2 min (A) or 5 min (B) in the presence or absence of the indicated stimuli. The cells were homogenized by sonication in a Ca^{2+} chelator containing buffer and the microsomal fraction prepared as described in detail previously [22]. PKC activity was measured after partial purification of the enzyme by polyacrylamide gel electrophoresis [22]. The results are given as % control and are the mean ± SE of the number of observations indicated in parentheses.

fraction. The subcellular redistribution of PKC is considered as an index of activation of the enzyme. Such redistribution was not observed when either islets [28,39] or the insulin secreting cell line HIT [40] were incubated in the presence of stimulatory concentrations of glucose.

There are several possible explanations for these results: first, the DAG formed by de novo synthesis contributes only to a minor extent to the total DAG mass [25], and is therefore insufficient to activate the enzyme. Second, the DAG produced from the glycolytic intermediates has a different location in the cell (not at the plasma membrane) compared to that formed by PIP_2 hydrolysis and can therefore not interact with the enzyme. Third, the DAG species originating from de novo synthesis and the PIP_2 turnover are distinct in their fatty acid composition [24,41] and have different efficacy of PKC activation [40,42]. Thus, several studies have concluded that PKC is not involved in glucose stimulated insulin secretion. However, some questions still remain. It is, for instance, not known, at present, if the association of PKC with the membrane is an absolute requirement for the activation of this enzyme. Indeed conditions can be found where a cell-permeable DAG analog, 1-oleyl-2-acetylglycerol (OAG), while causing maximal secretion, did not increase the amount of PKC associated with the membrane (Table I). OAG at 50 μg/ml stimulates insulin release from 19 $\pm$ 3 (mean $\pm$ SE, $n = 10$) to 39 $\pm$ 4 ng/10^6 cells ($n = 12$) and increases the amount of PKC recovered in the membrane fraction. However, OAG at 5 μg/ml while enhancing secretion to the same extent as 50 μg/ml (36 $\pm$ 3 ng/10^{16} cells, $n = 12$) causes no significant changes in PKC distribution (Table I). These results suggest that either OAG can affect insulin secretion without activating PKC via an unknown mechanism, or that PKC can be activated without detectable increase in the proportion of the enzyme associated with the membrane. A generally accepted model for the activation of PKC by DAG, in contrast to phorbol esters, proposes the formation of a quaternary complex between the enzyme, phospholipids, DAG and Ca^{2+} [43]. Ca^{2+} is thought to permit the priming of the enzyme by bringing it in close apposition to the membrane. Experimental evidence has confirmed this model in intact cells [36], permeabilized cells [22] and red cell ghosts [44]. In permeabilized RINm5F cells endogenous DAG was produced by stimulating PLC with a non-hydrolysable GTP analog [22]. While a similar amount of DAG was generated at basal (10^{-7} M) and at higher Ca^{2+} concentrations ($\geq 10^{-6}$ M), the translocation of PKC only occurred in the presence of 10^{-6} M Ca^{2+} or higher [22]. A corollary of these findings is shown in Table B where cytosolic Ca^{2+} was raised by depolarizing the cells with KCl [45]. KCl alone does not promote association of PKC with cellular membranes but permits such an association to occur after stimulation with OAG. This is seen both under conditions where OAG is inefficient (5 μg/ml) and with the higher concentration (50 μg/ml) where OAG itself causes translocation. These results clearly demonstrate that an increase in cytosolic Ca^{2+} favours the DAG induced activation of PKC. In view of these findings it is conceivable that glucose, which raises cytosolic Ca^{2+} ($[Ca)^{2+}]_i$) both in the normal β-cell [46] and in HIT cells [40,47], primes PKC to a location in the proximity of the membrane. This hypothesis was tested by performing phorbol ester binding experiments on intact HIT cells. This approach has been shown to be useful for the monitoring of Ca^{2+} effects on PKC in astrocytoma cells [48]. The results of experiments in which [^{3}H]phorbol dibutyrate (PDBU) binding was measured after short exposure of insulin secreting HIT cells to the secretagogues glucose, bombesin and KCl are illustrated in Fig. 1. Glucose, like KCl, raises $[Ca^{2+}]_i$ by depolarizing the cells and promoting Ca^{2+} influx without generating sizeble amounts of DAG in the plasma membrane [25,40]. The $[Ca^{2+}]_i$ elevation in the absence of increased DAG production provides an increased number of PDBU binding sites on membrane-associated PKC. Bombesin stimulation also causes a $[Ca^{2+}]_i$ rise [9], but simultaneously generates DAG in the plasma membrane [9,40] which competes with PDBU for binding to PKC. This would explain why bombesin, in contrast to KCl and glucose, does not augment the PDBU binding (Fig. 1). This conclusion agrees with the findings of Trilivas and Brown, who observed that carbachol stimulated PDBU binding depends on the rise of $[Ca^{2+}]_i$ and indeed decreased at a

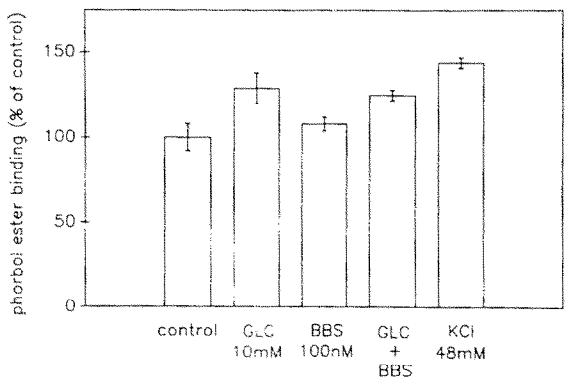

Fig. 1. Effect of glucose, bombesin and KCl on phorbol ester binding to intact HIT cells, HIT-T15 cells [40,47] were incubated for 3 h in spinner culture, washed twice in a modified Krebs-Ringer bicarbonate buffer [45] without glucose and preincubated in the same buffer at a concentration of about 4 $\times$ 10^6 cells/ml for 30 min. 20 nM [^{3}H]-phorbol dibutyrate (0.8 μCi/ml) was then added and the cells incubated for 10 min at 37C. The stimuli were present during the last minutes of incubation (1 min for bombesin and KCl; 3 min for glucose). The cell suspensions were transferred to GF/C microfibre filters and the latter rinsed 4-fold with ice-cold Krebs-Ringer buffer without glucose before counting. The results are expressed as % of control and are the mean $\pm$ SE of two independent experiments performed in triplicate. $P < 0.05$ for all conditions except for bombesin alone.

time when the muscarinic agonist increased the production of DAG [48]. Another method for the demonstration of PKC redistribution involves the localization of the enzyme by immunocytochemistry. Using this method, in leukemic cells the Ca^{2+} ionophore ionomycin induced the membrane association of PKC [49]. This association was not chelator-stable as assessed after cell fractionation [49]. Thus these findings with ionomycin involving the two approaches are similar to our results with glucose and KCl examining PDBU binding and chelator-stable membrane association of PKC.

6. POTENTIAL ROLE FOR PKC IN INSULIN SECRETION

In insulin secreting cells, as in other cell types, PKC appears to undergo activation in two stages. Under resting conditions the enzyme is cytosolic or only loosely attached to the plasma membrane. An increase in $[Ca^{2+}]_i$ converts the enzyme to a preactivated, prime state by association with the membrane (round symbol in Fig. 2). This stage can only be detected in intact cells (phorbol ester binding or immunohistochemistry) since the membrane association does not withstand subcellular fractionation in the presence of cation chelators. The third, membrane-intercalated stage of the enzyme requires increased production of DAG in the membrane. This chelator stable membrane association is observed when the cells are stimulated with Ca^{2+} mobilizing agonists such as carbachol and bombesin which stimulate DAG production from membrane phospholipids (Fig. 2, upper panel) [24,40,41]. In contrast, glucose and other fuel stimuli only generate small amounts of DAG in the plasma membrane following the limited activation of PLC secondary to the $[Ca^{2+}]_i$ rise [20] (not included in Fig. 2). Therefore, glucose is not expected to cause significant intercalation of PKC into the membrane agreeing with the experimental observations [28,39,40]. However, glucose and depolarizing concentrations of KCl appear to favour Ca^{2+}-mediated membrane association of the enzyme (Fig. 2, lower panel). In this case, the $[Ca^{2+}]_i$ rise is due to Ca^{2+} influx through voltage-gated channels. These channels are gated following the closure of ATP-sensitive K^+ channels by glucose, other carbohydrates and amino acids [15].

In summary, it is most unlikely that PKC plays a major role in mediating the action of glucose and other fuel stimuli on insulin secretion. At present other Ca^{2+}-sensitive enzymes, including protein kinases and phosphoprotein phosphatases, as well as other coupling facors such as acyl-CoA derivatives [50] are more likely to initiate the secretion. The main function attributed to PKC is the mediation of part or all of the effects of Ca^{2+} mobilizing hormones and neurotransmitters which are known to potentiate fuel-induced insulin

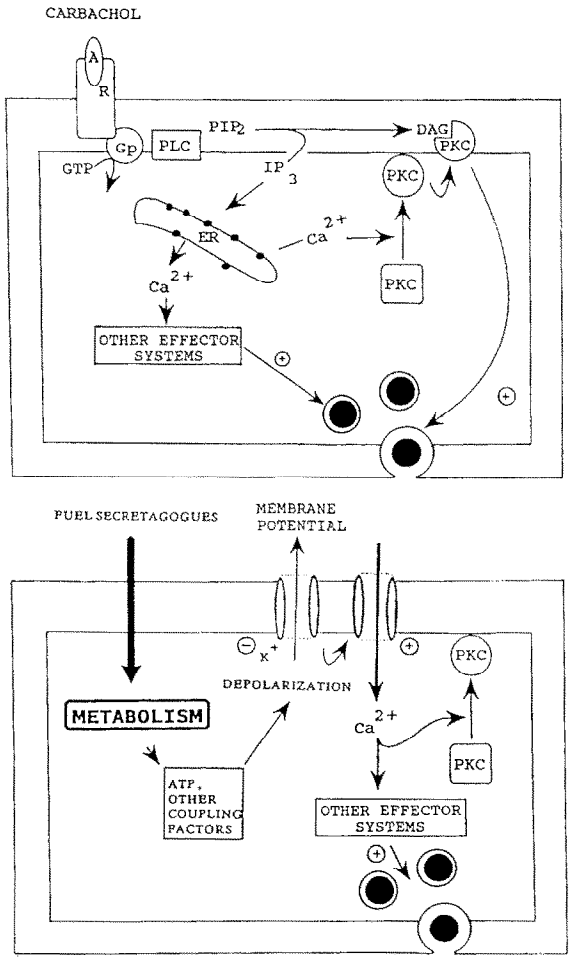

Fig. 2. Proposed mechanisms by which receptor agonists and fuel secretagogues affect protein kinase C insulin secretion. Protein kinase (PKC) undergoes a Ca^{2+}-dependent shift to the plasma membrane. Membrane intercalation of the enzyme requires an increase of the concentration of diacylglycerol (DAG) in the plasma membrane. A, agonist; R, receptor; G_p, phospholipase C (PLC) activating G-protein; PIP_2, phosphatidyl inositol-4,5-biphosphate; IP_3, inositol-1,4,5-trisphosphate; ER, endoplasmic reticulum.

secretion [2]. The synergistic action of glucose and muscarinic agonists, for example, could, at least in part, be due to the Ca^{2+}-mediated membrane apposition of PKC after membrane depolarization. Thus, despite extensive work, the detailed analysis of the nature of the involvement of PKC in insulin secretion remains to be established and probably requires new approaches.

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