Muscarinic activation of Ca^{2+}/calmodulin-dependent protein kinase II in pancreatic islets

Temporal dissociation of kinase activation and insulin secretion

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We have demonstrated previously that glucose activates the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) in isolated rat pancreatic islets in a manner consistent with a role of this enzyme in the regulation of insulin secretion [Wenham, Landt and Easom (1994) J. Biol. Chem. **269**, 4947–4952]. In the current study, the muscarinic agonist, carbachol, has been shown to induce the conversion of CaM kinase II into a Ca^{2+}-independent, autonomous form indicative of its activation. Maximal activation (2-fold) was achieved by 15 s, followed by a rapid return to basal levels by 1 min. This response was primarily the result of the mobilization of Ca^{2+} from intracellular stores since it was not affected by a concentration (20 μM) of verapamil that completely prevented the activation of CaM kinase II by glucose. Surprisingly, carbachol added prior to, or simultaneously with, glucose attenuated nutrient activation of CaM kinase II. This effect was mimicked by cholecystokinin-8 (CCK-8) and thapsigargin, suggesting its mediation by phospholipase C and the mobilization of intracellular Ca^{2+}. In contrast, carbachol, CCK-8 and thapsigargin markedly potentiated glucose (12 mM)-induced insulin secretion. These results suggest that CaM kinase II activation can be temporally dissociated from insulin secretion but do not exclude the potential dependence of insulin exocytosis on CaM kinase II-mediated protein phosphorylation.

INTRODUCTION

An increased intracellular Ca^{2+} concentration is a critical signal in the regulation of insulin secretion from the pancreatic β-cell in response to D-glucose and is sufficient to initiate insulin secretion from permeabilized islets [1] or insulinoma cells [2,3]. The Ca^{2+}-dependent events that mediate exocytosis are not fully understood but probably encompass the activation of Ca^{2+}-dependent protein kinases [4–6]. Among those implicated in the distal steps of insulin secretion is the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II).

CaM kinase II is constituted by a family of four isoform classes (α, β, γ and δ) which are encoded by distinct genes that are differentially expressed in nervous and peripheral tissues [7]. Its multifunctional property to selectively phosphorylate a large number of substrates *in vitro* [7,8] predicts that this enzyme plays a regulatory role in a diverse array of cellular processes in response to an elevation in intracellular Ca^{2+}. These processes include secretory events and the best evidence for this *in situ* is provided by the demonstration that CaM kinase II-mediated phosphorylation of synapsin I accelerates neurotransmitter release from presynaptic nerve terminals in the hippocampus [9]. CaM kinase II also probably regulates neurotransmitter synthesis, and post-synaptic responsiveness via the phosphorylation of tyrosine hydroxylase [10] and the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors [11], respectively.

Enzymic activity characteristic of CaM kinase II has been detected in isolated islets [12,13] and cultured β-cells [14] and there is evidence that this activity is associated with the cytoskeleton [5]. Recently, molecular cloning has shown that the β-cell expresses a novel β_3 isoform that contains a proline-rich tandem repeat in the association domain [15]. A functional role of this enzyme in insulin secretion is supported by the demonstration that glucose activates CaM kinase II in a concentration-dependent manner that correlates with insulin secretion [12]. This conclusion is, however, challenged by pharmacological studies using the putative inhibitor of CaM kinase II, KN-62 [16]. Thus while KN-62 was proven capable of inhibiting nutrient-induced insulin secretion from isolated rat islets [17,18] and insulinoma (HIT) cells [19], this inhibitor failed to inhibit Ca^{2+}-induced insulin secretion from electropermeabilized β-cells (HIT) [19]. This result is not consistent with a mediatory role of CaM kinase II in Ca^{2+}-stimulated insulin secretion and has questioned the relevance of glucose-induced activation of CaM kinase II to the secretory process.

The implication that CaM kinase II is activated in glucose-stimulated islets for no purpose is unpalatable with respect to the energy economy of the β-cell. This study was therefore initiated to determine whether another insulin secretagogue that increases cytosolic Ca^{2+} in the β-cell, namely the muscarinic agonist carbachol, activates CaM kinase II in isolated pancreatic islets. Furthermore, in view of the established ability of muscarinic agonists to modulate glucose-induced insulin secretion [20–22] it was of interest to determine whether this response was associated with a similar modulation of the activation state of CaM kinase II induced by glucose.

EXPERIMENTAL

Materials

Male Wistar rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN, U.S.A.) and maintained on Tekland Rodent Diet (Indianapolis, IN, U.S.A.) *ad libitum* for 7–10 days prior to use. CMRL-1066, glutamine, streptomycin and fetal bovine serum were purchased from Life Technologies (Gaithersburg, MD, U.S.A.), and Hanks' Balanced Salt Solution (HBSS) was from Whittaker Bioproducts (Walkersville, MD, U.S.A.). Ficoll,

Abbreviations used: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; CCK-8, cholecystokinin-8; PMA, phorbol 12-myristate 13-acetate.

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carbamylcholine (carbachol), ATP (disodium salt), atropine sulphate, leupeptin, bombesin and cholecystokinin-8 (CCK-8) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase P was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and glucose (Dextrose) was from the National Bureau of Standards (Gaithersburg, MD, U.S.A.). [γ-^{32}P]ATP was purchased from NEN Research Chemicals (DuPont, Boston, MA, U.S.A.). Autocamtide-2, sequence KKALRRQETVDAL [23], was synthesized by Bio-Synthesis, Inc. (Lewisville, TX, U.S.A.). All other chemicals were of the finest reagent grade available.

Isolation of islets

Pancreatic islets were isolated from male rats by collagenase P digestion and subsequent enrichment by centrifugation on a Ficoll gradient as described previously [24,25]. Following isolation, islets were cultured in CMRL-1066 containing 5.5 mM glucose and supplemented with 2 mM L-glutamine, 10 % (v/v) heat-inactivated fetal bovine serum, 100 units/ml streptomycin and 100 μg/ml penicillin overnight at 24 °C under an atmosphere of 95 % air/5 % CO_2. Immediately before experimentation, the islets were incubated at 37 °C for a minimum of 60 min.

Assay of CaM kinase II activity

CaM kinase II activity was assayed using the determination of ^{32}P incorporation into an exogenously added selective peptide substrate, autocamtide-2, by a method described previously [12]. Briefly, islets (300–400/tube) were then preincubated in 500 μl of KRB basal medium (25 mM Hepes, pH 7.4/115 mM NaCl/24 mM $NaHCO_3$/5 mM KCl/2.5 mM $CaCl_2$/1 mM $MgCl_2$ containing 3 mM glucose and 0.1 % BSA) for 15 min at 37 °C followed by incubations in the presence of the test secretagogue for the indicated times. The incubations were terminated by sonication in ice-cold buffer (20 mM Tris/HCl, pH 7.5, 0.5 mM EGTA, 1.0 mM EDTA, 2.0 mM dithiothreitol, 10 mM sodium pyrophosphate, 0.4 mM ammonium molybdate, 100 μg/ml leupeptin, 75 μl; 10 pulses, setting 3, 30 % duty cycle). CaM kinase II activity was assayed in a reaction mixture containing 50 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 0.1 mg/ml BSA (fraction V), 10 μM autocamtide-2, 20 μM ATP (specific radioactivity, 40 Ci/mmol), and either 0.5 mM $CaCl_2$/5 μg/ml calmodulin for Ca^{2+}-stimulated activity or 0.9 mM EGTA for Ca^{2+}-independent activity. The total reaction volume was 50 μl. The assay was initiated by the addition of 10 μl of islet homogenate and continued for 30 s at 30 °C before termination by the addition of ice-cold trichloroacetic acid (25 μl, 15 %). After centrifugation (12000 ***g*** for 1 min), 35 μl of the resulting supernatant was spotted on to strips (3 cm × 0.5 cm) of phosphocellulose paper (Whatman P81), washed five times in 500 ml of distilled water and dried at 110 °C for 15 min. [^{32}P]P_i incorporation into autocamtide-2 was determined by Čerenkov radiation (Beckman). In the described experiments, [^{32}P]P_i incorporation into autocamtide-2 in the absence of Ca^{2+}/calmodulin (autonomous CaM kinase II activity) is expressed as a percentage of incorporation in the presence of these cofactors (Ca^{2+}-dependent CaM kinase II activity).

Insulin secretion

For static secretion experiments, islets were counted (20 per tube) in 12 mm × 75 mm siliconized borosilicate tubes and preincubated for 30 min at 37 °C with gentle shaking in KRB basal medium containing 3 mM glucose and 0.1 % BSA (200 μl) under an atmosphere of oxygen/CO_2 (95/5 %). The medium was replaced with fresh KRB basal medium alone or supplemented with stimulatory concentrations of glucose in the absence and presence of modulatory agonists (carbachol, CCK-8, bombesin and thapsigargin) and the incubation continued for a further 30 min. The incubation was terminated by the removal of the medium. Insulin content of incubation media was determined by a double-antibody radioimmunoassay [26].

Statistical treatment of data

Data are presented as means ± S.E.M. determined from at least three independent observations unless otherwise stated. Statistical significance was assessed by Student's *t*-test.

RESULTS

On activation by Ca^{2+}/calmodulin, CaM kinase II undergoes rapid autophosphorylation and conversion into an autonomous, Ca^{2+}-independent kinase form [27] which can be detected using a selective peptide substrate, autocamtide-2 [23]. This property has been followed as an indicator of enzyme activation in numerous cell types [7] including glucose-stimulated isolated rat islets [12]. In the current study, a maximally effective concentration of glucose (28 mM) was demonstrated to similarly activate CaM kinase II, as indicated by an increased proportion of the enzyme in an autonomous, Ca^{2+}-independent form (17.05 ± 2.89 % versus 4.57 ± 1.51 %; 28 mM versus 3 mM glucose, respectively). This activation of CaM kinase II was totally prevented by the co-incubation of isolated islets with a selective inhibitor of the voltage-sensitive, L-type Ca^{2+} channel, verapamil (20 μM); under these conditions (i.e. 28 mM glucose in the presence of verapamil) the proportion of enzyme in an autonomous form was 3.47 ± 1.15 %. The inclusion of 20 μM verapamil had no significant effect on CaM kinase II activation state in basal KRB medium containing 3 mM glucose. These data provide evidence that glucose-induced activation of CaM kinase II is mediated via the promotion of Ca^{2+} influx through L-type Ca^{2+} channels.

To further evaluate whether the mobilization of Ca^{2+} from intracellular stores was capable of activating CaM kinase II, pancreatic islets were stimulated with carbachol, a stable analogue of the neurotransmitter, acetylcholine. This muscarinic agonist has been shown previously to promote, in pancreatic β-cells, phospholipase C-mediated hydrolysis of membrane phosphoinositides to generate Ins(1,4,5)P_3 [28,29] and diacylglycerol [29a]. Ins(1,4,5)P_3 has been demonstrated to mobilize Ca^{2+} from the endoplasmic reticulum in permeabilized islets [30] or β-cells [31,32] and diacylglycerol has been shown to activate Ca^{2+}/phospholipid-dependent protein kinase C [21,33]. As demonstrated in Figure 1, carbachol (500 μM) in the presence of basal concentrations of glucose induced a rapid activation of CaM kinase II. At the earliest time point measurable, 15 s, the percentage of enzyme in an autonomous form was determined to be 8.82 ± 0.66 %, significantly greater than that of islets incubated in 3 mM glucose alone (4.23 ± 0.21 %, $P < 0.001$). As the stimulation period was extended the activation state of CaM kinase II rapidly declined such that by 2.5 min the activation state had returned to pre-stimulation levels (4.49 ± 0.92 %). Carbachol (500 μM)-induced activation of CaM kinase II at 15 s was abolished by the co-incubation of the muscarinic receptor antagonist, atropine (10 μM)(results not shown). Furthermore, carbachol-induced CaM kinase II activation at 15 s was not significantly suppressed by verapamil added to both pre-incubation and incubation media; the proportion of enzyme in an

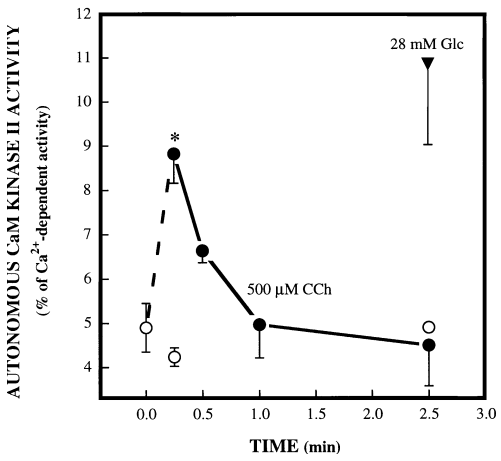

Figure 1 Carbachol (CCh) transiently activates CaM kinase II in isolated rat islets

Islets (300–400/tube) were incubated with 3 mM glucose (○), 3 mM glucose supplemented with 500 μM CCh (●) or 28 mM glucose (Glc, ▼) at 37 °C for the times indicated. Autonomous CaM kinase II activity was determined as described in the Experimental section and expressed as a percentage of Ca^{2+}-dependent CaM kinase II activity. *, $P < 0.001$, independent *t*-test.

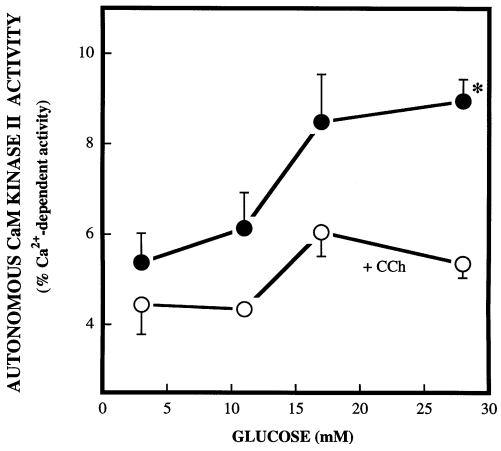

Figure 2 Carbachol (CCh) suppression of CaM kinase II activation is independent of glucose concentration

Islets (300–400/tube) were incubated at 37 °C with increasing concentrations of glucose (3–28 mM) in the absence and presence of 500 μM CCh. After 2.5 min, islets were homogenized and CaM kinase II activity determined as described in the Experimental section. (*: $P < 0.005$ and $P < 0.0005$ versus 3 mM glucose and 28 mM glucose plus CCh, respectively).

autonomous form was determined to be 4.18 ± 0.21 % in control conditions (3 mM glucose) and 9.30 ± 2.30 % and 10.3 ± 0.22 % in carbachol-stimulated islets in the absence and presence of verapamil (20 μM), respectively ($n = 2$, mean $\pm$ S.D.). These observations suggested that activation of carbachol was probably mediated via the mobilization of Ca^{2+} from intracellular stores.

Since carbachol has been demonstrated to potentiate glucose-induced insulin secretion [22,34] it was of interest to determine whether this effect was accompanied by a potentiation of CaM kinase II activation. However, as shown in Table 1, the co-incubation of islets with stimulatory concentrations of glucose (28 mM) and carbachol (500 μM) for 2.5 min resulted in the suppression of CaM kinase activation relative to glucose alone. Glucose (28 mM) induced the 2.4-fold increase in the proportion of CaM kinase II in the autonomous form. In the presence of carbachol, no activation of CaM kinase II was evident in response to stimulatory concentrations of glucose (28 mM). This effect of carbachol in suppressing glucose-induced activation of CaM kinase II was characterized further by evaluating the relationship to glucose concentration. Isolated islets were incubated for 2.5 min with increasing concentrations of glucose in the absence and presence of 500 μM carbachol. Glucose induced the dose-dependent activation of CaM kinase II with a sigmoidal-like relationship similar to that reported previously [12] (Figure 2). At each of the concentrations studied (3, 11, 17 and 28 mM) glucose-induced activation of CaM kinase II was markedly suppressed by the presence of carbachol (Figure 2). Furthermore, the pre-incubation of islets with carbachol for 5 min prior to the addition of a stimulatory concentration (28 mM) of glucose produced a similar suppression to that observed when glucose and carbachol were added simultaneously (Table 1). No activation by glucose was apparent at earlier times (i.e. 30 s and 1 min, results not shown) eliminating the possibility that a transient and rapid activation of CaM kinase II by glucose had occurred and returned to a basal level by 2.5 min. These observations therefore demonstrated that the stimulation of the β-cell by carbachol, either prior to, or simultaneously with, a glucose stimulation resulted in the suppression of CaM kinase II activation by the sugar.

To test the hypothesis that this response was mediated by the activation of phospholipase C, the ability of other agonists of this enzyme (i.e. CCK-8 [35,36] and bombesin [37]) to mimic

Table 1 Carbachol (CCh) suppresses glucose-induced activation of CaM kinase II in isolated rat islets

Islets (300–400 per tube) were preincubated in basal KRB (3 mM glucose) with or without CCh (500 μM) for 5 min prior to exposure to KRB containing 3 mM glucose, 28 mM glucose or 28 mM glucose in the presence of CCh. After 2.5 min, islets were homogenized and assayed for CaM kinase II activity. (* $P < 0.05$ versus 3 mM Glc; ** and ***, $P < 0.01$ and $P < 0.05$ versus 28 mM Glc, respectively).

Pre-incubation	Stimulation	Autonomous CaM kinase II activity (% of Ca^{2+}-dependent activity)
3 mM Glucose	3 mM Glucose	4.57 ± 1.51 (4)
3 mM Glucose	28 mM Glucose	10.85 ± 1.83* (4)
3 mM Glucose	28 mM Glucose + 500 μM CCh	4.83 ± 0.45** (5)
3 mM Glucose + 500 μM CCh	28 mM Glucose	4.24 ± 0.58*** (4)

Table 2 Reciprocal relationship of CaM kinase II activation and insulin secretion from isolated rat islets

Determination of autonomous CaM kinase II activity: islets were incubated in KRB medium containing 3 mM glucose (Control) or 28 mM glucose (Glucose) in the absence and presence of carbachol (500 μM), CCK-8 (100 nM), bombesin (BBS, 100 nM) or thapsigargin (2 μM) for 2.5 min at 37 °C. Insulin released: islets (20/tube) were incubated in KRB containing 3 mM glucose (Control) or 12 mM glucose (Glucose) in the absence and presence of carbachol, CCK-8, BBS or thapsigargin. When added alone, carbachol and thapsigargin were included in KRB basal medium containing 3 mM glucose. In experimental conditions utilizing thapsigargin, islets were pre-incubated for 10 min in the presence of the inhibitor prior to determination of enzyme activation or insulin secretion. (ND; not determined in this series of experiments).

Condition	Autonomous CaM kinase II activity (% of Ca^{2+}-dependent activity)	Insulin released (μ-units/islet per 30 min)
Control	4.73 ± 0.42 (4)	2.40 ± 0.34 (8)
500 μM Carbachol	ND	3.60 ± 0.28 (8)
Glucose	9.14 ± 0.25 (6)	8.22 ± 0.82 (8)
Glucose + 500 μM carbachol	5.25 ± 0.23 (4)	39.19 ± 5.57 (8)
Glucose + 100 nM CCK-8	5.32 ± 0.19 (4)	25.05 ± 4.19 (8)
Glucose + 100 nM BBS	8.37 ± 0.77 (4)	10.15 ± 2.87 (8)
2 μM Thapsigargin	ND	2.12 ± 0.32 (8)
Glucose + 2 μM thapsigargin	5.48 ± 0.69 (4)	27.16 ± 3.48 (8)

Table 3 Effects of phorbol ester on glucose-induced activation of CaM kinase II

Islets (300–400 per tube) were incubated for 15 min prior to exposure to KRB containing 3 mM glucose or 28 mM glucose in the absence or presence of PMA. After 2.5 min, islets were homogenized and assayed for CaM kinase II activity. For experimental conditions in which PMA was included, the phorbol ester was also added to the pre-incubation medium. (* $P < 0.0005$ versus 3 mM glucose; NS, not significant versus 28 mM glucose).

Condition	Autonomous CaM kinase II activity (% of Ca^{2+}-dependent activity)
3 mM Glucose	4.73 ± 0.42
3 mM Glucose + 100 nM PMA	5.89 ± 0.65
28 mM Glucose	$9.06 \pm 0.59^{*}$
28 mM Glucose + 100 nM PMA	7.31 ± 0.61^{NS}

carbachol were evaluated. As demonstrated in Table 2, the co-exposure of islets to glucose and CCK-8 (100 nM) resulted in the prevention of glucose-induced activation of CaM kinase II that was equivalent to the effect of carbachol. These results suggested that the activation of a phospholipase C pathway by carbachol 'shielded' CaM kinase II from activation by Ca^{2+} influx promoted by glucose. In secretion studies, carbachol or CCK-8 demonstrated similar abilities to potentiate insulin secretion induced by a submaximal glucose concentration (12 mM, 4.78- and 3.05-fold, for carbachol and CCK-8, respectively, Table 2) as previously reported [36,37]. Interestingly, and in contrast, bombesin failed to suppress glucose-induced activation of CaM kinase II and also had no effect on glucose-induced insulin secretion (Table 2).

The mechanism by which carbachol suppressed glucose-induced activation of CaM kinase II was reasoned to be the result of the activation of protein kinase C, the mobilization of intracellular Ca^{2+} pools or another undefined action on the β-cell. Carbachol has been shown to activate protein kinase C in isolated islets [21,38]. Therefore to evaluate the potential involvement of protein kinase C, islets were preincubated in the presence of a potent activator of this enzyme, phorbol ester [phorbol 12-myristate 13-acetate (PMA), 100 nM] [39] for 15 min and then exposed to basal and stimulatory concentrations of glucose for 2.5 min. As demonstrated in Table 3, PMA had no significant effect on basal levels of autonomous CaM kinase II activity in islets and only modestly suppressed glucose-induced activation of CaM kinase II. By contrast, an inhibitor of the endoplasmic reticulum Ca^{2+}/ATPase, thapsigargin [40], completely suppressed glucose-induced activation of CaM kinase II in a manner that closely mimicked carbachol and CCK-8 (Table 2). Furthermore, thapsigargin also markedly potentiated glucose-induced insulin secretion (Table 2). Thapsigargin is known to initially promote an increased intracellular concentration of Ca^{2+} [41] and therefore the close correlation of these effects with those of carbachol suggest that it was the mobilization of Ca^{2+} from the intracellular pools that was responsible for the prevention of glucose-induced activation of CaM kinase II, rather than the activation of protein kinase C.

DISCUSSION

CaM kinase II has emerged as a prominent regulator of cellular responses to an elevation in intracellular Ca^{2+} and is predicted to play a role in a diverse array of cellular functions that range from the regulation of ion fluxes through the plasma membrane to gene expression events in the nucleus [7]. That this enzyme may play a role in insulin secretion is supported by the previous demonstration that CaM kinase II is activated in both islets [12] and RINm5F cells [14] by insulin secretagogues. In the former study, the maximal activation of CaM kinase II induced by glucose was achieved at 2.5 min and mimicked by depolarizing concentrations of K^{+}, suggesting that it was mediated by the activation of Ca^{2+} influx. This was confirmed in the current study by the observation that glucose-induced activation of CaM kinase II was completely prevented by concentrations of verapamil known to inactivate β-cell L-type Ca^{2+} channels [42].

Novel to this study, however, was the demonstration that the stimulation of isolated rat islets with the muscarinic agonist, carbachol, also activates CaM kinase II. This activation contrasted with that induced by glucose since it was rapid (i.e. maximal by 15 s or sooner) and characterized by a sharp decline to basal levels by 1 min. As such, this temporal profile closely reflects that of a carbachol (or acetylcholine)-induced increase in cytosolic Ca^{2+} concentrations in islets [43,44] or individual β-cells [45,46] and is consistent with receptor-mediated modulations in Ca^{2+} homoeostasis. Indeed, this profile closely corresponded to the activation of CaM kinase II induced by bradykinin in PC-12 cells which is mediated by the stimulation of membrane phospho-

inositide hydrolysis [47]. While still a matter of debate, the elevation of cytosolic Ca^{2+} induced by high concentrations of carbachol, such as used in the current study (i.e. $> 100\ \mu M$), is thought to be the consequence of both the mobilization of Ca^{2+} from intracellular stores as well as influx from the extracellular medium [43]. The activation of CaM kinase II induced by carbachol, however, was predominantly the result of the mobilization of intracellular Ca^{2+} since it was not significantly affected by verapamil.

The most intriguing observation from this study was that carbachol prevented the activation of CaM kinase II by glucose. This effect was independent of whether carbachol was added prior to, or simultaneously with, glucose and was probably the result of the activation of phospholipase C since it was mimicked closely by CCK-8. (The observed inability of bombesin to mimic this effect of carbachol and CCK-8 is not totally understood but predicted to be the result of insufficient expression of specific receptors on the β-cell.) These results suggest either: (i) that the muscarinic stimulation of phospholipase C compromised the pool of Ca^{2+} required for the activation of CaM kinase II by glucose; or (ii) that the rapid activation of CaM kinase II by carbachol resulted in an enzymic form that was refractory to the subsequent Ca^{2+} influx induced by glucose. Such a mechanism is feasible since *in vitro* experiments have demonstrated that autonomous CaM kinase II is capable of catalysing Ca^{2+}-independent phosphorylation of threonine residues (Thr-305/306) in the calmodulin-binding domain of the enzyme, rendering it insensitive to further activation by Ca^{2+}/calmodulin [48,49]. The involvement of this mechanism is not, however, supported in the current study since no significant loss of Ca^{2+}-dependent phosphorylation of autocamtide-2 was detected in homogenates of carbachol- or glucose-stimulated islets. In fact, the autophosphorylation of CaM kinase II at these sites has yet to be demonstrated in any cell type *in situ*.

In the presence of stimulatory concentrations of glucose, muscarinic agonists have been reported to increase cytosolic Ca^{2+} [43,50], modulate oscillatory patterns [43] or have no effect [51] in single-cell or islet models. A systematic dissection of the effects of acetylcholine stimulation of intact mouse islets in the presence of stimulatory concentrations of glucose (15 mM) has revealed differential, concentration-dependent effects on initial and steady-state cytosolic Ca^{2+} concentration [43]. Of particular interest here is that high concentrations (i.e. 100 μM) of acetylcholine markedly reduced the steady-state level of cytosolic Ca^{2+} as a consequence of the attenuation and acceleration of Ca^{2+} influx and efflux, respectively. Given that islet CaM kinase II has a low affinity for Ca^{2+}/calmodulin (~ 300 nM) [52] relative to other Ca^{2+}-activated kinases (e.g. myosin light chain kinase, ~ 1 nM) [53], such a response under the current experimental conditions could conceivably reduce cytosolic Ca^{2+} to a level insufficient to maintain CaM kinase II in an activated state. This could be achieved, for example, by the phosphorylation and negative modulation of the L-type Ca^{2+} channel by carbachol-activated CaM kinase II. While there is some evidence that the α1-subunit of Ca^{2+} channels may be phosphorylated by CaM kinase II in non-β-cell types [54] and consensus sites exist on the α-subunit of the β-cell channel [55], there is as yet no evidence for the functional modulation of channel activity by this enzyme.

A consequence of carbachol stimulation of intact islets is the production of diacylglycerol [29a,57] and the activation of protein kinase C [21,58]. Phorbol ester activators of this enzyme have been shown to negatively modulate CaM kinase II activation following K^+-induced membrane depolarization in PC12 cells as a result of the suppression of Ca^{2+} influx [59]. Phorbol esters may have similar effects on Ca^{2+}-influx in insulinoma cells [60], although the impact of protein kinase C [46,61] or diacylglycerol itself [62] on Ca^{2+} channel function in β-cells is controversial. However, the possibility that diacylglycerol and/or activation of protein kinase C mediate carbachol attenuation of glucose activation of CaM kinase II are not entirely supported in the current study, since it was not accurately mimicked by the phorbol ester, PMA. Furthermore, preliminary experiments have suggested that pharmacological intervention of protein kinase C by bisindoylmaleimide did not compromise the effect of carbachol (results not shown). In contrast to the phorbol ester, thapsigargin produced an effect on glucose-induced activation of CaM kinase II and insulin secretion that was essentially identical to carbachol. Because this inhibitor of endoplasmic reticulum Ca^{2+}/ATPase initially invokes a mobilization of intracellular Ca^{2+} stores in isolated islets followed [41] by their depletion, these results suggest that the inactivation of CaM kinase II induced by phospholipase C agonists is the result of the elevation of cytosolic Ca^{2+} concentrations or the depletion of intracellular Ca^{2+} stores. The precise mechanism involved is not clear, although there is evidence for communication between the extent of depletion/repletion of intracellular stores and membrane potential within the β-cell [63].

A primary objective of this study was to evaluate the role of CaM kinase II in regulation of insulin secretion. In this respect, this study suggests that there may not be a direct temporal correlation between insulin secretion and CaM kinase II activation, but rather that conditions exist where these two parameters can be dissociated. Thus, insulin secretion was potentiated by carbachol, CCK-8 and thapsigargin in conditions similar to those in which glucose activation of CaM kinase II was suppressed. The pre-exposure of islets to carbachol [20] or CCK-8 [46] has previously been shown to result in a substantial increase in insulin secretion induced by a subsequent glucose stimulation. Superficially, these results suggest that insulin secretion is not absolutely dependent on the momentary presence of activated CaM kinase II or, alternatively, that this enzyme may play a negative role to restrain insulin secretion. It is considered unlikely that the activation of CaM kinase II contributes to the potentiation of insulin secretion by carbachol previously ascribed to combinatory effects on Ca^{2+} homoeostasis and the protein kinase C-mediated sensitization of the secretory machinery to Ca^{2+} [22,44,64]. These results do not, however, eliminate the possibility that CaM kinase II plays an important positive role in the secretory process of the β-cell. Regardless of whether carbachol is added prior to or simultaneously with glucose, it is possible that a transient muscarinic activation of CaM kinase II is sufficient to initiate insulin secretion and/or facilitate this process in the presence of glucose even after the return of the enzyme to basal autonomy/activation levels. Such a function could be mediated by the phosphorylation of a single or multiple β-cell protein(s) that play(s) a key 'gating' role in the exocytotic process. The proteins targeted by CaM kinase II in the β-cell are, however, unidentified.

In summary, this study has demonstrated that CaM kinase II is activated in isolated rat islets by carbachol-induced mobilization of intracellular Ca^{2+} stores as well as glucose-induced influx of Ca^{2+} from the extracellular medium. This property places CaM kinase II in a central position in the β-cell to be able to co-ordinate the cellular response to multiple signals that may result from nutrient and receptor stimulation of this cell and further implies a central role for CaM kinase II in β-cell function. The existing controversy surrounding the postulated role of CaM kinase II in insulin secretion is not totally clarified by this study but a fundamental role of this enzyme in this process is by no means eliminated. Rather, this study raises fundamental

questions with respect to the temporal association of CaM kinase II activation and insulin secretion under physiological conditions represented by glucose in combination with incretins such as carbachol (acetylcholine), and experimental models are being developed to directly evaluate this.

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