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PRESENCE AND POSSIBLE INVOLVEMENT OF Ca/CALMODULIN-DEPENDENT PROTEIN KINASES IN INSULIN RELEASE FROM THE RAT PANCREATIC ß CELL

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ABSTRACT Roles of Ca/calmodulin-dependent protein kinase II (Ca/CaM kinase II) and myosin light chain kinase (MLCK) in insulin release from rat pancreatic islets were investigated. Western blotting using polyclonal antibody to Ca/CaM kinase II suggested the presence of this kinase in the pancreatic islets. Extracts of pancreatic islets phosphorylated exogenous myosin light chain, which was inhibited by ML-9, an inhibitor of MLCK. KN-62 and KN-93, inhibitors of Ca/CaM kinase II, and ML-9 at μM concentrations inhibited insulin release stimulated by glucose or high K^+. KN-62 and KN-93, but not ML-9, inhibited insulin release increased by glucose and forskolin, an activator of adenylate cyclase. These inhibitors had no effect on insulin release evoked by 12-*O*-tetradecanoyl phorbol-13-acetate, an activator of Ca^{2+}-sensitive, diacylglycerol-dependent protein kinase. These results suggest that Ca/CaM kinase II and MLCK may participate in the control of insulin release.

Calmodulin, one of the most well-characterized Ca^{2+}-binding proteins, exists in a wide variety of tissue such as the pancreatic ß-cell (1). Calmodulin was suggested to play a positive role in insulin release, since calmodulin inhibitors such as trifluoperazine or W-7 inhibited insulin release induced by glucose (1-3). The Ca^{2+}-binding protein is also considered to be involved in an increase in cAMP in

The abbreviations used here are: MLCK, myosin light chain kinase; Ca/CaM kinase II, Ca/calmodulin-dependent protein kinase II; protein kinase C, Ca^{2+}-sensitive, diacylglycerol-dependent protein kinase; protein kinase A, cAMP-dependent protein kinase; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; DMSO, dimethylsulphoxide; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis-(ß-aminoethyl ester)-N,N,N',N'-tetraacetic acid; KN-62, 1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; KN-93, 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzene sulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; ML-9, 1-(5-chloronaphtalenesulfonyl)-1-H-hexahydro-1,4-diazepine; PVDF, polyvinyldene difluoride.

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response to glucose via activating calmodulin-dependent adenylate cyclase (4). Binding of Ca^{2+} to calmodulin causes protein phosphorylation through several protein kinases in the brain (5). In this paper, the roles of two Ca/calmodulin-dependent protein kinases, Ca/calmodulin-dependent protein kinase II (Ca/CaM kinase II) and myosin light chain kinase (MLCK), in insulin release were investigated by the use of their specific inhibitors.

Materials and Methods

Materials

Collagenase (Type V), leupeptin and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) were purchased from Sigma (St. Louis, MO, U.S.A.). Forskolin was from Wako pure chemical (Osaka, Japan). Bovine serum albumin (fraction V) was from Iwai Kagaku (Tokyo, Japan). 1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 2-[N-(2-hydroxyethyl)-*N*-(4-methoxybenzene sulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) and 1-(5-chloronaphtalenesulfonyl)-1-H-hexahydro-1,4-diazepine (ML-9) were synthesized as in Refs (6,7,8), respectively. KN-62 and KN-93 were prepared in dimethylsulphoxide (DMSO) at a concentration of 10mM. TPA and ML-9 was dissolved in DMSO at 1.62mM and 50mM, respectively. Forskolin (10mM) was dissolved in ethanol. Polyvinyldene difluoride (PVDF) membrane was Imobilon P (0.45μm pore size) from Millipore, MA, USA. Horseradish peroxidase-linked anti-rabbit IgG was from Medical and Biological Lab., Nagoya, Japan. EIA kit Mitsui II used for insulin assay was from Mutsui Seiyaku, Tokyo, Japan. The other substances used here were of the purest grade.

Insulin release

Pancreatic islets were isolated from male Wistar rats (250-300g) fed *ad lib* using collagenase digestion. Groups of size-matched 5 islets were preincubated at 37°C for 1hr in 1ml of Hepes-buffered Krebs-Ringer solution containing (mM): NaCl 119, KCl 4.75, $NaHCO_3$ 5, $CaCl_2$ 2.54, $MgSO_4$ 1.2, KH_2PO_4 1.2 and Hepes 20 (pH 7.4 with NaOH). When the concentration of K^+ was heightened, Na^+ was reduced isoosmotically. After preincubation, the islets were incubated for either 30min or 1hr in various conditions with or without the inhibitors. 0.1% DMSO was added to the control. At the end of the incubation, the amount of insulin in the media was measured by enzyme-linked immunoassay using rat insulin as a standard. None of these substances used here affected the assay.

Immunoblot analysis

The polyclonal antibody to Ca/CaM kinase II was prepared as described previously (6) using synthetic polypeptides of rat brain Ca/CaM kinase II (amino acid residues 39-70). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to PVDF membranes in 48mM Tris, 39mM glycine, 1.3mM SDS, 20% (V/V) methanol (pH 9.2) at 15V for 30min. For immunodetection, procedure of Burnette (9) was followed, except that the second antibody was horseradish peroxidase-linked anti-rabbit IgG. Antigen-antibody complex was visualized by reacting the bound peroxidase with diaminobenzidine. Purified Ca/CaM kinase II from rat brain as described in (6) was used as a control.

MLCK activity in the soluble fraction from islet homogenates

MLCK activity was assayed as described in (10). Approx. 6000 pancreatic islets were sonicated on ice in a 1ml buffer containing 50mM Tris·HCl (pH 7.5), 2mM EGTA, 2mM dithiothreitol and 0.01% leupeptin. After spinning at 108,000g for 60min at 4°C, 20μl of the supernatant was incubated in 200μl (final volume) for 0.5-5 min at 30°C with 30μM [γ-^{32}P]ATP (250cpm/pmol), 0.4mg/ml chicken gizzard myosin light chain, 0.2mM $CaCl_2$, 0.4μg/ml calmodulin and ML-9 at concentrations shown in the figure. Proteins in the mixture were precipited and washed by 10% trichloroacetic acid. Radioactivity from [^{32}P]-phosphate incorporated into the precipitate was counted.When Ca^{2+} were omitted from the mixture, $CaCl_2$ and calmodulin were replaced with 2mM EGTA.

Statistical analysis

Statistical significance was assessed by unpaired Student's *t*-test.

Results

Immunological detection of Ca/CaM kinase II

In the immunoblot analysis with islet homogenates, a 50kD band was immunopositive with the polyclonal antibody to Ca/CaM kinase II. The antibody used here reacted with the two bands at 50kD and 60kD when Ca/CaM kinase II purified from rat brain was applied in parallel (Fig 1).

Inhibition by ML-9 of phosphorylation of exogenous myosin light chain by islet extracts

Fig.2 shows the effect of ML-9 on phosphorylation of chicken gizzard myosin light chain by the soluble fraction from islet homogenates. ML-9 (5-50μM) dose-dependently inhibited [^{32}P]-incorporation into chicken gizzard myosin light chain. IC_{50} for the inhibition assessed with the counts at 1min was approx. 22μM.

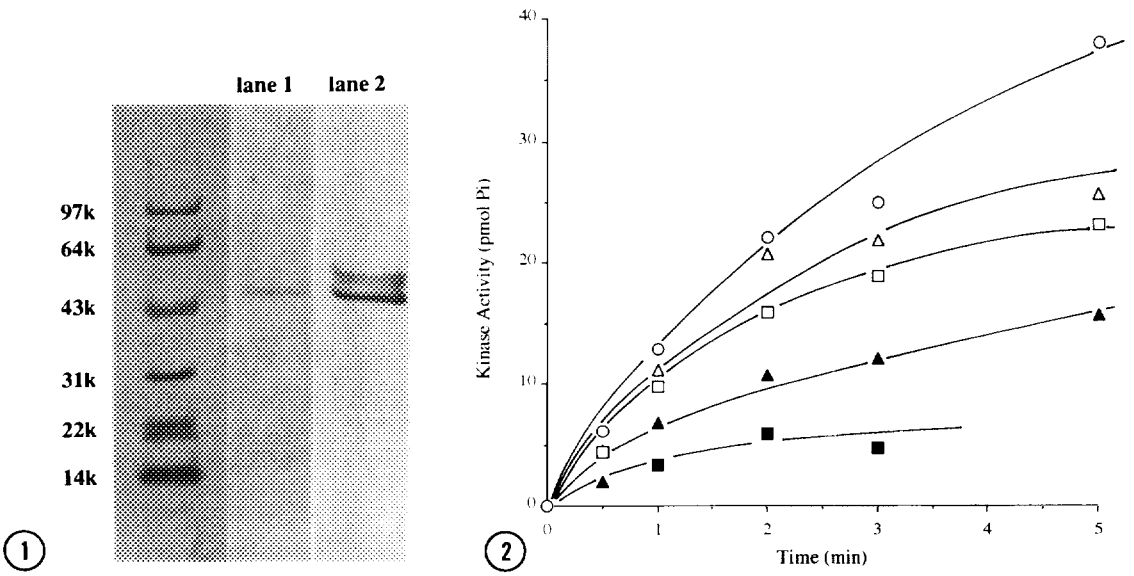

Fig 1. Immunoblot analysis of Ca/CaM kinase II in islets homogenates.

50 rat pancreatic islets were homogenized, separated by SDS-PAGE and blotted to PVDF membrane, followed by treatment with 2μg/ml antibody to rat brain Ca/CaM kinase II. The immuno-positive bands were visualized by peroxidase reaction (lane 1). Purified rat brain Ca/CaM kinase II was applied in parallel as a positive control (lane 2).

Fig 2. Inhibition by ML-9 of [^{32}P]-phosphate incorporation into exogenous myosin light chain by islet extracts.

The soluble fraction from islet extracts was incubated in the presence of Ca/calmodulin, [γ-^{32}P]ATP, chicken gizzard myosin light chain and ML-9 at 0 (O), 5 (Δ), 10 (□), 20 (▲) and 50μM (■). After precipitation using trichloroacetic acid, the radioactivity from [γ-^{32}P]-phosphate in the precipitate was counted.

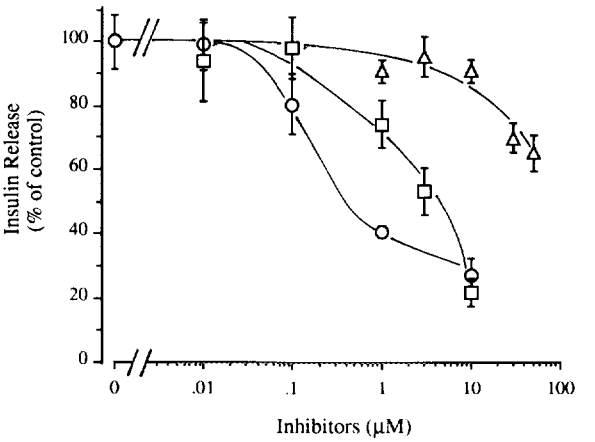

Fig 3. Dose-dependent inhibition by KN-62 (O), KN-93 (□) and ML-9 (Δ) of glucose-induced insulin release from rat pancreatic islets.

Groups of 5 isolated rat pancreatic islets were incubated at 37°C for 1hr in 1ml Hepes-buffered Krebs-Ringer solution with 10mM glucose in the presence or absence of various inhibitors as described above. Each value is the mean ± SEM for 4-8 observations and expressed as a percentage of the release in the absence of the inhibitors.

Effects of KN-62 , KN-93 and ML-9 on glucose-induced insulin release from rat pancreatic islets

As shown in Fig 3, insulin release induced by 10mM glucose was significantly inhibited by KN-62 over 1μM (11.8±1.48ng · 5 islets^{-1} · 1hr^{-1} for control vs 5.19±0.84ng · 5 islets^{-1} · 1hr^{-1} for 1μM KN-62, $p<0.01$, n=5). KN-93 also inhibited glucose-induced insulin release, whereas the threshold of KN-93 for the inhibition was between 1μM and 3μM (9.3±1.5ng · 5 islets^{-1} · 1hr^{-1} for control vs 5.1±0.7ng · 5 islets^{-1} · 1hr^{-1} for 3μM KN-93, $p<0.05$, n=5). None of these inhibitors affected insulin release with a non-stimulating concentration (3mM) of glucose (data not shown). Higher concentrations of ML-9 were required to inhibit glucose-induced insulin release (10.9±0.7ng · 5 islets^{-1} ·60min^{-1} for control vs 7.6±0.5ng · 5 islets^{-1} · 60min^{-1} for 30μM ML-9, $p<0.05$, n=5).

Inhibition by KN-62, KN-93 and ML-9 of insulin release elicited by high K^+ , glucose plus forskolin, and TPA

Table 1 shows the effects of KN-62, KN-93, ML-9 on insulin release stimulated by high K^+, glucose plus forskolin, and TPA. 20mM K^+ elicited a 4-fold increase in insulin release from pancreatic islets. Addition of 10μM KN-62, KN-93 or 50μM ML-9 caused a significant decrease in insulin release stimulated by 20mM K^+. In the presence of 10mM glucose, 5μM forskolin approx 3-

Table 1. Effects of KN-62, KN-93, and ML-9 on insulin release from rat pancreatic islets stimulated by high K^+, glucose plus forskolin, and TPA

Glucose (mM)	Additions	Insulin Release (ng/5islets during a 30min or 60min incubation)			
			Inhibitors		
		No Inhibitors	10μM KN-62	10μM KN-93	50μM ML-9
3	20mM K^+	0.83 ± 0.06 (5)	0.52 ± 0.02 (5)**	0.61 ± 0.07 (5)*	0.61 ± 0.05 (5)*
10	5μM forskolin	47.2 ± 4.2 (10)	13.3 ± 1.8 (5)***	12.5 ± 2.5 (5)***	47.0 ± 5.0 (5)
3	162μM TPA	4.35 ± 0.30 (10)	3.70 ± 0.32 (10)	3.77 ± 0.22 (10)	3.88 ± 0.22 (14)

Groups of 5 pancreatic islets were incubated at 37°C for 30min in 0.5ml high K^+ (20mM) Hepes-buffered Krebs-Ringer solution or for 60min in 1ml normal Hepes-buffered Krebs-Ringer solution with various substances. The amount of insulin in the media was measured using enzyme-linked immunoassay. Each value is the mean ± SEM for the number of observations shown in the parentheses.

Significance: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ as compared with the parallel condition in the absence of the inhibitors.

folds enhanced insulin release from the pancreatic islets. KN-62 and KN-93 at 10μM caused a potent inhibition of insulin release increased by glucose plus forskolin. 50μM ML-9, however, failed to influence the release under the parallel condition. Insulin release stimulated by 162nM TPA was not inhibited by KN-62 or KN-93 at 10μM, or ML-9 at 50μM.

Discussion

In the pancreatic ß cell, at least two different calmodulin-dependent protein kinases were suggested to exist. Penn et al (11) reported that islet homogenates retain an activity to phosphorylate myosin light chain. Ca/CaM kinase II in rat pancreatic islets was demonstrated by Fukunaga et al (12) by an immunohistochemical approach. Our results are in good agreement with their findings, indicating that these two protein kinases are present in rat pancreatic islets. Although α (50kD) and ß (60kD) subunits of Ca/CaM kinase II from rat brain were detected by the antibody used here, only α subunit was found in the islets in the present study. It is known that Ca/CaM kinase II from various types of tissue has a different composition of its subunits. It seems that Ca/CaM kinase II from the secretory glands prefers a single band on immunoblot analysis (13).

Isoquinoline derivatives exhibit distinct specificity to inhibit various protein kinases and have been widely used to investigate the functions of protein kinases. One of the authors (H.H.) recently synthesized specific inhibitors of MLCK, ML-9 (8) and of Ca/CaM kinase II, KN-62 and KN-93 (6, 7). KN-93 was reported to reduce dopamine content in PC12h cells (7). ML-9 inhibited serotonin secretion from platelet induced by collagen (10).

Glucose is the most important secretagogue in insulin release. Closure of the ATP-sensitive K^+ channel by ATP as a result of glucose metabolism depolarizes ß-cell plasma membrane, leading to opening the voltage-dependent Ca^{2+} channel. Ca^{2+} influx, then, occurs which is a key event in stimulus-secretion coupling in the ß cell. Depolarization of plasma membrane is also mimicked by a high concentration of extracellular K^+. In the present experiments, KN-62, KN-93 and ML-9 inhibited insulin release induced by glucose or high K^+, suggesting that Ca/CaM kinase II and MLCK may play positive roles in insulin release in response to an increase in intracellular Ca^{2+}. Li et al (14) recently reported that insulin release induced by mixed nutrients or high K^+ from a pancreatic ß cell line, HIT T15, was inhibited by KN-62. They argued, however, that Ca/CaM kinase II may not be involved in insulin release, because KN-62 failed to inhibit Ca^{2+}-induced insulin release from permeabilized HIT cells. Neveretheless, the secretory processes in the ß cell could be altered by the treatments for permeabilization. Moreover, the presence of Ca/CaM kinase II has not been demonstrated in HIT cells.

Although KN-62 and KN-93 does not inhibit cAMP-dependent protein kinase (preotein kinase A) (6, 7), these inhibitors inhibited insulin release increased by glucose and forskolin. Since cAMP enhances insulin release evoked by an increase in intracellular Ca^{2+}(15), inhibition by KN-62 and KN-93 of insulin release by coincubation with glucose and forskolin may result from the action of these inhibitors on the Ca^{2+}-dependent process. Interestingly, we could not find any effect of ML-9 on insulin release by glucose and forskolin. One of the explanation could be that different types of protein kinases may share common substrate(s), since myosin light chain was also reported to be phosphorylated by protein kinase A in smooth muscle (16).

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