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A possible role for Ca^{2+}/calmodulin-dependent protein kinase IV during pancreatic acinar stimulus–secretion coupling

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Abstract

Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are important intracellular mediators in the mediation of stimulus–secretion coupling and excitation–contraction coupling in a wide variety of cell types. We attempted to identify and characterize the functional roles of CaMK in mediating pancreatic enzyme secretion. Immunoprecipitation and immunoblotting studies using a CaMKII or CaMKIV antibody showed that rat pancreatic acini expressed both CaMKII and CaMKIV. Phosphotransferase activities of CaMKs were measured by a radioenzyme assay (REA) using autocamtide II, peptide γ and myosin P-light chain as substrates. Although CaMKII and CaMKIV use autocamtide II as a substrate, peptide γ is more efficiently phosphorylated by CaMKIV than by CaMKII. Intact acini were stimulated with cholecystokinin (CCK)-8, carbachol (CCh) and the high-affinity CCK-A receptor agonist, CCK-OPE, and the cell lysates were used for REA. CCK-8, CCh and CCK-OPE caused a concentration-dependent increase in CaMKs activities. When autocamtide II was used, maximal increases were 1.5–1.8-fold over basal (20.2 ± 2.0 pmol/min/mg protein), with peaks occurring at 20 min after cell stimulation. In separate studies that used peptide γ, CCK-8, CCh and CCK-OPE dose-dependently increased CaMKIV activities. Maximal increases were 1.5–2.4-fold over basal (30.7 ± 3.2 pmol/min/mg protein) with peaks occurring at 20 min after cell stimulation. Peak increases after cell stimulation induced by peptide γ were 1.8–2.8-fold higher than those induced by autocamtide II. CCK-8, CCh and CCK-OPE also significantly increased phosphotransferase activities of myosin light chain kinase (MLCK) substrate (basal: 4.4 ± 0.7 pmol/min/mg protein). However, maximal increases induced by MLCK substrate were less than 10% of those occurring in peptide γ. Characteristics of the phosphotransferase activity were also different between autocamtide II and peptide γ. When autocamtide II was used, elimination of medium Ca^{2+} in either cell lysates or intact cells resulted in a significant decrease in the activity, whereas it had no or little effect when peptide γ was used. This suggests that Ca^{2+} influx from the extracellular space is not fully required for CaMKIV activity and Ca^{2+} is not a prerequisite for phosphotransferase activity once CaMKIV is activated by either intracellular Ca^{2+} release or intracellular Ca^{2+} oscillations. The specific CaMKII inhibitor KN-62 (50 μM) had no effect on the CaMKIV activity and pancreatic enzyme secretion elicited by CCK-8, CCh and CCK-OPE. The specific MLCK inhibitor, ML-9 (10 μM), also did not inhibit CCK-8-stimulated pancreatic amylase secretion. In contrast, wide spectrum CaMK inhibitors, K-252a (1 μM) and KT5926 (3 μM), significantly inhibited CaMKIV activities and enzyme secretion evoked by secretagogues. Thus, CaMKIV appears to be an important intracellular mediator during stimulus–secretion coupling of rat pancreatic acinar cells.

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1. Introduction

Ca^{2+} serves as a second messenger for the activation of a broad group of protein kinases that are calmodulin (CaM)-dependent (reviewed in [1–3]). CaM-dependent protein kinases (CaMKs) can phosphorylate a wide range of substrates and modulate many cellular responses stimulated by an increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). CaMKs are kept fairly inactive in unstimulated cells by the presence of an autoinhibitory domain which can be overcome by the binding of Ca^{2+}/CaM complex. The conformation change in CaMKs by their binding to Ca^{2+}/CaM allows them to phosphorylate the serine and threonine residues of cellular substrates. In addition to myosin light chain kinase (MLCK), which activates smooth muscle contraction, and phosphorylase kinase, which activates glycogen breakdown, five other distinct CaMKs termed types I, II, III, IV and V are presently known. These kinases phosphorylate serines and threonines in proteins and the response of a target cell to an increase in $[Ca^{2+}]_i$ depends on which CaMK-regulated target proteins are present in the cell (reviewed in [4–7]).

CaMKI (molecular weight (MW): 37–42 kDa) was first identified and purified from bovine brain and phosphorylates the synaptic vesicle protein, synapsin I and II, and cAMP response element binding protein on the consensus sequence Hyd-XRRXS(T)-XXX-Hyd, where Hyd represents a hydrophobic amino acid. A Ca^{2+}/CaMK-dependent 52 kDa kinase, which can activate and phosphorylate CaMKI, has recently been purified and cloned (CaMKI kinase). CaMKII is a well known multifunctional kinase, which is widely distributed in many tissues especially in the brain. CaMKII functions in synthesis, long-term potentiation, neurotransmission and formation of spatial learning. CaMKII is composed of several subunits ranging from 55 to 60 kDa in size (α, β, β', γ, δ) and phosphorylates many substrates. They include calcineurin, GABA-A receptor, inositol 1,4,5-trisphosphate receptor (IP_3R), microtubule-associated protein 2, phospholipase A_2, synapsins, tau, tyrosine hydroxylase, and AMPA-type glutamate receptor on the consensus sequence RXXS(T). This sequence is also found in CaMKII itself, thus allowing an autophosphorylation at Thr-286 in the regulatory subunit upon binding Ca^{2+}/CaM, leading to the activation of enzyme in a Ca^{2+}-independent manner. CaMKIII has been purified from rabbit reticulocytes and rat pancreas (MW: 95–103 kDa). A Ca^{2+}/CaM-dependent autophosphorylation on a serine residue(s) is required for activation of CaMKIII and for the induction of a Ca^{2+}/CaM-independent activity. This enzyme is distributed in many different tissues and due to its restricted substrate specificity (only elongation factor 2 (EF-2, MW: 100 kDa)), the name EF-2 kinase rather than CaMKIII is gaining wide acceptance. CaMKIV (M_r: 60 kDa), which was initially named CaMK-Gr (granule), is a more recently discovered monomeric kinase. It is mainly distributed in cerebellum, thymus and testis. In contrast to other CaMKs, CaMKIV does not show stoichiometric autophosphorylation for its full activation and it appears to require another kinase (68 kDa CaMK kinase). CaMKIV has high sequence homologies with the catalytic and regulatory domains of CaMKII and has an autoinhibitory domain that can suppress kinase activity in the absence of Ca^{2+}/CaM. Although CaMKII and CaMKIV share an apparent similarity in consensus site for phosphorylation (RXXS(T)), sequences from the ribosomal S6 protein, CaMKIIγ (peptide γ), and Rab-1b protein are more efficiently phosphorylated by CaMKIV than by CaMKII. CaMKV, a monomer of 41 kDa, has several properties similar to CaMKI. The CaMKII inhibitor KN-62 also potently inhibits CaMKV activity. These distinct CaMKs are differentially distributed and function in different tissues (reviewed in [4–7]).

The objective of this study was to identify and characterize the functional roles of CaMK during stimulus–secretion coupling of pancreatic acinar cells. This was because our preliminary data showed that the specific CaMKII inhibitor KN-62 [8] did not inhibit pancreatic enzyme secretion elicited by cholecystokinin (CCK) analogues and carbachol (CCh), even when the maximal doses of KN-62 were used (50–100 μM) [9]. In control experiments, we showed that KN-62 significantly and dose-dependently inhib-

ited CCh-induced and Ca^{2+}-mediated gastric acid secretion from rabbit parietal cells with an IC_{50} of 20 μM; KN-62 in turn did not affect cAMP-mediated acid secretion [10]. One report showed that the CaM inhibitor (W-7, 100 μM) and KN-62 (10 μM) reduced rat pancreatic amylase secretion stimulated by CCK, CCh, Ca^{2+} ionophore, phorbol ester or GTPγs, suggesting that CaM and CaMKII are involved in Ca^{2+}-dependent pancreatic exocytosis [11]. However, the inhibitory effects of KN-62 were only 10–20%, whereas W-7 caused a 75% reduction, suggesting that other CaMKs may be involved in Ca^{2+}-dependent secretion. Using autocamtide II, a substrate for CaMKII, it has been reported that in rat pancreatic acini CaMKII was rapidly activated by a large increase in $[Ca^{2+}]_i$ stimulated by CCK, CCh and bombesin [12]. Thus current evidence addressing the role of CaMKII in mediating pancreatic enzyme secretion is conflicting, though affinity chromatographical studies provided evidence that CaM (19 kDa) is present and functions in stimulus–secretion coupling of pancreatic acinar cells in various species [13–17].

Three approaches were performed in this study: (1) immunoprecipitation (IP) and immunoblotting (IB) studies using different antibodies of CaMKs; (2) radioenzyme assay (REA) using different substrates for CaMKs; (3) functional studies using several CaMK inhibitors. Results obtained suggest that CaMKIV appears to be a major enzyme in mediating Ca^{2+}-dependent pancreatic exocytosis.

2. Materials and methods

2.1. Materials

Chemicals were purchased from the following sources: CCh, CCK-8 and soybean trypsin inhibitor (SBTI) from Sigma (St. Louis, MO, USA); CCK-OPE from Research Plus (Bayonne, NJ, USA); fura-2/AM, W-7, KN-62, KN-93, K-252a, KT5926, ML-9 and ophiobolin A from Calbiochem (San Diego, CA, USA); anti-CaMKII mouse monoclonal antibody and anti-CaMKIV mouse monoclonal antibody (N-terminus) from Transduction Laboratories (Lexington, KY, USA); anti-CaMKIV rabbit polyclonal antibody (C-terminus) and autocamtide II from Upstate Biotechnology (Lake Placid, NY, USA); peptide γ and MLCK substrate (myosin P-light chain) from Biomol (Plymouth Meeting, PA, USA). All chemicals were dissolved in either dimethyl sulfoxide, ethanol, methanol, physiological salt solution (PSS) or D_2O, the final concentration being 0.01–0.1%; these concentrations of solvents did not affect the cell response.

2.2. Isolation of pancreatic acini and measurements of amylase secretion and intracellular Ca^{2+} concentration

Isolated rat pancreatic acini were prepared by collagenase digestion with pancreas obtained from male Sprague–Dawley rats [18]. Acini were suspended in PSS. The PSS contained 0.1% bovine serum albumin, 0.1 mg SBTI, and in mM: 137 NaCl, 4.7 KCl, 0.56 Mg_2Cl_2, 1.28 $CaCl_2$, 1 NaH_2PO_4, 10 *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES), Eagles' minimum essential amino acids neutralized with NaOH, 2 L-glutamine and 5.5 D-glucose. The PSS was adjusted to pH 7.35 and equilibrated with 100% O_2. Isolated acini were preincubated for 40 min at 37°C in 40 ml PSS, washed twice and resuspended in fresh PSS. Aliquots (2 ml) were distributed into flasks, preincubated with or without inhibitors for 10 min and further coincubated with secretagogues for 60 min at 37°C. The incubation was terminated by centrifugation at 10 000 rpm for 50 s at 4°C in a microfuge. The amylase released into the supernatant and remaining in the pellet was assayed, using a Procion yellow starch as a substrate [19]. Amylase secretion was expressed as a percentage of the total content in each sample. Measurement of $[Ca^{2+}]_i$ was performed as described previously [20]. Briefly, isolated acini were incubated with 2 μM fura-2/AM at 37°C in 10 ml PSS for 30 min, washed with 30 ml PSS and resuspended in 10 ml fresh PSS. All experiments were performed using a dual wavelength (340/380 nm emitted at 505 nm) modular fluorometry system (SPEX Fluorolog 2) coupled to a Nikon Diaphot inverted microscope (×40). Acini were placed on a zero cover glass, mounted in a closed chamber and superfused by an eight-chambered reservoir (1 ml/min). A fluorescence ratio was converted to $[Ca^{2+}]_i$ according to in vitro calibration with an external standard (Calcium kit II, Molecular Probes,

Eugene, OR, USA) and 50 μM fura-2 potassium salt.

2.3. IP

Acinar cells (2×10^6) in 1 ml PSS were incubated with reagents for 20 min at 37°C. The incubation was terminated with 1 ml chilled 8 mM HEPES (buffer A), containing (in mM) 1 sodium orthovanadate, 0.5 NaH_2PO_4, 109.5 NaCl, 4.7 KCl and 1.13 $MgCl_2$. The suspension was immediately centrifuged (10 000 rpm) for 15 s at 4°C. The supernatant was discarded and the resultant pellet was resuspended in chilled lysis buffer (buffer B; pH 7.4), containing (in mM) 25 β-glycerophosphate, 0.2 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), 5 EDTA, 1 dithiothreitol (DTT), 150 NaCl, 50 tris(hydroxymethyl)aminoethane (Tris), 25 sodium fluoride, 0.2% Triton X-100, 10 μg/ml leupeptin and 0.05 trypsin inhibitor units (TIU)/ml aprotinin. Each suspension was sonicated, vortexed for 30 s and centrifuged (10 000 rpm) for 10 min at 4°C. The suspension was diluted with buffer B to 1 mg protein/ml. Protein concentrations were measured using the Bio-Rad protein assay reagent kit (Hercules, CA, USA). 500 μl samples were incubated with the anti-CaMKIV monoclonal antibody (10 μg/ml) overnight at 4°C and with 50 μl of protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 2 h at 4°C. The immunoprecipitates were washed three times with 100 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl and 1.5 ml/l Tween 20, and then analyzed by sodium dodecyl sulfate (SDS)–PAGE and Western blotting.

2.4. Western IB

Acinar cells (2×10^6) in 0.5 ml PSS were incubated with secretagogues for the indicated periods. The incubation was terminated with buffer A. The suspension was immediately centrifuged (10 000 rpm) for 15 s at 4°C. The supernatant was discarded and the resultant pellet was resuspended in 150 ml chilled lysis buffer, which consists of (in mM) 66.7 β-glycerophosphate, 1 sodium orthovanadate, 1 PMSF, 1.5 EGTA, 1 DTT, 1% Triton X-100, 10 μg/ml leupeptin and 0.05 TIU/ml aprotinin (buffer C; pH 7.4). Each suspension was sonicated, vortexed for 30 s at 4°C and centrifuged at 10 000 rpm for 10 min. The supernatant (20–25 μl; 30–37.5 μg protein) was mixed with 2.5 ml of 100% β-mercaptoethanol and 12.5 ml of Laemmli buffer (buffer D), containing 62.5 mM Tris-base, 2.3% SDS, 7.5% glycerol and 0.1% bromophenol blue. The solution was heated at 95°C for 5 min, separated by SDS–PAGE on 15% polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (NM; Bio-Rad) for 1 h at 4°C. IB was performed using either an anti-CaMKIV monoclonal antibody (4 μg/ml), anti-CaMKII monoclonal antibody (2 μg/ml) or anti-CaMKIV polyclonal antibody (1 μg/ml). The membrane was then incubated with the peroxidase (0.3 μg/ml)-conjugated goat anti-mouse IgG (1:5000 dilution). The blots were developed and visualized with a chemiluminescent horseradish peroxidase substrate (ECL, Amersham, Arlington Heights, IL, USA).

2.5. Measurement of Ca^{2+}/CaMK activities

For measurement of CaMK activities, the REA was performed, using a CaMKII assay kit with changes in CaMK substrates (UBI). Aliquots (0.5 ml) of the acinar cell suspension (2×10^6 cells/PSS) were incubated with reagents for 20 min at 37°C. The incubation was terminated with 1 ml chilled PSS, and the aliquots were immediately centrifuged (10 000 rpm) for 50 s in a microfuge. The supernatant was removed and the resultant pellet was resuspended in 50 μl chilled 50 mM HEPES buffer (pH 7.4), containing 50 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 150 mM NaCl, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 25 μg/ml leupeptin and 25 μg/ml aprotinin (extraction buffer). Each suspension (30 samples) was immediately frozen in liquid nitrogen and stored at −70°C overnight. The sonicates were vortexed and allowed to settle for 10 min at 4°C, then centrifuged (10 000 rpm) for 15 min at 4°C. Each 10 μl sample of the supernatant (~50 μg protein) was combined with 20 μl of substrate solution, which contained 10 μg peptide γ (CaMKIV substrate), 1 μM protein kinase A inhibitor peptide, 1 μM protein kinase C (PKC) inhibitor peptide, 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT and 1 mM $CaCl_2$ (assay dilution buffer; ADB). In separate ex-

periments, autocamtide II (10 μg) and MLCK substrate (10 μg) were used as substrates for CaMKII and MLCK, respectively. For the control reactions, 10 μl of the supernatant from corresponding cell extracts was combined with 20 μl ADB without peptide γ, autocamtide II or MLCK substrate. All procedures were performed at 4°C. Aliquots (10 μl each) of [γ-^{32}P]ATP (1 μCi) (Amersham) dissolved in ADB, 500 μM ATP and 75 mM $MgCl_2$ were added to each sample at 30°C. The final volume per incubation was 40 μl. The final incubation buffer contained 5 mM EGTA and 0.75 mM $CaCl_2$ (Ca^{2+}-free). After incubation for 10 min at 30°C, a 25 μl aliquot was spotted onto p81 phosphocellulose papers, which were mounted on a piece of aluminum foil. Each disc was placed in a glass scintillation vial containing 15 ml of 0.75% phosphoric acid. After 30 min mixing at room temperature, the washing reagent was decanted. Each disc was then mixed with 10 ml of 0.75% phosphoric acid, followed by 10 ml acetone, for 10 min, respectively, at room temperature. Each reagent was decanted after each washing. Finally, 10 ml of scintillant (Cytoscint, ICN, Costa Mesa, CA, USA) was added and the radioactivity remaining on each binding paper was counted in a liquid scintillation counter. Non-specific binding of [γ-^{32}P]ATP to the binding paper without substrates (peptide γ, autocamtide II or MLCK substrate) was subtracted from each control sample that included the substrate. Non-specific binding of [γ-^{32}P]ATP was ~30% of total binding. It was ~6% when substrates, but not cell extracts, were added. Ca^{2+}-independent CaMK activities were expressed as pmol/min/mg protein.

3. Results

3.1. Expression of CaMKII and CaMKIV in rat pancreatic acini

IP and IB studies utilizing CaMKII and CaMKIV monoclonal antibodies in acinar cell lysates showed that rat pancreatic acini expressed both CaMKII and CaMKIV at the location of 60 kDa (Fig. 1). The immunodetectable CaMKIV band required IP and IB, whereas CaMKII was immunodetected by IB alone. A polyclonal antibody of CaMKIV was capable of detecting several CaMKs by IB. Probing Western blot with the polyclonal antibody immunodetected four major proteins ranging from 40 kDa to 60 kDa (Fig. 2). Among these, p50 which is yet to be identified (third band from the top), is quantitatively close to p51 CaMK present in rat pancreatic acini; p51 CaMK phosphorylates p29 ribosomal protein S6 [21,22]. The upper band (p60) may correspond to CaMKIV or CaMKII, as A431 cell lysates expressed a single band at the same location. The CaMKIV polyclonal antibody may recognize both CaMKII and CaMKIV because of their structural similarities. Note that each figure (Figs. 1–9) presented in this study and statistical analyses were prepared from data conducted on the same series of experiments.

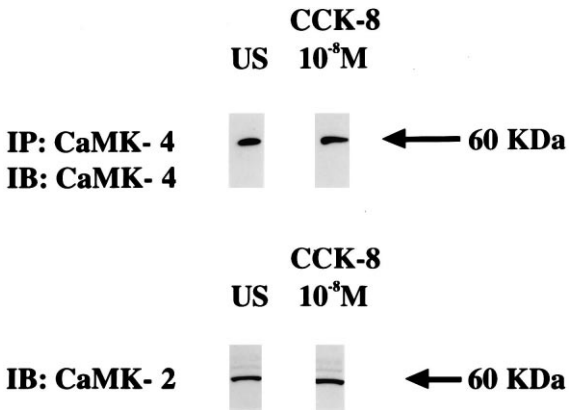

Fig. 1. IP and IB of CaMKII and CaMKIV in rat pancreatic acinar cell lysates. Isolated pancreatic acini were treated with or without CCK-8 for 20 min at 37°C. Proteins from Triton X-100-soluble fractions (10 000 rpm supernatant of cell sonicates) were immunoprecipitated with monoclonal anti-CaMKIV antibody (immunogen: CaMKIV N-terminus), analyzed by SDS–PAGE and immunoblotted with monoclonal anti-CaMKIV antibody (upper panel). Cell lysates were resolved in SDS–PAGE gels and transferred to nitrocellulose membrane electrophoretically. Membrane was blotted with monoclonal anti-CaMKII antibody, whose immunogen is the regulatory domain of CaMKII (lower panel). Similar results were observed in seven experiments. Positive control: Jurkat cell lysates (not shown). Abbreviations: US, unstimulated cells; IP, immunoprecipitation; IB, immunoblotting.

3.2. Phosphotransferase activities of CaMKII, CaMKIV and MLCK

The objective of this study was to identify and characterize the functional roles of CaMK in mediating pancreatic enzyme secretion. This is because

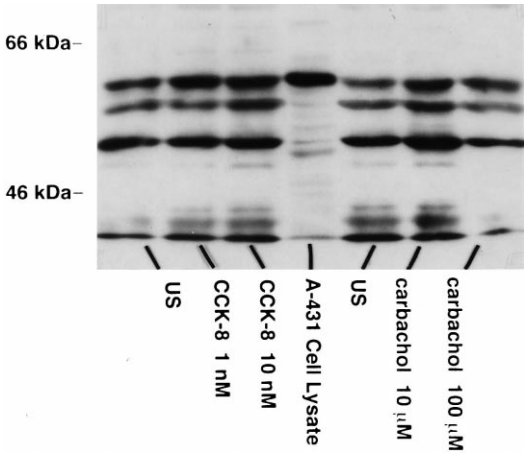

Fig. 2. Western IB of CaMKIV with polyclonal anti-CaMKIV antibody. Pancreatic acini were treated with or without CCK-8 or CCh for 20 min at 37°C. Cell lysates were resolved in 7.5% SDS–PAGE and transferred to polyvinylidene difluoride for IB. The primary CaMKIV polyclonal antibody (immunogen: CaMKIV C-terminus) was detected using a goat anti-rabbit IgG linked to horseradish peroxidase developed with ECL. Four major bands (p60, p57, p50 and p40) were observed. Positive control: A431 cell lysates. Similar results were observed in three experiments.

our preliminary data showed that the specific CaMKII inhibitor KN-62 [8] did not inhibit amylase secretion elicited by Ca^{2+}-mobilizing secretagogues [9]. In separate studies, we showed that KN-62 significantly inhibited acid secretion from parietal cells [10]. These results suggest the possibility that other CaMK(s) may be involved in stimulus–secretion coupling of rat pancreatic acinar cells. Peptide γ (KSDGGVKKRKSSSS), a sequence from CaKIIγ (345–358), is specific for CaMKIV ($K_m = 8$ μM) and is a poor substrate for CaMKII [23]. Autocamtide II (KKALRRQETVDAL), a sequence derived from CaMKII autophosphorylation site RQETVD plus basic residues to facilitate phosphocellulose binding, is a highly selective CaMKII substrate ($K_m = 2$ μM) [24]. Substrates for CaMKII require the consensus sequence RXXS(T) and underlines mean phosphorylated amino acids. MLCK substrate (KKRAARATSNVFA-NH_2), a sequence from chicken gizzard smooth muscle myosin P-light chain (MLC11–23) with Pro to Ala-14 and Gln to Ala-15, is specific for MLCK ($K_m = 7.5$–8.6 μM) [25,26]. Basal phosphotransferase activities (20 min incubation without stimulation) of CaMK in rat pancreatic acini were as follows (pmol/min/mg protein): 30.7 ± 3.2 (peptide γ, $n = 12$), 20.2 ± 2.0 (autocamtide II, $n = 18$) and 4.4 ± 0.7 (MLCK substrate, $n = 18$). These basal activities were 7–123-fold higher than those of Src/protein tyrosine kinase activities (0.25–0.63 pmol/min/mg protein) [27], suggesting a quantitative predominance of Ser/Thr kinases in pancreatic acini as well as other eukaryotic cells. Stimulation of intact acini with CCK-8, a high-affinity CCK-A receptor agonist CCK-OPE and CCh for 20 min dose-dependently increased the phosphotransferase activity of CaMKIV (Fig. 3A). As shown in Fig. 3B,C, these secretagogues also increased phosphotransfer-

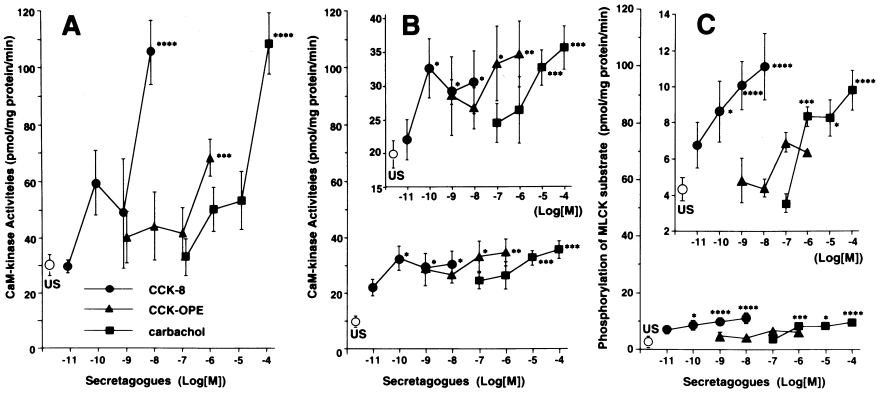

Fig. 3. Phosphotransferase activities of CaMK using different substrates. Acini were treated with or without CCK-8, CCK-OPE and CCh for 20 min at 37°C and CaMK phosphotransferase activities of the cell lysates were measured. A: peptide γ (CaMKIV substrate); B: autocamtide II (CaMKII substrate); C: myosin light chain kinase (MLCK) substrate. Insets of B and C are enlarged figures from originals. A, B and C are the mean ± S.E.M. from six, three and three separate experiments, respectively, each performed in duplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ and $^{****}P < 0.001$ compared with basal values (US) by two-tailed unpaired t tests.

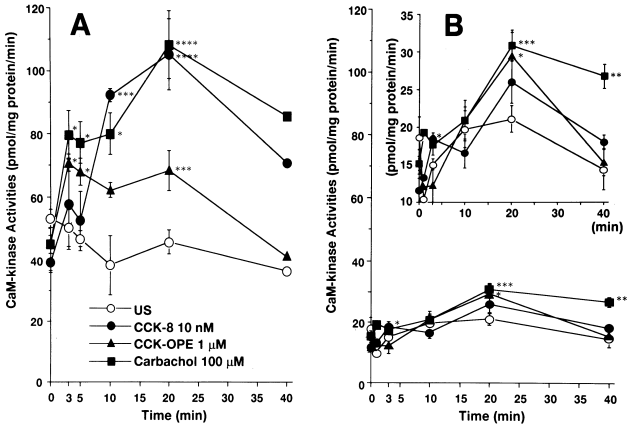

Fig. 4. Time course studies of CaMK activities using different substrates. Acini were treated with or without CCK-8 (10 nM), CCK-OPE (1 μM) and CCh (100 μM) for indicated time periods at 37°C and CaMK phosphotransferase activities of the cell lysates were measured. Note that 100 pM CCK-8 was used for CaMKII activities in this figure and Figs. 5 and 6, because this concentration caused a maximal response (see Fig. 3B). A: peptide γ; B: autocamtide II. An inset of B is the enlarged figure from the original. A and B are the mean ± S.E.M. from seven and eight separate experiments, respectively, each performed in duplicate. $*P<0.05$, $**P<0.01$, $***P<0.005$ and $****P<0.001$ compared with the zero time by unpaired t tests.

ase activities of CaMKII and MLCK. The maximal activities were however approximately 30% (CaMKII) and less than 10% (MLCK) compared with those occurring in CaMKIV activities. Time course studies showed that the maximal activity of CaMKIV occurred at 20 min following stimulation with secretagogues accompanied with the first initial peak occurring at 3–10 min (Fig. 4A). A similar phenomenon was observed with CaMKII activity (Fig. 4B).

3.3. Requirements of calcium on CaMKII and CaMKIV activities

We next examined whether the phosphotransferase activities of CaMKII and CaMKIV in pancreatic acinar lysates are Ca^{2+}-dependent or not. This is because CaMKII causes an autophosphorylation at Tyr-286 in the regulatory subunit upon binding Ca^{2+}/CaM, leading to the activation of enzyme in a Ca^{2+}-independent manner [28–32]. On the other hand, CaMKIV does not appear to be significantly activated by autophosphorylation. Intact acini were stimulated with secretagogues for 20 min in the presence of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and the cell lysates were resuspended with either the extraction buffer ($[Ca^{2+}]$-free) or the assay buffer, which contained 1 mM $CaCl_2$ (see Section 2). Note that all other experiments except Fig. 5 were performed in the cell lysates suspended in a $[Ca^{2+}]$-free extraction buffer to measure Ca^{2+}-independent CaMK activities. As shown in Fig. 5A, CaMKIV activities were not reduced, but rather enhanced, by eliminating $[Ca^{2+}]$, whereas elimination of $[Ca^{2+}]$ in the cell lysates resulted in a 70–75% reduction of CaMKII activities (Fig. 5B). These results suggest that once CaMKIV is activated, it does not require $[Ca^{2+}]$ for the subsequent substrate phosphorylation. On the other hand, Ca^{2+}-independent activities of CaMKII were 25–30% of total activities occurring in the presence of $[Ca^{2+}]$. Distinct characteristics between CaMKII and CaMKIV were also observed in intact cells. Fig. 6 depicts the effects of $[Ca^{2+}]_o$ on CaMK activities in intact acini. Elimination of $[Ca^{2+}]_o$ (zero $CaCl_2$ plus 1 mM EGTA in PSS) did not significantly alter the CaMKIV activity (only 4–13% inhibition), whereas this maneuver significantly reduced the CaMKII activity. Thus Ca^{2+} influx from the extracellular space appears to be not fully required for the activation of CaMKIV. Intracellular Ca^{2+} release from the stores elicited by CCK-8 or

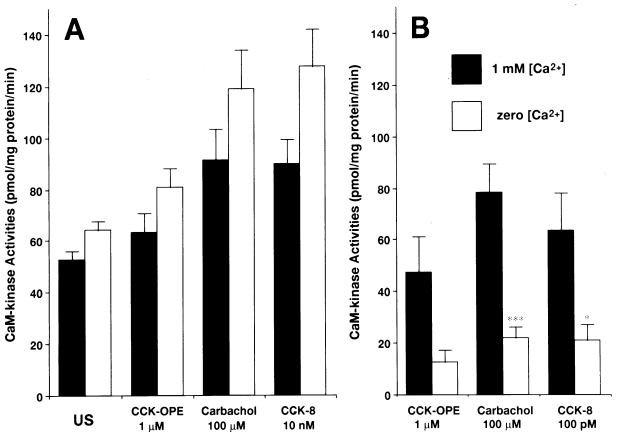

Fig. 5. Effects of Ca^{2+} on CaMK activities. Acini were treated with or without CCK-8, CCK-OPE and CCh for 20 min at 37°C and phosphotransferase activities of CaMK were measured in the presence (1 mM $CaCl_2$) or the absence (5 mM EGTA plus 0.75 mM $CaCl_2$, see Section 2) of $[Ca^{2+}]$. A: peptide γ; B: autocamtide II. A and B are the mean ± S.E.M. from two ($n=7$ for each column) and two ($n=5$ for each column) separate experiments, respectively. $*P<0.05$ and $***P<0.005$ compared with 1 mM $[Ca^{2+}]$ in each stimulation (unpaired t tests).

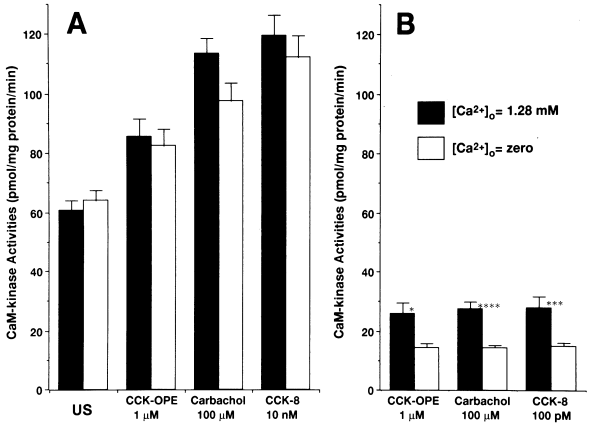

Fig. 6. Effects of extracellular Ca^{2+} on CaMK activities. Acini were treated with or without CCK-8, CCK-OPE and CCh for 20 min at 37°C in the presence (1.28 mM $CaCl_2$ in PSS) or the absence (1 mM EGTA plus zero $CaCl_2$ in PSS) of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and CaMK phosphotransferase activities of the cell lysates were measured. Note that CaMK activities were measured in the presence of $[Ca^{2+}]_o$ (intact cells) and the absence of $[Ca^{2+}]$ (cell lysates) in all experiments except Fig. 5 and this figure. A: peptide γ; B: autocamtide II. A and B are the mean ± S.E.M. from two ($n=8$ for each column) and three ($n=12$ for each column) separate experiments, respectively. $^{*}P<0.05$, $^{***}P<0.005$ and $^{****}P<0.001$ compared with zero mM $[Ca^{2+}]_o$ in each stimulation (unpaired t tests).

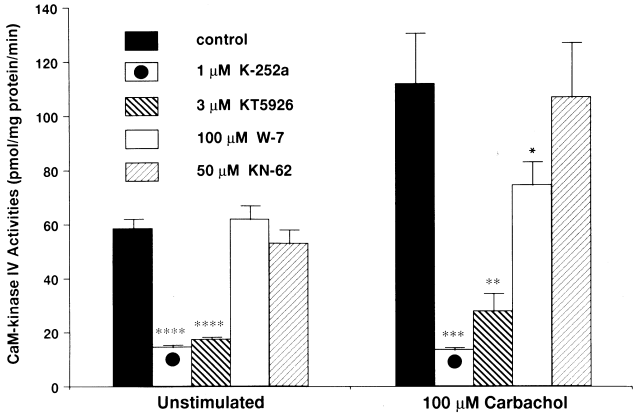

Fig. 7. Effects of CaM and CaMK inhibitors on CaMK activities. Acini were pretreated with or without various inhibitors for 10 min and further coincubated with or without CCh for 20 min at 37°C, and phosphotransferase activities of CaMKIV (peptide γ as a substrate) of the cell lysates were measured. Data are the mean ± S.E.M. from two separate experiments ($n=5$ for each column). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ and $^{****}P<0.001$ compared without inhibitors (control) by unpaired t tests.

CCh and intracellular Ca^{2+} oscillations induced by CCK-OPE may be sufficient to activate CaMKIV. Conversely, Ca^{2+} influx was partly (40–50%) required for the activation of CaMKII.

3.4. Effects of CaMK inhibitors on CaMK activities

As the specific inhibitor of CaMKIV is not available at present, we performed the random screening of potential inhibitors of CaMKs and MLCK. Pre-treatment of intact acini with wide spectrum inhibitors of CaMKs and MLCK, K-252a (1 μM) and its derivative KT5926 (3 μM) [33], for 10 min and the following stimulation with or without CCh (100 μM) for 20 min resulted in a significant inhibition of CaMKIV activities (Fig. 7). K-252a also significantly inhibited basal and CCK-8- or CCK-OPE-stimulated CaMKIV activities in rat pancreatic acini [34]. The CaM inhibitor, W-7 (100 μM), reduced CCh-induced CaMKIV activity (CCh alone: 113.6 ± 19.1 vs. CCh+W-7: 75.8 ± 8.4 pmol/min/mg protein, $n=10$, $P<0.05$ by unpaired t tests). In contrast, the specific inhibitor of CaMKII, KN-62 [8], had no effect on the CaMKIV activity even at the maximal dose used (50

Table 1
Effects of K252-a and ML-9 on CCK-8-stimulated pancreatic amylase secretion

		Control	+K-252a (3 μM)	+ML-9 (10 μM)
US		10.4 ± 1.7 (10)	13.5 ± 2.2 (8)	18.0 ± 2.1 (4)
CCK-8	10^{-11} M	31.0 ± 4.4 (10)	20.8 ± 2.1 (8)**	43.7 ± 3.2 (4)
	10^{-10} M	39.2 ± 2.0 (10)	26.4 ± 1.6 (8)***	48.2 ± 2.5 (4)
	10^{-9} M	30.0 ± 1.9 (10)	21.5 ± 2.2 (7)*	30.3 ± 1.9 (4)
	10^{-8} M	19.8 ± 1.1 (10)	21.6 ± 2.5 (8)	21.3 ± 1.3 (4)

Isolated acini were pretreated with or without K-252a and ML-9 for 10 min and further coincubated with or without CCK-8 for 60 min for 37°C. Data are the mean ± S.E.M. from 2–5 separate experiments expressed in % of total/60 min. Numbers in parentheses indicate the number of determinations. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ compared without K252-a (CCK alone) by two-tailed unpaired t tests. Abbreviation: US, unstimulated cells.

μM). Results indicate that K-252a and KT5926 may be useful as potent inhibitors for CaMKIV.

3.5. Effects of the CaM inhibitor W-7 and CaMKII inhibitor KN-62 on pancreatic enzyme secretion

We next compared the potency of the CaM inhibitor W-7 and CaMKII inhibitor KN-62 on pancreatic enzyme secretion. Fig. 8A shows that W-7 at 100 μM significantly inhibited amylase secretion evoked by CCK-8 (1–100 pM), CCK-OPE (1–1000 nM) or CCh (1–100 μM). Similar to the effects of KN-62 on the CaMKIV activity, KN-62 at 50 μM had no effect on secretion (Fig. 8B), suggesting that CaMKII is not involved in stimulus–secretion coupling of pancreatic acini. This dose of KN-62 in turn significantly inhibited acid secretion from rabbit parietal cells, serving as a positive control [10].

3.6. Effects of K-252a on secretagogues-stimulated Ca^{2+} spiking and amylase secretion

Fig. 9A shows that K-252a (1 μM), a likely CaMKIV inhibitor candidate, did not affect the sustained $[Ca^{2+}]_i$ plateau evoked by CCh, suggesting that it acts on post Ca^{2+} signal events. K-252a at 3 μM

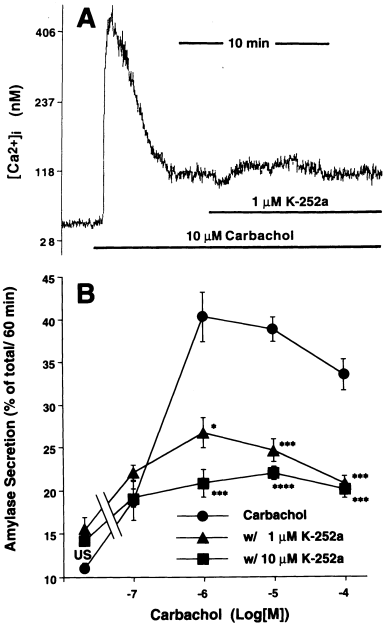

Fig. 9. Effects of the CaMK inhibitor K-252a on CCh-evoked Ca^{2+} spiking and pancreatic amylase secretion. A is a representative tracing of four determinations. B: Acini were pretreated with K-252a for 10 min and further coincubated with CCh for 60 min at 37°C. B is the mean ± S.E.M. from two separate experiments, each performed in duplicate. $^{*}P<0.05$, $^{***}P<0.005$ and $^{****}P<0.001$ compared without K-252a (CCh alone) by unpaired t tests.

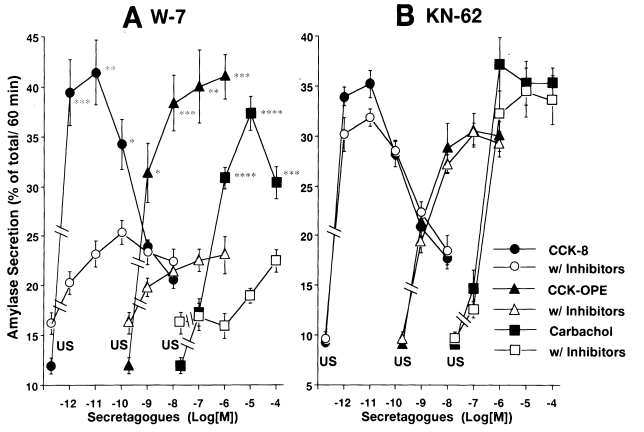

Fig. 8. Effects of W-7 (CaM inhibitor) and KN-62 (CaMKII inhibitor) on secretagogues-stimulated pancreatic amylase secretion. Acini were pretreated with or without W-7 (100 μM, A) and KN-62 (50 μM, B) for 10 min and further coincubated with CCK-8, CCK-OPE and CCh for 60 min at 37°C. A and B are the mean ± S.E.M. from four and seven separate experiments, respectively, each performed in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ and $^{****}P<0.001$ compared with inhibitors by unpaired t tests.

also did not alter Ca^{2+} influx elicited by CCK-8 [34]. Alternatively, K-252a inhibited intracellular Ca^{2+} oscillations induced by CCK-OPE, suggesting that activated CaMKIV acts as a positive feedback regulator on intracellular Ca^{2+} oscillations activated by the high-affinity CCK-A receptor [34]. Activated PKC, in turn, acts as a negative feedback regulator on CCK-8 or CCK-OPE-induced Ca^{2+} oscillations [20,35]. It is also interesting to note that the PTK inhibitor genistein selectively inhibited Ca^{2+} influx evoked by CCK-8 and CCh, whereas it had no effect on Ca^{2+} oscillations stimulated by CCK-8, CCK-OPE and CCh [27,34]. This suggests that PTK positively mediates extracellular Ca^{2+} influx. K-252a (1–10 μM) significantly inhibited enzyme secretion evoked by CCh or CCK-8 (Fig. 9B and Table 1). Because a supramaximal concentration of CCK-8 caused a reduction of amylase secretion probably due to negative feedback regulation by PKC [35,36], K-252a had no effect on the action stimulated by 10 nM CCK-8. K-252a also significantly

inhibited amylase secretion stimulated with CCK-OPE ranging from 10 nM to 1 μM [34]. Because CCK-8 or CCh caused a small but significant increase in MLCK activities (see Fig. 3C), we examined the effects of the specific MLCK inhibitor ML-9. ML-9 (10 μM), however, did not inhibit but enhanced amylase secretion elicited by CCK-8 (see Table 1), suggesting that MLCK appears to be not involved in pancreatic enzyme secretion.

We finally performed pharmacological screenings of CaMK inhibitors, which may be available for CaMKIV inhibitors (Table 2). A fungal metabolite ophiobolin A (*Helminthosporium maydis*) is a cell-permeable antagonist of CaM and inhibits the activation of Ca^{2+}/CaM phosphodiesterase (IC_{50} = 9 μM) [37]. This compound is, however, not useful at least in rat pancreatic acini, because 3 and 10 μM ophiobolin A with and without CCh toxically secreted amylase with concomitant decreases in cellular amylase (86–95% of total/60 min). KN-93 is a potent inhibitor of CaMKII (IC_{50} = 370 nM) [38]. However, 1 and 3 μM KN-93 did not inhibit amylase secretion induced by 0.1–100 μM CCh. These concentrations were in turn sufficient to inhibit the CCh-induced parietal cell secretion [38]. KT5926 (1–10 μM), an analogue of K-252a, significantly inhibited pancreatic secretion stimulated by CCh (0.1–300 μM), a manner similar to that of K-252a (not shown). Therefore, K-252a and KT5926 seem to be useful as CaMKIV antagonists at present, though they also act on MLCK [33]. Because the CaMKII inhibitor KN-62 also inhibits CaMKIII and CaMKV activities and because K-252a does not affect CaMKIII activity [39], it appears that, in addition to CaMKII, CaMKIII and CaMKV may not be involved in pancreatic acinar stimulus–secretion coupling.

Table 2
Effects of ophiobolin A and KN-93 on CCh-stimulated pancreatic amylase secretion

		Control	+Ophiobolin A		+KN-93	
			3 μM	10 μM	1 μM	3 μM
US		8.8 ± 0.8	87.2	93.2	9.4	8.5 ± 0.3
CCh	10^{-7} M	19.0 ± 1.1	88.0	94.6	16.5	18.3 ± 0.4
	10^{-6} M	33.6 ± 1.4	86.3	93.5	34.2	32.3 ± 0.7
	10^{-5} M	34.9 ± 1.3	89.3	93.7	33.2	31.4 ± 1.3
	10^{-4} M	26.6 ± 1.2	88.1	93.5	28.0	25.5 ± 0.7

Isolated acini were pretreated with or without ophiobolin A and KN-93 for 10 min and further coincubated with or without CCh for 60 min at 37°C. Data are the mean ± S.E.M. from three separate experiments (n = 4–6) expressed in % of total/60 min. No S.E.M. denotes two determinations.

4. Discussion

Several previous reports using different approaches indicated that CaM, Ca^{2+}/CaMKs and Ca^{2+}/CaM-dependent protein phosphatases (types 1, 2A and 2B) are involved in stimulus–secretion coupling of mammalian pancreatic acinar cells [11–17,21,22,40–43]. Regarding CaMKs in rat pancreatic acini, a single component of 51 kDa CaMK, which phosphorylates the ribosomal S6 protein, was purified using hydrophobic chromatography followed by gel filtration and affinity chromatography [21,22]. A 92 kDa cytosolic protein was greatly increased by Ca^{2+}/CaM in rat pancreatic acini, whereas phosphatidylserine-dependent kinase activity most heavily phosphorylated proteins of 62 kDa and 40 kDa [40]. CaMKIII (MW: 95–103 kDa), which only phosphorylates the endogenous 100 kDa protein identified as EF-2 that catalyzes the translocation of peptidyl-tRNA on the ribosome, was purified from the cytosol fraction of the rat pancreas [44,45]. Intracellular Ca^{2+} inhibits protein synthesis via CaMKIII-catalyzed phosphorylation of EF-2 [45]. Thus it appears that several types of CaMK exist in pancreatic acini and function in exocytosis, cell differentiation, and cell growth and proliferation. However, the type of CaMK, which is involved in pancreatic enzyme secretion, is unclear at present. Among these, we first expected that the multifunctional CaMKII is an important mediator for pancreatic signal transduction and exocytosis. This was because CCK and CCh, well known Ca^{2+}-mobilizing reagents that stimulate pancreatic amylase secretion, were capable of stimulating CaMKII activity [12]. Our preliminary data, however, showed that the specific CaMKII inhibitor KN-62, which significantly inhibited CCh-induced parietal cell acid secretion, had no effect on CCK- and CCh-evoked pancreatic enzyme secretion [8–10]. Therefore, we aimed to identify and characterize the type of CaMK on pancreatic acinar stimulus–secretion coupling.

IP and Western IB studies using monoclonal antibodies of CaMKII and CaMKIV showed that rat

pancreatic acini expressed both CaMKII and CaMKIV. However, Ca^{2+}-independent phosphotransferase activity of CaMKIV was significantly higher than that of CaMKII and MLCK in both basal and stimulated levels. As well as CCK-8 and CCh, a high-affinity CCK-A receptor agonist CCK-OPE at 1 μM significantly increased activities of both CaMKII and CaMKIV. In this regard, a previous study showed that, in contrast to CCK-8 and CCh, another high-affinity CCK-A receptor agonist JMV-180 (1 μM) failed to stimulate CaMKII activity, suggesting that Ca^{2+} oscillations elicited by JMV-180 are not enough to switch CaMKII to a maintained Ca^{2+}-independent form in pancreatic acini [12]. This may be a misinterpretation, as they measured CaMKII activity after 30 s JMV-180 stimulation. Ca^{2+} oscillations required approximate 2 min lag time to initiate the first Ca^{2+} spike [35]. Our study showed that CCK-OPE stimulated CaMKIV activity at 3 min after cell stimulation, indicating that intracellular Ca^{2+} oscillations are capable of activating CaMK. It is, however, true that CCK-OPE-stimulated CaMKIV (or MLCK) activity was less than that elicited by either CCK-8 or CCh.

Endogenous CaMKs, which phosphorylated peptide γ and autocamtide II, were apparently distinct entities because of their different $[Ca^{2+}]$-dependency. While the activity of CaMKIV in acinar cell lysates did not require $[Ca^{2+}]$, Ca^{2+}-independent activity of CaMKII only occupied 25–30%. Therefore, CaMKIV seems not to require $[Ca^{2+}]$ to phosphorylate peptide γ in cell lysates, once it is activated by Ca^{2+}/CaM in intact cell levels. CaMKIV activities were rather inhibited by 1 mM $[Ca^{2+}]$ and, therefore, the total activity was less than the Ca^{2+}-independent activity (see Fig. 5A). The mechanism by which $[Ca^{2+}]$ inhibits CaMKIV activity remains unknown. CaMK IV did not require the presence of $[Ca^{2+}]_o$ in intact acini, whereas CaMKII required the presence of $[Ca^{2+}]_o$ at least 50% for its full activation. Therefore, it appears that the initial intracellular Ca^{2+} release (evoked by CCK-8 and CCh) or sustained intracellular Ca^{2+} oscillations (evoked by CCK-OPE) may be sufficient to activate CaMKIV. Because sustained pancreatic amylase secretion elicited by CCK-8 and CCh, but not by CCK-OPE, requires the presence of $[Ca^{2+}]_o$ [46], CaMKIV may act on the initial step of pancreatic exocytosis, which is triggered by the IP_3-mediated initial intracellular Ca^{2+} release coupled to the low-affinity CCK-A receptor and the muscarinic m3 receptor.

Although the specific inhibitor of CaMKIV is not available at present, our study suggested that the wide spectrum inhibitor K-252a is useful as a CaMKIV inhibitor. This is because: (1) K-252a abolished CaMKIV activity in both basal and stimulated states; (2) K-252a inhibited CCK-8 or CCh-stimulated pancreatic enzyme secretion without affecting the sustained $[Ca^{2+}]_i$ plateau; (3) K-252a did not affect secretagogues-stimulated second messenger levels [34]. Because the CaMKII inhibitor KN-62, which apparently lacked the ability to inhibit CaMKIV activity and pancreatic enzyme secretion evoked by CCK-8 or CCh, also inhibits CaMKIII and CaMKV in other cell systems [39], it seems unlikely that CaMKIII and CaMKV are involved in pancreatic exocytosis. CaMKIII, which is highly expressed in the pancreas, may be involved in Ca^{2+}-dependent protein synthesis [44,45]. While it is clear that CaMKII exists in rat pancreatic acini and that CCK-8, CCK-OPE and CCh increased CaMKII activity, the functional role of CaMKII is unknown at present. It has been shown that the α isoform of CaMKIV expressed using recombinant baculovirus/Sf9 cells preferentially phosphorylated syntide-2 and autocamtide [47]. Peptide γ, reported to be a specific substrate for purified brain CaMKIV [23], was barely phosphorylated by expressed CaMKIV. In contrast to our study, this study [47] also showed that KN-62 strongly inhibited CaMKIV activity. This may suggest that different subtypes of CaMKIV exist in different tissues. Since the monoclonal antibody of CAMKI is not available at present, we do not know whether CaMKI exists and functions in pancreatic acini. Furthermore, MLCK appears not to be involved in pancreatic acinar stimulus–secretion coupling, as the MLCK inhibitor ML-9 did not inhibit CCK-8-induced secretion.

These things considered together, it is conceivable that CaMKIV is an important serine/threonine kinase that participates in pancreatic acinar stimulus–secretion coupling. This is surprising because a previous study reported that only brain showed significant CaMKIV activity using peptide γ and other tissues including lung, heart, skeletal muscle and adrenal gland showed very little, if any, activity

[23]. However, our studies clearly showed that CaMKIV exists and functions in pancreatic acinar stimulus–secretion coupling. CaMKIV was initially named CaMK-Gr, as it is enriched in cerebellar granule cells [48,49]. The presence of extended sequences enriched in glutamate residues may influence the subcellular distribution of this kinase [48]. As pancreatic acini possess abundant zymogen granules, there is a possibility that CaMKIV may be located in these areas. CaMKIV was first demonstrated in mouse brain by cDNA cloning and its characteristics from rat brain have been extensively studied [48–51]. CaMKIV is thought to play a pivotal role in Ca^{2+}-mediated neuronal communication [48–51]. Our study further suggested that CaMKIV functions in Ca^{2+}-mediated pancreatic exocytosis.

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