# CONVERSION TO $Ca^{2+}$-INDEPENDENT FORM OF $Ca^{2+}$/CALMODULIN PROTEIN KINASE II IN RAT PANCREATIC ACINI

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**SUMMARY:** CCK rapidly converted $Ca^{2+}$/calmodulin kinase II (CaMK II) to a $Ca^{2+}$-independent form with peak action at 30 sec followed by decline to the basal level at 10 min. The threshold concentration of CCK for this action was 30 pM and maximum effect occurred at 1 nM, which induced a 6-10-fold increase. Bombesin and carbachol similarly induced CaMK II autonomous activity, whereas secretin and JMV 180 did not. Ionomycin induced a more stable elevation of CaMK II autonomous activity and the intracellular $Ca^{2+}$ chelator, BAPTA/AM, blocked the effect of CCK. In conclusion, pancreatic CaMK II is rapidly activated by a large increase in $[Ca^{2+}]_i$ generated by either stimulation of   phosphatidylinositol pathway or by an influx of extracellular $Ca^{2+}$.   © 1994 Academic Press, Inc.

The increase in $[Ca^{2+}]_i$  in pancreatic acinar cells following   activation of phospholipase C  is considered  an important signal for regulating cellular  function of these cells (1). A leading candidate  for mediating the  action of $Ca^{2+}$ in acinar and other cells  is $Ca^{2+}$/calmodulin protein kinase II (CaMK II), which is widely distributed, and   has multiple substrates (2,3). In vitro studies using purified CaMK II have demonstrated that after  binding  $Ca^{2+}$/calmodulin, the kinase undergoes  auto-phosphorylation that converts it from a $Ca^{2+}$-dependent to a $Ca^{2+}$-independent form (4-6). Recently, the activation of CaMK II in several cell types  has also been studied, and   $Ca^{2+}$-independent  activity   was shown to be induced by some hormones, transmitters and  depolarization (7-10), indicating the important signaling action of this enzyme. Although the existence of CaMK II in exocrine pancreatic cells has been reported (11,12),  and an involvement of CaMK II in pancreatic function has been suggested (1,13,14),  no direct evidence exists as to  the activation of this enzyme in pancreatic cells.  In the present work we  studied the response of CaMK II to various stimuli in pancreatic acini and found that a large  increase in $[Ca^{2+}]_i$ was necessary and sufficient to induce $Ca^{2+}$-independent or autonomous activity.

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## MATERIALS AND METHODS

**Materials** The following reagents were purchased: calmodulin from Calbiochem (La Jolla, CA); autocamtide 2 (KKALRRQETVDAL) from University of Michigan Protein Facility (Ann Arbor, MI); carbachol, and secretin from Sigma Co. (St. Louis MO); bombesin from Bachem (Torrance CA); BAPTA/AM from Molecular Probes, (Eugene, OR); collagenase from Worthington Biochemicals (Freehold NJ); essential amino acids from GIBCO (Grand Island NY) and JMV180 from Research Plus (Natrick, MA). CCK was a gift from the Squibb Research Institute (Princeton NJ).

**Preparation of acini and stimulation** Rat pancreatic acini were prepared by collagenase digestion as reported previously (15). Acini were suspended in HEPES-buffered Ringer (HR) solution supplemented with 1.28 mM $CaCl_2$, 11.1 mM glucose, Eagle's minimal amino acids, 0.1 µg/ml soybean trypsin inhibitor, and 10 mg/ml bovine serum albumin, and preincubated at 37 °C for 30 min. One ml aliquots (about 1 mg acinar protein) were then transferred to 17x100 mm polystyrene tubes and stimulated with agonists at 37 °C. In some experiments, acini were washed twice with and resuspended in $Ca^{2+}$-free HR buffer containing 1 mM EGTA and immediately stimulated with CCK for various times. When effect of BAPTA/AM was studied, acini were preincubated in HR buffer containing 50 µM BAPTA/AM for 30 min, and were then centrifuged, washed and resuspended in fresh HR buffer and immediately stimulated with CCK for 30 sec. The stimulation was terminated by addition of 2 ml ice-cold homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 10 µg/ml leupeptin, 10 mM sodium pyrophosphate and 0.4 mM ammonium molydate, followed by sonication for 10 sec. The sonicated mixture was centrifuged at 4 °C for 2 min in a microcentrifuge and the supernatant was assayed for CaMK II activity.

**CaMK II activity assay** CamK II activity was assayed according to Jefferson et al (6) by use of autocamtide 2 as a specific substrate. For $Ca^{2+}$-stimulated activity, 5 µg of protein was mixed with assay buffer in a final volume of 25 µl containing 50 mM PIPES pH 7.0, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 µg/ml calmodulin, 10 µM autocamtide 2, 40 µM Mg ATP, and 5 µCi [$\gamma$-$^{32}$P] ATP. The $Ca^{2+}$-independent autonomous activity was measured in the same buffer except that $CaCl_2$ and calmodulin were omitted and 1 mM EGTA was added. The reaction preceded at 30 °C for 10 min, since preliminary experiments showed both $Ca^{2+}$-dependent and autonomous activities were linear over this time. The reaction was stopped by the addition of ice-cold TCA to 5%. After centrifugation, 15 µl of supernatant was spotted onto phosphocellulose paper (Whatman p81), rinsed four times with 1% phosphoric acid and the radioactivity retained on the paper was determined by liquid scintillation counting. The autonomous activity was expressed as the percentage of the $Ca^{2+}$-stimulated activity. The maximum $Ca^{2+}$-dependent activity did not change significantly under our experimental conditions.

## RESULTS AND DISCUSSION

Activation of CaMK II in pancreatic acini was studied by observing the ability of secretagogues to induce $Ca^{2+}$-independent or autonomous activity. After preincubation in HR buffer, acini were resuspended in the same buffer or the buffer without $Ca^{2+}$ containing 1 mM EGTA, followed by stimulation with CCK. The $Ca^{2+}$-independent activity in the quiescent acini was very low, being 3.7 ± 1.0 % of maximal activity measured in the presence of $Ca^{2+}$ and calmodulin (n=4). CCK, in both
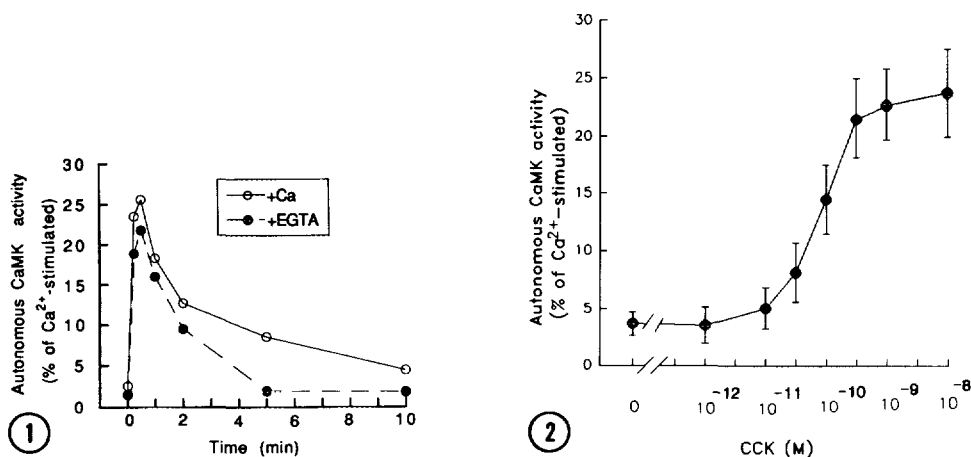
**Fig.1.** Time-course of CCK-induced activation of CaMK II in presence and absence of extracellular $Ca^{2+}$. Acini were suspended in HR solution (1.28 mM $Ca^{2+}$) or without $Ca^{2+}$ containing 1 mM EGTA and stimulated with CCK (1 nM). Acini were then sonicated in homogenization buffer and both $Ca^{2+}$-dependent and independent CaMK II activities assayed. Autonomous activity was expressed as % of $Ca^{2+}$-stimulated activity. Similar results were obtained from two experiments.

**Fig. 2.** Concentration-dependent effect of CCK on CaMK II autonomous activity. Acini were incubated with CCK for 30 sec and then sonicated and autonomous activity in the supernatant was determined. Results are means ± SE of 4 separate experiments.

presence and absence of $[Ca^{2+}]_o$, rapidly augmented autonomous activity within 15 sec; the maximum 10-fold increase was reached at 30 sec and then declined (Fig.1). In the absence of extracellular calcium, CCK was only slightly less effective then control. These results clearly demonstrate the importance of mobilization of $Ca^{2+}$ from intracellular stores by CCK for converting CaMK II to an autonomous form. Nevertheless, the basal autonomous activity was lower and the decline of elevated autonomous activity was accelerated in the absence of extracellular calcium, indicating the influx of $Ca^{2+}$ caused by CCK may play a role in maintaining the autonomous activity. The decline of autonomous activity may be a result of dephosphorylation, since previous studies on purified CaMK II from brain showed that protein phosphatase 1 and phosphatase 2A could reverse the $Ca^{2+}$-independence of CaMK II (16) and that okadaic acid increased the basal and stimulated $Ca^{2+}$-independent activity (17).

This effect of CCK was concentration-dependent. CCK increased $Ca^{2+}$-independent activity, with the threshold concentration being about 30 pM and the maximum effect observed at 1 nM, at which point autonomous activity was increased 6 fold over control (Fig.2). Previous studies from our laboratory found that CCK at a concentrations lower than 30 pM induced only $Ca^{2+}$ oscillations, whereas at concentrations of 100 pM or higher caused a transient large increase in $[Ca^{2+}]_i$ (15,18). Thus it seems that $Ca^{2+}$ oscillations are not strong enough to switch CaMK II
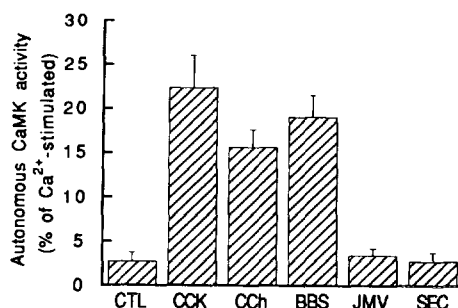
**Fig. 3**. Effects of various agonists on autonomous CaMK II activity. Acini were incubated with CCK (1 nM), bombesin (BBS, 10 nM), carbachol (CCh, 0.1 mM), JMV180 (JMV, 1 μM), secretin (SEC, 0.1 μM) or only HR buffer (CTL) for 30 sec. Acini were then sonicated and CaMK II activity was assayed. Results are means ± SE of 3 separate experiments.

to a maintained $Ca^{2+}$-independent form in pancreatic acinar cells. To further confirm this, we studied the action of JMV180, which activates the high affinity state of CCK receptor and stimulates amylase release, but only induces $[Ca^{2+}]_i$ oscillations (15, 19). JMV180 at 1 μM had little effect on CaMK II autonomous activity (Fig.3), therefore supporting the important role of a sharp increase in $[Ca^{2+}]_i$ in generation of a $Ca^{2+}$-independent form of CaMK II.

Similar to CCK, other secretagogues particularly bombesin and carbachol, upon binding their receptors on acinar cells, also activate phospholipase C, generate $IP_3$ and induce a large increase in $[Ca^{2+}]_i$ (1, 20). Both were found to increase CaMK II autonomous activity (Fig.3). Secretin which stimulates pancreatic cells through adenylate cyclase pathway was unable to convert CaMK II to an autonomous form (Fig.3). These results suggest that the activation of G protein coupled phospholipase C is an important mechanism for activation of CaMK II. However, activation of phospholipase C generates two second messengers, one is $Ca^{2+}$ and the other is diacylglycerol that stimulates protein kinase C. In contrast to $Ca^{2+}$, protein kinase C may have no direct effect on converting CaMK II to a $Ca^{2+}$-independent form, since stimulation of acini with TPA failed to generate the autonomous CaMK II activity (data not shown).

The importance of $[Ca^{2+}]_i$ in the generation of autonomous CaMK II activity was further studied by experimental alteration of $[Ca^{2+}]_i$ levels. First we used the $Ca^{2+}$ ionophore, ionomycin to enhance the influx of extracellular $Ca^{2+}$ as well as to mobilize intracellular $Ca^{2+}$, and found an enhancement of $Ca^{2+}$-independent activity of CaMK II (left panel of Fig. 4). Differing from the transient increase in autonomous CaMK II activity stimulated by CCK, ionomycin induced a more stable elevation of $Ca^{2+}$-independent activity. These differences may be attributable to different $Ca^{2+}$ responses to CCK and ionomycin, since CCK induced a transient sharp increase of
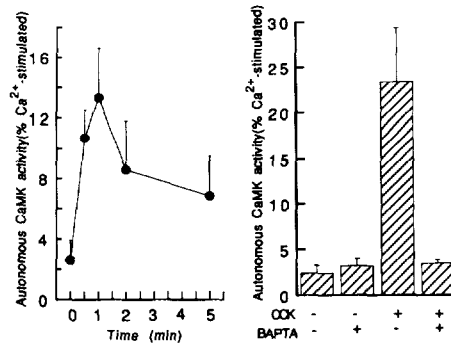
**Fig.4.** Left panel: effects of ionomycin on autonomous CaMK II activity. Acini were incubated in HR solution followed by stimulation with ionomycin (2 µM) for various times. Acini were then sonicated and CaMK II activity was assayed. Right panel: effect of BAPTA/AM on effect of CCK. Acini were preincubated with or without BAPTA/AM (50 µM) for 30 min, followed by stimulation with CCK (1 nM) for 30 sec. Acini were then sonicated and autonomous CaMK II activity was determined. Results are means ± SE of 3 separate experiments.

$[Ca^{2+}]_i$ (about 500 nM) followed by a rapid decline; whereas ionomycin stimulation resulted in a large increase of $[Ca^{2+}]_i$ (about 400 nM) followed by a elevated plateau (about 250 nM) for more than 10 min, when measured using fura-2 loaded acini (data not shown). Finally whether chelation of $[Ca^{2+}]_i$ would block the conversion of autonomous CaMK II activity generated by CCK was studied. We found that a 30 min preincubation with 50 µM BAPTA/AM, an intracellular $Ca^{2+}$ chelator (21) to reduce intracellular $Ca^{2+}$, completely abolished CCK-induced elevation of autonomous CaMK II activity (right panel of Fig. 4). This concentration of BAPTA/AM has previously been shown to block CCK-stimulated amylase release (15). These data of this study together with previous work in acini measuring changes in $[Ca^{2+}]_i$ (1) indicate that activation of CaMK II and conversion to a $Ca^{2+}$-independent form is driven by the large transient increase in $[Ca^{2+}]_i$ induced by high concentrations of secretagogues. Whether the lower concentrations of agonists activate CaMK II within the cell without conversion to an autonomous form requires further investigation.

## ACKNOWLEDGMENTS

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