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Muscarinic stimulation of calcium/calmodulin-dependent protein kinase Ⅱ in isolated rat pancreatic acini[1]

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KEY WORDS atropine; bethanechol; calcium; calmodulin; islets of Langerhans; protein kinases; sincalide; vasoactive intestinal peptide

AIM: To study whether M_3 receptor occupation would lead to activation of calcium/calmodulin-dependent protein kinase Ⅱ (CaM kinase Ⅱ). **METHODS**: In this study, we isolated rat pancreatic acini by collagenase digestion; measured the Ca^{2+}/calmodulin-independent activity of autophosphorylated form of the CaM kinase Ⅱ both before and after stimulation of the acini with muscarinic secretagogue bethanechol (Bet). **RESULTS**: Bet stimulated the activation of, or generation of Ca^{2+}-independent activity of, this kinase, in a concentration ($0.0001-1$ mmol·L^{-1}) and time ($5-300$ s)-dependent manner; with Bet of 100 μmol·L^{-1}, Ca^{2+}-independent activity increased from an unstimulated level of 4.5 ± 0.3 ($n=4$) to 8.9 ± 1.3 ($n=4$, $P<0.05$) at 5 s. Another Ca^{2+} mobilizing secretagogue cholecystokinin (CCK) also activated the kinase; at 1 μmol·L^{-1}, CCK increased Ca^{2+}-independent kinase activity to 12.9 ± 0.5 ($n=6$, $P<0.05$). Vasoactive intestinal peptide (VIP) at 1 μmol·L^{-1} did not produce significant Ca^{2+}-independent kinase activity (from control 3.90 ± 0.28 to 4.53 ± 0.47, $n=6$, $P>0.05$). Atropine completely blocked Bet activation of the kinase. **CONCLUSION**: CaM kinase Ⅱ plays a pivotal role in digestive enzyme secretion, especially during the initial phase of amylase secretion.

The muscarinic receptors (M_3) in rat pancreatic acinar cells belong to G protein-coupled serpentine receptors. There are 3 subtypes of muscarinic receptors: M_1, M_2, and M_3[1]. Five subtypes (m_1, m_2, m_3, m_4, m_5) have been molecularly cloned and functionally expressed, and M_1/m_1, M_2/m_2, and M_3/m_3 are identical[2]. The muscarinic receptors in the rat pancreatic acini belong to the M_3 subtype[3], which are coupled to a Gq-protein[4] to stimulate phospholipase C_β, to generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1, 2-sn-diacylglycerol (DAG)[5]. Intracellular Ca^{2+} is elevated both by release from intracellular calcium stores after stimulation of IP_3 receptors and through receptor-operated calcium channels on the plasma membrane[6,7]. Elevated $[Ca^{2+}]_i$ may in turn bind to calmodulin and trigger various cellular processes, eg, stimulate calcium/calmodulin-dependent protein kinases, to finally lead to exocytosis.

In the present work, we studied the muscarinic activation of calcium/calmodulin-dependent protein kinase Ⅱ (CaM kinase Ⅱ), and investigated its role in digestive enzyme secretion.

MATERIALS AND METHODS

Pancreatic acini were prepared from Sprague-Dawley rats[8] by collagenase (Sigma) digestion (37 ℃). The standard incubation buffer was composed of NaCl 118, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.0, NaH_2PO_4 1.16, glucose 5.6 mmol·L^{-1}, bovine serum albumin 2 g·L^{-1}, soybean trypsin inhibitor 0.1 g·L^{-1}, HEPES 25 mmol·L^{-1}, MEM formula mixture of amino acids (Gibco), and pH 7.3 with NaOH 1 mol·L^{-1}. The solution was gassed with O_2 for at least 30 min before use. The acini were resuspended in 10 mL gassed buffer after preparation and incubated for 30 min before resuspension in 10 mL gassed buffer, 0.25 mL aliquots were then added to each well of a 48-well tissue culture plate; stimulus was applied to each well for a set time point and Ca^{2+}-independent kinase activity determined.

To determine the activation of CaM kinase Ⅱ, bethanechol (Bet) 0.25 mL (Sigma), or sincalide (CCK-8), vasoactive intestinal peptide (VIP) (both from Cambridge Research Biochemicals) in incubation buffer was added to each well containing acini 0.25 mL, and action stopped with 1 mL ice-cold stop solution (mmol·L^{-1}): Tris·HCl 20, egtazic acid 0.5, edetic acid 1, $Na_2P_2O_7$ 10, Na molybdate 2, *DL*-dithiothreitol 2, leupeptin (Calbiochem) 1 mg·L^{-1}, soybean trypsin inhibitor (Sigma) 0.2 g·L^{-1}, followed immediately

[1] This paper is dedicated to Prof Tomio Kanno, on the occasion of his retirement

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by sonication (100 W for 15 s). Then 50 μL of the sonicate was taken to assay for Ca^{2+}-dependent and Ca^{2+}-independent activities of CaM kinase Ⅱ[9]. Briefly, to each 1.5 mL microcentrifuge tube was added the following (on ice): 10 μL reaction buffer (piperazine-*N*-*N*′-bis[2-ethanesulfonic acid] (PIPES) or *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) 500 mmol·L^{-1}, $MgCl_2$ 100 mmol·L^{-1}, bovine serum albumin 1 g·L^{-1}), 10 μL $CaCl_2$ 5 mmol·L^{-1} and 10 μL calmodulin (Calbiochem) 0.3 g·L^{-1} or 10 μL egtazic acid 10 mmol·L^{-1} and 10 μL distilled water, 10 μL substrate peptide (CaM kinase Ⅱ α 280-293) 0.2 g·L^{-1}, 10 μL [^{32}P]ATP (Amersham) 40 μmol·L^{-1} (specific activity 37 GBq·mmol^{-1}). The mixture was first incubated at 30 ℃ for 1 min, and reaction initiated by the addition of 50 μL sonicate; 3 min later the reaction was terminated with 5 μL trichloroacetic acid (TCA) 100 %. The reaction mixture was centrifuged in microfuge (IEC Centra-M) for 2 min, and supernatant applied to spin column (with 125 μL Dowex AG 1×8, Biorad) sequentially in 40 μL aliquots twice. The final eluate (70 μL) was spotted on phosphocellulose paper (Whatman P_{81}, 2×2 cm^2), air-dried, and rinsed with slow-running tap water for 15-20 min before Cerenkov counting. All cpm was subtracted from blank (with [^{32}P]ATP alone without either substrate or sonicate) and unless stated otherwise results expressed as the percentage of cpm from Ca^{2+}-independent reaction over the cpm from Ca^{2+}-dependent reaction (the reaction rate was linear under our experimental conditions, and all experiments were done at least three times). It should be noted that CaM kinase Ⅱ activity is sometimes expressed in absolute terms as dpm·min^{-1} or mol·min^{-1} of [^{32}P] transferred, for clarity, the Ca^{2+}-independent activity here is expressed as % of total activity. All data were expressed as $\bar{x} \pm s$, and t test was used to determine statistical significance.

RESULTS

Bet 100 μmol·L^{-1} elicited a rapid generation of Ca^{2+}-independent CaM kinase Ⅱ activity, the earliest time point detected was 5 s and the Ca^{2+}-independent activity increased from 4.5 ± 0.3 (n=4) to 8.9 ± 1.1 (n=4). The activity declined gradually in the continuous presence of Bet and was down to 5.30 ± 0.18 (n = 4) by min 5 (Fig 1).

The generation of Ca^{2+}-independent CaM kinase Ⅱ activity by Bet was concentration-dependent. Ca^{2+}-independent CaM kinase Ⅱ activity was determined 60 s after addition of Bet. At 1 mmol·L^{-1} the Ca^{2+}-independent CaM kinase Ⅱ activity increased from 4.5±0.6 (n=3) to 8.6 ± 0.6 (n = 3). Significant activation was seen only from 100 μmol·L^{-1} (7.5 ± 0.3, $P<0.05$). Detectable activation was seen from 10 μmol·L^{-1} (5.1±0.4, n = 3; $P>0.05$). Bet did not have any effects below 1 μmol·L^{-1} (4.2 ± 0.6, n = 3; $P>0.05$) (Fig 1).

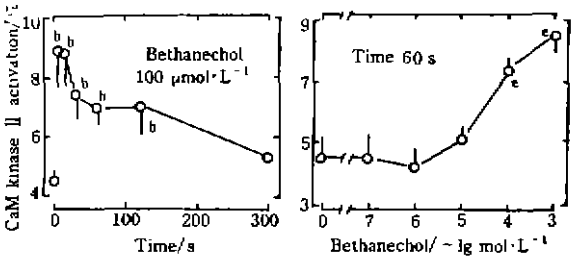

Fig 1. Generation of Ca^{2+}-independent CaM kinase Ⅱ activity by bethanechol. n = 4 rats, $\bar{x} \pm s$. $^bP<0.05$ *vs* 0 s. n = 3 rats for each bethanechol concentration, $\bar{x} \pm s$. $^eP<0.05$ *vs* 0 mol·L^{-1}.

Atropine 10 μmol·L^{-1} completely abolished the effect of Bet 100 μmol·L^{-1}, indicating that the muscarinic receptors were solely responsible for the activation of CaM kinase Ⅱ by Bet. Atropine (10 μmol·L^{-1}) was added 2 min before addition of Bet 100 μmol·L^{-1} to acini, percentage of Ca^{2+}-independent kinase activity was determined 15 s after addition of Bet. In this set of experiments (n =6), Ca^{2+}-independent CaM kinase Ⅱ activity in control acini was 3.64 ± 0.47. Atropine alone had no effect (5.2 ± 1.2, $P>0.05$), but completely blocked the Bet effect, to reduce it from 14.5±2.1 to 4.0±0.4, $P<0.05$ (Tab 1).

Besides M_3 receptors, both CCK and VIP

Tab 1. Generation of Ca^{2+}-independent CaM kinase Ⅱ activity. n = 6 rats, $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$ *vs* control; $^dP>0.05$ *vs* atropine.

Treatment	CaM kinase Ⅱ activation/%
Control	3.6±0.5
Bet 100 μmol·L^{-1}	14.5±2.1^b
Atropine 10 μmol·L^{-1}	5.2±1.2^a
Bet + atropine	4.0±0.4^d
Control	3.9±0.3
Sincalide 1 μmol·L^{-1}	12.9±0.5^b
VIP 1 μmol·L^{-1}	4.5±0.5^a

receptors can stimulate amylase secretion in isolated acini. Buffer alone (control), sincalide (1 μmol $\cdot L^{-1}$), VIP (1 μmol $\cdot L^{-1}$) was added to acini, and percentage of Ca^{2+}-independent CaM kinase Ⅱ activity was determined 15 s after addition. VIP 1 μmol $\cdot L^{-1}$ did not produce any Ca^{2+}-independent CaM kinase Ⅱ activity (4.53 ± 0.47 *vs* control 3.90 ± 0.28, $P > 0.05$). In contrast, the Ca^{2+}-mobilizing hormone sincalide 1 μmol $\cdot L^{-1}$, produced a 2-fold (3 fold of control) net increase of Ca^{2+}-independent activity (12.9 ± 0.5, $P < 0.05$) (Tab 1).

DISCUSSION

M_3 receptor stimulation in the isolated pancreatic acinar cells has been shown to lead to IP_3-dependent Ca^{2+} release from intracellular Ca^{2+} stores, and direct Ca^{2+} influx from extracellular space. However, what happens next is not clear. It is known that $[Ca^{2+}]_i$ increases together with DAG triggers protein kinase C translocation from the cytosol to cell membranes[10], but whether $[Ca^{2+}]_i$ increases also stimulate other protein kinases is not known. In this study, we have taken advantage of the fact that, CaM kinase Ⅱ, once stimulated, undergoes instantaneous autophosphorylation to generate partial Ca^{2+}-independent activity[11]. By measuring the changes in the Ca^{2+}-independent activity, we have demonstrated that muscarinic receptor activation directly leads to the activation of CaM kinase Ⅱ. The rapid nature of the onset of activation of CaM kinase Ⅱ (Fig 1) indicates that this kinase plays an important role in the initiation rather than the maintenance of amylase secretion.

The maximal concentration of Bet for stimulating amylase secretion has been reported to be around 100 μmol $\cdot L^{-1}$[12], and we report here that at 10 μmol $\cdot L^{-1}$, Bet was just beginning to stimulate the activation of CaM kinase Ⅱ and at 100 μmol $\cdot L^{-1}$, the activation was near-maximal. To our knowledge, this is the first report demonstrating a direct correlation of activation of CaM kinase Ⅱ and amylase secretion. At 1 mmol $\cdot L^{-1}$, Bet starts to significantly inhibit amylase secretion, therefore generating the typical "bell-shaped" dose-response curve[8,12]. But at 1 mmol $\cdot L^{-1}$, Bet continues to stimulate the activation of CaM kinase Ⅱ (Fig 1), indicating that something other than CaM kinase Ⅱ must be responsible for the supra-maximal inhibition of amylase secretion. This could be some inhibitory second messenger(s).

We have shown here that the Ca^{2+}-mobilizing sincalide, like Bet, also activated CaM kinase Ⅱ. VIP, which acts through the generation of cAMP[13], did not have any effects (Tab 1). This together with the fact that atropine blocked the effects of Bet (Tab 1) indicates that the activation of CaM kinase Ⅱ is indeed physiological and functional. The concentration of sincalide and VIP used here, 1 μmol $\cdot L^{-1}$, may seem high in comparison with the dose-response relationship for amylase secretion[12,14]; but judging from the very rapid nature of its appearance (Fig 1), it is possible that the Ca^{2+}-independent activity at shorter time-periods (1 or 2 s, which was impossible to detect in the present study) is much higher than 3-fold. A more rapid method will allow studies at lower sincalide concentrations. Nonetheless, we have unequivocally demonstrated the specific activation of CaM kinase Ⅱ in isolated pancreatic acinar cells.

Autophosphorylation of purified CaM kinase Ⅱ (to homogeneity) generally generates partial Ca^{2+}-independent activity. Using myosin light chain to measure enzyme activity, Miyamoto *et al* reported that maximal percentage of the calcium-independent activity was about 25 %[15]. If indeed this is the case, the percentage of Ca^{2+}-independent activity seen in the present study would represent half-maximal stimulation of CaM kinase Ⅱ under maximal stimulation of the pancreatic acinar cells.

Recent reports of the role of this kinase in insulin secretion in rat pancreatic islets and cultured β-cells (see Urquidi and Ashcroft, 1995) suggest that CaM kinase Ⅱ plays a role in the stimulus-secretion coupling in both exocrine and endocrine pancreas.

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分离大鼠胰腺腺泡细胞中对钙离子/钙调素依赖性蛋白激酶Ⅱ的M-型胆碱能激活

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关键词 阿托品；乌拉胆碱；钙；钙调素；胰岛；蛋白激酶类；辛卡利特；血管活性肠肽

胆碱能

目的：研究 M_3 型胆碱能受体与配体的结合可否导致钙离子/钙调素依赖性蛋白激酶Ⅱ (CaM kinase Ⅱ)的激活. 方法：用胶原水解酶法分离大鼠胰腺腺泡，并检测乌拉胆碱(Bet)刺激腺泡细胞前后自身磷酸化型的 CaM kinase Ⅱ 的活性. 结果：Bet 对该激酶的活化是时间(5 - 300 s)和浓度($0.0001 - 1\ mmol \cdot L^{-1}$)依赖性的，并可被阿托品所阻断. Bet 100 $\mu mol \cdot L^{-1}$ 在 5 s 内可以引起 CaM kinase Ⅱ 的激活(非钙离子/钙调素依赖性激酶活性从 4.5 ± 0.3，增加到 8.9 ± 1.3)；辛卡利特 1 $\mu mol \cdot L^{-1}$ 也导致该酶的激活(增加到 12.9 ± 0.5)，而血管活性肠肽 1 $\mu mol \cdot L^{-1}$ 无作用. 结论：CaM kinase Ⅱ 在消化酶的分泌中，尤其是消化酶分泌的始发期，起着重要作用.

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