Ryanodine-sensitive Ca\(^{2+}\) release mechanism of rat pancreatic acinar cells is modulated by calmodulin

Terutaka Ozawa *

Department of Physiology, Tohoku University School of Medicine Seiryocho 2-1, Aoba-ku, Sendai, 980-8575, Japan

Received 24 May 1999; received in revised form 5 October 1999; accepted 5 October 1999

Abstract

The effects of calmodulin (CaM) and CaM antagonists on microsomal Ca\(^{2+}\) release through a ryanodine-sensitive mechanism were investigated in rat pancreatic acinar cells. When caffeine (10 mM) was added after a steady state of ATP-dependent \(^{45}\)Ca\(^{2+}\) uptake into the microsomal vesicles, the caffeine-induced \(^{45}\)Ca\(^{2+}\) release was significantly increased by pretreatment with ryanodine (10 \(\mu\)M). The presence of W-7 (60 \(\mu\)M), a potent inhibitor of CaM, strongly inhibited the release, while W-5 (60 \(\mu\)M), an inactive CaM antagonist, showed no inhibition. Inhibition of the release by W-7 was observed at all caffeine concentrations (5–30 mM) tested. The presence of exogenously added CaM (10 \(\mu\)g/ml) markedly increased the caffeine (5–10 mM)-induced \(^{45}\)Ca\(^{2+}\) release and shifted the dose–response curve of caffeine-induced \(^{45}\)Ca\(^{2+}\) release to the left. Cyclic ADP-ribose (cADPR, 2 \(\mu\)M)-induced \(^{45}\)Ca\(^{2+}\) release was enhanced by the presence of ryanodine (10 \(\mu\)M), cADPR (2 \(\mu\)M)- or ryanodine (500 \(\mu\)M)-induced \(^{45}\)Ca\(^{2+}\) release was also inhibited by W-7 (60 \(\mu\)M), but not by W-5 (60 \(\mu\)M), and was stimulated by CaM (10 \(\mu\)g/ml). These results suggest that the ryanodine-sensitive Ca\(^{2+}\) release mechanism of rat pancreatic acinar cells is modulated by CaM. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ca\(^{2+}\) release; Ryanodine; Calmodulin; Calmodulin antagonist; Pancreatic acinar cell; Endoplasmic reticulum

1. Introduction

In excitable cells, the ryanodine-sensitive Ca\(^{2+}\) release mechanism (ryanodine receptor), which is activated by caffeine, ryanodine or Ca\(^{2+}\) has been well characterized, and the channel protein has been purified [1] and cloned [2,3]. However, there have been very few reports concerning the properties of this mechanism in non-excitable cells. Recently, characterization of the ryanodine-sensitive Ca\(^{2+}\) release mechanism in rat parotid acinar cells was investigated in detail by using isolated microsomal vesicles [4,5]. It was shown that not only caffeine and ryanodine, but also cyclic ADP-ribose (cADPR), which is an endogenous regulator of the ryanodine receptor [6], and cAMP activate the mechanism. In pancreatic acinar cells, the presence of a Ca\(^{2+}\)-, caffeine- or cADPR-induced Ca\(^{2+}\) release mechanism has been suggested from the study of Ca\(^{2+}\)-dependent currents [7,8]. Characteristic features of the caffeine-sensitive Ca\(^{2+}\) store in pancreatic microsomal vesicles, which

Abbreviations: CaM, calmodulin; cADPR, cyclic ADP-ribose; ER, endoplasmic reticulum; IP\(_3\), inositol 1,4,5-trisphosphate; SR, sarcoplasmic reticulum; W-5, N-(6-aminoethyl)-1-naphthalenesulfonamide; W-7, N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide; W-12, N-(4-aminobutyl)-2-naphthalenesulfonamide; KN-62, 1-[N,O-bis(1, 5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazin
possesses a releasing mechanism activated by caffeine or Ca^{2+}, have been also described [9,10]. However, detailed information concerning the releasing mechanism, including ryanodine sensitivity to the release, has not yet been obtained.

Ryanodine receptor proteins in the sarcoplasmic reticulum (SR) of skeletal muscle are known to have several binding sites to calmodulin (CaM) from the study of the primary sequence of the receptor protein [11,12] and from a binding assay of ^{125}I-labeled CaM to the receptor [13]. In addition, the releasing mechanism is activated or inhibited by the presence of CaM depending on the Ca^{2+} concentration of the medium [14,15]. In sea urchin eggs, enhancement of Ca^{2+}-, caffeine- or cADPR-induced Ca^{2+} release by CaM has been demonstrated [16,17]. Recently, in rat permeabilized parotid acinar cells, it was shown that cADPR-mediated Ca^{2+} release was enhanced by CaM and was decreased by the CaM antagonist W-7 [18]. However, unlike for excitable cells, details of the CaM-dependent mechanism are unclear.

In the present study, ryanodine sensitivity to Ca^{2+} release induced by drugs that are known to activate the ryanodine-sensitive mechanism in excitable tissues and CaM involvement in the Ca^{2+} release were investigated in rat pancreatic acinar cells by using isolated microsomal vesicles. The present data clearly indicate that CaM can modulate the ryanodine-sensitive Ca^{2+} release mechanism that is present in the endoplasmic reticulum (ER) of pancreatic acinar cells and is activated by caffeine, ryanodine or cADPR.

2. Materials and methods

2.1. Materials

Creatine kinase and trypsin inhibitor were obtained from Boehringer Mannheim (Mannheim, Germany). Adenosine trisphosphate dipotassium salt (K_{2}ATP), creatine phosphate disodium salt, benzamidine, ruthenium red, CaM purified from the bovine brain, and the CaM antagonists (W-7, W-5 and W-12) were purchased from Sigma (St. Louis, MO, USA). CaM kinase II inhibitor KN-62 was from Seikagaku (Tokyo, Japan). cADPR was obtained from Wako Pure Chemical (Osaka, Japan). Ryanodine was obtained from Calbiochem (La Jolla, CA, USA). ^{45}CaCl_{2} (32.76 Ci/g) was purchased from New England Nuclear (Boston, MA, USA).

2.2. Preparation of microsomal vesicles

Pancreatic microsomal vesicles were prepared as described previously [9,19,20]. Briefly, isolated acinar cells from male Wistar rats (180–200 g) were homogenized in an ice-cold ‘mannitol buffer’ containing (in mM): 290 mannitol, 10 KCl, 5 HEPES, 1 MgCl_{2}, 1 benzamidine, 0.2 phenylmethylsulfonyl fluoride, and 20 μg/ml of trypsin inhibitor, adjusted with Tris to pH 7.0. After centrifugation of the cell homogenate at 11 000×g for 15 min, the ‘fluffy layer’ on top of the pellet, which is enriched by about two-fold in ER [19], was collected. The microsomal vesicles were kept frozen in liquid nitrogen until use. The protein concentration was measured by the method of Bradford [21] using bovine serum albumin as a standard.

2.3. Measurement of ^{45}Ca^{2+} uptake

Isolated membrane vesicles were preincubated for 15 min at a protein concentration of 1.0 mg/ml in 0.5 ml of an incubation buffer containing (in mM): 155 KCl, 5 HEPES, 0.15 CaCl_{2} (corresponding to 0.002 free Ca^{2+} concentration), 1.0 EDTA, 3.57 MgCl_{2} (corresponding to 1.0 free Mg^{2+} concentration), 10 NaN_{3}, 0.005 oligomycin, 5 μg/ml antimycin A, 10 creatine phosphate, 8 U/ml creatine kinase, and 1 μCi/ml of ^{45}CaCl_{2}, adjusted with Tris/HCl to pH 7.0 at 25°C. ^{45}Ca^{2+} uptake into the vesicles was initiated by the addition of K_{2}ATP at a final concentration of 2 mM. After a steady state of ^{45}Ca^{2+} uptake, caffeine, ryanodine or cADPR was added into the medium. At indicated times, ^{45}Ca^{2+} content of membrane vesicles was determined by a rapid filtration technique, as described previously [4]. The radioactivity was counted in a liquid scintillation counter (LS6500, Beckman).

2.4. Statistical analysis

Values are presented as mean ± S.E. Statistical analysis was performed using Student’s t-test.
3. Results

3.1. Caffeine-induced $^{45}\text{Ca}^{2+}$ release

A steady state of $^{45}\text{Ca}^{2+}$ uptake into pancreatic microsomal vesicles was reached 10–20 min after the addition of ATP (Figs. 1, 2A, 3 and 5) and remained stable until the end of observation (Fig. 1). The $^{45}\text{Ca}^{2+}$ content taken up into microsomal vesicles for 20 min after the addition of ATP was $4.6 \pm 0.1$ nmol/mg protein ($n = 139$). The effect of caffeine (10 mM) on $^{45}\text{Ca}^{2+}$ release from the vesicles at a steady state of $^{45}\text{Ca}^{2+}$ uptake was tested in the presence and absence of ryanodine (10 $\mu$M). Pretreatment of the vesicles with ryanodine did not affect the ATP-dependent $^{45}\text{Ca}^{2+}$ uptake before the addition of caffeine (Fig. 1) but, significantly enhanced caffeine (10 mM)-induced $^{45}\text{Ca}^{2+}$ release (Fig. 1) (% of released $^{45}\text{Ca}^{2+}$ over a 6-min period after the addition of caffeine to the $^{45}\text{Ca}^{2+}$ that had been taken up by the ATP-dependent mechanism: $10.0 \pm 0.8\%$ ($n = 9$) without ryanodine vs. $14.9 \pm 1.7\%$ ($n = 7$) with ryanodine ($P < 0.05$)). Previously, it was shown that caffeine-induced Ca$^{2+}$ release from the vesicles was inhibited by ruthenium red (10 $\mu$M), an inhibitor of the ryanodine-sensitive Ca$^{2+}$ release mechanism, but not by heparin (100 $\mu$g/ml), an inhibitor of the inositol 1,4,5-trisphosphate (IP$_3$)-sensitive mechanism [9]. This finding and the present result indicate that caffeine activates the ryanodine-sensitive Ca$^{2+}$ release mechanism, but not the IP$_3$-sensitive mechanism.

To investigate the involvement of endogenous CaM in caffeine-induced Ca$^{2+}$ release, the caffeine effect was tested in the presence and absence of the CaM antagonist W-7 [22]. Pretreatment of the vesicles with up to 60 $\mu$M of W-7 did not affect the ATP-dependent $^{45}\text{Ca}^{2+}$ uptake (Fig. 2A), but caffeine (10 $\mu$M)-induced $^{45}\text{Ca}^{2+}$ release was significantly ($P < 0.01$–0.001) inhibited by W-7 (30–120 $\mu$M) (Fig. 2B). The effect of W-5, an inactive analog of W-7 [22], was tested as a negative control. The presence of W-5 (60 $\mu$M) did not significantly ($P > 0.05$) inhibit the caffeine-induced $^{45}\text{Ca}^{2+}$ release (Fig. 2B). Another CaM antagonist, W-12 (60 $\mu$M), weakly inhibited the release of $^{45}\text{Ca}^{2+}$ (Fig. 2B), but compound 48/80 (50 $\mu$g/ml) did not inhibit the release (data not shown). Since CaM-dependent protein kinase II is involved in an activation of the release mechanism in pancreatic islets [23], the effect of KN-62, an inhibitor of CaM-dependent protein kinase II, on the caffeine-induced $^{45}\text{Ca}^{2+}$ release from pancreatic microsomes was also tested. KN-62 (10 $\mu$M) did not inhibit the caffeine (10 or 20 $\mu$M)-induced $^{45}\text{Ca}^{2+}$ release (data not shown). When the vesicles were preincubated with exogenously added CaM (10 $\mu$g/ml), the ATP-dependent $^{45}\text{Ca}^{2+}$ uptake was not affected by CaM (Fig. 2A), but caffeine (10 $\mu$M)-induced $^{45}\text{Ca}^{2+}$ release was significantly ($P < 0.01$) stimulated by CaM (Fig. 2C). The effects of W-7 (60 $\mu$M) and CaM (10 $\mu$g/ml) on the $^{45}\text{Ca}^{2+}$ release by caffeine are shown at each caffeine concentration in Fig. 2D. W-7 markedly inhibited the release at all caffeine concentrations (5–30 $\mu$M) tested. CaM significantly stimulated the release at lower concentrations (5–10 $\mu$M) of caffeine and shifted the dose–response curve of caffeine-induced $^{45}\text{Ca}^{2+}$ release to the left (Fig. 2D). These results indicate that endogenous CaM mediates caffeine-induced Ca$^{2+}$ release from pancreatic microsomes and that exogenously added CaM increases the caffeine sensitivity to the release.
3.2. Ryanodine-induced $^{45}\text{Ca}^{2+}$ release

When ryanodine (10–500 μM) was added to the medium after a steady state of $^{45}\text{Ca}^{2+}$ uptake, ryanodine released $^{45}\text{Ca}^{2+}$ from the vesicles in a concentration-dependent manner (Fig. 3) (% of released $^{45}\text{Ca}^{2+}$ over a 6-min period after the addition of ryanodine to the $^{45}\text{Ca}^{2+}$ that had been taken up: $2.3 \pm 1.0\%$ ($n=10$) at 10 μM, $4.3 \pm 1.3\%$ ($n=6$) at 50 μM, $5.4 \pm 0.7\%$ ($n=8$) at 100 μM and $7.7 \pm 1.0\%$ ($n=10$) at 500 μM). The effects of CaM and CaM antagonists on ryanodine-induced $^{45}\text{Ca}^{2+}$ release
were also tested. Ryanodine (500 μM)-induced 45Ca2+ release was significantly (P < 0.01) reduced by the presence of W-7 (60 μM), but not by W-5 (60 μM) (Fig. 4). The presence of CaM (10 μg/ml) significantly (P < 0.01) increased the release (Fig. 4).

3.3. cADPR-induced 45Ca2+ release

When a micromolar concentration of cADPR was added into the medium after a steady state of 45Ca2+ uptake, 2–10% of 45Ca2+ that had been taken up was released from the vesicles. An effect of ruthenium red or ryanodine on cADPR (2 μM)-induced 45Ca2+ release was observed. The presence of ruthenium red (30 μM) strongly inhibited the release (Fig. 5), while ryanodine (10 μM) significantly stimulated the release (Fig. 5) (% of released 45Ca2+ over an 11-min period after the addition of cADPR to the 45Ca2+ that had been taken up: 8.2 ± 0.9% (n = 7) without ryanodine vs. 15.8 ± 2.8% (n = 7) with ryanodine (P < 0.05)). The presence of heparin (200 μg/ml) did not affect the cADPR-induced 45Ca2+ release (data not shown). These results indicate that cADPR activates the ryanodine-sensitive Ca2+ release channel, but not the IP3-sensitive channel.

The effects of CaM and CaM antagonists on cADPR-induced 45Ca2+ release were also investigated. 45Ca2+ release by cADPR (2 μM) was significantly (P < 0.05) inhibited by W-7 (60 μM), but not
by W-5 (60 μM) (Fig. 6), and was significantly ($P < 0.05$) stimulated by CaM (10 μg/ml) (Fig. 6).

4. Discussion

In the present study, it was clearly demonstrated that the ryanodine-sensitive Ca$^{2+}$ release mechanism is present in the ER of rat pancreatic acinar cells and that the Ca$^{2+}$ release mechanism that is activated by caffeine, ryanodine or cADPR is CaM-dependent. The IP$_3$-sensitive mechanism may not be CaM-dependent, since IP$_3$ (5 μM)-induced 45Ca$^{2+}$ release from the vesicles was not inhibited by W-7 (80 μM) (data not shown). To the author’s knowledge, this is the first report of CaM sensitiveness to Ca$^{2+}$ release from microsomal vesicles prepared from non-excitable cells.

4.1. Characteristic features of ryanodine-sensitive Ca$^{2+}$ release

In excitable tissues, plant alkaloid ryanodine is known to lock the ryanodine-sensitive Ca$^{2+}$ release channel in an ‘open state’ and to activate the Ca$^{2+}$ channel [24-26]. The present data show that ryanodine induces Ca$^{2+}$ release from the ER of pancreatic acinar cells (Fig. 3). The data also show that caffeine- or cADPR-induced 45Ca$^{2+}$ release is increased by the presence of ryanodine (10 μM) (Figs. 1 and 5). Increment of the release is due to stimulation of the release mechanism not to inhibition of the reuptake of 45Ca$^{2+}$ into the vesicles after caffeine-, or cADPR-induced 45Ca$^{2+}$ release, since ryanodine (10 μM) did not affect the ATP-dependent 45Ca$^{2+}$ uptake (Figs. 1 and 5). Stimulation of Ca$^{2+}$- or caffeine-induced Ca$^{2+}$ release by pretreatment with low concentrations (≤10 μM) of ryanodine has been shown in skeletal muscle [24-27] and also in parotid acinar cells [4].

In some cases, ryanodine can lock the channel to a ‘closed state’ [24]. It has been demonstrated that Ca$^{2+}$-, caffeine- or cADPR-induced microsomal Ca$^{2+}$ release in excitable cells is inhibited by the presence of ryanodine at higher concentrations (≥50 μM) [6,24,28]. In parotid acinar cells, the microsomal 45Ca$^{2+}$ release is blocked by ryanodine (500 μM) in the presence of the Ca$^{2+}$-ATPase inhibitor thapsigargin [4]. It has also been shown that in pancreatic acinar cells, acetylcholine- or cADPR-evoked Ca$^{2+}$ spikes monitored as Ca$^{2+}$-dependent currents are inhibited by ryanodine even at 10 μM [8]. However, inhibition of caffeine- or cADPR-induced 45Ca$^{2+}$ release from pancreatic microsomes by ryanodine was not seen in the concentration range tested (10-500 μM) (data not shown). This may be due to the difference in experimental conditions. The effects of ryanodine are quite complicated, and it is known that stimulation or inhibition of Ca$^{2+}$ efflux is dependent on temperature, incubation time and Ca$^{2+}$ concentration as well as on ryanodine concentration [24]. In any case, the present results clearly indicate the presence of a ryanodine-sensitive Ca$^{2+}$ release mechanism in the ER of pancreatic acinar cells.

4.2. Modulation of the ryanodine-sensitive mechanism by CaM

CaM has been shown to regulate the ryanodine-sensitive Ca$^{2+}$ release channel in skeletal muscle [14,15] and in sea urchin eggs [16,17]. In the modulator model proposed by Lee et al. [17], endogenous
CaM modulates the Ca\(^{2+}\) or cADPR effect on the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel. It was demonstrated in pancreatic acinar cells that exogenously added CaM enhances the microsomal Ca\(^{2+}\) release caused by the ryanodine-sensitive Ca\(^{2+}\) release mechanism (Figs. 2, 4 and 6). Furthermore, in the case of caffeine, the added CaM increases the caffeine affinity to the release (Fig. 2D), as shown in sea urchin eggs [17].

It has been reported that in skeletal muscle endogenous CaM tightly binds to the SR proteins [29] and the CaM content of the heavy SR vesicles ranges from 15 to 33 pmol/mg protein [13]. The findings that 67 inhibited the \(^{45}\)Ca\(^{2+}\) release through the ryanodine-sensitive Ca\(^{2+}\) release mechanism (Figs. 2, 4 and 6) suggest that endogenous CaM that can modulate the ryanodine-sensitive Ca\(^{2+}\) channel is present in the ER of pancreatic acinar cells. The endogenous CaM, which is thought to be less than the level (~600 pmol/mg protein) of exogenous CaM added to the vesicles in the present study, may bind to the ER and play a crucial role in the regulation of the channel under physiological conditions. W-7 also inhibits the stimulatory effect of the ryanodine-sensitive Ca\(^{2+}\) release mechanism by exogenously added CaM, since the presence of both W-7 (60 \(\mu\)M) and CaM (10 \(\mu\)g/ml) reduced the caffeine (10 mM)-ryanodine (500 \(\mu\)M)- or cADPR (2 \(\mu\)M)-induced \(^{45}\)Ca\(^{2+}\) release from the vesicles to nearly the same level as that in the presence of W-7 alone (data not shown).

In skeletal muscle, it has been shown that in the absence of ATP, CaM activates the ryanodine-sensitive Ca\(^{2+}\) release from the SR vesicles at nanomolar to submicromolar Ca\(^{2+}\) concentrations and inhibits the release at micromolar to millimolar Ca\(^{2+}\) concentrations [15]. In the present study using pancreatic microsomes, CaM activation of the release was seen at 2 \(\mu\)M Ca\(^{2+}\) concentration in the presence of ATP. The discrepancy between activation and inhibition at micromolar Ca\(^{2+}\) concentrations may result from the difference in isoforms of ryanodine receptors or the difference in experimental conditions, including the presence of ATP.

It is known that cardiac or skeletal muscle SR Ca\(^{2+}\)-ATPase is phosphorylated by CaM-dependent protein kinase [30–32] and that the Ca\(^{2+}\) uptake into SR vesicles is inhibited by CaM inhibitors [31,33]. However, the effects of CaM and W-7 on the ER Ca\(^{2+}\)-ATPase of pancreatic acinar cells may be negligible in the present study, since CaM and W-7 did not affect the Ca\(^{2+}\) uptake into the ER vesicles (Fig. 2A).

Two ideas have been proposed concerning the modulation of the Ca\(^{2+}\) release mechanism by CaM. One is direct binding of CaM to the channel protein [13,15,17], and the other is phosphorylation by CaM-dependent protein kinase [23,34,35]. CaM-dependent protein kinase II may not be involved in the modulation of the ryanodine-sensitive mechanism in pancreatic acinar cells, since 10 \(\mu\)M of KN-62, an inhibitor of CaM-dependent protein kinase II, did not inhibit caffeine-induced \(^{45}\)Ca\(^{2+}\) release from pancreatic microsomes (data not shown).

It has been shown in sea urchin eggs that a low concentration of cADPR increases the Ca\(^{2+}\) or caffeine affinity in Ca\(^{2+}\)-induced Ca\(^{2+}\) release [36,37] and that cADPR, as well as CaM, can act also as a modulator for the channel [17]. In pancreatic microsomal vesicles, it has been shown that a low concentration (0.5 \(\mu\)M) of cADPR indeed increases caffeine (10 mM)-induced \(^{45}\)Ca\(^{2+}\) release (T. Ozawa, unpublished data). Further studies are needed in pancreatic acinar cells to clarify the properties of the ryanodine receptor protein, including the modulation mechanism by endogenous regulators, such as CaM and cADPR, and their physiological roles in Ca\(^{2+}\) mobilization.

References


[33] B.S. Tuana, D.H. MacLennan, Calmidazolium and compound 48/80 inhibit calmodulin-dependent protein phos-
phorylation and ATP-dependent Ca\textsuperscript{2+} uptake but not Ca\textsuperscript{2+}-

[34] D.R. Witcher, R.J. Kovacs, H. Schulman, D.C. Cefali, L.R. Jones, Unique phosphorylation site on the cardiac ryanodine

[35] M. Hohenegger, J. Suko, Phosphorylation of the purified
cardiac ryanodine receptor by exogenous and endogenous

[36] A. Galione, H.C. Lee, W.B. Busa, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} re-
lease in sea urchin egg homogenates: modulation by cyclic

[37] H.C. Lee, Potentiation of calcium- and caffeine-induced cal-
cium release by cyclic ADP-ribose, J. Biol. Chem. 268 (1993)
293–299.