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Characterization of Ryanodine-sensitive Ca^{2+} Release from Microsomal Vesicles of Rat Parotid Acinar Cells: Regulation by Cyclic ADP-ribose

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Abstract. We have measured ryanodine (caffeine)-sensitive $^{45}Ca^{2+}$ release from isolated microsomal vesicles of endoplasmic reticulum prepared from rat parotid acinar cells. After a steady state of ATP-dependent $^{45}Ca^{2+}$ uptake, the addition of caffeine (40 mM), ryanodine (10~500 μM) or an NAD^+ metabolite, cyclic ADP-ribose (cADPR, 4 μM) released about 10% of the $^{45}Ca^{2+}$ that had been taken up. The $^{45}Ca^{2+}$ release was not inhibited by heparin, an antagonist of IP_3 receptor. The effects of caffeine, ryanodine and cADPR on $^{45}Ca^{2+}$ release were also tested in the presence of thapsigargin (TG), an inhibitor of microsomal Ca^{2+}-ATPase. When caffeine (10~40 mM), ryanodine (10 μM) or cADPR (1~10 μM) was added in the medium with 100 nM TG, a significant $^{45}Ca^{2+}$ release was seen, while higher concentrations of ryanodine (>100 μM) did not cause any $^{45}Ca^{2+}$ release in the presence of TG. The initial rate of caffeine (40 mM)-induced $^{45}Ca^{2+}$ release was increased by a pretreatment with 10 μM ryanodine, whereas the caffeine-induced $^{45}Ca^{2+}$ release was strongly inhibited by the presence of a higher concentration (500 μM) of ryanodine. cADPR-induced $^{45}Ca^{2+}$ release was also inhibited by 500 μM ryanodine. Caffeine (40 mM)- or cADPR (4 μM)-induced $^{45}Ca^{2+}$ release was abolished by a presence of ruthenium red (50~100 μM). The presence of a low concentration (0.5 μM) of cADPR shifted the dose-response curve of caffeine-induced $^{45}Ca^{2+}$ release to the left. These results indicate the presence of a ryanodine sensitive Ca^{2+} release mechanism in the endoplasmic reticulum of rat parotid acinar cells that is distinct from the IP_3-sensitive Ca^{2+} channel and is activated by caffeine, cADPR and a low concentration (10 μM) of ryanodine, but is inhibited by higher concentrations (>100 μM) of ryanodine and ruthenium red. The properties of the ryanodine-sensitive mechanism are similar to that of the ryanodine receptor as described in muscle cells.

Key words: $^{45}Ca^{2+}$ efflux — Caffeine — Ruthenium red — Heparin — Endoplasmic reticulum — Thapsigargin

Introduction

In exocrine glands, mobilization of Ca^{2+} from intracellular Ca^{2+} stores plays a crucial role in triggering enzyme secretion. Receptor stimulation by hormones or neurotransmitters induces an increase in inositol 1,4,5-trisphosphate (IP_3), an intracellular messenger, which releases Ca^{2+} from IP_3-sensitive Ca^{2+} pools [3, 35]. The features of the IP_3-sensitive Ca^{2+} channel have been well characterized [9, 11]. In addition to the IP_3-sensitive Ca^{2+} release, caffeine- or Ca^{2+}-induced Ca^{2+} release has been demonstrated in pancreatic acinar cells [8, 32] and in lacrimal acinar cells [24]. As in sarcoplasmic reticulum of muscle tissues, a plant alkaloid ryanodine binds to the Ca^{2+} channel protein that is activated by caffeine or Ca^{2+}; the channel is referred to as a so-called ryanodine receptor. Ryanodine can lock the channel in either an open or closed state, dependeing upon the concentration of ryanodine [7, 25]. The channel protein has been purified, characterized [17, 18, 19] and cloned [30, 38] in skeletal and cardiac muscles. However, in nonexcitable tissues, very few results concerning the ryanodine-sensitive mechanism have been reported. Recently, it was shown that acetylcholine-induced Ca^{2+} oscillations or Ca^{2+}-ATPase inhibitor thapsigargin (TG)-induced Ca^{2+} oscillations were abolished by an addition of ryanodine in pancreatic acinar cells [40] or in parotid acinar cells [12], respectively. But, detailed information about the ryanodine receptor (channel) has not yet been obtained in exocrine glands.

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Evidence suggests that in excitable cells like muscles and neuronal tissues, cyclic ADP-ribose (cADPR), which is an endogenous NAD^+ metabolite, can induce Ca^{2+} release from a ryanodine-sensitive Ca^{2+} channel [27, 37, 42] and is also an intracellular messenger in addition to IP_3 [4, 22]. This compound also can cause repetitive Ca^{2+} spikes in pancreatic acinar cells [40] that are inhibited by ryanodine. In nonexcitable tissues, the origin of ryanodine or cADPR-sensitive changes remains unclear, since these drug-induced phenomena have been observed so far only on the level of whole cells.

In the present study, we have investigated the properties of the ryanodine-sensitive mechanism by parotid acinar cells in detail by using isolated microsomal vesicles preloaded with $^{45}Ca^{2+}$. Our results clearly indicate that the ryanodine-sensitive Ca^{2+} release mechanism is present in endoplasmic reticulum (ER) of parotid acinar cells. Not only caffeine but also cADPR can activate the ryanodine-sensitive mechanism and cADPR can modulate the caffeine effect. We also show that the effects of ryanodine on the mechanism depend on the concentration of ryanodine; that is, stimulation of the mechanism at a low concentration (10 μM) and inhibition at higher concentrations (>100 μM).

Materials and Methods

Creatine kinase and trypsin inhibitor were obtained from Boehringer Mannheim (Mannheim, FRG). Adenosine trisphosphate dipotassium salt (K_2ATP), antimycin A, creatine phosphate disodium salt, benzamidine, thapsigargin, oligomycin, heparin (Mr 4000–6000), IP_3 (potassium salt) and ruthenium red were purchased from Sigma Chemical (St. Louis, MO). Ryanodine was from Calbiochem (La Jolla, CA). Caffeine and collagenase were from Wako Pure Chemical (Osaka, Japan). $^{45}CaCl_2$ (18–20 Ci/g) was purchased from New England Nuclear (Boston, MA). Cyclic ADP-ribose, which was prepared enzymatically from NAD^+ with ADP-ribosyl cyclase as described previously [37], was kindly provided by Drs. S. Takasawa and H. Okamoto.

Preparation of Microsomal Vesicles

Parotid glands were removed from 8 male Wistar rats (180–200 g). Isolated acinar cells were prepared by collagenase digestion in a Krebs-Ringer solution containing (in mM): NaCl, 120; KCl, 4.7; KH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.0; Hepes, 10; glucose, 15; trypsin inhibitor, 0.1 mg/ml; bovine serum albumin, 0.2% (wt/vol) (pH 7.4) as described previously in the preparation of pancreatic acinar cells [36]. Microsomal vesicles were prepared from isolated cells as described previously [39]. Cells were washed twice in an ice-cold ''mannitol buffer'' containing (in mM): mannitol, 290; KCl, 10; Hepes, 5; $MgCl_2$, 1; benzamidine, 1; PMSF, 0.2; and trypsin inhibitor, 20 μg/ml adjusted with tris to pH 7.0. Homogenization and subsequent fractionation steps were performed at 4°C. Cells were homogenized in 20 ml of the ''mannitol buffer'' by 50 strokes at 1,000 rpm with a tightly fitting teflon homogenizer. The resulting homogenate was centrifuged for 5 min at 100 × *g* and the pellet rehomogenized. The combined homogenate was centrifuged at 1,000 × *g* for 15 min and the supernatant further centrifuged at 11,000 × *g* for 15 min in a Beckman model L8M centrifuge using a Beckman 70 Ti-fixed rotor. The 11,000 × *g* supernatant was centrifuged at 27,000 × *g* for 15 min in the 70 Ti rotor and the 27,000 × *g* pellet, enriched by approximately threefold in ER [39], was collected. The microsomal vesicles were kept frozen in liquid nitrogen until use. The protein concentration was measured by the method of Bradford (1976) [5] using bovine serum albumin as a standard.

Measurement of $^{45}Ca^{2+}$ Uptake

Isolated membrane vesicles were preincubated for 15 min at a protein concentration of 0.2 mg/ml in 0.6–1.0 ml of an incubation buffer containing (in mM): KCl, 155; Hepes, 5; $CaCl_2$, 0.15 (corresponding to 0.002 free Ca^{2+} concentration); EDTA, 1.0; $MgCl_2$, 3.57 (corresponding to 1.0 free Mg^{2+} concentration); NaN_3, 10; oligomycin, 0.005; antimycin A, 5 μg/ml; creatine phosphate, 10; creatine kinase, 8 U/ml; and 1 μCi/ml of $^{45}CaCl_2$, adjusted to pH 7.0 with tris/HCl at 25°C. $^{45}Ca^{2+}$ uptake was initiated by the addition of K_2ATP at a final concentration of 2 mM. After a steady state of $^{45}Ca^{2+}$ uptake, drugs that can induce Ca^{2+} release from ER, like IP_3 or caffeine, were added to the buffer. In some cases, TG microsomal Ca^{2+}-ATPase inhibitor was also given to inhibit a reuptake of the $^{45}Ca^{2+}$ that had been released into the medium. At indicated times, aliquots were removed from the incubation medium and vesicles were separated from the medium by filtering rapidly through cellulose nitrate filter with a pore size of 0.65 μM (Advantec Toyo, Tokyo, Japan), as described previously [2]. Filters were washed with 7 ml of an ice-cold solution containing (in mM): KCl, 140; Hepes, 5; $MgCl_2$, 1; $LaCl_3$, 0.1 adjusted to pH 7.0 with KOH. The radioactivity was quantitated using ''Clear-sol I®'' scintillator (Nacalai Tesque, Kyoto, Japan) in a liquid scintillation counter (LSC-1000, Aloka, Tokyo, Japan).

Statistics

Values are presented as mean ± SEM. P-values were calculated using *t*-test.

Abbreviations

cADPR:	cyclic ADP-ribose
EDTA:	ethylenediaminetetraacetic acid
ER:	endoplasmic reticulum
Hepes:	N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid
IP_3:	D-myo-inositol 1,4,5-trisphosphate
PMSF:	phenylmethylsulfonyl fluoride
SR:	sarcoplasmic reticulum
TG:	thapsigargin

Results

Comparison of IP_3- and Caffeine (Ryanodine)-sensitive $^{45}Ca^{2+}$ Release

A steady state of $^{45}Ca^{2+}$ uptake was reached 90~100 min after addition of ATP (Figs. 1 and 2). $^{45}Ca^{2+}$ content of microsomal vesicles 100 min after ATP was 439.0 ± 12.7 nmol/mg protein ($n = 25$). Figure 1 shows the effects of IP_3 (5 μM) and caffeine (40 mM) on $^{45}Ca^{2+}$ release from

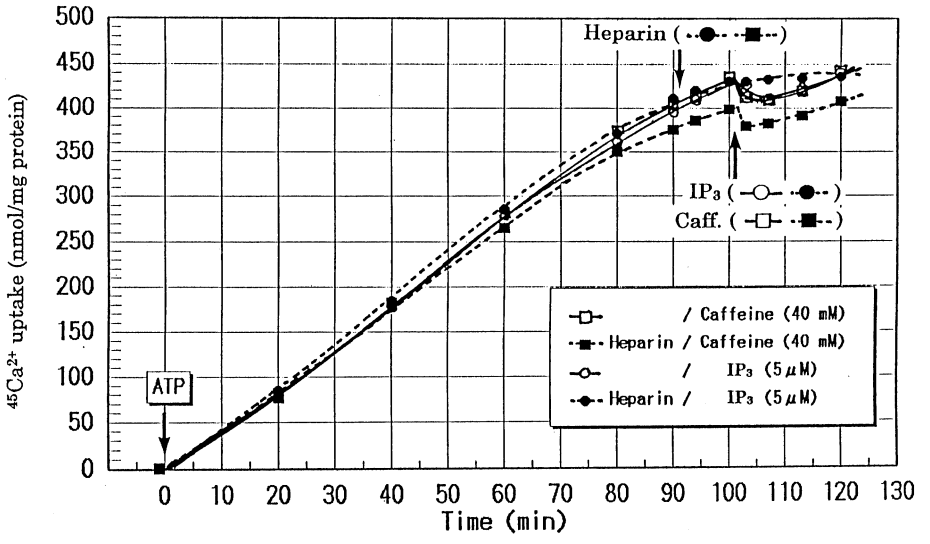

Fig. 1. Effects of heparin on IP_3- and caffeine-induced $^{45}Ca^{2+}$ release from microsomal vesicles prepared from parotid acinar cells. Vesicles (0.2 mg/ml) were preincubated for 15 min at 25°C in 1 ml of a KCl buffer containing $^{45}CaCl_2$ (1 μCi/ml). $^{45}Ca^{2+}$ uptake was initiated by the addition of 2 mM K_2ATP. Heparin (200 μg/ml) was added where indicated. IP_3 (5 μM) and caffeine (40 mM) were subsequently added. Note that heparin does not inhibit caffeine-induced $^{45}Ca^{2+}$ release.

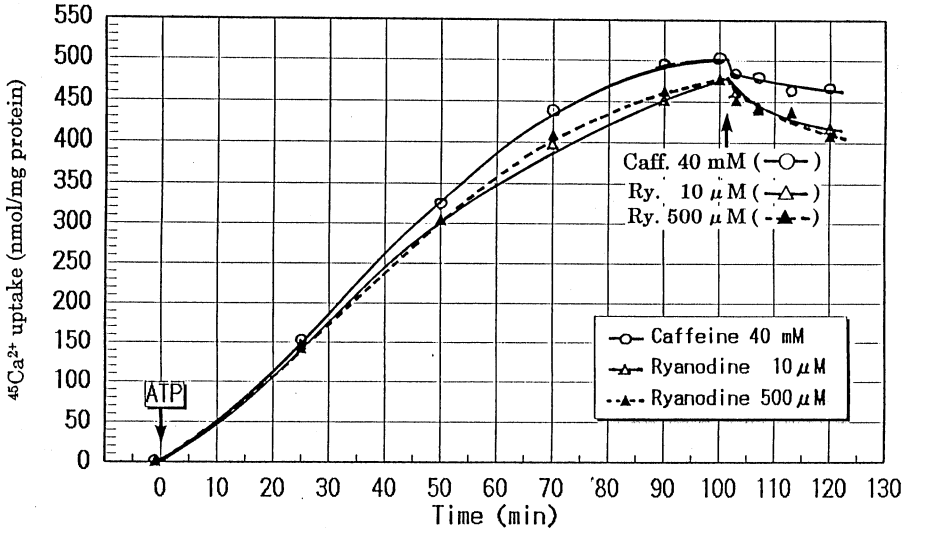

Fig. 2. Comparison of caffeine- and ryanodine-induced $^{45}Ca^{2+}$ release from microsomal vesicles. Experimental procedures were the same as described for Fig. 1. After a steady state of ATP dependent $^{45}Ca^{2+}$ uptake, caffeine (40 mM) and ryanodine (10, 500 μM) were added where indicated.

microsomal vesicles in the absence and presence of heparin (200 μg/ml), which is known to inhibit IP_3-mediated Ca^{2+} release [15, 16]. In the absence of heparin, both IP_3 and caffeine caused small $^{45}Ca^{2+}$ releases, which were approximately 10% of the $^{45}Ca^{2+}$ that had been taken up. But when heparin was added to vesicles prior to IP_3 or caffeine, the IP_3 effect was abolished, while the caffeine effect did not change (Fig. 1). Figure 2 shows the effect of ryanodine on microsomal $^{45}Ca^{2+}$ release. An addition of ryanodine (10 or 500 μM) at a steady state significantly released a $^{45}Ca^{2+}$ for 12 min; % of the $^{45}Ca^{2+}$ content before an addition of ryanodine was 6.5 ± 1.3% ($n = 7$, $P < 0.01$) at 10 μM or 8.4 ± 0.9% ($n = 4$, $P < 0.01$) at 500 μM. It was a similar amount of $^{45}Ca^{2+}$ as compared to caffeine (40 mM) (Fig. 2). Ryanodine-induced $^{45}Ca^{2+}$ release was not inhibited by the presence of heparin (*data not shown*). These results indicate that caffeine or ryanodine activate a mechanism that is distinct from the IP_3-sensitive Ca^{2+} channel.

Effects of Caffeine and Ryanodine in the Presence of TG

To exclude IP_3-sensitive mechanism involvement in the microsomal Ca^{2+} release, the following experiments were done in the presence of heparin (200 μg/ml). Thapsigargin (TG) microsomal Ca^{2+}-ATPase inhibitor was added at a steady state to inhibit a reuptake of $^{45}Ca^{2+}$ after drug-induced $^{45}Ca^{2+}$ release as shown in Fig. 1. Figure 3 shows a dose-dependent effect of caffeine on $^{45}Ca^{2+}$ release in the presence of 100 nM TG where $^{45}Ca^{2+}$ uptake into microsomal vesicles was completely inhibited in pancreatic acinar cells [33] or in pa-

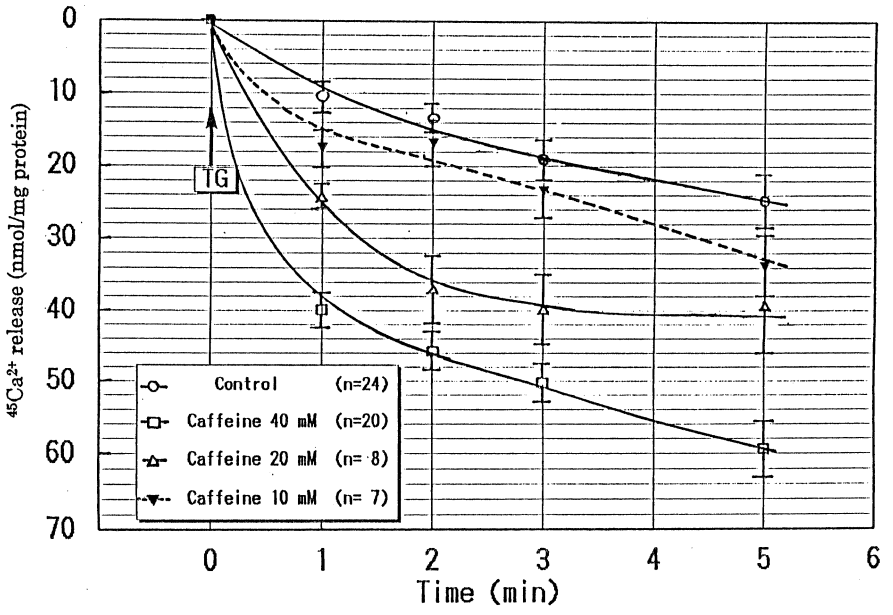

Fig. 3. Effect of caffeine on $^{45}Ca^{2+}$ release from microsomal vesicles in the presence of thapsigargin (TG). Vesicles (0.2 mg/ml) were incubated in 0.6 ml of the same medium as described for Fig. 1. One hundred minutes after an addition of ATP, caffeine (10~40 mM) was added with TG (100 nM) to inhibit a reuptake of $^{45}Ca^{2+}$ that had been released into the medium. Heparin (200 μg/ml) was also added 5 min before the addition of TG to block the IP_3-sensitive Ca^{2+} channel. Microsomal $^{45}Ca^{2+}$ content preceding the addition of TG was 452.8 ± 7.7 nmol/mg protein (n = 111), and the level of each curve was superimposed to zero. The decrease in $^{45}Ca^{2+}$ content after addition of TG (at 0 min) is represented on the vertical line. Caffeine-induced decrease with TG was compared to control with TG alone. Each point shows mean ± SE and n indicates number of experiments.

rotid acinar cells [13]. An addition of TG alone caused a slow decrease in $^{45}Ca^{2+}$ content (Fig. 3). When caffeine (10~40 mM) was added with TG, caffeine released $^{45}Ca^{2+}$ in a dose-dependent manner (Figs. 3 and 7). Forty millimolar of caffeine-induced $^{45}Ca^{2+}$ release for 2 min; the difference between caffeine-plus-TG-induced $^{45}Ca^{2+}$ decrease and TG-alone-induced $^{45}Ca^{2+}$ decrease was 33.0 ± 3.1 nmol/mg protein (n = 20, Fig. 3). Figure 4 shows the effect of ryanodine in the presence of heparin and TG. Ten micromolar of ryanodine induced a slower release of $^{45}Ca^{2+}$ (18.9 ± 4.2 nmol/mg protein for 2 min (n = 9, Fig. 4)) as compared to caffeine (40 mM). The reason may be due to slow ryanodine binding to the receptor, as reported in previous studies of sarcoplasmic reticulum (SR) of skeletal muscle [6, 20]. But, the decrease in $^{45}Ca^{2+}$ by 10 μM ryanodine was significantly ($P < 0.01$) larger than in the control with TG alone. On the other hand, 500 μM ryanodine did not induce a significant release of $^{45}Ca^{2+}$ (Fig. 4). One hundred micromolar of ryanodine also did not cause a significant release (*data not shown*). These results suggest that the ryanodine-sensitive Ca^{2+} channel is stimulated by a low concentration (~10 μM) of ryanodine and inhibited by higher concentrations (>100 μM) of ryanodine as described in previous studies [7, 25]. The effect of pretreatment of ryanodine on $^{45}Ca^{2+}$ release was also investigated. When microsomal vesicles were preincubated with low or high concentration of ryanodine, no effects of ryanodine on $^{45}Ca^{2+}$ uptake and TG-induced $^{45}Ca^{2+}$ decrease after a steady state were seen (*data not shown*). But, when caffeine (40 mM) was added with TG in the presence of 500 μM ryanodine, caffeine-induced $^{45}Ca^{2+}$ release was abolished (Fig. 5). The preincubation of 10 μM ryanodine significantly increased the initial rate of the caffeine-induced $^{45}Ca^{2+}$ release (Fig. 5, n = 5 of 9) (26.9 ± 5.1 nmol/mg/protein/min (n = 5) without ryanodine *vs.* 41.9 ± 3.6 nmol/mg/protein/min (n = 5) with ryanodine $P < 0.05$). The effect on the $^{45}Ca^{2+}$ release of ruthenium red, an inhibitor of caffeine- or Ca^{2+}-induced Ca^{2+} release in exocrine glands [8, 24] as well as in excitable tissues [26, 29], was also investigated. When 50 μM ruthenium red, which blocks the releasing mechanism in brain [26] and lacrimal gland [24], was added together with TG and caffeine (40 mM), the caffeine effect was abolished (Fig. 5).

Effects of Cyclic ADP-ribose

When micromolar concentration of cyclic ADP-ribose (cADPR), an endogenous regulator of ryanodine-sensitive Ca^{2+} release mechanism [27, 37, 42], was added to the medium after a steady state of $^{45}Ca^{2+}$ uptake in the presence of TG, $^{45}Ca^{2+}$ was released in a dose-dependent manner (Fig. 6*A*). The maximal effect was seen at 4 μM cADPR and no additional effect was observed at 10 μM cADPR. The dose-dependent effect of cADPR at concentrations of micromolar range was obtained also in the absence of TG (*data not shown*). Figure 6*B* shows that a pretreatment of 500 μM ryanodine completely inhibited the cADPR (4 μM)-induced $^{45}Ca^{2+}$ release. Ruthenium red (50 μM) also abolished the effect of 4 μM cADPR (*data not shown*). In sea urchin egg

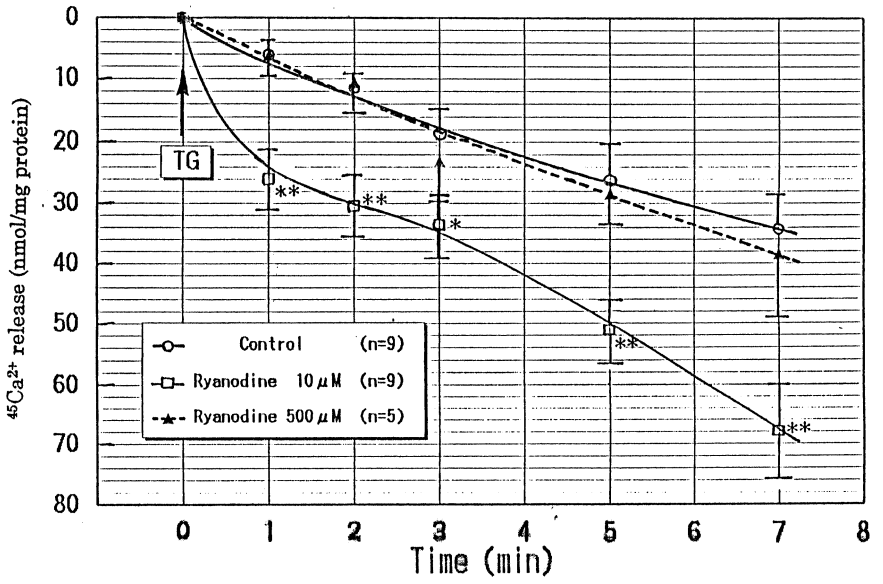

Fig. 4. Effects of ryanodine on $^{45}Ca^{2+}$ release from microsomal vesicles in the presence of TG and heparin. Experimental procedures were the same as described for Fig. 3. After a steady state of $^{45}Ca^{2+}$ uptake, ryanodine (10 and 500 μM) were added to the medium with TG. Ten micromolar of ryanodine caused a slow, but significant $^{45}Ca^{2+}$ release, while the addition of 500 μM ryanodine with TG decreased a small amount of $^{45}Ca^{2+}$, which was not significantly different from the control with TG alone. Each point shows mean ± SE and *n* is number of experiments. Asterisks give the level of significant difference as compared to the decrease obtained for control with TG alone at the corresponding time. $*P < 0.05$, $**P < 0.01$

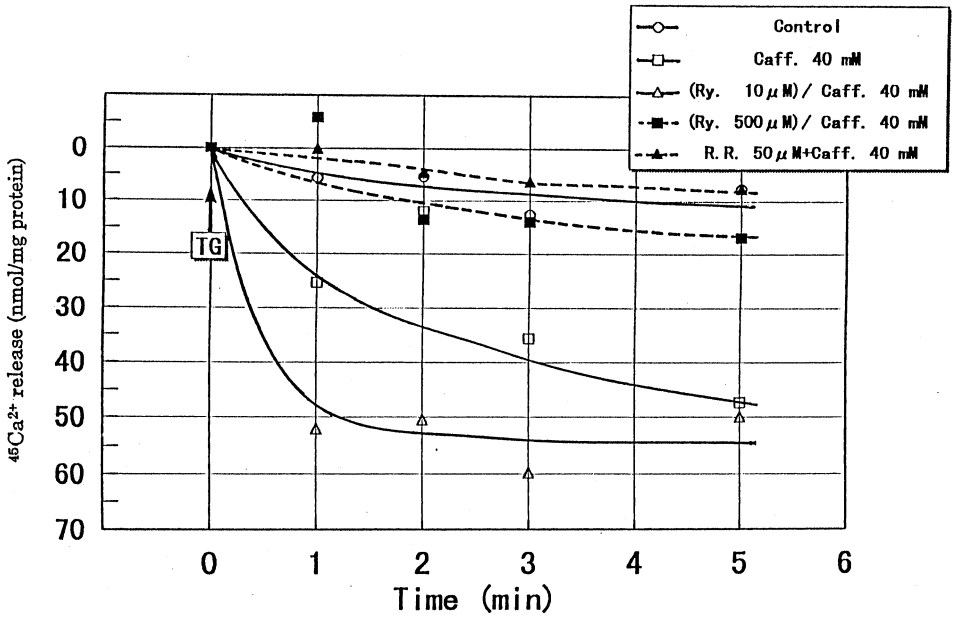

Fig. 5. Effects of ryanodine and ruthenium red on caffeine-induced $^{45}Ca^{2+}$ release in the presence of TG and heparin. After a steady state of $^{45}Ca^{2+}$ uptake, caffeine (40 mM) was added with TG in the absence and presence of ryanodine (Ry.: 10, 500 μM) or ruthenium red (R.R.: 50 μM). Ryanodine was present from the beginning of incubation and ruthenium red was given together with TG. Note that 10 μM ryanodine enhanced the caffeine-induced $^{45}Ca^{2+}$ release and ryanodine (500 μM) or ruthenium red strongly inhibited the caffeine effect. One out of three similar experiments.

homogenates, Ca^{2+}- or caffeine-induced Ca^{2+} release was potentiated by the presence of lower concentrations (20~40 nM) of cADPR, at which Ca^{2+} release was not induced by itself [21]. When 0.5 μM cADPR, which did not have a significant $^{45}Ca^{2+}$ release (*data not shown*), was added to the microsomal vesicles at a steady state prior to caffeine, the caffeine-induced $^{45}Ca^{2+}$ release was increased as compared to the release without cADPR. Figure 7 shows dose-response curves of caffeine-induced $^{45}Ca^{2+}$ release for 2 min in the absence and presence of 0.5 μM cADPR. The pretreament of 0.5 μM cADPR shifted the curve to the left. At 10 mM caffeine, 0.5 μM cADPR significantly stimulated the release (−0.8 ± 0.9 nmol/mg protein ($n = 4$) without cADPR *vs.* 18.7 ± 2.8 nmol/mg protein ($n = 4$) with cADPR $P < 0.001$, Fig. 7), while at higher concentrations of caffeine, the stimulation was not significant ($P > 0.05$).

Discussion

In the present study, we demonstrate and characterize the ryanodine (caffeine)-sensitive Ca^{2+} release mechanism in the ER of parotid acinar cells. To our knowledge, this is the first report of the ryanodine- and cADPR-induced Ca^{2+} release from microsomes prepared from nonexcitable tissues. Present data directly show that a low con-

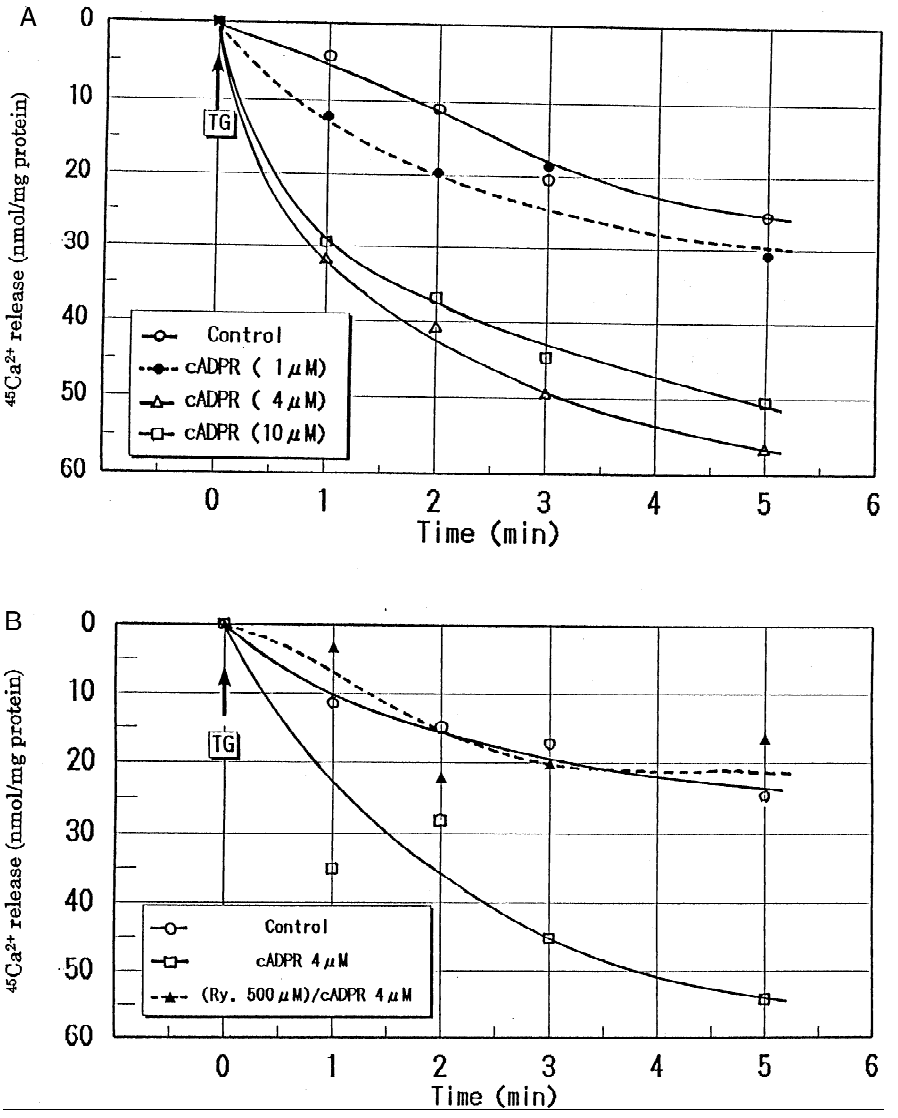

Fig. 6. (*A*) Dose-dependent effect of cyclic ADP-ribose (cADPR) on $^{45}Ca^{2+}$ release from microsomal vesicles in the presence of TG and heparin. After a steady state of $^{45}Ca^{2+}$ uptake, cADPR (1~10 μM) was added with TG. (*B*) Effect of ryanodine (Ry. 500 μM) on cADPR (4 μM)-induced $^{45}Ca^{2+}$ release. Treatments of ryanodine was the same as described for Fig. 5. Note that 4 μM cADPR had a maximal release and the release was completely abolished by the presence of ryanodine (500 μM). Each figure is one out of two or three similar experiments.

centration (10 μM) of ryanodine and cADPR as well as caffeine activate the IP_3-insensitive mechanism which is present in the ER membrane (Figs. 3, 4, and 6). In addition, ryanodine at higher concentrations (>100 μM) and ruthenium red inhibits the caffeine- or cADPR-induced $^{45}Ca^{2+}$ release (Figs. 5, 6 and *data not shown*). The properties of the releasing mechanism are similar to those of the ryanodine receptors in muscles [25, 27] and neuronal tissues [26, 42]. Our results also indicate that IP_3 and drugs that activate the ryanodine-sensitive mechanism, like ryanodine or caffeine, act on different mechanisms of the ER, since caffeine, ryanodine and cADPR are effective even in the presence of heparin (Figs. 3 to 7), and IP_3-induced $^{45}Ca^{2+}$ release is not inhibited by the presence of 500 μM ryanodine (*data not shown*). However, it is not clear from our results whether two different mechanisms are located in different Ca^{2+} pools or partially in the same pool as shown in pancreatic microsomal vesicles [32].

Characteristics of Ryanodine-sensitive Ca^{2+} Release

Plant alkaloid ryanodine can lock the ryanodine-sensitive Ca^{2+} channel to an ''open state'' at low concentrations (<10 μM). In this state, Ca^{2+}-induced Ca^{2+} release, which is induced by Ca^{2+} concentrations higher than 1 μM, is stimulated [25]. In parotid acinar cells, 10 μM ryanodine causes a $^{45}Ca^{2+}$ release from the microsomal vesicles in the presence of a micromolar free Ca^{2+} concentration of the medium (Figs. 2 and 4). Our results also show that 10 μM ryanodine increases an initial rate of the caffeine (40 mM)-induced $^{45}Ca^{2+}$ release (Fig. 5).

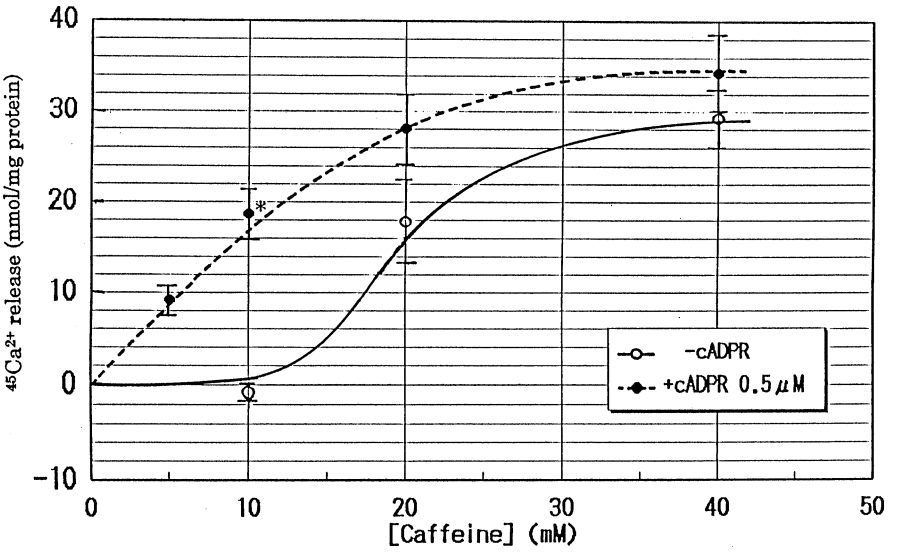

Fig. 7. Dose-response curves for caffeine-induced $^{45}Ca^{2+}$ release in the absence (○) and the presence of cADPR (0.5 μM) (●). Experimental procedures were the same as described for Fig. 3, except that cADPR (0.5 μM) was given in the medium 5 min before addition of TG. The caffeine-induced $^{45}Ca^{2+}$ release (for 2 min), which is represented on the vertical line, was determined at each caffeine concentration in the absence and presence of cADPR by subtracting the decrease in $^{45}Ca^{2+}$ content with TG alone from the decrease with caffeine plus TG. Each point shows mean ± SE from 3–5 paired experiments. Note that cADPR (0.5 μM) significantly (*P < 0.001) increased the release by 10 mM caffeine.

Stimulation of caffeine-induced Ca^{2+} release by 10 μM ryanodine has also been shown in excitable tissues based on the observation that a pretreatment of 10 μM ryanodine prior to the addition of caffeine leads to a complete discharge from the caffeine-sensitive store [1, 31]. On the other hand, ryanodine can lock the channel to a ''closed state'' at higher concentrations (>100 μM) [25]. Five hundred micromoles ryanodine does not induce the $^{45}Ca^{2+}$ release from the vesicles (Fig. 4) and strongly inhibits the $^{45}Ca^{2+}$ release activated by caffeine (Fig. 5) or cADPR (Fig. 6*B*). Previous studies have shown that caffeine- or cADPR-induced Ca^{2+} response is blocked by the presence of ryanodine at higher concentrations (≧50 μM) [26, 27], in some cases even at 10 μM [40]. We have a discrepancy in ryanodine effect at 500 μM between the absence and presence of TG. In the absence of TG, 500 μM ryanodine causes a $^{45}Ca^{2+}$ release as in the case of 10 μM (Fig. 2), while in the presence of TG, no $^{45}Ca^{2+}$ release is seen at 500 μM (Fig. 4). We do not know why 500 μM ryanodine induces the release when the microsomal Ca^{2+}-pump is not inhibited. Ryanodine effects are quite complicated and it is known that stimulation or inhibition of Ca^{2+} efflux are dependent on temperature, incubation time and Ca^{2+} concentration as well as ryanodine concentration [25]. In any case, our data show, for the first time, that ryanodine has a biphasic effect on microsomal Ca^{2+} release in nonexcitable cells.

Does cADPR Act as an Activator or a Modulator?

Intracellular messenger cADPR is synthesized from β-NAD^+ by the enzyme ADP-ribosyl cyclase, which can be activated by cGMP in sea urchin eggs [14]. The formation of cADPR leads to Ca^{2+} release through the ryanodine-sensitive release mechanism. A maximal cADPR concentration for cADPR-induced Ca^{2+} release is 100~250 nM in many excitable tissues [27, 37, 42], while in parotid acinar cells, cADPR is effective at a micromolar concentration (Fig. 6*A*). In addition, it also shows that cADPR activates the ryanodine-sensitive Ca^{2+} release mechanism, since both 500 μM ryanodine and 50 μM ruthenium red inhibit the cADPR-induced $^{45}Ca^{2+}$ release (Fig. 6*B* and *data not shown*). Quite recent studies have pointed out that in sea urchin eggs, cADPR not only directly activates the Ca^{2+} release channel, but can also play a modulator role by increasing Ca^{2+} and caffeine sensitivity of the release channel at lower concentrations [21, 23]. Present data show that 0.5 μM cADPR, which does not induce a significant $^{45}Ca^{2+}$ release (*data not shown*), increases the affinity to caffeine and shifts the dose response curve for caffeine-induced $^{45}Ca^{2+}$ release to the left (Fig. 7). In the modulator model proposed by Lee et al. (1995), cADPR modulates Ca^{2+} or caffeine effects on the ryanodine-sensitive Ca^{2+} channel at an endogenous level [23]. We do not know to what extent the intracellular level of cADPR in parotid acinar cells increases by receptor stimulation. However, an endogenous level of cADPR in rat brain, heart and liver has been reported to be 200 to 680 nM [41]. As cADPR potentiates the caffeine effect at a concentration of 500 nM, which is thought to be near the endogenous level of rat parotid acinar cells, there is a possibility that cADPR can increase the Ca^{2+} affinity of Ca^{2+}-induced Ca^{2+} release in physiological conditions.

Caffeine [10] and cADPR [21], which are known to increase Ca^{2+} affinity to Ca^{2+}-induced Ca^{2+} release, were

effective in the microsomal $^{45}Ca^{2+}$ release (Figs. 3 and 6). But we did not find direct evidence that the ambient Ca^{2+} activated the releasing mechanism. Further study concerning the involvement of the Ca^{2+} should be made by monitoring extravesicular Ca^{2+}.

In skeletal and cardiac muscles, the features of the ryanodine-sensitive Ca^{2+} channel (receptor) have been characterized not only from the study of Ca^{2+} efflux but also from the measurement of single channel currents by incorporating SR vesicles into planar lipid bilayers [34] and from binding assay of ^{3}H labeled ryanodine to the receptor protein [6, 28]. However, in nonexcitable tissues, very few reports about the channel (receptor) have been published. Further exploration is needed to understand the features of the ryanodine-sensitive mechanism on ER by using the isolated microsomal vesicles.

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