Identification of Cyclic ADP-ribose-dependent Mechanisms in Pancreatic Muscarinic Ca\textsuperscript{2+} Signaling Using CD38 Knockout Mice*

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Yasue Fukushi‡, Ichiro Kato§, Shin Takasawa‡, Tsukasa Sasaki‡, Boon Hooi Ong‡, Mika Sato‡, Atsushi Ohsga‡, Kozo Sato‡, Kunio Shirato§, Hiroshi Okamoto§, and Yoshio Maruyama‡

From the ‡Department of Physiology I, §Department of Internal Medicine, and ¶Department of Biochemistry, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan

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We showed that muscarinic acetylcholine (ACh)-stimulation increased the cellular content of cADPR in the pancreatic acinar cells from normal mice but not in those from CD38 knockout mice. By monitoring ACh-evoked increases in the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) using fura-2 microfluorimetry, we distinguished and characterized the Ca\textsuperscript{2+} release mechanisms responsive to cADPR. The Ca\textsuperscript{2+} response from the cells of the knockout mice (KO cells) lacked two components of the muscarinic Ca\textsuperscript{2+} release present in wild mice. The first component inducible by the low concentration of ACh contributed to regenerative Ca\textsuperscript{2+} spikes. This component was abolished by ryanodine treatment in the normal cells and was severely impaired in KO cells, indicating that the low ACh-induced regenerative spike responses were caused by cADPR-dependent Ca\textsuperscript{2+} release from a pool regulated by a class of ryanodine receptors. The second component inducible by the high concentration of ACh was involved in the phasic Ca\textsuperscript{2+} response, and it was not abolished by ryanodine treatment. Overall, we conclude that muscarinic Ca\textsuperscript{2+} signaling in pancreatic acinar cells involves a CD38-dependent pathway responsible for two cADPR-dependent Ca\textsuperscript{2+} release mechanisms in which the one sensitive to ryanodine plays a crucial role for the generation of repetitive Ca\textsuperscript{2+} spikes.

Cyclic ADP-ribose (cADPR),\textsuperscript{1} first found in sea urchin eggs, mobilizes Ca\textsuperscript{2+} by a mechanism independent of the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) pathway (1) and may act on the Ca\textsuperscript{2+} -induced Ca\textsuperscript{2+} release mechanism as an endogenous modulator (2–8). That both cADPR and its synthetic enzyme are ubiquitous signaling molecules, we have been ascribed to the activity of ADP-ribosyl cyclase of CD38 (12). By estimating the change in the cellular content of cADPR and monitoring the muscarinic Ca\textsuperscript{2+} response in pancreatic acinar cells, either from normal or CD38 knockout mice (13), we have successfully separated the CD38- and therefore cADPR-dependent component from the inositol phosphate-sensitive component. Thus, this paper provides insight into the mechanism of cADPR-dependent Ca\textsuperscript{2+} mobilization mediated by CD38 activities, which is independent of IP\textsubscript{3}-dependent Ca\textsuperscript{2+} mobilization in muscarinic acetylcholine (ACh) receptor signaling.

EXPERIMENTAL PROCEDURES

Mice—Mice lacking CD38 were generated by homologous recombination. The generation and genotyping of the mice have been described in detail previously (13). The mice used for each experiment were derived from ICR background and were from the same litter or the same family.

Cell Preparation—Fragments of pancreatic tissue from both normal ICR (CD38\textsuperscript{+/+}) and CD38 knockout ICR mice (CD38\textsuperscript{−/−}) were excised and treated with enzymes at 37 °C. Single pancreatic acinar cells used for the fluorescence and/or whole-cell current measurements were prepared using collagenase (200 units/ml; Wako, Osaka, Japan) for 3 min and trypsin (0.5 mg/ml; Sigma type XI) for 2 min and then with the same collagenase for 1 min, similar to the procedure described previously (14). The pancreatic acinar cells used for the measurements of IP\textsubscript{3} and cADPR were prepared using collagenase (1000 units/ml) alone for 10 min.

Experimental Solutions and Materials—We used a Ca\textsuperscript{2+}-free solution to avoid any contribution of external Ca\textsuperscript{2+} to the signaling in pancreatic acinar cells. The Ca\textsuperscript{2+}-free solution contained (in mM): 140 NaCl, 4.2 KCl, 1.13 MgCl\textsubscript{2}, 10 glucose, 10 HEPES (pH 7.2 adjusted with NaOH). Fluorescence experiments were carried out using a solution containing 0.5 mM EGTA. Pretreatment of the cells with ryanodine (Calbiochem) was performed at 37 °C for 5 min using the Ca\textsuperscript{2+}-free solution (15). The reagent was removed by washing the cells several times with the experimental solution before the fluorescence measurements (16).

Fura-2 Loading and Fluorescence Measurements—The single pancreatic acinar cells were incubated with 1 μM fura-2/AM (Dojin Chemical Institute, Kumamoto, Japan) for 40 min at 37 °C and then washed several times with the normal solution and kept at room temperature until use. The cells, attached to a glass coverslip, were placed in a small chamber (400 μl) mounted on the stage of an inverted microscope and perfused continuously (0.5 ml/min) with a stream of the experimental solution. This arrangement permitted a rapid exchange of the bathing solution (17). The fluorescence was measured by an epifluorescence inverted microscope system described previously (18, 19). Single acinar cells were alternately illuminated at excitation wavelengths of 340 and 380 nm by a rotating sector mirror at 3-ms—1-s intervals. The emission

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†To whom correspondence should be addressed: Dept. of Physiology I, Tohoku University Graduate School of Medicine, Seiryo-cho 2-1, Aobaku, Sendai 980-8575, Japan. Tel.: 81-22-717-8066; Fax: 81-22-717-8099; E-mail: maruyash@irc.tohoku.ac.jp.

§The abbreviations used are: cADPR, cyclic ADP-ribose; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; ACh, acetylcholine; [Ca\textsuperscript{2+}]\textsubscript{i}, cytosolic Ca\textsuperscript{2+} concentration; KO cells, CD38 knockout cells.

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was monitored at 510 nm. Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) changes were monitored as changes in the fluorescence ratio for excitation at 340 and 380 nm (F\(_{340}/F_{380}\)). The [Ca\(^{2+}\)]\(_{i}\) was determined from the ratio of fluorescence traces at the two excitation wavelengths (340/380 nm) (20).

Whole-cell Injection of cADPR, Ca\(^{2+}\), and IP3—The single pancreatic acinar cells were subjected to standard whole-cell patch-clamp recordings for the injection of reagents (IP\(_{3}\), cADPR, and Ca\(^{2+}\)), in a manner similar to the procedure described previously (21, 22). Patch pipettes were pulled from plain hematocrit glass capillaries by a two-stage puller (PP-83, Narishige, Tokyo) coated with beewax. The current signals were amplified by a List EPC7 amplifier (List Electronic, Darmstadt, Germany), appropriately low pass filtered (200–500 Hz) and displayed on an oscilloscope screen and a digital chart recorder. The cells were immersed with external solution containing (mM): 145 NaCl, 4 KCl, 1 CaCl\(_{2}\), 2 MgCl\(_{2}\), 10 HEPES at pH 7.2 (by NaOH). Patch pipettes were filled with a solution (pipette solution) containing (mM): 144 KCl, 2 MgCl\(_{2}\), 0.1 EGTA, 10 HEPES at pH 7.2 (by NaOH). For loading of the reagents, 20 μM cADPR (Molecular Probes), 20 μM IP\(_{3}\) (Wako, Osaka), or 200 μM CaCl\(_{2}\) were included in the pipette solution. In the last case, EGTA was eliminated from the pipette solution. The pipette resistance when filled with the pipette solution was 3–5 megohms. The establishment of the whole-cell recordings was monitored with repetitive voltage pulses superimposed on the holding potential, and the appearance of an additional current during the application of a train of short suction onto the patch pipette. The pulses were interrupted soon after the current responses started. The series resistance of the whole-cell recordings was 10–15 megohms, which was measured with a trimmer system of the amplifier after the current response evoked by the reagent recovered to the resting level. All the experiments were carried out at room temperature (24°C).

Western Blot Analysis—Proteins were extracted with 9 volumes of 100 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% (v/v) Triton X-100 supplemented with Complete\textsuperscript{TM} (one tablet/50 ml, Roche Diagnostics GmbH, Mannheim, Germany) from mouse tissues. The proteins (50 μg) were separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% skim milk, the membrane was incubated at room temperature for 1 h with an anti-cADPR polyclonal antibody raised against a peptide fragment of mouse CD38 (residues 283–301, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The concentration of antibody was 0.4 μg/ml with 5% skim milk. After rinsing, the membrane was further incubated at room temperature for 1 h with a secondary antibody labeled with hors eradish peroxidase and developed using an ECL detection system (Amersham Pharmacia Biotech) as described (6, 22).

Measurement of cADPR by Radioimmunoassay—The cADPR content was measured in either the presence or absence of Ach as described (23). Briefly, the suspension of enzymatically dispersed pancreatic acini (about 20 mg wet weight/ml) in 1-ml test tubes was incubated for 60 s with or without Ach, the supernatant was discarded after centrifugation at 600 × g for 1 min at 4°C, and the preparation was then stocked at −80°C. The acinar preparation was homogenized in 9 volumes of perchloric acid solution (2.5%, v/v). The homogenates were stored at −80°C and later defrosted and centrifuged for 10 min at 13,000 × g. The supernatant (330–350 μl) of the homogenates was then mixed with 150 μl of a solution of Norit A (27 mg/ml in H₂O, Nakalai tesque, Kyoto, Japan). After a 30-min incubation at 37°C, the samples were again centrifuged, and the supernatant was discarded. The pellet was washed three times with 1.0 ml of H₂O, resuspended in a pyridine-methanolic-H₂O mixture (10:50:40, v/v/v), and centrifuged for 10 min. An aliquot (10–20 μl) of the supernatant was immediately neutralized with a solution of 2 Tris base and subjected to the procedure of cADPR measurement. As a control, another aliquot was heated at 95°C for 10 min before being neutralized and analyzed by the radioimmunoassay. This heat treatment converted cADPR to ADP-ribose, resulting in no cross-reaction with the anti-cADPR antibody. The immunoreactivity of all the samples was abolished by the heat treatment. The recovery of cADPR, monitored by the recovery of [\(^{13}\)H]ADPR added in each homogenate, was 74.2 ± 2.92% (n = 8). Correction was introduced for the recovery of cADPR.

Measurement of IP₃—The cellular content of IP₃ was estimated with a d-lysino-isoinositol 1,4,5-[\(^{3}\)H]triphosphate assay system (Amersham Pharmacia Biotech) either in the presence or absence of Ach. The suspension of enzymatically dispersed pancreatic acini (about 20 mg wet weight/ml) in 1-ml test tubes was incubated for 60 s (with or without Ach), the supernatant was discarded after centrifugation at 600 × g for 1 min at 4°C, and the preparation was then stocked at −80°C. The cells were homogenized in 200 ml of ice-cold 10% (v/v) perchloric acid with an ultrasonic processor (Astron++, Heat Systems, Farmingdale, NY) for 10 s. Then, the samples were neutralized by the addition of 1.5 M KOH, 60 mM HEPES. After sedimentation of KClO₃, by centrifugation at 2000 × g for 15 min at 4°C, the supernatant was used for the IP₃ measurement. The assay was performed according to the manufacturer’s instructions.

Statistics—Statistical significance was analyzed with Student’s t test. Data are expressed as means ± S.E.

RESULTS

The CD38 Expression, cADPR Content, and the Changes of cADPR in Response to Ach in Pancreatic Acinar Cells from Wild (CD38\(^{+/+}\)) and Knockout Mice (CD38\(^{-/-}\))—By Western blot analysis using a polyclonal antibody against mouse CD38, we detected no CD38 expression in the pancreatic acinar cells from CD38 knockout mice, but its expression was detected from those of the wild type mice (Fig. 1A). In addition, by radioimmunoassay, we scarcely detected cADPR in the cells from the knockout mice, but it was detected in the wild type mice (Fig. 1B). Thus, the majority of the cellular CD38 content was caused by the activity of the ADP-ribosyl cyclase of CD38.

Changes in Cellular Content of cADPR and IP₃ in Response to Ach—Fig. 2 shows the changes in the cellular content of cADPR (Fig. 2A) and IP₃ (Fig. 2B) in response to Ach ranging from 40 nM to 4 μM. The cADPR content was increased by the Ach stimulation in the acini from wild mice but not in those from the knockout mice. In contrast, the IP₃ content was increased similarly in both types of acini. However, we were not certain that the method employed to measure the cADPR and IP₃ contents was sensitive enough to detect the difference in content at different Ach concentrations. The difference is
shown in later sections in terms of changes in the cellular \( \text{Ca}^{2+} \) concentration (\( \text{[Ca}^{2+}] \)) by fura-2 microfluorimetry.

**Effects of \( \text{Ca}^{2+} \), cADPR, and IP\(_3\) on \( \text{Ca}^{2+} \) Responses of Pancreatic Acinar Cells from CD38\(^{-/-}\) and CD38\(^{+/+}\) Mice**—Pancreatic acinar cells possess two types of \( \text{Ca}^{2+} \) activated ion channels (25). One is selective for monovalent cations (26), and the other for \( \text{Cl}^- \), and both are poorly dependent on membrane potentials between \(-60 \text{ mV}\) and \(-60 \text{ mV} \) but highly dependent on \( \text{[Ca}^{2+}] \) (27). Thus, increases and decreases of \( \text{[Ca}^{2+}] \), should be reflected by the activity of these channels. To demonstrate the presence of normal \( \text{Ca}^{2+} \) release mechanisms responsible for IP\(_3\) (28, 29), \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release (30, 31), and cADPR-mediated signaling, we injected these reagents directly into the single cells with the whole-cell recording technique and monitored the Ca\(^{2+}\)-dependent current activities (Ca\(^{2+}\) responses). Here, at a holding potential of \(-40 \text{ mV} \) achieved by the whole-cell recordings, Ca\(^{2+}\)-dependent current responses were expected to emerge as large inward currents carried by both \( \text{Cl}^- \) and monovalent cations. Shortly after the establishment of the whole-cell recordings (delay of 1–3 s) when the pipette included IP\(_3\), cADPR, or Ca\(^{2+}\), we observed a large deflection of the inward current at the membrane potential of

-40 mV (Fig. 3, 3/3 in each reagent injections). However, without the reagents there were no responses (3/3). That all the reagents successfully induced Ca\(^{2+}\) responses in the single acinar cells from both the wild and knockout mice indicated that the Ca\(^{2+}\)-releasing machinery was well preserved in both types of cells.

Close inspection of the records revealed that there might have been differences between the responses of the wild type and knockout cells to cADPR and/or IP\(_3\). That is, there was a longer response to cADPR and a shorter response to IP\(_3\) in the knockout cells than in those of the wild type. The reduction in cADPR hydrolysis activity in the knockout pancreatic acinar cells (13) may explain their prolonged response to cADPR. The cooperative action of cADPR for enhancing IP\(_3\)-induced Ca\(^{2+}\) responses (32) may explain the shortened response to IP\(_3\) because cADPR was eliminated in the knockout cells. However, further study is needed to determine the precise mechanisms of the reagent sensitivity.

**ACh-evoked \( \text{Ca}^{2+} \) Responses from Single Pancreatic Acinar Cells of Wild (CD38\(^{+/+}\)) and Knockout Mice (CD38\(^{-/-}\))**—Based on the above results, we employed fura-2 microfluorimetry and compared the ACh-induced pancreatic Ca\(^{2+}\) responses of the wild (CD38\(^{+/+}\)) and CD38 knockout mice (CD38\(^{-/-}\)) in a wide range of ACh concentrations from 10 to 20,000 nM. We used a Ca\(^{2+}\)-free solution containing 0.5 mM EGTA to avoid any contribution of external Ca\(^{2+}\) to the signaling in pancreatic acinar cells in the later experiments with fura-2 microfluorimetry. Table I summarizes the results in terms of the peak magnitude, the onset time after the application of ACh, and the
frequency of the periodic responses. The real traces of the responses are shown in Fig. 4 for 40 and 400 nM ACh.

The threshold concentration of ACh that induced a detectable Ca\(^{2+}\) response was 10 nM, and the response was a transient deflection with no significant difference observed in the two types of pancreatic acinar cells. A difference appeared at 40 nM ACh. Immediately after the application of 40 nM ACh, the cells from CD38\(^{+/+}\) mice showed a repetitive Ca\(^{2+}\) response (oscillations) during the ACh application, lasting for 2–3 min. In contrast, that of CD38\(^{-/-}\) was sporadic with the same ACh stimulation (Fig. 4A). However, at 400 nM ACh, the difference between the responses from CD38\(^{+/+}\) and CD38\(^{-/-}\) mice became less than that at 40 nM ACh, and both responses showed large phasic increases in the fura-2 signals (Fig. 4B).

The numerical data of the [Ca\(^{2+}\)]\(i\). increases in Table I were plotted against the ACh concentration (Ca\(^{2+}\) response curve) in Fig. 5, A and B. The magnitude response curve from the knockout mice (CD38\(^{-/-}\)) was smoothly graded with the increasing ACh concentrations. However, that of CD38\(^{+/+}\) showed two prominent phases separated at the concentration of 400 nM ACh. This feature was also seen in the Ca\(^{2+}\) index (18), which reflects the mixed information of both the magnitude and frequency of the response. Both response curves were replotted in Fig. 5, C and D, where the Ca\(^{2+}\) response from the knockout mice (CD38\(^{-/-}\)) was subtracted from that of the wild type (CD38\(^{+/+}\)) at each ACh concentration. Two prominent peaks were seen in the diagrams at the values of 40 and 4000 nM, indicating that two CD38-dependent phases, in other words, two cADPR-dependent phases, separated at 400 nM, are present in the ACh-induced release of internal Ca\(^{2+}\). One was induced by low ACh concentrations up to 400 nM (the first cADPR-dependent component) and the other by high concentration of ACh over 400 nM (the second cADPR-dependent component).

**Effect of Ryanodine on the ACh-induced Ca\(^{2+}\) Release in Single Pancreatic Acinar Cells from Wild Type (CD38\(^{+/+}\)) and Knockout (CD38\(^{-/-}\)) Mice**—Because cADPR has been postulated to be an endogenous modulator of the ryanodine-sensitive Ca\(^{2+}\) release mechanism (33–35), we next examined the effect of ryanodine on the pancreatic ACh-induced Ca\(^{2+}\) responses from normal cells. The results were compared with the Ca\(^{2+}\) responses from the knockout cells.

Fig. 6 shows the fura-2 signals in both types of cells treated either with or without ryanodine (500 μM) prior to the ACh stimulation (40 or 400 nM). The 40 nM ACh stimulation, without ryanodine, induced repetitive and sporadic transient responses in the wild and knockout mice, respectively, as described in Fig. 4A. In contrast, with ryanodine, the same stimulation induced a sporadic transient response and no response in the wild and knockout mice, respectively. Comparing these records, we noticed that the response to 40 nM ACh stimulation in the wild type cells with ryanodine resembled that of knockout mice without ryanodine (Fig. 6A). The diagram in Fig. 6C summarizes the effects of ryanodine on the ACh-induced Ca\(^{2+}\) responses in pancreatic acinar cells from the wild mice, showing

**Table I**

| ACh Concentration (nM) | CD38\(^{+/+}\) Magnitude | CD38\(^{-/-}\) Magnitude | CD38\(^{+/+}\) Frequency | CD38\(^{-/-}\) Frequency | CD38\(^{+/+}\) Onset | CD38\(^{-/-}\) Onset |
|------------------------|--------------------------|--------------------------|--------------------------|--------------------------|----------------------|----------------------|
| 40                     | 21.6 ± 6.1               | 14.5 ± 2.2               | 2.60 ± 0.32              | 0.6 ± 0.00              | 23.3 ± 3.9           | 55.1 ± 8.9           |
| 400                    | 100.5 ± 7.3              | 19.9 ± 3.7               | 3.00 ± 0.49              | 0.78 ± 0.04             | 19.4 ± 1.4           | 32.3 ± 4.9           |
| 4000                   | 228.8 ± 19.9             | 82.8 ± 5.2               | 1.93 ± 0.17              | 1.10 ± 0.17             | 19.4 ± 1.2           | 22.7 ± 1.8           |
| 2000                   | 230.4 ± 7.7              | 215.0 ± 11.6             |                        |                          | 10.6 ± 0.6           |                        |

* p < 0.001 different from control value.

* p < 0.01 different from control value.
that ryanodine abolished the first cADPR-dependent component but not the second component.

The complex effects of ryanodine on the knockout cells depended on the ACh concentration. That is, at concentrations of ACh from 10 to 400 nM, we could not detect any significant effect of ryanodine on the magnitude of the Ca$^{2+}$ response in knockout cells (Fig. 6D). In contrast, it enhanced the response at concentrations of ACh over 400 nM. It was noteworthy that the respective concentrations of ACh corresponded to the first and second cADPR-dependent components normally present in the wild type cells.

The effect of ryanodine at concentrations of ACh corresponding to the first cADPR-dependent component could be interpreted as indicating either that ryanodine treatment depletes the first cADPR-sensitive Ca$^{2+}$ pool or that it inhibits the Ca$^{2+}$ release from the pool. Either interpretation could be deduced from the result that ryanodine eliminated the first cADPR-dependent component from the wild cells and was without effect on the knockout cells. However, we prefer the first interpretation because the basal [Ca$^{2+}$] level was higher in the ryanodine-treated cells than that in both the wild and knockout cells without the treatment. The basal [Ca$^{2+}$], without ryanodine was 143.2 ± 3.2 nM (n = 269) in the wild cells and 151.3 ± 3.1 nM (n = 292) in the knockout cells. With ryanodine it was 234.6 ± 4.6 nM (n = 68) in the wild cells and 247.5 ± 2.6 nM (n = 87) in the knockout cells.

**DISCUSSION**

Comparing the ACh-induced Ca$^{2+}$ responses in pancreatic acinar cells from normal mice (CD38$^{+/+}$ wild type: normal cells) with those of CD38 knockout mice (CD38$^{-/-}$ type: KO cells), we distinguished the CD38- and therefore cADPR-dependent component from the overall muscarinic Ca$^{2+}$ signaling. The major findings are that: 1) ACh stimulation increased the cellular content of cADPR in the normal cells but not in KO cells; 2) the Ca$^{2+}$ response curve in the normal cells was separated into two phases at 400 nM ACh; 3) in contrast, the Ca$^{2+}$ response curve from KO cells was a smoothly graded one, and it lacked the two components inducible by ACh at concentrations below 400 nM (the first cADPR-dependent component) and over 400 nM (the second cADPR-dependent component) usually present in normal cells; 4) the first cADPR-dependent component contributed to the generation of repetitive Ca$^{2+}$ spikes; 5) ryanodine treatment eliminated the first cADPR-dependent component (Fig. 6).

**Physiological Significance of cADPR-dependent Ca$^{2+}$ Release**—IP$_3$ and cADPR are established Ca$^{2+}$-mobilizing messengers that activate internal IP$_3$ and ryanodine receptors, respectively (36–38). Because we scarcely detected cADPR formation in KO cells, the majority of Ca$^{2+}$ release in these cells could arise from Ca$^{2+}$ pools insensitive to cADPR but sensitive to IP$_3$ and/or yet unknown messengers. Thus, the IP$_3$-sensitive
pool obviously plays an important role in pancreatic Ca\textsuperscript{2+} responses, and it steadily contributes to the Ca\textsuperscript{2+} release in a wide range of ACh concentrations as evidenced by the smoothly graded response curve from KO cells in Fig. 5A. The superposition of cADPR on IP\textsubscript{3} and/or yet unknown messengers manifested two prominences on the curve, suggesting the presence of two subdivisions of cADPR-sensitive Ca\textsuperscript{2+} pools (the first and second cADPR-dependent components described above). The first cADPR-dependent Ca\textsuperscript{2+} pool is strictly sensitive to ryanodine, but the second one is rather resistant as evidenced by Fig. 6C.

A large part of the Ca\textsuperscript{2+} release is from the first cADPR-dependent pool at concentrations of ACh up to 40 nM, as shown in Table I and Fig. 4. The contribution of this pool promotes the sharp repetitive spikes (Fig. 4A), which could be advantageous for the fine control of Ca\textsuperscript{2+}-dependent cell function (39). That the repetitive spikes were severely impaired in the knockout mice suggests that they depend on cADPR formation. Thus, it could be that cADPR is one of the crucial messengers for maintaining the repetitive Ca\textsuperscript{2+} spikes. However, we cannot exclude the possible contribution of IP\textsubscript{3}. It has been reported in other cells that IP\textsubscript{3} is generated in an oscillatory manner (40). We do not know the detailed pattern of cADPR and IP\textsubscript{3} formation in our cells; however it is likely that the ACh stimulation generates both messengers in a repetitive or oscillatory fashion. The synchronized formation of these two messengers may generate

**Fig. 6.** Effects of ryanodine on ACh-induced Ca\textsuperscript{2+} responses in CD38\textsuperscript{+/+} and CD38\textsuperscript{-/-} pancreatic acinar cells. Traces of Ca\textsuperscript{2+} increases evoked by 40 nM (A) or 400 nM ACh (B) in single pancreatic acinar cells from CD38\textsuperscript{+/+} (left column) and CD38\textsuperscript{-/-} (right column) mice. Aa and Ba, control; A-b and B-b, after 500 \textmu M ryanodine treatment. Arrows indicate the start of ACh stimulation. C, diagram of ryanodine effect on ACh-induced Ca\textsuperscript{2+} responses in the wild (CD38\textsuperscript{+/+}) type cells. The peak magnitude of the evoked [Ca\textsuperscript{2+}], increases was plotted against the ACh concentration. Open circles (○) represent the wild (CD38\textsuperscript{+/+}) control response, and open triangles (△) are the wild with 500 \textmu M ryanodine. D, diagram of ryanodine effect on ACh-induced Ca\textsuperscript{2+} responses in the knockout (CD38\textsuperscript{-/-}) type cells. Filled circles (●) represent the knockout control cells (CD38\textsuperscript{-/-}) and filled triangles (▲) those with 500 \textmu M ryanodine. *** and ** represent the significance level \( p < 0.001 \) and \( < 0.01 \), respectively, for the test with ryanodine versus control.
various patterns of repetitive Ca$^{2+}$ spikes under cooperation with increased cellular Ca$^{2+}$, which is ascribed mainly to the first cADPR-dependent Ca$^{2+}$ pool. The critical concentration of ACh that switched the mode of the Ca$^{2+}$ response from the repetitive spike to the phasic deflection was 400 nM (Table I, “Frequency”). Based on the above findings, it could be that the messenger formation is no longer repetitive but rather phasic over this concentration of ACh. The significance of the second cADPR-dependent Ca$^{2+}$ pool could be that it serves as the resource of the phasic release of Ca$^{2+}$.

Ryanodine was able to induce Ca$^{2+}$ release at an exceedingly high rate (Fig. 6D) with concentrations of ACh over 400 nM in KO cells. This may have resulted from a complex mechanism that involves enhanced sensitivity to ryanodine in the second cADPR-sensitive Ca$^{2+}$ pool. Such an enhancement may have arisen from the constant lack of cADPR in the KO cells (Fig. 1B).

CD38 in the Ca$^{2+}$ Signaling of Muscarinic Receptor Stimulation—The presence of CD38 in the Ca$^{2+}$ release mechanism is of particular importance. The CD38-mediated Ca$^{2+}$ signaling may be associated directly with the receptor stimulation (Fig. 2A). This signaling pathway may be separated from that of IP$_3$ since both the formation of cADPR (Fig. 2A) and its contribution to the Ca$^{2+}$ release (Fig. 5A) are large at 40 nM ACh, at which concentration the contribution of IP$_3$ is still low. It is likely that muscarinic stimulation activates both signaling pathways in parallel and that they contribute synergistically to the overall Ca$^{2+}$ signaling in pancreatic acinar cells. However, the precise underlying mechanism of cADPR formation after the muscarinic ACh stimulation remains unknown.

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