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TIME- AND USE-DEPENDENT INHIBITION BY RYANODINE OF CAFFEINE-INDUCED CONTRACTION OF GUINEA-PIG AORTIC SMOOTH MUSCLE

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ABSTRACT: We examined how ryanodine interfered with Ca^{2+}-releasing action of caffeine in guinea-pig aorta. Ryanodine (10 μM) decreased the caffeine-induced contraction depending on the time of pretreatment with the agent. The development of ryanodine-effect with time was neither due to a slow access to its binding site nor due to the depletion of stored Ca^{2+}. Ryanodine more potently inhibited the second or the subsequent contraction due to caffeine than the first one even after the agent was removed from the bath after first caffeine. The enhancement of ryanodine-effect depended on the history of Ca^{2+} release but not on an increase in cytoplasmic Ca^{2+}. The data suggest that an opening of Ca^{2+} release channel enhances the interaction of ryanodine with the channel.

For these several years ryanodine gained reputations as a specific tool for the study of Ca^{2+} release mechanism of sarcoplasmic reticulum (SR) in striated muscles (1). In vascular smooth muscle, we showed that ryanodine inhibited the contractions due to intracellular Ca^{2+} release induced by caffeine or norepinephrine (2). This implies that the Ca^{2+} release mechanism in vascular smooth muscle, where SR is less specialized compared to striated muscles, shares some common property with that in skeletal or cardiac muscle.

Although the site of action of ryanodine is addressed to be a SR Ca^{2+} release channel (3-7), it is still argued how this agent interacts with the channel because the data on the effects of ryanodine on SR Ca^{2+} release are pretty conflicting depending on preparations and experimental conditions (1). The inconsistency of the ryanodine-effect among reports suggests that the interaction of ryanodine with SR Ca^{2+} release channel depends on the state of

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Abbreviations: SR, sarcoplasmic reticulum; PSS, physiological saline solution

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the channel. Here we examined the mode of action of ryanodine on caffeine-induced contraction of guinea pig aorta in order to know how this agent interacted with Ca^{2+} release channel.

METHODS AND MATERIALS

The aortic rings from guinea-pigs of 1-2 mm width were suspended in physiological saline solution (PSS) containing 2.5 mM $CaCl_2$ gassed with 95 % O_2 and 5 % CO_2. After the muscle was equilibrated in PSS for 1 hr with a basal tension of 1g, the external medium was changed to Mg^{2+}-free PSS in order to maximize the caffeine-induced contraction (8). Further 1 hr later the muscle was challenged by caffeine in Ca^{2+}-containing PSS or in nominally Ca^{2+}-free PSS. In preliminary experiments we learned that if the muscle was incubated in 2.5 mM Ca^{2+}-PSS for more than 5 min caffeine induced an equal size of contractions, so that the Ca^{2+}-loading was taken for at least 5 min. Stable responses to caffeine were obtained between 1 hr and 3 hrs after changing to Mg^{2+}-free PSS. As control caffeine was applied twice before ryanodine and the mean value was taken as 100%. Normal PSS contained (mM): NaCl 136.8, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $NaHCO_3$ 11.9 and glucose 5.5 (pH 7.2-7.4). Ryanodine (S.B. Penick, lot# 704RWP-1) was diluted into PSS from the stock solution (1mM). Caffeine (Wako Pure Chemicals) was directly dissolved in PSS. Data are expressed as a mean ± S.E. of 6-12 experiments.

RESULTS AND DISCUSSION

At first it was determined how long preincubation with ryanodine was needed to obtain the steady state effect. The Ca^{2+} store was loaded with Ca^{2+} by exposing the muscle to 2.5 mM Ca^{2+}-PSS for 15 min and then the muscle was exposed to Ca^{2+}-free PSS for 20 min until 10 mM caffeine was added. Ryanodine (10 μM) was present for a varied time before caffeine (Fig. 1A). As shown in

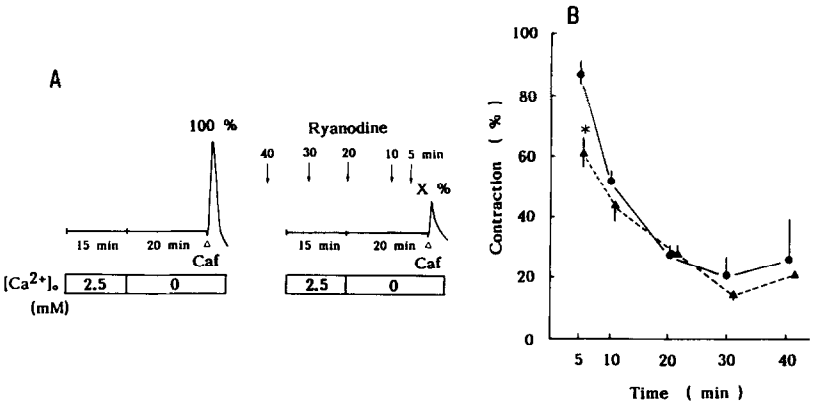

Fig. 1. Time-dependent inhibition by ryanodine of caffeine-induced contraction of guinea pig aorta. **A:** Protocol, the Ca^{2+} store of the muscle was loaded with Ca^{2+} by incubation with 2.5 mM Ca^{2+} PSS for 15 min. Then the medium was changed to Ca^{2+}-free PSS and 20 min later 10 mM caffeine was added. When present, 10 μM ryanodine was applied at a time indicated. For 40 min pretreatment with ryanodine, 2.5 mM Ca^{2+} PSS period was prolonged to 20 min. **B:** Decline of caffeine-contraction vs. the time with ryanodine. Circles; results obtained from the protocol shown in **A** (n=6). Triangles (broken line), experiments done in 2.5 mM Ca^{2+} PSS without Ca^{2+}-free PSS period (n=6). * $P<0.05$

Fig. 1B (circles), the caffeine-induced contraction decreased depending on the time with ryanodine. The period of incubation with ryanodine to attain the steady state effect was 20 min. To test whether the time dependency of ryanodine effect meant that it took a time for the agent to penetrate the plasma membrane, ryanodine was added in the bath for only 5 min, namely between 20 min and 15 min before caffeine. In this case, the decrease of the caffeine contraction was 74 $\pm$ 4 %, which was the same as the case that ryanodine was present throughout the period (72 $\pm$ 2 %). This suggests that ryanodine could pretty rapidly reach the intracellular site and it was not readily removed from the site.

It was reported that ryanodine enhanced the Ca^{2+} efflux from SR in intact muscles (9-13) or from isolated SR (14-16) under some conditions. Some groups postulated that this was a cause for the inhibition of contractions in cardiac muscle (9-11) and vascular smooth muscle (13). If ryanodine considerably accelerated the Ca^{2+} efflux from Ca^{2+} store in vascular smooth muscle, the Ca^{2+} content in the store of muscle incubated in Ca^{2+}-free PSS would decrease progressively with time. If this was a cause for the time-dependent inhibition by ryanodine, the decay of contraction should be faster when the muscle was incubated in Ca^{2+}-free PSS than in Ca^{2+}-containing PSS because the store might be replenished with Ca^{2+} to some extent under the latter condition. So we tested the time-dependent effect of ryanodine in the muscle incubated in 2.5 mM Ca^{2+}-PSS (Fig. 1, triangles). Contrary to the expectation, an inhibition of the caffeine contraction at 5 min treatment with ryanodine was greater when tested in 2.5 mM Ca^{2+}-PSS than in Ca^{2+}-free PSS and the time course of decay for the rest period was the same for both cases. This issue was re-examined by a protocol in which ryanodine-treatment was fixed to 20 min and Ca^{2+}-free period was varied (Fig. 2). It was expected that, if an acceleration of Ca^{2+} depletion was a cause for the time dependency, ryanodine should inhibit the caffeine-contraction depending on the time with Ca^{2+}-free PSS. To lessen the ryanodine effect, 3 μM ryanodine and 20 mM caffeine were used. As shown in Fig. 2 (B,C), a longer exposure to Ca^{2+}-free PSS rather attenuated the ryanodine effect.

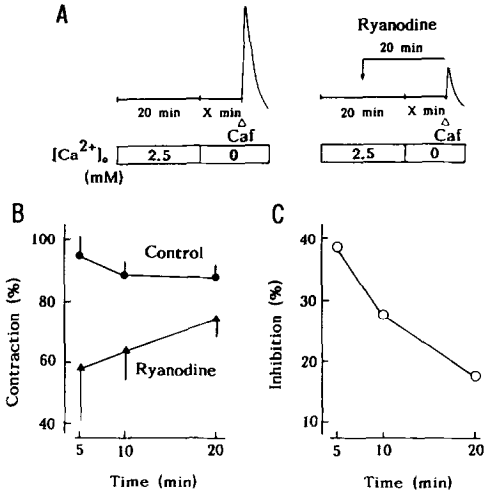

Fig. 2. Influence of incubation with Ca^{2+}-free PSS on inhibitory action of ryanodine on caffeine contraction. **A:** Protocol, 3 μM ryanodine was added 20 min before 20 mM caffeine. Ca^{2+}-free period was changed (5, 10 and 20 min). For 20 min Ca^{2+}-free PSS, ryanodine was added immediately after changing the medium to Ca^{2+}-free PSS. **B:** Caffeine contraction vs. the time of incubation with Ca^{2+}-free PSS. A 100 % in the ordinate represents the control caffeine contraction induced 2 min after changing to Ca^{2+}-free PSS. Abscissa, time with Ca^{2+}-free PSS. **C:** Percent inhibition by ryanodine to the respective control caffeine contraction. Abscissa, the same as **B**. n=8-12.

From the above results it is unlikely that the loss of stored Ca^{2+} was a major cause for time-dependent inhibition by ryanodine of caffeine-contraction. Existence of delay before the initiation of ryanodine action was also observed in cardiac cells (11,17), isolated SR (14,18) or SR Ca^{2+} release channel incorporated into lipid bilayers (6). In the latter two preparations the decline of Ca^{2+} content does not matter for the time dependency. As it was shown that ryanodine can reach the binding site within 5 min in intact aortas but it requires 20 min for the steady effect, it is likely that the binding itself does not mean the initiation of the action. The results shown in Fig. 2 suggest that the ryanodine effect also depends on the time of incubation in Ca^{2+}-containing PSS if the period of ryanodine treatment was fixed. This and the fact that the effect at 5 min treatment with ryanodine was greater when added in 2.5 mM Ca^{2+}-PSS than added in Ca^{2+}-free PSS (Fig. 1B) may be related to the observations that submicromolar or micromolar level of Ca^{2+} is necessary for the binding of ryanodine (3,15,18).

Next we observed the effect of this agent on the repeated contractions due to caffeine (Fig. 3). The protocol was as follows; the muscle was incubated in

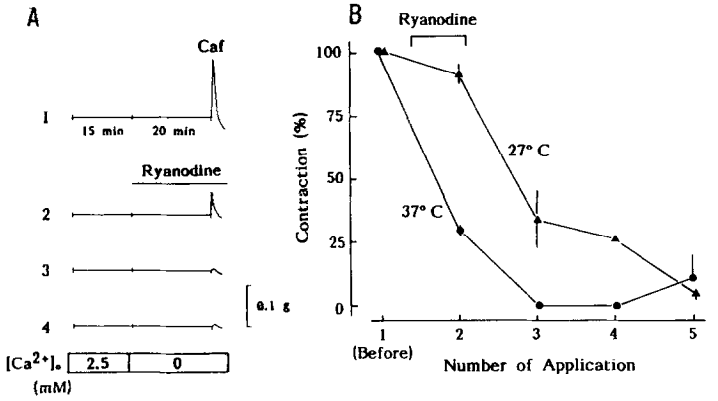

Fig. 3. Effect of ryanodine on repeated contractions due to caffeine. **A:** Protocol, after Ca^{2+}-loading for 15 min, the medium was changed to Ca^{2+}-free PSS and 20 min later 10 mM caffeine was added. After observing control caffeine contraction, 10 μM ryanodine was added to Ca^{2+}-free PSS for 20 min (panel 2). When caffeine was washed, ryanodine was also washed out. **B:** Contraction due to caffeine on successive applications. Experiments were done at 37° C (circles) or at 27° C (triangles). Control caffeine contraction (panel 1) under each condition was normalized to 100 %. n=6.

2.5 mM Ca^{2+}-PSS for 15 min for Ca^{2+} loading, then soaked in Ca^{2+}-free PSS for 20 min until 10 mM caffeine was added (Fig. 3A). When this protocol was repeated without ryanodine, reproducible responses were obtained. Ryanodine (10 μM) was present for 20 min when the muscle was incubated in Ca^{2+}-free PSS (Fig. 3A, panel 2). At 37° C ryanodine decreased the first caffeine-contraction to 25 % whereas it almost completely suppressed the second or third caffeine-contraction in spite of the absence of ryanodine in the medium (Fig. 3B, circles). Thus the ryanodine effect appeared to be use-dependent. It took about 3 hrs to restore the original size of caffeine-contraction. When the same experiment was performed at 27° C (Fig. 3B, triangles), the effect developed more slowly. Thus it seems that the effect of ryanodine developed depending on the history of challenges by caffeine.

To see whether the history of Ca^{2+} release might affect the action of ryanodine, we compared the effects of ryanodine, which was applied just before first caffeine or just after that, on the subsequent caffeine-induced contraction (Fig. 4). First caffeine contraction was induced in normal PSS. In A, ryanodine (10 μM) was applied just after the wash out of first caffeine and in B it was applied 1 min before the first caffeine. In both cases ryanodine was

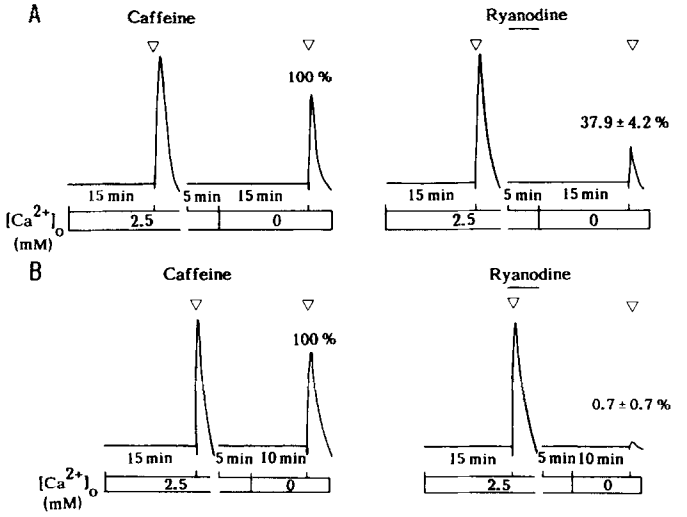

Fig. 4. Enhancement of ryanodine effect by Ca^{2+} release due to caffeine. First caffeine (10 mM) contraction was elicited in normal PSS and the second one was elicited 15 min (**A**) or 10 min (**B**) after changing to Ca^{2+}-free PSS. Ryanodine (10 μM) was present between 20 min and 15 min before the second caffeine although it was added just after first caffeine in **A** , while it was added 1 min before first caffeine in **B**. n=8.

present between 20 min and 15 min before the second application of caffeine. In B ryanodine slightly decreased the contraction due to first caffeine since the treatment with ryanodine was too short. However, it almost completely suppressed the contraction due to second caffeine. When ryanodine was applied just after the wash out of first caffeine it decreased the contraction due to second caffeine by 62 % (A). These results suggest that the opening of SR Ca^{2+} release channel or an increase in cytoplasmic Ca^{2+} enhances the action of ryanodine. To test the latter possibility ryanodine was added during a sustained phase of contraction due to 60 mM KCl which significantly increased the cytoplasmic Ca^{2+}. Ten min later the medium was changed to Ca^{2+}-free PSS (5.4 mM KCl) and further 20 min later 10 mM caffeine was added. Ryanodine inhibited caffeine-contraction by 78 $\pm$ 4 %. When ryanodine was added to 5.4 mM K^+-PSS, the contraction was inhibited by 78 $\pm$ 7 %. Namely an increase in cytoplasmic Ca^{2+} was not a cause for the greater inhibition on subsequent caffeine-contraction. Therefore we can conclude that an opening of Ca^{2+} release channel greatly enhances the action of ryanodine. This is consistent with observations of Meissner (15) on isolated SR that the conditions rendering Ca^{2+} release channel

open enhanced the action of ryanodine. The present study suggests that ryanodine easily binds to a receptive site but exerts a weak action when a large population of Ca^{2+} release channel is closed and that a ryanodine-receptor complex changes to a different form upon the channel opening.

Recent electrophysiological studies on Ca^{2+} release channel incorporated into lipid bilayers (6,7,19,20) revealed that ryanodine locked the channel in an open state whereas it reduced the channel conductance to about half. This means that ryanodine acts like a partial agonist, i.e. ryanodine may spill Ca^{2+} from SR under a resting condition through half-opened channels but antagonize the action of Ca^{2+} release channel activator such as caffeine. Further it was shown that a high concentration of ryanodine closed the channel (20) consistent with other biochemical data (15,16). If ryanodine acts similarly in vascular smooth muscle, it is possible that both a depletion of Ca^{2+} in the store and an antagonism to active Ca^{2+} release induced by caffeine contribute to a decrease of contraction due to Ca^{2+} release in the tissue. To see which is more important for the inhibition of caffeine-contraction it must be determined how much Ca^{2+} remains after the augmentation of ryanodine effect by an opening of Ca^{2+} release channel.

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