Effects of Ryanodine and 9,21-Didehydroryanodine on Caffeine-Induced Contraction of Rat and Guinea Pig Aortae

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Abstract—We compared the effects of ryanodine and 9,21-didehydroryanodine (DH-ryanodine), which are present in commercial preparations of 'ryanodine', on the contractions of rat and guinea pig aortae induced by 20 mM caffeine and tested the dependence of the action of each substance on external Ca++. With the first protocol, the aortae were incubated with ryanodine or DH-ryanodine for 20 min in Ca++-containing medium, and caffeine was added at 2 min incubation in Ca++-free medium. With the second protocol, each substance was added when the external medium was changed to Ca++-free medium, and 20 min later, caffeine was applied. Ryanodine and DH-ryanodine inhibited the caffeine-induced contractions in a similar way; i.e., with maximal effects at 3 nM and lesser effects at 10 nM. The potencies of inhibition by both substances were similar except that the effect of ryanodine at 1.5 nM was more potent than that of DH-ryanodine with the second protocol. The response by muscles previously loaded with Ca++ to a second application of caffeine was more greatly inhibited by both compounds (use-dependent effect). The inhibition of the contraction due to the first or second application of caffeine was greater when either agent was applied in Ca++-containing medium than in Ca++-free medium. These results indicate that ryanodine and DH-ryanodine are similar in their effects on caffeine-induced Ca++ release in vascular smooth muscle and that cellular Ca++ levels may affect the action of ryanodine.

Ryanodine is now widely used for studies of intracellular Ca++ release in muscles. The advantage of ryanodine over other agents is that this alkaloid does not affect the Ca++ influx from the extracellular fluid, easily permeates the plasma membrane and selectively affects the Ca++ release mechanism of the sarcoplasmic reticulum (SR), so that it can be used with intact as well as permeabilized muscles and cell-free preparations (1-3). However, a concern is that the commercially available 'ryanodine' is not pure and contains mostly ryanodine and 9,21-didehydroryanodine (DH-ryanodine, Fig. 1) together with a small quantity of other unknown components (4, 5). DH-ryanodine exists in a larger quantity than ryanodine. The presence of this impurity points out that when using commercial 'ryanodine', it is important to define the effects of both agents on the experimental system being investigated.

Although recent studies identified the ryanodine receptor in skeletal or cardiac muscle as specifically the Ca++ release channel at junctional SR (6-8), its actual effects on muscles appear to be very complicated because many factors influence the manifestation of ryanodine-action. Especially, its action greatly depends on the concentration of Ca++, probably that at its binding site, and on the opening of Ca++ release channels (9-11). Therefore, if these properties of ryanodine are different from those of DH-ryanodine, the data obtained by the use of a commercial 'ryanodine' would become variable according to experimental conditions. In this paper, we
Fig. 1. Structures of ryanodine and DH-ryanodine (A) and separation of fractions from Penick-ryanodine and Lvog-ryanodine (B). In B, ryanodine and DH-ryanodine are labeled as Ry and DH-Ry, respectively. Numbers in the charts represent the elution volume (ml).

tested the pharmacological effects of purified ryanodine and DH-ryanodine on caffeine-induced contraction of aortic smooth muscles isolated from rats and guinea pigs and examined the dependency of each component on external Ca\(^{2+}\) and the history of Ca\(^{2+}\) release.

**Materials and Methods**

The thoracic aorta isolated from male
Wistar rats (350–450 g) or Hartley guinea pigs (400–500 g) was cut into rings and then cut open to rectangular strips (3–4 mm in width and 8–10 mm in length). The endothelial layer was removed by rubbing to eliminate any possible effect of ryanodine on the endocellular reticulum of endothelial cells. The strip was suspended in 5 ml physiological salt solution (PSS) aerated with 95% O₂+5% CO₂ at 37°C. PSS used for the tests of drugs was Mg²⁺-free PSS containing: 136.8 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl₂, 11.9 mM NaHCO₃ and 5.5 mM glucose (pH 7.3–7.4). For only the equilibration period, 1 mM MgCl₂ was included in PSS. The muscle was allowed to equilibrate for 1 hr under a resting tension of 1 g prior to experimentation. The isometric tension was recorded with a force-displacement transducer (Nihon Kohden, SB-1T). After this equilibration period, the muscle was challenged by 60 mM KCl twice and 1 μM noradrenaline to stabilize the contractility, then exposed to Mg²⁺-free PSS to maximize the caffeine-induced contraction (12), and again left for 1 hr to equilibrate the intracellular Mg²⁺ concentration.

The effects of ryanodine and DH-ryanodine were tested with two protocols. With the first protocol (protocol-1, Fig. 2A), rat aortae were incubated in Mg²⁺-free PSS (2.5 mM [Ca²⁺]₀) for 20 min, switched to Ca²⁺-free PSS for 2 min and then challenged with 20 mM caffeine. Ca²⁺- and Mg²⁺-free PSS contained 0.1 mM EGTA but not CaCl₂. When this protocol was repeated in the absence of ryanodine, caffeine induced reproducible contractions (39.5±1.5% of the maximal contraction induced by 60 mM KCl in normal PSS, n=58). After the control response to caffeine was observed two times, ryanodine or DH-ryanodine was applied only in Mg²⁺-free PSS period. When the medium was exchanged to Ca²⁺-free PSS 20 min later, the test substance was removed from the medium. In the second protocol (protocol-2, Fig. 3A), rat aortae were incubated in Mg²⁺-free PSS for 10 min, switched to Ca²⁺-free PSS for 20 min, at which time 20 mM caffeine was applied. Caffeine-contraction was stable when this protocol was repeated in the absence of ryanodine (32.5±2.7% of the maximal contraction induced by 60 mM KCl in normal PSS, n=41). When tested, ryanodine or DH-ryanodine was added just after the medium was switched to Ca²⁺-free PSS, i.e., 20 min before caffeine.

After treatment with ryanodine or DH-ryanodine, the caffeine-induced contractions were observed two times. For the observations, the same protocol (exposure to 2.5 mM Ca²⁺-PSS and then to Ca²⁺-free PSS) was repeated. Because caffeine was not readily soluble in water, 5 ml Ca²⁺-free PSS containing caffeine at a two-fold greater concentration than ultimately desired was added to
Fig. 3. Effects of ryanodine and DH-ryanodine which were added in Ca²⁺-free PSS on caffeine-induced contraction of rat aorta. A, Protocol-2. The muscle was loaded with Ca²⁺ in normal PSS for 10 min, then exposed to Ca²⁺-free PSS for 20 min until 20 mM caffeine was added. Test substance was added just after the exchange of the medium to Ca²⁺-free PSS. B, inhibition of contraction due to first caffeine by ryanodine and DH-ryanodine. C, inhibition of contraction due to second caffeine (use-dependent effect). After the first caffeine was washed out, the same Ca²⁺ loading and exposure to Ca²⁺-free PSS were repeated. In B and C, % inhibition was calculated from the control response to caffeine prior to application of test drugs and the response after the treatment. n=5–8. *: Significantly different from ryanodine.

5 ml Ca²⁺-free PSS. The results are expressed as the mean with S.E.

For HPLC analysis of commercial 'ryanodine', a column (4.6 mm φ×150 mm) packed with Wakosil 5C18 (reversed phase, Wako Pure Chemicals, Osaka) was eluted with methanol/H₂O (50/50, v/v). The flow rate was 1.0 ml/min at 30°C. The compounds were monitored at 268 nm (13). The amounts of the products were calculated from their peak areas on the chromatogram.

The following drugs were used: noradrenaline (Tokyo Kasei, Tokyo), caffeine (Wako Pure Chemicals, Osaka), commercial 'ryanodine', i.e., Penick-ryanodine (S.B. Penick, Lyndhurst, NJ, lot#: 704RWP-1) and Lvog-ryanodine (Lvog-Agrisystems, Wind Gap, PA, lot#: 87-2049-1), ryanodine and 9,21-didehydroryanodine (DH-ryanodine, Fig. 1) both purified by HPLC. After purification, ryanodine and DH-ryanodine were recovered into methanol/H₂O (50/50). The purity was 99.7% and 99.3% for ryanodine and DH-ryanodine, respectively. Since we found that methanol at above 1% made SR leaky to Ca²⁺, methanol was removed by freeze-drying the eluted samples, and then each sample was dissolved in distilled water. On the other hand, Penick-ryanodine was dissolved in distilled water, and Lvog-ryanodine was dissolved in 30% methanol to make a concentration of 1 mM since Lvog-ryanodine did not completely dissolve in distilled water. Methanol at 0.3%, which was the maximum concentration used when diluted from the stock solution of Lvog-ryanodine, did not affect the Ca²⁺ release.
Results

Contents of ryanodine and 9,21-didehydroryanodine in commercial preparations: The contents of ryanodine and DH-ryanodine in commercial preparations were 36.1% and 61.4%, respectively, for Penick-ryanodine, and 16.1% and 57.7%, respectively, for Lvog-ryanodine. The chromatography revealed that 5 other small peaks (24.0% in total) could be distinguished in Lvog-ryanodine, while 3 small peaks (2.4% in total) were observed in Penick-ryanodine (Fig. 1B). In Lvog-ryanodine, further small fractions were present, each of which was less than 0.5%.

Effects of ryanodine and 9,21-didehydroryanodine on caffeine-induced contraction: First, the effects of ryanodine and DH-ryanodine on caffeine-induced contractions were tested with protocol-1, where each substance was applied in Ca2+-containing PSS. Figure 2, B and C, show the effects of both substances at various concentrations on contractions induced by 20 mM caffeine on first and second applications, respectively. Ryanodine and DH-ryanodine quite similarly inhibited the first caffeine-induced contraction. The effect was maximal at 3 μM, and it decreased at 10 μM. Even if ryanodine or DH-ryanodine was washed out 5 min after its application or was present until caffeine was added, the potency of inhibition was equal to that shown in Fig. 2B. Notwithstanding that both substances were absent in the medium, the caffeine-induced contraction on second application was more greatly inhibited (P<0.05 for every concentration), exhibiting a 'use-dependent' effect (11).

Figure 3, B and C, show the effects of ryanodine and DH-ryanodine tested on protocol-2, where each substance was applied in Ca2+-free PSS. Ryanodine and DH-ryanodine similarly decreased the 20 mM caffeine-induced contraction, except that at 3 μM ryanodine was slightly more potent than DH-ryanodine. The inhibition by each substance was less potent as compared to the results with protocol-1 (P<0.05 for every concentration). If ryanodine or DH-ryanodine was washed out 5 min after its application, the potency of inhibition was the same as that in the case where the substance was present for 20 min. Therefore, for both protocols, once the muscle was exposed to ryanodine or DH-ryanodine for at least 5 min, the inhibitory effect of the substance on caffeine-induced contraction was the same whether or not the substance was present in the medium at the time of caffeine application. Like in the previous protocol, the effect was maximal at 3 μM and less at 10 μM. These substances also exhibited a 'use-dependent' effect on the second caffeine-induced contraction, although the effects were less potent at 0.3 and 1 μM as compared with the respective use-dependent effect in protocol-1 (P<0.05, Fig. 3C).

Table 1 summarizes the effects of purified

|                      | Protocol-1 | Protocol-1 | Protocol-2 |
|----------------------|------------|------------|------------|
|                      | Guinea pig | Rat        | Rat        |
| Ryanodine            | 79.5±1.6  | 81.5±2.7   | 49.9±1.6   |
| DH-ryanodine         | 72.2±2.4  | 73.7±3.1   | 40.9±2.8   |
| Penick-ryanodine     | 73.0±2.2* | 68.0±6.8   | 38.3±1.8*  |
| Lvog-ryanodine       | 48.4±4.6**| 65.6±4.6*  | 26.0±3.5** |

Protocol-1 and protocol-2 are shown in Fig. 2A and Fig. 3A, respectively. Briefly, for protocol-1 drugs were put in 2.5 mM Ca2+ PSS while they were put in Ca2+-free PSS for protocol-2. The comparison was made on the same weight basis because commercial 'ryanodine' was a mixture of several components. Data are expressed as a mean of percent inhibition with S.E. calculated from the response to caffeine prior to the application of test drugs and that after the application. The number of determinations is shown in parentheses. Statistical significance was tested between inhibition by purified ryanodine or DH-ryanodine and that by the commercial 'ryanodine' (P<0.05). *Significantly different from ryanodine. **Significantly different from DH-ryanodine.
ryanhodine, DH-ryanodine and commercial ‘ryanodine’ at a concentration of 1.5 μg/ml (approximately 3 μM as ryanodine) on 20 mM caffeine-induced contraction when tested with protocol-1 and protocol-2 on rat and guinea pig aortae. Penick-ryanodine was slightly less potent than ryanodine, and Lvog-ryanodine was significantly less potent than ryanodine or DH-ryanodine. As mentioned above, the total content of ryanodine and DH-ryanodine in Lvog-ryanodine was 73.8% so that the content of both substances which were tested in this experiment was 1.1 μg/ml. The measured potency of Lvog-ryanodine was lower than the potency expected from the dose-response curves for ryanodine and DH-ryanodine (Figs. 2B and 3B).

Next, we tested the effect of ryanodine on the concentration-response relationship of caffeine in guinea pig aorta using protocol-1. As we found in the above experiments that ryanodine and DH-ryanodine were pharmacologically similar when tested with protocol-1, we used Penick-ryanodine at a nominal concentration of 10 μM in this experiment. As shown in Fig. 4, ryanodine inhibited the caffeine-induced contraction in a non-competitive manner.

Discussion

Biochemical reports suggest that the binding of ryanodine to its receptor requires sub-micromolar or micromolar level of Ca2+ and the opening of Ca2+ release channels (9, 10, 14, 15). Therefore, we tested the effects of ryanodine and DH-ryanodine with two different protocols, where cytoplasmic Ca2+ should have been maintained at high (protocol-1) or low (protocol-2) concentrations, to see the dependency on cytoplasmic Ca2+, and on two successive caffeine-induced contractions to see the ‘use-dependency’. Another idea for adapting the two protocols was to determine if the two substances had different effects on SR membrane Ca2+ permeability that affected Ca2+ release oppositely. Such effects should have appeared differently in the two protocols because SR could lose Ca2+ rapidly in a muscle incubated in Ca2+-free milieu (protocol-2) where cytoplasmic Ca2+ was low and Ca2+ was not replenished to SR. Namely, the relative potency of the Ca2+ leaking component should have been larger in protocol-2 than in protocol-1.

In this study, ryanodine and DH-ryanodine exhibited similar characteristics such as the dose-related effect, the dependency on external Ca2+ and the ‘use-dependent’ effect, indicating that the pharmacological properties of ryanodine and DH-ryanodine are qualitatively similar rather than opposite in regard to the effect on caffeine-induced contraction of the vascular preparations tested here. Quantitatively, DH-ryanodine may be slightly less potent than ryanodine since ryanodine was significantly more potent than DH-ryanodine at 3 μM for protocol-2 (Fig. 3B). If any, the difference in potency between two substances should be small. Ryanodine and DH-ryanodine have been found to be equipotent in their functional effects on skeletal and cardiac muscle preparations and isolated SR membrane Ca2+ permeability (13, 16). In addition, these agents bind with similar affinities to isolated SR membranes (K. Murakami and J.L. Sutko, unpublished observations). Therefore the biphasic action of ryanodine observed in some studies (9, 15, 25).
17–20) is not due to a co-existence of DH-ryanodine.

The inhibition by ryanodine or DH-ryanodine of caffeine-induced contraction was maximal at 3 μM and reduced at 10 μM. Such a deviation from the dose-dependence at a high concentration was also observed in cardiac (20, 21) and skeletal muscles (22). This suggests a possibility that each substance has a dual action on Ca\(^{2+}\) release, which depends on the concentration. However, at present, we are not sure whether each substance at low concentrations enhances Ca\(^{2+}\) leakage from the SR and at high concentrations, prevents Ca\(^{2+}\) release as suggested in some biochemical reports (9, 14, 18). Further study is needed to clarify whether ryanodine can either enhance or inhibit Ca\(^{2+}\) release in intact tissues depending on the concentration or experimental conditions.

The action of ryanodine or DH-ryanodine applied in the presence of Ca\(^{2+}\) in the medium was larger than that obtained in the absence of Ca\(^{2+}\). This provides a possibility that cytoplasmic Ca\(^{2+}\) concentration affects the binding of ryanodine to a site(s). This assumption would be supported by the biochemical studies that micromolar Ca\(^{2+}\) was required for the ryanodine binding or for the existence of a particular channel conformation that binds ryanodine (10, 14, 15).

However, there is another possibility that Ca\(^{2+}\)-induced Ca\(^{2+}\) release affected the potency of ryanodine-action. As our previous study revealed (11), the ryanodine-action depends on the history of opening of the Ca\(^{2+}\) release channels. Since it is considered that a small amount of Ca\(^{2+}\)-induced Ca\(^{2+}\) release occurs with a resting level of Ca\(^{2+}\), as in the case of cardiac cells (23), this spontaneous release could render ryanodine bound at an inactive site into an 'active form' during incubation with normal external Ca\(^{2+}\), while this could not occur if the muscle was incubated in Ca\(^{2+}\)-free PSS. However, this may not be a major cause for the greater inhibition in protocol-1 than in protocol-2 for the following reason: Caffeine at 20 mM would open most of the Ca\(^{2+}\) release channels present as suggested from the data of Fig. 4, thereby exerting the use-dependent effect on the subsequent application of caffeine. Therefore, if ryanodine equally bound to its site in both conditions, its use-dependent effect on the second application of caffeine should have appeared similarly irrespective of the different protocols. Contrary to this expectation, the inhibition of second caffeine-induced contraction was still smaller in protocol-2 than in protocol-1. This means that the extent of initial binding of ryanodine, which might be dependent on cytoplasmic Ca\(^{2+}\), is a major determinant for the greater potency of ryanodine-action in protocol-1. In this study, we observed the effects of ryanodine and DH-ryanodine in Mg\(^{2+}\)-deficient medium to maximize the caffeine-induced contraction. Some papers suggested that Mg\(^{2+}\) competes with the promoting action of Ca\(^{2+}\) for ryanodine binding to skeletal muscle SR vesicles (9, 15). Therefore, if tested in Mg\(^{2+}\)-containing PSS, the potency of ryanodine might be lower than the present results.

The present data show that the actions of ryanodine and DH-ryanodine are comparable and similarly dependent on external Ca\(^{2+}\) and the 'use'. When calculated from the content of both substances in commercial preparations, the potency of Lvo-r-ryanodine should have been larger than that shown in Table 1. This apparently lower potency of the commercial 'ryanodine' suggests that components other than ryanodine and DH-ryanodine in the preparation may interfere with the action of the active components. The influences of other small fractions must be clarified in a future study.

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