Effects of Ryanodine and Nifedipine on Contractions Induced by Norepinephrine, Acetylcholine, and Potassium in the Isolated Vas Deferens of the Guinea Pig

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(Received March 22, 1990)

In the present study we tried to determine the relative contributions of sarcoplasmic reticulum (SR) calcium release and gated calcium entry through voltage-dependent calcium-channels (VDC) to the contractions induced by norepinephrine (NE, 10 μ M) and acetylcholine (ACh, 10 μ M) in the vas deferens of the guinea pig. NE and ACh at 10 μ M caused contractions composed of three phases: a prephasic, a phasic and a tonic component. Nifedipine strongly inhibited the contractions induced by potassium (K, 100 mM) and abolished phasic components of the contractions induced by NE or ACh. However, the tonic components of NE- or ACh-induced contractions were slightly attenuated by nifedipine. Pretreatment with ryanodine in combination with caffeine attenuated prephasic and tonic components of the contractions induced by NE and ACh in a dose-dependent manner. The inhibitory effects of ryanodine and caffeine on the phasic component of NE-induced contractions were divided into two types: 1) abolishment of the phasic contractions and 2) a delayed appearance of the phasic contractions without attenuation of magnitude. However, ryanodine with caffeine minimally affected phasic component by ACh- and K-induced contractions. Ryanodine pretreatment in combination with NE, ACh or K had no effect on the contractions induced by each agonist. These results suggest that 1) SR Ca release is essential for the initiation and/or the maintenance of the tonic components of NE- or ACh-induced contractions as well as for the initiation of the prephasic components, 2) the phasic components by NE depend on both the SR Ca release and gated Ca entry but those induced by ACh depend only on gated Ca entry, 3) the Ca-induced Ca-release mechanism may be scarcely involved in SR Ca release induced by NE and ACh.

Keywords — ryanodine; nifedipine; norepinephrine; acetylcholine; calcium release; guinea pig vas deferens

Introduction

Contractions of the smooth muscles have been considered to be caused by an elevation in the cytosolic free Ca concentration. The relative contribution of the Ca entry mechanisms and sarcoplasmic reticulum (SR) Ca release for the contractions are likely to vary with the types of stimulants and smooth muscles. In the smooth muscle of the vas deferens, contractions induced by potassium (K) depend on extracellular Ca influx through voltage-dependent Ca-channels (VDC).^{1,2)} However, conflicting experimental results have been reported regarding Ca sources utilized for the contractions induced by norepinephrine (NE) in the smooth muscle of the vas deferens. Khoiyi *et al.* suggested that NE did not stimulate Ca influx in the vas deferens of the rat and guinea pig.^{3,4)} However, we found, by measuring uptake of Mn used as a Ca analogue, that NE activates VDC in the vas deferens of

guinea pig.⁵⁾ It was also reported that the gated Ca influx was important for the initiation of NE-induced contractions⁶⁻⁸⁾ or for maintenance of tonic contractions induced by NE.⁸⁻¹⁰⁾ Moreover, Ca sources utilized for contractions induced by acetylcholine (ACh) have not been clarified in the vas deferens.

Recently ryanodine has been shown to interfere with SR function by acting on Ca release channels and is widely used to remove the function of SR. In this study, the effects of ryanodine and nifedipine on the contractions induced by NE, ACh and K were examined in the vas deferens of the guinea pig in order to determine the relative contributions of SR Ca release and gated Ca entry to NE- and ACh-induced contractions.

Materials and Methods

Male guinea pigs of the Hartley strain weigh-

ing 400 to 550 g were sacrificed by a blow to the neck. The vasa deferentia were isolated and the connective tissues were removed. A strip of 8 to 10 mm in length was prepared from the epididymal portion of the vas deferens. The preparations were mounted under 0.3 g load in organ baths containing aerated HEPES-Locke-Ringer solution (normal medium) of the following composition (mM): NaCl, 154; KCl, 5.6; MgCl_2 , 2.1; CaCl_2 , 2.2; glucose, 2.8; HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), 4.4, adjusted to pH 7.4 at 30 °C with 1N NaOH. Isotonic high-K medium (100 mM K) was prepared by replacing 94.4 mM NaCl in the normal medium with equimolar concentration of KCl.

The preparations were allowed to equilibrate in the normal medium for 60 min. Isotonic contractions in response to 10 μM NE, 10 μM ACh and 100 mM K were recorded for 5 min with 12.5 times magnification with an isotonic transducer (Nihon Kohden, Model TD-112S). The agonists at these concentrations caused maximal phasic and about 90% of maximal tonic contractions. One kind of agonist was applied to one preparation. NE- and ACh-induced contractions were initiated by bolus application of agonists. K-Induced contractions were initiated by substitution of the bathing medium with the high-K medium. Agonists were usually applied every 30 min except after the treatment of ryanodine with caffeine as described below.

Ryanodine (1.5 or 15 $\mu\text{g}/\text{ml}$) was applied with caffeine (50 mM) 25 min after the control contractions were obtained. The preparation used was exposed to ryanodine for 3 min. After a 27 min resting period, the test contractions were elicited by NE, ACh, or K to assess the effects of ryanodine. Caffeine did not inhibit the contractions when it was applied alone according to the above procedure. In some experiments, ryanodine was applied with NE (10 μM), ACh (10 μM) or K (100 mM) instead of caffeine. Ryanodine used in this study is a mixture of ryanodine and 9,21-didehydroryanodine. Thus, the concentration of ryanodine was not exactly expressed, but the real concentration of ryanodine is supposed to be less than 3 and 30 μM at the dosage of 1.5 or 15 $\mu\text{g}/\text{ml}$, respectively. The

potency of ryanodine and 9,21-didehydroryanodine has been shown to be comparable and similarly dependent on external Ca and the method of application.¹¹⁾

Nifedipine (10 nM—1 μM) was applied 20 min prior to addition of agonists.

The magnitudes of the prephasic and phasic components of the contractions were expressed by the peak heights of those components. The magnitudes of the tonic components of contractions were expressed by the heights 5 min after the application of agonists. They were measured from the resting level just before an application of an agonist. Unless otherwise stated, each component of contractions was expressed as the percentage of the magnitude of each components of the control contraction in the same preparation. The results were expressed as mean values $\pm$ S.E.M. Statistical analysis was made using the paired *t*-test and was considered to be significant at the level of 5% probability.

The drugs used were: *l*-norepinephrine (Nacalai Tesque), acetylcholine chloride (Daiichi-seiyaku), caffeine anhydrous (Nacalai Tesque), ryanodine (Wako), nifedipine (Yodogawa-seiyaku). NE was dissolved in 0.1 N HCl and diluted with 0.1% NaHSO_3 solution. ACh was dissolved in deionized and distilled water. Caffeine was dissolved in normal medium before use. Ryanodine and nifedipine were dissolved in ethanol. The final concentration of ethanol in the bathing medium was 0.3%. At this concentration, the vehicle had no effect on the contractions.

Results

Effects of Nifedipine on the Contractions Induced by Norepinephrine, Acetylcholine and Potassium

NE (10 μM) or ACh (10 μM) caused a contraction composed of three components which are the first, small transient phase (prephasic component), the second large phase (phasic component), and the third ensuing phase (tonic component) (see Fig. 1, control). High K-induced contraction was composed of phasic and tonic components (see Fig. 1, control).

The phasic and tonic component of the con-

Fig. 1. The Effects of Nifedipine on Each Component of the Contractions Induced by Norepinephrine, Acetylcholine and Potassium in the Isolated Vas Deferens of the Guinea Pig

Nifedipine (Nif; 10 nM—1 μM) was applied 20 min before each contraction elicited. A) norepinephrine (10 μM). B) acetylcholine (10 μM). C) potassium (100 mM).

tractions induced by NE or ACh were inhibited by nifedipine in a dose-dependent manner (10 nM—1 μM, Fig. 1). Nifedipine in a higher concentration (1 μM) abolished phasic components. The maximal inhibitory effect of nifedipine on the tonic components induced by NE and ACh was obtained at 0.1 μM of nifedipine, and the extent of the inhibition was approximately 15%. The prephasic components of the contractions were not inhibited by nifedipine, but they were significantly increased by nifedipine (1 μM) to $130.1 \pm 6.1\%$ and $114.8 \pm 5.8\%$, respectively.

Nifedipine (10 nM—1 μM) markedly inhibited the contractions induced by K in a dose-dependent manner (Fig. 1).

Effects of Ryanodine on the Contractions Induced by Potassium, Norepinephrine and Acetylcholine

Ryanodine (1.5 or 15 μg/ml) applied for 3 min alone did not cause a contraction and had no effect on the contractions caused by the three agonists which were applied 30 min after the treatment with ryanodine. Ryanodine treatment in combination with caffeine for 3 min also did not inhibit the contraction induced by K. However, the same treatment reduced the contractions induced by NE or ACh (Fig. 2).

Ryanodine applied in combination with caffeine significantly and dose-dependently inhibited the prephasic components and the tonic components of the contractions induced by NE (Fig. 2a). The inhibitory effects of ryanodine (1.5 μg/ml) applied with caffeine on the phasic component of the NE-induced contraction were grouped into two types (Fig. 3). In 5 of 12 preparations, the phasic component of the contraction

Fig. 2. The Effects of Ryanodine on the Contractions Induced by Norepinephrine (a) and Acetylcholine (b) in the Isolated Vas Deferens of the Guinea Pig

Columns represent the magnitude of each component of the contractions induced by NE (10 µM, a) or ACh (10 µM, b) after the treatment of ryanodine as below.

A, ryanodine (1.5 µg/ml) applied with caffeine (50 mM); B, ryanodine (15 µg/ml) applied with caffeine (50 mM); C, ryanodine (15 µg/ml) applied with an agonist. The magnitude of each component of the control contractions was regarded as 100%. The effects of ryanodine (A, B) on phasic components of NE-induced contractions were not shown (see Fig. 3). Vertical bars indicated S.E.M. a) means significant decrease from control; $p < 0.05$. $n = 8-12$.

induced by NE was abolished (Fig. 3, type I). In the remaining 7 preparations, the magnitude of the phasic component was not attenuated but the lag time of the appearance of phasic component

became significantly longer from 32.1 ± 1.7 s to 53.6 ± 4.1 s (Fig. 3, type II). By increasing the concentration of ryanodine to 15 µg/ml, the number of preparations showing the type I ef-

Fig. 3. Two Types of the Inhibitory Effects of Ryanodine on the Contractions Induced by Norepinephrine. Ryanodine was applied in combination with caffeine (50 mM). A, control; B, after ryanodine treatment.

fects were increased (6 of 8 preparations).

Ryanodine applied in combination with caffeine also inhibited the prephasic and tonic component of the ACh-induced contractions in a dose-dependent manner, but did not affect the phasic component (Fig. 2b).

When ryanodine (15 $\mu\text{g/ml}$) was applied with NE (10 μM) or ACh (10 μM) instead of caffeine, the treatments did not show any inhibitory effects on the subsequent contractions which were induced by NE or ACh, respectively (Fig. 2). Further, ryanodine (15 $\mu\text{g/ml}$) treatment in combination with K (100 mM) also had no effects on the subsequent contractions induced by NE or ACh.

Discussion

NE- and ACh-induced contractions of the guinea pig vas deferens were composed of a prephasic component, a phasic component, and a tonic component and each can be clearly differentiated. The tonic components of the contractions induced by agonists were sustained for more than 5 min.

The phasic components of the NE-contractions have been suggested to be strongly dependent on the gated Ca entry through VDC.¹²⁾ In fact, nifedipine (10 nM—1 μM) markedly inhibited the K-induced contractions and the phasic components of the contractions induced by NE and ACh. However, the prephasic components were not inhibited but were slightly increased by nifedipine used in the highest concentration. The mechanism of potentiation of prephasic components is unknown. The tonic components of the contractions induced by NE and ACh were slightly reduced by nifedipine. These results suggest that gated Ca entry through VDC scarcely participates in the initiation of the prephasic components and the maintenance of tonic components of the contractions induced by these agonists.

It has been reported that ryanodine inhibits the contractions of smooth muscle cells. The inhibition is considered to be due to the depletion of the intracellular calcium stores by locking Ca-induced Ca-release (CICR) channels to the open state.¹³⁻¹⁷⁾ The inhibitory effect is enhanced by

caffeine which enhances CICR mechanism. Ryanodine does not affect the Ca influx from extracellular fluid.^{11,17)} In fact, in this study, the contractions induced by K were not affected by the application of ryanodine with caffeine. The pretreatment of ryanodine together with caffeine also did not affect the phasic component of contractions induced by ACh. This is consistent with the above suggestion that gated Ca entry through VDC is mainly involved in the phasic components of ACh-induced contractions. However, ryanodine applied with caffeine abolished the phasic component of NE-induced contractions or delayed the appearance of those NE-induced contractions without any attenuation of the magnitude. The inhibition by ryanodine indicates that the SR Ca release is involved in the initiation of the phasic component of NE-induced contractions, even though the detailed mechanism of this all-or-none like phenomenon remains unclear. Further, ryanodine applied with caffeine abolished the prephasic components and greatly decreased the tonic components of the contractions induced by these agonists. These results indicate that the prephasic and the tonic components of the contractions induced by NE and ACh are strongly dependent on the SR Ca release.

The reduction of the tonic components by ryanodine was unexpected because it has been supposed that such a sustained contraction is due to Ca influx from extracellular fluid. Recently, new theories have been proposed to explain the sustained tonic contractions. One is the mechanism of latch and another is the cycling of Ca into and out of SR.¹⁸⁻²⁰⁾ The strong inhibitory effects of ryanodine on the tonic components of agonist-induced contractions are not in conflict with the involvement of such mechanisms in the contractions of the guinea pig vas deferens induced by NE and ACh.

Ryanodine pretreatment with NE or ACh did not have significant effects on the NE- and ACh-induced contractions. This result suggests that the mechanisms of SR Ca release induced by NE and ACh are different from those induced by caffeine. Recently, Iino *et al.* proposed the presence of two kinds of Ca stores in the smooth muscle.¹³⁾ One of them, named S_{α} , has both

CICR and inositol triphosphate (IP_3)-induced Ca-release (IICR) mechanisms, while $S\beta$ has only IICR mechanism. Ryanodine does not have any appreciable effect on the IICR channels even if ryanodine is applied when these channels are open.¹³⁾ The inhibitory effects of ryanodine applied in combination with caffeine on the contractions induced by NE and ACh suggest that the contractions induced by NE and ACh are dependent on Ca release from $S\alpha$ in the smooth muscle of the guinea pig vas deferens. The lack of inhibitory effects of ryanodine applied in combination with NE or ACh on the contractions induced by these agonists suggests that these agonists open only IICR channels but not CICR channels. It has been reported that the activation of α_1 -adrenergic receptor stimulates phosphatidylinositol turnover and IP_3 formation in vas deferens of the guinea pig²¹⁾ and the rat.^{3,22)} It seems strange that CICR channels do not open in spite of the increase of intracellular Ca concentration by NE, ACh or K which stimulate approximately maximum contractions. A possible explanation is that even in these conditions, the intracellular Ca concentration does not exceed $1\ \mu M$, which is the threshold to activate the CICR mechanism. Unfortunately, we do not have methods to precisely determine the intracellular Ca concentration. Another possible explanation of the ineffectiveness of ryanodine applied with agonists is that ryanodine could interact with CICR channels only in the presence of caffeine. The latter possibility seems to be unlikely because it has been reported that ryanodine without caffeine, can interact with Ca release channels of SR^{23,24)} and inhibit the contractions.^{25,26)}

In conclusion, the present results show that in the vas deferens of the guinea pig, 1) the prephasic and tonic components of contraction induced by NE and by ACh markedly depend on Ca release from $S\alpha$, 2) the phasic component of the contraction induced by NE depends on both Ca release from $S\alpha$ and gated Ca entry through VDC, 3) the phasic component of the contraction induced by ACh depends on gated Ca entry through VDC, 4) CICR mechanism is little involved in the SR Ca release induced by NE and ACh.

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