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Ryanodine-sensitive intracellular Ca^{2+} stores in isolated rabbit penile erectile tissue

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Abstract Norepinephrine release from adrenergic nerve terminals leads to a rise in intracellular Ca^{2+}, which promotes penile smooth muscle contraction and detumescence. Ca^{2+} sources are the extracellular space and sarcoplasmic Ca^{2+} stores. To elucidate the role of intracellular stores strips from rabbit erectile tissue were investigated in an organ bath study. Contractions were elicited by phenylephrine (PE) and electrical stimulation. Incubation in Ca^{2+}-free solution as well as exposure to nifedipine did not abolish electrical or PE-induced contraction. Ryanodine (10^{-5} mol/l), a functional blocker of sarcoplasmic Ca^{2+} channels, significantly reduced PE response. In the presence of caffeine (10^{-3} mol/l) the effect was significantly enhanced. Addition of nifedipine nearly abolished the contraction. These results provide evidence for intracellular Ca^{2+} pools in cavernosal tissue and indicate that the α_1-adrenoceptor-induced contraction requires the opening of voltage-gated Ca^{2+} channels and the release of Ca^{2+} from intracellular stores.

Key words Penile erection · Smooth muscle
Calcium channel blocker · Intracellular calcium
Ryanodine

Smooth muscle contraction is induced by an increase in the cytosolic free Ca^{2+}. The membrane systems involved in the control of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in the smooth muscle are (1) the plasmalemma, which is under the control of the membrane potential and various agonists, and (2) the sarcoplasmic reticulum, which is regulated by second messengers [4]. Plasmalemmal Ca^{2+} permeability is mainly controlled by voltage-gated Ca^{2+} channels and by chemically gated receptor operated Ca^{2+} channels. Since, in vascular smooth muscle, neurotransmitters can elicit transient submaximal contractions in the absence of extracellular Ca^{2+}, intracellular Ca^{2+} stores have been postulated. The sarcoplasmic reticulum has been identified as the main intracellular Ca^{2+} source [2, 4] and two separate types of Ca^{2+}-release channels have been demonstrated: Ca^{2+}-activated channels and inositol 1,4,5-triphosphate-activated channels [1, 18].

It has been shown that contraction in human penile erectile tissue is mediated mainly by neuronally released norepinephrine [16] and is highly dependent on the concentration of extracellular Ca^{2+} [7, 20]. However, electrically and norepinephrine-induced contraction could only partly be inhibited by aministration of the Ca^{2+} channel blocker nifedipine in vitro [7, 10].

This finding suggests that mechanisms other than voltage-gated Ca^{2+} channels are important in increasing $[Ca^{2+}]_i$. Thus norepinephrine-induced contractions in the presence of nifedipine may be attributable to the mobilization of intracellular Ca^{2+} stores or to activation of receptor-operated Ca^{2+} channels [1, 7]

To investigate intracellular Ca^{2+} stores, ryanodine, a naturally occurring plant alkaloid [9], is a useful tool [3, 6, 11, 17, 19]. In the rat vas deferens ryanodine-binding sites have been found in endoplasmic reticulum [3, 22], and ryanodine abolished mainly the initial phasic component of the nerve-mediated contraction, which suggests interference with the intracellular Ca^{2+} store [8, 11]. Ryanodine selectively depletes Ca^{2+} from the sarcoplasmic reticulum by locking Ca^{2+} release channels in a subconductance state. Ryanodine does not affect plasma membrane Ca^{2+} channels, nor does it affect the contractile apparatus or the sarcolemmal Ca^{2+} transport mechanisms [17].

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Materials and methods

Tissue preparation

Adult male New Zealand white rabbits, weighing 2.5–3.5 kg, were put to death by i.v. pentobarbital injection and exsanguination. The

penis was dissected free in toto and immediately placed in Krebs' solution (for composition see below). The cavernosal smooth muscle was bilaterally dissected free from the tunica albuginea and then each muscle strip was cut into two pieces measuring approximately $1 \times 2 \times 5$ mm. The cavernous strips were mounted between two metal hooks in an organ bath chamber containing 10 ml of a Krebs' solution.

Tension measurements

Tension was maintained by an adjustable connection to a Grass Instruments FTO3C force-displacement transducer for recording of isometric tension. A Grass 7D polygraph (Grass, Quincy, Mass., USA) was used for recording. Tension of the preparations was adjusted to 5 mN for the erectile tissue and equilibration was allowed for 1 h.

Experimental procedure

During the equilibration period repeated adjustment of tension was performed. No changes in tension were made after the experiment was started. At the end of the equilibration period, high-K^+ solution was added to the organ bath in order to test the contractile capacity of each strip. One tissue strip always served as a control.

Drugs and solutions

Krebs' solution was composed as follows: NaCl 118 mmol/l, $NaHCO_3$ 24 mmol/l, KCl 4.6 mmol/l, KH_2PO_4 1.6 mmol/l, $CaCl_2$ 1.2 mmol/, $MgSO_4$ 1.2 mmol/l and glucose 11 mmol/l. The solution was aerated with 5% CO_2 and 95% O_2, maintainng pH at 7.4. A thermoregulated water circuit maintained the temperature at 37 °C. Isotonic high-K^+ solution (124 mmol) was prepared by replacing the NaCl in the normal Krebs' solution by equimolar amounts of KCl. Ca^{2+}-free solution contained 0.1 mml/l ethylene glycol-bis (β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA, Sigma) and $CaCl_2$ was omitted. In Ca^{2+}-free, K^+-rich solution NaCl was again replaced by equimolar KCl in EGTA-containing Ca^{2+}-free solution.

The following drugs were used: phenylephrine (Sigma), nifedipine (Sigma), caffeine (Sigma) and ryanodine (ICN). Stock solutions and subsequent dilutions in Krebs' solution were prepared daily for phenylephrine (PE) and caffeine. A stock solution of nifedipine and ryanodine (10^{-2} mol/l) was prepared with ethanol and further dilutions were performed in distilled water. Both in separate experiments and in the permanent controls ethanol in the concentrations used showed no effects on the tissue. Direct exposure of nifedipine to light was avoided.

Phenylephrine stimulation

To obtain a PE dose relation to the K^+ (124 mmol/l)-induced contraction, PE was added cumulatively (10^{-8}–10^{-4} mol/l) to the tissue after each level of contraction had stabilized. To study the effect of nifedipine the tissue was incubated with increasing concentrations of nifedipine for 15 min and then PE was added cumulatively. Responses after nifedipine treatment were expressed as a percentage of the maximum untreated PE response. For all other experiments a PE concentration of 10^{-4} mol/l was applied and the data were compared with the pretreatment PE response.

Transmural electrical stimulation

A Grass S44 stimulator (Grass, Quincy, Mass., USA) and a current amplifier were used. The tissue strips were mounted between two parallel platinum electrodes (5 mm apart). Square waves at a voltage of 20 V with a duration of 0.8 ms and variable frequencies were provided in trains of 5 s. The stimulation interval was 120 s. Initially frequency-dependent contractions (1–100 Hz) after transmural electrical stimulation were recorded. At 20 Hz reproducible contractions of $60 \pm 8\%$ of the maximum response were seen and this frequency was chosen for the further study (data not shown). The responses were often biphasic with a small relaxation preceding the contraction and for the purpose of this study only the contractile element was measured. Amplitude of electrically induced contractions following treatment with Ca^{2+}-free solution or nifedipine was compared with the averaged amplitude of three pretreatment contractions, which was considered as 100%.

Statistical analysis

Results are expressed as mean value $\pm$ standard error of the mean. For all reported data at least five experiments in different animals ($=n$) were performed. EC_{50} values (concentration of PE showing half-maximum contraction) and IC_{50} values (concentration of nifedipine producing half-maximum inhibition) were determined graphically. Student's *t*-test was applied to compare responses. Comparison of responses of the same strip was analyzed as paired data, whereas for different strips the unpaired *t*-test was used. $P < 0.05$ was considered as significant.

Results

High K^+ and PE response

After equilibration high-K^+ solution (124 mmol/l) was applied to the strips and following washout PE dose response curves were recorded and the contractile responses were compared (Fig. 1). Maximum response to PE at a concentration of 10^{-3} mol/l was $152 \pm 6.3\%$ of the high-K^+-induced peak. The EC_{50} value for PE was 9.1 ± 0.7 µmol/l.

Ca^{2+}-free solution

Ca^{2+}-free solution reduced the baseline 2.6 mN (0.1–0.4 mN, $n=17$) and always eliminated spontaneous contractions, which were seen in 72% of strips. Incubation in Ca^{2+}-free solution time dependently abolished electrical stimulation (Fig. 2); however, after 5 min a contraction of $41 \pm 11\%$ ($n=5$) was still elicited. After 15 min in all but one strip the contraction was totally abolished. Readmission of Ca^{2+}-containing Krebs' solution always restored the response to electrical stimulation to the initial level within 5–7 min.

PE (10^{-4} mol/l)-induced contractions were only partially reduced to $65 \pm 5.7\%$ after 5 min incubation in Ca^{2+}-free solution ($n=7$). After 30 min a contractile response of $11 \pm 2\%$ ($n=5$) was still seen (Fig. 3). If now – in the presence of PE – the Ca^{2+}-containing Krebs' solution was administered the contraction slowly recovered. If after 10 min the bath solution was changed from Ca^{2+}-free to K^+-rich, Ca^{2+}-free solution in five of six experiments a small contraction of $23 \pm 6\%$ was still recorded ($n=6$).

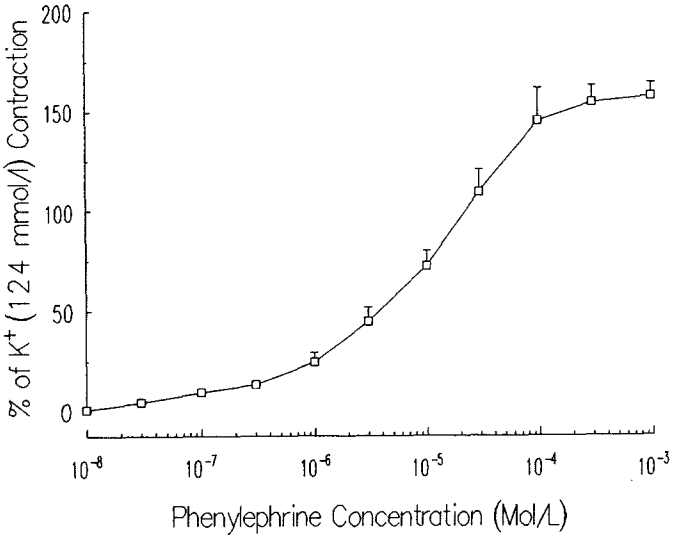

Fig. 1 Concentration response curves for phenylephrine (PE) expressed as percentages of K^+-induced (124 mmol/l) contraction. Each point represents mean ± standard error of mean of 12 determinations

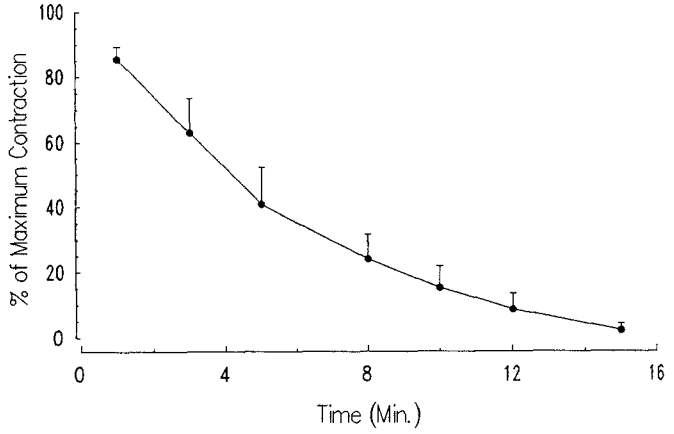

Fig. 2 Contractile response to electrical stimulation (20 Hz) after various periods of incubation in Ca^{2+}-free solution. Each point represents mean ± standard error of mean of five determinations

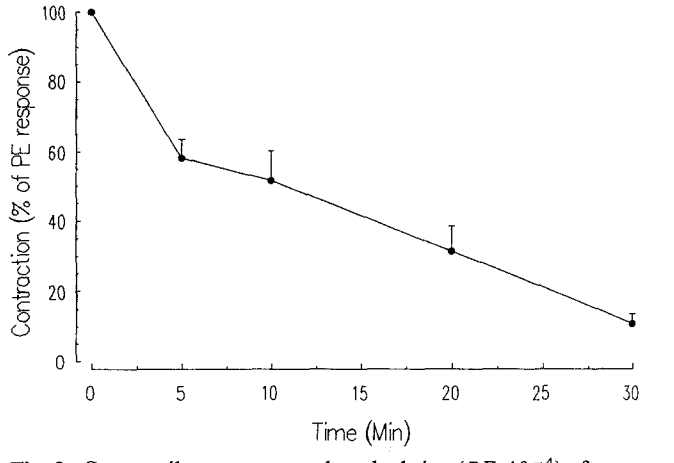

Fig. 3 Contractile response to phenylephrine (*PE*, 10^{-4}) after various periods of incubation in Ca^{2+}-free solution. Each point represents mean ± standard error of mean of five to seven determinations

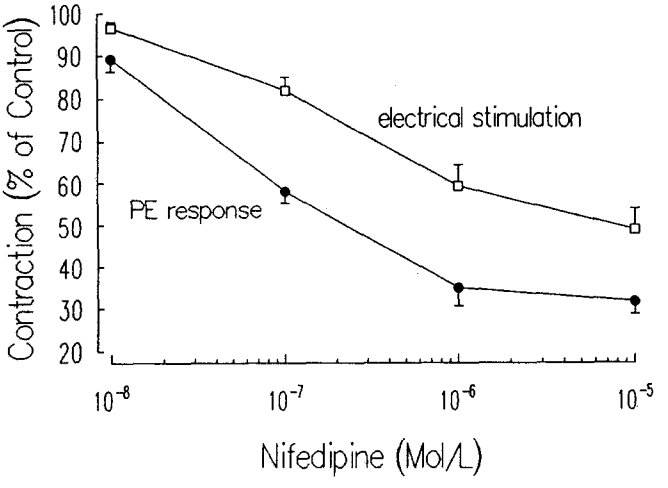

Fig. 4 Incubation of cavernosal tissue strips with nifedipine dose dependently reduces the contractile response to electrical stimulation (20 Hz) and phenylephrine (*PE*). Each point represents mean ± standard error of mean of four to six determinations

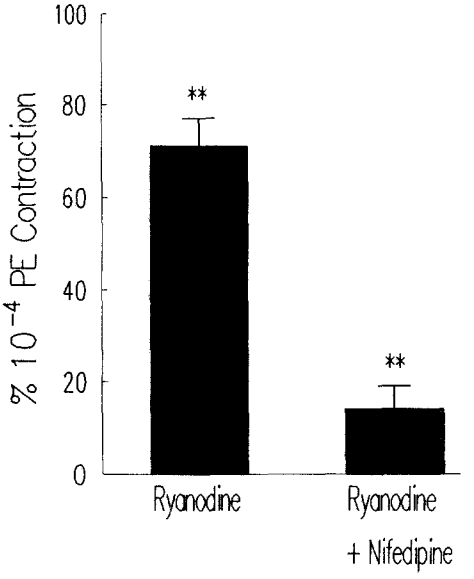

Fig. 5 Ryanodine (10^{-5} mol/l) significantly reduced contractile response to 10^{-4} mol/l phenylephrine (*PE*). Incubated together with nifedipine (10^{-5} mol/l), the results again show a significant suppression of PE response compared with the ryanodine effect. *Bars* represent mean ± standard error of mean of five to six determinations; ** $P < 0.01$

Nifedipine

Preincubation for 15 min with increasing concentrations of nifedipine was effective in inhibiting subsequent PE response (Fig. 4). A slight inhibition to 89 ± 3% was seen at 10^{-8} mol/l nifedipine, and at 10^{-5} mol/l the response to 10^{-4} mol/l PE was reduced to 31 ± 3% ($n = 6$). The log IC_{50} value for nifedipine was -6.63 ± 0.16. Administration of 10^{-5} mol/l nifedipine to tissue precontracted with 10^{-4} PE reduced the contraction to 40 ± 5% ($n = 5$). Nifedipine never totally abolished PE-induced contractions.

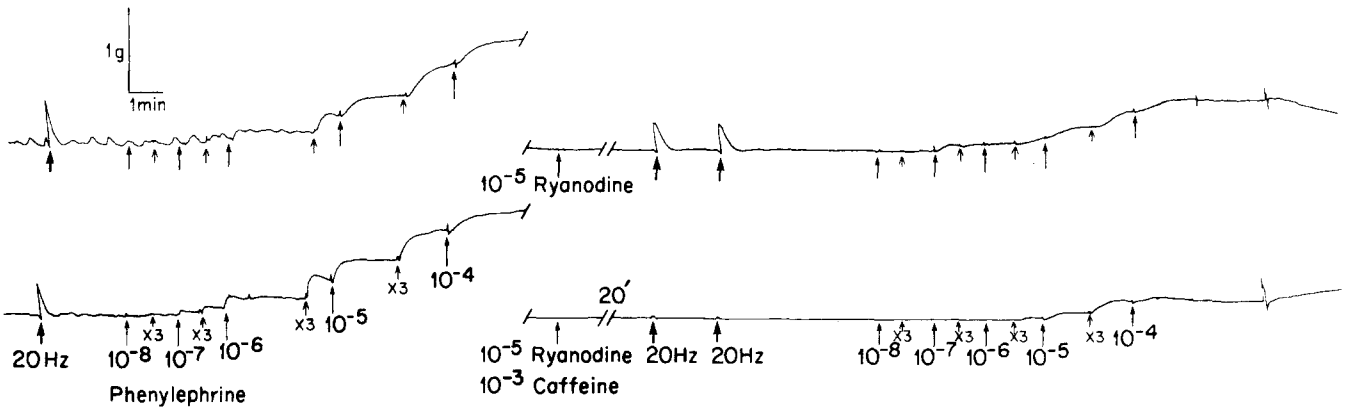

Fig. 6 Drawing from original recording. Phenylephrine-induced contraction and electrical stimulation are reduced after incubation (20 min) with 10^{-5} mol/l ryanodine (*upper tracing*). Addition of 10^{-3} mol/l caffeine further suppressed and nearly abolished the contractile response to either stimulus

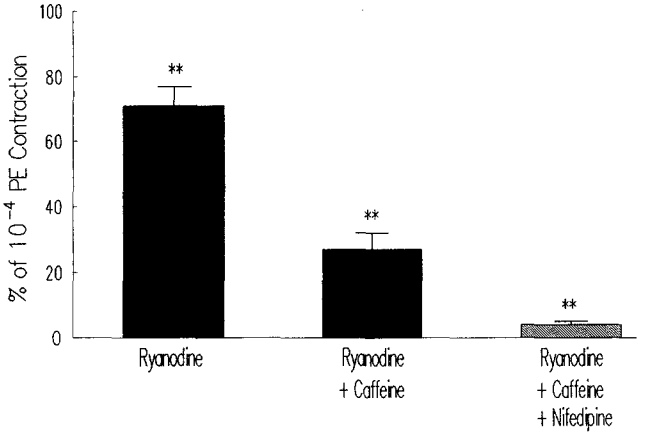

Fig. 7 Ryanodine significantly reduced contractile response to 10^{-4} mol/l phenylephrine (*PE*). Addition of caffeine (10^{-3} mol/l) and nifedipine (10^{-5} mol/l) furthermore significantly suppressed the PE-induced contraction. *Bars* represent mean ± standard error of mean of five to six determinations; ** $P<0.01$

The effect of nifedipine on electrically induced contractions is shown in Fig. 4. Again nifedipine concentration dependently depressed the response to electrical field stimulation; however, it was not able to abolish contractions. Maximum inhibition at 10^{-5} mol/l was 50 ± 7% ($n=4$).

Incubation of the strips with 10^{-5} mol/l nifedipine resulted in an almost complete inhibition of high-K^+-induced contraction ($n=5$).

Caffeine

In three out of four otherwise untreated strips, 10^{-3} mol/l caffeine induced a decrease in the baseline value of 2 mN ($n=4$) and reduced spontaneous contractions. Subsequent responses to PE were not changed after application of caffeine at this concentration. Caffeine in a dose of 30 mmol/l elicited a spike contraction before relaxing baseline tension, However, as caffeine at this concentration markedly inhibited the next PE response, only the low concentration of caffeine was used.

Ryanodine

Ryanodine (10^{-5} mol/l) abolished spontaneous contractions. After 20 min of incubation in ryanodine-containing Krebs' solution, the subsequent PE response (10^{-4} mol/l) was reduced to 71 ± 6% ($n=5$), and the additional administration of 10^{-5} nifedipine further reduced the PE-induced contraction to 14 ± 5% ($n=5$, Fig. 5). To empty the intracellular Ca stores, 10^{-3} mol/l caffeine was applied 5 min prior to ryanodine. The subsequent PE-induced contraction was reduced to 27 ± 5% ($n=5$, Fig. 6). In four strips the same experiment was repeated in the presence of 10^{-5} nifedipine and contraction was then nearly abolished to 4 ± 1% (Fig. 7).

Discussion

Contraction of penile smooth muscle is mainly mediated by a release of norepinephrine [16] from prejunctional adrenergic nerve fibers. The contraction increases the resistance to arterial inflow into the intracavernous sinuses and thus physiologically leads to detumescence [14]. In penile erection a high arterial inflow leads to tumescence and with rising intracavernous pressure the veno-occlusive mechanism is activated [14, 21]. Penile erection depends on the integrity of cavernosal smooth muscle relaxation and a blockade of adrenergic neurotransmission. In chronic erectile failure one causative factor may be an increased smooth muscle tone leading to an imbalance between relaxing and contractile forces [14]. In older patients a significantly increased corporal tissue sensitivity to phenylephrine has been described [5].

At the cellular level cavernosal smooth muscle relaxation is characterized by an increase in cytosolic Ca^{2+},

which depends on Ca^{2+} influx from the extracellular space [7, 10, 20] and possibly on the presence and size of intracellular Ca^{2+} stores.

Evidence for the importance of the latter in cavernosal tissue as in vascular smooth muscle derives from the observation that blockade of extracellular Ca^{2+} influx by nifedipine does not abolish electrically or PE-induced contractions [7, 10]. Another proof is that after short-term incubation in Ca^{2+}-free solution contractile response to electrical and adrenergic stimulation were still recorded [7, 20]. Experiments on isolated human corpus cavernosum suggested that papaverine relaxes cavernous tissue by inhibition of extracellular Ca^{2+} influx and by inhibition of storage or release of intracellular Ca^{2+} [12].

In the present experiments after incubation in nominally Ca^{2+}-free solution both electrical stimulation and α_1-adrenoceptor stimulation with the selective α_1-receptor agonist phenylephrine elicited marked contractile responses, which decreased with time. This first finding suggests the presence of intracellular Ca^{2+} stores; the time-dependent decrease of contractile force in Ca^{2+}-free solution may be due to a continuous depletion of the intracellular stores [4, 19].

Additional support for an intracellular Ca^{2+} source is provided by the results of experiments with nifedipine. Preincubation with this L-type Ca^{2+}-channel blocker as well as cumulatively added nifedipine did not completely relax PE-induced contractions. Also electrically induced contractions were not abolished by maximum doses of nifedipine. It has been shown that nifedipine does not selectively block Ca^{2+} influx from the extracellular space but also inhibits Ca^{2+} release from the sarcoplasmic reticulum [13]. However, the concentrations used were higher than in our study, and nonspecific effects are possible at these high concentrations. Since nifedipine – in accordance with the literature [7] – markedly blocked the high-K^+-induced contractions in the present study, the reduced PE-induced contraction after incubation with nifedipine may be attributed to a blockade of plasmalemmal voltage-gated Ca^{2+} channel. After exposure to nifedipine, remaining high-K^+-induced contractions may be due to release of norepinephrine from cavernosal adrenergic nerves. This might also explain high-K^+-induced contraction in Ca^{2+}-free solution. Intracavernosal injection of Ca^{2+}-channel blockers has been discussed as a treatment option in chronic erectile failure [7, 10]. However, in the presence of activated intracellular stores, blockade of Ca^{2+} channels might be not sufficient to induce penile erection.

Caffeine acts directly on the sarcoplasmic reticulum and enhances Ca^{2+}-induced Ca^{2+} release. It has not been reported to release Ca^{2+} from any other organelle [4]. Caffeine-induced contractions when applied in high concentrations clearly demonstrated the effects on Ca^{2+} release. Unfortunately caffeine also inhibits cyclic AMP phosphodiesterases, thereby eliciting a rise in cyclic AMP levels and smooth muscle relaxation [17]. Therefore we used caffeine at a relatively low concentration of 1 mmol/l, which did not affect the subsequent PE response.

The ryanodine receptor is the channel through which Ca^{2+} is released during normal excitation-contraction coupling (electromechanical coupling) in skeletal and cardiac muscle. In smooth muscle the inositol 1,4,5-triphosphate receptor is the major functional Ca^{2+} release channel involved in pharmacomechanical coupling [18], but smooth muscle cells may also contain ryanodine receptors [15, 18]. The number of ryanodine-binding sites varies considerably between different tissues [22]. The effect of ryanodine can be described as a stimulation of Ca^{2+} leakage from intracellular stores, so that the drug behaves as a functional Ca^{2+} store blocker through the depletion of Ca^{2+} in the store [8]. In the rabbit cavernosal smooth muscle ryanodine (10^{-5} mol/l) significantly inhibited the PE response, and the addition of caffeine further significantly enhanced the inhibitory effect of ryanodine. Incubation of ryanodine together with nifedipine markedly decreased the PE-induced contraction, and if ryanodine, caffeine and nifedipine were combined the contraction was nearly abolished.

These data confirm the well-known importance of Ca^{2+} influx from extracellular space for cavernous smooth muscle contraction and support evidence for Ca^{2+} release from intracellular stores in this tissue. We furthermore suggest that PE mobilizes Ca^{2+} from a ryanodine-sensitive Ca^{2+} store and that ryanodine-sensitive Ca^{2+}-release channels may play a role in cavernosal smooth muscle signal transduction.

It has been shown that small increases in contractility can produce disproportionately larger decreases in relaxation [6]. As normal erectile function strongly depends on a delicate balance between endogenous vasoconstrictors and vasorelaxants, discrete functional changes in intracellular Ca^{2+} stores could play a role in chronic erectile failure. However, the exact mechanisms of signal transduction leading to activation of intracellular Ca^{2+} stores in cavernosal tissue remain to be clarified.

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