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Agonist-dependent modulation of Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle

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Abstract. The effects of the stable thromboxane analogue U46619, the α_1-adrenergic agent phenylephrine and depolarization with high K^+ on cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) and force development were determined in rabbit pulmonary artery smooth muscle. Following stimulation with each of the excitatory agents, the time course of the $[Ca^{2+}]_i$/force relationship described counter-clockwise hysteresis loops with the rise and fall in $[Ca^{2+}]_i$ leading, respectively, contraction and relaxation. The rank order of the force/$[Ca^{2+}]_i$ ratios evoked by the different methods of stimulation was: U46619 > phenylephrine high K^+. The difference between the actions of U46619 and phenylephrine was due to the lesser Ca^{2+}-releasing and greater Ca^{2+}-sensitizing action of U46619. Both U46619 and phenylephrine also released intracellular Ca^{2+} in intact (non-permeabilized) preparations. The effects of the two agonists on force, at constant free cytoplasmic $[Ca^{2+}]$ maintained with EGTA, were also determined in preparations permeabilized with staphylococcal α-toxin, in which intracellularly stored Ca^{2+} was eliminated with A23187. Sensitization of the contractile response to Ca^{2+} by agonists was indicated by the contractile responses of permeabilized muscles to U46619 and to phenylephrine, in the presence of constant, highly buffered $[Ca^{2+}]_i$. These contractions were inhibited by GDP[βS] and could also be elicited by GTP. We conclude that, in addition to changing $[Ca^{2+}]_i$, pharmacomechanical coupling can also modulate contraction by altering the sensitivity of the regulatory/contractile apparatus of smooth muscle to $[Ca^{2+}]_i$, through a G-protein-coupled mechanism.

Key words: $[Ca^{2+}]_i$ – Ca^{2+} sensitivity – Smooth muscle – Pharmacomechanical coupling – Thromboxane – G proteins

Introduction

The extent and mechanisms of the modulation of Ca^{2+} sensitivity are major current problems of contractile regulation in smooth muscle. There are several lines of evidence, obtained with the aid of Ca^{2+} indicators and through studies of permeabilized smooth muscle fibers, indicating the variable relationship between force and the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a given smooth muscle (see Discussion and reviewed in Rüegg 1986; Somlyo and Somlyo 1990). This variability can be a function both of time (the duration of contraction) and of the mode of stimulation. Thus, desensitization of the regulatory/contractile apparatus to Ca^{2+}, with the consequent reduction in the force/calcium ratio due to the decreased response to $[Ca^{2+}]_i$with the duration of contraction, is thought to be responsible for some of the phasic properties of "phasic" smooth muscles (Somlyo et al. 1969; Himpens et al. 1989; Somlyo et al. 1989). Conversely, the greater force/calcium ratio observed following stimulation with agonists compared to that following depolarization with high K^+ (Morgan and Morgan 1984; Himpens and Casteels 1987; Rembold and Murphy 1988), because of sensitization of the regulatory/contractile apparatus to Ca^{2+} (Kitazawa et al, 1989), appears to be one of the mechanisms of pharmacomechanical coupling (Somlyo and Somlyo 1968).

Earlier studies, showing the greater force responses to agonists than to high K^+-induced increases in $[Ca^{2+}]_i$ and suggesting that agonists may even stimulate contractions without a rise in free cytoplasmic Ca^{2+}, were based on the use of aequorin as the Ca^{2+} indicator (Morgan and Morgan 1984; Bradley and Morgan 1987). However, although this photoprotein has been very useful for studies of some aspects of excitation/concentraction coupling (reviewed in Blinks 1986), its limitations (Somlyo and Himpens 1989) necessitated considerable caution in the interpretation of these results, and suggested the desirability of their reexamination with a different Ca^{2+} indicator. Fluorescent indicators, while not without their limitations, such as intracellular binding and uncertainty of K_d (reviewed in Somlyo and Himpens 1989), are more sensitive to Ca^{2+} and less sensitive to Mg^{2+} and more readily loaded into cells than aequorin, which, furthermore, is consumed in the luminescence reaction with Ca^{2+}. The purpose of the present study was to determine, with the fluorescent Ca^{2+} indicator,

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Fura-2 (Grynckiewicz et al. 1985; Himpens and Somlyo 1988), the relationship between $[Ca^{2+}]_i$ and force, following stimulation of vascular smooth muscle with the α_1-adrenergic agent, phenylephrine, and the stable thromboxane derivative, U46619 (9,11-dideoxy-9α,11α-methanoepoxy-PG $F_{2\alpha}$). These two agonists have been previously reported to produce contractions with particularly low aequorin signal/force ratios (Bradley and Morgan 1987). We compared the responses to these agonists with those to depolarization with high K^+, and searched for evidence of hysteresis in the Ca^{2+}/force relationship (Chatterjee and Murphy 1983; Yagi et al. 1988), as a measure of time-dependent desensitization or sensitization. In addition, we examined the efficacy of the above agonists in altering the force output at a given $[Ca^{2+}]_i$ that was clamped with EGTA, in preparations permeabilised with staphylococcal α-toxin. Our results are consistent with the notion that modulation of Ca^{2+} sensitivity by agonists can play a major role in pharmacomechanical coupling.

Materials and methods

Male and female New Zealand white rabbits weighing approximately 3 kg were instantaneously killed by cervical dislocation by a humane procedure as approved by the Institutional Animal Care and Use Committee. The heart and lungs were removed and transferred to a Krebs solution. The left and the right pulmonary arteries were excised and cleaned of their periarterial connective tissue and the endothelium was gently scraped away. Small strips of approximately 5 mm × 10 mm were cut along the direction of the circular muscle layer of the left and right branch of the pulmonary artery (Himpens and Casteels 1987; Himpens and Somlyo 1988).

Fura-2 experiments. The protocols for loading with Fura-2AM and for making the fluorescence measurements have been described by Himpens and Somlyo (1988). Briefly, the procedure consists of loading the strip in a cuvette containing 1 ml HEPES-buffered Krebs solution containing 1.2 mM Ca^{2+} and 2 μM Fura-2AM dissolved in dimethylsulfoxide (final concentration 0.5%) premixed with Pluronic F127 (final concentration 0.01%). Strips were loaded at room temperature with the cuvettes rotating at 30 r/min, transferred from this loading solution to a fresh Krebs solution and then washed for 1.5 h. The method of mounting and a description of the apparatus have been published (Himpens and Somlyo 1988). The instrumentation consisted essentially of an illuminating light source with a rotating wheel containing the interference filters of 340 nm and 380 nm connected to it. The UV light of the two wavelength was then passed through one end of a bifurcated light pipe to the muscle sheet, which was mounted in the tissue chamber, and was attached to a force transducer. The fluorescence emitted by the Fura-2 in the cell was transferred to a photomultiplier through the other end of the light pipe and an interference filter of 510 nm. The two signals were then separated and digitized for further data analysis.

Cytosolic Ca^{2+} concentrations were calculated using an internal calibration and using the formula described by Grynkiewicz et al. (1985). The minimum fluorescence was obtained by superfusing the muscle with a 140 mM K^+, Ca^{2+}-free solution containing 2 mM EGTA at pH 8.6 to optimize the ionomycin effects (Himpens et al. 1989). Ionomycin (50 μM) was added 5 min after superfusion with this solution. After determining the minimum signal ratio (R_{min}), the tissue was superfused with an excess of calcium (10 mM Ca^{2+} solution at pH 8.6), which gave the maximal signal ratio, R_{max}. The autofluorescence was determined after each experiment at the two excitation wavelengths, in order to subtract the values from the total fluorescence and obtain the net Ca^{2+}-sensitive Fura-2 fluorescence. This was done by superfusing the strip with a 20 mM Mn^{2+} Krebs solution after the R_{min} and R_{max} ratios had been determined. The remaining fluorescence represents the proportion that had to be subtracted from each signal before making the ratio signal. The above calibration was used in each of the experiments in which absolute values of $Ca^{2+}$are indicated in the figures. Owing to some uncertainties in the value of the K_d (reviewed by Somlyo and Himpens 1989) the absolute ratio is also shown in the figures showing individual experiments in addition to the computed $[Ca^{2+}]_i$. The calculated figures were obtained by averaging the records for 2.5 s every 20 s. Since this method of averaging resulted in peak values lower than the values of the raw data, we also sampled the latter at 2 Hz as an indication of the true peak rise in $[Ca^{2+}]_i$ following the respective stimulations. In each experiment 0% force was arbitrarily defined as the force of the resting muscle in normal Krebs solution. Maximum tension was normalized for each smooth muscle strip to the maximum force (100%) obtained after 15 min stimulation with 140 mM K^+ and 1.2 mM Ca^{2+}.

No quantitative comparison should be made between the widths of the hysteresis loops described by the responses to the three different stimuli (U46619, phenylephrine and high K^+) in Fig. 2, because the relaxation phases of these loops were initiated by removing excitatory stimuli of varying durations. Furthermore, even if the duration of exposure to high K^+, U46619 and phenylephrine had been identical, other uncontrolled (e.g. diffusion, repolarization, off-rate of agonists from their receptors and inactivation of second messengers) factors that terminate excitation and so influence the fall in $[Ca^{2+}]_i$ would have affected hysteresis.

All values are means ± SE, and n is the number of observations. Comparisons were made using Student's t-test. Supramaximal concentrations of phenylephrine and U46619 were used to compare the relative potencies of these agonists. The standard physiological solution was a HEPES-buffered modified Krebs solution at pH 7.3 containing (mM): Na^+ 135.5, K^+ 5.9, Ca^{2+} 1.2, Mg^{2+} 1.2, Cl^- 143.8, HEPES 11.6 and glucose 11.6. Solutions with increased $[K^+]_o$ were obtained by replacing Na^+ by an equivalent amount of K^+. Experiments were performed at room temperature to reduce the accelerated leak of Fura-2 acid from cells at 37 °C (Himpens and Somlyo 1988).

Skinning procedure. Small strips (150–200 μm wide and 2 mm long) were dissected from rabbit pulmonary arteries, cleaned of their periarterial connective tissue, scratched on the luminal surface to remove the endothelium and stretched to about 1.5 times the slack length. Isometric force was measured with a force transducer (AE801; AME, Horten, Norway) in a chamber on a bubble plate (Kitazawa et al. 1989). After measuring steady contractions induced by 154 mM K^+ and agonists (U46619 and phenylephrine), single strips were briefly incubated in relaxing solution containing 4.5 mM MgATP and 1 mM EGTA. The skinning of strips was done by incubation at 25 °C in 2 mg/ml crude staphylococcal α-toxin at pCa 7 buffered with 10 mM EGTA for 30 min. Thereafter, the Ca^{2+}-accumulating and -releasing functions of the sarcoplasmic reticulum were eliminated by treatment with the Ca^{2+} ionophore, A23187 (10 μM) for 20 min in the relaxing solution. The composition of the relaxing solution has been published (Kitazawa et al. 1989). The activating solution always contained 10 mM EGTA to clamp the free Ca^{2+} concentration. The combined use of high concentrations of EGTA and of A23187 has been shown to eliminate Ca^{2+} transients, detected with Fluo-3, due to Ca^{2+} release (Somlyo et al. 1990). The experiments were done at 25 °C.

Materials. Fura-2AM was obtained from Molecular Probes, Pluronic F127 from BASF Wyandotte Corporation and U46619 from Cayman Chemical (Mich., USA). Crude *Staphylococcus aureus* α-toxin, ryanodine and inositol 1,4,5-triphosphate ($InsP_3$) were from Calbiochem GTP[γS] from Sigma and GDP[βS] from Boehringer Mannheim.

Results

Response to stimulation in a Ca^{2+}-containing solution with 140 mM K^+ or U46619

Figure 1 shows an example and the averaged data of the measurements with 140 mM K^+ and 0.1 μM U46619 in the

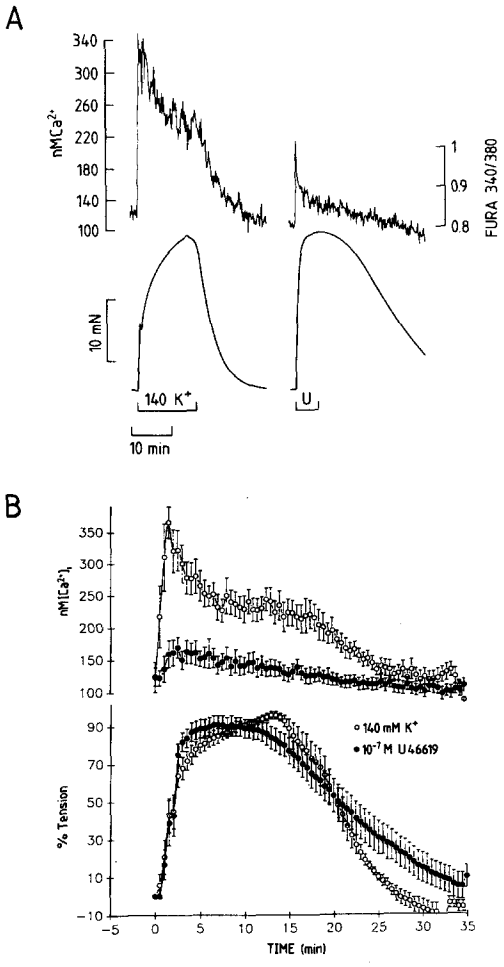

Fig. 1. A Example of a stimulation of a smooth muscle strip for 15 min with 140 mM K^+ or for 5 min with 0.1 μM U46619. The *upper trace* represents the $[Ca^{2+}]_i$, the *lower* the force. **B** Mean value obtained with data from nine smooth muscle strips. The force is normalized to the peak tension during stimulation with 140 mM K^+. ○, the high K^+ stimulation; ● the U46619 stimulation

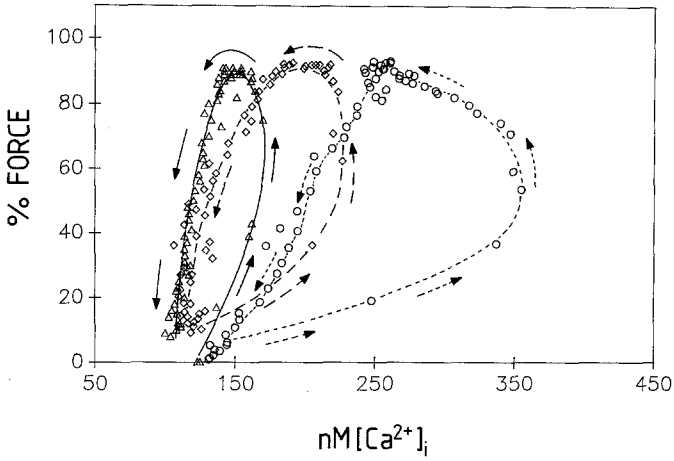

Fig. 2. Ca^{2+} versus force relationship sampled at 20 s intervals in the pulmonary artery stimulated with 140 mM K^+ (n = 20), 0,1 mM phenylephrine (n = 11) or 0,1 μM U46619 (n = 9) in normal Krebs/HEPES solution. The *arrows* indicate the sequence of contraction and relaxation. The ○ and *dashed arrows* represent the high-K^+ stimulation; the ◊ and *semi-dashed arrows* the phenylephrine stimulation and the △ and *solid lines* indicate the U46619 stimulation

same smooth muscle strip (n = 9). As shown in Himpens et al. (1988), $[Ca^{2+}]_i$ rose within 1–2 min after depolarization with 140 mM K^+ at room temperature to a maximum peak (366 ± 25 nM) (n = 9). Thereafter it decreased to a lower plateau level of around 240–250 nM, where it remained as long as the stimulus was maintained. Force rose with a slower time course and continued to increase while $[Ca^{2+}]_i$ was already declining (Himpens et al. 1988). The force was only 43 ± 9% of its maximum amplitude at the time of the maximal $[Ca^{2+}]_i$ spike. The half-time fo relaxation was reached 5 min after the return to the normal Krebs solution.

U46619 (0.1 μM) was applied to the same smooth muscle strips for 5 min (Fig 1). The $[Ca^{2+}]_i$ rose after 2–3 min of stimulation to a maximum of only 170 ± 16 nM. Thereafter, a slow decline in $[Ca^{2+}]_i$ was observed towards its resting value. Tension, normalized to the maximum force obtained during the 140 mM K^+ stimulation, had already reached its maximum value after 3–4 min. Maximum tension was 91 ± 5% and is therefore comparable to the maximum force obtained during K^+ stimulation, despite the significantly lower Ca^{2+} concentrations. The effects of U46619 washed out very slowly with a $t_{1/2}$ of relaxation of 15 min after the drug was removed.

The calcium/force curves evoked by two stimuli (140 mM K^+ and U46619) in identical muscles were generated by plotting the calculated $[Ca^{2+}]_i$ against the force observed during each recorded interval of contraction and relaxation (n = 9) and are shown in Fig. 2. The tension is normalized to the maximal force observed during the K^+ depolarization (n = 20). A counter-clockwise hysteresis is present in the calcium/tension curve for both stimuli during the contraction/relaxation cycle. The response to K^+ stimulation describes a hysteresis loop consisting of three components. The initially disproportionally large increase in $[Ca^{2+}]_i$ is followed by a second phase in which the tension increases, while $[Ca^{2+}]_i$ is already decreasing to a lower value. Finally a steady state is reached during continued stimulation. During the relaxation following removal of the stimulus, a rather linear decline of force and $[Ca^{2+}]_i$ can be seen. The relaxation can be fitted by a linear regression (EC_{50} = 189 nM; r = 0.96).

The hysteresis loop evoked by U46619 also appears counter-clockwise. However, the whole loop is shifted to the left as compared to the K^+ contraction and has only two components: a rising component and a declining part associated with the washout of U46619. The relaxation can again be fitted by a linear regression (EC_{50} = 120 nM; r = 0.94). Repetitive stimulation with high K^+ always resulted in the same counter-clockwise hysteresis loop. No shift in the direction of the loop could be observed. The pattern of the response to K^+ after stimulation with U 46619 was similar to that prior to exposure to the endoperoxide analogue (data not shown).

The effect of the α_1-adrenergic stimulation on the calcium/force relationship in the pulmonary artery and comparison with depolarization and U46619

The relationship between Ca^{2+} and force was also investigated by comparing in 11 other strips the responses to stimulation with 140 mM K^+ or phenylephrine (Fig. 3). The data obtained with 140 mM K^+ are similar to those described above. The $[Ca^{2+}]_i$ increased upon stimulation induced by 0.1 mM phenylephrine to a value of 246 ± 16 nM (n = 11). Thereafter a steady decline in $[Ca^{2+}]_i$ is observed. The force peak was, however, comparable to the tension obtained in the same strip with 140 mM K^+. The contractile response to phenylephrine (0.1 mM) also described a counter-clockwise hysteresis curve (Fig. 2) formed by two components: a rising phase followed by the relaxing phase with an EC_{50} of 138 nM, as estimated by a linear regression fit (r = 0.86).

Owing to the method of averaging every 20 s (see Materials and methods and Figs. 1, 3) the peak $[Ca^{2+}]_i$ reported above are somewhat lower than the true peak values. The peak $[Ca^{2+}]_i$ obtained, from the data sampled at 2 Hz, were: 371 ± 31 nM for K^+, 264 ± 25 nM for phenylephrine and 191 ± 21 nM for U46619. Thus, whether averaged at 0.05 Hz or at 2 Hz, the data show the following rank order of effectiveness for increasing $[Ca^{2+}]_i$: K^+ > phenylephrine > U46619. Using the $[Ca^{2+}]_i$ sampled at 2 Hz and forces normalized to 100% of the maximal contractions stimulated with high K^+ the force/$[Ca^{2+}]_i$ ratios, obtained in normal Ca^{2+}-containing solutions [force (%)/Ca^{2+} (nM)] are K^+: 0.27, phenylephrine: 0.38 and U46619: 0.48. Thus, the force output at a given level of $[Ca^{2+}]_i$ is U46619 > phenylephrine > high K^+.

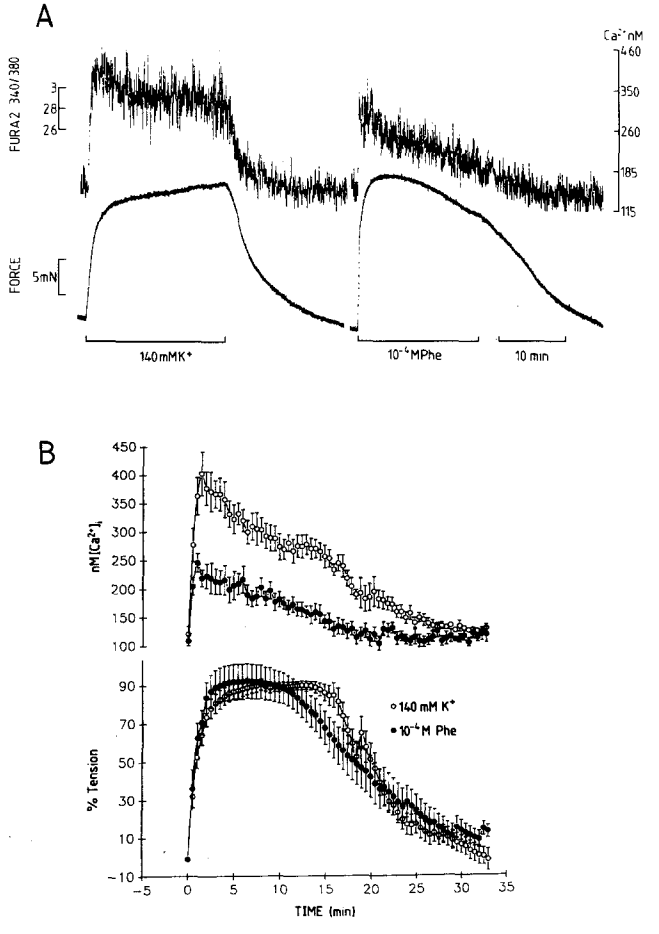

Fig. 3. A Example of a stimulation of a smooth muscle strip for 15 min with 140 mM K^+ or also for 15 min with 0.1 mM phenylephrine. The *upper trace* represents the $[Ca^{2+}]_i$, the *lower* the force. **B** Mean value obtained from 11 smooth muscle strips. The force is normalized to the peak tension during stimulation with 140 mM K^+. ○, the K^+ stimulation; ● the phenylephrine effect.

The effects of U46619 and phenylephrine in Ca^{2+}-free solution

After stimulation for 20 min with 140 mM K^+ solution containing 1.2 mM Ca^{2+}, the solution was changed for 15 min to a Ca^{2+}-free solution containing 2 mM EGTA (n = 6) (Fig. 4). Thereafter 0.1 μM U46619 was added for 5 min, followed by reperfusion with the Ca^{2+}-free solution for another 15 min. Then 0.1 μM U46619 was added a second time. The $[Ca^{2+}]_i$ increased during the K^+ depolarization from 116 ± 9 nM to a peak of 355 ± 16 nM and this was followed by a fall to 240 ± 17 nM; during Ca^{2+}-free perfusion it further declined to 75 ± 3 nM. Stimulation with 0.1 μM U46619 induced a transient increase to 108 ± 4 nM that was statistically ($P < 0.05$) higher than the previous value in Ca^{2+}-free, but not different from the resting value in normal Ca^{2+}-containing solution. The second superfusion with U46619 in the Ca^{2+}-free medium resulted in no detectable increase of $[Ca^{2+}]_i$ from the initial value in Ca^{2+}-free solution (43 ± 11 nM vs 36 ± 16 nM). Also when

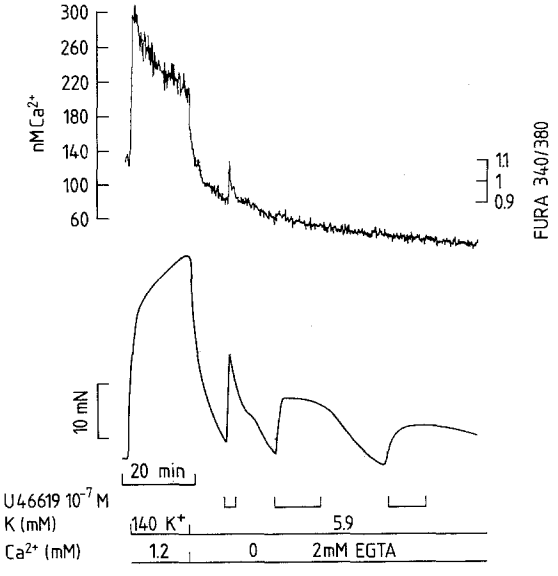

Fig. 4 Effect of incubation in Ca^{2+}-free solution on the responses to U46619. After loading the store with Ca^{2+} during a high K^+ contraction, the solution was changed to a Ca^{2+}-free solution containing 2 mM EGTA. After 15 min, the muscle was stimulated for 5 min with 0.1 μM U46619. Thereafter U46619 was washed out for another 15 min. This was followed by renewed stimulation in this Ca^{2+}-free solution, which resulted in tonic tension, maintained throughout the duration of the stimulation, without a detectable increase in $[Ca^{2+}]_i$

phenylephrine instead of U44619 was used as the second stimulus, it did not increase the $[Ca^{2+}]_i$ ($n = 3$), but could cause a small contraction (Fig. 4). A third stimulation with U46619 in Ca^{2+}-free solution also induced a small contraction without a change in $[Ca^{2+}]_i$.

During perfusion with Ca^{2+}-free solution, force was close to resting levels. The first stimulation with U46619 induced a peak of 49 ± 3% of the maximum, which then slowly declined to its value in Ca^{2+}-free solution. Force, however, remained enhanced much longer than the increase in $[Ca^{2+}]_i$. The second stimulation induced only a tonic increase in force (14 ± 1% of the peak) without any initial transient peak. The reduced contraction rate, as well as the amplitude, is consistent with the interpretation that the second response is due to a Ca^{2+}-sensitizing effect: this mechanism is activated by a much longer delay than Ca^{2+} release (Somlyo et al. 1989). The U46619-induced force during prolonged superfusion with the Ca^{2+}-free solution was slightly lower than the normal resting tension in Ca^{2+}-containing Krebs solution, while $[Ca^{2+}]_i$ declined to 36 ± 16 nM.

In summary, following removal of $[Ca^{2+}]_o$, the first exposure to U46619 resulted in a small Ca^{2+} transient accompanied by disproportionally large force development. Subsequent stimulation with the drug evoked contractions without detectable changes in $[Ca^{2+}]_i$.

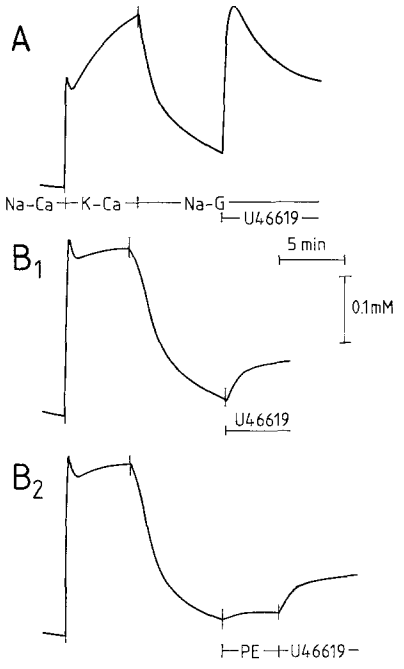

Fig. 5 A, B_1, B_2. Persistence of the agonist-induced responses of intact rabbit pulmonary artery after treatment with ryanodine in the absence of Ca^{2+}. **A** The contractile response to U46619 in the absence of added Ca^{2+} and in the presence of 2 mM EGTA before treatment with ryanodine. The strip (200 μm wide and 2 mm long) was first stimulated with a Ca^{2+}-containing high-K^+ solution (*K-Ca*) and then relaxed by a Ca^{2+}-free, EGTA-containing low-K^+ and high-Na^+ solution (*Na-G*). 1 μM U46619 produced a large transient contraction followed by a sustained phase even in the absence of Ca^{2+}. **B** The contractile responses to 1 μM U46619 ($\mathbf{B_1}$ and $\mathbf{B_2}$) and 100 μM phenylephrine (*PE*) ($\mathbf{B_2}$) in the absence of Ca^{2+} and after treatment with 20 μM ryanodine and 30 mM caffeine for 20 min. U46619 produced a larger tonic contraction after ryanodine treatment than did phenylephrine

The effect of ryanodine on the response to U46619 and phenylephrine and sensitization to $[Ca^{2+}]_i$ by U46619 and by phenylephrine in pulmonary artery permeabilized with staphylococcal α-toxin

In order to verify further that agonists could cause contraction by modifying the sensitivity of the regulatory/contractile apparatus to Ca^{2+} even in the absence of Ca^{2+} release, we determined the responses to agonists in intact smooth muscles, in which the intracellular calcium stores had been eliminated with ryanodine (Iino et al. 1988), and in permeabilized smooth muscles, in which the intracellular stored Ca^{2+} was removed with A23187, and Ca^{2+} could be "clamped" with high concentrations of EGTA.

Representative responses of ryanodine-treated pulmonary artery smooth muscle to U46619 and to phenylephrine are shown in Fig. 5. Prior to depletion of Ca^{2+} from the sarcoplasmic reticulum, but in the absence of extracellular Ca^{2+}, U46619 evoked contractions comparable to the maximum K^+ contractures (Fig. 5A). Following treatment with ryanodine, the responses in the absence of extracellular Ca^{2+} were very much reduced, and the rate of force development was slower than that prior to ryanodine treatment (Fig. $5B_1$ and B_2). Phenylephrine evoked a similar, but smaller contraction (Fig. $5B_2$). It should also be noted that unequal Ca^{2+}-sensitizing effects were evoked by supramaximal concentrations of the two agonists (Figs. 5, 6). In any event, agonists could induce contractions in these preparations in which both Ca^{2+} influx and Ca^{2+} release were eliminated. The relative sensitivities to, respectively, phenylephrine and U46619 varied from animal to animal, but were comparable in different strips obtained from the same animal (data not shown).

The responses of muscles permeabilized with staphylococcal α-toxin and pretreated with A23187 are shown in Fig. 6. These preparations were highly sensitive to GTP and also Ca^{2+}. In the presence of 1 μM GTP, significant force was produced at 100 nM Ca^{2+}, and further marked increase in force was produced by the addition of 1 μM U46619 (Fig. 6A–C). The response to the combined effects of GTP and U46619 was partially reversed by 1 mM GDP[βS] (Fig. 6A). Phenylephrine also potentiated, to a variable extent, the contractile response to a fixed (pCa = 7) concentration of free Ca^{2+} (Fig. 6B), while 100 μM $InsP_3$ had no effects (Fig. 6C).

The persistent, while reduced, contractile response to agonists in preparations in which Ca^{2+} release was absent and free Ca^{2+} was "clamped" at submaximal contractile levels, in comparison with the response evoked in preparations in which Ca^{2+} release could occur, indicates that these agonists can exert their effects through the combined actions of increasing $[Ca^{2+}]_i$ and modulating the sensitivity of the regulatory/contractile apparatus to Ca^{2+}.

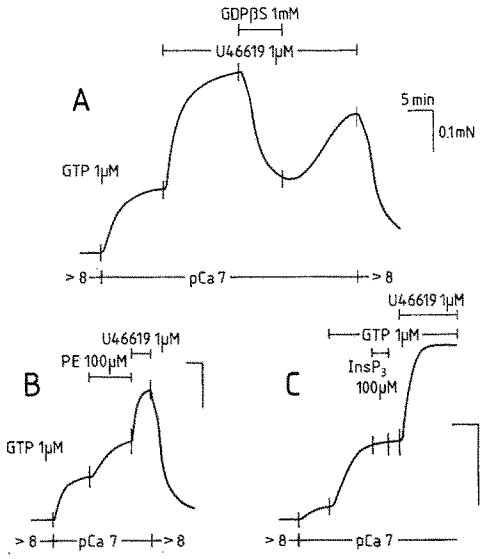

Fig. 6 A–C. Modulation by U46619, phenylephrine (*PE*) and GTP, but not inositol 1,4,5-trisphosphate (*InsP_3*), of the contractile response to Ca^{2+} in rabbit pulmonary artery permeabilized with staphylococcal α-toxin and treated with A 23187. Each muscle from different animals was contracted submaximally with pCa 7 solution buffered with 10 mM EGTA. 1 μM GTP was present in **A** and **B** throughout the experiments. The addition of 1 μM U46619 (**A, B, C**) produced additional force in the presence of constant Ca^{2+}, while 100 μM phenylephrine had a significant, but lesser effect (**B**) and InsP_3 was ineffective (**C**). Added GTP itself (1 μM) superimposed an additional contraction on the initial Ca^{2+}-induced one (**C**). 1 mM GDP[βS] reversibly inhibited the potentiation of the contraction by U46619 (**A**)

Discussion

Ca^{2+} sensitization by U46619 and phenylephrine

The major new findings of this study are the contractile effects of the thromboxane analogue, U46619, at constant "cytoplasmic" Ca^{2+} in permeabilized smooth muscles, the inhibition of this Ca^{2+} -sensitizing effects by GDB[βS] and the quantitative dissociation between, respectively, Ca^{2+}-releasing and Ca^{2+} -sensitizing effects of different agonists. These and similar experiments provide evidence of the ability of this and other muscarinic (Kitazawa et al. 1989; Kobayashi et al. 1989) and α-adrenergic (Kitazawa et al. 1989; Nishimura et al. 1988) agonists to modify the sensitivity of the regulatory/contractile apparatus to $[Ca^{2+}]_i$. A major advantage of permeabilizing smooth muscles with α-toxin or with β-escin, a saponin ester (Kobayashi et al. 1989), is that these methods, unlike saponin permeabilization, retain plasma membrane receptors coupled to their effector systems, while providing the rigorous control of $[Ca^{2+}]_i$ with high concentrations of EGTA. The ability of certain agonists, compared to high K^+, to increase the Ca^{2+} sensitivity of the regulatory apparatus to Ca^{2+} was also suggested by the greater force/Ca^{2+} ratio observed during agonist-induced contractions compared to high-K^+ depolarization-induced contractions (Morgan and Morgan 1984; Bradley and Morgan 1987; Himpens and Casteels 1987; Rembold and Murphy 1988; Sato et al. 1988; present study), but the conclusions drawn from such observations were subject to uncertainties concerning the intracellular behavior of Ca^{2+} indicators. The limitations of aequorin, used in previous studies, differ from and are probably more restrictive at low $[Ca^{2+}]_i$ than those of Fura-2 used in the present study (reviewed in Somlyo and Himpens 1989).

The Ca^{2+} sensitizing action of agonists is probably mediated by G proteins, as it can be mimicked by GTP[γS] (Fujiwara et al. 1989; Kitazawa et al 1989; present study) and inhibited by GDP[βS] (Kitazawa et al. 1989; present study). This potentiation is not mediated by InsP_3 (Fig. 6) and is insensitive to the effect of heparin, an inhibitor of InsP_3-induced Ca^{2+} release (Kobayashi et al. 1989).

An interesting observation made in the course of the present study was the lack of correlation between the Ca^{2+}-releasing and the Ca^{2+}-sensitizing actions of the two agonists studied. Thus, U46619 caused greater sensitization to Ca^{2+} but less Ca^{2+} release than did phenylephrine, resulting in a higher force/$[Ca^{2+}]_i$ ratio in the muscles stimulated with the thromboxane analogue. This result suggests that the Ca^{2+}-sensitizing action of agonists may be mediated by some mechanism other than hydrolysis of phosphatidylinositol bisphosphate (PtdInsP_2) by phospholipase C. One of the products of this reaction, InsP_3 (Berridge 1988), is the messenger responsible for pharmacomechanical Ca^{2+} release (Somlyo et al. 1990). Other products of this reaction, such as diacylglycerol, should be produced at concentrations at least proportional to that of InsP_3. Therefore, any simple mechanism of sensitization through the phosphatidylinositol cascade would be expected to show a positive correlation between the Ca^{2+}-releasing and the sensitizing action of different agonists. More definitive proof of this conclusion, however, will require measurements of the products of PtdInsP_2 hydrolysis and, preferably, identification of the messenger(s) responsible for the sensitizing action of agonists. At present, we can not exclude the possibility that receptors mediating high force but little Ca^{2+} release are distant from the sarcoplasmic reticulum and the Ca^{2+}-releasing effects due to InsP_3 are disproportionally reduced through diffusion and hydrolytic inactivation of this messenger, whereas the "sensitizing messenger" does not require proximity to the sarcoplasmic reticulum.

Both of the agonists used in this study, the α_1-adrenergic agent phenylephrine and the stable thromboxane analogue, U46619, could release intracellular Ca^{2+}, as indicated by the rise of $[Ca^{2+}]_i$ during stimulation of muscles in Ca^{2+}-free solutions (Fig. 4). In this regard, our results differ from those of Bradley and Morgan (1987), who found "little or no detectable rise" in aequorin signals following stimulation of porcine coronary artery with U46619. Direct comparison of the results of these authors with ours is difficult,not only because of the differences in species and blood vessels examined, but also because the concentration of U46619 used was not indicated in that study. However, in view of the different properties of aequorin and Fura-2 as Ca^{2+} indicators (reviewed in Somlyo and Himpens 1989), we suspect that the negative results of Bradley and Morgan (1987) reflect factors other than species – or organ-specific effects, such as small responses (contractions equivalent to those evoked by 33 mM K^+) and the relative insensitivity of aequorin. A calcium-mobilizing effect of U46619 has also been observed in cultured smooth muscles cells studied with

methods other than aequorin (Fukuo et al. 1986; Dorn et al. 1987). However, we did find that when muscles were stimulated *repeatedly* in Ca^{2+}-free solutions to deplete intracellular Ca^{2+}, both phenylephrine and U46619 could cause contractions in the absence of a detectable change in fluorescence. We can not fully exclude the possibility of a small, undectected change in Ca^{2+} within the vicinity of the myofilaments, but it is more likely that such contractions are due to the "sensitizing" action of agonsits, without any change in $[Ca^{2+}]_i$. In guinea pig pulmonary artery, force equivalent to a high K^+-induced contraction develops at 200–300 nM $[Ca^{2+}]_i$ (Kitazawa, cited in Somlyo and Himpens 1989), and such high intrinsic sensitivity to Ca^{2+}, together with a relatively high $[Ca^{2+}]_i$ maintained in the absence of $[Ca^{2+}]_o$, as occurs in rabbit pulmonary artery (Himpens et al. 1989), could account for contractions due to agonist-induced "sensitization" without a change in $[Ca^{2+}]_i$. This conclusion is also supported by the response of toxin-permeabilized preparations to agonists in the presence of "clamped" low Ca^{2+} levels (present study).

Pharmacomechanical coupling was originally defined as the mechanism through which agonists could alter the level of force in smooth muscles without a necessary change in membrane potential; while ascribing this largely to "chemical" modulation of cytoplasmic Ca^{2+}, it was also suggested that "some stimuli may also release a potentiator which augments the contractile force developed at any given free calcium" (Somlyo and Somlyo 1968). Our present findings and others reviewed here suggest that such mechanism does, indeed, operate in smooth muscle, and also raise the possibility that the different maximal contractions produced by different agonists (Somlyo and Somlyo 1968) could reflect the different "efficacies" of agonists in increasing Ca^{2+} influx, releasing Ca^{2+} or altering the Ca^{2+} sensitivity.

The time course of Ca^{2+} and force transients

In addition to the greater force at a given $[Ca^{2+}]_i$ during stimulation with agonists than during depolarization with high K^+, there is frequently also a major temporal dissociation between the time courses of, respectively, $[Ca^{2+}]_i$ and force transients in smooth muscles. In the tonic pulmonary artery smooth muscle, the temporal dissociation is manifested as a counter-clockwise hysteresis loop: force continues to increase during the declining phase of the Ca^{2+} transient (this study; Himpens et al. 1988). A certain degree of dissociation between the time course of $[Ca^{2+}]_i$ and contraction is to be expected, as the force response to a rapid rise in Ca^{2+} will show a variable lag depending on the rate-limiting steps of the contractile regulatory mechanism and of the crossbridge cycle. Myosin light-chain phosphorylation, the primary mechanism of regulation in smooth muscle (reviewed by Rüegg 1986; Hartshorne 1987), is relatively slow and indirect, and it is also the rate-limiting process of contraction (Somlyo et al. 1988; Horiuti et al. 1989, reviewed in Somlyo and Somlyo 1990). Prephosphorylation reactions and/or mechanical properties of muscles and the kinetics of a multistage mechanism can give rise to a significant delay (0,2–0.5 s) between the rise in $[Ca^{2+}]_i$ and force development and contribute to an "inertial" Ca^{2+} force hysteresis loop. Modulation of the light-chain kinase/light-chain phosphatase activity ratios, resulting in Ca^{2+} sensitization/desensitization (Somlyo et al. 1989), probably plays a more important role, as myosin light-chain phosphorylation is more closely coupled than $[Ca^{2+}]_i$ to force development (Rembold and Murphy 1988).

Myosin light-chain phosphorylation alone, however, cannot fully account for a counter-clockwise hysteresis, since the rate of relaxation can exceed that of dephosphorylation (Driska et al. 1989), and conversely force can be maintained by smooth muscle while both $[Ca^{2+}]_i$ and myosin light-chain phosphorylation are declining. The mechanism of this "latch" state (Hai and Murphy 1988), whether cooperative reattachment (Somlyo et al. 1988), slow detachment of dephosphorylated bridges that require rephosphorylation to be reattached (Hai and Murphy 1988; Driska et al. 1989) or some thin-filament-regulated process, is outside the scope of the present study (reviewed in Somlyo and Somlyo 1990). A clockwise hysteresis or its equivalent decrease in force in the presence of constant $[Ca^{2+}]_i$, indicative of "desensitization" to Ca^{2+} in phasic smooth muscles (Yagi et al. 1988; Himpens et al. 1989; Kitazawa et al. 1989) may be due to rapid dephosphorylation of myosin light chains (Himpens et al. 1988) and can be reversed by a phosphatase inhibitor (Somlyo et al. 1989).

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