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Effects of verapamil on the contractions of guinea-pig tracheal muscle induced by Ca, Sr and Ba

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1 A comparison was made of contractions produced by calcium (Ca), strontium (Sr) or barium (Ba) in guinea-pig tracheal smooth muscle after the preparation had been relaxed in Ca-free medium. Most of the experiments were carried out in the presence of indomethacin (5 μM) to inhibit endogenous prostaglandin synthesis. In 40 mM K^+ solution, the Ca, Sr and Ba concentrations which produced 50% of maximum tension responses were 0.07 mM, 1 mM and 2 mM, respectively. Maximum tension of a similar size was produced by either 2.4 mM Ca, 9.6 mM Sr or 9.6 mM Ba.

2 The Ca-induced contraction in 5.9 mM K solution, which is probably due to the presence of endogenous prostaglandins, was not significantly affected by verapamil. When the external K concentration was increased to 40 mM, the Ca-induced contraction became susceptible to inhibition by verapamil. Similarly, contractions induced by Sr and Ba in excess K solution were strongly suppressed by verapamil.

3 In the presence of prostaglandin (PG) $F_{2\alpha}$ (1.4 μM) or carbachol (5 μM), Ca, Sr and Ba produced contractions in both the 5.9 mM K and 40 mM K solutions. Contractions produced by $PGF_{2\alpha}$ or carbachol in the presence of Ca were little affected by 10 μM verapamil, whereas those in the presence of Sr or Ba were strongly suppressed by verapamil in both the 5.9 and 40 mM K solutions.

4 A strong suppressant effect of verapamil on the K-induced contraction, but a weak effect on the drug-induced contraction, in the presence of Ca can be explained by assuming that verapamil blocks voltage-operated Ca channels, but not receptor-operated Ca channels. However, this theory cannot account for the effect of verapamil on drug-induced contractions in the presence of Sr or Ba. It may be that susceptibility to verapamil is determined by the relative affinity of the divalent cations and verapamil for the Ca channels, both for voltage- and receptor-operated channels.

Introduction

It is generally assumed that calcium ion (Ca^{2+}) influx from the extracellular medium is mainly responsible for contraction of smooth muscle as, in most smooth muscles, contractions are quickly reduced or abolished by removal of external calcium or administration of 'calcium antagonists'; inhibitions which are ascribed to a reduction in the transmembrane influx of calcium ions (Fleckenstein, 1983). However, in some smooth muscles, for example some vascular or airways smooth muscles, the removal of external Ca^{2+} does not abolish the contraction induced by stimulating agents as rapidly as it prevents the contraction induced by increasing the external K^+ concentration (Hudgins & Weiss, 1968; Kirkpatrick *et al.*, 1975; Ito *et al.*, 1977; for review see Bolton, 1979). Furthermore, 'calcium antagonists' such as verapamil or D-6000 (methoxyverapamil) have little effect on the contractions induced by agonists at cholinoceptors or adrenoceptors, although they suppress high K^+-induced contractions (Golenhofen & Hermstein, 1975; Coburn, 1977; Golenhofen, 1981; Meisheri *et al.*, 1981). This could be due to the fact that, in these muscles, the contractions induced by the agonists are dependent on intracellular Ca^{2+} release (Kuriyama, 1981), and/or on influx of Ca^{2+} through receptor-operated Ca channels, which are different from the voltage-operated channels activated by depolarization of the membrane by excess K^+ (Bolton, 1979).

The guinea-pig tracheal smooth muscle exhibits spontaneous tone in normal Krebs solution that is probably maintained by prostaglandins synthesized by the tracheal muscle itself (Orehek *et al.*, 1975). Although this tone is readily blocked in calcium-free

solution, it is little affected by verapamil at a concentration up to 10^{-5} M (Duncan & Douglas, 1984; Kawanishi *et al.*, 1984). In airways smooth muscle, contractions induced by acetylcholine or prostaglandins are considered to be much less sensitive to calcium antagonists than those in other types of smooth muscle (Coburn, 1977; Himori & Taira, 1980; Triggle, 1983).

In the present studies, the effects of verapamil on the contraction induced by either carbachol or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were further investigated in the guinea-pig tracheal muscle under conditions in which calcium was substituted by strontium (Sr) or barium (Ba). It is known that Sr^{2+} and Ba^{2+} can produce action potentials and contractions in several types of smooth muscles, probably by carrying the inward current through the Ca channel (Bülbring & Tomita, 1969; Kuriyama & Tomita, 1970; Sakamoto, 1970; 1971; Uvelius *et al.*, 1974; Takata, 1979; Hotta & Yamamoto, 1983).

It was hoped that the combination of the effects of a calcium antagonist and substitution of Ca^{2+} with other divalent cations might be a useful approach to clarify the role of calcium pathways in tracheal smooth muscle contraction.

Methods

Guinea-pigs weighing 250–350 g were stunned and bled, and the trachea removed. The muscle strip contained in one cartilage ring was carefully dissected out with the short cartilage attached each side of the muscle strip, and it was fixed vertically in an organ bath (1 ml volume) for the recording of isometric tension. The preparation was superfused with test solutions at a constant rate (1.5 ml min^{-1}). After equilibrating the preparation in a normal bathing solution for 30 min, isoprenaline (2 μM) was applied to produce complete relaxation. Under these conditions the tension was adjusted to 250 mg by stretching the preparation and the isoprenaline was washed out. The experiment was started after the resting tension had developed again and had reached an approximately constant level.

The normal bathing solution had the following composition (mM): NaCl 137, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2 and glucose 11.8, aerated with 99% O_2 and 1% CO_2. When Ca^{2+} was substituted by Sr^{2+} or Ba^{2+}, their concentration was usually 9.6 mM, 2.4 mM $CaCl_2$ and 10.8 mM NaCl were replaced with the divalent cation to maintain the osmolarity of the solution. EGTA (ethyleneglycol-bis-(β-aminoethyl-ether) N,N'-tetraacetic acid) 0.5 mM was always added to 'Ca-free' solution, except in the experiments where the concentration-response relationship of divalent cations was studied. All experiments were carried out at 35°C.

The drugs used were indomethacin, isoprenaline, carbachol, EGTA and prostaglandin (PG) $F_{2\alpha}$. All drugs were obtained from Sigma, except $PGF_{2\alpha}$ (Ono Pharmaceutical Co.) and verapamil (Knoll A.G.). Contamination by calcium, of the Ca-free solutions, was less than 1 μM when measured by atomic absorption spectroscopy.

Results

The concentration-response relationship for Ca^{2+} ion was compared with those for Sr^{2+} and Ba^{2+} ions. In these experiments, the preparations were first exposed to Ca-free 40 mM K^+ solution containing indomethacin (5 μM) and then Ca was applied cumulatively from 0.019 to 9.6 mM to produce a steady state of contraction at each concentration. Indomethacin was added in all of these experiments, but the results were essentially the same as those obtained when indomethacin was not present.

Similarly, Sr and Ba were applied to the same preparation in this order at intervals of 30 min; the results are summarized in Figure 1. The threshold concentration for Ca was much lower than that for Sr and Ba. The concentration-response curves were similar for Sr and Ba. The concentration which produced 50% of maximum tension was about 0.07 mM for Ca, 1 mM for Sr and 2 mM for Ba. In some experiments, concentration-response curves were obtained using separate preparations for each divalent cation but no significant difference was found

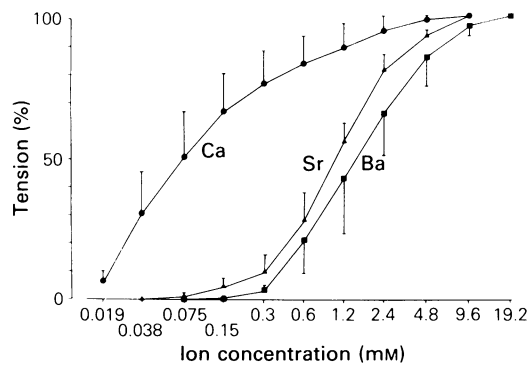

Figure 1 Concentration-response relationships of Ca (●), Sr (▲) and Ba (■) in Ca-free, 40 mM K^+ solution containing indomethacin (5 μM) in guinea-pig tracheal muscle. The divalent cations were applied cumulatively to the same preparation in the order Ca, Sr and Ba at intervals of 30 min, for at least 20 min, by which time a steady state of contraction had been reached. Ordinate: percentage of the maximum tension for each divalent cation; each point shows the mean ($n = 4$) and vertical lines s.d.

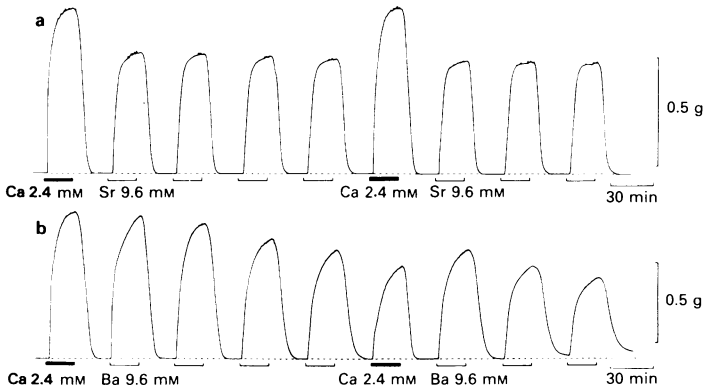

Figure 2 Contractions produced by repeated application of 9.6 mM Sr (a) and 9.6 mM Ba (b). The preparations were first exposed to Ca-free 40 mM K^+ solution containing 0.5 mM EGTA and 5 μM indomethacin, and control responses to Ca (2.4 mM) were observed twice. The trace started from the second response to Ca. EGTA was present throughout, except for 10 min before and throughout each Ca application. Note the nearly constant responses to Sr but a slow decrease in Ba responses and also that Ca treatment did not affect subsequent Sr responses (a), but potentiated subsequent Ba responses (b).

from those shown in Figure 1. Thus, treatment with one divalent cation did not affect the tension development in the presence of other cations if the interval between applications was about 30 min.

Maximum tension was produced by approximately 2.4 mM Ca, 9.6 mM Sr and 9.6 mM Ba, and their absolute values were 0.73 ± 0.02 g for Ca, 0.64 ± 0.07 g for Sr and 0.80 ± 0.20 g for Ba (mean ± s.d., $n = 4$). When expressed relative to that for Ca, the values were $86.6 \pm 8.2\%$ for Sr and $108 \pm 26.2\%$ for Ba.

In Figure 2, Sr (a) and Ba (b) were repeatedly applied to Ca-free 40 mM K^+ solution (containing 0.5 mM EGTA) for 20 min at 25 min intervals after observing a control response to the readdition of Ca^{2+}. The maximum tension induced by Sr (9.6 mM) was smaller than that induced by Ca (2.4 mM), but it remained nearly constant during the successive applications of Sr, and was not significantly affected by interposing a Ca-induced contraction between the Sr-induced responses. The tension induced by Ba (9.6 mM) decreased gradually and linearly on succes-

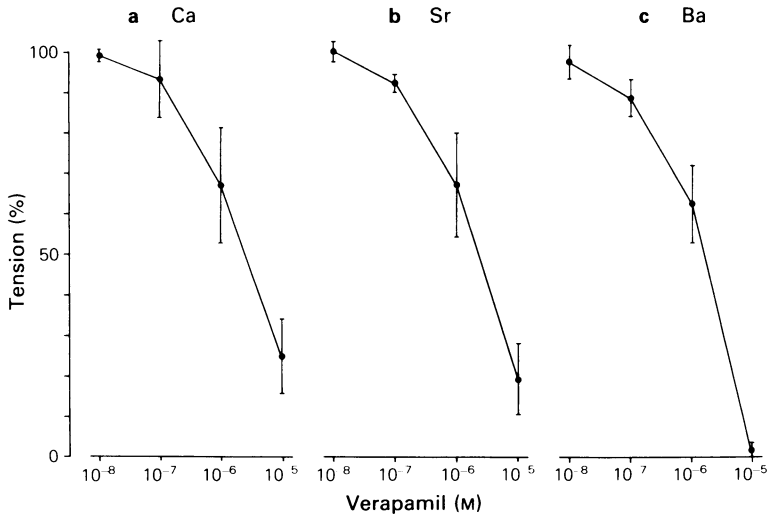

Figure 3 Effects of verapamil on the contractions induced by 2.4 mM Ca (a), 9.6 mM Sr (b) and 9.6 mM Ba (c) in 40 mM K^+ solution containing indomethacin (5 μM). EGTA (0.5 mM) was added to Sr and Ba solutions. Different preparations were used for each concentration-response curve and the maximum responses before the addition of verapamil were taken as 100%. Each point is the mean of 4 different preparations and vertical lines represent the s.d.

sive applications of this ion, and the 4th response was about 75% of the 1st response. When Ca was added after the 4th response to Ba, the tension produced was much smaller than the control response to Ca but this treatment resulted in a partial recovery of the subsequent Ba response. The rate of relaxation on removal of the divalent cations was faster for Ca and Sr than for Ba and it became gradually slower when Ba was repeatedly applied.

Effects of verapamil on the contractions produced by Ca (2.4 mM), Sr (9.6 mM) and Ba (9.6 m) were compared and the results shown in Figure 3. Verapamil had little effect on Ca-induced contraction in 5.9 mM K^+ solution, whereas it suppressed Ca-induced contractions significantly in 40 mM K^+ solution (Figure 3a), as shown previously (Kawanishi *et al.*, 1984). The contractions induced by Sr and Ba were similarly suppressed by verapamil in 40 mM K^+ medium. There was no clear difference in the effect of verapamil on the contractions induced by the three divalent cations.

In Figure 4, the contractions induced by the different divalent cations were compared in the presence of $PGF_{2\alpha}$ (1.4 mM) or carbachol (5 μM). In these experiments, the preparations were first exposed to Ca-free solution, and $PGF_{2\alpha}$ (1.4 μM) or carbachol (5 μM) was added. No clear contraction was observed when these two agonists were added 15 min after the removal of Ca. These experiments were also carried out in the presence of indomethacin (5 μM) to abolish the basal tone. The concentration-response curves in the presence of $PGF_{2\alpha}$ were roughly parallel for all three divalent cations and the sensitivity was Ca > Ba > Sr. In the presence of carbachol, the concentration-response curves for Ca and Ba were shifted to left and the curves were steeper compared

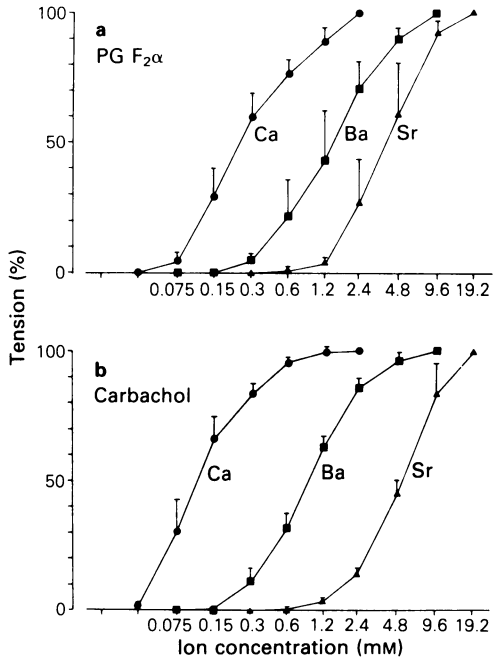

Figure 4 Concentration-response curves for Ca (●), Ba (■) and Sr (▲) in the presence of (a) prostaglandin (PG)$F_{2\alpha}$ (1.4 μM) and (b) carbachol (5 μM). Experimental procedure was the same as Figure 1 and Ca, Sr and Ba were applied in this order without EGTA. The maximum responses to 2.4 mM Ca, 9.6 mM Ba and 19.2 mM Sr were taken as 100%. Each point represents the mean of 4 different preparations and vertical lines indicate s.d.

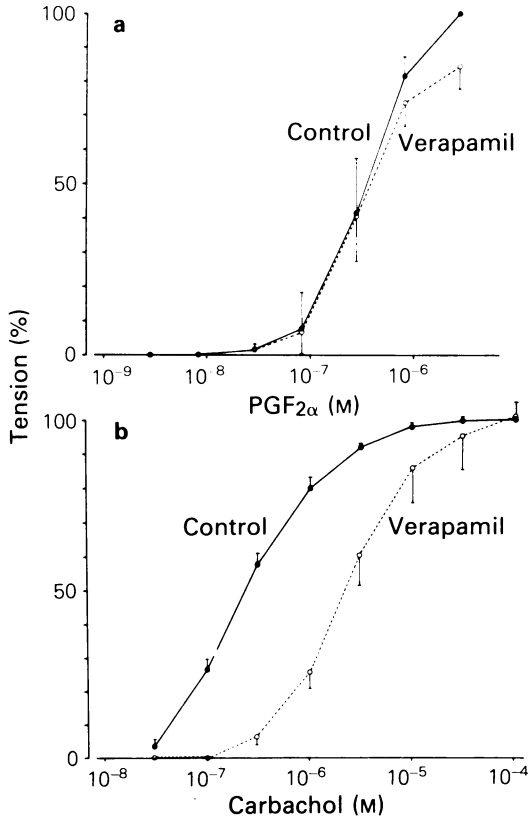

Figure 5 Effects of verapamil on the responses to prostaglandin (PG) $F_{2\alpha}$ (a) and carbachol (b) in normal bathing solution containing 2.4 mM Ca and 5 μM indomethacin. Ordinates: tension expressed as percentage of the response to 2.8 μM $PGF_{2\alpha}$ and 0.1 mM carbachol, before the addition of verapamil. Abscissae: concentrations of $PGF_{2\alpha}$ (a) or carbachol (b). After monitoring the control response twice at a 30 min interval, verapamil (10 μM) was added and 30 min later a concentration-response curve was again obtained. Each point represents the mean of 4 preparations and vertical lines indicate s.d.

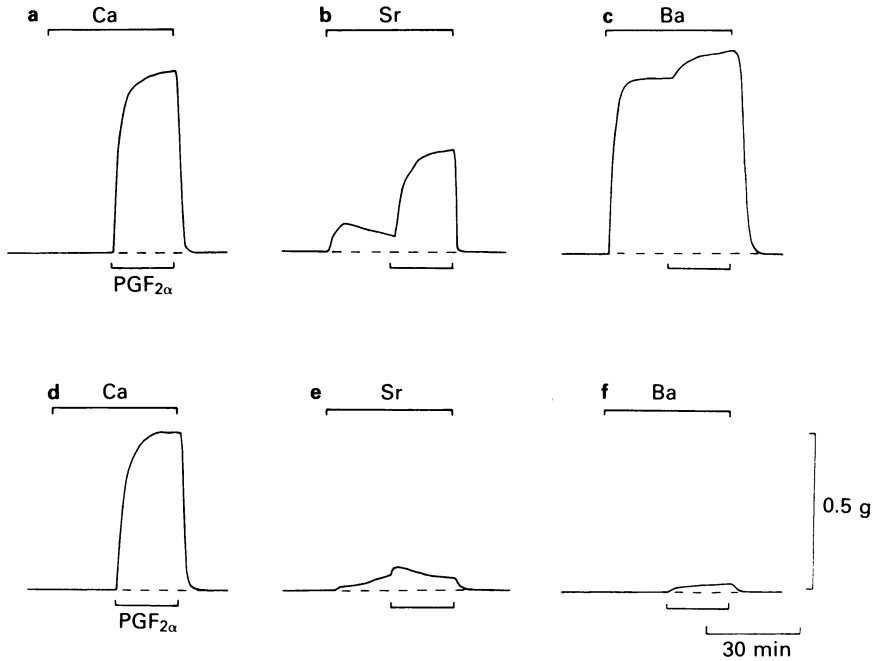

Figure 6 Comparison of responses to prostaglandin (PG) $F_{2\alpha}$ (1.4 μM) in the presence of Ca (2.4 mM), Sr (9.6 mM) and Ba (9.6 mM), and effects of verapamil (10^{-5}M) on these responses. The preparation was first treated with Ca-free solution containing 5.9 mM K, 5 μM indomethacin and 0.5 mM EGTA. EGTA was removed 10 min before and throughout Ca application. (a to f) Successive responses at 40 min intervals obtained from the same preparation, and (d to f) responses in the presence of verapamil (10 μM).

with those in the presence of $PGF_{2\alpha}$. The contractions produced by Sr in the presence of $PGF_{2\alpha}$ were not so markedly different from those produced in the presence of carbachol. The concentration which resulted in 50% maximum tension was 0.24 mM for Ca, 1.5 mM for Ba and 4.4 mM for Sr in the presence of $PGF_{2\alpha}$, and 0.1 mM for Ca, 0.9 mM for Ba and 5.6 mM for Sr in the presence of carbachol. Thus, the relative sensitivity to different divalent cations was not so greatly different between $PGF_{2\alpha}$ and carbachol.

The contractions produced by cumulative administration of $PGF_{2\alpha}$ (3 nM–3 μM) or carbachol (30 nM–0.1 mM) were compared in solutions containing 2.4 mM Ca and 5 μM indomethacin, in the presence and in the absence of verapamil (10 μM) (Figure 5). Verapamil had no depressant effect on contractions induced by $PGF_{2\alpha}$ except at very high concentrations (0.8 – 3 μM) (Figure 5a), whereas the contractions produced by low concentrations (less than 1 μM) of carbachol were very markedly suppressed by verapamil. As the concentration of carbachol was increased, however, the suppression became less significant and the effect disappeared at 0.1 mM carbachol (Figure 5b). It has also been found that verapamil (up to 131 μM) has little effect on the histamine (50 μM)-induced contraction (Duncan & Douglas, 1984).

In the experiment shown in Figure 6, the preparation was first relaxed by exposing it to Ca-free Krebs solution containing indomethacin (5 μM) and EGTA (0.5 mM), then either Ca (2.4 mM), Sr (9.6 mM) or Ba (9.6 m) was applied (in this order) for 40 min. In the presence of indomethacin Ca produced no response (Figure 6a), Sr a weak contraction (Figure 6b), and Ba a strong contraction (Figure 6c). This suggests that the contribution of prostaglandins is larger to Ca- or Sr-induced contractions than to Ba-induced contractions. However, the underlying mechanism for the contractions induced by Sr and Ba was not investigated in the present experiment. Addition of $PGF_{2\alpha}$ (1.4 μM) produced contractions in the presence of these divalent cations, as expected from Figure 4. In the presence of verapamil (10 μM) the same type of experiments was carried out. The contraction induced by $PGF_{2\alpha}$ in the presence of Ca was only slightly reduced by verapamil (Figure 6d). On the other hand, verapamil strongly suppressed not only the contractions induced by either Sr or Ba, but also those produced by $PGF_{2\alpha}$ in the presence of these two ions (Figure 6e,f).

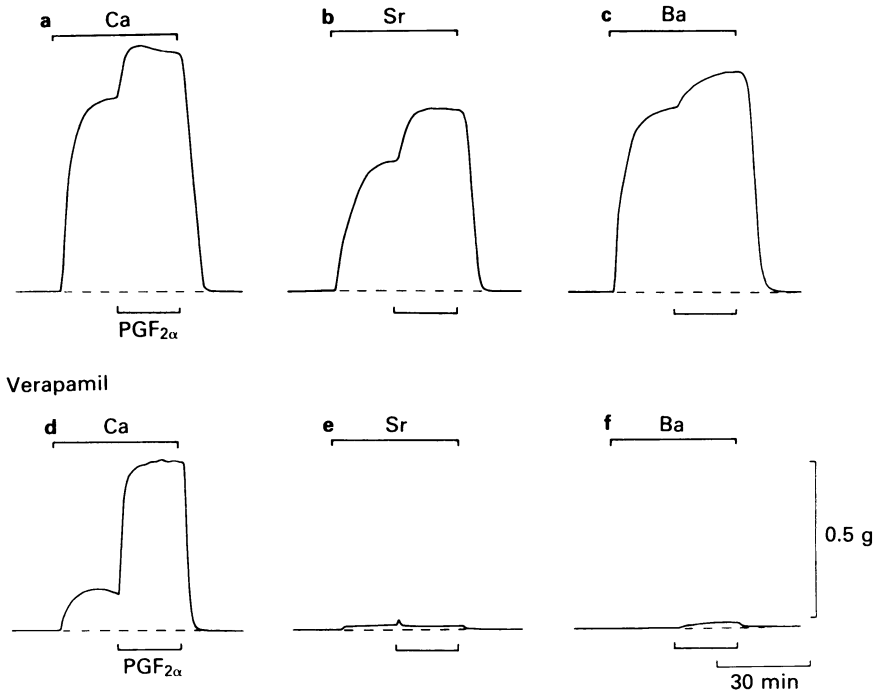

Figure 7 The same type of experiment as Figure 6, but the responses to prostaglandin (PG) $F_{2\alpha}$ (1.4 μM) in the presence of Ca (2.4 mM), Sr (9.6 mM) and Ba (9.6 mM), and the effects of verapamil (d–f) on these responses, were compared in Ca-free solution containing 40 mM K^+ (cf 5.9 mM K^+ in Figure 6), 5 μM indomethacin and 0.5 mM EGTA.

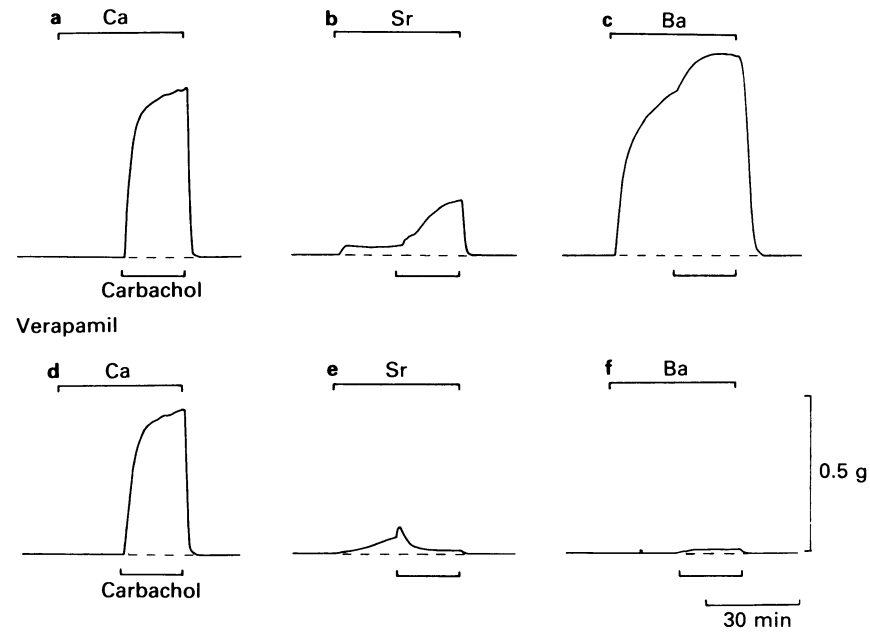

Figure 8 The same type of experiment as Figure 6 but the responses to carbachol (5 μM) (cf. prostaglandin $F_{2\alpha}$) were compared in the presence of Ca (2.4 mM), Sr (9.6 mM) and Ba (9.6 mM) and the effects of 10^{-5} M verapamil (d–f) on these responses. The bathing solution contained 5.9 mM K^+, 5 μM indomethacin and 0.5 mM EGTA.

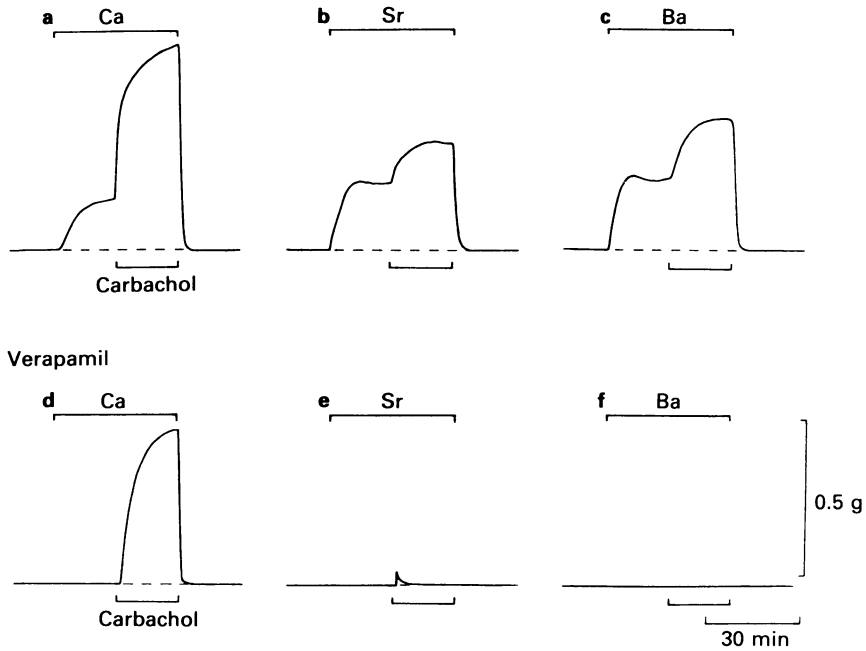

Figure 9 The same type of experiment as Figure 8 but responses to carbachol (5 μM) in the presence of Ca (2.4 mM), Sr (9.6 mM)) and Ba (9.6 mM), and the effects of 10^{-5}M verapamil (d–f) on these responses, were compared in Ca-free solution containing 40 mM K^+ (cf. 5.9 mM K^+), 5 μM indomethacin and 0.5 mM EGTA.

The same type of experiment as that shown in Figure 6 was repeated in 40 mM K^+ medium (Figure 7). Under these conditions, Ca, Sr and Ba all produced contractions of a similar size in the presence of indomethacin. Thus, the main difference between the response patterns in normal and excess K^+ media was found in the response to Ca and Sr. Addition of $PGF_{2\alpha}$ further increased the tension produced by the divalent cations. Verapamil suppressed only the response induced by Ca itself, the contraction to $PGF_{2\alpha}$ in the presence of Ca being little affected, as in 5.9 mM K^+ medium (Figure 7d). However, as in 5.9 mM K^+ medium, verapamil suppressed not only the contractions produced by Sr and Ba but also those by $PGF_{2\alpha}$ in the presence of Sr and Ba (Figure 7e,f).

The effects of verapamil on the responses to carbachol (5 μM) were essentially the same, both in normal (5.9 mM) K^+ (Figure 8) and in excess (40 mM) K^+ (Figure 9), as those on the responses to $PGF_{2\alpha}$. The responses to carbachol in the presence of Ca were resistant (Figures 8d and 9d), while those in the presence of Sr and Ba were very susceptible, to verapamil (e,f, in Figures 8 and 9).

Discussion

In the guinea-pig tracheal muscle, Sr and Ba are less effective in producing a contraction than Ca. An approximately 20 times higher concentration of Sr or Ba was necessary to produce 50% maximum tension and about 4 times higher for maximum tension development. However, the relative tension induced by these divalent cations in the depolarized condition in excess (40 mM) K^+ solution was similar, being 1:0.9:1.1 for Ca, Sr and Ba, respectively. It is difficult to explain the underlying mechanism of activation of the contractile elements by Sr and Ba. According to an experiment on the chicken gizzard, sensitivity of myosin B to Sr and Ba is 1/20 and 1/210, respectively, in comparison to Ca (Ebashi & Endo, 1968). If this relationship can also be applied to the tracheal muscle, one must assume that Sr and Ba are much more permeant ions than Ca and/or that Sr and Ba release intracellular Ca.

In other smooth muscles, such as guinea-pig vas deferens (Jurkiewicz *et al.*, 1975), ileum (Antonio *et al.*, 1973) and taenia coli (Karaki *et al.*, 1967), the

contraction produced by Ba gradually disappears in Ca-free solution, but it recovers after a short treatment with Ca. In these experiments, the contractions are interpreted as being due to Ca release from an intracellular store by Ba. On the other hand, in the rat portal vein (Uvelius *et al.*, 1974), and the rat ileum (Taniyama *et al.*, 1977), it has been shown that Sr and Ba can still produce a significant contraction in a depolarized preparation exposed for more than 2 h to a Ca-free solution containing EGTA, observations which suggest that the contractile machinery can, at least partly, be directly activated by these divalent cations. In the present experiments on tracheal muscle, the Sr-induced contracture remained nearly constant for 3 h, and this was not affected by the readdition of Ca. This result cannot be explained by Sr-induced intracellular Ca release. The response to Ba slowly decreased in Ca-free solution but the reduction was only 25% in 3 h and the contraction produced by Ca was also similarly reduced after the Ba treatment. This suggests that the reduction of the Ba-induced contracture is not due to depletion of intracellular Ca but due to some deleterious effect of Ba which is probably related to a slowing of the relaxation. Since carbachol and $PGF_{2\alpha}$ do not produce contractions in Ca-free solution, influx of Ca (or other divalent contractile cations) is considered to be essential for the production of a response to these agonists. Thus, in the guinea-pig tracheal muscle, it is most likely that Sr and Ba are able to activate directly the contractile protein with an efficiency similar to Ca, although Ca release from some intracellular store cannot be completely ruled out.

In some smooth muscles, it is known that verapamil more readily blocks K^+-induced contractions than drug-induced contractions (Coburn, 1977; Bolton, 1979; Golenhofen, 1981). When a drug-induced contraction is resistant to removal of external Ca, the contraction is likely to be caused by intracellular Ca release. However, there are some smooth muscles in which the drug-induced contraction is easily inhibited by Ca removal, although it is resistant to verapamil (Golenhofen, 1981). Such a contraction is probably due to Ca influx through some pathway which is not affected by verapamil. This supports the idea that two different Ca pathways exist; i.e., voltage-operated and receptor-operated Ca channels which have different affinities for verapamil (Bolton, 1979).

The Ca-induced contraction in the presence of carbachol or $PGF_{2\alpha}$ is also very resistant to verapamil in the guinea-pig tracheal muscle. If the verapamil sensitivity is related to the difference between voltage-dependent and receptor-operated Ca channels, one would expect that the contractions induced by drugs in the presence of Sr or Ba would be similarly resistant to verapamil blockade but they are easily abolished by verapamil. This result suggests that the susceptibility of the Ca^{2+} channel to Ca antagonists does not depend on the type of Ca channel involved.

It may be argued that Sr and Ba utilize the voltage-operated Ca channel even when the contraction is induced by drugs. However, since the maximum K-induced contracture in the presence of Sr or Ba can be still further increased by the addition of prostaglandin $F_{2\alpha}$ or carbachol, the Ca pathway opened by drugs seems to be different from the voltage-operated Ca channel, similarly for Sr and Ba. Another possibility is that in the presence of Sr or Ba, the contribution of the voltage-operated channel to the drug-induced contraction may be relatively larger than in the presence of Ca. However, this does not explain the nearly complete inhibition of drug-induced contractions by verapamil in the presence of Sr or Ba.

Our tentative hypothesis is that the affinity of the Ca channel, both voltage-operated and receptor-operated, to different cations and to verapamil varies depending on the experimental conditions and that the intensity of the blocking action of verapamil is determined by the relative affinity of verapamil and the cations for the channel. In the depolarized condition, verapamil has a stronger affinity for the channel than the divalent cations, while in the presence of the two agonists studied Ca has a stronger affinity than verapamil but Sr and Ba have a weaker affinity than verapamil. However, more direct evidence is necessary to advance this hypothesis.

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