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Effects of verapamil on the response of the guinea-pig tracheal muscle to carbachol

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1 The effects of verapamil on the contraction of the guinea-pig tracheal smooth muscle induced by calcium (Ca^{2+}) or barium (Ba^{2+}) were investigated in three different conditions: (a) in excess K solution, (b) in the presence of carbachol, and (c) in excess K solution containing carbachol. In order to clarify the contractions, the effects of removal and readdition of the divalent cations were also investigated.

2 In Ca^{2+}-loaded tissues, application of carbachol in Ca-free medium produced a transient contraction, the magnitude of which decreased the longer the duration of exposure to Ca^{2+}-free solution.

3 In Ca^{2+}-depleted, Ba^{2+}-loaded tissues, application of carbachol in a Ba^{2+}- and Ca^{2+}-free medium produced a transient contraction the magnitude of which decreased the longer the duration of exposure to the Ba^{2+}- and Ca^{2+}-free solution.

4 After exposure to Ca^{2+}-free solution for 40 min, the sensitivity of the tissue to Ca^{2+} was greater in the presence of 30 μM carbachol (ED_{50} = 0.06 mM) than in the presence of 40 mM K^+ (ED_{50} = 0.3 mM). The Ca^{2+}-sensitivity in the presence of 30 μM carbachol plus K^+ (40 mM) was not different from that in the presence of 30 μM carbachol alone.

5 In Ca^{2+}-free solution, the sensitivity of the tissue to Ba^{2+} in the presence of 40 mM K^+ (ED_{50} = 1.4 mM) was not different from that observed in the presence of 30 μM carbachol (ED_{50} = 1.3 mM).

6 After exposure to Ca^{2+}-free solution, verapamil produced a parallel rightward shift in the concentration-response curves to added Ca^{2+} and Ba^{2+} in the presence of either 40 mM K^+, 30 μM carbachol or 40 mM K^+ plus 30 μM carbachol.

7 The pA_2 values of verapamil against Ca^{2+} responses in the presence of 40 mM K^+, 30 μM carbachol and 40 mM K^+ plus 30 μM carbachol were 7.0, 6.5 and 6.5, respectively. The pA_2 values of verapamil against Ba^{2+} responses under these conditions were 7.1, 7.0 and 7.1, respectively.

8 It is concluded that the sustained contraction produced by carbachol requires the influx of Ca^{2+} and that Ba^{2+} can substitute for Ca^{2+} in this process. Furthermore, the ionic channels which admit Ca^{2+} may be modified by carbachol to different degrees depending on the presence of Ca^{2+} or Ba^{2+}. Such changes alter the affinity of the channel to verapamil.

Introduction

Ca^{2+} influx from the external medium is generally considered to be very important for smooth muscle contraction. It has been assumed that there are two different types of Ca channel in most smooth muscles and one of these, activated by membrane depolarization, is termed voltage-operated channel (VOC). The other seems to be opened by agonist-receptor interaction, and is termed the receptor-operated Ca channel (ROC) (Bolton, 1979; Meisheri *et al.*, 1981). The former is usually susceptible to Ca channel blockers while the latter is resistant to these drugs. For example, Ca-blockers readily suppress K-induced contractions, but have only weak effects on noradrenaline- or carbachol-induced contractions. However, in some vascular smooth muscles, the ROC seems highly sensitive to Ca-blockers (Bevan, 1981; Cauvin *et al.*, 1982; Walus *et al.*, 1981). Furthermore, it has been shown in rabbit aorta that Ca channels become more

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resistant to Ca-blockers as the concentration of noradrenaline is increased, probably due to a larger contribution of intracellular Ca release (Cauvin *et al.*, 1983).

We have previously shown in the guinea-pig tracheal muscle that in the presence of Ca^{2+} the carbachol-induced contraction is very resistant to verapamil compared with the K-contracture. On the other hand, when Ca^{2+} was substituted by Ba^{2+} or Sr^{2+}, both carbachol- and K-induced contractions were strongly suppressed by verapamil, suggesting that the susceptibility to verapamil is also modified by divalent cations present in the external medium (Baba *et al.* 1985).

Thus, the sensitivity of Ca channels to Ca-blockers seems to vary from one type of smooth muscle to another and is probably modified by the type of agonist, its concentration and the external divalent cation concentration. It is possible that Ca channels are fundamentally the same in all smooth muscles, but that apparent differences may appear as a result of modification of their properties. In the present experiments, this problem was further investigated in the guinea-pig tracheal smooth muscle by continuing our study of the effects of verapamil on the contractions induced by Ca^{2+} or Ba^{2+} in the presence of excess K^+ and/or carbachol.

Methods

Guinea-pigs (300–450 g) of either sex were stunned and bled, and the trachea removed. The muscle strip contained in one cartilage ring was prepared for isometric recording as described by Baba *et al.* (1985). Four identical strips were dissected from a single trachea.

The normal solution had the following composition

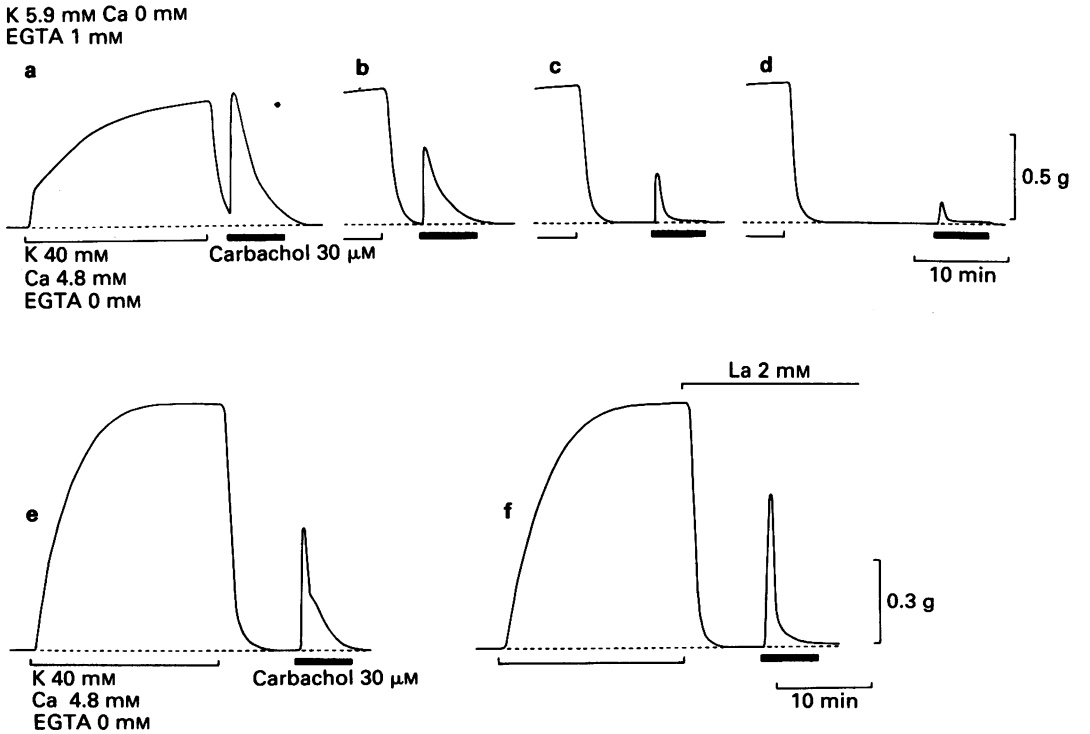

Figure 1 Carbachol-induced contractions in Ca-free solution in guinea-pig tracheal muscle. (a–d) The tissue was loaded with Ca^{2+} for 20 min in 40 mM K^+ solution containing 4.8 mM Ca^{2+} and then exposed to Ca-free solution containing 1 mM EGTA and 5.9 mM K^+. Carbachol (30 μM) was applied for 6 min at different times (2, 4, 8 and 16 min) after Ca^{2+} removal, as shown by horizontal bars. Ca^{2+} loading was repeated at 40 min intervals in the same preparation. (e–f) Similar experiments were carried out on another preparation with (f) and without La^{3+} (e). Carbachol was applied for 6 min, 8 min after removal of Ca^{2+}. In (f), 2 mM La^{3+} was added when Ca^{2+} was removed. Note transient contraction by carbachol in Ca-free solution, even in the presence of La^{3+}, and also note its progressive decrease with longer intervals between Ca^{2+} removal and carbachol application.

(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 the ionic concentration was modified the osmolarity was adjusted by substituting NaCl. In some experiments in which lanthanum chloride was applied, $KHCO_3$ was replaced with KCl and Tris-Cl (20 mM) (pH 7.4 at 35°C) was used as a buffer. EGTA (ethyleneglycol-bis-β-aminoethylether N,N'-tetraacetic acid) (1 mM) was always added to Ca-free solution, except for the experiments with Ba^{2+} in which 0.01 mM EGTA was added to eliminate contamination with Ca^{2+}. A possible contribution of prostaglandins to mechanical changes was excluded by adding indomethacin (5 μM) throughout the experiments.

The drugs used were indomethacin, isoprenaline, EGTA, carbachol and verapamil. All drugs were obtained from Sigma except verapamil (Knoll A.G.). For pharmacological analyses, the Hill plot and the Schild plot were employed using a computer programme (Tallarida & Murray, 1981). All data were represented as means ± s.e.mean (n). Statistical comparisons were made using Student's t test, and P values smaller than 0.05 were considered to be significant.

Results

Effects of the removal and readdition of Ca^{2+} or Ba^{2+} on responses to carbachol

When the preparation was exposed to Ca-free solution containing 1 mM EGTA, the resting tension quickly disappeared. Following equilibration for at least 40 min in Ca-free solution, tissues were loaded with Ca^{2+} for 20 min in 4.8 mM Ca^{2+}, 40 mM K^+ medium. Muscles were then again exposed to Ca-free solution, and carbachol (30 μM) was applied for 6 min at 2, 4, 8 or 16 min after removal of Ca^{2+}, as shown in Figure 1 (a–d). Contractions due to addition of 4.8 mM Ca^{2+}, and rapid relaxation on removal of Ca^{2+}, were reproducible many times. A transient contraction was produced by application of carbachol in Ca-free solution. The rate of relaxation of the transient contraction varied in different preparations and occasionally decayed in two phases (e.g. Figure 1e), but it became faster as the slow phase was lost on repeated exposure to carbachol. Although the tension rapidly decreased, a small tonic contraction always

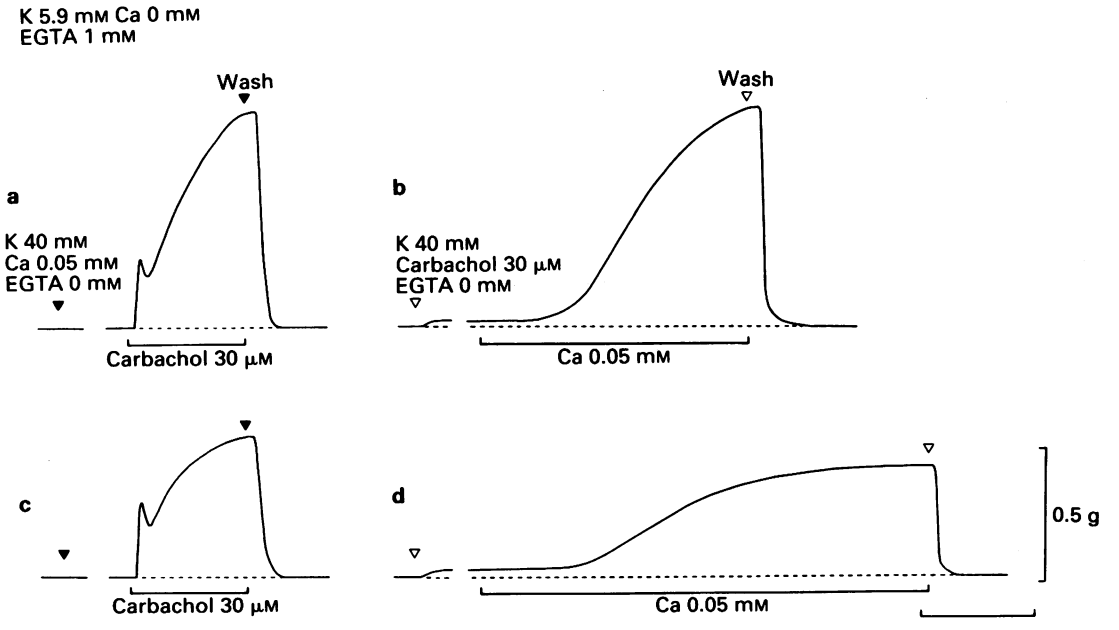

Figure 2 (a) Responses to carbachol (30 μM) in the presence of 0.05 mM Ca^{2+}, and (b) responses to Ca^{2+} (0.05 mM) in the presence of 30 μM carbachol. (c and d) The same experiments as (a) and (b), but in the presence of verapamil (0.1 μM), in the same preparation. The preparation was first treated with Ca-free solution containing 1 mM EGTA for 40 min. The pretreatment in each solution was for 15 min, but verapamil was applied 40 min before application of carbachol shown in (c), and present through (c) and (d).

remained for as long as carbachol was present. As the administration of carbachol was delayed after Ca removal, the transient response decreased roughly exponentially, the time constant being about 8 min. The rate of decrease was approximately 6 times faster than that for the noradrenaline response in the rabbit ear artery (Droogmans *et al.*, 1977).

Similar transient contractions were obtained even when La^{3+} (2 mM) was added to Ca-free solution. A comparison of the responses to carbachol in Ca-free solution in the absence and presence of 2 mM La^{3+} is shown in Figure 1e,f. The carbachol responses were fundamentally similar in the absence and presence of La^{3+}. In the presence of Ca^{2+} in the external medium, the tension produced by carbachol was sustained. When the external Ca^{2+} was removed or 2 mM La^{3+} was added to Ca-containing solution, most of the sustained contraction induced by carbachol was quickly abolished, leaving a small maintained tension. Therefore, Ca influx seems necessary for the sustained contraction and the transient contraction in Ca-free solution is likely to be due to Ca^{2+} released by carbachol from a limited intracellular store, the amount of which is slowly reduced during exposure to Ca-free solution.

Figure 2 shows responses to carbachol (30 μM) in the presence of 0.05 mM Ca^{2+} (a) and to Ca^{2+} (0.05 mM) in the presence of 30 μM carbachol (b). Addition of 0.05 mM Ca^{2+} (0 mM EGTA), after equilibration in Ca-free solution, did not produce a noticeable contraction by itself, but subsequent application of carbachol produced an early phasic contraction followed by a slowly developing, maintained contraction (a). Pretreatment with carbachol in Ca-free solution produced a small increase in tension whilst addition of Ca^{2+} in the presence of carbachol resulted in the development of a tonic contraction (b), the maximum being the same as that produced by the reversed procedure (a).

The same experiments were repeated in the presence of verapamil (0.1 μM) using the same preparation (Figure 2c,d). Verapamil suppressed the tonic contractions produced by both procedures to the same extent (approximately 40%). However, the phasic contraction induced by carbachol in the presence of Ca^{2+} (c) and the small tonic component of the carbachol-induced contraction observed in the absence of Ca^{2+} (d) were not affected by verapamil.

As previously described (Baba *et al.*, 1985), contractions of a similar magnitude could be evoked when Ca^{2+} was replaced by Ba^{2+} (4.8 mM). However, the rate of relaxation after removal of Ba^{2+} was slightly slower compared with the contraction caused by Ca^{2+}. As observed in the Ca experiments, a transient response to carbachol could also be produced following removal of Ba^{2+}, even in the presence of 2 mM La^{3+}. Furthermore, essentially the same results were obtained when the experiments shown in Figure 2a,b were carried out with Ba^{2+}, although the effect of verapamil was much greater than with Ca^{2+}, as previously shown (Baba *et al.*, 1985).

Concentration-response relationship for Ca^{2+} and Ba^{2+}

After incubation in Ca-free solution containing EGTA (1 mM) for 40 min, K^+ (40 mM), carbachol (30 μM), or K^+ plus carbachol were applied without EGTA, and steady state cumulative concentration-response curves were obtained to Ca^{2+} and Ba^{2+}. When the same experiment was repeated at an interval of 90 min with the same preparation, there was no difference between the Hill coefficients computed from the first and second concentration-response curves.

The results of these experiments are shown in Figure 3. Preparations were more sensitive to Ca^{2+} than to Ba^{2+} under all conditions. The maximum

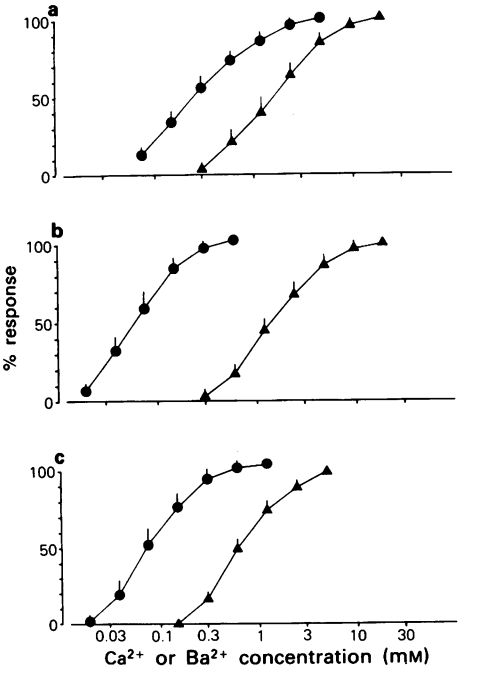

Figure 3 Concentration-response relationships for Ca^{2+} (●) and Ba^{2+} (▲) in the presence of excess (40 mM) K^+ (a), 30 μM carbachol (b), and excess K^+ (40 mM) plus 30 μM carbachol (c). The responses were obtained by cumulatively increasing Ca^{2+} or Ba^{2+} concentrations and repeated 90 min later with the same divalent cation in each preparation. Each curve was obtained by averaging the data from 6 preparations, and vertical lines indicate s.e.mean.

Table 1 Analyses of concentration-response curves for Ca^{2+} and Ba^{2+}

Solution	*Maximum tension* (g)	$-\log ED_{50}$ (M)	*Hill coefficient*	pA_2 *(verapamil)*
		Ca^{2+}		
40 mM K^+	0.8 ± 0.1 (16)	3.7 ± 0.1 (16)	1.5 ± 0.1 (16)	7.0 ± 1.0 (12)
Carbachol (30 μM)	1.4 ± 0.1 (15)*	4.2 ± 0.2 (15)*	2.1 ± 0.1 (15)*	6.4 ± 0.04 (15)*
40 mM K^+ + carbachol	1.4 ± 0.1 (14)*	4.4 ± 0.1 (14)*	2.0 ± 0.1 (14)*	6.5 ± 0.1 (14)*
		Ba^{2+}		
40 mM K^+	1.1 ± 0.1 (16)	2.9 ± 0.1 (16)	1.8 ± 0.1 (16)	7.1 ± 0.04 (15)
Carbachol (30 μM)	1.1 ± 0.04 (15)	2.9 ± 0.1 (15)	2.0 ± 0.1 (15)	7.0 ± 0.03 (15)
40 mM K^+ + carbachol	1.5 ± 0.1 (14)*	3.3 ± 0.1 (14)*	1.9 ± 0.1 (14)	7.1 ± 0.1 (14)

pA_2 values were obtained from the Schild plots.
*Significantly different ($P < 0.05$) from the value in 40 mM K^+ solution. All data are presented as means ± s.e.mean (n).

tension produced by Ba^{2+} was about 20% larger than that produced by Ca^{2+} in 40 mM K^+ solution, 20% smaller in the presence of carbachol, and nearly the same as that produced by Ca^{2+} in 40 mM K^+ solution containing carbachol. The concentration-response curve for Ca^{2+} was shifted to the left when carbachol was present, and this was independent of the K^+ concentration (b,c). The concentration-response curve for Ba^{2+} was not modified by carbachol alone (b), but it was slightly shifted to left in 40 mM K^+ solution containing carbachol (c). Increasing the external K^+ concentration to 70 mM did not affect the concentration-response curve obtained compared to that obtained in 40 mM K^+ solution. The maximum tension developed and the ED_{50} values obtained in the various conditions are summarized in Table 1.

The Hill coefficient for the response to Ca was 1.5 in 40 mM K^+ solution, but clearly larger (about 2) in the presence of carbachol, a value which was independent of the external K^+ concentration. The Hill coefficient for the response to Ba was 1.8 in 40 mM K^+ solution, and this was not significantly altered by carbachol (Table 1).

Effects of verapamil on the concentration-response relationship for Ca^{2+} and Ba^{2+}

Tissues were exposed to verapamil (0.1–3 μM) for 40 min before re-examining the responses to Ca^{2+} and Ba^{2+} in the continuing presence of verapamil. The maximum contraction obtained in the first concentration-response curve was taken as 100%, and subsequent responses were expressed as a percentage of this value.

Figure 4 shows the effects of verapamil (0.1–3 μM) on the concentration-response curve for Ca^{2+} in the presence of 40 mM K^+ (a), 30 μM carbachol (b), and

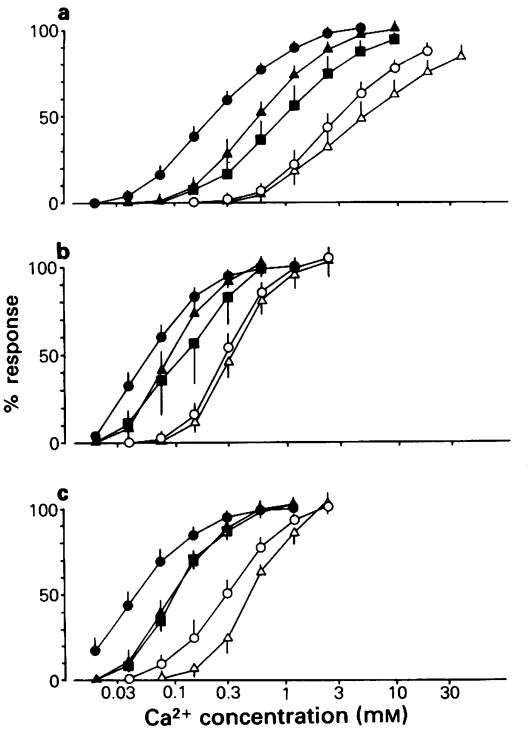

Figure 4 Effects of verapamil on Ca-induced contractions in the presence of 40 mM K^+ (a), 30 μM carbachol (b) and 40 mM K^+ plus 30 μM carbachol (c). Each panel shows concentration-response curves in the absence (control, ●) and presence of verapamil 0.1 μM (▲), 0.3 μM (■), 1 μM (○) and 3 μM (△). Each point represents the mean of 14–16 experiments for the control and of 3–4 experiments in the presence of verapamil. The maximum of the control response was taken as 100% under each condition.

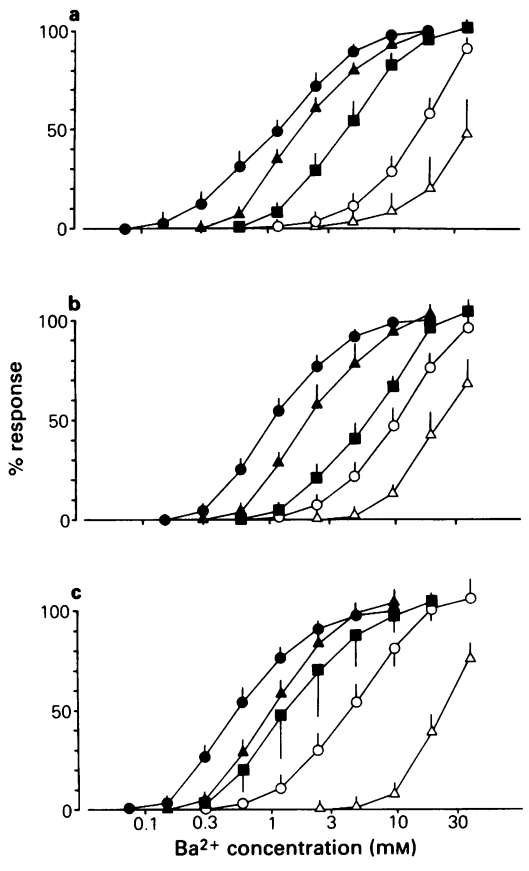

Figure 5 Effects of verapamil on the concentration-response curves for Ba^{2+} in the presence of 40 mM K^+ (a), 30 μM carbachol (b) and 40 mM K^+ plus 30 μM carbachol (c). Concentration-response curves in the absence (control, ●) and presence of verapamil 0.1 μM (▲), 0.3 μM (■), 1 μM (○) and 3 μM (△) are shown. Experimental conditions were the same as those in Figure 4.

40 mM K^+ plus 30 μM carbachol (c). Verapamil (0.1–1 μM) produced a dose-dependent, parallel displacement of the Ca^{2+} concentration-response curve to the right. However, when the concentration was increased to 3 μM, the maximum tension was reduced and the Hill coefficient became significantly smaller (1.1) than that for the control concentration-response curve (1.5).

In the presence of carbachol (30 μM), verapamil shifted the concentration-response curve to the right without reducing the maximum response (b), and the Hill coefficient remained roughly the same (being 1.9–2.2), indicating a parallel shift. The effect of verapamil in the presence of carbachol plus K^+ 40 mM was not different from that seen in the presence of carbachol alone (c).

Verapamil (0.1–1 μM) produced a parallel rightward shift of the Ba^{2+} concentration-response curves (Figure 5). Although Ba^{2+} concentrations higher than 38.4 mM were not tested, the maximum response to Ba^{2+} did not seem to be suppressed by verapamil even at 3 μM. The Hill coefficient of the Ba response in the presence of verapamil (1.7–2.2) was not significantly different from the control (1.8–2.0) in any of the three solutions.

The Schild plots of verapamil suppression on the Ca- and Ba-induced contractions under various conditions gave regression lines with slopes close to unity, as shown in Figure 6. The pA_2 values of verapamil, obtained from standard Schild plots, were not significantly different from those calculated using the constrained Schild plot by assuming unity slope (Tallarida & Murray, 1981). The latter values are summarized in Table 1. The pA_2 value of verapamil against Ca^{2+} was significantly smaller in the presence of carbachol. pA_2 values for verapamil against Ba^{2+} were 7.0–7.1 and were found to be independent of the presence or absence of carbachol. These values were the same as those for Ca^{2+} in excess K^+ solution without carbachol.

Discussion

It is generally believed that in airway smooth muscles Ca^{2+} influx from the external medium is mainly involved in K-contracture whilst Ca^{2+} release from intracellular stores is the trigger for carbachol-induced contractions (Kirkpatrick *et al.*, 1975; Farley & Miles, 1978; Creese & Denborough, 1981; Ahmed *et al.*, 1984; Ito & Itoh, 1984). In the present experiments, it was demonstrated that carbachol can produce a contraction in Ca-free solution, supporting the view that carbachol can release Ca^{2+} from an internal store. However, since the carbachol-induced contraction was transient in Ca-free solution or in the presence of La^{3+}, an influx of external Ca^{2+} is essential for the maintenance of the subsequent tonic contraction. When Ca^{2+} was completely replaced with Ba^{2+} fundamentally the same response was observed, except that there was a lower affinity for Ba^{2+} than for Ca^{2+}. Thus, an influx of Ba^{2+} also seems to be able to maintain the carbachol-induced contraction.

The experiments on readdition of Ca^{2+} to Ca^{2+}-free solution indicated that the sensitivity of the tissues to Ca^{2+} was about 5 times greater in the presence of 30 μM carbachol (ED_{50} = 0.06 mM) than it was in the presence of 40 mM K^+ alone (ED_{50} = 0.3 mM). Furthermore, the sensitivity to Ca^{2+} in the presence of carbachol was not modified by increasing the external K^+ concentration to 40 mM. The increase in sensitivity

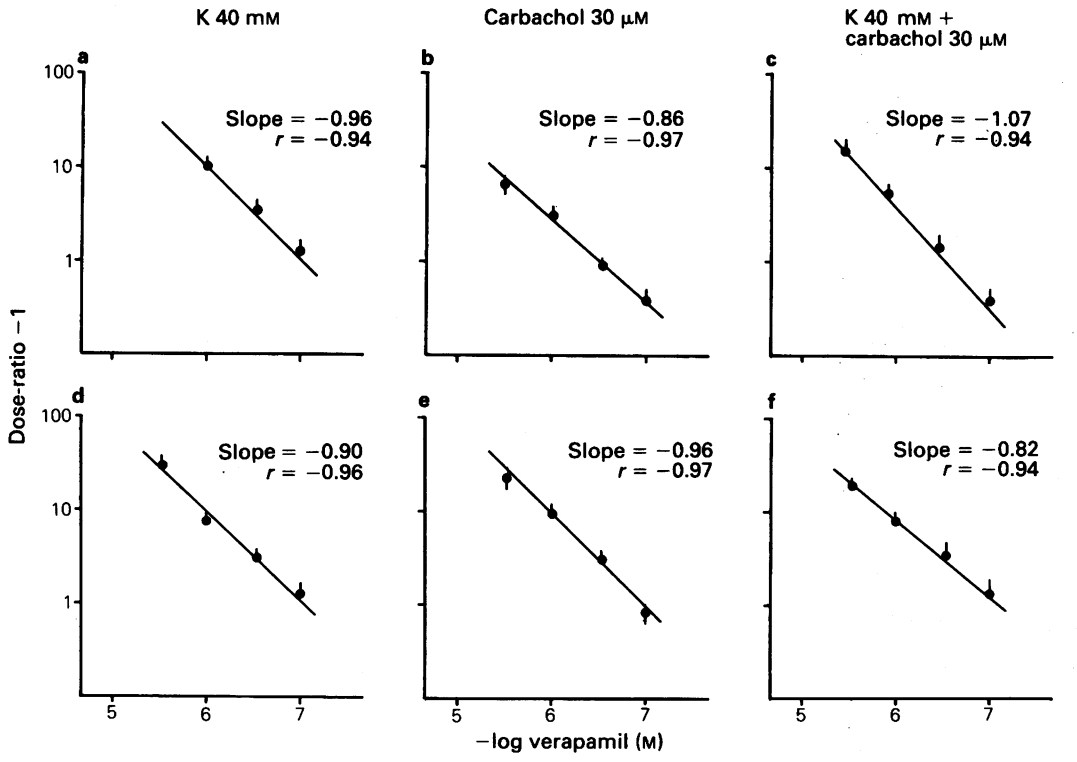

Figure 6 Schild plots obtained from Figures 4 and 5, to show the antagonism by verapamil of responses to Ca^{2+} (a–c) and Ba^{2+} (d–f). The point for 3 μM verapamil in the Ca-response in 40 mM K^+ solution (a) was omitted because of non-parallel shift, as seen in Figure 4(a). The slope of the regression line and the correlation coefficient (r) are indicated in each panel.

to Ca^{2+} produced by carbachol was accompanied by an increase in the Hill coefficient of the Ca^{2+} concentration-response curve from 1.5 to about 2. Such observations suggest that carbachol modifies the kinetics of the Ca channel. However, the sensitivity to Ba^{2+} was not altered by carbachol alone, although it was increased about two fold in the presence of excess (40 mM) K^+ plus carbachol (30 μM). Similarly, the Hill coefficient of the Ba^{2+} concentration-response curve was not significantly affected by carbachol or even by excess K^+ plus carbachol. This suggests that the presence of extracellular Ca^{2+} (but not Ba^{2+}) is necessary to modify the kinetics of the Ca channel.

It has been shown using skinned fibres of the guinea-pig taenia coli that verapamil (up to 100 μM) has no effect on the contractile machinery (Spedding, 1983). When the verapamil concentration is more than 10 μM, it may affect smooth muscle muscarinic receptors (Cheng *et al.*, 1985), or cardiac muscle sarcoplasmic function (Entman *et al.*, 1972; Watanabe & Besch, 1974; Colvin *et al.*, 1982). In the present experiments, however, it is likely that at concentrations below 3 μM verapamil is acting on Ca channels in the plasma membrane. Maximum mechanical responses were not affected by verapamil, except for the response to Ca in 40 mM K^+ solution at 3 μM. This suppression is probably due to the fact that high concentrations of Ca^{2+} exert a stabilizing action on the membrane, as observed in vascular smooth muscle (Högestätt & Andersson, 1983).

In airway smooth muscles, organic Ca channel blockers such as verapamil have little effect on contractions induced by acetylcholine or carbachol, whereas they suppress the contractions induced by excess K^+, although both are dependent on the external Ca^{2+} (Coburn, 1979; Baba *et al.*, 1985). This difference could be explained by the hypothesis that a receptor-operated Ca channel (ROC) is more resistant to Ca-blockers than the voltage-operated Ca channel (VOC) (Bolton, 1979; Van Breemen *et al.*, 1979).

However, we have previously shown in guinea-pig tracheal muscle that contractions produced by 5 μM carbachol in the presence of 2.4 mM Ca^{2+} are little affected by 10 μM verapamil, but that those in the

presence of 9.6 mM Ba^{2+} are strongly suppressed in both 5.9 and 40 mM K^+ solutions (Baba *et al.*, 1985). This clear difference was confirmed in the present experiments, and the high verapamil sensitivity of carbachol-induced contractions in Ba^{2+} solution is difficult to explain by the hypothesis which assumes two different Ca channels, the VOC and the ROC.

In the present study, verapamil (0.1–1 μM) behaved like a competitive antagonist of both Ca^{2+}- and Ba^{2+}-induced contractions. For this reason it was felt that the use of Schild plots for the analysis of the results was justified. In the presence of carbachol, low pA_2 values (6.4–6.5) for verapamil against Ca^{2+} were obtained compared with those (7.0) obtained in 40 mM K^+ solution. Since muscarinic agonists depolarize the tracheal muscle (Ahmed *et al.*, 1984), both the VOC and the hypothetical ROC might be activated during a carbachol-induced contraction. Furthermore, if two types of channel having different affinities for verapamil were involved, the slope of Schild plot would be expected to be different from unity. However, Schild plot analysis produced regression lines having slopes close to unity, with a significant correlation coefficient (0.94–0.97). These results suggest that in the presence of carbachol (and carbachol + excess K^+) only one type of Ca channel is involved, but that the affinity of the channel for verapamil is decreased when the receptor is activated by carbachol, independent of the external K^+ concentration.

When Ca^{2+} was completely substituted by Ba^{2+}, the effect of verapamil was not affected by carbachol. The Ba^{2+} concentration-response curves were shifted in parallel to the right by verapamil with the same pA_2 value in excess K^+ solution in the absence or presence of carbachol. This suggests that the sensitivity of the channel to verapamil is not affected by carbachol in the presence of Ba^{2+} (and absence of Ca^{2+}).

The results obtained in the present experiments are inconsistent with the assumption that the postulated receptor-operated Ca channel is less susceptible to Ca-blockers. It is therefore possible that Ca channels are basically of only one type, but that their properties can be modified by agonist-receptor interaction and that this modification itself varies depending on the divalent cations present in the external medium. Such a mechanism is likely to be responsible for the differences in verapamil sensitivity observed in the present study. The change induced by carbachol in the Hill coefficients of Ca^{2+} concentration-response curves, but not in those for Ba^{2+}, strongly suggests a modification of the channel kinetics.

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