Br. J. Pharmac. (1986), **87**, 15–22

Carbachol-induced contraction in the circular muscle of guinea-pig stomach in calcium-free solution

F. Ashoori, H. Hidaka* & T. Tomita[1]

Department of Physiology, Nagoya University, Nagoya 466 and Department of Pharmacology*, Mie University, Tsu 514, Japan

1 In the circular muscle of the fundic part of the guinea-pig stomach, a small tonic contraction could be repeatedly produced by carbachol in Ca-free solution containing 2 mM EGTA.

2 The carbachol-induced response was gradually increased during prolonged exposure to Ca-free solution for 50 h, whereas a short treatment with 0.1–0.2 mM Ca suppressed the subsequent carbachol response in Ca-free solution. The response was not essentially modified by increasing the external K^+ concentration to 40 mM.

3 Calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W-7) and trifluoperazine selectively suppressed the carbachol response in the presence of Ca (0.05 mM) and the contraction induced by Ca (0.1 mM), but they had little effect on the response to carbachol in Ca-free solution at a concentration of less than 10 μM.

4 A vasodilator agent, N-(2-guanidinoethyl)-5-isoquinoline sulphonamide (HA-1004), inhibited the carbachol response both in the presence and absence of Ca, as well as the Ca-induced contraction, to a similar extent, provided that the external Ca concentration was less than 0.1 mM.

5 These results led us to propose that the contraction evoked by carbachol in the absence of external Ca is mediated by a process independent of the Ca-calmodulin system.

Introduction

It has been found in several smooth muscle tissues that a small tonic contraction can be induced repeatedly without decay during prolonged exposure to a Ca-free solution. These include vascular smooth muscle tissues (rabbit ear, pulmonary and saphenous artery: Casteels *et al.*, 1981; rat aorta: Heaslip & Rahwan, 1982) and rat vas deferens (Ashoori & Tomita, 1983) in response to noradrenaline; rat myometrium in response to oxytocin (Sakai & Uchida, 1980; Sakai *et al.*, 1981; 1982; Ashoori *et al.*, 1985b), acetylcholine, prostaglandin E_2 or vanadate (Mironneau *et al.*, 1984); and canine stomach muscle in response to acetylcholine (Golenhofen *et al.*, 1982). Although these contractions are generally assumed to be due to intracellular Ca release (e.g., Daniel, 1984; Mironneau *et al.*, 1984), it is possible that they are produced by some Ca-independent process, as proposed by Casteels *et al.* (1981).

Since the contraction of smooth muscle is thought to be initiated by a Ca-calmodulin reaction in phosphorylation of myosin (Hartshorne & Siemankowski, 1981), a calmodulin antagonist would be expected to suppress a Ca-mediated contraction. On the other hand, if the contraction is independent of Ca, the contraction may not be affected by a calmodulin antagonist. In previous studies, a calmodulin antagonist, W-7, was used for the vas deferens and rat myometrium (Ashoori & Tomita, 1983; Ashoori *et al.*, 1985b). This compound is known to be one of the most specific antagonists of calmodulin (Hidaka *et al.*, 1978; 1980; Asano *et al.*, 1982). However, no conclusive results were obtained concerning the involvement of Ca.

It has recently been shown that a vasodilator compound, HA-1004, which has a similar structure to the calmodulin antagonist, W-7, but does not have an anticalmodulin action (Asano & Hidaka, 1984), suppressed preferentially the contractions observed in Ca-free solution compared with those in the presence of Ca (Ashoori *et al.*, 1985a). Thus, in the present experiments, the effects of W-7 and HA-1004 were further studied in the circular muscle of guinea-pig stomach which also produces a contraction resistant to Ca-removal in response to carbachol.

[1] Author for correspondence.

Methods

Guinea-pigs (250–350 g) of either sex were stunned and bled, and the stomach isolated. A piece of the smooth muscle (about 1 mm wide and 7 mm long) was dissected in a circular direction from the fundic part of the stomach after the mucosa had been carefully removed. Isometric tension was measured with a strain gauge from the preparation vertically mounted in an organ bath (1 ml capacity) and superfused with a physiological solution kept at 35°C, at a constant rate of about 2.5 ml min^{-1}. Each experiment was repeated at least four times with different preparations, confirming the same results.

The normal physiological solution had the following composition (mM): NaCl 136.9, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 11.8. When Ca^{2+} was removed, 2 mM EGTA was added to the Ca^{2+}-free solution and K^+ was increased to 12 or 40 mM by replacing NaCl with KCl on an equimolar basis, because the basal tension tended to increase slowly in Ca^{2+}-free solution containing less than 6 mM K^+. In the experiments in which Ca^{2+} was reintroduced to the external medium, EGTA was not added to the Ca^{2+}-free solution.

W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide) and HA-1004 (N-(2-guanidinoethyl)-5-isoquinoline sulphonamide) were obtained from Kaken Kagaku and Asahi Kasei (Japan), respectively. Their chemical properties have been described previously (W-7: Hidaka *et al.*, 1978; HA-1004: Asano & Hidaka, 1984).

Results

Figure 1 shows a typical example of mechanical responses to different concentrations of carbachol in the presence (a–e) and absence of the external Ca ion (f–j). In the normal solution, the circular muscle of the fundic part of the guinea-pig stomach developed a maintained tension with slow irregular fluctuations, but usually lacking rhythmic activity, typical of a circular muscle obtained from the antrum region (Ohba *et al.*, 1976). The response pattern to carbachol differed from preparation to preparation, and also depended on its concentration. When the concentration was low, the response was usually a simple increase in tension and a slow relaxation beyond the control level was observed following washout (a). As the concentration was increased, the contractile response became transient and the tension was reduced below the control level even during the application of carbachol (c–e).

In the absence of external Ca^{2+}, the muscle strip lost basal tone, but carbachol produced a slow tonic response in a dose-dependent manner (f–j). Although the developed tension was less than 10% of the response produced by the same concentration of carbachol in the presence of Ca^{2+}, the sensitivity to carbachol did not seem to be modified significantly by Ca^{2+} removal. The maximum response was obtained at abouit 100 μM, but due to a slow increase in the response even at a constant concentration, it was difficult to obtain reliable dose-response curves. In the following experiments, 30 μM carbachol was generally used. In the experiment shown in Figure 1, the external K^+ concentration was increased to 40 mM when Ca^{2+} was removed, but almost the same results were obtained in the presence of 5.9–12 mM K^+, except that the basal tone tended to increase after a few hours in Ca^{2+}-free solution containing 5.9 mM K^+ or less.

The response to carbachol in Ca^{2+}-free medium slowly increased with time, as observed for the oxytocin response in the rat myometrium (Ashoori *et al.*, 1985b). The maximum increase was about 4 times, that originally obtained, after continuous perfusion with Ca^{2+}-free solution containing 2 mM EGTA for nearly 50 h. An example of the carbachol response during continuous perfusion with Ca^{2+}-free solution containing 2 mM EGTA is shown in Figure 2A, and the results from the same experiments on two different preparations are shown in Figure 2B. When the administration of doses of carbachol at 20 min intervals was resumed after interruption for a few hours, the first two to three consecutive responses were smaller than the steady state size that was eventually reached.

The rate of increase in peak tension following Ca^{2+} removal varied in different preparations, and was usually faster at the early period than that after 24 h, as shown in Figure 2B, but this time course was not carefully studied. In some preparations, the increase was already significantly slowed down after an exposure to Ca-free solution for 2 h.

The carbachol response slowly increased following removal of Ca^{2+} from the external medium, as already described. On the other hand, administration of small amounts of Ca^{2+} decreased the following carbachol response in Ca^{2+}-free solution. In Figure 3, after observing the response to carbachol (30 μM) in normal solution (a), the administration of carbachol was resumed at 20 min intervals 100 min after exposure to Ca^{2+}-free solution (b). At the time of the 7th dose of carbachol (at 244 min in Ca^{2+}-free solution), 0.1 mM Ca^{2+} was added for 4 min, instead of carbachol. This treatment of the preparation with Ca^{2+} (not shown in the figure) suppressed the next carbachol response (e), which recovered slowly (f, g). When a higher concentration of Ca^{2+} (0.2 mM) was similarly administered for 4 min, the suppression of the following carbachol response was greater (h).

Figure 4 shows effects of a calmodulin antagonist, W-7, on the responses induced by carbachol (30 μM) in

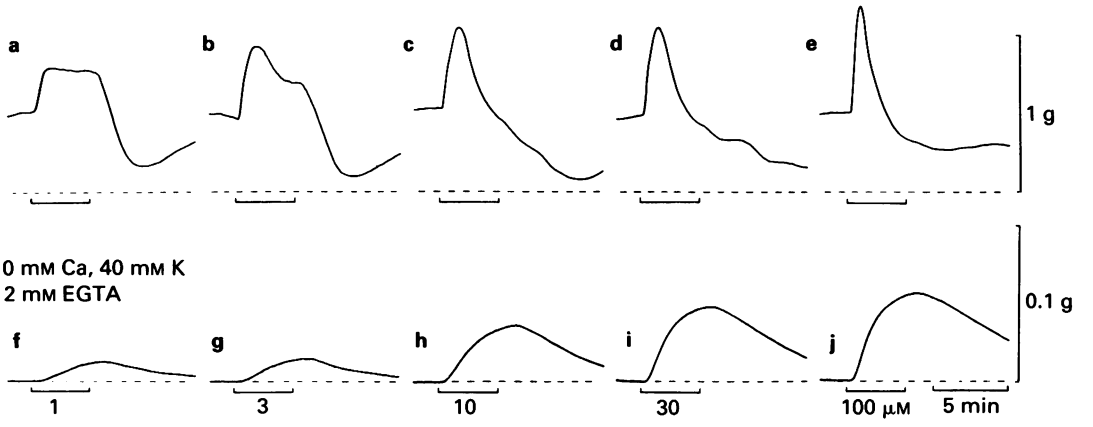

Figure 1 Mechanical responses of the guinea-pig stomach muscle to different concentrations of carbachol in the presence and absence of Ca^{2+}. Increasing concentrations of carbachol (1 – 100 μM) were added for 4 min at 20 min intervals, before (a–e) and after Ca^{2+} removal (f–j) in the same preparation. Carbachol application is indicated by the horizontal bar below each record. Note the difference in gain for tension recording.

Ca^{2+}-free solution (a–h) and the responses to 0.1 μM carbachol in the presence of 0.05 mM Ca^{2+} (i–p). The concentrations of carbachol and Ca^{2+} were adjusted to give tension responses (i, j) comparable to those evoked by 30 μM carbachol in the absence of Ca^{2+} (a, b). W-7, up to 10 μM, had no effect on the response in the absence of Ca^{2+} (d), and only slightly suppressed it at a concentration of 30 μM (e). On the other hand, the responses in the presence of 0.05 mM Ca^{2+} were markedly inhibited by W-7 (k–m). The recovery of the responses from the effects of W-7 were good in Ca^{2+}-free solution (f–h), but very poor in the presence of Ca^{2+} (n–p). The contraction induced by administration of 0.1 mM Ca^{2+} in Ca^{2+}-free 40 mM K^+ solution was also susceptible to W-7 (Figure 5). After observing more of less constant responses to carbachol (a) and to 0.1 mM Ca^{2+} (f) in Ca^{2+}-free solution, 3 μM (b, g) and 10 μM (c, h) W-7 were added cumulatively, each for 20 min. The carbachol response was scarcely affected, while the Ca^{2+}-induced contraction was greatly reduced by W-7. The Ca^{2+}-induced response recovered very slowly after washout of W-7 (i, j). Another calmodulin antagonist, trifluoperazine had effects similar to those of W-7 and their potencies were approximately equal, but the recovery of the Ca-induced contraction from trifluoperazine treatment was poorer.

A vasodilator compound, HA-1004, has been shown to lack anticalmodulin action (Asano & Hidaka, 1984). This compound was examined using the experimental procedures similar to those shown in Figures 4 and 5. In contrast to W-7, HA-1004 suppressed not only the contraction induced by 0.1 mM Ca^{2+} in Ca^{2+}-free/40 mM K^+ solution, but also the carbachol response in this Ca^{2+}-free solution, as shown in Figure 6. Both responses were reduced to a similar extent. The antagonistic effect of HA-1004 was also observed on the response to 0.1 μM carbachol in the solution containing 0.05 mM Ca^{2+} and 12 mM K^+.

Discussion

The circular muscle of the fundic part of the guinea-pig stomach can produce a contraction repeatedly in response to carbachol in Ca-free solution, as described for the canine stomach muscle (Golenhofen *et al.*, 1982). This contraction slowly increases after Ca^{2+} removal and is suppressed by a short treatment with a low concentration of Ca^{2+}. The underlying mechanism of the carbachol-induced contraction is considered to be essentially the same as that produced in other smooth muscles (blood vessels, myometrium and vas deferens) in response to their respective agonists, such as noradrenaline or oxytocin (see Introduction for references).

It has been shown that in the rat vas deferens, the noradrenaline-induced contraction in Ca^{2+}-free solution is suppressed by W-7. However, the effective concentration is more than 100 μM and at this concentration, the Ca-induced contraction is also markedly inhibited (Ashoori & Tomita, 1983). Similarly, phenothiazine derivatives, such as trifluoperazine, chlorpromazine or fluphenazine, which are known to have an anticalmodulin action, inhibit both the noradrenaline response and the Ca-induced response (Tomita *et al.*, 1985), but this result is difficult to interpret because of the α-adrenoceptor blocking actions of the phenothiazine derivatives (Takayanagi, 1964; Cocks *et al.*, 1981).

In the rat myometrium, the response to oxytocin in Ca-free solution and the Ca-induced contraction are

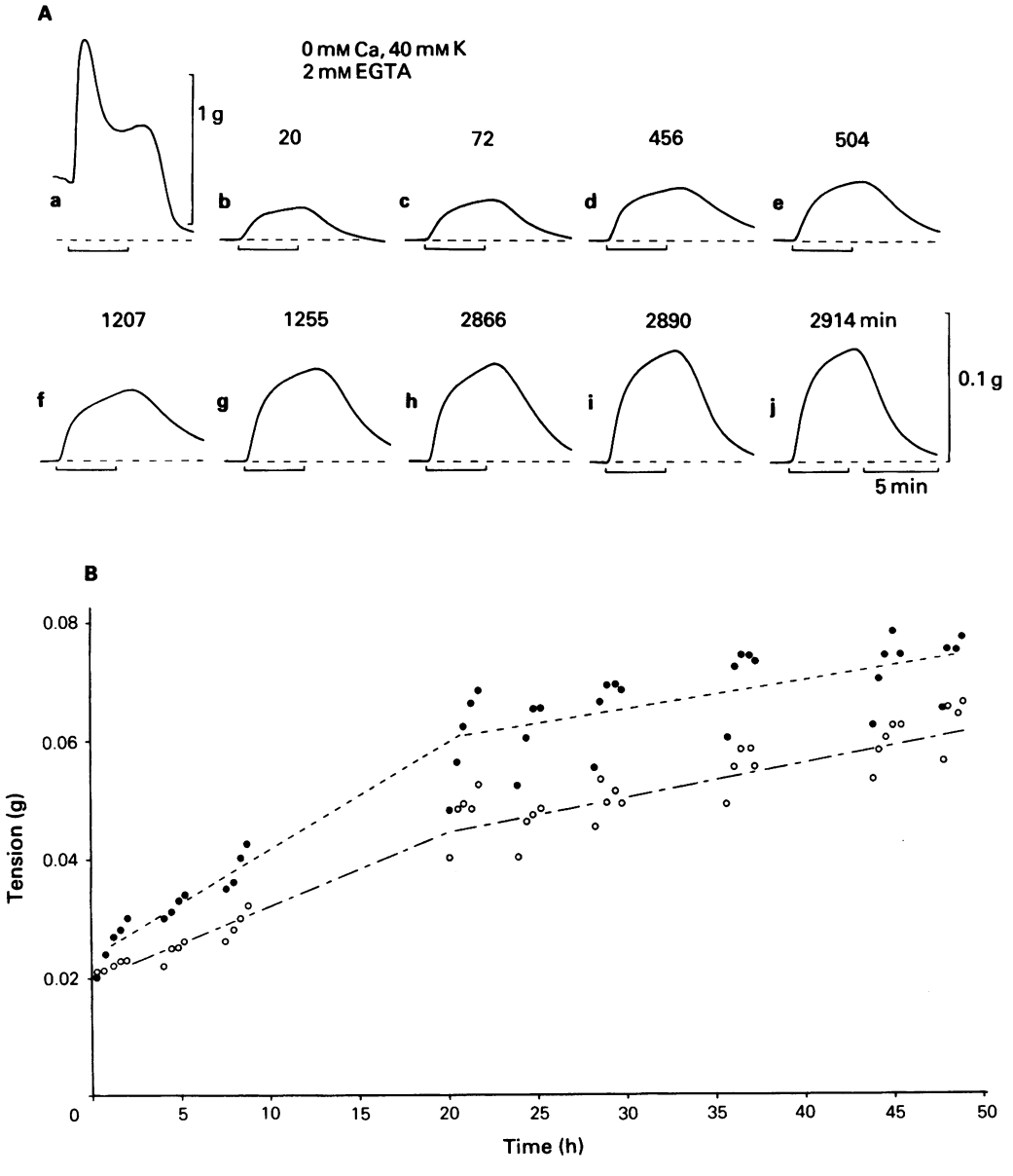

Figure 2 (A) Responses to carbachol (30 μM) during prolonged exposure to Ca-free solution. After observing the carbachol response in normal solution (a), continuous superfusion with Ca-free solution was started. Time (in min) of carbachol application (horizontal bars) after Ca removal is indicated by the records. (B) Peak tension of carbachol (30 μM) responses plotted against the time of exposure to Ca-free solution. Responses in two different preparations are shown.

suppressed to a similar degree by W-7 at a high concentration (100 μM). On the other hand, other calmodulin antagonists (phenothiazine derivatives), such as trifluoperazine, selectively inhibit the Ca-induced contraction, while at a 10 μM they only weakly suppress the oxytocin response in Ca-free solution (Ashoori *et al.*, 1985b). In the present experiments on the guinea-pig stomach muscle, trifluoperazine

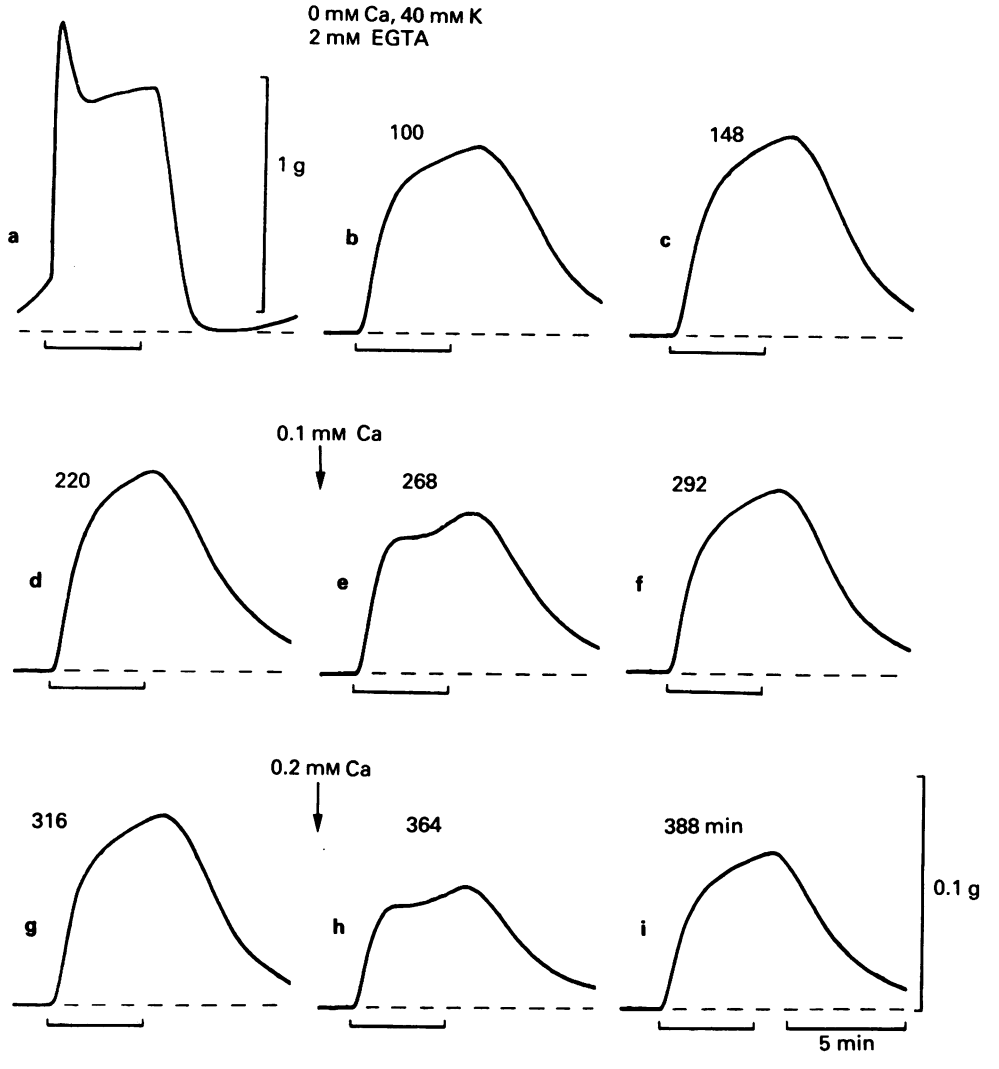

Figure 3 The antagonistic effects of a short treatment with Ca on the carbachol response. After observing the carbachol (30 μM) response in normal solution (a), Ca was removed and 100 min later carbachol was added for 4 min at 20 min intervals. The 1st (b), 3rd (c) and 6th response (d) are shown with the time in min in Ca-free solution. At 244 min, 0.1 mM Ca was added for 4 min and 20 min later carbachol application was resumed (e–g). Similarly, 0.2 mM Ca was applied for 4 min at 340 min in Ca-free solution and then carbachol administered at 20 min intervals (h, i). The application of carbachol is represented by the horizontal bars.

(10 μM) also preferentially blocked the Ca-induced contraction without a significant effect on the carbachol response in Ca-free solution.

Thus, from the results with the calmodulin antagonists which are phenothiazine derivatives, the contractions observed in Ca-free solution are likely to be mediated by some process independent of the Ca-calmodulin reaction. In the guinea-pig stomach, when 10 μM W-7 was used, it scarcely affected the carbachol response observed in Ca-free solution but markedly inhibited the Ca-dependent contraction. In previous experiments, on the rat vas deferens and myometrium, W-7 may have exerted some actions other than an anticalmodulin effect due to the high concentrations of W-7 used (more than 100 μM).

It has been shown in the vas deferens and

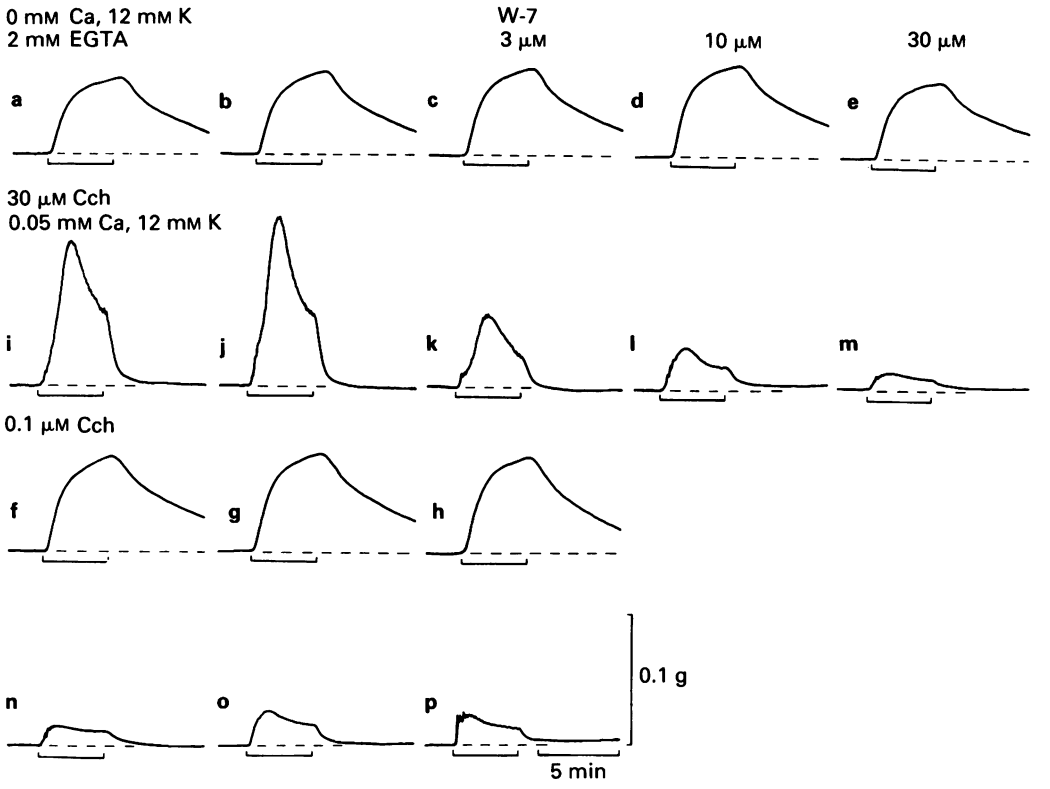

Figure 4 Effects of W-7 on carbachol (Cch) responses in the absence of Ca (a–h) and in the presence of 0.05 mM Ca (i–p). Carbachol concentration was 30 μM in Ca-free solution, while 0.1 μM was used in 0.05 mM Ca solution in order to produce responses of roughly comparable size in two different preparations. The responses were evoked at 20 min intervals. (a, b) and (i, j): the 2nd and the 4th responses after exposure to Ca-free and 0.05 mM Ca solution, respectively. W-7 was applied cumulatively from 3 to 30 μM (c–e, and k–m) 10 min before carbachol as indicated above the records. After observing (e) and (m), W-7 was washed out, and the subsequent responses are shown in (f–h) and (n–p), respectively.

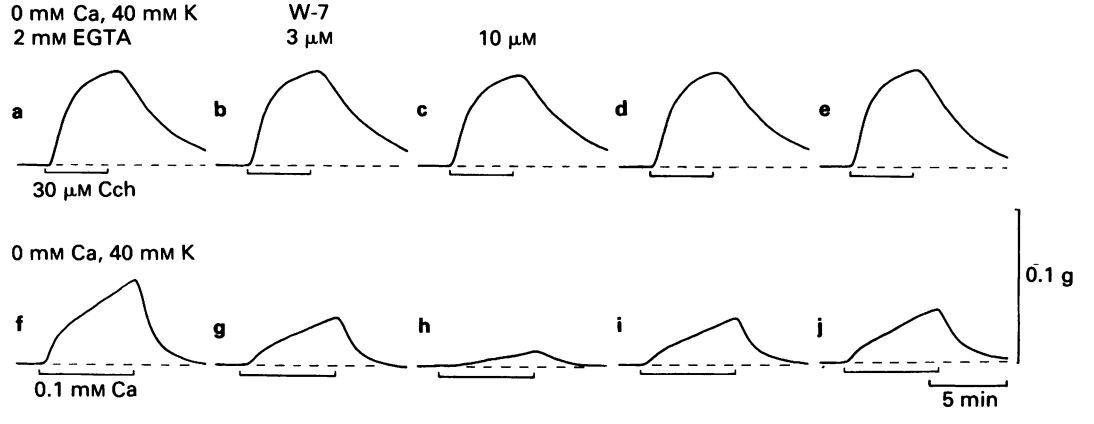

Figure 5 Effects of W-7 on the response to carbachol (Cch) in Ca-free solution (a–e) and on the contraction induced by the addition of Ca into this medium (f–j) in two different preparations. Carbachol (30 μM for 4 min) and Ca (0.1 mM for 6 min) were applied at 20 min intervals. W-7 was added cumulatively 10 min before carbachol (b, c) and Ca (g, h), respectively. Between (c) and (d), and similarly between (h) and (i), W-7 was washed out.

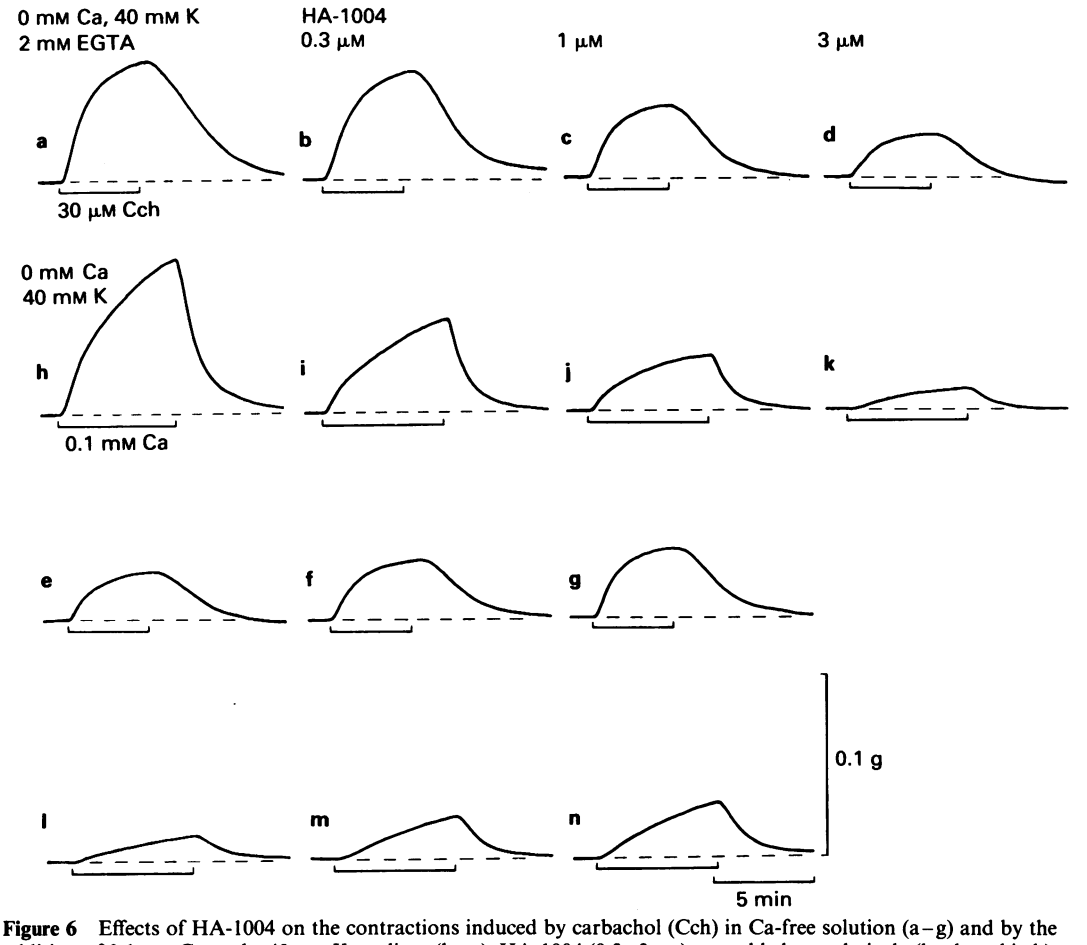

Figure 6 Effects of HA-1004 on the contractions induced by carbachol (Cch) in Ca-free solution (a–g) and by the addition of 0.1 mM Ca to the 40 mM K medium (h–n). HA-1004 (0.3–3 μM) was added cumulatively (b–d, and i–k). Traces (e–g) and (l–n) show recovery after washout in the absence and presence of Ca, respectively.

myometrium that HA-1004 preferentially inhibits the response in Ca-free solution (Ashoori *et al.*, 1985a). However, when the Ca concentration was reduced so as to produce a response of a size comparable to that induced by carbachol in Ca-free solution, the HA compound suppressed both the responses roughly in parallel to those in the stomach muscle.

HA-1004 (3 μM) has been shown to relax the rabbit aortic strip contracted by a Ca-ionophore, A 23187 and by phenylephrine in Ca-free solution, whereas the Ca-channel blockers, diltiazem (30 μM) and verapamil (30 μM) have no such effect. Furthermore, the guinea-pig atria and papillary muscle are not affected by HA-1004. These results strongly suggest that the HA compound is not interfering with Ca influx at the plasma membrane, but acting intracellularly. Thus, it is possible that HA-1004 blocks some intracellular process in common with the Ca-dependent and Ca-independent contractions. Since W-7 and HA-1004 are comparable in structure, it may be that W-7 also has a similar action to HA-1004, in addition to its anticalmodulin effect, when used at a high concentration. Low concentrations (3–10 μM) of W-7 and trifluoperazine block only the Ca-dependent contraction; they have no effect on the carbachol response in Ca-free solution. If the effect of calmodulin antagonists on these responses is due to blockade of the Ca-calmodulin interaction in myosin light chain kinase, the carbachol-induced contraction in Ca-free solution is likely to be mediated by a Ca-independent process. The slow increase in the size of the response during 50 h of perfusion with a Ca-free solution containing 2 mM EGTA is difficult to explain by the very effective recycling of intracellular Ca. It is more reasonable to assume that the response is mediated by some Ca-independent process. This idea can also be applied to the similar contraction in Ca-free solution observed in other smooth muscle tissues.

References

ASANO, T. & HIDAKA, H. (1984). Vasodilatory action of HA-1004 N-(2-guanidinoethyl)-5-isoquinolinesulfonamide, a novel calcium antagonist with no effect on cardiac function. *J. Pharmac. exp. Ther.*, **231,** 141–145.

ASANO, M., SUZUKI, Y. & HIDAKA, H. (1982). Effects of various calmodulin antagonists on contraction of rabbit aortic strips. *J. Pharmac. exp. Ther.*, **220,** 191–196.

ASHOORI, F., HIDAKA, H., TAKAI, A. & TOMITA, T. (1985a). Contraction of smooth muscle in Ca-free solution. *Jap. J. Smooth Muscle Res.*, **21,** 57–69.

ASHOORI, F., TAKAI, A. & TOMITA, T. (1985b). The response of non-pregnant myometrium to oxytocin in Ca-free solution. *Br. J. Pharmac.*, **84,** 175–183.

ASHOORI, F. & TOMITA, T. (1983). Mechanical response to noradrenaline in calcium-free solution in the rat vas deferens. *J. Physiol.*, **338,** 165–178.

CASTEELS, R., RAEYMAEYKERS, L., SUZUKI, H. & VAN ELDERE, J. (1981). Tension response and Ca-45 release in vascular smooth muscle incubated in Ca-free solution. *Pflügers Arch.*, **392,** 139–145.

COCKS, T.M., DILGER, P. & JENKINSON, D.H. (1981). The mechanism of the blockade by trifluoperazine of some actions of phenylephrine on liver and smooth muscle. *Biochem. Pharmac.*, **30,** 2873–2875.

DANIEL, E.E. (1984). Cellular calcium mobilization. *J. cardiovasc. Pharmac.*, **6** (Suppl. 4), S622–S629.

GOLENHOFEN, K., HOHNSBEIN, J., LAMMEL, E., LUKANOW, J. & MANDREK, K. (1982). Contractile tone of canine gastric fundus: Evidence for a chemical, non-electrical control. In *Motility of the Digestive Tract*, ed. M. Wienbeck. pp. 95–102. New York: Raven Press.

HARTSHORNE, D.J. & SIEMANKOWSKI, R.F. (1981). Regulation of smooth muscle contraction. *A. Rev. Physiol.*, **43,** 519–530.

HEASLIP, R.J. & RAHWAN, R.G. (1982). Evidence for the existence of two distinct pools of intracellular calcium in the rat aorta accessible to mobilization by norepinephrine. *J. Pharmac. exp. Ther.*, **221,** 7–13.

HIDAKA, H., ASANO, M., IWADARE, S., MATSUMOTO, I., TOTSUKA, T. & AOKI, N. (1978). A novel vascular relaxing agent, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide which affects vascular smooth muscle actomyosin. *J. Pharmac. exp. Ther.*, **207,** 8–15.

HIDAKA, H., YAMAKI, T., NAKA, M., TANAKA, T., HAYASHI, H. & KOBAYASHI, R. (1980). Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. *Mol. Pharmac.*, **17,** 66–72.

MIRONNEAU, C., MIRONNEAU, J. & SAVINEAU, J.P. (1984). Maintained contractions of rat uterine smooth muscle incubated in a Ca^{2+}-free solution. *Br. J. Pharmac.*, **82,** 735–743.

OHBA, M., SAKAMOTO, Y. & TOMITA, T. (1976). Spontaneous rhythmic activity of the smooth muscle of the guinea-pig stomach and effects of ionic environment. *INSERM*, **50,** 301–316.

SAKAI, K., HIGUCHI, K., YAMAGUCHI, T. & UCHIDA, M. (1982). Oxytocin-induced Ca-free contraction of rat uterine smooth muscle: effects of preincubation with EGTA and drugs. *Gen. Pharmac.*, **13,** 393–400.

SAKAI, K. & UCHIDA, M. (1980). A calcium reversal phenomenon: differentiation of excitatory and inhibitory roles of Ca in uterine smooth muscle contraction. *Jap. J. Pharmac.*, **30,** 394–396.

SAKAI, K., YAMAGUCHI, T. & UCHIDA, M. (1981). Oxytocin-induced Ca-free contraction of rat uterine smooth muscle: effects of divalent cations and drugs. *Arch. int. Pharmacodyn.*, **250,** 40–54.

TAKAYANAGI, I. (1964). Phenothiazine derivatives. Relationship between peripheral and central actions. *Arzneimittel-Forsch.*, **14,** 694–698.

TOMITA, T., ASHOORI, F. & TAKAI, A. (1985). Contraction in vas deferens and myometrium during prolonged exposure to calcium free solution. In *Calmodulin Antagonists and Cellular Physiology*, ed. Hidaka, H. & Hartshorne, D.J. pp. 363–378. New York: Academic Press.

(Received July 9, 1985.
Revised September 10, 1985.
Accepted September 24, 1985.)