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Ketamine-induced relaxation in intact and skinned smooth muscles of the rabbit ear artery

[1]Y. Kanmura, *J. Yoshitake & R. Casteels

Laboratorium voor Fysiologie, Campus Gasthuisberg, K.U.L., B-3000, Leuven, Belgium and *Department of Anaesthesiology, Faculty of Medicine, Kyushu University, Fukuoka, 812, Japan

1 The effects of ketamine, an intravenous anaesthetic, on the rabbit ear artery were investigated by measuring the tension in intact and saponin-treated skinned smooth-muscle fibres.

2 Ketamine dose-dependently inhibited contractions of intact smooth-muscle fibres induced by high K^+ solution and by noradrenaline (NA) or histamine in Krebs solution. This drug similarly attenuated both phasic and tonic contractions induced by high K^+ solution.

3 Ketamine also inhibited NA- or histamine-induced contractions in Ca^{2+}-free solution containing 2 mM EGTA, but it did not affect the caffeine-induced contraction in this solution.

4 Because the pCa-tension relationship of saponin-treated skinned smooth-muscle fibres was not affected, it can be proposed that ketamine does not have an effect on the contractile proteins.

5 In the presence of 5 mM NaN_3, 20 μM inositol 1,4,5-trisphosphate ($InsP_3$) or 25 mM caffeine produced a contraction in skinned smooth-muscle fibres after accumulation of Ca^{2+} by intracellular stores. Analysis of the $InsP_3$- or caffeine-induced contractions indicates that ketamine does not have an effect on the Ca^{2+} accumulation into and Ca^{2+} release from the intracellular stores.

6 These results indicate that the relaxant effects produced by ketamine in the rabbit ear artery are not likely to be due to an intracellular action. The inhibitory effects of ketamine could be caused by a decrease of the Ca^{2+} influx through the plasma membrane or interference with the process of signal transduction between receptors on the plasma membrane and intracellular stores.

Introduction

Ketamine, an intravenous anaesthetic, has various effects on the cardiovascular system. Amongst them, ketamine is known for its hypertensive properties (Hug, 1979). However, this drug has also been demonstrated to produce biphasic blood-pressure responses (initially hypotensive, later hypertensive) in man, rats and dogs (Domino *et al.*, 1965; Virtue *et al.*, 1967; Dowdy & Kaya, 1968) or profound hypotension in certain species including rabbits (Clanachan *et al.*, 1976). The mechanisms of the hypertensive actions are thought to be via the central nervous system, baroreceptors and/or vascular sympathetic neurotransmission (Fukuda *et al.*, 1986). However, the hypotension is thought to be caused by a direct effect on vascular smooth muscles.

The contraction and relaxation of vascular smooth muscles depends on changes of the intracellular free Ca^{2+} concentration. The cytoplasmic Ca^{2+} is increased by an influx of external Ca^{2+} through voltage-dependent or receptor-operated channels and by the release of this ion from intracellular stores, mainly from the sarcoplasmic reticulum (SR) (Kuriyama *et al.*, 1982). The inhibitory effects of ketamine on vascular contraction have been shown to be due to an interference with transmembrane Ca^{2+} influx (Altura *et al.*, 1980; Fukuda *et al.*, 1983), but other mechanisms, such as effects on contractile proteins and on intracellular stores, were not excluded.

The present studies were therefore undertaken to investigate further the effects of ketamine on vascular smooth muscles using intact and skinned smooth-muscle fibres of the rabbit ear artery.

Methods

Preparation

Rabbits of either sex, weighing 2 to 3 kg were stunned and exsanguinated. The central ear artery was dissected out of the ear and cleaned of its periarterial connective tissues under a binocular microscope while being kept in warmed and oxgenated Krebs solution. Thin circular strips (0.3–0.5 mm in

[1] Author for correspondence at Department of Anaesthesiology, Faculty of Medicine, Kyushu University, Fukuoka, 812, Japan.

length, 0.05–0.08 mm in width and 0.02–0.03 mm in thickness) were prepared under a binocular microscope by use of fine forceps and small knives made from pieces of razor blades. In all experiments, the endothelium was carefully removed with the small knives as indicated previously (Itoh *et al.*, 1985).

Recording of mechanical activity

Mechanical activity of intact and skinned smooth-muscle fibres was measured by attaching a circular strip to a strain gauge (U-gauge, Shinko, Tokyo, Japan). Each end of the strip was knotted by a fine silk fibre which was then fixed to a piece (about 1 mm × 1 mm) of Scotch double-sided adhesive tape (3M Co., St. Paul, MN, U.S.A.). One tape was fixed to a side of the chamber and the other tape to the strain gauge. The chamber had a volume of 0.9 ml. The solutions were rapidly injected by a syringe from one end of the chamber and simultaneously siphoned off from the other end. The tissue was not superfused constantly but remained exposed to the solution in the chamber until the next solution was injected (Kanmura *et al.*, 1988).

Chemically skinned muscle fibres were obtained by exposing the fibres to saponin (35 $\mu g\,ml^{-1}$) in relaxing solution for 20 min (Itoh *et al.*, 1981). The Ca^{2+}-induced contractions were recorded at various concentrations of free Ca^{2+} buffered by ethyleneglycol *bis*-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). The intracellular Ca^{2+} stores were loaded by exposing the skinned fibres to a solution containing 0.6 μM free Ca^{2+} (buffered with 4 mM EGTA). The release of Ca^{2+} from these stores was then examined by recording the tension induced by inositol 1,4,5-trisphosphate ($InsP_3$) or by caffeine in Ca^{2+}-free solution supplemented with 0.1 mM EGTA. NaN_3 5 mM was present throughout the experiments in order to prevent Ca^{2+} accumulation by the mitochondria. To prevent the deterioration of the function of the contractile proteins, 0.1 μM calmodulin was present throughout the experiments. The relaxation of Ca^{2+}-induced contraction was accelerated by supplementing the relaxing solution needed for washing away the Ca^{2+}-containing solution with 6 mM K phosphate (Itoh *et al.*, 1986). The temperature was kept at 25°C (Iino, 1981; Itoh *et al.*, 1981) because skinned fibres deteriorate more rapidly at higher temperatures.

Solutions

The composition of Krebs solution was as follows (mM): NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11.5 and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) 11.6. It was bubbled with O_2 and the pH was adjusted to 7.3 with NaOH. High K^+ solution was prepared by replacing NaCl with KCl. In Ca^{2+}-free solutions, $CaCl_2$ was replaced with $MgCl_2$ and 2 mM EGTA was added. The relaxing solution used for skinned fibres contained (mM): K methanesulphonate (KMs) 114, Tris maleate 20, $Mg(Ms)_2$ 5.1, adenosine 5'-triphosphate (ATP) 5.2 (10.4 mM Na^+ as Na_2-ATP) and EGTA 4. The free Ca^{2+} concentration was changed by adding appropriate amounts of $Ca(Ms)_2$ to EGTA. A value of $10^6\,M^{-1}$ was used for the apparent binding constant of Ca^{2+} to EGTA at pH 6.8 and 25°C (Itoh *et al.*, 1981). To observe the effects of ketamine on Ca^{2+} release from intracellular stores, the concentration of EGTA was reduced to 0.1 mM.

Drugs

Chemicals used were: ketamine hydrochloride from Parke Davis (Bornem, Belgium), noradrenaline (NA) and EGTA from Fluka (Switzerland), ATP from Boehringer (Manheim, F.R.G.). $InsP_3$ from Sigma Chemical Co. (St. Louis, MO, U.S.A.), saponin from ICN Pharmac. Inc. (Cleveland, OH, U.S.A.), caffeine from BDH Chemicals (Poole, England), HEPES and histamine from Merck (Darmstadt, F.R.G.). Calmodulin was prepared from bovine brain according to Gopalakrishna & Anderson (1982).

Results

Effects of ketamine on mechanical responses induced by high K^+ or agonists in intact smooth-muscle fibres

In intact muscle strips of the rabbit ear artery, high K^+ solution evoked a rapid phasic and sustained tonic contraction. Ketamine (100 μM and 300 μM) inhibited these phasic and tonic responses to the same extent (Figure 1Aa). Figure 1B shows the effects of various concentrations of ketamine on the phasic and tonic responses of the high K^+-induced contraction. The IC_{50} values measured for the action of ketamine on the phasic and tonic responses evoked by 138 mM K^+ were 176 μM and 145 μM, respectively. In this tissue, 10 μM NA produced the maximal contraction and the amplitude of contraction evoked by 138 mM K^+ solution was 0.45 ± 0.05 times that of the 10 μM NA-induced contraction ($n = 5$). Fifty nM NA, 500 nM histamine and 138 mM K^+ produced approximately the same amplitude of contraction, and we investigated the effects of ketamine on these similar contractions. It was observed that NA- or histamine-induced contractions in Krebs solution were also inhibited by ketamine (Figure 1Ab,c). Figure 1C shows the dose-dependent

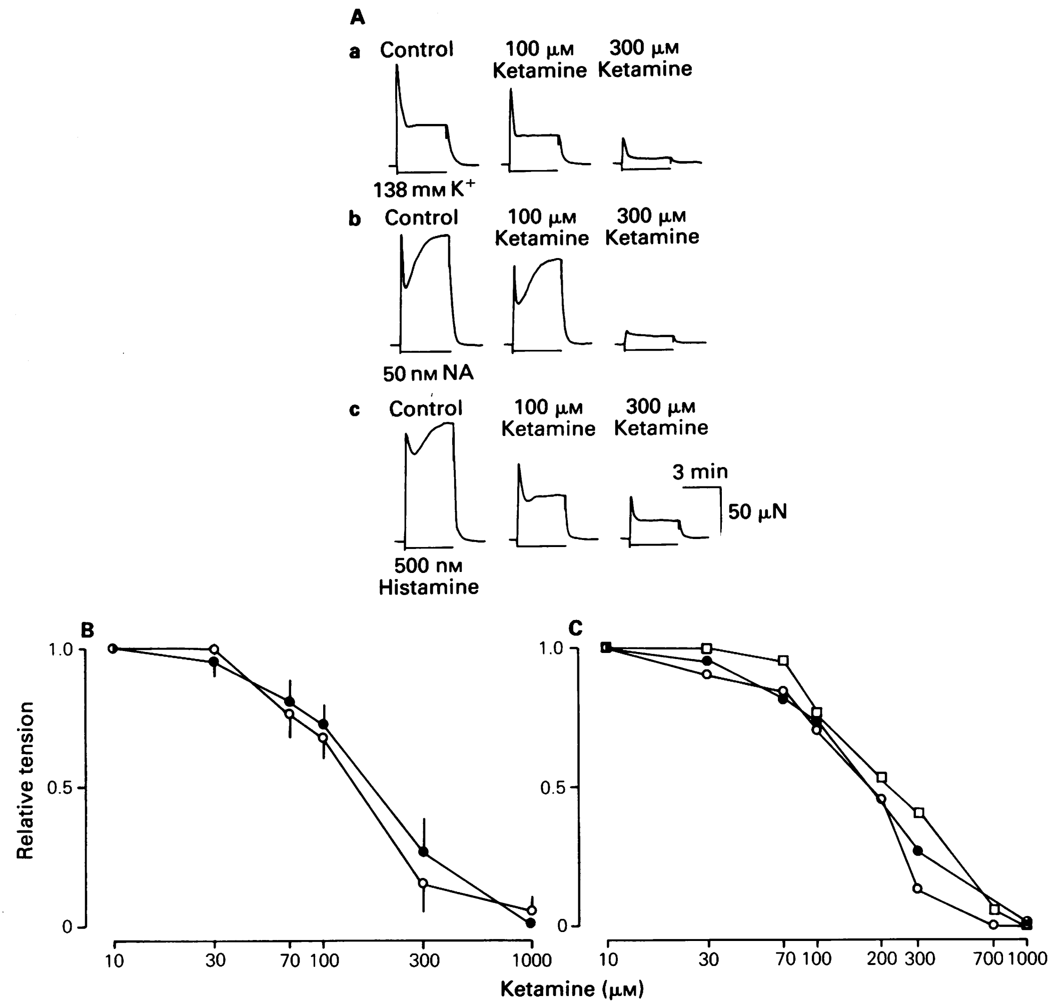

Figure 1 Effects of ketamine on the contraction evoked by 138 mM K^+, 50 nM noradrenaline (NA) or 500 nM histamine in intact smooth-muscle fibres of the rabbit ear artery. (A) Typical example of effects of 100 and 300 μM ketamine on the contraction evoked by K^+ (Aa), NA (Ab) or histamine (Ac). (B) Concentration-response curves of ketamine on the phasic (●) and tonic (○) responses of the 138 mM K^+-induced contraction. The amplitude of phasic or tonic responses evoked in the absence of ketamine was normalized as 1.0. Vertical bars indicate s.d., $n = 4$. Curves were fitted by eye. (C) Concentration-response curves of ketamine on contraction evoked by 138 mM K^+ (●), 50 nM NA (○) or 500 nM histamine (□). Amplitude of the phasic contraction evoked by each stimulant in the absence of ketamine was normalized as 1.0. Vertical bars which represent s.d. were omitted to make curves clear, $n = 4$. Curves were fitted by eye.

action of ketamine on the phasic response of contractions evoked by 138 mM K^+, 50 nM NA or 500 nM histamine. The maximum amplitude of contraction evoked by each agent in the absence of ketamine was normalized to 1.0. The IC_{50} values for 138 mM K^+-, NA- and histamine-induced contractions were 176 μM, 170 μM and 200 μM, respectively.

Effects of ketamine on contractions of intact muscle fibres induced by NA and caffeine in Ca^{2+}-free solution

In the rabbit ear artery, in Ca^{2+}-free solution containing 2 mM EGTA, NA produces a contraction due to the release of Ca^{2+} from intracellular stores

(Casteels & Droogmans, 1981). Caffeine also produces a contraction due to the release of Ca^{2+} from intracellular stores that are closely related to or are the same as those on which NA acts. We further investigated the effects of ketamine on these agonist-induced contractions in Ca^{2+}-free solution.

After releasing the stored Ca^{2+} completely by repeated application of 10 mM caffeine in Ca^{2+}-free solution containing 2 mM EGTA, the tissues were exposed to 1.5 mM Ca^{2+} for 10 min. It is known that the intracellular Ca^{2+} stores are completely filled by this procedure (Casteels & Droogmans, 1981). Thereupon the tissues were superfused for 3 min with Ca^{2+}-free solution and 50 nM NA was then applied for 3 min. Ketamine was applied before and during the application of NA. Ketamine 300 μM significantly inhibited this NA-induced contraction (0.35 $\pm$ 0.05 times the control, $n = 4$), and at 1 mM ketamine this contraction was completely blocked (Figure 2A). Ketamine also inhibited the histamine-induced contraction in Ca^{2+}-free solution (data not shown). In contrast, ketamine had little effect on the contraction evoked by 10 mM caffeine (Figure 2B). Ketamine (up to 300 μM) applied only during the application of Ca^{2+} did not inhibit the subsequent contraction induced by NA, but 1 mM ketamine in Ca^{2+}-containing solution slightly inhibited the contraction induced by NA in Ca^{2+}-free solution (data not shown).

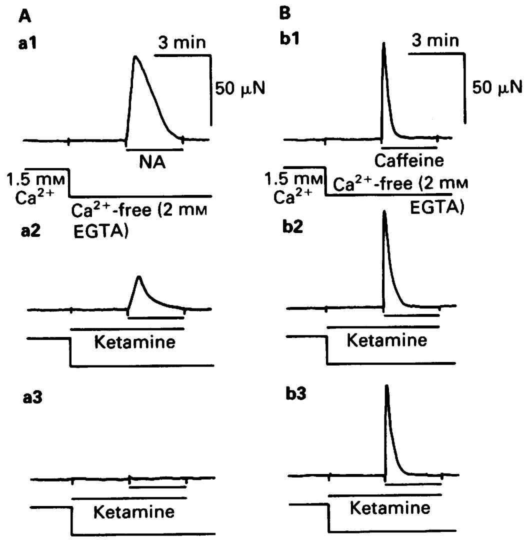

Figure 2 Effects of ketamine on noradrenaline (NA)- and caffeine-induced contraction in Ca^{2+}-free solution containing 2 mM EGTA. (A) After 1.5 mM Ca^{2+} was applied for 10 min, the smooth muscle fibres were washed with Ca^{2+}-free solution containing 2 mM EGTA and subsequently 50 nM NA was applied. (B) 10 mM caffeine was applied in Ca^{2+}-free solution instead of NA in (A). (a1 and b1) Control; (a2 and b2) 300 μM ketamine was applied before and during application of stimulants in Ca^{2+}-free solution; (a3 and b3) 1 mM ketamine was applied. These results are typical of 4 experiments.

To see whether this inhibitory effect of ketamine is due to the inhibition of Ca^{2+} release from intracellular stores or to an enhancement of the Ca^{2+} extrusion through the plasma membrane, the effects of ketamine were investigated on contractions induced in Ca^{2+}-free solution by successive applications of NA and caffeine. After eliciting a contraction to 50 nM NA, a small contraction could still be induced by subsequent application of 10 mM caffeine. However, if a contraction was first induced by 10 mM caffeine, neither 50 nM NA nor 10 mM caffeine could induce a further contraction. Treatment with 300 μM ketamine significantly inhibited the NA-induced contraction (0.43 $\pm$ 0.08 times the control, $n = 4$), but enhanced the caffeine-induced contraction (1.66 $\pm$ 0.32 times the control, $n = 4$) (Figure 3). This result indicates that ketamine probably inhibits the NA-induced Ca^{2+}-release from stores rather than enhancing the Ca^{2+} extrusion through the plasma membrane.

Effects of ketamine on the contractile proteins of skinned smooth-muscle fibres

The minimum concentration of Ca^{2+} required to produce a contraction in saponin-treated skinned muscle fibres of the rabbit ear artery was 0.3 μM and

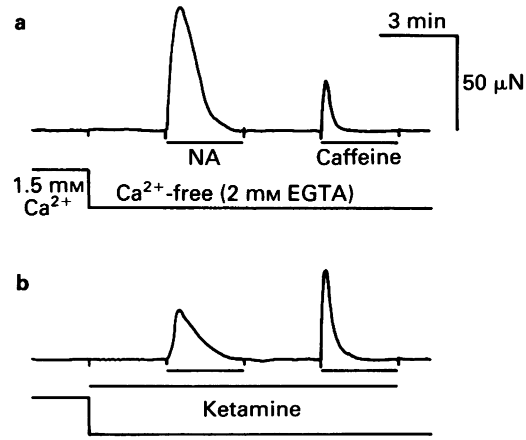

Figure 3 Effects of ketamine on successive noradrenaline (NA)- and caffeine-induced contractions in Ca^{2+}-free solution containing 2 mM EGTA. After 1.5 mM Ca^{2+} was applied for 10 min, 50 nM NA and 10 mM caffeine were applied successively for 3 min with 3 min interval. (a) Control; (b) 300 μM ketamine was applied in Ca^{2+}-free solution. These results were typical of 4 experiments.

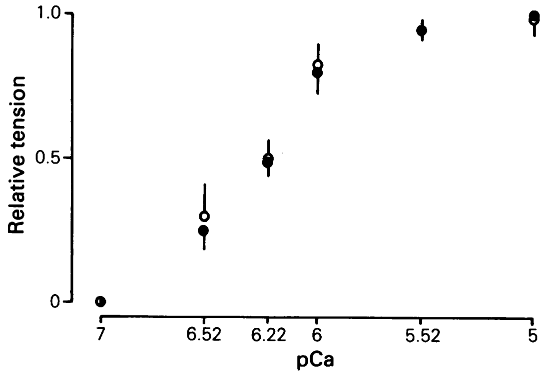

Figure 4 Effects of 1 mM ketamine (○) on the pCa-tension relation in saponin-treated skinned smooth-muscle fibres (●, control). The amplitude of contraction evoked by 10 μM Ca^{2+} in the absence of ketamine was normalized to 1.0. Vertical bars indicate s.d. ($n = 3$ to 5).

the maximum amplitude of contraction was reached in 10 μM Ca^{2+}. After the Ca^{2+}-induced contraction had reached a steady state, 1 mM ketamine was applied. Ketamine did not exert any effect on these Ca^{2+}-induced contractions (Figure 4).

Effects of ketamine on caffeine- and $InsP_3$-induced contractions in skinned smooth-muscle fibres

To study the effects of ketamine on the release of Ca^{2+} from intracellular stores directly, its effects on caffeine- and $InsP_3$-induced contractions were observed in saponin-treated skinned smooth-muscle fibres. After skinning, 0.6 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min, the tissue was rinsed with relaxing solution containing 0.1 mM EGTA and 25 mM caffeine was added (Figure 5a). On the basis of the findings of Endo (1977) and of Itoh *et al.* (1981), it is accepted that high concentrations of caffeine release most of the Ca^{2+} present in intracellular stores, and that the amplitude of caffeine-induced contraction can be used as an indication of the amount of Ca^{2+} present in the stores.

When 1 mM ketamine was added to the relaxing solution containing 0.1 mM EGTA before and during application of 25 mM caffeine, the amplitude of this caffeine-induced contraction remained the same as that of the control (Figure 5b). When 1 mM ketamine was added to the solution containing 0.6 μM Ca^{2+} which was used to load the stores, the subsequent caffeine-induced contraction was not affected (Figure 5c).

$InsP_3$, the hydrolytic product of phosphatidylinositol 4,5-bisphosphate (PIP_2), is thought to act as a second messenger in vascular smooth muscles, by releasing Ca^{2+} from intracellular stores

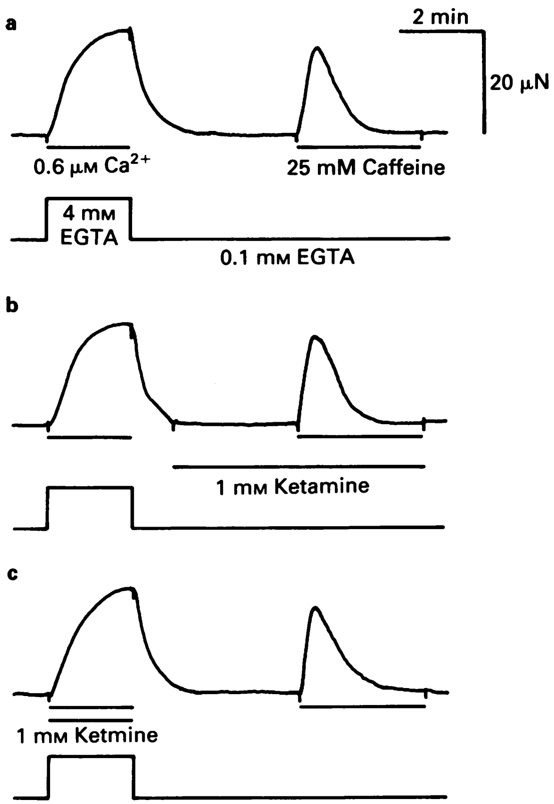

Figure 5 Effects of ketamine on caffeine-induced contraction in saponin-treated skinned smooth-muscle fibres. Caffeine 25 mM was applied after a 2 min exposure to a solution with 0.6 μM Ca^{2+} and a 4 min wash in the relaxing solution with 0.1 mM EGTA. (a) Control; (b) 1 mM ketamine was added 3 min before the application of caffeine; (c) 1 mM ketamine was applied during application of 0.6 μM Ca^{2+}. These results were typical of 4 experiments.

(Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986). We investigated the effects of ketamine on this $InsP_3$-induced contraction in skinned smooth-muscle fibres. After accumulation of Ca^{2+} in the stores, application of 20 μM $InsP_3$ for 3 min elicited a transient contraction (Figure 6a). Treatment with 1 mM ketamine after or during Ca^{2+} loading did not inhibit the $InsP_3$-induced contractions (Figure 6b,c). These results suggest that ketamine does not act on the final step of the mechanism responsible for the release of Ca^{2+} by agonists.

Discussion

Ketamine is known for its hypertensive properties (Hug, 1979), and this effect has been ascribed to its

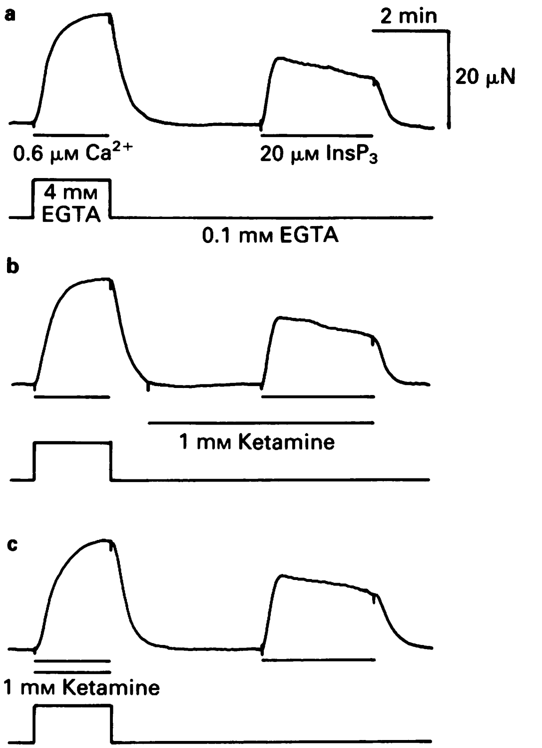

Figure 6 Effects of ketamine on inositol 1,4,5-trisphosphate ($InsP_3$)-induced contraction of saponin-treated skinned smooth-muscle fibres. $InsP_3$ 20 μM was used instead of 25 mM caffeine used in Figure 5. Other procedures were the same as in Figure 5.

action on the central nervous system (Wong & Jenkins, 1974), on the baroreceptors (Dowdy & Kaya, 1968) or on vascular sympathetic neuromuscular junctions (Fukuda *et al.*, 1986). However, *in vitro*, this drug has been shown to inhibit contractions of vascular smooth muscles (Altura *et al.*, 1980; Fukuda *et al.*, 1983) and the mechanism of this inhibitory action has been thought to be due to its interference with transmembrane influx of Ca^{2+} in a way similar to that of Ca antagonists. However, the present results indicate that ketamine possesses different characteristics from Ca antagonists.

In vascular tissues Ca antagonists inhibit preferentially tonic responses rather than phasic responses (Itoh *et al.*, 1984). In contrast, the inhibitory effects of ketamine on contraction induced by high K^+ were similar for both the phasic and the tonic responses. Moreover, ketamine inhibited agonist-induced contractions in the same manner as contractions induced by high K^+ (Figure 1C). In the rabbit ear artery, NA and histamine can cause contractions without modifying the membrane potential (Droogmans *et al.*, 1977). It is therefore unlikely that the voltage-dependent Ca^{2+} influx plays an important role in these contractions. From these results, we suspect that the inhibitory effects of ketamine are not limited to the voltage-dependent Ca^{2+} influx.

Agonists induce contractions of the rabbit ear artery in Ca^{2+}-free solution by releasing Ca^{2+} from intracellular stores, mainly from sarcoplasmic reticulum. Ketamine inhibits agonist-induced contractions in Ca^{2+}-free solution, but not the contractions induced by caffeine. This difference could be due to the fact that caffeine acts directly on intracellular stores. It can therefore be proposed that ketamine blocks some step(s) between receptor occupancy on the plasma membrane and the Ca^{2+} release from intracellular stores.

The mechanism responsible for Ca^{2+} release from intracellular stores by agonists is thought to be due to $InsP_3$, one of the hydrolytic products of PIP_2. Receptor occupancy by agonists induces the activation of phosphodiesterase (phospholipase C), which causes hydrolysis of PIP_2. Guanine nucleotide binding proteins (G-proteins) are assumed to be involved in this process.

It is possible that ketamine could block the receptor occupation by agonists, but because it exerts its inhibitory action on many different kinds of agonist-induced contractions (Altura *et al.*, 1980), this hypothesis is unlikely. Because ketamine penetrates rapidly into cells (Cohen *et al.*, 1973), an intracellular action of ketamine has been suggested (Altura *et al.*, 1980). However, intracellular application of ketamine in skinned smooth-muscle fibres did not reveal any effect on contractile proteins or on $InsP_3$- or caffeine-induced Ca^{2+} release from intracellular Ca^{2+} stores. It is therefore possible that ketamine could interfere with the functions of G-proteins and/or phosphodiesterase.

Recently, hydrolysis of PIP_2 has been shown to be induced not only by agonists but also by high K^+ solutions in guinea-pig ileum smooth muscles (Best & Bolton, 1986; Sasaguri & Watson, 1988). Part of the inhibition of ketamine of high K^+-induced contractions could therefore be due to an attenuated activity of G-proteins and/or phosphodiesterase. Further investigations about the effects of ketamine on the hydrolysis of PIP_2 by agonists or high K^+ solution are needed.

In conclusion, ketamine inhibits high K^+- and agonist-induced contractions in the presence of Ca^{2+} and agonist-induced contractions in Ca^{2+}-free solutions. Ketamine does not exert an effect on contractile proteins or on intracellular Ca^{2+} stores in skinned smooth-muscle fibres. The main site of the inhibitory effects of ketamine may be the process of hydrolysis of PIP_2.

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