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The mechanisms of α_2-adrenoceptor agonist-induced contraction in longitudinal muscle of the porcine uterus

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Abstract

The aim of the present study was to clarify the cellular mechanisms underlying the α_2-adrenoceptor-mediated contraction of porcine myometrium (nonvascular smooth muscle). Acetylcholine (3 nM–1 μM), clonidine (1 nM–10 μM) and 5-bromo-*N*-[2-imidazolin-2-yl]-6-quinoxalinamine (UK14304) (1 nM–10 μM) in Krebs solution caused a concentration-dependent contraction in the longitudinal muscles of the porcine uterus with similar EC_{50} values and maximum responses. A lowered external Ca^{2+} concentration and verapamil (10 nM–10 μM) decreased the contractile response to clonidine and UK14304 more markedly than the response to acetylcholine. However, in Kumagai solution, neither clonidine nor UK14304 caused contractile responses, but acetylcholine remained effective. The effects of α_2-adrenoceptor agonists on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and smooth muscle force were measured simultaneously using fura-PE3-loaded muscle preparations. Clonidine and UK14304 caused increases in $[Ca^{2+}]_i$ and force of the longitudinal muscle. The increases in $[Ca^{2+}]_i$ and muscle force were markedly inhibited by verapamil and in Ca^{2+}-free solution (EGTA, 1 mM). In the absence of external Ca^{2+}, clonidine caused only a small increase in $[Ca^{2+}]_i$ in Ca^{2+}-loaded preparations compared with those increases caused by carbachol, histamine, and oxytocin. Ca^{2+} (2.5 mM) caused increases in $[Ca^{2+}]_i$ and force of the longitudinal muscles in a Ca^{2+}-free high K^+ solution. Clonidine concentration dependently potentiated the Ca^{2+}-induced contraction without significantly changing the increase in $[Ca^{2+}]_i$, and this potentiation was inhibited by yohimbine. These results suggested that clonidine increases the Ca^{2+} sensitivity of the contractile elements through activation of α_2-adrenoceptors. During the development of the contractile response to clonidine (1 μM, 0–5 min), tissue cyclic AMP levels did not change significantly. In vitro treatment with pertussis toxin (1 μg/ml for 2 h) significantly decreased the contraction induced by clonidine without affecting the responses to carbachol and high K^+. The present results indicate that in porcine myometrium, α_2-adrenoceptor stimulation caused contraction of the longitudinal muscles by mechanisms largely dependent on the influx of extracellular Ca^{2+}, probably through voltage-dependent Ca^{2+} channels (VDCCs), and that the potentiation of the Ca^{2+} sensitivity of the contractile elements is another mechanism of the contractile responses. These actions involve a pertussis-toxin-sensitive G protein (probably G_i type) in the signal transduction pathway.

Keywords: Myometrium (porcine); α_2-Adrenoceptor; Clonidine; Ca^{2+}, intracellular; cAMP; Ca^{2+} sensitivity

1. Introduction

Adrenergic nerves (sympathetic innervation) regulate the motility of the uterus of several mammals (pig, cow, guinea-pig, cat, rat, and human) through activation of α(excitatory)- and β(inhibitory)-adrenoceptors (Digges, 1982; Bülbring and Tomita, 1987; Wray, 1993; Taneike et al., 1995, 1999). β-Adrenoceptors have been subdivided into β_1 and β_2 subtypes and α-adrenoceptors into α_1 and α_2 subtypes on the basis of responsiveness to various adrenoceptor agonists and antagonists and of the binding characteristics of selective radioligands. In most species, the β-adrenoceptors involved in uterine inhibition are the β_2-subtype (Johansson et al., 1980; Lefkowitz et al., 1983). However, there are considerable species differences in the α-adrenoceptor subtype involved in the catecholamine-induced response. α_1-Adrenoceptors are considered to be responsive to the excitatory response to catecholamines in the rabbit (Hoffman et al., 1981), rat (Kaulenas et al., 1991) and guinea-pig myometrium (Haynes and Pennefather, 1993). On the other hand, the involvement of α_2-adrenoceptors has been reported in the ovine (Marnet et

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al., 1987), bovine (Ko et al., 1990; Taneike et al., 1999) and porcine myometrium (Taneike et al., 1995).

At present, it is known that α_1-adrenoceptors are coupled to the phospholipase C-inositol 1,4,5-trisphosphate (IP_3)-diacylglycerol pathway and that α_1-adrenoceptor agonists contract the vascular smooth muscles by causing the release of stored intracellular Ca^{2+} and/or an influx of extracellular Ca^{2+} through Ca^{2+} channels. In addition, diacylglycerol causes the activation of protein kinase C (Ruffolo et al., 1991). In contrast, the cellular mechanisms underlying the contractile action of α_2-adrenoceptor activation are not well understood. α_2-Adrenoceptors are coupled to a class of heterotrimeric GTP-binding proteins (G-proteins), known as G_i, and activation of the receptor inhibits adenylate cyclase activity (Ruffolo et al., 1991). However, there is little evidence to suggest that inhibition of the basal generation of cyclic AMP causes an increase in smooth muscle contraction (Wright et al., 1995), and it seems likely that some other mechanisms are involved in α_2-adrenoceptor-induced contractions. α_2-Adrenoceptor activation is linked to the stimulation of phospholipase A_2 in some cell types (Fraser, 1991; Nebigil and Malik, 1992), and arachidonic acid released from phospholipids by phospholipase A_2 has been reported to induce contraction in vascular smooth muscle (Jancar et al., 1987) and has been proposed as a modulator of the Ca^{2+} sensitivity of the contractile elements through the inhibition of myosin light chain phosphatase (Gong et al., 1992; Karaki et al., 1997). α_2-Adrenoceptor mediated slow depolarization (saphenous vein; Cheung, 1985) and the induction of dihydropyridine Ca^{2+} channel antagonist-sensitive contraction by α_2-adrenoceptor agonists (subcutaneous resistance arteries; Parkinson and Hughes, 1995) have also been reported. These results suggest the involvement of voltage-dependent Ca^{2+} channels (VDCCs) in the α_2-adrenoceptor-mediated contraction of the vascular smooth muscles.

In our previous study, we demonstrated that α_2-adrenoceptors mediate contractile responses induced by clonidine and norepinephrine in the porcine myometrium (Taneike et al., 1995), but the mechanisms of the contraction remained to be clarified. While investigating the effects of clonidine and acetylcholine in Kumagai solution (a low-temperature and low-Ca^{2+} nutrient solution used to decrease spontaneous contractile activity of the myometrium; Kumagai et al., 1952), we found that acetylcholine, but not clonidine, causes contraction of the longitudinal muscles of the porcine myometrium. These results suggest a high extracellular Ca^{2+} dependence of α_2-adrenoceptor-mediated contraction. Because most studies on the mechanisms of α_2-adrenoceptor-mediated contraction have used vascular smooth muscle preparations, it was considered interesting to analyze the mechanisms of the α_2-adrenoceptor-mediated contraction in nonvascular smooth muscle and to compare them with those in vascular smooth muscles. The present study was designed to clarify the cellular mechanisms of α_2-adrenoceptor agonist (clonidine and 5-bromo-*N*-[2-imidazolin-2-yl]-6-quinoxalinamine (UK14304))-induced contraction in the porcine myometrium. For this purpose, we used isolated myometrial strips loaded with a Ca^{2+} indicator, fura-PE3, and simultaneously measured smooth muscle force and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The effect of clonidine on tissue cyclic AMP production was also examined in order to investigate the relationships between contractile response and tissue cyclic AMP level.

2. Materials and methods

2.1. Tissue preparations

Fresh uteri, with the ovaries intact, from 120 sexually mature, crossbred virgin gilts (about 6 months old) were provided by a local abattoir and were used in experiments on the day of slaughter. According to gross examination of the follicle size and appearance of the corpora lutea, the uteri of gilts were judged as proestrus (McDonald, 1975). We used longitudinal muscle in the present study, because only the longitudinal muscle was sensitive to clonidine and norepinephrine (Taneike et al., 1995). The longitudinal muscle was peeled out gently from the antimesometrial coat of the adtubal region (10 cm distal from the apex) in either the left or right cornu. Smooth muscle strips (10×1 mm) were isolated and suspended vertically in an organ bath (5 ml) containing 37°C Krebs solution (in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.5) or 28°C Kumagai solution (in mM: NaCl 150.6, KCl 5.4, $CaCl_2$ 0.4, $MgCl_2$ 0.2, Na_2HPO_4 0.6, KH_2PO_4 0.1, $NaHCO_3$ 4.76, and glucose 2.8) gassed with 95% O_2 + 5% CO_2 (pH, 7.4). In the Kumagai solution, at a low temperature and low concentration of Ca^{2+}, the spontaneous contractile activity of the myometrium was completely abolished. A force-displacement transducer (SB-1T, Nihon Kohden), equipped with a pen-writing recorder (Recticorder, Nihon Kohden) was used to measure the mechanical activity of the preparations. Each strip was loaded at an initial tension of 0.2 g and was allowed to equilibrate for 60 min.

After steady tonus of the longitudinal muscle preparations (observation of spontaneous contraction in Krebs solution) was obtained, contractile agents were applied cumulatively (at 3-min intervals) in the organ bath, and concentration–response curves were made in both Krebs and Kumagai solutions on the basis of the increase in smooth muscle tonus (amplitude of the contraction was normalized using 100 μM acetylcholine-induced response and expressed as a percentage). The EC_{50} value (concentration of agents that caused 50% of 100 μM acetylcholine-induced contraction) was determined by least squares nonlinear regression analysis of the concentration–response curves.

2.2. Simultaneous measurement of muscle force and $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ of the porcine myometrial strips was measured as previously reported by Kitazawa et al. (1999a) with the fluorescent Ca^{2+} indicator, fura-PE3. In brief, small longitudinal muscle strips (5 mm long, 1 mm wide) were loaded with the acetoxymethyl ester of fura-PE3 (fura-PE3/AM, 8 μM, Wako) for 12 h at room temperature in the dark. The incubation medium also contained a noncytotoxic detergent, cremophor EL (0.04%, Nacalai Tesque), to increase the solubility of fura-PE3/AM. After loading, each muscle strip was washed with HEPES-buffer (in mM: NaCl 136.9, KCl 5.4, $MgCl_2$ 1.0, HEPES 20, $CaCl_2$ 2.5, glucose 5.5, and EDTA 0.01; neutralized with NaOH to pH 7.4) at 37°C for 20 min to remove uncleaved fura-PE3/AM, and finally was placed horizontally in a warmed 5-ml organ bath (37°C). One end of the muscle strip was connected to a force displacement transducer to measure muscle force. The muscle strip was illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm). The emission at 500 nm (F340 and F380) was measured with a spectrophotometer (CAF-110, Jasco, Tokyo). The ratio of F340 to F380 was calculated as an indicator of $[Ca^{2+}]_i$. The absolute Ca^{2+} concentration was not calculated in this experiment because the dissociation constant of the fluorescent indicator for Ca^{2+} in the cytosol may be different from that obtained in vitro (Konishi et al., 1988). Therefore, the ratios obtained in the resting and high K^+-stimulated (50 mM) smooth muscle were taken as 0% and 100%, respectively. The high K^+ solution (50 mM) was made by replacing 50 mM NaCl of the bathing solution by equimolar KCl. In some experiments, a high K^+ solution in which all NaCl (136.9 mM) was replaced by equimolar KCl was used to cause Ca^{2+}-induced contraction and then to load Ca^{2+} into the intracellular stores.

2.3. Measurement of cyclic AMP level

Isolated fresh longitudinal muscle strips weighing approximately 20–30 mg were used in the cyclic AMP study. After equilibration in Krebs solution gassed with a 95% O_2 + 5% CO_2 mixture for 1 h at 37°C, the myometrial strips were treated with 1 μM clonidine for either 0.5, 1, 2 or 5 min, and the effect of clonidine on the tissue cyclic AMP level was examined. After incubation, the smooth muscle strips were frozen quickly in liquid nitrogen and stored at −80°C until assay. Each muscle strip was homogenized in 6% cold trichloroacetic acid solution with a Polytron (PT-10-35, Kinematica, Switzerland). After centrifugation (3000 rpm, twice), trichloroacetic acid in the supernatant was removed by washing with water-saturated ether (three times), and the extracted cyclic AMP was assayed by means of enzyme immunoassay kits (Amersham). Tissue cyclic AMP levels were expressed as picomoles per gram tissue wet weight.

2.4. Chemicals

The following chemicals were used in this experiment: acetylcholine chloride (Wako), carbachol hydrochloride (Wako), clonidine hydrochloride (Sigma), forskolin (Wako), histamine hydrochloride (Wako), oxytocin (Peptide Ins.), pertussis toxin (RBI), UK14304 (RBI), yohimbine hydrochloride (Sigma), ethylenediaminetetraacetic acid tetrasodium salt (EDTA, Dojin), ethyleneglycol bis (β-aminoethylether) tetraacetic acid (EGTA, Dojin), and *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES, Dojin). All drugs except forskolin and UK14304 were dissolved in distilled water immediately before use and diluted with the respective nutrient solutions. UK14304 and forskolin were dissolved in dimethyl sulfoxide and ethanol, respectively. The maximum concentrations of dimethyl sulfoxide and ethanol in the bathing solution were set below 0.2% and 0.1%, respectively, and these concentrations did not change either myometrial tonus or spontaneous contractile activity of the myometrium.

2.5. Statistical analysis

The results of the experiments are expressed as the means or means ± S.E.M of more than four individual experiments. Statistical analysis was carried out by means of paired and unpaired *t*-tests, with $P < 0.05$ as the criterion of statistical significance.

3. Results

3.1. Contractile responses to acetylcholine, clonidine and UK14304 in Krebs and Kumagai solutions

In Krebs solution, acetylcholine (3 nM–1 μM), clonidine (1 nM–10 μM), and UK14304 (1 nM–10 μM) caused contractions of the porcine uterine longitudinal muscle in a concentration-dependent manner. The EC_{50} values and the maximum amplitudes of the contractions were 35 nM and 107% (acetylcholine), 41 nM and 92% (clonidine), and 57 nM and 111% (UK14304), respectively (Fig. 1A). As previously demonstrated (Taneike et al., 1995; Kitazawa et al., 1999b), atropine (1 μM) inhibited the response to acetylcholine, and yohimbine (100 nM–1 μM) but not prazosin (1 μM) inhibited the responses to clonidine and UK 14304, indicating the involvement of a muscarinic cholinoceptor or α_2-adrenoceptor in the respective contractile responses. Fig. 1B shows the concentration–response curves for acetylcholine, clonidine, and UK14304 in Kumagai solution. Neither clonidine nor UK14304 induced a contraction of the longitudinal muscles, even at high concentrations, but acetylcholine was effective to cause a contractile response (EC_{50}, 82 nM).

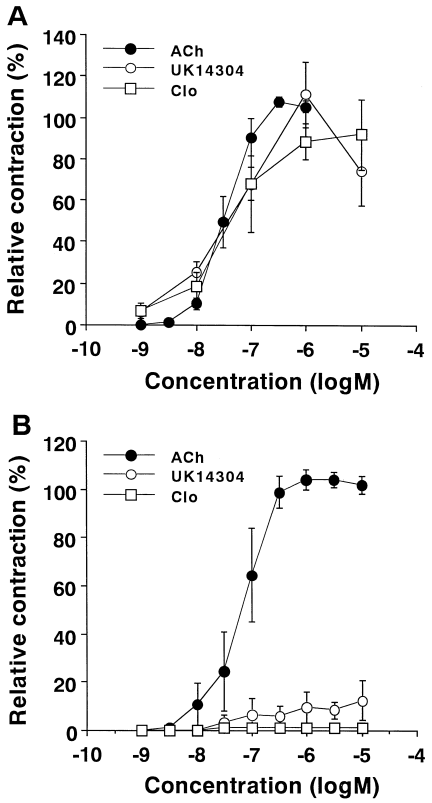

Fig. 1. Concentration–response curves for acetylcholine, clonidine, and UK14304 in the longitudinal muscle of the porcine uterus. Each symbol shows the contractile response to acetylcholine (ACh, ●), UK14304 (○), and clonidine (Clo, □) in Krebs solution (37°C, A) and Kumagai solution (28°C, B). Ordinate: relative amplitude of contraction expressed as percentage of the contraction induced by 100 μM acetylcholine. Abscissa: concentration of agonists (log *M*). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

3.2. Effects of lowering Ca^{2+} concentration and verapamil

Since the Ca^{2+} concentration of Krebs solution (2.5 mM) is different from that of Kumagai solution (0.4 mM), we next examined the effects of lowering the Ca^{2+} concentration of Krebs solution (1.0, 0.5 and 0 mM) on the responses to acetylcholine (100 nM), clonidine (1 μM), and UK14304 (100 nM), which had caused almost the same amplitude of contractions. As indicated in Fig. 2A, a decrease in the external Ca^{2+} concentration inhibited the contractile responses to the three stimulants, but the inhibition of the clonidine- and UK14304-induced contractions was stronger than that of the acetylcholine-induced contraction. In the Ca^{2+}-free solution, the contractions induced by clonidine and UK14304 were almost completely inhibited (UK14304, $1.4 \pm 0.7\%$; clonidine, $1.0 \pm 1.0\%$, $n = 6$) but part of the acetylcholine-induced contraction persisted ($26.0 \pm 6.5\%$, $n = 6$). These results suggest that the porcine uterine longitudinal muscles have a contractile response to α_2-adrenoceptor agonists highly dependent on external Ca^{2+}.

Studies on porcine myometrial cells and rabbit blood vessels have demonstrated the opening of VDCCs by α_2-adrenoceptor stimulation (Dunn et al., 1991; ZhuGe et al., 1997). This possibility was also examined in the porcine myometrial strips, using a VDCC blocker, verapamil. Verapamil inhibited the contractions induced by clonidine (1 μM) and UK14304 (100 nM) in a concentration-dependent manner and abolished them at a high concentration (10 μM), as with high K^+-induced (50 mM) contractions (Fig. 2B). High K^+ stimulation is known to depolarize the cytoplasmic membrane and activate VDCCs in the smooth muscle cells (Karaki et al., 1997). The IC_{50} values (the concentration of verapamil that decreased the contraction induced by the stimulants by 50% of the control) and the maximum inhibition of clonidine-,

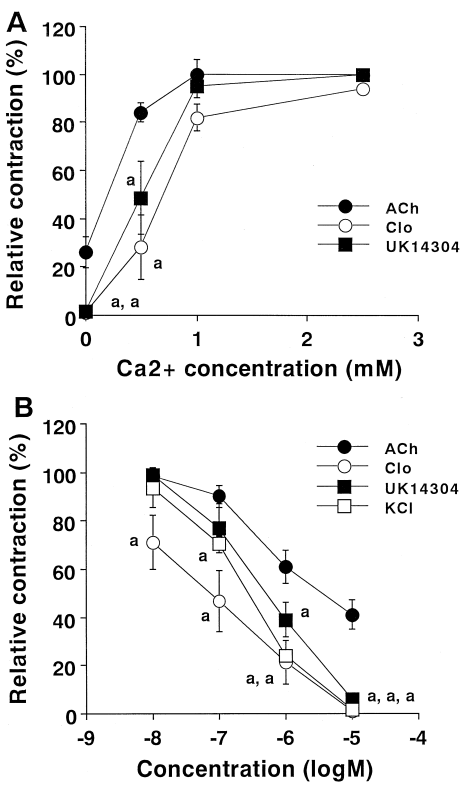

Fig. 2. Effects of external Ca^{2+} concentration and verapamil on the contractile responses to acetylcholine, UK14304, clonidine, and high K^+ in the longitudinal muscle of the porcine uterus. (A) Ca^{2+} concentration in Krebs solution was lowered from 2.5 to 1.0, 0.5, 0 mM, and the relationships between Ca^{2+} concentration and the contractile responses to acetylcholine (ACh, 100 nM, ●), UK14304 (100 nM, ■), and clonidine (Clo, 1 μM, ○) were investigated. Ordinate: relative amplitude of contraction expressed as percentage of the contraction induced by 100 nM acetylcholine. Abscissa: Ca^{2+} concentration (mM). (B) Inhibition of contractile responses to acetylcholine (ACh, 100 nM, ●), UK14304 (100 nM, ■), clonidine (Clo, 1 μM, ○) and high K^+ (KCl, 50 mM, □) by verapamil (10 nM–10 μM). Ordinate: relative contraction expressed as percentage of the responses before the application of verapamil. Abscissa: concentration of verapamil (log *M*). Points represent the means of four or more experiments with S.E.M. shown by vertical lines. (a) Significantly different ($P < 0.05$) from the corresponding value for acetylcholine (●).

UK14304-, and high K^+-induced contractions were 260 ± 20 nM and 99 ± 1% ($n = 4$), 560 ± 180 nM and 94 ± 1.2% ($n = 5$), and 280 ± 70 nM and 98 ± 3.4% ($n = 4$), respectively. On the other hand, although verapamil inhibited the acetylcholine-induced contraction, the inhibition was not complete at 10 μM (59 ± 6.2%, $n = 5$), and the IC_{50} value (2300 ± 903 nM, $n = 5$) was significantly higher than those for clonidine, UK14304 and high K^+.

3.3. Effects of clonidine and UK14304 on the $[Ca^{2+}]_i$ of the longitudinal muscle

The application of clonidine (1 μM) caused transient increases in muscle force and $[Ca^{2+}]_i$ of the longitudinal muscle of the porcine myometrium loaded with fura-PE3 (Fig. 3A). UK14304 produced similar increases in $[Ca^{2+}]_i$ and uterine muscle force (data not shown). Fig. 3B shows the concentration–response relationships of the clonidine-induced increases in $[Ca^{2+}]_i$ and muscle force. The EC_{50} value of clonidine for increasing the $[Ca^{2+}]_i$ (72 nM) was close to that for causing muscle contraction (61 nM). The muscle contraction and $[Ca^{2+}]_i$ relationships of clonidine (Fig. 3C) indicated that although the $[Ca^{2+}]_i$ increase induced by clonidine was lower than that induced by 50 mM of high K^+, the contractile response was the same as that induced by high K^+. A similar relationship between muscle force and $[Ca^{2+}]_i$ was observed for UK 14304.

Fig. 4 shows the effects of verapamil (10 μM) and of the Ca^{2+}-free solution (EGTA, 1 mM) on the clonidine-induced increases in $[Ca^{2+}]_i$ and muscle force. Verapamil (10 μM) inhibited both the $[Ca^{2+}]_i$ increase and muscle contraction almost completely, as with high K^+-induced responses. Ca^{2+}-free solution also abolished the clonidine-induced increases in $[Ca^{2+}]_i$ and muscle force.

3.4. Effect of clonidine on Ca^{2+} release from intracellular Ca^{2+} stores

The marked inhibition of the clonidine-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free solution and by verapamil suggests that extracellular Ca^{2+} has an important role in the response to clonidine. Recently, however, ZhuGe et al. (1997) reported that α_2-adrenoceptor activation stimulates the release of Ca^{2+} from intracellular stores. To clarify whether clonidine is able to mobilize intracellular Ca^{2+}, the effects of clonidine and some drugs (carbachol, histamine, and oxytocin) on the $[Ca^{2+}]_i$ of the myometrial strips were examined in the Ca^{2+}-free solution (EGTA, 1 mM). The intracellular Ca^{2+} stores were filled with Ca^{2+} by stimulation with a high K^+ (136.9 mM) solution con-

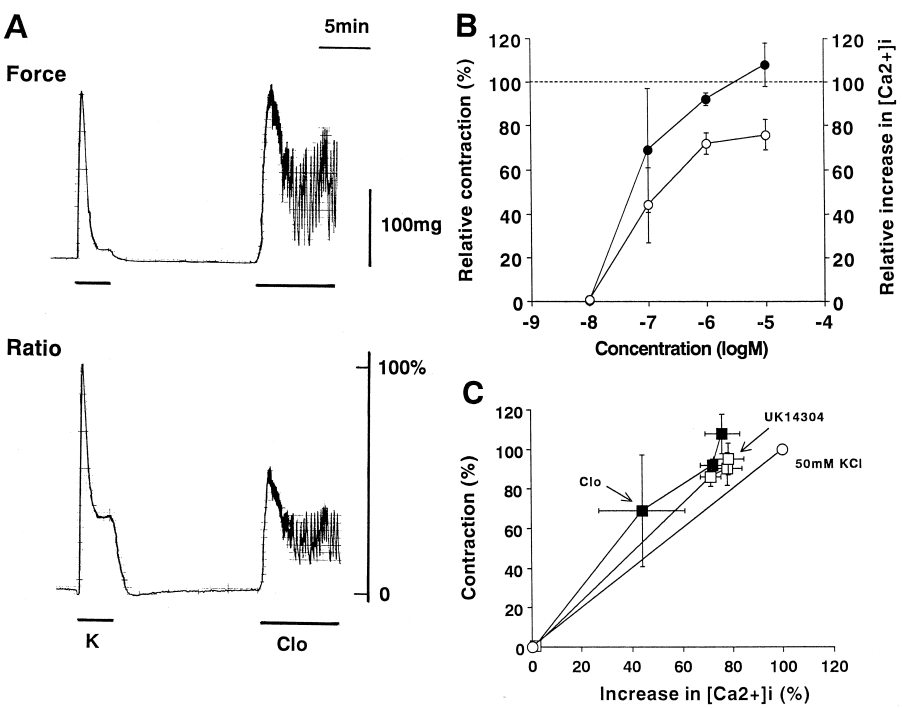

Fig. 3. Effects of α_2-adrenoceptor agonists on $[Ca^{2+}]_i$ and muscle force in the longitudinal muscle of the porcine uterus. (A) Typical effects of high K^+ (K, 50 mM) and clonidine (Clo, 1 μM) on smooth muscle force (upper) and $[Ca^{2+}]_i$ (ratio, lower). (B) Concentration–response curves for the effect of clonidine on smooth muscle force and $[Ca^{2+}]_i$. Ordinate: smooth muscle contraction (left, ●) and $[Ca^{2+}]_i$ (right, ○) are expressed as percentages of the responses induced by 50 mM high K^+. Abscissa: clonidine concentration (log *M*). (C) Relationships between $[Ca^{2+}]_i$ and contractile force in the presence of clonidine (■) or UK14304 (□). Smooth muscle contraction (*Y*-axis) and $[Ca^{2+}]_i$ (*X*-axis) are expressed as a percentage of the responses induced by 50 mM high K^+ (○). Points represent the means of four experiments with S.E.M. shown by vertical lines.

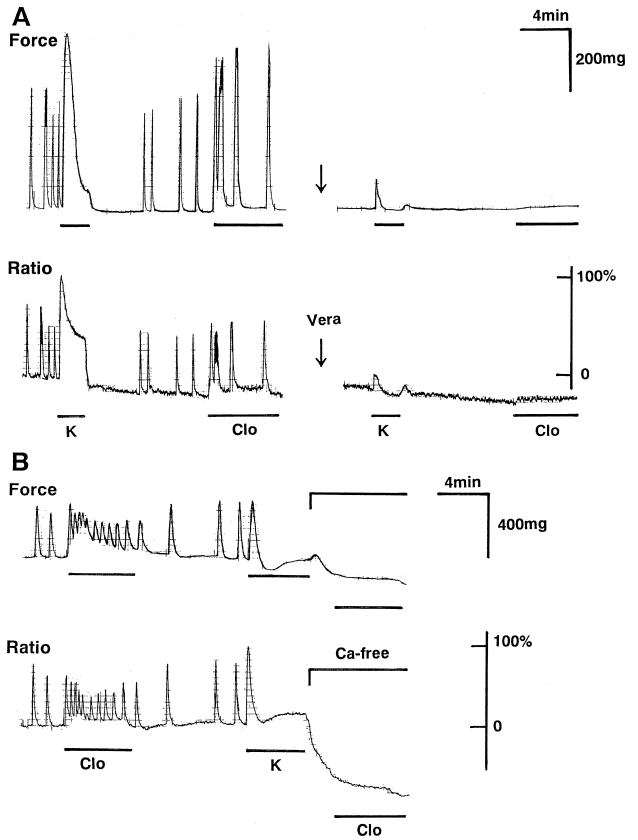

Fig. 4. Typical effects of verapamil and Ca^{2+}-free solution on clonidine-induced increases in muscle force and $[Ca^{2+}]_i$ in the longitudinal muscle of the porcine uterus. (A) Inhibition by verapamil (Vera, 10 μM) of clonidine- (Clo, 1 μM) and high K^+-induced (K, 50 mM) contraction (upper) and $[Ca^{2+}]_i$ increase (ratio, lower). (B) Inhibition by a Ca^{2+}-free solution (EGTA, 1 mM) of clonidine-induced (Clo, 1 μM) contraction (upper) and $[Ca^{2+}]_i$ increase (ratio, lower).

taining 2.5 mM Ca^{2+} for 5 min beforehand. Application of carbachol (10 μM), histamine (10 μM), and oxytocin (1 μM) caused transient increases in $[Ca^{2+}]_i$, the amplitudes of which were 30–50% of the high K^+-induced increase (carbachol, $33 \pm 8\%$, $n = 4$; histamine, $47 \pm 12\%$, $n = 5$; oxytocin, $49 \pm 15\%$, $n = 4$). On the other hand, clonidine (10 μM) caused only a small increase in $[Ca^{2+}]_i$ under the same conditions ($5 \pm 1.5\%$, $n = 8$) and some preparations (4 out of 12, 33%) did not respond to clonidine (Figs. 5 and 6A). The small increase in $[Ca^{2+}]_i$ induced by clonidine (10 μM) was significantly reduced by pretreatment with yohimbine (1 μM, $1.9 \pm 1.2\%$, $n = 4$).

3.5. Effect of clonidine on Ca^{2+}-induced contraction in the depolarized muscle strips

We investigated the effect of clonidine on Ca^{2+}-induced responses ($[Ca^{2+}]_i$ and muscle force) and examined whether clonidine increases the Ca^{2+} sensitivity of the contractile elements. In a Ca^{2+}-free, high K^+ (136.9 mM) solution, Ca^{2+} (2.5 mM) caused increases in $[Ca^{2+}]_i$ and muscle force (a transient contraction), which were reproducible with applications at 15-min intervals. As indicated in Fig. 6A, 5 min pretreatment with clonidine (1 μM) potentiated the Ca^{2+}-induced contraction without affecting the increase in $[Ca^{2+}]_i$. The potentiation was still observed after washout of clonidine for 10 min, but complete recovery was obtained 30–40 min later (data not shown). Fig. 6B shows the concentration–response relationships of the clonidine-induced potentiation. Potentiation of the Ca^{2+}-induced contraction was marked at relatively high concentrations (control = 100%; 1 μM, $127 \pm 8.5\%$, $n = 5$; 10 μM, $185 \pm 9.0\%$, $n = 6$). Yohimbine (1 μM) inhibited the potentiation by clonidine significantly (10 μM, $104 \pm 11\%$, $n = 5$) but this antagonist itself did not have any effect on Ca^{2+}-induced increases in $[Ca^{2+}]_i$ and muscle force.

3.6. Effect of clonidine on tissue cyclic AMP

The effect of clonidine on myometrial cyclic AMP production was examined in order to analyze the involvement of the α_2-adrenoceptor-mediated inhibition of adenylate cyclase in the contractile response. As indicated in

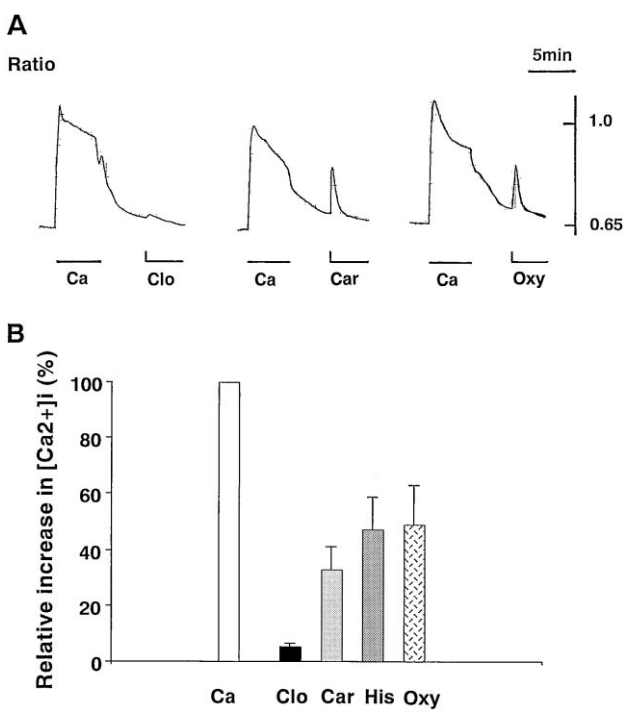

Fig. 5. Effect of clonidine on $[Ca^{2+}]_i$ of the uterine longitudinal muscle in the absence of external Ca^{2+}. (A) Longitudinal muscle strips were incubated in Ca^{2+}-free solution (EGTA, 1 mM) and then the intracellular Ca^{2+} store was filled by stimulation with a 136.9 mM high K^+ solution (2.5 mM Ca^{2+}) for 5 min (Ca). After the wash with Ca^{2+}-free solution for 5 min, the effects of clonidine (Clo, 10 μM), carbachol (Car, 10 μM), and oxytocin (Oxy, 1 μM) on $[Ca^{2+}]_i$ were examined. (B) Comparison of the Ca^{2+} releasing action of clonidine (Clo, 10 μM), carbachol (Car, 10 μM), histamine (His, 10 μM), and oxytocin (Oxy, 1 μM) in the absence of external Ca^{2+}. Ordinate: $[Ca^{2+}]_i$ increases caused by the various agents are expressed as percentages of the increase in $[Ca^{2+}]_i$ induced by 2.5mM Ca^{2+} (Ca). Columns represent the means of four or more experiments with S.E.M. shown by vertical lines.

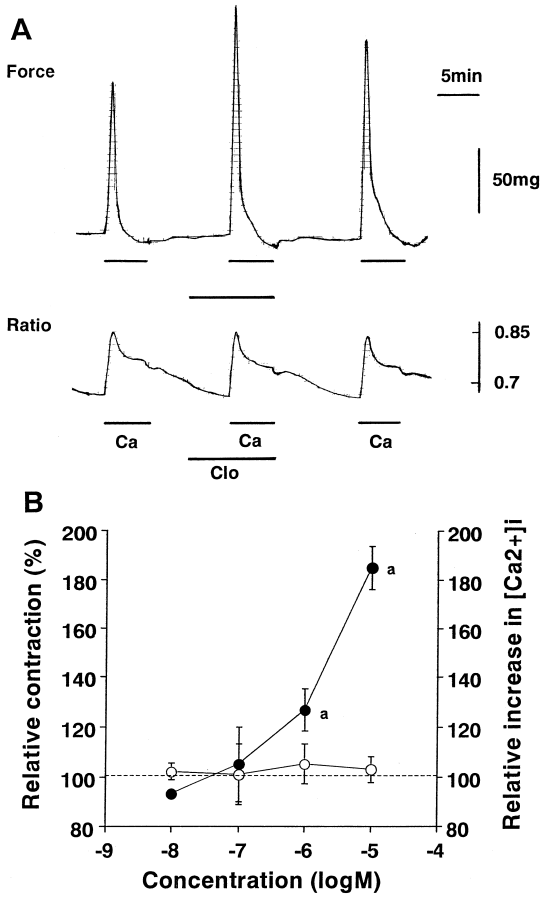

Fig. 6. Potentiation of Ca^{2+}-induced contraction by clonidine in the longitudinal muscle of the porcine uterus. (A) Typical effects of clonidine (Clo, 1 μM) on 2.5 mM Ca^{2+}-induced (Ca) contraction (force, upper) and $[Ca^{2+}]_i$ increase (ratio, lower) in a Ca^{2+}-free, high K^+ (136.9 mM) solution. After washout of clonidine for 10 min, the potentiation of the contraction was still observed. (B) Concentration–response curves for the effect of clonidine on the Ca^{2+}-induced contraction and $[Ca^{2+}]_i$ increase. Ordinate: smooth muscle contraction (●, left) and $[Ca^{2+}]_i$ increase (○, right) are expressed as a percentage of the responses induced by 2.5 mM Ca^{2+} in the absence of clonidine (control = 100%). Points represent the means of four or more experiments with S.E.M. shown by vertical lines. Abscissa: concentration of clonidine (log *M*). (a) Significantly different from the value obtained in the absence of clonidine ($P < 0.05$).

Fig. 7, the contractile response to clonidine (1 μM) developed quickly was sustained for 1 min, and then faded gradually. The relative amplitudes of the contraction after incubation with clonidine for 2, 3, 4 and 5 min were 69.1 ± 12%, 45.4 ± 13%, 24 ± 12.3%, and 11.5 ± 6.5% ($n = 5$) of the maximal contraction, respectively. However, the tissue cyclic AMP level (control: 493 ± 50 pmol/g tissue wet weight, $n = 10$) did not significantly change during the contractile response.

3.7. Contractile response to clonidine in the presence of forskolin

The contractile response to clonidine was examined using histamine-precontracted muscle strips relaxed by forskolin (an adenylate cyclase activator). If α_2-adrenoceptor activation inhibits adenylate cyclase activity, this inhibition might attenuate the relaxation induced by forskolin and restore the contractile responses to histamine. This setup is a modification of a pharmacological approach to the analysis of the contractile response of muscarinic M_2 receptors (coupled with the inhibition of adenylate cyclase activity) in isolated smooth muscle strips (Thomas and Ehlert, 1996). In the absence of histamine and forskolin, clonidine (10 nM–10 μM) caused concentration-dependent contractions as described in Section 3.1. Next, the longitudinal muscle preparation was contracted with histamine (3 μM) then relaxed with forskolin (3 μM). Under these conditions, clonidine failed to attenuate the forskolin-induced inhibition in the histamine-precontracted strips; i.e., clonidine did not cause contraction. To exclude the possible nonselective inhibition of muscle contractility by forskolin, the effect of acetylcholine was tested. Acetylcholine was effective to cause a contraction, the amplitude of which, however, was only slightly less than that of the control (Fig. 8).

3.8. Inhibition of clonidine-induced contraction by pertussis toxin

The effects of in vitro treatment with pertussis toxin (5 μg/ml for 2 h) on the contractile responses to high K^+ (50 mM, 100%), carbachol (1 μM, 108 ± 6%, $n = 5$) and clonidine (1 μM, 98 ± 6.3%, $n = 5$) were tested in order to determine the involvement of the G-protein-coupled signal transduction pathway in the response to clonidine. The clonidine-induced (1 μM) contraction was signifi-

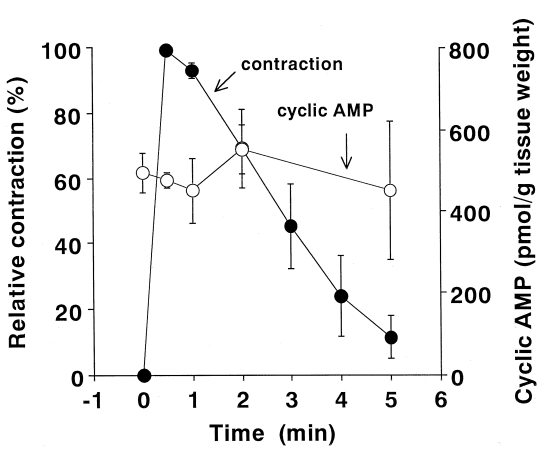

Fig. 7. Effect of clonidine on cyclic AMP production in the longitudinal muscle of the porcine uterus. Time course of smooth muscle tonus (0, 0.5, 1, 2, 3, 4, 5 min, ●) and tissue cyclic AMP levels (0, 0.5, 1, 2, 5 min, ○) during the contractile response to clonidine (1 μM). Ordinate left: response was expressed as a percentage of the maximum amplitude of the contraction induced by clonidine (1 μM). Ordinate right: tissue cyclic AMP level (pmol/g tissue wet weight). Abscissa: time after the application of clonidine (min). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

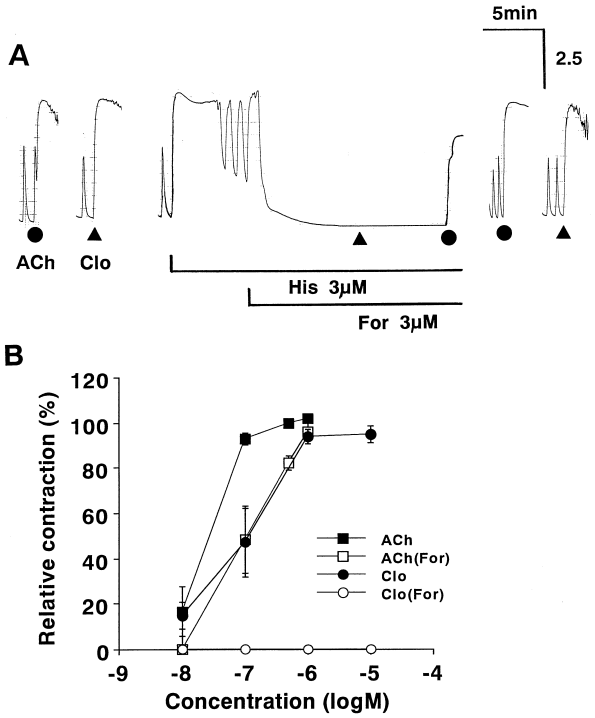

Fig. 8. Clonidine-induced response of the porcine uterine longitudinal muscles in the presence of forskolin and histamine. (A) Protocol and typical responses. After control responses to acetylcholine (ACh, 500 nM, ●) and clonidine (Clo, 1 μM, ▲) were obtained, the preparation was contracted with histamine (His, 3 μM) and then relaxed with forskolin (For, 3 μM). Contractile responses to clonidine and acetylcholine were investigated in the presence of histamine and forskolin. After washout of histamine and forskolin, the recovery of the contractile response was observed. (B) Concentration–response curves for clonidine (Clo, ●, ○) and acetylcholine (ACh, ■, □) in the absence (●, ■) and presence (○, □) of histamine and forskolin (For). Ordinate: relative contraction expressed as percentage of the contraction induced by 500 nM acetylcholine. Abscissa: concentration of agonists (log *M*). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

cantly inhibited (relative amplitude; $24 \pm 10\%$, $n = 5$) in the pertussis-toxin-treated muscle preparations but the carbachol- and high K^+-induced contractions were unaffected (relative amplitude: carbachol, $112 \pm 10\%$; high K^+, $106 \pm 3.2\%$, $n = 5$).

4. Discussion

α_2-Adrenoceptors are known to mediate the contractile responses to norepinephrine and clonidine in the porcine myometrium (Taneike et al., 1995) but the cellular mechanisms of the contractile responses are not yet completely understood. Recent studies on vascular smooth muscles using Ca^{2+}-sensitive indicators (aequorine and fura-2) indicated that UK14304 increased $[Ca^{2+}]_i$ and caused phosphorylation of myosin light chain in the rabbit saphenous vein (Aburto et al., 1993), and that azepexole (an α_2-adrenoceptor agonist) caused increases in $[Ca^{2+}]_i$ and smooth muscle tension in the isolated human subcutaneous resistance arteries (Parkinson and Hughes, 1995). An α_2-adrenoceptor-mediated increase in $[Ca^{2+}]_i$ has been demonstrated in both muscle strips and dispersed smooth muscle cells (Lepretre and Mironneau, 1994). These reports clearly show the essential role of the increase in $[Ca^{2+}]_i$ in the α_2-adrenoceptor-mediated contraction of vascular smooth muscles. In the present study, the fact that the contractile responses to clonidine and UK14304 were associated with a rise in $[Ca^{2+}]_i$ in the porcine myometrium, suggested that the activation of α_2-adrenoceptors in nonvascular smooth muscle also increases $[Ca^{2+}]_i$ and causes muscle contraction. Both the contraction and increase in $[Ca^{2+}]_i$ were markedly inhibited in the Ca^{2+}-free solution and by a dihydropyridine Ca^{2+}-channel antagonist, verapamil, in the porcine myometrium. Therefore, it is thought that the increase in $[Ca^{2+}]_i$ caused by α_2-adrenoceptor stimulation is largely dependent on the influx of extracellular Ca^{2+}, probably through VDCCs rather than stored Ca^{2+}. The important contribution of extracellular Ca^{2+} and VDCCs to the α_2-adrenoceptor-mediated increase in $[Ca^{2+}]_i$ and smooth muscle contraction has been also demonstrated in vascular smooth muscles such as the isolated human subcutaneous resistance artery (Parkinson and Hughes, 1995), canine and rabbit saphenous vein (Jim and Matthews, 1985; Dunn et al., 1991; Aburto et al., 1993), rat tail artery (Li et al., 1993), and rabbit aorta (Nebigil and Malik, 1993). Electrophysiological studies with voltage-clamped smooth muscle cells (porcine myometrial cells and rat portal vein myocytes) have indicated an increase in voltage-activated inward current (Ca^{2+} current) due to α_2-adrenoceptor activation (Lepretre and Mironneau, 1994; ZhuGe et al., 1997), and Cheung (1985) reported a slow depolarization evoked by α_2-adrenoceptor agonists in the rat saphenous vein. Based on these reports and the present study, it is suggested that the activation of α_2-adrenoceptors causes depolarization of smooth muscles, activates the VDCCs, increases Ca^{2+} influx and $[Ca^{2+}]_i$, and eventually contracts porcine myometrium. However, the ionic mechanisms of the depolarization induced by α_2-adrenoceptor activation need clarification.

In the present study, the activation of α_2-adrenoceptors caused only a small increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} but carbachol (a muscarinic M_3 receptor; Kitazawa et al., 1999b), histamine (a histamine H_1 receptor; Kitazawa et al., 1997), and oxytocin (an oxytocin receptor) were considerably more effective to increase $[Ca^{2+}]_i$ under the same experimental conditions. These three receptors are positively coupled with the phospholipase C-IP_3-diacylglycerol pathway, and IP_3 stimulates the release of Ca^{2+} from intracellular stores (Soloff, 1990; Eglen et al., 1996; Hill et al., 1997). These results suggest that α_2-adrenoceptor stimulation does not mobilize intracellular Ca^{2+} in the porcine myometrium. A relatively minor contribution of the intracellular Ca^{2+} mobilizing action to the α_2-adrenoceptor-mediated Ca^{2+} response has been demonstrated in the rat portal vein (Lepretre and

Mironneau, 1994) and isolated human subcutaneous resistance artery (Parkinson and Hughes, 1995). However, these reports and the present results are contrary to those from studies on the human subcutaneous artery and the rabbit ear vein, which suggest the Ca^{2+} mobilization by α_2-adrenoceptor stimulation (Daly et al., 1990; Nielsen et al., 1992). There have been inconsistent results concerning the effects of α_2-adrenoceptor activation on the accumulation of inositol phosphates (probably IP_3). No accumulation of IP_3 after α_2-adrenoceptor stimulation was demonstrated in the rat portal vein (Lepretre and Mironneau, 1994), but an α_2-adrenoceptor-mediated rise in the synthesis of inositol phosphates was reported in the human digital artery (Stevens and Moulds, 1990). The causes of the discrepancy are unclear at present. There might be species- or smooth muscle tissue (vascular or nonvascular, region, vein or artery)-related variations in the synthesis of IP_3 and in the responsiveness of the Ca^{2+} stores to IP_3. Interestingly, using dispersed cells of the porcine myometrium, ZhuGe et al. (1997) demonstrated an α_2-adrenoceptor-mediated release of stored Ca^{2+}. Differences in the type of smooth muscle preparation (muscle strips vs. isolated muscle cells) or in oestrus cycle (proestrus vs. luteal phase) might explain the difference in the contribution of the stored Ca^{2+} after α_2-adrenoceptor activation. A similar discrepancy has also been observed when the Ca^{2+} mobilizing action of caffeine in muscle strips (Kitazawa et al., 1999a) and in dispersed myocytes (ZhuGe and Hsu, 1995) was compared.

Clonidine potentiated the Ca^{2+}-induced contraction without affecting the increase in $[Ca^{2+}]_i$ in the depolarized muscle preparation, and clonidine caused a greater contraction than did 50 mM high K^+ at a given $[Ca^{2+}]_i$. Therefore, it is suggested that the mechanisms of the contraction induced by clonidine are not limited by an increase in $[Ca^{2+}]_i$ and that, in addition to stimulating Ca^{2+} influx, clonidine increases the sensitivity of contractile elements to Ca^{2+}. An α_2-adrenoceptor-mediated increase in Ca^{2+} sensitivity has been reported for rabbit saphenous vein (Aburto et al., 1993) and human subcutaneous resistance artery (Parkinson and Hughes, 1995). This Ca^{2+} sensitization may represent an important role in the generation of uterine tone without an increase in $[Ca^{2+}]_i$ and in the stimulating action of agonists in the porcine myometrium. Although the mechanisms by which α_2-adrenoceptor agonists increase the sensitivity of the contractile elements to Ca^{2+} are not yet clearly understood, several possibilities have been discussed. In smooth muscle tissues, the elevation of intracellular cyclic AMP decreases the Ca^{2+} sensitivity of contractile elements (Nishimura and Van Breemen, 1989; Karaki et al., 1997). As α_2-adrenoceptors are known to inhibit adenylate cyclase activity by coupling with G_i (Ruffolo et al., 1991), the activation of α_2-adrenoceptors might decrease the tissue cyclic AMP level and attenuate the cyclic AMP-dependent inhibitory action on Ca^{2+} sensitivity. However, clonidine did not change the cytoplasmic cyclic AMP level significantly during the development of the contractile response in the present study. Therefore, the increase in Ca^{2+} sensitivity could not be explained by the decrease in cytoplasmic cyclic AMP level. Protein kinase C has been reported to increase the Ca^{2+} sensitivity of contractile elements in various smooth muscle tissues (Karaki et al., 1997). Diacylglycerol, an endogenous activator of protein kinase C, is synthesized equimolarly with IP_3 from the hydrolysis of membrane phospholipids by phospholipase C (Nishizuka, 1984). In the porcine myometrium, clonidine only weakly mobilized intracellular Ca^{2+} and this indirectly indicated the presence of very low levels of the synthesized IP_3 and diacylglycerol. Thus, it seems unlikely that the protein-kinase-C-dependent Ca^{2+}-sensitizing pathway is activated by α_2-adrenoceptor stimulation in the porcine myometrium. Another possible mechanism to explain the Ca^{2+} sensitization is the involvement of arachidonic acid. α_2-Adrenoceptor activation has been reported to release arachidonic acid in vascular smooth muscle (Nebigil and Malik, 1992, 1993). Arachidonic acid inhibits myosin light chain phosphatase and increases the Ca^{2+} sensitivity of contractile elements (Gong et al., 1992; Karaki et al., 1997). Further studies with porcine myometrium should clarify the involvement of arachidonic acid in α_2-adrenoceptor induced Ca^{2+} sensitization.

The significant and selective inhibitory effect of pertussis toxin on the contractile response to clonidine indicated the participation of a pertussis-toxin-sensitive G-protein in the action of α_2-adrenoceptors in the porcine myometrium. Pertussis toxin-induced inhibition of α_2-adrenoceptor-mediated responses has been reported for the contraction of dog (Miller et al., 1991) and human vascular muscles (Parkinson and Hughes, 1995), and increases in $[Ca^{2+}]_i$ in dispersed muscle cells of the porcine myometrium (ZhuGe et al., 1997) and of the rat portal vein (Lepretre and Mironneau, 1994). Recent studies indicated the involvement of G_i proteins, in part, in the α_2-adrenoceptor-mediated activation of VDCCs (Nebigil and Malik, 1993; Lepretre and Mironneau, 1994; ZhuGe et al., 1997). These findings suggest the following cascade of events for α_2-adrenoceptor activation in the porcine myometrium: α_2-adrenoceptor activation $\rightarrow$ activation of $G_i \rightarrow$ opening of VDCCs with an increase in the influx of $Ca^{2+} \rightarrow$ increase of $[Ca^{2+}]_i \rightarrow$ smooth muscle contraction. However, regarding this cascade, the question of whether G-protein activates VDCCs directly or indirectly still needs to be studied.

Although α_2-adrenoceptors are well known to be coupled with the inhibition of adenylate cyclase activity by G_i (Ruffolo et al., 1991), in the present study, clonidine caused the contraction of the uterine smooth muscle without changing the cytoplasmic cyclic AMP level. The present finding is consistent with results of a previous study on the porcine palmar vein (Wright et al., 1995) and indicates that the α_2-adrenoceptor-mediated contraction is dissociated from the reduction in cytoplasmic cyclic AMP

(inhibition of adenylate cyclase activity). Failure of clonidine to restore the inhibitory response to forskolin in histamine-precontracted preparations also supports the notion that inhibition of adenylate cyclase activity is not responsible for the initiation of contraction by α_2-adrenoceptor stimulation.

In conclusion, in the porcine myometrium, α_2-adrenoceptor agonists cause contraction of the longitudinal muscle by mechanisms largely dependent on the influx of extracellular Ca^{2+}, probably through VDCCs. The increase in Ca^{2+} sensitivity of the contractile elements is the additional mechanism of contractile responses. This Ca^{2+} sensitization might play an important role in the regulation of porcine uterine tone by adrenergic nerves. These α_2-adrenoceptor-induced contractions involve a pertussis-toxin-sensitive G-protein (probably G_i type) in the signal transduction pathway.

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