J. Pharm. Pharmacol. 2000, 52: 1377–1385
Received February 10, 2000
Accepted May 19, 2000

Inhibitory Effects of Amlexanox on Carbachol-induced Contractions of Rabbit Ciliary Muscle and Guinea-pig Taenia Caecum

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Abstract

Instillation of amlexanox, an anti-allergic drug, over a long period improves myopia in some allergy patients and in monkeys. The relaxing effect of amlexanox on persistent contraction of ciliary muscle may be involved in the improvement of myopia. In this study, the mechanism of the noncompetitive inhibition of carbachol-induced contractions by amlexanox (1–100 μM) was investigated in isolated smooth muscle preparations of the rabbit ciliary body and guinea-pig taenia caecum.

In ciliary muscles, amlexanox (100 μM) inhibited both the phasic and tonic components of carbachol-induced contractions even in the presence of cyclopiazonic acid (10 μM) where the function of the sarcoplasmic reticulum was impaired, while diltiazem (3·2, 32 μM) did not. In taenia caecum, diltiazem (3·2 μM) slightly inhibited the phasic component and abolished the tonic component of carbachol-induced contractions. Amlexanox also abolished the tonic component, but it did not decrease the $^{45}Ca^{2+}$ uptake into taenia caecum smooth muscle cells induced by carbachol. Amlexanox did not increase the cyclic adenosine monophosphate (cyclicAMP) content of ciliary muscles in the presence of 3-isobutyl-1-methylxanthine (10 μM), while forskolin (1 μM) did. Gel-shift assay showed that the inhibition of carbachol-induced contractions by amlexanox was accompanied by a decrease in phosphorylation of the 20-kDa myosin light chain in taenia caecum tissue preparations. Amlexanox had no effect on calmodulin activity, whereas it inhibited phosphorylation of the myosin light chain by purified myosin light-chain kinase from chicken gizzard.

These results suggested that amlexanox may not affect either Ca^{2+} mobilization or calmodulin activity, although it inhibits myosin light-chain kinase, which may inhibit carbachol-induced contraction.

Although its aetiology remains to be elucidated, near work is generally accepted as one of the causes of the onset and progression of myopia. Since persistent contraction of the ciliary muscles due to continuous near work can result in tonic accommodative myopia, drugs that cause relaxation of ciliary muscles may be useful for treatment of some myopic changes. Recently, we found that instillation of the anti-allergic drug amlexanox over a long period improved myopia in some allergy patients treated with the drug (unpublished data) and in monkeys (Watanabe et al 1999). Ciliary muscles have dense cholinergic innervation and have a high concentration of muscarinic receptors (Barany et al 1982; Konno & Takayanagi 1985; Stahl et al 1991). Therefore, the dominant neurotransmitter is acetylcholine, and muscarinic agonists induce contraction. Since amlexanox inhibits carbachol-induced contraction in rabbit ciliary muscles (Watanabe et al 1998), the relaxing effect may be involved in the improvement of myopia. The mechanism of this inhibition by amlexanox is still unknown, although we found that muscarinic receptor blockade did not mediate the relaxing

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effect of amlexanox (Watanabe et al 1998). Many muscarinic antagonists also relax ciliary muscles. However, this action is accompanied by potent mydriasis. Unlike these agents, amlexanox induces minimal mydriasis and thus may be available for clinical use.

In this study, we investigated the effects of amlexanox on cyclic adenosine monophosphate (cyclicAMP) content, Ca^{2+} mobilization and myosin phosphorylation to clarify the mechanism for the inhibitory effects of amlexanox on carbachol-induced contractions in rabbit ciliary muscles and guinea-pig taenia caecum.

Materials and Methods

Chemicals

Amlexanox (2-amino-7-(1-methylethyl)-5-oxo-5*H*-(1)benzopyrano(2,3-b)-pyridine-3-carboxylic acid) was provided by Takeda Chemical Industries (Osaka, Japan). [γ-^{32}P]ATP (specific activity > 5000 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). $^{45}CaCl_2$ (specific activity 17–56 mCi mg^{-1}) was from NEN Life Science Products (Boston, MA). Calyculin A, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Wako Pure Chemicals (Osaka, Japan). Calmodulin, carbachol chloride, cyclopiazonic acid, diltiazem, methylene blue, thapsigargin and wortmannin were from Sigma (St Louis, MO). All other chemicals were of the highest reagent grade available.

Contractility measurement

Female Dutch Belted rabbits, 1·7–2·6 kg, were anaesthetized and killed by intravenous injection of pentobarbital sodium (100 mg kg^{-1}). The eyes were excised and bisected 3 mm behind the limbus. With the corneal side downward, each hemisphere was cut into three sectors to prepare three strips of ciliary muscle from the circular ciliary body. Incisions were made on either side of the ciliary body to remove the choroid and iris. Then, the ciliary muscle was detached from the sclera with microscissors. A ciliary muscle strip approximately 2 mm wide and 10 mm long was mounted vertically in an organ bath filled with Krebs-Ringer solution containing (mM): 118 NaCl, 4·7 KCl, 1·2 $MgCl_2$, 1·2 $CaCl_2$, 11 glucose, 1·2 KH_2PO_4 and 25 $NaHCO_3$. The medium was maintained at 37°C and aerated with 95% O_2-5% CO_2 (pH 7·6). The resting tension of the muscle was adjusted to be equivalent to a 20–30-mg load, and contractions stimulated by carbachol were recorded isometrically.

Taenia caecum strips (1·5 mm wide, 10 mm long) were prepared from male Hartley guinea-pigs, 430–610 g. Isotonic contractions induced by carbachol were recorded with 500-mg load in HEPES-Tyrode solution ((mM)137 NaCl, 2·7 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 5·6 glucose and 4·2 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES), adjusted to pH 7·4 at 37°C with 1 N NaOH).

After equilibration for 60 min, 100 μM carbachol was applied and the contraction evoked was designated the standard contraction. In both preparations, this concentration of carbachol induced maximal contraction. Each inhibitor was applied after the standard contraction. Unless otherwise stated, each contraction is expressed as a percentage of the magnitude of the standard contraction.

$^{45}Ca^{2+}$ uptake measurement

After equilibration for 60 min in Krebs-Ringer solution or HEPES-Tyrode solution at 37°C, the strips of ciliary muscle and taenia caecum prepared as described above were exposed to $^{45}Ca^{2+}$ (1 μCi mL^{-1}) in the respective solution, with or without carbachol (100 μM) for 5 min. In the experiments to examine the effects of amlexanox (100 μM) and diltiazem (3·2 μM), these agents were added 10 min before $^{45}Ca^{2+}$. After incubation in medium containing $^{45}Ca^{2+}$, the tissues were immediately transferred to Ca^{2+}-free medium, and subsequently immersed in ice-cold La^{3+} solution (Karaki & Weiss 1979) containing (mM) 80·8 $LaCl_3$, 11·0 glucose and 6·0 Tris, adjusted to pH 6·8 at 4°C with 1 N HCl. After a 30-min immersion, the strips were blotted, weighed, and solubilized with Protozol (E.I. DuPont de Nemours Co. Inc, Boston, MA). The radioactivity was measured by scintillation spectrometry. Tissue counts were converted into tissue calcium uptake using the specific activity of the medium and the results are expressed as μmol/kg wet weight of tissue.

Measurement of cyclicAMP

After equilibration for 30 min in Krebs-Ringer solution at 37°C, ciliary muscle preparations were exposed to IBMX (10 μM) for 5 min. Then, carbachol (100 μM) was added, and after 5 min when carbachol-induced contractions appeared as the tonic component, amlexanox (1–100 μM) was added. Preparations were exposed to amlexanox for 30 s or 15 min in the presence of carbachol and IBMX. As a positive control, forskolin (1 μM) was added instead of amlexanox for 30 s or 5 min. The

difference in the exposure time between amlexanox and forskolin was due to the differences in the rate of relaxation of the tonic component of carbachol-induced contractions by these inhibitors to the same extent. Immediately after the incubation, the preparations were frozen rapidly in acetone-dry ice slurry.

The frozen preparations were powdered quickly in a stainless-steel mill that was pre-chilled in liquid nitrogen. Ice-cold 6% trichloroacetic acid containing 1 mM EDTA was added to the frozen powder to extract cyclicAMP and the suspension was centrifuged at 10 000 *g* for 15 min at 0–4°C. The pellets were reconstituted in 2 N NaOH and the protein content was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). The supernatant fractions were extracted 4 times with water-saturated ether to remove trichloroacetic acid. The amount of cyclicAMP in each extracted supernatant fraction was determined using a [^{125}I]cyclicAMP RIA Kit (Yamasa Co., Chiba, Japan). Each sample was assayed in duplicate, and the mean values were taken as the results.

Calmodulin activity assay

The calmodulin assay is based on the ability of calmodulin to activate 3′,5′-cyclic nucleotide phosphodiesterase, which converts adenosine 3′, 5′-cyclicAMP to AMP. The 5′-phosphate is subsequently cleaved from the newly formed AMP by the addition of 5′-nucleotidase. To start the reaction, phosphodiesterase (0·1 IU mL^{-1}) was added to the reaction mixture consisting of 150 IU mL^{-1} bovine brain calmodulin, 8 mM cyclicAMP, 100 μM $CaCl_2$, 3 mM $MgCl_2$ and 80 mM imidazole-HCl (pH 6·9), and incubated for 60 min at 37°C. Then, the reaction was stopped by a 3-min incubation in boiling water. $MnCl_2$ (4·5 mM) and 5′-nucleotidase (0·56 IU mL^{-1}) were added and incubated for 30 min. The concentration of inorganic phosphate produced was determined by the malachite green method (Itaya & Ui 1966).

Determination of 20-kDa myosin light-chain phosphorylation in strips of guinea-pig taenia caecum

Strips of taenia caecum were frozen by immersion in acetone-dry ice slurry containing 10% trichloroacetic acid and 10 mM dithiothreitol, 0, 0·5 and 10 min after the application of carbachol (100 μM) with or without amlexanox (100 μM). The phosphorylation levels of 20-kDa myosin light chain (MLC_{20}) were determined using the method described by Seto et al (1990). Briefly, myosin was extracted from the strips by a 14-h incubation in sample buffer containing 20 mM Tris base, 23 mM glycine, 10 mM dithiothreitol, 8 M urea and 0·1% bromophenol blue, pH 8·6. The phosphorylated and unphosphorylated MLC_{20} were separated by urea-glycerol polyacrylamide gel electrophoresis (Persechini et al 1986), blotted to Clear Blot Membrane P (ATTO Co., Tokyo, Japan), and labelled with mouse anti-myosin light-chain immunoglobulin M. The separated MLC_{20} was visualized using biotinylated goat anti-mouse immunoglobulin G and streptavidin-alkaline phosphatase. The area of each MLC_{20} band was determined with a scanning densitometer. The extent of MLC_{20} phosphorylation was expressed as a percentage of the total MLC_{20}.

Protein purification

Smooth muscle myosin was prepared from frozen chicken gizzard (50 g) by the method of Ikebe & Hartshorne (1985). The yield was 1·1 mg g^{-1}, and the purified myosin was dissolved in medium (0·5 M KCl, 1 mM $NaHCO_3$, 1 mM dithiothreitol) at a concentration of 5 mg mL^{-1}. Then, 50% glycerol was added, and the stock solution was stored at −80°C.

Myosin light-chain kinase (MLCK) was also prepared from fresh chicken gizzard (50 g) by a modification of the method of Walsh et al (1983). The yield was 0·31 mg g^{-1}, and the purified myosin was dissolved in medium (10 mM Tris-HCl, 50 mM NaCl, 0·2 mM dithiothreitol, 5% sucrose) at a final concentration of 0·31 mg mL^{-1}. The enzyme solution was stored at −80°C.

Myosin light-chain kinase activity assay

The amount of radioactive inorganic phosphorus (^{32}P) incorporated into MLC_{20} by MLCK was determined as an index of MLCK activity. Phosphorylation of MLC_{20} at 30°C was initiated by adding [γ-^{32}P]ATP (12·5 μCi/tube, final concentration 1·0 mM) to assay tubes containing 250 μL of reaction mixture (0·5 mg mL^{-1} myosin, 15 μg mL^{-1} MLCK, 4 μg mL^{-1} bovine brain calmodulin, 1 μM calyculin A, 0·1 mM $CaCl_2$, 4 mM $MgCl_2$, 60 mM KCl, 20 mM Tris-HCl, pH 7·5). The reaction was also initiated without adding $CaCl_2$. Amlexanox (1–100 μM) and wortmannin (10 μM) were added 3 min before [γ-^{32}P]ATP. The reaction was stopped after 60 s by adding 60% trichloroacetic acid. The mixture was left for 10 min at room temperature and centrifuged at 350 *g* for 10 min. The pellet was washed twice with acetone. Then, 10 μL of sample buffer (36·1 mM sodium

lauryl sulphate, 141·9 mM 2-mercaptoethanol, 50·4 mM Tris-HCl (pH 6·8), 2·7 M glycerol, 0·01% bromophenol blue) for SDS-polyacrylamide gel electrophoresis was added. The mixture was left overnight and subjected to SDS-polyacrylamide gel electrophoresis (15% acrylamide) according to the method of Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R-250. The area of the MLC_{20} band was determined with a scanning densitometer. Then, for autoradiography, the gel was dried and exposed to an imaging screen (Molecular Imaging Screen-BI, Bio-Rad, Cambridge, MA) for 42 h. The amount of ^{32}P incorporated in the MLC_{20} was determined as a pixel density with an image analyser (Molecular Imager system GS-363, Molecular Analyst, Bio-Rad, Cambridge, MA). The level of phosphorylation (the ratio of the amount of ^{32}P to the area of the MLC_{20} band) is shown as a percentage of the level in the presence of Ca^{2+} without amlexanox or wortmannin.

Statistical analysis

The results are expressed as mean values ± s.e.m. Student's *t*-test, analysis of variance and multiple comparisons with Sheffe's F-test were used for statistical analysis, with $P < 0.05$ considered significant.

Results

Effects of various inhibitors on contractions induced in rabbit ciliary muscle and guinea-pig taenia caecum

In ciliary muscle, carbachol (1–100 μM) induced biphasic contractions consisting of a phasic and a tonic component in a concentration-dependent manner. As reported previously (Watanabe et al 1998), amlexanox (1–300 μM) that was applied cumulatively during the tonic component of the maximal contraction induced by carbachol (100 μM) significantly inhibited this component at concentrations above 3 μM and reduced it to 10% at 100 μM. Amlexanox noncompetitively inhibited the concentration–response relationship of carbachol (Figure 1). The inhibition was reversible and reproducible.

When amlexanox (100 μM) was applied 10 min before a bolus application of carbachol (100 μM), it decreased the phasic and tonic components by 50% and 60%, respectively. Methylene blue (100 μM) did not affect the inhibitory effect of amlexanox. Cyclopiazonic acid (10 μM) applied 15 min before carbachol did not affect the resting tension of the ciliary muscle preparations but inhibited both components of the carbachol-induced contractions by 30%. Amlexanox and cyclopiazonic acid additively inhibited both components (Table 1). However, diltiazem (3·2 and 32 μM) did not affect carbachol-induced contractions.

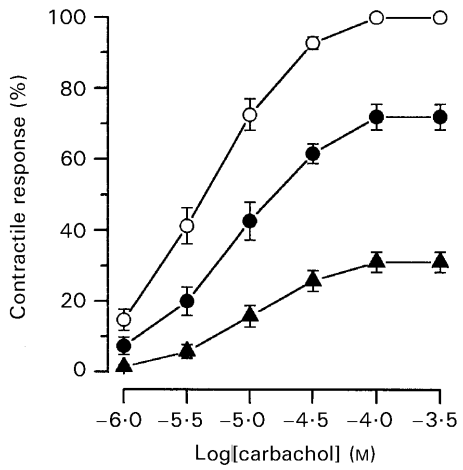

Figure 1. Inhibitory effects of amlexanox on cumulative concentration–response curves for carbachol-induced contractions in rabbit ciliary muscles. Amlexanox (●, 30 μM; ▲, 100 μM) or Krebs-Ringer solution (○, vehicle) was applied 10 min before cumulative application of carbachol (1–300 μM). The magnitude of contraction is expressed as a percentage of the response to the maximal concentration of carbachol alone. Values are mean ± s.e.m., n = 6.

Similar to their effects in ciliary muscle, carbachol induced a biphasic contraction in guinea-pig taenia caecum, and amlexanox inhibited the contractions in a concentration-dependent manner. Furthermore, amlexanox also inhibited K^+-induced contractions similarly to those induced by carbachol (Figure 2).

In taenia caecum, the magnitude of the inhibition of the tonic component of carbachol-induced contraction by amlexanox was markedly greater than that of the phasic component, and 100 μM amlexanox abolished the tonic component (Figure 3A). Diltiazem (3·2 μM) also abolished the tonic component and slightly inhibited the phasic component (Figure 3B).

Thapsigargin (1 μM) slowly increased the resting tension level of the taenia caecum preparations. The contraction induced by thapsigargin obscured its inhibitory effect on carbachol-induced contractions. Since diltiazem (3·2 μM) completely inhibited the contractile response to thapsigargin, as well as the tonic component of the carbachol-induced contraction in guinea-pig taenia caecum, these contractions may be dependent on the influx of extracellular Ca^{2+} via L-type Ca^{2+}-channels. Thus, the effect of deprivation of sarcoplasmic reticulum

Table 1. Additive inhibitory effects of cyclopiazonic acid and amlexanox on the phasic and tonic components of carbachol-induced contractions in rabbit ciliary muscles.

	Inhibition (%)	
	Phasic component	Tonic component
Vehicle	−0·9 ± 3·2	−13·7 ± 2·8
Cyclopiazonic acid	29·9 ± 6·6	32·5 ± 6·5
Amlexanox	46·9 ± 5·8	60·0 ± 1·1
Cyclopiazonic acid + amlexanox	76·0 ± 3·5	87·3 ± 3·6

After a 15-min exposure to cyclopiazonic acid (10 μM), carbachol (100 μM) was applied in the presence of cyclopiazonic acid. When the effect of amlexanox was examined, amlexanox (100 μM) was applied 5 min after the application of either cyclopiazonic acid or vehicle (0·1% DMSO) and then after 10 min contractions were induced by a bolus application of carbachol. The magnitude of inhibitory effects on contraction is expressed as a percentage of the respective component of the standard contraction induced by carbachol (100 μM) alone. Values are means ± s.e.m., n = 8.

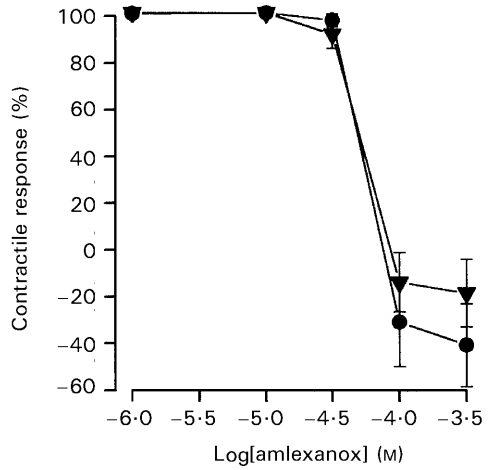

Figure 2. Inhibition of carbachol- and K^+-induced contractions by amlexanox in guinea-pig taenia caecum. Amlexanox was applied cumulatively during the tonic component of the contractions induced by carbachol (●, 100 μM) and K^+ (▼, 100 mM). The magnitude of contraction is expressed as a percentage of the tonic component just before the application of amlexanox. Values are mean ± s.e.m., n = 3.

function by thapsigargin on the carbachol-induced contractions was examined in the presence of diltiazem in taenia caecum preparations. In this experiment, the contractions were induced by application of carbachol (100 μM) three times every 35 min in the presence of either thapsigargin or vehicle (0·01% DMSO) that was applied 5 min before the first application of carbachol. Diltiazem was applied 10 min before each application of carbachol. Thapsigargin reduced the diltiazem-resistant phasic component of carbachol-induced contractions in guinea-pig taenia caecum. The magnitude of inhibition increased depending on number of applications of carbachol. Thapsigargin had little effect on the phasic component of the first contraction, while it inhibited that of the third contraction to 28·3 ± 6·7% (n = 4). Vehicle alone showed no significant effects. Wortmannin (1 μM) inhibited the tonic component of carbachol-induced contractions to a greater extent than the phasic component in taenia caecum, while in ciliary muscle it inhibited both components equally. The magnitude of inhibition of the tonic component in taenia caecum was stronger than that in ciliary muscle (Figure 4). The manner of inhibition was similar to that of amlexanox (Table 1, ciliary muscle; Figure 3A, taenia caecum).

Effects of amlexanox and diltiazem on $^{45}Ca^{2+}$ uptake induced by carbachol

In ciliary muscle, carbachol (100 μM) induced no significant increase in $^{45}Ca^{2+}$ uptake. Amlexanox had no effect on the $^{45}Ca^{2+}$ uptake in either the basal or stimulated state (Table 2).

In taenia caecum, carbachol significantly increased $^{45}Ca^{2+}$ uptake. Diltiazem (3·2 μM) abolished this increase completely, while amlexanox showed no effect (Table 2). Neither diltiazem nor amlexanox reduced the basal $^{45}Ca^{2+}$ uptake.

Effects of amlexanox and forskolin on cyclicAMP level

In the resting state, the cyclicAMP level of ciliary muscle was 39·8 ± 11·2 pmol (mg protein)$^{-1}$ (n = 3) in the presence of IBMX (10 μM). Carbachol (100 μM) did not affect the cyclicAMP level. The data shown in Table 3 were analysed with repeated two-way analysis of variance and Sheffe's F-test ($P < 0·05$). Forskolin (1 μM) significantly increased the amount of cyclicAMP. Amlexanox (1–100 μM) did not significantly affect the cyclicAMP level, although at the highest concentration applied a slight increase was observed (Table 3). The magnitude of the inhibition of carbachol-induced contractions by amlexanox (100 μM) was similar to that by forskolin (1 μM) in ciliary muscle.

Effects of amlexanox on calmodulin activity

Ca^{2+} (100 μM) significantly increased the inorganic phosphate level from 113 ± 8·7 μg mL^{-1} (n = 5) to 343·5 ± 10·0 μg mL^{-1} (n = 5). Amlexanox (100 μM) did not inhibit calmodulin activated by Ca^{2+} (inorganic phosphate level: 379·1 ± 27·2 μg mL^{-1}, n = 5).

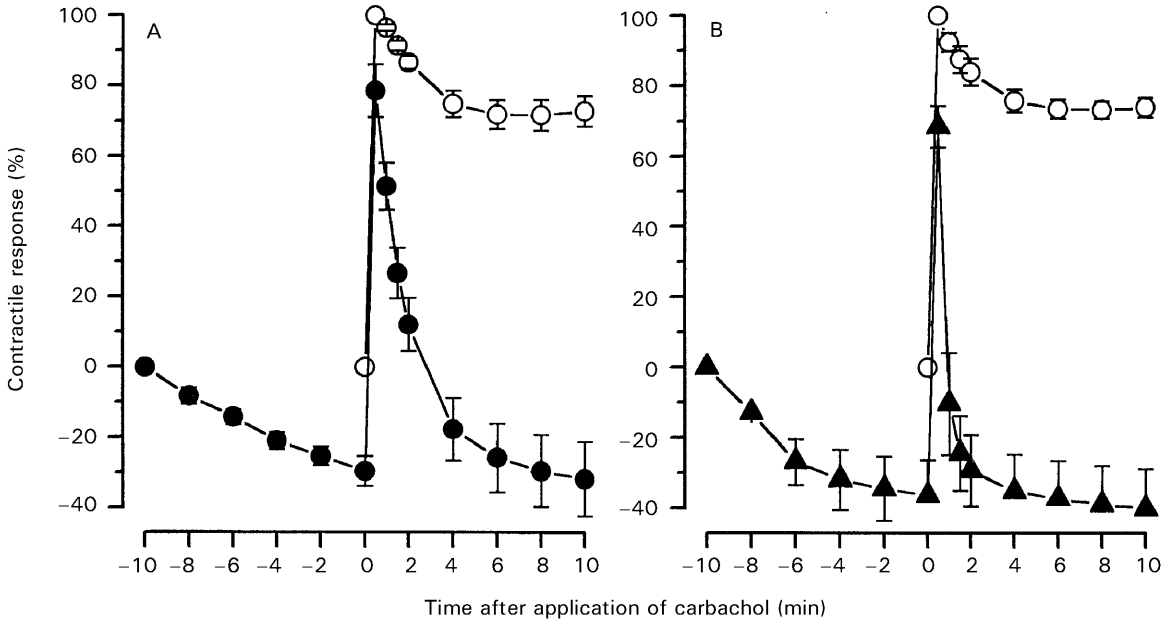

Figure 3. Time course of carbachol-induced contractions before (open symbol) and after (solid symbols) exposure to amlexanox (100 μM, A) and diltiazem (3·2 μM, B) for 10 min in guinea-pig taenia caecum. Carbachol (100 μM) was applied in the presence of the inhibitors. The magnitude of the contraction is expressed as a percentage of the phasic component of the standard contraction induced by carbachol (100 μM) alone. Values are means ± s.e.m., n = 5.

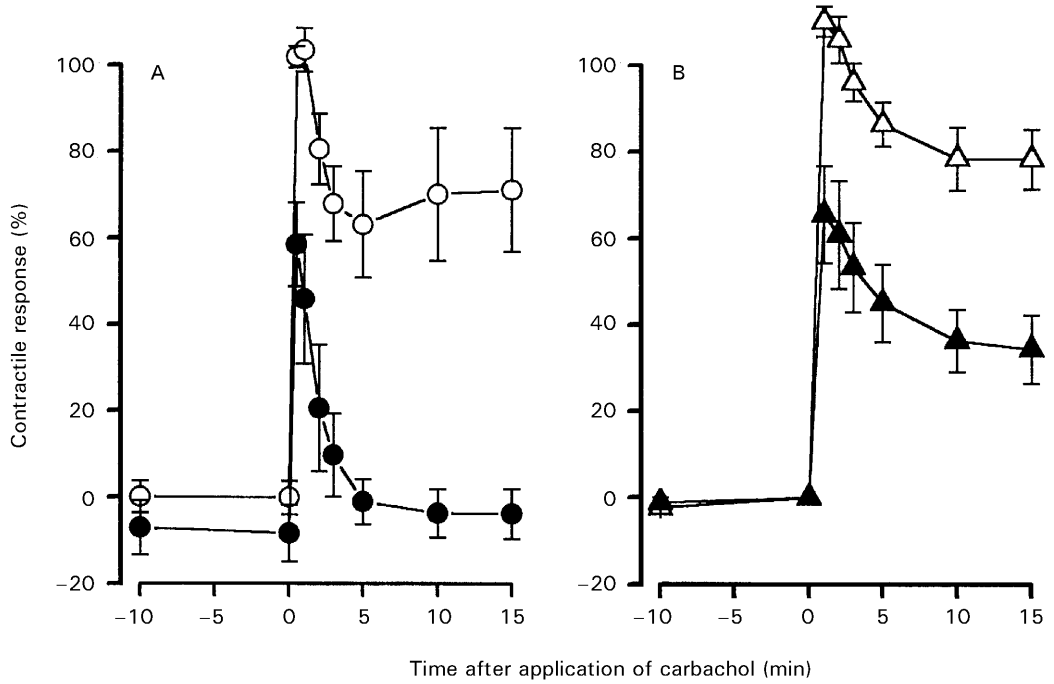

Figure 4. Effects of wortmannin (1 μM) on carbachol-induced contractions in guinea-pig taenia caecum (A) and rabbit ciliary muscle (B). Carbachol (100 μM) was applied 30 min after the application of wortmannin (solid symbols) or vehicle (0·1% DMSO, open symbols). The magnitude of the contractions is expressed as a percentage of the phasic component of the standard contraction induced by carbachol (100 μM) alone. Values are means ± s.e.m., n = 5.

Effects of amlexanox on MLC_{20} phosphorylation

The level of MLC_{20} phosphorylation in strips of taenia caecum increased significantly within 30 s of application of carbachol (100 μM). The increase was slightly reduced 10 min after the application of carbachol. Amlexanox (100 μM) inhibited the phosphorylation (Table 4).

Ca^{2+} increased ^{32}P uptake in purified MLC_{20} in the presence of purified MLCK and calmodulin. The uptake in the absence of Ca^{2+} was 1·9 ± 0·4%

Table 2. Effects of amlexanox and diltiazem on Ca^{2+} uptake by rabbit ciliary muscle and guinea-pig taenia caecum.

	Calcium uptake (μmol kg-wet weight^{-1})	
	Ciliary muscle	Taenia caecum
Basal	125·8 ± 22·9 (12)	61·4 ± 4·9 (18)
Carbachol	152·0 ± 23·8 (13)	94·7 ± 7·5* (17)
Diltiazem		68·1 ± 10·5 (11)
Diltiazem + carbachol		71·6 ± 10·0 (12)
Amlexanox	110·7 ± 34·6 (9)	69·3 ± 13·4 (19)
Amlexanox + carbachol	135·0 ± 29·6 (12)	99·0 ± 19·3*† (18)

$^{45}Ca^{2+}$ (1 μCi mL^{-1}) was applied for 5 min with or without carbachol (100 μM). Amlexanox (100 μM) or diltiazem (3·2 μM) was applied 10 min before the application of carbachol. After incubation in medium containing $^{45}Ca^{2+}$, preparations were washed in ice-cold La^{3+} solution and then solubilized. Tissue counts determined by scintillation spectrometry were converted into tissue Ca^{2+} uptake using the specific activity of the medium, and the results are expressed as μmol/kg wet weight of tissue. Values are means ± s.e.m. The experimental numbers are given in parentheses. *$P < 0.05$ vs basal level of Ca^{2+} uptake. †$P < 0.05$ vs Ca^{2+} uptake in the presence of amlexanox alone.

Table 3. Effects of amlexanox on cyclicAMP levels in the presence of IBMX and carbachol in rabbit ciliary muscles.

		CyclicAMP (pmol (mg protein)$^{-1}$)	
		30-s Incubation	15-min Incubation[a]
Vehicle		39·7 ± 4·0 (4)	39·2 ± 9·8 (4)
Amlexanox	1 μM	30·6 ± 6·9 (3)	38·0 ± 3·9 (3)
	10 μM	34·5 ± 6·9 (3)	40·6 ± 20·3 (3)
	100 μM	55·5 ± 12·7 (4)	49·7 ± 7·0 (5)
Forskolin	1 μM	70·4* (2)	106·0* (2)

[a]When forskolin was applied, the incubation time was shortened to 5 min. After a 5-min exposure to IBMX (10 μM), carbachol (100 μM) was applied in the presence of IBMX for 5 min. Subsequently, at the time point corresponding to the tonic component of the contraction, amlexanox (1–100 μM), forskolin (1 μM) or vehicle (0·1% ethanol) was applied in the presence of IBMX and carbachol. After an appropriate incubation time, preparations were frozen rapidly to stop the reaction. The cyclicAMP content was determined using a radio-immuno assay kit. Values are means ± s.e.m. The experimental numbers are given in parentheses. *$P < 0.05$ vs basal level of Ca^{2+} uptake (Sheffe's F-test).

(n = 4) of that in the presence of Ca^{2+}. Amlexanox (100 μM) significantly reduced the uptake induced by Ca^{2+} to 46·0 ± 5·7% (n = 4), although it was less potent than wortmannin (10 μM), which decreased the uptake to 23·1 ± 6·6% (n = 4).

Discussion

Amlexanox noncompetitively inhibited carbachol-induced contractions in both rabbit ciliary muscle and guinea-pig taenia caecum. Furthermore, amlexanox also inhibited K^+-induced contractions in taenia caecum in a similar manner. These results indicated that the inhibitory effect of amlexanox was not specific to ciliary muscle, and the inhibitor probably acts on a general mechanism common to smooth muscle contractions.

Table 4. Inhibitory effects of amlexanox on carbachol-induced phosphorylation of 20-kDa myosin light chain (MLC_{20}) in guinea-pig taenia caecum.

Time (min)	MLC_{20} phosphorylation (%)	
	Vehicle	Amlexanox
0	1·3 ± 0·8 (11)	2·1 ± 0·6 (11)
0·5	20·0 ± 2·4 (18)	10·5 ± 1·8* (14)
10	14·4 ± 3·6 (15)	4·7 ± 2·2* (13)

Amlexanox (100 μM) or Krebs-Ringer solution (vehicle) was applied 10 min before application of carbachol (100 μM). Phosphorylation of MLC_{20} was determined 0, 0·5 and 10 min after application of carbachol. The degree of phosphorylation is expressed as a percentage of the total amount of phosphorylated and non-phosphorylated MLC_{20} in each preparation. Values are means ± s.e.m. The experimental numbers are given in parentheses. *$P < 0.05$ vs phosphorylation level of MLC_{20} at the corresponding time point without amlexanox.

Makino et al (1987) reported that amlexanox exerts its anti-allergenic action by increasing the intracellular cyclicAMP content of mast cells. In general, an increase in the cyclicAMP content of smooth muscle cells suppresses contractions (Silver & Krafte 1996). In this study, however, forskolin inhibited the carbachol-induced contractions by accelerating adenylate cyclase activity and increasing the cyclicAMP content of ciliary muscle cells, while amlexanox did not increase the cyclicAMP content. Thus, it is unlikely that cyclicAMP contributes to the inhibitory effects of amlexanox on carbachol-induced contractions in ciliary muscles.

An increase in the cyclicGMP content may also induce relaxation in smooth muscles. However, methylene blue did not affect the inhibitory effect of amlexanox. The increase in cyclicGMP by activation of guanylate cyclase may not be involved.

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in smooth muscle is a major determinant of smooth muscle contractility. $[Ca^{2+}]_i$ is primarily increased by Ca^{2+} influx via voltage-dependent L-type Ca^{2+} channels and non-selective cation channels in various smooth muscle cells. In taenia caecum, diltiazem abolished the tonic component of carbachol-induced contractions. The phasic component was resistant to diltiazem, but was markedly reduced by thapsigargin. Thus, the phasic component may

depend predominantly on Ca^{2+} release from the sarcoplasmic reticulum, whereas the tonic component may depend predominantly on Ca^{2+} influx via L-type Ca^{2+} channels. Similarly to diltiazem, high concentrations of amlexanox also abolished the tonic component, but slightly inhibited the phasic component. However, amlexanox did not inhibit the increase in $^{45}Ca^{2+}$ uptake stimulated by carbachol in taenia caecum, while diltiazem did. This suggested that amlexanox may not inhibit contraction via inhibition of Ca^{2+} influx.

In ciliary muscle, neither a significant carbachol-induced increase nor an amlexanox-induced decrease in $^{45}Ca^{2+}$ uptake was detected. Diltiazem did not inhibit carbachol-induced contractions. Thus, similar to human (Stahl et al 1991) and bovine ciliary muscle (Lepple-Wienhues et al 1991; Fujii et al 1997), L-type Ca^{2+}-channels may play a minor role in carbachol-induced contractions in rabbit ciliary muscle.

Ca^{2+} released from the sarcoplasmic reticulum also increases $[Ca^{2+}]_i$. Cyclopiazonic acid, which specifically inhibits Ca^{2+} pumps on the sarcoplasmic reticulum membrane to deplete Ca^{2+} in the sarcoplasmic reticulum at 0·1–10 μM (Suzuki et al 1992; Uyama et al 1992), partially reduced the carbachol-induced contraction in ciliary muscles. Thus, carbachol-induced contractions may be dependent on both Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} influx via non-L-type channels in ciliary muscles. Amlexanox inhibited the contraction even in the presence of cyclopiazonic acid where the sarcoplasmic reticulum function was lost, suggesting that it does not act via inhibition of sarcoplasmic reticulum Ca^{2+} transport. This was supported by the observation that the phasic component of carbachol-induced contraction in taenia caecum depended mainly on Ca^{2+} release from the sarcoplasmic reticulum and was only slightly inhibited by amlexanox.

One consequence of increases in $[Ca^{2+}]_i$ is the activation of calmodulin-dependent MLCK and phosphorylation of MLC_{20}, resulting in smooth muscle contractions. In agreement with the results of a previous study (Oyama et al 1997), amlexanox did not interfere with calmodulin activity. Although we tried to examine the effects of amlexanox on MLC_{20} phosphorylation in ciliary muscles, the antibody used did not react with ciliary muscle MLC_{20}. In contrast, amlexanox inhibited MLC_{20} phosphorylation of guinea-pig taenia caecum. Thus, it is likely that amlexanox inhibited MLCK or activated myosin light-chain phosphatases. To examine the effects of amlexanox on MLCK activity, we determined the ^{32}P uptake into chicken gizzard myosin MLC_{20} induced by purified MLCK. Amlexanox decreased the Ca^{2+}-dependent ^{32}P uptake. Although it did not inhibit MLCK as strongly as wortmannin, the effective concentration range of amlexanox matched the inhibitory effect on contraction. Wortmannin inhibited carbachol-induced contractions in both ciliary muscle and taenia caecum in a similar fashion to amlexanox. These results suggested that the inhibitory effect of amlexanox on MLCK may be involved in suppressing contractions in taenia caecum and probably in ciliary muscles.

In summary, our results suggested that unlike its effect on mast cells amlexanox may not increase the cyclicAMP content in smooth muscle cells. Our results also suggested that amlexanox may not affect either Ca^{2+} mobilization or calmodulin activity, but it may inhibit MLCK resulting in the inhibition of carbachol-induced contraction.

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