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European Journal of Pharmacology 528 (2005) 169–175
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Rho kinase expression and its central role in ovine gallbladder contractions elicited by a variety of excitatory stimuli

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Received 28 April 2005; received in revised form 21 October 2005; accepted 26 October 2005

Abstract

Rho kinase has contractile activity, which induces Ca^{2+} sensitization in various cells. Several receptors are linked to the Rho/Rho-kinase pathway. Therefore, in this study we aimed to demonstrate the central importance of this novel pathway for diverse excitatory stimuli in the smooth muscle of the sheep gallbladder. Accordingly, the effects of a Rho kinase inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, 10^{-8}–3×10^{-5} M), were investigated on cholecystokinin-8 (CCK-8, 10^{-8} M), endothelin-1 (10^{-8} M), carbachol (10^{-6}–10^{-5} M), 5-hydroxytryptamine (5-HT, 10^{-6}–10^{-5} M), histamine (10^{-6}–10^{-5} M), phenylephrine (10^{-5}–10^{-4} M), neurokinin A (10^{-7}–10^{-6} M), electrical field stimulation (40 V, 0.5 ms, 2, 4, 8, 16, 32 Hz, 15 s, 3 min intervals) and potassium chloride (KCl, 25–50 mM)-induced contractions as well as spontaneous contractile activity. Electrical field stimulation evoked tetrodotoxin (3×10^{-6} M)-sensitive reproducible contractions, which were inhibited by atropine (2×10^{-6} M) and potentiated by eserine (5×10^{-7} M). EFS-induced contraction was significantly inhibited by Y-27632 (10^{-5} M). In addition, spontaneous contractile activity was suppressed in the presence of the compound (10^{-6}–10^{-5} M). This Rho kinase inhibitor also dramatically decreased the contractions elicited by 5-HT, neurokinin A and carbachol. KCl-induced contraction, which was not atropine-sensitive, was also conspicuously attenuated by Y-27632. Moreover, Y-27632 (10^{-8}–3×10^{-5} M) relaxed gallbladder strips that were contracted by histamine, endothelin-1, CCK-8 and phenylephrine in a concentration-dependent manner. pEC_{50} values for Y-27632 were 6.25±0.10, 5.79±0.12, 5.83±0.09 and 5.70±0.13 for the contraction elicited by histamine, CCK-8, endothelin-1 and phenylephrine, respectively. Furthermore, we also demonstrated Rho kinase protein expression (ROCK-1 and ROCK-2) by Western blot analysis. In conclusion, ROCK is expressed in the smooth muscle of the ovine gallbladder, and it has a central role in the contractile activity induced by diverse excitatory stimuli.

Keywords: Electrical field stimulation; Rho kinase; Sheep gallbladder smooth muscle; Y-27632

1. Introduction

The gallbladder emptying and refilling cycle depends on an interplay between contractions and relaxations of the gallbladder smooth muscle (Portincasa et al., 2004). Spontaneous muscle activity controls gallbladder smooth muscle tone (Portincasa et al., 2004). Moreover, numerous mediators regulate the physiological control of gallbladder smooth muscle tone, for instance, cholecystokinin-8 (CCK-8) (Stasiewicz et al., 1977), acetylcholine (Shaffer, 2000), endothelin-1 (Al-Jiffry et al., 2001), platelet-activating factor (PAF) (Parkman et al., 2000), tachykinins (Maggi et al., 1989), neurotensin (Yamasato and Nakayama, 1988; Walker et al., 1985), histamine (Jankovic and Beleslin, 1991), 5-hydroxytryptamine (5-HT) (Cox et al., 1988), prostaglandins, (Xiao et al., 2000), noradrenaline, and bradykinin (Johnson, 2003) could act as excitatory stimuli; however, nitric oxide (McKirdy et al., 1994; Sanger et al., 1999), vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase-activating peptide (PACAP), which is also excitatory (Greaves et al., 2000a,b), calcitonin gene-related peptide (CGRP) (Kline and Pang, 1997), thyrotropin releasing hormone (TSH) (Lenz et al., 1993) function as inhibitory mediators. It has been reported that impaired gallbladder motility and increased stasis are key factors in the pathogenesis of biliary cholesterol crystallisation and gallstones (Portincasa

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doi:10.1016/j.ejphar.2005.10.055

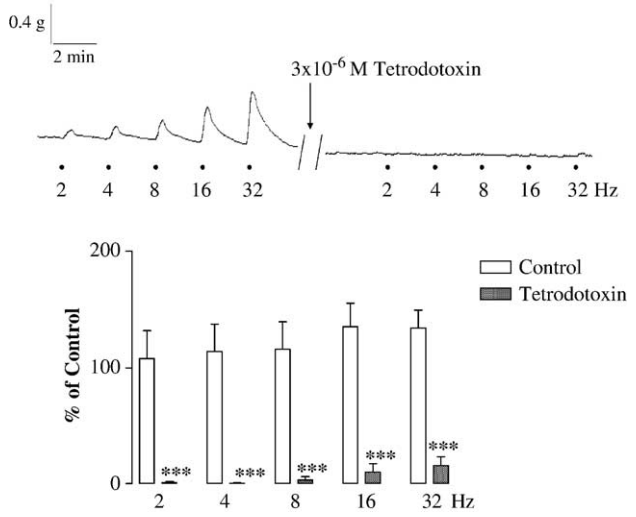

Fig. 1. Original tracing and bar graphs showing the effect of electrical field stimulation (40 V, 0.5 ms, 2, 4, 8, 16, 32 Hz, 15 s, 3 min intervals) on gallbladder smooth muscle and abolition of electrical field stimulation-elicited contraction in the presence of tetrodotoxin (3×10^{-6} M, $n=4$). The second series of responses are expressed as percentages of the responses in the first series. Data show means±S.E.M. Comparison was made by one-way of ANOVA followed by Bonferroni post hoc test. ***$P<0.001$. The break represents the incubation period of 30 min.

et al., 2000). Although many mediators are involved in the control of gallbladder tone and motility, the exact mechanism by which these mediators induce contraction or relaxation, and thus how the emptying and refilling cycle is regulated, has not been clearly explained.

Rho kinase is expressed in diverse types of smooth muscle as well as non-muscle cells, and it mediates calcium (Ca^{2+}) sensitization, whereby contractile force does not parallel the increase in intracellular Ca^{2+} level (Somlyo and Somly, 1994). This can be achieved by inhibition of myosin phosphatase, which dephosphorylates myosin light chain kinase, and consequently results in the inhibition of smooth muscle contraction (Fukata et al., 2001). There are several Rho kinase inhibitors, namely, fasudil and Y-27632 (Uehata et al., 1997; Swärd et al., 2000). Y-27632 in particular has been used to investigate the involvement of this enzyme in cellular events, basically the contraction of smooth muscle and non muscle cells (endothelial, epithelial, blood cells and tumoral cells) because this inhibitor is more selective for ROCK than fasudil (Davies et al., 2000). Recently, ROCK inhibitors have been proposed as smooth muscle relaxants and also potential therapeutic agents in certain pathological states in which smooth muscle tone is increased (Wettschureck and Offermanns, 2002).

Consequently, the aim of this preliminary study was to explore the possible involvement of Rho/Rho kinase signalling in spontaneous phasic activity and in the contractions triggered by different kinds of excitatory stimuli, such as electrical field stimulation, several peptide and non-peptide agonists and potassium chloride. We used Y-27632 for the inhibition of Rho-kinase because it is more selective than fasudil (Davies et al., 2000). Moreover, we demonstrated the expression of Rho-kinase isoforms in sheep gallbladder smooth muscle by Western blotting.

Parts of this study were presented at the National Neuroscience Meeting (Şahan-Fırat et al., 2005).

2. Materials and methods

2.1. Animals and tissue preparation

Sheep gallbladders were obtained from a local slaughterhouse in Mersin, Turkey. Strips (about 3–4 cm long and 2–3 mm wide) were prepared and suspended between two ring electrodes connected to a Biopac stimulator (Biopac system Inc., CA, USA) in organ baths filled with Krebs' solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$, 25, KH_2PO_4 1.2, glucose 11, Na_2EDTA 0.3) gassed with 95% O_2 and 5% CO_2 under an initial tension of 1 g. The bath temperature was maintained at 37 °C. Tension was recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac system Inc., CA, USA).

2.2. Organ bath experiments

Following an equilibration period of 1 h, strips of sheep gallbladder were contracted by electrical field stimulation (EFS, 40 V, 0.5 ms, 2, 4, 8, 16 and 32 Hz, for 15 s, 3 min intervals), carbachol (10^{-6}–10^{-5} M), 5-hydroxytryptamine (5-HT, 10^{-6}–10^{-5} M), neurokinin A (10^{-7}–10^{-6} M) or potassium chloride

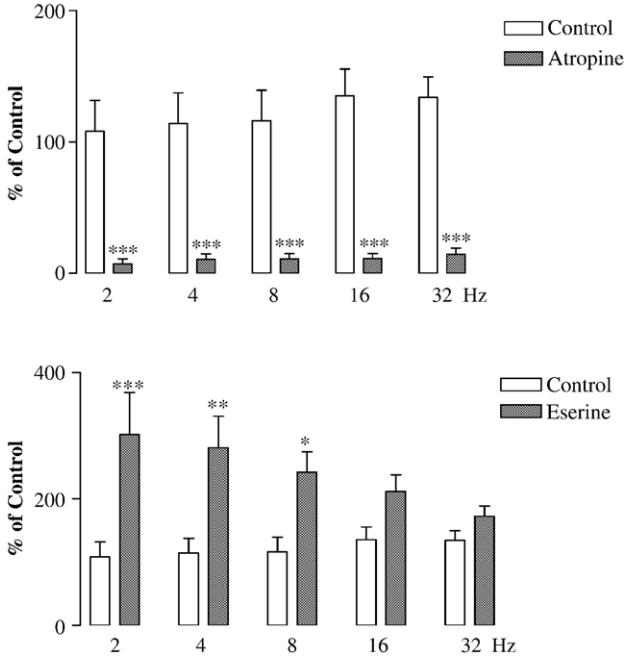

Fig. 2. Inhibition and potentiation of electrical field stimulation-induced contraction by atropine (2×10^{-6} M, $n=6$, upper panel) and eserine (5×10^{-7} M, $n=5$, lower panel), respectively. The second series of responses are expressed as percentages of the responses in the first series. Data show means±S.E.M. Comparison was made by one-way of ANOVA followed by Bonferroni post hoc test. *$P<0.05$, **$P<0.01$, **$P<0.001$.

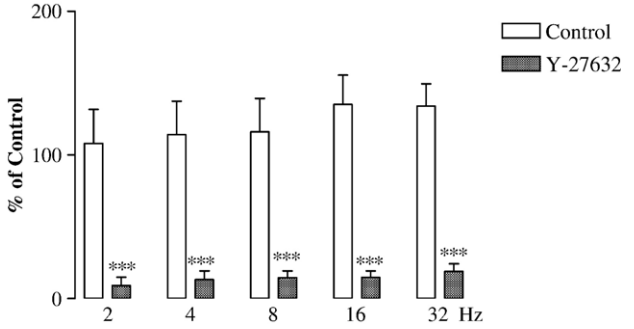

Fig. 3. Effect of a Rho kinase inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, 10^{-5} M, $n=6$) on electrical field stimulation-evoked contractile responses. The second series of responses are expressed as percentages of the responses in the first series. Data show means ± S.E.M. Comparison was made by one-way of ANOVA followed by Bonferroni post hoc test. ***$P<0.001$.

(KCl, 25–50 mM). These contractions were regarded as the first contraction series. After being washed with fresh Krebs solution, the strips were incubated for 1 h. Thereafter they were contracted in the same manner (the second series). In another series of experiments between the first and the second series, the strips were incubated with Y-27632 (10^{-5} M, 30 min), atropine (2×10^{-6} M for 30 min), eserine (5×10^{-7} M, for 30 min) or tetrodotoxin (3×10^{-6} M for 30 min). In another series of experiments, the effects of Y-27632 (10^{-8}–3×10^{-5} M, cumulatively) were also tested on histamine (10^{-5} M), CCK-8 (10^{-8} M), phenylephrine ($5\times0\,10^{-5}$ M) and endothelin-1 (10^{-8} M)-induced contractions. Carbachol, phenylephrine, neurokinin A and 5-HT did not induce stable tonic contractions, so the strips were incubated with Y-27632 for 30 min between the two series. Some strips had spontaneous contractile activity. In such preparations, Y-27632 (10^{-6} and 10^{-5} M) was tested on this contraction. The concentrations and incubation duration of the agents used in this study were determined after preliminary experiments.

2.3. Western blot analysis for Rho-kinase

The sheep gallbladder smooth muscle was homogenized with lysis buffer (composition in mM; Tris–HCl (pH = 7.4) 50 mM, NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulfonyl fluoride 10 μM, leupeptin 10 μg/ml, pepstatin 1 μg/ml, benzamidine 1 mM). The homogenate was centrifuged at 2000 ×*g* for 10 min at 4 °C to remove nuclei and unlysed cells, and the supernatant was removed. It was then used for protein analysis (with Lowry method) and Western blot analysis. Equal amounts of protein (250 μg) were loaded in wells, electrophoresed on 8% polyacrylamide-sodium dodecyl sulphate (SDS) gels and then transferred to a nitrocellulose membrane overnight. The membrane was blocked with the blocking agent of the enhanced chemiluminescence (ECL advance) kit (Amersham Biosciences, Freiburg, Germany) in Tris-buffered solution containing 0.05% Tween-20 (TBS-T) for 1 h. It was then probed with primary antibodies raised against ROCK-1 (ROKβ) or ROCK-2 (ROKα, Polyclonal IgG, Santa Cruz Biotechnology Inc., CA, USA) at 1 : 200 dilution followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1 : 1000, Santa Cruz Biotechnology Inc., CA, USA). The blots were then detected with the advanced chemiluminescence detection kit (Amersham Biosciences, Freiburg, Germany) and visualized on commercial X-ray film.

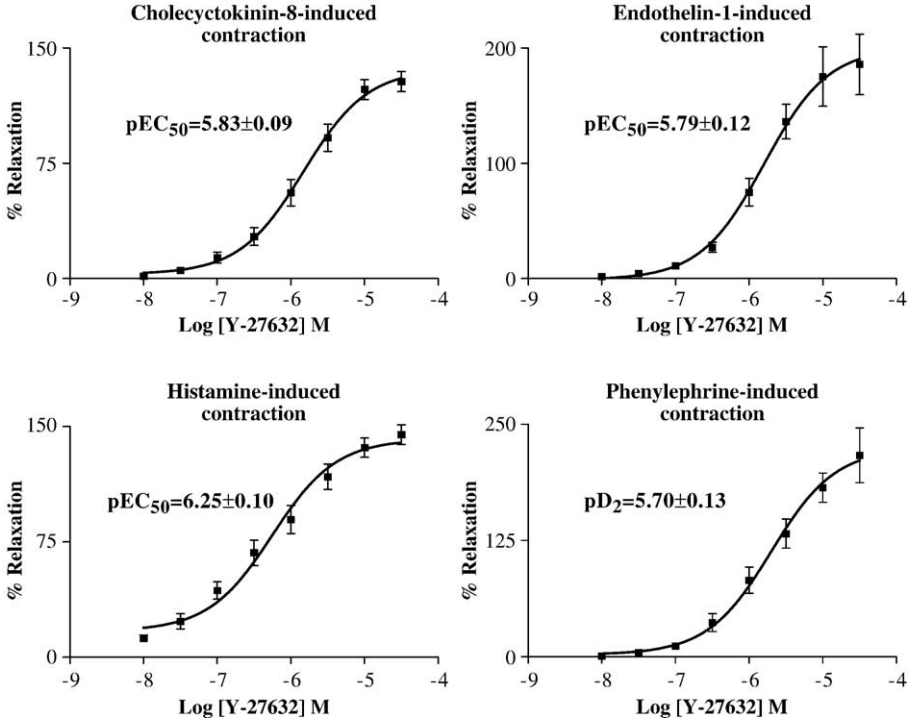

Fig. 4. Relaxant effects of Y-27632 (10^{-8}–3×10^{-5} M, cumulatively, $n=7$–10) on different excitatory agent-induced tone. The responses are expressed as percentage reduction of the tonic contracture induced by CCK-8 (10^{-8} M), endothelin-1 (10^{-8} M), histamine (10^{-5} M), phenylephrine (5×10^{-5} M). Data show means ± S.E.M.

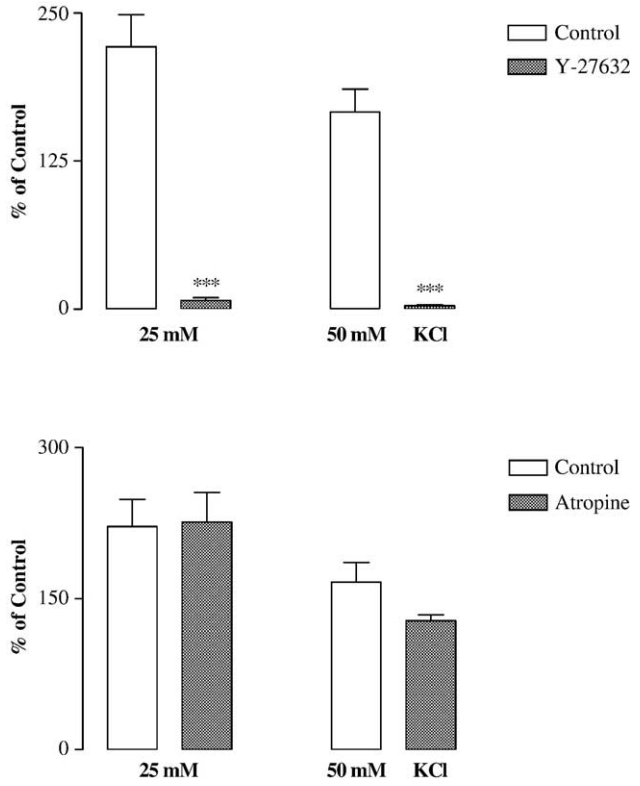

Fig. 5. Effects of Y-27632 (10^{-5} M, $n=10$ and atropine (2×10^{-6} M, $n=6$) on KCl (25 and 50 mM)-induced contractions. The second series of responses are expressed as percentages of the responses in the first series. Data show means± S.E.M. Comparison was made by one-way of ANOVA followed by Bonferroni post hoc test. ***$P<0.001$. Note that KCl-induced contractions were inhibited by Y-27632 but not by atropine.

2.4. Chemicals used

Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA) disodium, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), leupeptin, benzamidine, Tris–HCl, carbamylcholine chloride (carbachol), histamine dihydrochloride, eserine (physostigmine salicylate), 5-hydroxytryptamine bitartarate, phenylephrine HCl and atropine sulfate were obtained from Sigma Chemical Co (St. Louis, USA). (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632), cholecystokinin-8 sulphate (CCK-8S), endothelin-1 were obtained from Tocris Cookson Ltd (Bristol, UK). Potassium chloride (KCl), glycine and dodecyl sulphate sodium salt (SDS) were purchased from Merck Co (Darmstadt, Germany), and tetrodotoxin from Alomone (Jerusalem, Israel). Primary antibody for ROCK-2 and HRP-conjugated secondary antibody were obtained from Santa Cruz Biotechnology Inc. (CA, USA). ECL advance kit was purchased from Amersham Biosciences (Freiburg, Germany). The kit was used according to the manufacturer's guide. Potassium chloride, cholecystokinin-8 sulphate, endothelin-1, phenylephrine HCl, 5-hydroxytryptamine bitartarate, histamine 2HCl and Y-27632 were dissolved in distilled water.

2.5. Statistical evaluations

All data represent means±standard error of the mean (S.E.M.) of *n* observations. The second series of contractions are expressed as a percentage of the first series of contractions. Relaxations in response to Y-27632 were evaluated as percent reductions of active tone induced by the agonists. For statistical comparison, one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or Student *t* test, if appropriate, was used. A *P* value less than 0.05 was considered significant. Graphs were drawn by use of GraphPad Prism 3.0 program (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Effects of EFS on the gallbladder smooth muscle

EFS (40 V, 0.5 ms, 2, 4, 8, 16, 32 Hz, 15 s, 3 min intervals) induced frequency-dependent reproducible contractions, which were abolished by tetrodotoxin (3×10^{-6} M, $n=4$, Fig. 1). These

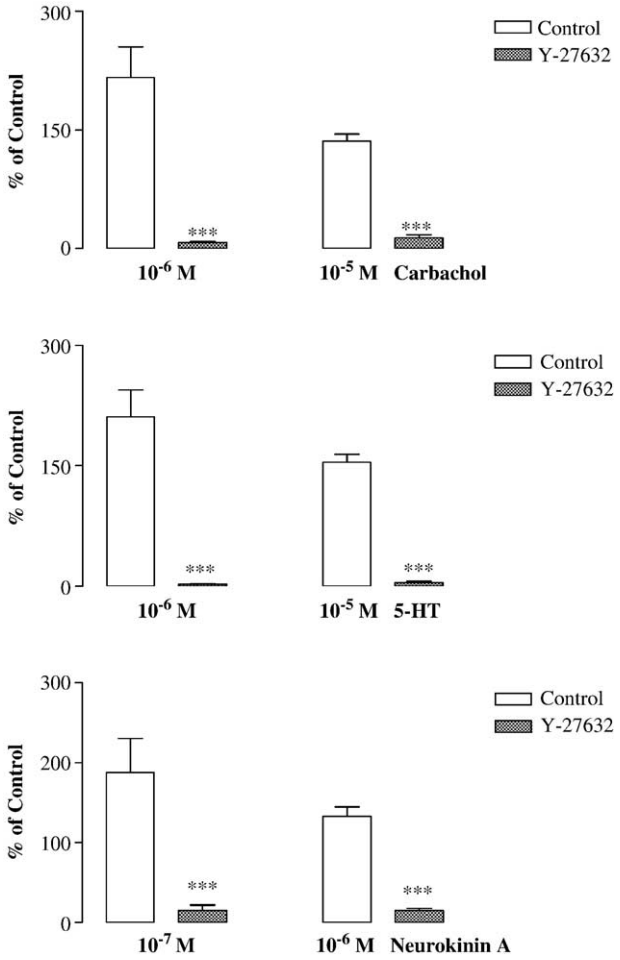

Fig. 6. Effects of Y-27632 (10^{-5} M) on carbachol (upper panel), 5-hydroxytryptamine (5-HT, central panel) and neurokinin A (lower panel)-elicited contractions. The second series of responses are expressed as percentages of the responses in the first series. Data show means±S.E.M. Comparison was made by one-way of ANOVA followed by Bonferroni post hoc test. ***$P<0.001$.

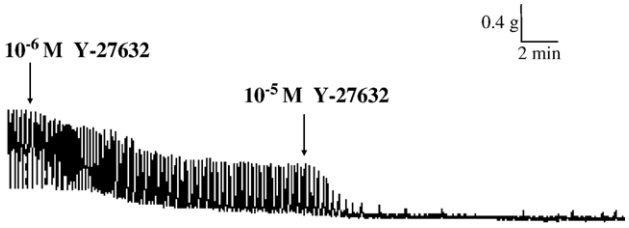

Fig. 7. Original tracing showing the effects of (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, 10^{-6}–10^{-5} M) on spontaneous contractile activity in the sheep gallbladder. Note that Y-27632 not only abolished the phasic contractions but also decreased the resting tone of the tissue.

contractions were significantly suppressed by atropine (2×10^{-6} M, $n=7$) and potentiated by eserine (5×10^{-7} M, $n=5$), respectively (Fig. 2).

3.2. Effects of Y-27632 on spontaneous rhythmic activity and on electrical field stimulation, 5-hydroxytryptamine, histamine, carbachol, cholecystokinin-8, phenylephrine, neurokinin A, endothelin-1 and potassium chloride-induced contractions

The Rho-kinase inhibitor, Y-27632 (10^{-5} M), produced a significant inhibition of EFS-elicited contractions (Fig. 3). It (10^{-8}–3×10^{-5} M) relaxed gallbladder strips constricted by histamine, cholecystokinin-8 (CCK-8), phenylephrine and endothelin-1 in a concentration-dependent manner (Fig. 4). In addition, Y-27632 substantially inhibited the contraction induced by KCl, which was not changed in the presence of atropine (2×10^{-6} M) (Fig. 5). The ROCK inhibitor almost abolished the contractions elicited by neurokinin A, 5-HT and carbachol (Fig. 6). Some gallbladder strips spontaneously produced phasic contractile activity. In such tissues, Y-27632 also suppressed this spontaneous rhythmic contraction at 10^{-6}–10^{-5} M (Fig. 7).

3.3. Expression of two isoforms of Rho-kinase in the sheep gallbladder smooth muscle

Western blot analysis revealed that both ROCK-1 and ROCK-2 were expressed in the homogenates of sheep gallbladder (Fig. 8).

4. Discussion

The most important findings of the present study were the demonstration of Rho-kinase (ROCK-1 and -2) expression in the ovine gallbladder and the central role of this enzyme in the contraction elicited by a diverse array of excitatory stimuli. In this study, the observation that the EFS-induced reproducible contraction was abolished by tetrodotoxin supports the existence of neurally mediated contraction of gallbladder smooth muscle. The EFS-elicited contraction was also significantly suppressed by atropine and potentiated by eserine, suggesting that cholinergic nerves could mediate EFS-induced responses. The ROCK inhibitor, Y-27632, also dramatically attenuated this contraction. This is of physiological relevance since the gallbladder emptying and filling cycle appears to be largely neurally regulated (Johnson, 2003). Furthermore, Y-27632 not only suppressed the amplitude of spontaneous contractile activity but it also reduced the resting tone of the gallbladder. A cholinergic agent, carbachol, also induced contraction of the sheep gallbladder, but this contraction was not sustained. Y-27632 effectively inhibited the contraction, indicating that cholinergic receptors are coupled with Rho signalling, as was previously reported in the mouse gastric fundus (Büyükafşar and Levent, 2003). It has been reported that gallbladder smooth muscle possesses at least four different muscarinic receptor subtypes, i.e., M_1, M_2, M_3 and M_4 (von Schrenck et al., 1993; Oktay et al., 1998). However, the main muscarinic receptors mediating gallbladder contractility are M_3 and to a lesser extent M_2 subtypes (Stengel and Cohen, 2002). Although M_3 muscarinic receptors are coupled with the formation of inositol phosphates to cause contraction of gallbladder smooth muscle (von Schrenck et al., 1994), we suggest that muscarinic cholinoceptors could also be linked to Rho signalling.

It has been reported that cholecystokinin is the major determinant of gallbladder emptying, acting on both preganglionic cholinergic nerves and smooth muscle (Behar and Biancani, 1980). The receptor mediating CCK-8-induced contraction of the human gallbladder smooth muscle seems to be the CCK_1 subtype (Morton et al., 2002). However, we did not investigate which type of CCK (CCK_1 and/or CCK_2) receptor is responsible for the CCK-8 effect, which is sensitive to Y-27632 inhibition. We also did not investigate whether CCK-8 could release acetylcholine from cholinergic nerve endings. However, it is clear that the CCK-8-induced contraction can be suppressed by the ROCK inhibitor, regardless of

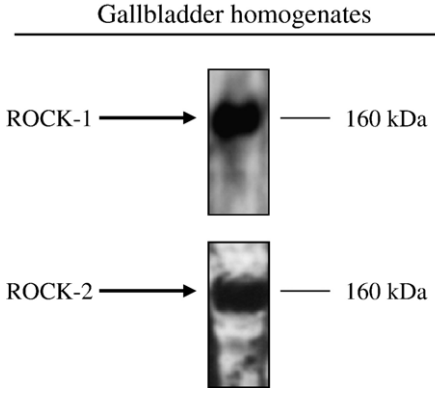

Fig. 8. Demonstration of the expression of ROCK-1 and ROCK-2 in the sheep gallbladder homogenates by Western blotting. The homogenates were submitted to sodium dodecyl sulphate (SDS)–PAGE with 8% polyacrylamide and then transferred to a nitrocellulose membrane (0.22 μm, Santa Cruz). The membrane was blocked with the blocking agent of the enhanced chemiluminescence advance kit (ECL advance) in Tris-buffered solution containing 0.05% Tween-20 (TBS-T) for 1 h. It was then probed with a primary antibody raised against ROCK-1 and ROCK-2 (Polyclonal IgG, Santa Cruz) at 1 : 200 dilution, followed by horseradish peroxidase-conjugated secondary antibody (donkey antigoat, 1 : 1000). Blots were then detected with an Enhanced Chemiluminescence Detection Kit (ECL Advance, Amersham Bioscience) and visualized on commercially available X-ray films.

whether CCK-8 affects gallbladder smooth muscle directly or indirectly.

Endothelin-1 induced slow-developing, stable contractile activity in this study. It has been suggested that ET_A and ET_B receptors mediate contraction in human and possum gallbladder smooth muscle (Al-Jiffry et al., 2001). The Rho kinase inhibitor relaxed the endothelin-1-induced contraction, suggesting that Rho/Rho-kinase signalling is involved in endothelin-1-elicited signal transduction, as we have recently reported (Büyükafşar et al., 2004). Tachykinins have been also reported to be excitatory mediators; in guinea pig gallbladder, both neurokinin A and substance P produced a concentration-related contraction (Maggi et al., 1989). Although in the sheep gallbladder only substance P induced a comparable contraction to that induced by carbachol (Tucci et al., 2003), in this study neurokinin A also induced a substantial contraction, and this contraction was conspicuously suppressed in the presence of Y-27632. This may show that tachykininergic receptors are associated with the Rho/Rho kinase pathway.

Histamine is an inflammatory mediator present in mast cells, which are abundant in the wall of the gallbladder, and mediates contraction via H_1 histaminergic receptors (Hemming et al., 2000). In this study, histamine also induced contraction and this response was antagonized by Y-27632, indicating that Rho/Rho-kinase signalling is involved in the histaminergic excitatory receptor(s)-induced action. Another contractile agent is 5-HT in this study, it elicited phasic contractile activity, which was substantially inhibited by the ROCK inhibitor. The receptors mediating 5-HT-induced contraction in the gallbladder have yet to be investigated. We did not classify which subtypes of 5-HT receptors mediated the serotonin-induced contraction in gallbladder smooth muscle, and this should be investigated in a detailed study, given the diversity of 5-HT receptors. Nevertheless, as indicated by others (Cox et al., 1988; Emre-Aydingöz et al., 2001), 5-HT induced contraction, and this contraction was considerably attenuated in the presence of Y-27632, revealing excitatory 5-HT receptors coupled with this pathway. Phenylephrine also induced tonic contraction in this study, and Y-27632 relaxed the strips. Excitatory α-adrenoceptors are proposed to exist in the gallbladder (Nakata and Kurahashi, 1981). Therefore, these receptors may be coupled with the Rho/Rho kinase pathway, as is also suggested to occur in nonvascular smooth muscle (Büyükafşar et al., 2003; Levent and Büyükafşar, 2004). Although it has been reported that pituitary adenylyl cyclase-activating peptide and sodium nitroprusside elicit contraction in guinea pig and canine gallbladder (Greaves et al., 2000a,b; Alcon et al., 2001), these compounds up to 10^{-7} and 10^{-2} M, respectively, did not induced contraction at all in this study (data not shown).

In conclusion, we have for the first time demonstrated that ROCK isoforms are highly expressed in the ovine gallbladder, and that this enzyme plays a central role in the actions of a diverse array of excitatory stimuli, such as endothelin-1, cholecystokinin-8, histamine, 5-hydroxytryptamine, neurokinin A, phenylephrine, potassium, electrical field stimulation as well as spontaneous contractile activity. Moreover, the results of this study provide not only new insight into the physiological control of gallbladder smooth muscle contractility but also clues for the formation of gallstones, because poor contraction and impaired pumping of the gallbladder ingredients are implicated in gallstone formation (Portincasa et al., 2004).

Acknowledgement

This work has been supported by the Turkish Academy of Sciences, in the framework of the Young Scientist Award Program (K.B./TÜBA-GEBİP/2002-1-5).

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