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Contribution of Rho-kinase in human gallbladder contractions

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Abstract

Rho/Rho-kinase-mediated pathway has been involved in a variety of physiological processes, including Ca^{2+} sensitization, which enhances smooth muscle contraction. In this study, first of all we investigated the expression of Rho-kinase (ROCK-2) and then the role of this protein in the control of smooth muscle contraction in the isolated human gallbladder. For this purpose, we examined the effects of a selective Rho-kinase inhibitor, (+)- (*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, $10^{-8}-3\times10^{-5}$ M) on carbachol (10^{-8}–10^{-4} M), cholecystokinin-8 (10^{-8} M), endothelin-1 (10^{-8} M), histamine (10^{-5} M), neurokinin A (10^{-7}–10^{-6} M), 5-hydroxytryptamine (10^{-6}–10^{-5} M) and potassium chloride (KCl, 25–50 mM)-induced contractions as well as spontaneous contractile activity. Y-27632 (10^{-5} M) significantly reduced 5-hydroxytryptamine, neurokinin A and KCl-induced contractions. Moreover, this Rho-kinase inhibitor ($10^{-8}-3\times10^{-5}$ M, cumulatively) relaxed the contractions produced by cholecystokinin-8, endothelin-1 and histamine in a concentration-dependent manner, being the pEC_{50} values for Y-27632 5.74±0.12, 5.33±0.09 and 5.95±0.18, respectively. Carbachol (10^{-8}–10^{-4} M) pfroduced concentration-dependent contractions, which were also inhibited significantly by Y-27632. In addition, the spontaneous contractile activity was suppressed in the presence of Y-27632 (10^{-6}–10^{-5} M). Moreover, Western blot analysis has revealed that Rho-kinase is expressed in homogenates of the human gallbladder. Taken together, these results show that Rho-kinase is expressed in the human gallbladder, and it has an essential role in agonists and depolarization-induced contractions as well as spontaneous contractile activity.

Keywords: Carbachol; Cholecystokinin; Human gallbladder; Rho-kinase; Y-27632

1. Introduction

Gallbladder is an alimentary organ that stores and transfers hepatic bile into the cystic duct during the digestive process (Shaffer et al., 1980). Physiologically, gallbladder contractions and relaxations are regulated by different kinds of mediators, such as tachykinines (Maggi et al., 1989), 5-hydroxytryptamine (Cox et al., 1988), histamine (Jankovic and Beleslin, 1991), cholecystokinin-8 (Stasiewicz et al., 1977), endothelin-1 (Al-Jiffry et al., 2001), prostaglandins (Xiao et al., 2000), nitric oxide (McKirdy et al., 1994; Sanger et al., 1999), acetylcholine (Shaffer, 2000), calcitonine gene-related peptide (Kline and Pang, 1997), tryrotropin releasing hormone (Lenz et al., 1993), bradykinin (O'Riordan et al., 2001; Johnson, 2003), noradrenaline (Feeley et al., 1987), platelet-activating factor (Guarino et al., 2003; Parkman et al., 2000), neurotensin (Guo et al., 1989a,b), pituitary adenylyl cyclase-activating peptide (Parkman et al., 1997) and progesterone (Kline and Karpinski, 2005). However, post receptor events, which are coupled with the receptors for these mediators have yet to be fully understood.

The small GTPase Rho, a member of the Rho subfamily of the Ras superfamily of monomeric GTPases, is responsible for the Ca^{2+} sensitization. This small G protein stimulates its downstream effector, Rho-kinase (ROCK, ROK), a serine–threonine kinase. It phosphorylates to inhibit myosin phosphatase, which is liable to dephosphorylate the phosphorylated myosin light chain to induce the inhibition of smooth muscle contraction (Fukata et al., 2001). It has been reported that the Rho/Rho-kinase pathway could be involved in the contractile activity of various nonvascular tissues, such as gastric fundus (Büyükafşar and Levent, 2003), vas deferens (Büyükafşar et al., 2003a,b), corpus cavernosum (Büyükafşar and Ün, 2003), uterus (Tahara et al., 2002), urinary bladder (Wibberley et al., 2003) and ureter (Levent and

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Büyükafşar, 2004). However, there are no papers regarding the role of Rho/Rho-kinase cascade in the contractile activity of the human gallbladder. For this purpose, we first demonstrated the expression of Rho-kinase protein by Western blot analysis and then obtained functional data with a selective Rho-kinase inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) by organ bath experiments.

2. Materials and methods

2.1. Tissue preparation

The protocol of this study was approved by the local ethical committee of the Medical Faculty, Mersin University. Human gallbladders were obtained from the Department of General Surgery, Research and Application Hospital of Medical Faculty, Mersin University. They were obtained from the patients with no malignancy (female and male, age: 60±8 years old, $n=28$) undergoing laparoscopic cholecystectomy for gallstone disease. Connective and fat tissues were cleared off. Strips (about 3–4 cm long and 2–3 mm wide) were prepared from fundal part of the tissue 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 0.01) 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. Experimental protocol

Following an equilibration period of 1 h, strips of the human gallbladder were contracted by 5-hydroxytryptamine (5-HT, 10^{-6}–10^{-5} M), neurokinin A (10^{-7}–10^{-6} M) or potassium chloride (KCl, 25–50 mM). These contractions were regarded as the first contraction series. After washing baths with Krebs solution, the strips were left to equilibrate for 1 h and then they were contracted again 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). In another series of experiments, relaxant effect of Y-27632 ($10^{-8}-3\times10^{-5}$ M, cumulatively) was also tested on histamine (10^{-5} M), cholecystokinin-8 (10^{-8} M) and endothelin-1 (10^{-8} M)-induced contractions cumulatively. Y-27632 (10^{-6} and 10^{-5} M) was also tested on strips having spontaneous contractile activity. In other group of experiments, carbachol-elicited concentration-dependent (10^{-8}–10^{-4} M) contraction. This contraction was evaluated in the presence of Y-27632 (10^{-5} M). The concentrations and incubation duration of the agents used in this study were determined after preliminary experiments.

2.3. Western blotting for Rho-kinase

Small pieces of the human gallbladder were homogenized with a 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, and the supernatant was removed. It was then used for protein analysis (with Lowry method) and Western blot analysis. Equal amounts of the 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 for 1 h. It was then probed with a primary antibody raised against ROCK-2 (ROKα, Polyclonal IgG, Santa Cruz Biotechnology Inc., CA, USA) at 1:200 dilution followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (donkey antigoat, 1:1000, Santa Cruz Biotechnology Inc., CA, USA). Protein blots were then detected with the advanced chemiluminescence detection kit (Amersham Biosciences, Freiburg, Germany) and visualized on commercial X-ray film.

2.4. Drugs and chemicals

5-hydroxytryptamine bitartarate, histamine dihydrochloride and carbamylcholine chloride (carbachol) were obtained from Sigma Chemical Co (St. Louis, USA). (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632), endothelin-1, cholecystokinin-8 sulphate (CCK-8S) were obtained from Tocris Cookson Ltd (Bristol, UK). Primary antibody for ROCK-2 and HRP-conjugated secondary antibody were obtained from Santa Cruz Biotechnology Inc. (CA, USA). The ECL Advance kit was purchased from Amersham Biosciences (Freiburg, Germany). The kit was used according to the manufacturer's guide. Y-27632, potassium chloride, 5-hydroxytryptamine bitartarate, endothelin-1, histamine dihydrochloride and cholecystokinin-8 sulphate 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 were expressed as percentages of the first series of contractions. Relaxations to Y-27632 were evaluated as percent reductions of active tone induced by agonists. For statistical comparison, one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or Student's *t* test, if appropriate, was used. A *P* value less than 0.05 was considered significant. Graphs were drawn by the use of a GraphPad Prism 3.0 program (GraphPad software, San Diego, CA, USA).

3. Results

3.1. Expression of Rho-kinase (ROCK-2 isoform) in human gallbladder

ROCK-2 protein (approximately 160 kDa) expression was demonstrated by Western blot analysis in homogenates of the human gallbladder (Fig. 1).

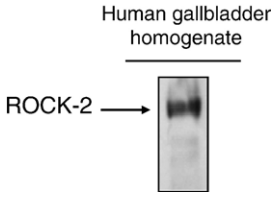

Fig. 1. Western blotting for Rho-kinase (ROCK-2, ROKα) in human gallbladder. Homogenates of the tissue were submitted to sodium dodecyl sulphate (SDS)-PAGE with 8% polyacrylamide and then transferred to a nitrocellulose membrane (0.22 μm, Santa Cruz, USA). The membrane was blocked with an enhanced chemiluminescence (ECL) blocking agent in Tris-buffered solution containing 0.05% Tween-20 (TBS-T) for 1 h. Thereafter, it was probed with a primary antibody raised against ROCK-2 (Polyclonal IgG, Santa Cruz) at 1:200 dilution, followed by horseradish peroxidase (HRP)-conjugated secondary antibody (donkey antigoat, 1:1000). Blots were then detected with the ECL detection Kit (Amersham Bioscience) and visualized on commercially available X-ray films.

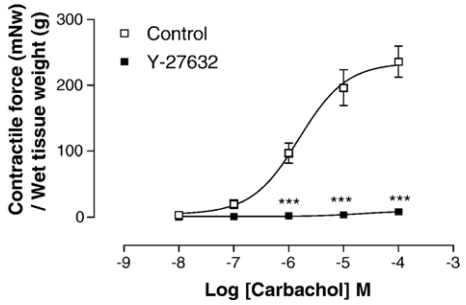

Fig. 3. Effect of the Rho-kinase inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, 10^{-5} M) incubation (for 45 min) on a cholinergic agent, carbachol-induced contractions. The contractions are expressed as mN of the wet tissue weight (g). Data show means±S.E.M. ***: $P<0.001$ and comparison was made by one-way ANOVA, followed by the Bonferroni post hoc test (for lower panel).

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

Y-27632 (10^{-6}–10^{-5} M) significantly suppressed spontaneous rhythmic contractile activity (Fig. 2). Carbachol-elicited concentration-dependent contractions were inhibited by Y-27632 (10^{-5} M) (Fig. 3). This Rho-kinase inhibitor ($10^{-8}-3\times10^{-5}$ M) also produced relaxation on histamine (10^{-5} M), endothelin-1 (10^{-8} M) and cholecystokinin-8 (10^{-8} M)-induced contractions in a concentration-dependent manner (Fig. 4). Furthermore, Y-27632 (10^{-5} M) considerably inhibited the contractions produced by 5-hydroxytryptamine (10^{-6}–10^{-5} M) and neurokinin A (10^{-7}–10^{-6} M) (Fig. 5). In addition, KCl-induced contraction was markedly attenuated by Y-27632 (Fig. 5).

4. Discussion

In the present study, we investigated possible involvement of the Rho/Rho-kinase pathway in spontaneous contractile activity, depolarization-induced contraction as well as several excitatory agonists-induced contractions of the human gallbladder. Furthermore, we demonstrated the expression of Rho-kinase (ROCK-2 isoform) by Western blotting.

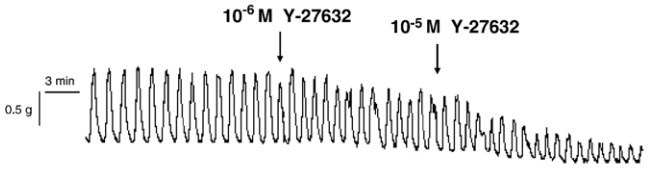

Fig. 2. The original tracing showing the effects of a Rho-kinase inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632, 10^{-6}–10^{-5} M) on spontaneous contractile activity in human gallbladder. It has both suppressed the resting tone of the spontaneous contractile activity and the amplitude of the gallbladder contraction.

It has been known that spontaneous rhythmic activity of the gallbladder is tetrodotoxin-insensitive and myogenic in origin (Mancinelli et al., 1989). This rhythmic activity is substantially attenuated by the ROCK inhibitor in this study. Furthermore, Y-27632 also suppressed basal tone of the tissue. This seems to be of physiological relevance in gallbladder emptying and refilling process, although the exact nature of the activity has yet to be established. Moreover, carbachol-elicited gallbladder contraction was also significantly attenuated by Y-27632, suggesting that cholinergic receptors are coupled with Rho A and its downstream effector, Rho-associated kinase as previously reported in the ovine gallbladder (Şahan-Fırat et al., 2005). Muscarinic M_1, M_2, M_3 and M_4 receptor subtypes modulate gallbladder contraction by directly acting on smooth muscle and by regulating release of acetylcholine from cholinergic nerves (Parkman et al., 1999). It has recently demonstrated that muscarinic M_3 receptors could be linked to Rho and Rho-associated kinase in human urinary bladder (Schneider et al., 2004). However, we did not investigate which subtypes are related with the Rho signalling.

Cholecystokinin is an important mediator of the gallbladder motor function (Morton et al., 2002). It has been reported that cholecystokinin has a presynaptic facilitator effect on fast synaptic transmission acting on preganglionic cholinergic nerves and, this effect is mediated by presynaptic cholecystokinin CCK_1 receptors (Mawe, 1991). The mediator also appears to cause contraction by direct action on the gallbladder smooth muscle (Behar and Biancani, 1980). However, in this study it has not been examined if cholecystokinin could release acetylcholine from the cholinergic nerve endings and which type of cholecystokinin receptors mediate gallbladder smooth muscle contraction. However, the significant relaxation by Y-27632 of the contractions elicited by cholecystokinin indicates that the Rho/Rho-kinase signalling could be associated with cholecystokinin receptors in the human gallbladder. Consistently, cholecystokinin CCK_1 receptors activate Rho primarily through G_{13} protein (LePage et al., 2003).

In addition to guinea pig gallbladder, it was demonstrated that human gallbladder possesses both endothelin ET_A and ET_B receptors, cooperating to mediate smooth muscle contraction

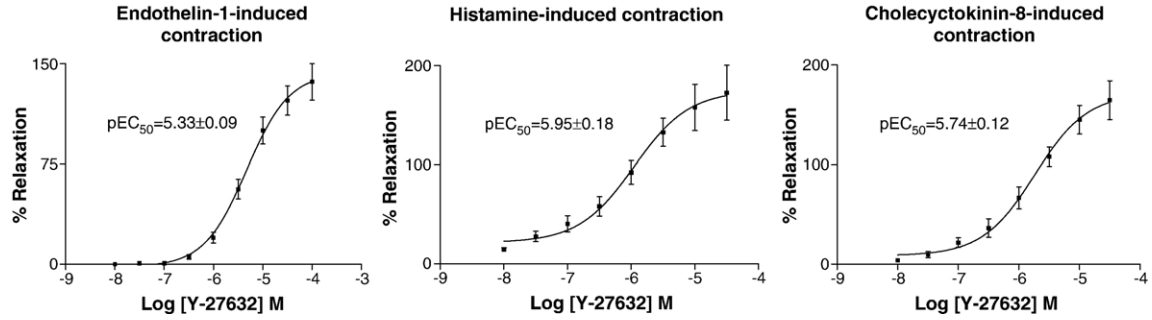

Fig. 4. Relaxant effects of Y-27632 ($10^{-8}-3\times10^{-5}$ M, cumulatively) on histamine (10^{-5} M, $n=5$), endothelin-1 (10^{-8} M, $n=5$) and cholecystokinin (10^{-8} M, $n=5$)-induced contractions. The responses are expressed as percentage reductions of the tonic contractures.

(Huang et al., 2001). The relaxation of endothelin-1-induced contractions by Y-27632 demonstrates that the Rho/Rho-kinase pathway has an important role in this contraction. However, we had no data suggesting which subtypes of endothelin receptors are in association with Rho/Rho-kinase signalling cascade. On the other hand, we previously demonstrated that endothelin-1 receptors may be coupled with Rho signalling (Büyükafşar et al., 2003a,b).

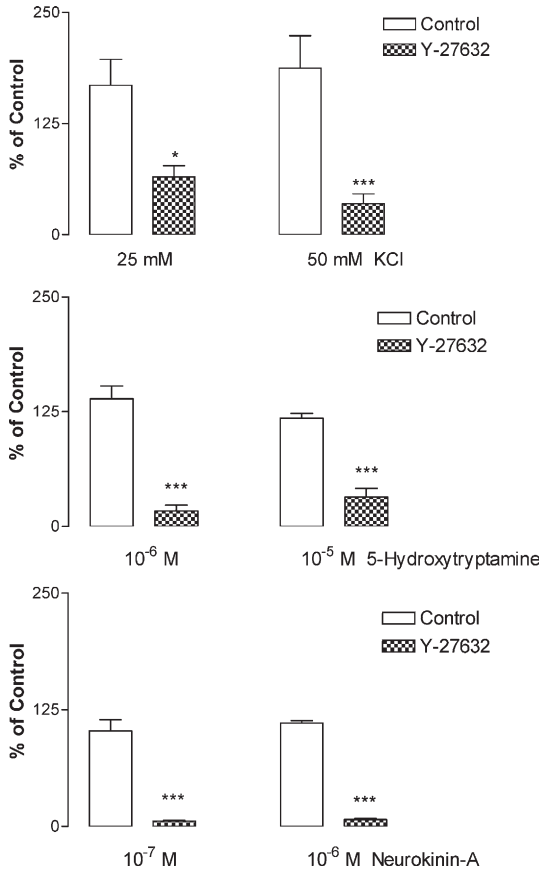

Fig. 5. Suppression of KCl (25–50 mM, upper panel, $n=4$–5), 5-hydroxytryptamine (10^{-5} M, central panel, $n=5$), and neurokinin A (lower panel, $n=5$)-induced contractions by Y-27632 (10^{-5} M, for 45 min). The second series of responses are expressed as percentages of the responses in the first series. Data show means±S.E.M. Comparison was made by one-way of ANOVA followed by the Bonferroni post hoc test. *: $P<0.05$, ***: $P<0.001$.

An inflammatory mediator, histamine presents abundantly in mast cells of the gallbladder and acts on gallbladder smooth muscle inducing contraction via histamine H_1 excitatory receptors (Hemming et al., 2000). The inhibition of histamine-evoked contractions by Y-27632 may show that the Rho/Rho-kinase signalling pathway could be linked with excitatory histamine receptors. Tachykinins such as substance P, neurokinin A and neurokinin B act as excitatory mediators in guinea pig gallbladder (Guo et al., 1989a,b). They exert their biological functions in the ovine gallbladder through receptor types termed tachykinin NK_1 and NK_2 (Tucci et al., 2003). In this study, neurokinin A induced a substantial contraction, which was diminished by the Rho-kinase inhibitor, showing that the receptors responsible for smooth muscle contraction for this peptide are coupled with this novel signalling. Another autacoid, which was employed in this study, is 5-hydroxytryptamine. It induced concentration-dependent contractions, and these contractions were considerably diminished by Y-27632 as has been recently reported in the ovine gallbladder (Şahan-Fırat et al., 2005). This may indicate that the Rho/Rho-kinase pathway mediates 5-hydroxytryptamine-evoked contractions. However, we had no data about which types of 5-hydroxytryptamine receptors mediating its responses in this study. Other gastrointestinal peptides, such as motilin, secretin, bombesin, neurotensin, glucagon, vasopressin, vasoactive intestinal peptide (VIP) and somatostatin were not examined in this study because these mediators were demonstrated to have no affect on human gallbladder contractility (Feeley et al., 1987).

It has long been known that KCl could directly stimulate smooth muscle cells by increasing intracellular Ca^{2+} concentration, independently of plasma membrane receptor activation. Interestingly, however, it has been proposed that K^+ also enhances Ca^{2+} sensitivity in coronary vascular smooth muscle (Yanagisawa and Okada, 1994). The contraction induced by high K^+ was inhibited in the presence of Y-27632 in this study as previously reported in mouse gastric fundus (Büyükafşar and Levent, 2003), mouse vas deferens (Büyükafşar et al., 2003a,b) and ovine gallbladder (Şahan-Fırat et al., 2005). Inhibition of KCl-induced contractions by the Rho-kinase inhibitor, Y-27632 reveals that Ca^{2+} ions may activate the Rho/Rho-kinase signalling. We previously termed this as "Ca^{2+}-induced Ca^{2+}-sensitization" (Büyükafşar and Levent, 2003). The mechanism underlying K^+-evoked Ca^{2+} sensitivity has not been understood yet. However, a complex set of events may be involved in this

phenomenon, such as activation of calmodulin, voltage-operated ion channels, a number of signal transduction molecules and enzymes. Consequently, this may lead Rho-kinase activation, as has recently been proposed (Urban et al., 2003; Sakurada et al., 2003).

It has been reported that alterations in gallbladder motility may play a role in pathological conditions, such as cholesterol gallstone formation (Pozo et al., 2004). Hypersecretion of heõpatic cholesterol, chronic supersaturation of bile with cholesterol and rapid precipitation of cholesterol crystals from cholesterol-enriched vesicles in the gallbladder with abnormal smooth musculature contractility, impaired gallbladder motility, and increased stasis, all play an important role in the pathogenesis of cholesterol gallstones (Portincasa et al., 2003; Pozo et al., 2004). Therefore, the activity and/or expression of this enzyme, which seems most likely to be involved in the gallbladder emptying and refilling cycle should be examined in the smooth muscle of stone-diseased and stone-free human gallbladders to make an important insight into the pathogenesis of gallstone formation in human beings.

In conclusion, we have demonstrated that Rho-kinase protein (ROCK-2 isoform) is expressed and plays an important role in the human gallbladder motor function.

Acknowledgements

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