Evidence for the involvement of the cyclooxygenase-metabolic pathway in diclofenac-induced inhibition of spontaneous contraction of rat portal vein smooth muscle cells

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Abstract

The effects of diclofenac, a cyclooxygenase (COX) inhibitor, were investigated on spontaneous phasic contractions of longitudinal preparations of the rat portal vein. Diclofenac produced a concentration-dependent decrease in the amplitude of these spontaneous phasic contractions. Diclofenac (30 µM) decreased the amplitude of the spontaneous phasic increase in the F340/F380 ratio of Fura PE3, an indicator of intracellular Ca²⁺ concentration. It also reduced the number of action potentials in each burst discharge without changing the resting membrane potential of longitudinal smooth muscle cells. The extent of the distribution of Lucifer Yellow injected into a smooth muscle cell was decreased in the presence of diclofenac (30 µM). Both AH6809, a prostanoid EP receptor antagonist, and SQ22536, an adenylate cyclase inhibitor, decreased the amplitude of the spontaneous contractions. On the other hand, neither ozagrel, a thromboxane synthase inhibitor, nor SQ29548, a prostanoid TP receptor antagonist, significantly affected spontaneous contractions. These results indicate that diclofenac inhibits the amplitude of spontaneous contractions of the rat portal vein through inhibition of electrical activity, which may be related to an inhibition of the cyclooxygenase pathway.

Key words: diclofenac, portal vein, contraction, intracellular Ca²⁺, membrane potential

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Introduction

Longitudinal preparations of the rat portal vein develop spontaneous phasic myogenic contractions which are accompanied by bursts of action potentials (Funaki and Bohr, 1964; Axelsson et al., 1967; Johansson et al., 1967). Because such contractions are very sensitive to extracellular calcium, the rat portal vein has been used to examine the effects of drugs on vascular contraction (Sutter, 1990). Products of the cyclooxygenase (COX) pathway have been shown to be involved in the regulation of physiological activities (Wright et al., 2001). Spontaneous contractions of the rat portal vein have been shown to be potentiated by prostaglandin E1 (PGE1) (Miwa et al., 1997) and arachidonic acid (Vidulescu et al., 2000). On the other hand they are inhibited by meclofenamate, a COX inhibitor (Enero, 1979), although the mechanism involved in this inhibition has not been determined. In this study, we have examined the effect of diclofenac, another COX inhibitor (Mitchell et al., 1994), on spontaneous contractions of smooth muscle cells of the rat portal vein, as well as on their membrane potential and intracellular Ca2+ concentration. Products of the COX pathway act on prostanoid receptors, which use different intracellular mediators (Wright et al., 2001). To identify the product involved in this spontaneous contraction, we examined the effects of prostanoid receptor antagonists and an adenylate cyclase inhibitor on these spontaneous contractions.

Cell-to-cell coupling has been shown to play an important role in propagation of excitation and therefore the coordination of vascular responses (Christ et al., 1996). Therefore, we have examined the effect of diclofenac on the distribution of Lucifer Yellow injected intracellularly, as this has been used to evaluate intercellular communication (Beny, 1990). The results obtained indicate that COX may play a role in the generation of spontaneous contractions in the rat portal vein.

Materials and Methods

Male Wistar rats weighing 200–300 g were anesthetized using CO2, and treated according to the Guiding Principles for the Care and Use of Laboratory Animals Approved by the Japanese Pharmacological Society. The hepatic portal vein was isolated and preparations of the longitudinal smooth muscle layer dissected under a binocular microscope and placed in a modified Tyrode’s solution. The luminal surface of the tissue was rubbed with paper to remove the endothelium. The composition of the modified Tyrode’s solution (in mM) was as follows: 137 NaCl, 5.4 KCl, 2.0 CaCl2, 1.0 MgCl2, 0.4 NaH2PO4, 11.9 NaHCO3, 5.6 glucose, with a pH of 7.3. The high K+-Tyrode’s solution was made by replacing NaCl with an equimolar concentration of KCl. All experiments were conducted at 37°C in Tyrode’s solution aerated continuously with a mixture of 95% O2 - 5% CO2. In the contractile force recording experiments, each preparation was mounted vertically in an organ bath. The isometric contractile force of preparations was measured using a force-displacement transducer (U-gage 10G, Minebea, Karuizawa, Japan) equipped with a strain amplifier (AS2102, NEC, Tokyo, Japan) and recorded with a thermal-pen recorder (Linear recorder WR3310, Graphtec, Tokyo, Japan).

Changes in intracellular Ca2+ concentrations were estimated according to our previous report (Shimamura et al., 2003). The surfaces of all materials used for the Ca2+-indicator-
experiment were coated with silicon using Siliconizer L-25 (Fuji System, Tokyo, Japan). The small adventitia and endothelium-removed longitudinal strips of rat portal vein were incubated with Fura-PE3/AM (Wako, Osaka, Japan) 20 µM dissolved in dimethyl sulfoxide (DMSO) and 0.08% Pluronic F-127 in Tyrode’s solution for 2 hours at room temperature. The preparations were then mounted adventitial-side down on a silicon rubber in the temperature-controlled chamber of a fluorometer (CAF 100, JASCO, Tokyo, Japan). Intracellular Ca\(^{2+}\) concentrations were estimated from the luminescence intensity ratio \((F_{340}/F_{380})\) when excited at wavelengths of 340 nm \((F_{340})\) and 380 nm \((F_{380})\). One end of each preparation was connected to a force-displacement transducer so that changes in tension could be measured simultaneously. Isometric tension and fluorescence were recorded on a pen recorder (Yokogawa LR4220, Tokyo, Japan) and stored in a PCM recorder (RD101-T, TEAC, Tokyo, Japan). Mirror images in both \(F_{340}\) and \(F_{380}\) were considered to be a marker of the successful measurement of \(F_{340}/F_{380}\) without movement interference. After each experiment, preparations were first exposed to 40 mM K\(^+\) Tyrode’s solution and then subsequently exposed to 2 mM EGTA in a Ca\(^{2+}\)-free Tyrode’s solution to determine the minimum level of intracellular Ca\(^{2+}\). Changes in the fluorescence ratio were expressed as a percentage of the elevation induced by the 40 mM K\(^+\) Tyrode’s solution. The contraction induced by the 40 mM K\(^+\) Tyrode’s solution had a stable amplitude which was slightly, but not significantly, less than that induced by the 50 mM K\(^+\) Tyrode’s solution.

The membrane potential was measured by the microelectrode technique as reported previously (Shimamura et al., 2003). Adventitia and endothelium-removed longitudinal strips were mounted adventitial-side up on a silicon rubber bed in a chamber that was continuously perfused with warmed Tyrode’s solution at a flow rate of 5 ml/min. A pulled glass capillary microelectrode (PN-3, Narishige, Tokyo, Japan), filled with 3M KCl and with a tip resistance of 40MΩ, was impaled from the adventitial side. Membrane potentials were monitored using a microelectrode amplifier (Intra 767 Electrometer, World Precision Instruments, Sarasota, FL, USA) and recorded through both a thermal pen recorder (Graphtec Lineacorder WR3310) as well as a PCM recorder (RA125T, TEAC, Tokyo, Japan). Data were retrieved using both Axotape (Axon Instruments, Foster city, USA) and Labmaster (Scientific Solutions, Inc., Mentor, OH, USA) software in an IBM-compatible PC.

Intercellular diffusion capability was estimated by detection of the fluorescent signals of neighboring cells following injection of Lucifer Yellow (Sigma Chemical Co. St. Louis, MO, USA) as reported by others (Beny and Connat, 1992; Sakai et al., 1992). A 4% solution of Lucifer Yellow (Lucifer Yellow dissolved in 150 mM Lithium chloride) was back-filled into the microelectrodes using a resistance of 200–300 MΩ. The dye was considered to be successfully loaded when a stable membrane potential of below –40 mV was recorded with action potentials of more than 30 mV in amplitude both before and after the Lucifer Yellow injection. During impalement of single longitudinal smooth muscle cells of the rat portal vein, dye was injected with a hyperpolarizing direct current (0.35 nA) for 2 minutes. When dye injection was complete, preparations were suspended vertically in Tyrode’s solution for 10 minutes and the tissue then fixed with 4% paraformaldehyde. In experiments with Lucifer Yellow, diclofenac 30 µM was added to the perfusate 5 min prior to dye injection. To evaluate the extent of intercellular dye diffusion, preparations were examined with a fluorescence microscope (DMIRB/E, Leica
Microsystem, Wetzlar, Germany). Preparations were excited at 480 nm and the resulting fluorescence recorded at 520 nm with a CCD camera (Hamamatsu, Japan) and the data stored on a computer hard disk.

**Drugs**

Diclofenac sodium, prostaglandin E₂ (PGE₂) and 1H-[1,2,4] oxadiazolo [4,3-a] guinoxalin-1-one (ODQ) were from Wako Pure Chemical (Osaka, Japan); 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809) and [1s-[1α, 2α (Z), 3α, 4α]-7-[3-[(phenylamino) carbonyl] hydrazino] methyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-heptenoic acid (SQ29548) from Cayman Chemical (Ann Arbor, MI, USA); 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536) from Calbiochem (San Diego, CA, USA) and ozagrel HCl and DMSO were from Sigma Chemical Co. (St. Louis, MO, USA). AH6809 was dissolved in 37.7 mM Na₂CO₃ to make a 10 mM stock solution. PGE₂ was dissolved in ethanol to make 1 mM stock solution. ODQ was dissolved in DMSO to make a 10 mM stock solution. SQ29548 was dissolved in DMSO to make a 1 mM stock solution. In experiments with the above drugs, the final concentration of DMSO and ethanol was less than 0.1% and 0.001%, respectively. These concentrations of DMSO and ethanol did not affect the contraction of the rat portal vein.

**Data analysis**

Results are given as the mean ± SEM with the number of preparations in parenthesis. Statistical significance was assessed using the Student t-test. Paired t-test’s were used when appropriate. \( P \) values <0.05 were considered to be statistically significant.

**Results**

**Effects of diclofenac on spontaneous contraction**

The rat portal vein exhibited spontaneous phasic contractions with a stable frequency of 4.1 ± 0.6 cpm (n=15). The amplitude of these spontaneous phasic contractions was stable at 1.4 ± 0.1 mN (n=14) and was 41.6 ± 2.7% (n=9) of that developed in 50 mM K⁺-Tyrode’s solution. Five to 10 min after administration, diclofenac 10 \( \mu \)M lowered the amplitude of the spontaneous contractions of the rat portal vein by 59.4 ± 2% (n=10) without a marked change in the contraction frequency. The inhibition of the contractile amplitude by diclofenac was concentration-dependent (Fig. 1). The amplitude of contraction induced by 50 mM K⁺ in the presence of 30 \( \mu \)M diclofenac was 96.2 ± 1.9% (n=5) of that in the absence of diclofenac. When the amplitude of spontaneous contractions was decreased by 10 \( \mu \)M diclofenac, administration of 10 nM PGE₂ restored the amplitude to 94.1 ± 4.9% (n=7) of that before administration of diclofenac.

**Effects of diclofenac on intracellular Ca²⁺ concentration**

During the simultaneous recording of the Fura PE3 F₃₄₀/F₃₈₀ ratio and the force of contraction, we observed that spontaneous phasic contractions were generated synchronously with a phasic increase in the F₃₄₀/F₃₈₀ signal. The amplitude of these spontaneous contractions...
was 39.6 $\pm$ 8.7% (n=7) of that developed in 40 mM K+ Tyrode’s solution. The amplitude of the contractions induced by 40 mM K+ Tyrode’s solution were not significantly different from those in 50 mM K+ Tyrode’s solution. The maximum intensity of the F340/F380 signals was 37.0 $\pm$ 3.0% (n=7) of that developed in 40 mM K+ Tyrode’s solution. The increase of the ratio was inhibited by incubation of the nominally Ca2+-free Tyrode’s solution. The amplitude of the Fura PE3 F340/F380 ratio in the control was 40.2 $\pm$ 10.0% (n=5) and it was decreased by 30 $\mu$M diclofenac to 8.8 $\pm$ 4.1% (n=5) (Fig. 2).

Effects of diclofenac on the membrane potential

The spontaneous action potential bursts that appeared periodically in the rat portal vein preparations could be inhibited by reduction of the Ca2+ concentration in the Tyrode’s solution or by application of 1 $\mu$M nicardipine (data not shown). Diclofenac (30 $\mu$M) did not change the resting membrane potential (control, $-43.6 \pm 1.3$ mV, n=19; 30 $\mu$M diclofenac, $-46.0 \pm 1.2$ mV, n=20). However, it markedly inhibited the number of action potentials in each burst (Fig. 3). The number of action potentials in each burst was $7.0 \pm 1.3$ (n=11) in the absence of diclofenac and $2.0 \pm 0.6$ (n=11) in the presence of 30 $\mu$M diclofenac.

Effects of a thromboxane synthesis inhibitor and of prostanoid receptor antagonists on spontaneous contraction

Neither ozagrel, a thromboxane synthase inhibitor, nor SQ29548, a prostanoid TP receptor
antagonist, changed the amplitude of spontaneous contractions (data not shown). An EP prostanoid receptor antagonist, AH6809, reduced the amplitude of spontaneous contractions in a concentration-dependent manner (Fig. 4). In the presence of 30 µM AH6809, the amplitude of spontaneous contractions decreased to 61.8 ± 7.3% (n=8) of that in control, while the 50 mM K+-induced contracture in the presence of 30 µM AH6809 was 101.7 ± 4.1% (n=4) of that in control.

**Effects of cyclic nucleotide synthesis inhibitors**

The role of cAMP in spontaneous contraction was examined by using SQ22536, an adenylate cyclase inhibitor. The amplitude of spontaneous contractions in the presence of 100 µM SQ22536 was 52 ± 3% (n=8) of that in control. The inhibitory effect of SQ22536 was
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concentration-dependent between 30 μM and 100 μM (Fig. 5). The 50 mM K⁺-induced contracture in the presence of 100 μM SQ22536 was 98.9 ± 7.0% (n=4) of that in control.

The role of cGMP in spontaneous contraction was examined by using ODQ, a guanylate cyclase inhibitor. The amplitude of spontaneous contraction in the presence of 10 μM ODQ was 98.1 ± 1.9% (n=5) of that in control.

Effects of diclofenac on Lucifer Yellow dye distribution

After Lucifer Yellow dye was injected into a longitudinal muscle cell from the microelectrode, its intercellular diffusion occurred predominantly in the direction of the longitudinal muscle layer of the preparation. When the area and length of the dye-stained regions were compared between untreated preparations and those treated with 30 μM diclofenac, the area and longitudinal distance of dye staining were significantly smaller in the diclofenac-treated preparations when compared to the untreated preparations (Fig. 6).

Discussion

COX inhibitors have been reported to inhibit the spontaneous tonic contractions of the smooth muscle of the cat esophagus (Cao et al., 1999), the spontaneous rhythmic contractions in the rat renal pelvis (Davidson and Lang, 2000), and the twitch contractions in the rat gastric
Fig. 5. Effect of the adenylate cyclase inhibitor, SQ22536, on spontaneous contractions of longitudinal preparations of the rat portal vein. A: A typical trace showing the inhibitory effect of 100 µM SQ22536 on spontaneous contractions. B: A summary plot showing the concentration-dependent depression of the spontaneous contraction amplitude by SQ22536. Each point represents the mean of 4 to 6 preparations.

Fig. 6. Changes in Lucifer Yellow dye distribution in longitudinal preparations of the rat portal vein following a 2 min electric current injection from the microelectrode. The area of distribution (A) and the distance of distribution in both the longitudinal and circular directions are compared. Open columns, absence of diclofenac; filled columns, presence of 30 µM diclofenac. Data shows the mean of 6 preparations in each experiment. Asterisks indicate statistical significance (P<0.05).
It has also been indicated that products of the COX pathway are important in the regulation of vascular smooth muscle contraction (Wright et al., 2001). While meclofenamate, a COX inhibitor, has been reported to inhibit spontaneous contractions of the rat portal vein (Enero, 1979), the detailed mechanism of action is not clear. In the present study, we have observed that diclofenac, another COX inhibitor (Mitchell et al., 1994), decreased the amplitude of spontaneous phasic contractions of longitudinal preparations of the rat portal vein without affecting 50 mM K⁺-induced contractions. As nicardipine, an L-type Ca²⁺ channel inhibitor abolished both spontaneous and 50 mM K⁺-induced contractions in this preparation, the inhibition by diclofenac does not involve voltage-dependent Ca²⁺ channels or nonspecific mechanisms. A relationship between contraction and free intracellular Ca²⁺ concentration in smooth muscle cells has been reported on the basis of studies using calcium indicators (Morgan and Morgan, 1984). Very little information is available concerning the relationship between contraction and intracellular free Ca²⁺ concentration in smooth muscle cells of the rat portal vein (Swärd et al., 1993). In the present study, we have observed that each spontaneous phasic contraction was accompanied by a phasic increase in the Fura PE3 F_{340}/F_{380} ratio. Since diclofenac decreased both the amplitudes of the spontaneous phasic contractions and the phasic increases in the intensities of the Fura PE3 F_{340}/F_{380} ratio in a similar manner, it would appear that the diclofenac-induced inhibition of the spontaneous contractions was mediated by a decrease in the intracellular Ca²⁺ concentrations. It has been shown that spontaneous contractions of the rat portal vein are accompanied by bursts of action potentials (Funaki and Bohr, 1964; Axelsson et al., 1967; Johansson et al., 1967). Both contraction and action potentials are dependent on an influx of extracellular calcium through L-type Ca²⁺ channels (Sutter, 1990; Kamishima and McCarron, 1996). We observed that diclofenac decreased the number of spikes in each burst of action potentials with an accompanying decrease in the Ca²⁺ influx. As this agent did not affect the high-K⁺-induced contraction, it was considered that diclofenac influenced pathways other than voltage-dependent Ca²⁺ channels. Further investigation would be needed to discriminate between the effects of diclofenac and nicardipine, an L-type channel inhibitor, on action potentials.

Elevation of cAMP increased intercellular communication in both cardiomyocytes, (Burt and Spray, 1988) and osteocytes (Cherian et al., 2003). Thus, formation of cAMP is involved in both electrical cell-to-cell coupling and in spontaneous contractile activity. Generally cell-to-cell conductance has been shown to be increased by elevation of cAMP and decreased by elevation of cGMP (Brink and Barr, 2000). These responses may be mediated via the phosphorylation of connexin, however, its relationship to change in intercellular communication is not clear (Sáez et al., 2003). Several studies have suggested that the inhibition of spontaneous contraction by COX inhibitors is mediated by a decrease in intercellular communication. An earlier study has shown that dye transfer through gap junctions in osteocyte-like MLO-Y4 cells was inhibited by indomethacin and that PGE₂ facilitated gap junction-mediated communication in these cells (Cheng et al., 2001). In the canine trachealis muscle, PGE₂ or PGI₂ increased gap junction formation (Agrawal and Daniel, 1986). It has also been shown that an increase in intercellular communication enhanced the amplitude of spontaneous contractions (Garfield et al., 1988; 1992), while inhibition of intercellular communication reduced myogenic contraction in cerebral
arteries (Lagaud et al., 2002). However, in cardiac muscle cells, cyclooxygenase metabolites were not involved in gap junction conductance (Schmilinsky-Fluri et al., 1997). In the rat myometrium, generation of gap junctions was inhibited by indomethacin and increased by PGH2 and arachidonic acid (Garfield et al., 1980). The reduction in the distribution of Lucifer Yellow induced by diclofenac observed in the present study is consistent with the findings of the aforementioned studies on smooth muscle. However, in rat myometrium, it was reported that 2-deoxy-D-glucose diffusion was reduced by an increase in intracellular cAMP or isoproterenol and PGE2 administration (Cole and Garfield, 1986). This difference in the myometrium might indicate the possible presence of a different regulation by cyclooxygenase pathway from that in the smooth muscle cells of the rat portal vein. In the present study, our results have indicated that the physiological level of cAMP plays an important role in the maintenance of spontaneous contractions. The marked decrease in the distribution of Lucifer Yellow by diclofenac was observed in the longitudinal direction but not in the circular direction. This observation was compatible with the results of the mechanical and electrical recordings from longitudinal preparations.

We observed that ozagrel and SQ29548 did not affect the amplitude of spontaneous contractions; thus, prostanoid TP receptor agonists such as thromboxane and PGF2α do not appear to play a role in mediating the contractions. On the contrary, an EP prostanoid receptor antagonist, AH6809 (Janssen et al., 2000; Woodward et al., 1995; Coleman et al., 1985), inhibited the amplitude of spontaneous contractions in the rat portal vein. Since AH6809 did not inhibit contractions induced by 50 mM K+, its inhibitory effect on spontaneous contraction appears to involve neither L-type Ca2+ channel-mediated nor nonspecific mechanisms. Thus, it would appear that prostanoid EP receptors are involved in spontaneous contractions of the rat portal vein.

In the present study, SQ22536, an adenylate cyclase inhibitor (Gao and Raj, 2001), decreased the amplitude of the spontaneous contractions of the rat portal vein. Since SQ22536 did not inhibit the 50 mM K+-induced contracture, the inhibition did not appear to involve L-type Ca2+ channel-mediated or nonspecific mechanisms. Since cAMP serves as a second messenger for PGE2, the inhibition of spontaneous contractions by adenylate cyclase inhibition was compatible with the effect of a PGE2 receptor antagonist. As only 50% of the spontaneous contraction was inhibited by SQ22536, other mechanisms might also be involved in spontaneous contraction. The contribution of cGMP was excluded since ODQ did not affect spontaneous contraction.

In conclusion, these results indicate that diclofenac inhibits the spontaneous contraction of the rat portal vein by decreasing electrical activity. The inhibition of spontaneous contraction may be mediated by the COX pathway. Decreases in the production of cAMP and PGE2, as well as cell-to-cell coupling, would appear to be involved in the inhibition. In the rat portal vein, intrinsic PGE2 plays an important role in the maintenance of spontaneous electrical and mechanical activities.

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