ROLE OF POTASSIUM CHANNELS IN RABBIT INTESTINAL MOTILITY DISORDERS INDUCED BY 2, 2´-AZOBIS (2-AMIDINOPROPANE) DIHYDROCHLORIDE (AAPH)

Oxidative stress appears to play a role in the pathogenesis of several inflammatory gastrointestinal diseases. Changes in intestinal motility have been reported in different models of intestinal inflammation. The initiating factor of altered motility could be an alteration of gut redox status. The aim of this study was to investigate the effect of oxidative stress evoked by 2, 2´-azobis (2-aminopropane) dihydrochloride (AAPH) on the intestinal motility of rabbit duodenum and the possible contribution of different K⁺ channels in mediating this response. Whole thickness segments of rabbit duodenum were suspended in the direction of the longitudinal or circular smooth muscle fibres in an organ bath to study the effects of AAPH alone, or in the presence of different K⁺ channel blockers on the amplitude, frequency and tone of spontaneous contractions. In circular muscle, AAPH 20 mM induced a reduction of the amplitude, the frequency and tone of the spontaneous contractions. In longitudinal muscle, AAPH 10 mM induced a reduction of the amplitude and tone of the spontaneous contractions. The reduction of the amplitude and tone induced by AAPH was reverted by BaCl₂ (1 mM) and TEA (5 mM). Charybdotoxin (100 nM) and iberiotoxin (100 nM) only reverted the reduction of the tone induced by AAPH. In conclusion, our results show that the peroxyl radicals released by AAPH reduced the amplitude and the tone of the spontaneous contractions of the longitudinal smooth muscle from rabbit small intestine. Inward rectifier and intermediate and large-conductance Ca²⁺-activated K⁺ channels could be involved in these effects.

Key words: 2, 2´-azobis (2-aminopropane) dihydrochloride (AAPH), intestine, motility, oxidative stress, peroxyl radicals, smooth muscle

INTRODUCTION

Oxidative stress appears to play a role in the pathogenesis of several gastrointestinal diseases including inflammatory bowel disease (IBD) (1-3), ischemia/reperfusion induced intestinal injury (4) and colon cancer (5-7). Oxidases found in resident phagocytic cells, microvascular endothelium and mucosal epithelium appear to be the major source of reactive oxygen species (ROS) in the intestine. Although there are numerous antioxidant enzymes and scavengers that protect from ROS in the intestine, if the rate of production of ROS exceeds the capacity of antioxidant defence, tissue injury may ensue.

Changes in intestinal motility have been reported in different experimental models of colitis induced by DSS (8) or endotoxemia induced by LPS (9) in rats. The initiating factor in the etiology of altered motility could be an early alteration of gut redox status, suggesting the importance of experimental models of gut contractility under oxidative stress. Two reactive oxygen species generating systems, H₂O₂ (10, 11) and cumene hydroperoxide (12), and the lipid peroxidation product 4-hydroxy-2,3-trans-1 nonenal (13) inhibited the intestinal motility and contractility in rat. In the gastrointestinal tract, activated macrophages increase the level of H₂O₂ in the muscle layers (11) and a potential target of ROS is likely to be the intrinsic neurons of the intestine that control secretion and absorption at the mucosa, and motility in the muscle (14).

It has been reported significant variations in the enzymatic and non-enzymatic antioxidant profiles of the gastric and duodenal mucosa of rat, rabbit, cat and pig (15). As these variations have potentially important implications in the interpretation and comparison of data from oxidative stress experimental models when differences in species are involved, we chose 2,2´-azobis (2-aminopropane) dihydrochloride (AAPH) as the free radical generating system to study the effect of oxidative stress on the intestinal motility of rabbit duodenum. This compound decomposes uni-molecularly, without enzymes or biotransformation to yield molecular nitrogen (N₂) and two carbon radicals (R·). The carbon radicals are formed in pairs at close proximity, some recombining to give stable products (R-R), though many diffuse and react rapidly with oxygen molecules to yield peroxyl radicals (ROO•). Carbon-centred and peroxyl radicals generated by AAPH, damage lipids, proteins and DNA (16). The rate of decomposition of AAPH is determined primarily by temperature. At 37°C and pH 7, the half-life of AAPH is about 175 h, which means that the rate of radicals generation is virtually reproducible and constant for the first few hours (17). In addition, AAPH generates peroxyl radicals directly without generating H₂O₂ as an intermediate, thus rendering its effect independent of tissue content in enzymatic antioxidant. Intrarectal administration of AAPH causes erythema, edema and histologically verifiable mucosal inflammation in rat colon (18).
A variety of inwardly rectifying, voltage-, Ca²⁺-, and ATP-dependent K⁺ channels have been identified in the gastrointestinal tract (19). Different reactive oxygen species such as O₂⁻, H₂O₂, and ONOO⁻ have been reported to modulate the activity of large conductance Ca²⁺-activated, ATP- and voltage-dependent K⁺ channels in vasculature and cardiac myocytes (20). However, the contribution of K⁺ channels in the response evoked by oxidative stress in the gastrointestinal smooth muscle has hardly been studied.

The aim of this study was to investigate the effect of oxidative stress evoked by AAPH on the intestinal motility of rabbit duodenum and the possible contribution of different K⁺ channels in mediating this response.

MATERIALS AND METHODS

The handling, equipment used and the sacrifice of animals all complied with European Council legislation 86/609/EEC concerning experimental animal protection. Male New Zealand rabbits weighing 2-2.5 kg were maintained at a constant room temperature (22°C) with free access to water and standard rabbit fodder. Experimental protocols were approved by the Ethics Committee of the University of Zaragoza (Spain).

Preparation of smooth muscle segments

After 24 hours of fasting, the rabbits were humanely killed by a blow to the head. Pieces of duodenum (1-6 cm distal pylorus) were removed, washed, freed from mesenteric attachment and cut into smaller segments. Whole thickness segments (10 mm long and 5 mm wide) were suspended in the direction of the longitudinal or circular smooth muscle fibres in a thermostatically controlled (37°C) organ bath (10 ml capacity) containing Krebs solution and continuously gassed with 95% O₂ and 5% CO₂. Each segment of duodenum was connected to an isometric force transducer (Pioden UF1, Graham Bell House, Canterbury, U.K.) and stretched passively to an initial tension of 20 mN. Signal output of mechanical activity was amplified, recorded on a computer for later analysis using The Mac Lab

Fig. 1. Recordings of the effects of AAPH (1, 5, 10 and 20 mM) and papaverine (0.6 mM) on spontaneous contractions in the longitudinal smooth muscle of rabbit duodenum.
System/8e computer program (AD Instruments Inc., Milford MA, U.S.A) and digitized at two samples per second per channel. Before testing, segments were allowed to equilibrate in Krebs solution for 60 min. During that time, the nutrient solution was changed every 20 min.

Each experimental protocol was systematically performed on four segments of longitudinal and circular smooth muscle of duodenum taken from each of three or four rabbits. Segments that did not show spontaneous activity were discarded. After the equilibration period, spontaneous contractions in longitudinal or circular smooth muscle of small intestine were recorded in the absence (control period) or presence of different drugs.

In order to study the effect of carbon-centered and peroxyl radicals on spontaneous contractions, longitudinal and circular smooth muscle segments were incubated for 5 min with 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) at 1, 5, 10 and 20 mM. We also compared the effects of AAPH with the effect evoked by papaverine (0.6 mM, 5 min), a well known gastrointestinal smooth muscle relaxant.

To determine the participation of the different types of K+ channels on AAPH induced changes in spontaneous contractions, longitudinal smooth muscle segments were incubated with different blockers of K+ channels for 15 min before the addition of AAPH (10 mM, 5 min). The blockers of K+ channels were: apamin, (1 µM, a blocker of small-conductance Ca2+-activated K+ channels); charybdotoxin (100 nM, a selective blocker of intermediate- and large-conductance Ca2+-activated K+ channels); iberiotoxin (100 nM, a blocker of large-conductance Ca2+-activated K+ channels); tetrodotoxin (5 mM, a non-specific K+ channel blocker); 4-aminopyridine (0.1 mM, blocker of voltage-sensitive K+ channels); glibenclamide (1 µM, a blocker of ATP-sensitive K+ channels); and BaCl2 (1 mM, a blocker of inward rectifier K+ channels). The concentrations of the different blockers of K+ channels are based on the concentrations used by our group in a previous work (21).

Data analysis

Most segments showed spontaneous contractions. For each segment of smooth muscle, the mean amplitude (in mN) of contractions was calculated as the average of peak-to-peak differences over 5 min. The frequency of contractions was expressed as the number of contractions per minute (cpm) in a 5-min period, and the tone of spontaneous contractions as the mean of the tension values recorded in a 5-min period. Amplitude, frequency and tone of spontaneous contractions in the presence of AAPH or K+ channel blocker + AAPH are expressed as a percentage of the values recorded in the absence of drugs (control period) (21, 22). Each preparation served as its own control.

![Fig. 2. Effects of AAPH (1, 5, 10 and 20 mM) and papaverine (0.6 mM), on the amplitude (A), frequency (B) and tone (C) of spontaneous contractions in the longitudinal smooth muscle of rabbit duodenum. Columns are mean percentages of control values of spontaneous contractions (100%) and vertical bars indicate S.E.M. ***P<0.001 vs. control.](image-url)
Values are expressed as mean ±SEM. Comparisons between means were made using one-way analysis of variance (ANOVA) tests and P-values were determined using the Bonferroni test. Differences with P-values <0.05 were considered statistically significant. Statview for Windows version 5.0.1 (SAS Institute Inc., Cary NC, U.S.A.) was used for the analyses.

Solutions and substances

The Krebs solution contained the following (in mM): NaCl 120, KCl 4.70, CaCl₂ 2.40, MgSO₄ 1.20, NaHCO₃ 24.50, KH₂PO₄ 1.00 and glucose 5.60 at 37°C to achieve pH 7.4.

2, 2´-Azobis (2-amidinopropane) dihydrochloride (AAPH), apamin, charybdotoxin (ChTX), iberiotoxin (IbTX), tetraetylammonium chloride (TEA), 4-aminopyridine (4-AP), glibenclamide (GB) and papaverine were purchased from Sigma (Madrid, Spain). BaCl₂ was from Merck (Madrid, Spain). All chemicals were analytical grade.

Stock solutions of AAPH (2 M) were prepared in ethanol 1% (v/v) and they were used to obtain a concentration of 20 mM in the organ bath. The rest of the solutions of AAPH were prepared in distilled water. Stock standard solutions of 4-AP (1 mg/ml) and glibenclamide (0.2 mg/ml) were prepared in dimethyl sulphoxide (DMSO). Stock solutions of apamin (0.2 mg/ml) were prepared in acetic acid. All the other drugs were dissolved in distilled water. All solutions were stored at -20°C and fresh dilutions were made daily.

RESULTS

Effect of AAPH on spontaneous contractions of longitudinal and circular smooth muscle

Longitudinal (n=60) and circular (n=57) smooth muscle segments of rabbit duodenum exhibited cyclic, phasic and rhythmic spontaneous contractions with an amplitude of 21.1±1.6 and 2.1±0.1 mN, frequency of 11.4±0.3 and 10.3±0.5 cpm and tone of 22.3±1.2 and 10.7±0.4 mN, respectively.

When the duodenal longitudinal smooth muscle segments (n=12) were incubated in the presence of AAPH (1-20 mM, 5 min), an inhibition of the spontaneous contractions was observed (Fig. 1, 2). This inhibitory response was dose dependent and characterized by a reduction of the amplitude, frequency and tone of these spontaneous contractions (Fig. 1, 2). The amplitude was not altered by AAPH 1 and 5 mM but it was concentration dependently reduced by AAPH 10 and 20 mM (Fig. 2A). The frequency of the spontaneous contractions was reduced significantly only by AAPH 20 mM (Fig. 2B). The tone of the spontaneous contractions was reduced significantly by AAPH (5-20 mM) (Fig. 2C). We also compared the effect of AAPH with the effect induced by papaverine, a well-known smooth muscle relaxant. Papaverine (0.6 mM, 5 min) reduced the amplitude and frequency of the spontaneous contractions in a similar manner to AAPH 20 mM (Fig. 1, 2A,B) but induced a higher reduction of the tone (Fig. 2C).

![Fig 3. Effects of AAPH (1, 5, 10 and 20 mM) and papaverine (0.6 mM), on the amplitude (A), frequency (B) and tone (C) of spontaneous contractions in the circular smooth muscle of rabbit duodenum. Columns are mean percentages of control values of spontaneous contractions (100%) and vertical bars indicate S.E.M. ***P<0.001 vs. control.](image-url)
When the circular intestinal segments (n=12) were incubated in the presence of AAPH (20 mM, 5 min), a reduction of the amplitude and frequency of the spontaneous contractions was observed. However, the concentrations of 1, 5 and 10 mM did not modify these parameters (Fig. 3A,B). The tone was significantly reduced by AAPH (1-20 mM) and this effect was not dose dependent (Fig. 3C). Papaverine (0.6 mM, 5 min) induced a higher reduction of the amplitude and frequency of the spontaneous contractions than AAPH 20 mM (Fig. 3A,B) and reduced the tone similarly (Fig. 3C).

**Effect of K⁺ channel blockers on the inhibitory response induced by AAPH in longitudinal smooth muscle**

As we obtained a higher effect of AAPH on the longitudinal than on circular muscle, we focused the study on the effect of K⁺ channel blockers on the inhibitory response induced by AAPH in the longitudinal muscle of rabbit duodenum. We also decided to use the concentration of AAPH at 10 mM because at this concentration we obtained a repetitive effect of inhibition of the spontaneous motility.

The duodenal segments were incubated with different blockers of K⁺ channels for 15 min before the addition of AAPH (10 mM, 5 min). The reduction of the amplitude and tone of the spontaneous contractions induced by AAPH (10 mM) was reverted by BaCl₂ (1 mM, n=8) and TEA (5 mM, n=1) (Fig. 4B, 5A,B). Glibenclamide (1 µM, n=11), 4-aminopyridine (0.1 mM, n=10) and apamin (1 µM, n=10) did not modify the inhibitory response induced by AAPH on the amplitude and tone of the spontaneous contractions (Fig. 4C, 5A,B). Charybdotoxin (100 nM, n=8) and iberiotoxin (100 nM, n=8) reverted the AAPH-induced reduction of the tone but not the amplitude (Fig. 5A,B).

**DISCUSSION**

In this study we used 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) as a carbon-centered and peroxyl radicals generating system to study the effect of oxidative stress on the intestinal motility of rabbit duodenum. AAPH induced a relaxing response, characterized by a reduction of the amplitude, frequency and tone of the spontaneous contractions in longitudinal and circular smooth muscle. The effects of AAPH, used as a radical initiator, are rapid and reproducible. Therefore, this compound could be a useful tool for reproducing oxidative stress in diseases.

The effect of AAPH on gastrointestinal smooth muscle has hardly been reported but our results agree with another study in which AAPH induced an inhibition of the contractility and motility in isolated segments of guinea pig ileum and rabbit jejunum (17). Other effects of AAPH on the gastrointestinal tract include the induction of lipid peroxidation in intestinal brush-border membrane vesicles of guinea pig and the resulting decrease in the function of the intestinal sodium-dependent glucose transporter (23). AAPH also produces erythema, edema and mucosal inflammation in rat colon (18).
A number of other reactive oxygen species generating systems such as H$_2$O$_2$ (10, 11) and cumene hydroperoxide (12), and the lipid peroxidation product 4-hydroxy-2,3-trans-1 nonenal (13) were also reported to inhibit the intestinal motility and contractility in rat.

We studied the possible contribution of different K$^+$ channels in mediating the relaxing response induced by the oxidative stress generated by AAPH in the smooth muscle of rabbit intestine (Table 1). The reduction of the amplitude and tone of the spontaneous contractions induced by AAPH was reverted by BaCl$_2$ (a blocker of inward rectifier K$^+$ channels) and TEA (a non-specific K$^+$ channel blocker). Charybdotoxin (a selective blocker of intermediate- and large-conductance Ca$^{2+}$-activated K$^+$ channels) and iberiotoxin (a blocker of large-conductance Ca$^{2+}$-activated K$^+$ channels) reverted and even increased the reduction of the tone induced by AAPH. These results suggest that carbon-centered and peroxyl radicals released by AAPH relax intestinal smooth muscle by hyperpolarization through inward rectifier and large-conductance Ca$^{2+}$-activated K$^+$ channels activation. In fact, it has been described the presence of large-conductance Ca$^{2+}$-activated K$^+$ channels in neurons such as the human glioma cell line LN229 (24) and smooth muscle cells such as human umbilical vein endothelial cells (HUVECs) and mouse aortic smooth muscle cells (MASMCs) (25). Inward rectifier K$^+$ channels have been found in neurons such as differentiated NG108-15 cell line (26). Glibenclamide (a blocker of ATP-sensitive K$^+$ channels), 4-aminopyridine (blocker of voltage-sensitive K$^+$ channels) and apamin (a blocker of small-conductance Ca$^{2+}$-activated K$^+$ channels) did not modify the inhibitory response induced by AAPH on the amplitude and tone of the spontaneous contractions, indicating that ATP-sensitive, voltage-sensitive and small-conductance Ca$^{2+}$-activated K$^+$ channels are not involved in the relaxation induced by AAPH in the rabbit intestine.

Different types of K$^+$ channels have been reported to mediate the effects of free radicals on the contractility of several smooth muscles. However, their role varies, depending on the free radical and the tissue involved. Data on the effect of oxidative stress on large-conductance Ca$^{2+}$-activated K$^+$ channel activity in vascular smooth muscle cells suggest that O$_2^-$ and H$_2$O$_2$ enhance this channel activity, whereas ONOO$^-$ decreases it (20). H$_2$O$_2$ induces coronary arteriolar dilation in pig and this dilation was attenuated by iberiotoxin, indicating that H$_2$O$_2$ directly relaxes smooth muscle by hyperpolarization through large-conductance Ca$^{2+}$-activated K$^+$ channels activation. In contrast, the H$_2$O$_2$ dilation was insensitive to glibenclamide and BaCl$_2$, suggesting that ATP-sensitive and inward rectifier K$^+$ channels were not involved in this response (27).

In the gastrointestinal smooth muscle, AAPH inhibited the BaCl$_2$ induced contractions of guinea pig ileum and this effect was reversed by Bay-K8644, which promotes the opening of the voltage operated Ca$^{2+}$ channels (17). Peroxyl radicals' inhibition of contractions seems also to be exerted via the inhibition of Ca$^{2+}$ influx into the cells through the L-type Ca$^{2+}$ channels, according to the literature and data showing that ROS (xanthine/xanthine oxidase, H$_2$O$_2$ and cumene hydroperoxide) inhibits the voltage sensitive L-type Ca$^{2+}$ channels (28).

A potential target of ROS is likely to be the intrinsic neurons of the intestine that control secretion and absorption at the mucosa, and motility in the muscle (14). Vogalis et al. investigated the actions of H$_2$O$_2$ on the excitability of intestinal neurons that generate slow after-hyperpolarizing potentials (AH neurons) (29). The H$_2$O$_2$ induced a hyperpolarization of the AH neurons that included the activation of an inwardly rectifying outward current, which was blocked by glibenclamide and TEA.

In conclusion, our results show that the peroxyl radicals released by AAPH reduced the amplitude and the tone of the spontaneous contractions of the longitudinal smooth muscle from rabbit small intestine. Inward rectifier and intermediate and large-conductance Ca$^{2+}$-activated K$^+$ channels could be involved in these effects.

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Table 1. Summary of the effects of K⁺ channel blockers on the reduction of amplitude and tone of spontaneous contractions induced by AAPH in longitudinal muscle from rabbit duodenum.

| Channel blockers | Action                                               | Does the channel blocker revert the AAPH-induced effects in longitudinal muscle?
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<tr>
<td></td>
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<td>Amplitude</td>
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<tr>
<td>BaCl₂</td>
<td>Inward rectifier K⁺ channels blocker</td>
<td>Yes</td>
</tr>
<tr>
<td>TEA</td>
<td>Non-specific K⁺ channel blocker</td>
<td>Yes</td>
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<tr>
<td>Glibenclamide</td>
<td>ATP-sensitive K⁺ channels blocker</td>
<td>No</td>
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<tr>
<td>4-aminopyridine</td>
<td>Voltage-sensitive K⁺ channels blocker</td>
<td>No</td>
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<tr>
<td>Apamin</td>
<td>Small-conductance Ca²⁺-activated K⁺ channels blocker</td>
<td>No</td>
</tr>
<tr>
<td>Charybdotoxin</td>
<td>Intermediate- and large-conductance Ca²⁺-activated K⁺ channels blocker</td>
<td>No</td>
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<tr>
<td>Iberiotoxin</td>
<td>Large-conductance Ca²⁺-activated K⁺ channels blocker</td>
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