The Effects of H_2O_2 on Electromechanical Activity of the Ileum Longitudinal Muscle

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Abstract. The effects of H_2O_2 on electrical and mechanical activity of the longitudinal layer from the guinea-pig ileum were studied using sucrose-gap technique and the influence of H_2O_2 on ionic current was investigated in single smooth muscle cells by the patch-clamp method. In most of the preparations tested, the spontaneous activity observed was composed of slow waves with superimposed action potentials (APs). Both were resistant to tetrodotoxin and atropine. H_2O_2 (1 mmol/l) evoked sustained 3–5 mV membrane depolarisation, doubled the amplitude of the slow waves and increased their frequency, augmented the APs and reduced their splitting. These changes were accompanied with significant contraction, which had an amplitude comparable to that of the tonic component of 50 mmol/l K^+-induced contraction. Calcium-free solution caused membrane depolarisation, reduction of the slow wave amplitude and frequency, disappearance of APs and decreased the mechanical tension of the preparations. Application of H_2O_2 (1 mmol/l) into the zero-calcium bath solution recovered the APs, which was accompanied by a low amplitude contraction. H_2O_2 (up to 1 mmol/l) increased the L-type calcium current (I_{Ca}) both under conventional whole-cell patch-clamp configuration and under amphotericin-perforated patches by $16 \pm 3\,\%$. These data demonstrated that contractile response of the ileum longitudinal smooth muscle preparation evoked by H_2O_2 was mainly due to the enhanced electrical activity.

Key words: H_2O_2 — Ileum — Longitudinal muscle — Calcium current

Introduction

At present time, the involvement of reactive oxygen species (ROS) in the inflammatory reaction is well documented and broadly accepted by many laboratories (for review see Bauer and Bauer 1999; Hensley et al. 2000). Some ROS are capable of

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long living and of diffusion over great distances from the site of origin, potentially modifying physiological functions of the tissues affected. The molecule of hydrogen peroxide (H_2O_2), a product of the spontaneous or enzymatically-mediated dismutation of two molecules of superoxide anion radical, is a typical example of such a compound. In patients with inflammatory diseases (Vermillion et al. 1993; Bauer and Bauer 1999) or in the proposed experimental animal models (Dieleman et al. 1997; Martinolle et al. 1997) of the small intestine, motility disturbances were observed. Their underlying mechanisms are not well known as yet. Rhythmic mechanical activity is characteristic of many (if not most) smooth muscles of the gastrointestinal tract (Horowitz et al. 1999). Slow waves (periodic oscillations of the membrane potential) accompanied by one or more action potentials are responsible for this contractile activity (Tomita 1981). Slow waves are generated continuously and are independent of neural activity. It has been suggested that interstitial cells of Cajal (ICC) are putative pacemaker cells responsible for the slow waves in the gastrointestinal tract (for review see Farrugia 1999). There is a striking difference between the guinea-pig small intestine and most of the smooth muscles of the gastrointestinal tract. In the guinea-pig small intestine no marked "spontaneous" slow waves occur without the application of intraluminal pressure (Kuriyama et al. 1967). In the longitudinal muscle layer of the guinea-pig ileum the spontaneous electrical activity consists of one or more action potentials carried on slow waves of depolarisation. Cholinergic nerves clearly contribute to the generation of these slow waves, since in contrast to other tissues, they are abolished by atropine (Hukuhara and Fukuda 1968) or tetrodotoxin (Kuriyama et al. 1967). According to Bolton (1981) and Cousins et al. (1993), these results are consistent with the possibility that most, if not all, the slow wave activity seen in longitudinal muscle of guinea-pig small intestine is caused by the spontaneous release of acetylcholine within the preparation.

The aim of the present work was to describe the effects of H_2O_2 on spontaneous electrical and mechanical activity of the longitudinal layer from the guinea-pig ileum by simultaneous recording of membrane potential and mechanical tension using single sucrose-gap technique. To elucidate the ionic mechanisms involved, we decided to apply patch-clamp technique.

Materials and Methods

Sucrose-gap technique recording

Male guinea-pigs (400–550 g) were sacrificed by stunning and exsanguination. A length of ileum was taken at a distance greater than 20 cm from the ileocaecal junction and immersed in a preparation chamber containing bicarbonate-buffered physiological saline solution (PSS) of the following composition (mmol/l): NaCl, 121.9; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 15.5; KH_2PO_4, 1.2; glucose, 11.5. The solution was gassed with 95 % O_2–5 % CO_2. The zero-calcium solution was prepared from PSS by substitution of 2.5 mmol/l $CaCl_2$ by $MgCl_2$. The longitudinal

muscle with the adhering myenteric plexus of about 20 mm length and about 2 mm width was dissected gently away from the underlying circular muscle. The preparations were trimmed down to have lengths of 15 mm and widths of 1.0 mm.

The single sucrose-gap method was used to record simultaneously force and relative changes in membrane potential. Sucrose-gap was made by using rubber membranes, as described by Berger and Barr (1969). After setting up the preparations in the sucrose gap chamber, they were continuously perfused by PSS with a flow rate of 1 ml·min^{-1} (PSS node volume 0.5 ml) and maintained at 36 ℃. Passive tension was adjusted over a 10 min equilibration period to 10 mN. In high K^+ (50 mmol/l) solution, NaCl was replaced by KCl on a mole-for-mole basis. Contractile responses of the strips were measured with a strain gauge transducer (SG-01D, Experimetria, Budapest, Hungary). Membrane potential was measured with a home-made preamplifier (input resistance 1 GΩ, frequency band 0–1 kHz). Mechanical and electrical recordings were filtered at 50 Hz and sampled at 200 Hz using TL-1 acquisition system (Axon Instruments). Data analysis was performed with the pCLAMP6 (Axon Instruments) and Origin 5.0 (Microcal Software, Inc.) softwares.

Preparation of cells

Single smooth muscle cells were isolated from the longitudinal layer of the guinea-pig ileum using a combination of collagenase (2 mg·ml^{-1}) and pronase (0.05–0.1 mg·ml^{-1}) with fatty-acid-free albumin (3 mg·ml^{-1}), similarly as described by Kohda et al. (1997). The cells were suspended in Krebs-HEPES solution (for composition see below) containing 0.3 mmol/l Ca^{2+} and kept at 4 ℃ until use on the same day.

Conventional whole-cell and perforated patch recordings

A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (0.2 ml) mounted on the stage of an inverted microscope. Myocytes were allowed to adhere to the bottom of the chamber for 10 to 15 minutes and were then superfused at the rate of 1.2 ml·min^{-1} by Krebs-HEPES. Membrane current recordings were performed at room temperature (23–25 ℃) using standard patch-clamp techniques (Hamill et al. 1981). For amphotericin-perforated patch recording, a stock solution was made by sonication of 1 mg amphotericin B (Sigma, St. Louis, MO, USA) in 40 μl dimethyl sulfoxide. Immediately prior to each experiment, 8 μl amphotericin stock was added to 1 ml of pipette solution (for composition see below) and sonicated. Data acquisition and voltage protocol were carried out using an Axopatch 1-D Patch-Clamp amplifier and TL-1 interface (Axon Instruments) coupled to IBM-compatible computer equipped with pCLAMP software (Axon Instruments). Currents were filtered at 2 kHz and digitised at a sampling rate of 10 kHz. The indifferent electrode was an Ag-AgCl plug electrically connected to the bath. During acquisition, correction of leakage current was made by digital subtraction of currents produced by small hyperpolarizing pulses (10 mV).

Solutions

The standard extracellular Krebs-HEPES solution had the following composition (mmol/l): NaCl, 130; KCl, 6; $CaCl_2$, 2.5; $MgCl_2$, 1; glucose, 10; HEPES, 10 (titrated to pH 7.4 with NaOH). Cs^+-based pipette solution had the following composition (mmol/l): CsCl, 140; $MgCl_2$, 2.5; Na_2ATP, 2; EGTA, 0.2; HEPES, 10 (titrated to pH 7.3 with NaOH).

Chemicals

Drugs and chemicals used, namely collagenase (type XI), bovine serum albumin (Fraction V, essentially fatty acid free), disodium adenosine 5′-triphosphate (Na_2-ATP), ethyleneglycol-bis-(β-aminoethylether)-tetraacetic acid (EGTA), N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid (HEPES), sucrose and nimodopine, were from Sigma (St. Louis, USA) and hydrogen peroxide (30 %) was from Lachema (Brno, Czech Republic).

Statistical analysis

The values in the text are expressed as means ± S.D. (n = sample size). Statistical significance was tested using unpaired Student's t-test and differences were considered significant when $p < 0.05$.

A Fast Fourier Transform (FFT) was used to analyse the acquired time-domain (amplitude *versus* time) signals from sucrose-gap technique in the frequency domain (amplitude *versus* frequency). Spectral leakage was minimised by windowing of the original signal by a Blackman window. The two-sided amplitude spectrum was converted to the one-sided amplitude spectrum and scaled by dividing the windowed array by the coherent gain of the Blackman window (Smith 1997). The high-amplitude direct current spectral component at 0 Hz was eliminated by filtrating the original signal using high-band filter ($f_{cutoff} = 0.1$ Hz).

Results

Electrical and mechanical activity of guinea-pig ileum longitudinal muscle

When isolated preparations of longitudinal muscle of the guinea-pig ileum were set up in sucrose gap chamber with a resting force of 10 mN, various shapes of spontaneous electrical and mechanical activities were observed. About 70 % of all the preparations tested ($n = 60$) generated periodic oscillations of the membrane potential with a fundamental frequency ranging from 0.3 to 3.0 Hz. A waveform of each cycle consisted of the so-called slow wave with a superimposed action potential/s (AP). The total changes of the membrane potential were within the range of 3–4 mV. Sometimes a notch appeared on the AP, or the AP was split into two spike-like potentials. Rhythmic muscle contractions appeared in preparations when slow waves were accompanied by APs. The slow waves were of various amplitudes (from 1 to 3 mV). The waveform of the electrical activity of the preparations recorded using sucrose-gap technique (our experiments) was basically the same as

that recorded using microelectrodes in longitudinal layer of the guinea-pig ileum (Bauer and Kuriyama 1982a,b) or of opossum jejunum (Hara et al. 1986). Neither tetrodotoxin (3×10^{-7} mol/l) nor atropine (10^{-6} mol/l) affected the amplitude and frequency of slow waves ($n = 5$). The slow wave frequency determined the frequency of the spontaneous muscle contractions (see time-expanded scales in Fig. 1A). The amplitude of these mechanical oscillations was from 0.5 to 2.5 mN. In some preparations (about 40% of the tested) the frequency of slow waves was higher than 2 Hz. These preparations had no mechanical oscillation but a tetanic-like contraction was observed (Fig. 4, top trace).

According to the Fourier theorem, if the signal is periodic with the frequency f, the only frequencies composing the signal are integer multiples of f, namely f, $2f$, $3f$, $4f$, etc. These frequencies are called harmonics. The first harmonic (fundamental frequency) is f, the second harmonic is $2f$, the third harmonic is $3f$, and so forth. An amplitude spectrum of a pure sine wave will give a single peak of first harmonic f in the frequency domain. If the sine wave has been distorted on the top (asymmetrical distortion), the frequency domain is composed of the original peak plus even and odd harmonics (Smith 1997). In our case, the waveform of the slow wave may be considered in the first approximation as a sine wave and the APs as its asymmetrical distortions. Fig. 1B shows the amplitude spectrum of the original electrical signal presented in Fig. 1A (bottom trace). There is one large peak f characterising slow waves, and two small peaks, $2f$, $3f$, representing the asymmetrical distortion of slow waves by APs.

Application of nimodipine (1 μmol/l), an L-type calcium channel blocker, resulted in membrane depolarisation (4.2 ± 0.4 mV, $n = 6$) and in disappearance of APs, without any influence on the slow wave amplitude and frequency. The alteration of electrical activity was accompanied with a decline of the initial tension by 4.0 ± 0.5 mN ($n = 6$) and abolishment of mechanical oscillations (Fig. 1A). In accordance with the effects described above, nimodipine decreased the amplitude of peaks $2f$ and $3f$ without noticeably affecting the first harmonic f.

Effect of H_2O_2 on mechanical and electrical activity of guinea-pig ileum longitudinal muscle

The above mentioned observations served as a basis for our further experimental work studying effects of H_2O_2 on spontaneous electrical and mechanical activity of the longitudinal layer from guinea-pig ileum.

Fig. 2 shows simultaneous long-lasting recordings of changes in contractile activity and membrane potential in response to H_2O_2 and high K^+. Application of H_2O_2 (1 mmol/l) evoked sustained membrane depolarisation by 3–5 mV and doubled the amplitude of the slow waves ($n = 16$). APs were transformed to more pronounced and less split ones (see time-expanded scale of the membrane potential trace in Fig. 2A). Due to the presence of notches and splittings in APs before H_2O_2 application, we could not determine the exact value of the H_2O_2-induced increase of the AP amplitude. High K^+ (50 mmol/l) solution elicited biphasic membrane depolarisation. The initial phase of about 30–35 mV was followed by a sustained

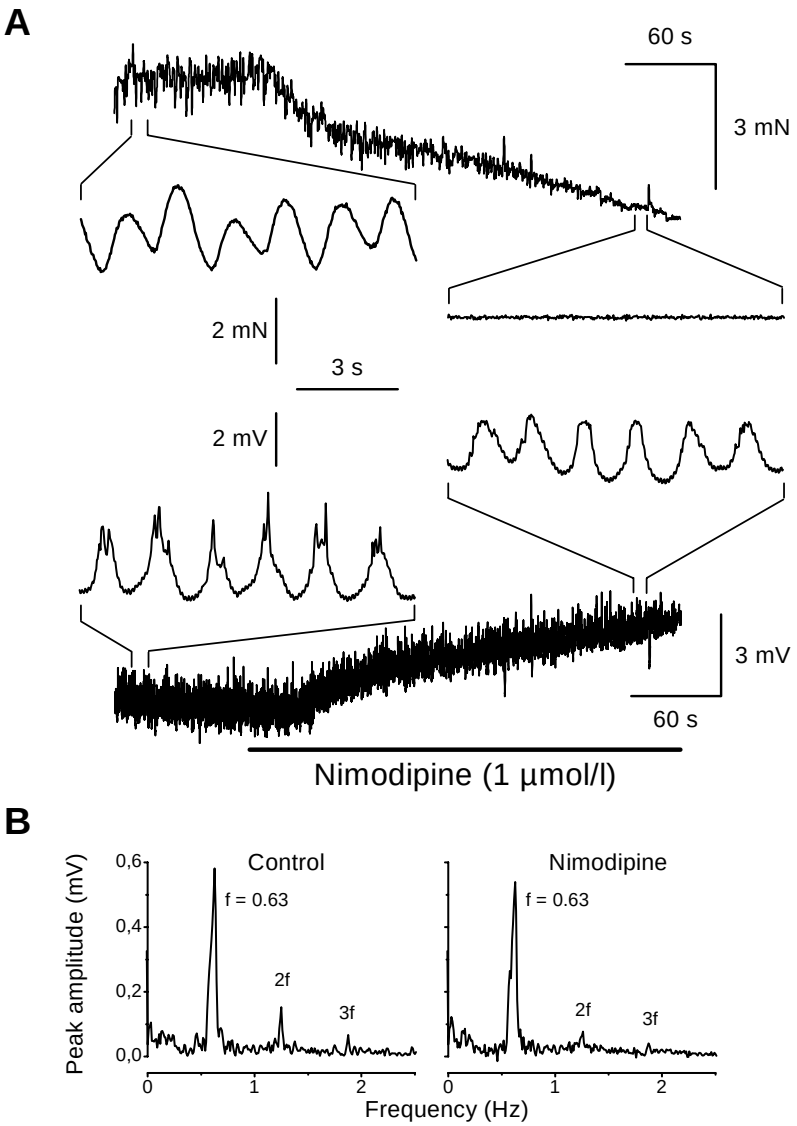

Figure 1. Spontaneous electro-mechanical activity of guinea-pig ileum longitudinal smooth muscle preparation. Simultaneous long-lasting recording of contractile tension (A, top trace) and membrane potential (A, bottom trace) was performed using the single sucrose-gap technique. The effect of nimodipine, namely inhibition of APs (see lower time-expanded scale) exhibited by a decline of mechanical tension and disappearance of tension oscillations (see upper time-expanded scale), illustrates a close relationship between electrical and mechanical activity of the preparation. The amplitude spectrum of membrane potential (B) under control conditions (left part) and in the presence of nimodipine (right part), obtained by applying FFT, revealed the first (peak f) and the second ($2f$) and third harmonics ($3f$).

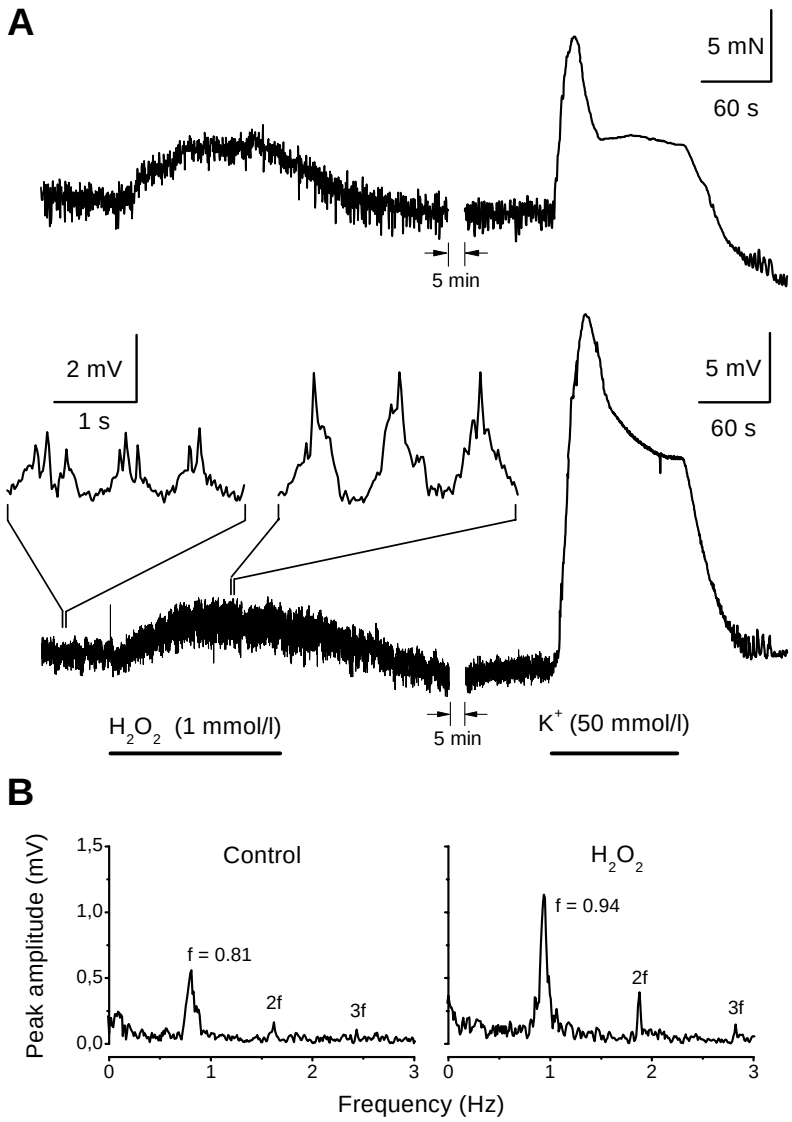

Figure 2. Effect of hydrogen peroxide on guinea-pig ileum longitudinal smooth muscle preparation. Mechanical tension (A, top trace) and membrane potential (A, bottom trace) were simultaneously recorded using single sucrose gap technique. A – H_2O_2 (1 mmol/l) evoked contraction whose amplitude was comparable to that of the tonical component of high K^+-induced contraction. In addition to moderate depolarisation, H_2O_2 increased the amplitudes of slow waves and of APs, as demonstrated in the time-expanded scale of the membrane potential trace. The amplitude spectrum of membrane potential (B) illustrates that in addition to a small increase of the frequency of the first harmonic, H_2O_2 significantly increased the amplitude of the first harmonic (peak f) and of the second ($2f$) and third harmonics ($3f$).

one with the amplitude of about 20 mV. H_2O_2-evoked changes of electrical activity were accompanied with significant contractile response (6 ± 1 mN, $n = 16$) of the preparations (Fig. 2A, top trace). The amplitude of H_2O_2-induced contraction was comparable to that of tonic component of 50 mmol/l K^+-induced contraction performed after a 5–10 min washout of H_2O_2.

As shown in Fig. 2B, H_2O_2 significantly increased the amplitude of the first harmonic (peak f) and of the second and third harmonics (peak $2f$ and $3f$, respectively), with a small increase of the frequency of the first harmonic.

The findings of the increased slow wave amplitude, pronounced APs and depolarisation of the preparation, along with its contractile response after H_2O_2, suggest that these H_2O_2-mediated effects on the guinea-pig ileum longitudinal muscle layer are likely due to the increased entry of calcium ions through L-type calcium channels. We therefore decided to elucidate the role of external calcium ions in the H_2O_2 effects observed under the experimental conditions of blocking the L-type channels or removing the current carrier (Ca^{2+}) from the bath solution (zero-calcium).

Role of external calcium ions in the H_2O_2-induced effects in guinea-pig ileum longitudinal muscle

In contrast to the above described action of nimodipine in its short lasting presence (3–4 min, Fig. 1), its longer (more then 10 min) presence in the bathing fluid completely abolished the slow waves (Fig. 3). Under these conditions, addition of H_2O_2 (1 mmol/l) elicited further depolarisation (Fig. 3, bottom trace), but did not affect the mechanical tension (Fig. 3, top trace).

Exposure of the muscle preparation to calcium-free solution resulted in membrane depolarisation, reduction of slow wave amplitude and frequency, and disappearance of APs (Fig. 4, bottom trace). These changes in the electrical activity were accompanied by decreasing mechanical tension (Fig. 4, top trace; notice the tetanus-like mechanical activity of the preparation). In contrast to the H_2O_2-mediated effect in the presence of nimodipine, application of H_2O_2 (1 mmol/l) into the zero-calcium bath solution recovered the slow wave amplitude and APs, but only partially the slow wave frequency (Fig. 4, bottom trace). Mechanical activity of the preparations revealed a tendency to increase muscle tension after H_2O_2 application (Fig. 4, top trace).

Effect of H_2O_2 on calcium current in single muscle cells

Pronounced APs under H_2O_2 conditions may be also due to the increased calcium current (I_{Ca}). For confirmation, we studied the influence of H_2O_2 on I_{Ca} using the patch-clamp technique.

Our study showed that H_2O_2 (up to 1 mmol/l) affected I_{Ca} both under conventional whole-cell configuration of patch-clamp and under amphotericin-perforated patches. The cells were held under voltage clamp and dialysed with a CsCl-based pipette solution to block K^+ currents. I_{Ca} was evoked by stepping from the holding potential -60 mV to 0 mV for 100 ms at the frequency of 0.1 Hz. H_2O_2 (1 mmol/l) increased the peak amplitude of I_{Ca} by $16 \pm 3\,\%$ ($n = 10$), as shown in Fig. 5A. The

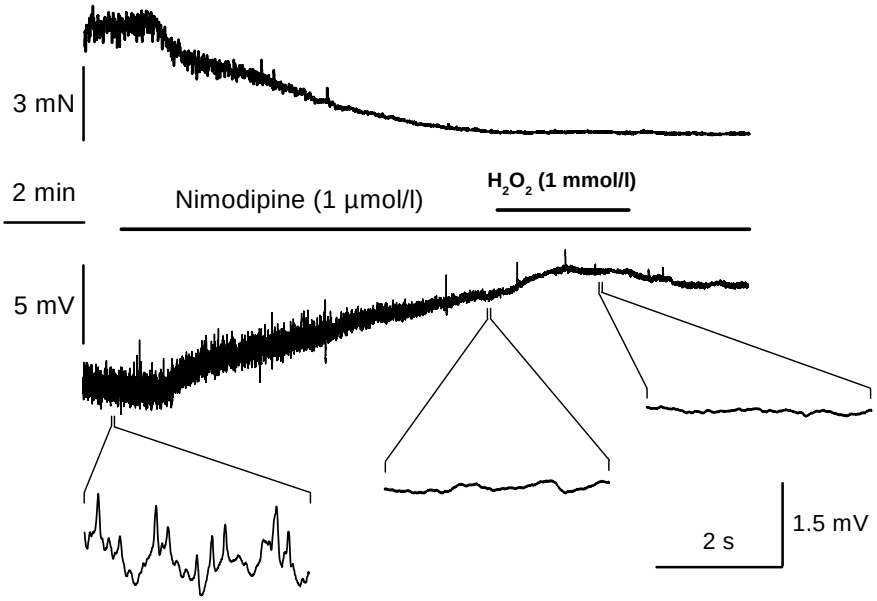

Figure 3. Effects of nimodipine on hydrogen peroxide action in guinea-pig ileum longitudinal smooth muscle preparation. Representative traces of mechanical tension (top trace) and membrane potential (bottom traces) of the guinea-pig ileum longitudinal muscle preparation were recorded simultaneously using single sucrose gap technique. Nimodipine (1 μmol/l) was applied 5 min before and was present during the action of H_2O_2 (1 mmol/l). Note that in the presence of nimodipine slow waves gradually disappeared and H_2O_2 evoked depolarisation without recovery of spontaneous electrical and mechanical oscillations and tension.

H_2O_2-induced enhancement of I_{Ca} amplitude was sustained and it was reversible after washout under perforated patch conditions (Fig. 5B, open circles) while under conventional whole-cell condition it had transient character (Fig. 5B, full circles). H_2O_2 did not induce any shift in the maximum of the current-voltage relationship under either of the conditions tested.

Discussion

In our experimental conditions, using sucrose gap technique, the recorded amplitudes of the slow waves and the APs were very small, both in control and H_2O_2 solutions. Why were they so small? One of the obvious explanations is that this may result from a spatial non-uniformity of nodal potential caused by cable properties of the muscle, and a short-circuit factor of sucrose gap (Bolton et al. 1981). Presumably, other factors also come into play. When e.g. membrane depolarisation is evoked by a KCl solution under sucrose-gap condition, the recorded changes are

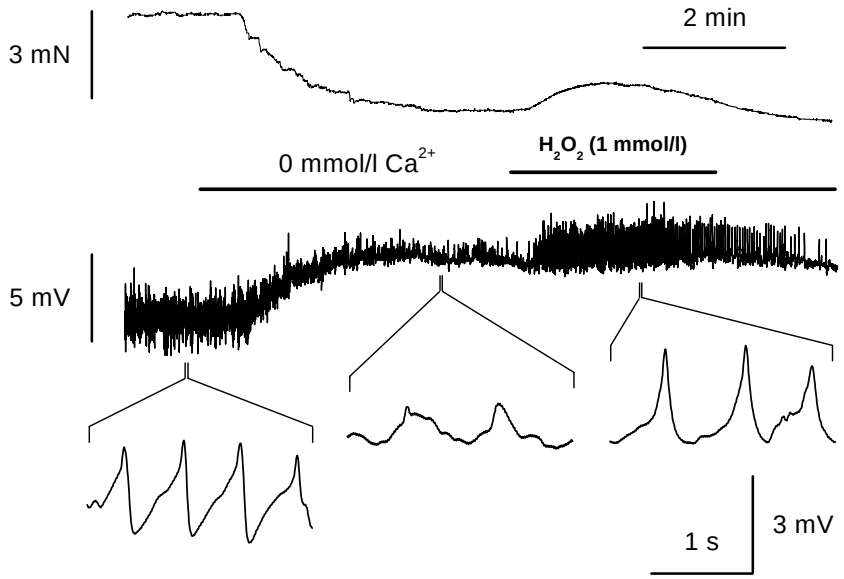

Figure 4. Effect of H_2O_2 in zero calcium (EGTA free) solution on guinea-pig ileum longitudinal smooth muscle preparation. Representative traces of mechanical tension (top trace) and membrane potential (bottom traces) of the guinea-pig ileum longitudinal muscle preparation were recorded simultaneously using single sucrose gap technique. Note that H_2O_2 restored the spike activity but caused only slight contraction and marginal depolarisation.

reflecting not only those described by Bolton et al. (1981) but also the changes in the real membrane potential and in the diffusion potential. Since the diffusion potential cannot be measured under sucrose-gap condition we had to evaluate it. Considering the interface as circular disk, the concentration profile of the ions in solutions was found by solving the steady state diffusion equation (Crank 1975):

$$X^2 C(x, y, z) = 0$$

with boundary conditions for our Krebs and high K^+ solution.

Substitution of this concentration profile to generalised Henderson equation (Barry and Diamond 1970) gave the changes of the diffusion potential of about 5 mV. Thus the change of the real steady state membrane depolarisation caused by 50 mmol/l K^+ solution in our case was 15 mV (Fig. 4), which was comparable to the amplitude (20–25 mV) recorded by the microelectrode technique (Dieleman et al. 1997). Thus under our sucrose-gap condition, spatial non-uniformity of nodal potential caused by cable properties of the muscle, short-circuit factor and diffusion potential changes play a minor role. Attenuation was about 20–30 %.

Several recent results suggest another possible explanation of the described phenomena. Hennig et al. (1999) have observed rhythmic local longitudinal move-

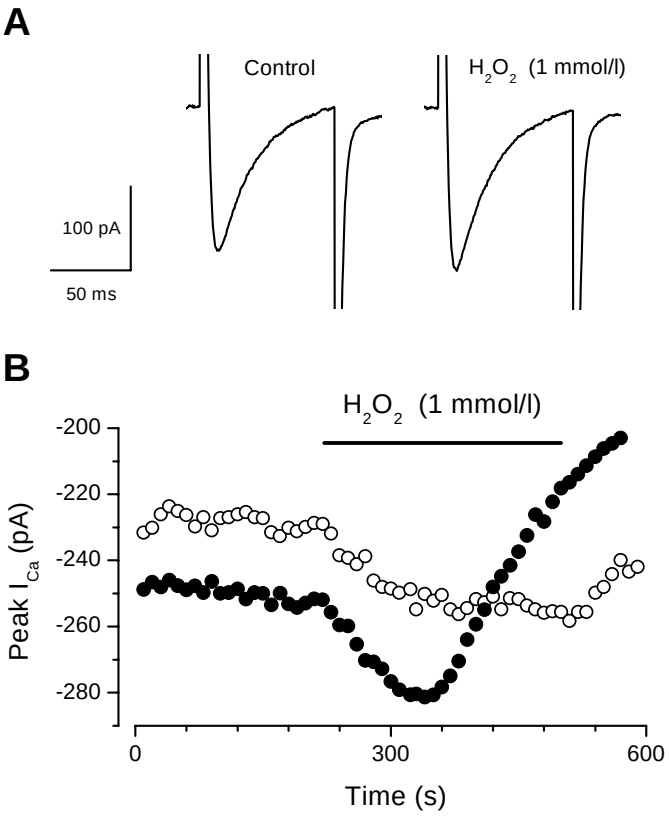

Figure 5. Hydrogen peroxide reversibly enhances Ca^{2+} current in guinea-pig ileum longitudinal single smooth muscle cells. Amphotericin-perforated and whole-cell patch technique was applied to record I_{Ca} in response to 100-ms depolarising pulses from holding potential −60 mV to 0 mV with frequency of 0.1 Hz. Recording pipettes contained Cs^+-based solution, bath contained PSS. **A** – representative traces of I_{Ca} under control conditions and in the presence of 1 mmol/l H_2O_2. **B** – time course of the H_2O_2-elicited changes in I_{Ca} amplitude showing maintained and reversible enhancement of peak current under amphotericin-perforated patch (open circles) and transient enhancement under whole-cell conditions (full circles).

ments in the guinea-pig small intestine using spatio-temporal maps. Myogenic contractions originating in discrete, very localised regions (dominant pacemakers), were observed also in rat ileum (Bercik et al. 2000). In guinea-pig ileum longitudinal smooth muscle Ca^{2+} oscillations were spontaneously produced at specific cells in a fixed location of the tissue (pacemaker site of Ca^{2+} oscillations). These Ca^{2+} oscillations propagated along the short axes of the cells and were unaffected by tetrodotoxin and atropine (Satoh et al. 2000). These observations, along with our electrophysiological results, are in favour of the existence of a large electrical by-

pass of the dominant pacemakers by the surrounding silent tissue. Thus the small amplitude of slow waves and APs might be explained by this mechanism.

It has been suggested that ICC act as pacemaker cells in the small intestine and are responsible for the generation of slow-wave electrical activity of the preparations (Kadlec et al. 1989; Komuro et al. 1996). Toma et al. (1999) described the pacemaker function of ICC and responding smooth muscle cells, both present in the guinea-pig ileum. The absence of L-type calcium channels in ICC of the small intestine (Lee et al. 1999) gives reasonable explanation why the slow-wave activity remained unchanged after nimodipine application in our tissue. On the other hand, the role of the L-type channels in guinea-pig ileum smooth muscle cells in the generation of APs has been well documented (Kohda et al. 1997), and thus blocking of these channels removed APs, which resulted in reduced tension and disappearance of mechanical oscillations.

As mentioned above, ICC are the main candidates for pacemaker cells in most gastrointestinal smooth muscles. The morphological evidence of this statement for the guinea-pig ileum was given by Komuro et al. (1996). They demonstrated the presence of myenteric ICC as a cellular network independent of the myenteric plexus and further ICC associated with deep muscular plexus, using the zinc iodide-osmic acid method and immunohistochemistry for vimentin and c-Kit receptor tyrosine kinase. Moreover, recently Toma et al. (1999) showed the presence of the c-Kit positive cellular network of ICC within the longitudinal muscle layer as well as in the subserosal layer, which were not associated with nerve fibres. In contrast to the observations of Kuriyama et al. (1967) and Bolton (1981) on the one hand, and Stevens et al. (2000) on the other, who observed atropine sensitive spontaneous activity and Ca^{2+} waves, respectively, our electrophysiological findings that neither tetrodotoxin nor atropine affected the amplitude and frequency of slow waves are in favour of the involvement of ICC in the pace-making mechanism in the guinea-pig ileum longitudinal muscle. Very recent results of Satoh et al. (2000) who recorded Ca^{2+} oscillations propagated along the short axes of the cells, which were unaffected by tetrodotoxin and atropine, support this suggestion. Thus, probably depending on the procedure of isolation of muscle preparations and on the experimental conditions, the longitudinal muscle-myenteric plexus of the ileum may exhibit spontaneous activity of the slow-waves type, stochastic type, or their combination.

Since the longitudinal muscle-myenteric plexus preparations of the ileum are unhomogeneous tissue consisting of muscle layer, the myenteric plexus and ICC, H_2O_2 could affect all the components of these preparations. There were several alterations in the electrical activity recorded in our experiments in the presence of H_2O_2 due to its actions on the pacemaker activity and action potentials, which could explain the observed contraction. The pacemaker activity is believed to be determined by ICC in the intestinal smooth muscle; yet, our knowledge on the mechanisms of slow wave regulation is incomplete (Malysz et al. 2001). In our opinion, the observed increase of the first harmonic amplitude of the slow waves may be caused by the direct action of H_2O_2 on ICC. The enhancement of the

slow wave amplitude in sucrose gap, however, could be explained by the direct influence of H_2O_2 on ICC, electrical coupling between ICC and smooth muscle cells, and/or both. Using this technique, it is not possible to separate the three possible mechanisms because the electrical signal recorded is obtained from the smooth muscle cells and involves the activity of both the ICC and smooth muscle cells. On the other hand, the action potentials recorded in sucrose gap were generated in the smooth muscle cells. Enhancement of their amplitude by H_2O_2 might reflect the increased rate of the rise of the depolarisation during the enlarged slow waves and the direct action of H_2O_2 on smooth muscle cells. One of the direct actions of H_2O_2 on smooth muscle cells found in our experiments under voltage clamp conditions was the enhancement of the evoked inward L-type calcium current. This finding is in accordance with our previous results and conclusion (Pucovský et al. 1999) that H_2O_2 increases the open probability of calcium-sensitive potassium channels as the result of an enhanced calcium concentration in the internal vicinity of the plasma membrane. Recently Liu and Gutterman (2002) also described a H_2O_2-induced increased activation of BK_{Ca}. The fact, that in spite of this in the smooth muscle preparation H_2O_2 evoked depolarisation, might be explained only by involvement other conductances, namely cationic or/and chloride one in the mechanism of membrane depolarisation. The difference between the characteristics of calcium current enhancement under conventional whole-cell and perforated patch conditions suggests that some intracellular component/s which is/are dialysed under the conventional whole-cell condition is/are essential for its maintenance. Such enhancement was recorded also in other tissues. Pei et al. (2000) have reported activation of Ca^{2+}-permeable channels in the plasma membrane of Arabidopsis guard cells by H_2O_2, Guo et al. (2000) have observed enhancement of the L-type Ca^{2+} current in rabbit sinoatrial node cells, the latter being mediated by a PKC-dependent pathway and Gen et al. (2001) have mentioned except other mechanisms also a Ca^{2+} influx through routes other than the voltage-dependent Ca^{2+} channels in cardiomyocytes. H_2O_2 was described to increase the intracellular Ca^{2+} both in the absence and presence of external Ca^{2+} in cat tracheal smooth muscle cells (Bauer et al. 1997). The possible mechanism by which H_2O_2 may evoke contraction under calcium free conditions might be Ca^{2+} and myosin light chain phosphorylation-independent contraction, as observed in vascular muscle (Palaez et al. 2000), involvement of mitochondria (Michelakis et al. 2002a,b), or release of Ca^{2+} from intracellular store sites as described by Favero et al. (1995) in skeletal muscle and Gen et al. (2001) in cardiomyocytes. Our result, however, excludes this possibilities because H_2O_2 did not evoke contraction under voltage-dependent calcium channels being blocked by the presence of nimodipine. Our result is in favour of a Ca^{2+}-dependent mechanism. This is supported by the enhancement of the action potentials in the presence of extracellular Ca^{2+} and their recovery under the Ca^{2+}-free conditions. Mobilisation of Ca^{2+} from the extracellular matrix, resulting in an increase of the effective concentration of Ca^{2+} in the vicinity of the calcium channels, may provide one of the possible explanations of the recovery of the APs in the absence of extracellular Ca^{2+}.

Thus, in the sequence of events leading to the contractile response of the longitudinal smooth muscle of the guinea-pig ileum caused by H_2O_2, the changes of the electrical activity of the preparations play a crucial role.

Acknowledgements. The linguistic help and critical reading of the manuscript by Prof. M. Kouřilová are gratefully acknowledged. This study was supported by the grants: Slovak Grant Agencies GAV 2/2052/22 and APVT-20–020802.

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Final version accepted: January 22, 2003