Hypoxia-induced inhibition of calcium channels in guinea-pig taenia caeci smooth muscle cells

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1. The effects of hypoxia on whole-cell current in single smooth muscle cells and on a high K^+-induced contraction of strips of the guinea-pig taenia caeci were studied.
2. In physiological salt solution (PSS) and K^+-based pipette solution, hypoxia ($P_{O_2} = 20$ mmHg) reversibly inhibited both the inward Ca^{2+} current (I_{Ca}) and outward Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) components of the whole-cell current.
3. In PSS and Cs^+-based pipette solution, hypoxia reversibly suppressed I_{Ca} by $30 \pm 5\%$ at 0 mV.
4. When Ba^{2+} was used as a charge carrier, the I_{Ba} was suppressed by hypoxia in a potential-dependent manner, with the maximum of $40 \pm 7\%$ at +10 mV. Alterations of concentrations of EGTA, GDBβS or ATP in the pipette solution did not change the inhibitory effects of hypoxia on I_{Ca} and I_{Ba}.
5. In PSS with 2 mM $CaCl_2$ replaced by $CoCl_2$, hypoxia did not affect the Ca^{2+} influx-independent potassium current.
6. In cells voltage clamped at −20 mV hypoxia reversibly inhibited the spontaneous transient outward currents.
7. The response of high K^+-contracted taenia caeci to hypoxia was composed of an initial rapid relaxation followed by a small transient contraction and slow relaxation. The transient contraction was blocked by atropine (1–10 μM), while relaxations were unaffected by atropine and guanethidine (10 μM).
8. The results show that hypoxia reversibly inhibits I_{Ca} and secondarily suppresses $I_{K(Ca)}$ due to decreased Ca^{2+} influx through Ca^{2+} channels.
9. It is suggested that inhibition of I_{Ca} was responsible for the rapid relaxation, whereas transient contraction may have been due to release of acetylcholine from nerve terminals upon hypoxia.

Effects of hypoxia are rather variable among different smooth muscles (for review see Wadsworth, 1994). Hypoxia is known to relax a high K^+-induced contraction in the taenia caeci of the guinea-pig (Bose & Bose, 1975; Knull & Bose, 1975; Nasu, Yui, Nakagawa & Isida, 1982). Two general classes of mechanisms have been proposed to explain the relaxing effects of hypoxia. On the one hand, the capacity of the cell to produce ATP may be limited by hypoxia. As the actin–myosin ATPase is a major site of ATP utilization, contraction may be limited directly by the ATP synthetic capacity of the cell under hypoxia (Isida, Takagi & Urakawa, 1984). On the other hand, the second class involves a type of oxygen-sensing mechanisms which would inhibit contraction at some stage of excitation–contraction coupling. An example of these would include alterations in Ca^{2+} permeability (Ashoori, Takai, Tokuno & Tomita, 1984).

Recently it has become clear that plasma membranes of various tissues can sense the lack of oxygen, not only indirectly via alterations in the intracellular milieu (such as ATP, Ca^{2+} and pH) but also directly through unknown mechanisms that involve plasma membrane potassium and calcium channels (for review see Haddad & Jiang, 1997). Oxygen-sensitive calcium channels were observed in dispersed smooth muscle cells of the celiac, femoral (Franco-Obregón, Ureña & López-Barneo, 1995) and proximal pulmonary arteries of rabbits (Franco-Obregón & López-Barneo, 1996).

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The present work was undertaken to clarify whether oxygen-sensitive channels are also present in the plasma membrane of the taenia caeci myocytes and to investigate in detail the hypoxia-induced relaxation of the taenia caeci contracted by high K^+. We used the whole-cell patch voltage clamp technique to record currents from taenia caeci myocytes. Our results suggest that at least two mechanisms may be involved in the relaxing effects of hypoxia. One of them is the reversible inhibition of the macroscopic Ca^{2+} current of taenia caeci myocytes, which is responsible also for the reduction of $I_{K(Ca)}$. The mechanism underlying the slow phase of hypoxia-induced relaxation is probably of myogenic origin.

METHODS

Preparation of cells

Male guinea-pigs (400–550 g) were killed by stunning and exsanguination; the method was approved by the ethical committee of the Institute. Single smooth muscle cells were dispersed using a combination of collagenase (1 mg ml^{-1}) and fatty acid-free albumin (1 mg ml^{-1}) as described earlier (Bauer, Rekalov & Ito, 1994). The cells were suspended in physiological salt solution (PSS; for composition, see below) containing 0·3 mM Ca^{2+} and were kept at 4 °C until use on the same day.

Recording of membrane currents

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 10–15 min and were then superfused at the rate of 1·2 ml min^{-1} with PSS. Whole-cell membrane current recordings were made at room temperature (23–25 °C) using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigword, 1981). Patch pipettes had resistances of 2·5–4 MΩ when filled with K^+-based pipette solution (for composition, see below). Data acquisition and the voltage protocols were carried out using an Axopatch 1-D patch-clamp amplifier and TL-1 interface (Axon Instruments) coupled to an IBM-compatible computer equipped with pCLAMP software (Axon Instruments). Currents were filtered at 2 kHz and digitized 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 and drugs

The standard extracellular (bath) solution (PSS) had the following composition (mM): NaCl, 130; KCl, 6; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 10; Hepes, 20 (titrated to pH 7·4 with NaOH). The concentration of Hepes in the PSS was 20 mM because pH was changed by N_2 bubbling at a Hepes concentration of 10 mM. When Ba^{2+} was used as a charge carrier for I_{Ca}, the $CaCl_2$ was replaced with an equimolar concentration of $BaCl_2$. In Ca^{2+}-free PSS, $CaCl_2$ was replaced with $MgCl_2$ and EGTA (1 mM) was added. For Co^{2+} bath solution, 2 mM $CaCl_2$ was replaced with 2 mM $CoCl_2$ in PSS. K^+-based pipette solution had the following composition (mM): KCl, 140; $MgCl_2$, 2·5; Na_2ATP, 2; EGTA, 0·2; Hepes, 10 (titrated to pH 7·3 with KOH). Cs^+-based pipette solution was prepared by replacement of KCl in K^+-based solution with an equimolar concentration of CsCl. The calculated free Ca^{2+} concentration was < 10 nM, assuming that contamination of Ca^{2+} in the pipette solution did not exceed 20 μM. In some experiments, 0·2 mM EGTA was replaced with a Ca–EGTA buffering system (0·6 mM EGTA and 0·4 mM $CaCl_2$) giving 100 nM Ca^{2+} in the pipette solution (Hu, Yamamoto & Kao, 1989).

The following drugs and chemicals used were obtained from Sigma: collagenase (type XI), bovine serum albumin (Fraction V, essentially fatty acid free), Na_2ATP, lithium guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), atropine sulphate, guanethidine sulphate and EGTA.

Oxygen tension

External solutions were equilibrated with either air or 100% N_2 in glass reservoirs for at least 20 min before the onset of each experiment. Due to the small size of the perfusion chamber, it was impossible to measure directly the P_{O_2} during whole-cell recording. A preliminary experiment was therefore carried out for monitoring P_{O_2} in the perfusion chamber using an O_2-sensing electrode (model SOPS-31, Chemoprojekt, Prague, Czech Republic). The rate of perfusion was 1·2 ml min^{-1} in this and the subsequent experiments. Under these experimental conditions, the P_{O_2} in the experimental bath reached a stable level of approximately 20 mmHg within 1·5 min after switching to external solution equilibrated with 100% N_2.

Tension recording

Male guinea-pigs (400–550 g) were killed by stunning and exsanguination. The taenia was dissected free from the caecum and cut into strips about 15 mm long. The strips were suspended in a tissue bath containing 20 ml bicarbonate-buffered PSS of the following composition (mM): 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 maintained at 37 °C and gassed with 95% O_2–5% CO_2 for normoxic conditions, or with 95% N_2–5% CO_2 for hypoxic conditions. The pH of the solution was 7·3–7·4. P_{O_2} in a tissue bath was 14 ± 3 mmHg ($n = 4$) when gassed with 95% N_2–5% CO_2, possibly due to the open tissue bath and contamination of O_2 in the gas mixture. This value was ~6 mmHg lower than that recorded in the cell bath. In high-K^+ (80 mM) solution, NaCl was replaced by KCl on a mole for mole basis. When the taenia was subjected to hypoxic conditions, the oxygenated solution was exchanged for a solution pre-equilibrated with nitrogen and the aeration gas was concomitantly switched from oxygen to nitrogen.

Contractile responses of the taenia were recorded with a strain gauge transducer (SG-01D, Experimetria, Budapest, Hungary) electrically connected to a chart recorder (TZ 4200, Laboratorní Přístroje, Prague, Czech Republic). A digitizing tablet (model 4611, Océ Graphics, Créteil, France) transferred the data from a recorder to a computer. The taenia was equilibrated under aerobic conditions for more than 60 min before the actual experiment started.

Statistics

The values in the text are expressed as means ± S.D. (n = sample size). Statistical significance was tested using Student's unpaired t test and differences were considered significant when $P < 0·05$.

RESULTS

Effect of hypoxia on whole-cell current

To record the membrane current, PSS extracellular solution and K^+-based pipette solution were used. Depolarizing pulses from the holding potential of −60 mV to the test potential of 0 mV of 50 ms duration were applied with a frequency of

0·067 Hz. Depolarization of the membrane potential to 0 mV was used to minimize possible contamination from Cl^- current (chloride equilibrium potential, E_{Cl}, was 0 mV). Under such experimental conditions in smooth muscle cells of taenia caeci, the voltage-dependent inward current is mainly the dihydropyridine-sensitive L-type calcium current, I_{Ca} (Yoshino, Somea, Nishio & Yabu, 1988). The outward component of the whole-cell membrane current is almost exclusively due to the opening of large-conductance, voltage-activated Ca^{2+}-sensitive K^+ channels, $I_{K(Ca)}$ (Hu *et al.* 1989).

After establishing the whole-cell configuration, the recorded membrane currents were small but increasing progressively, to reach a nearly stable level after several minutes (Fig. 1*A* and *B*, point *a*). Hypoxic external solution applied to the bath at this time produced a decrease of the inward and outward components of the membrane current (Fig. 1, point *b*). On switching the superfusing solution back to the normoxic one, the membrane currents were found to recover (Fig. 1, point *c*). In these whole-cell membrane currents a considerable overlap of their inward and outward components was found (Yamamoto, Hu & Kao, 1989*a*) and a fragment of $I_{K(Ca)}$ in taenia caeci myocytes was reported to be somewhat reduced whenever calcium influx was decreased (Yamamoto, Hu & Kao, 1989*b*). Thus investigation of the effect of hypoxia on isolated components of the whole-cell membrane current appeared to be important.

Figure 1. The effect of hypoxia on whole-cell current in a single smooth muscle cell isolated from guinea-pig taenia caeci

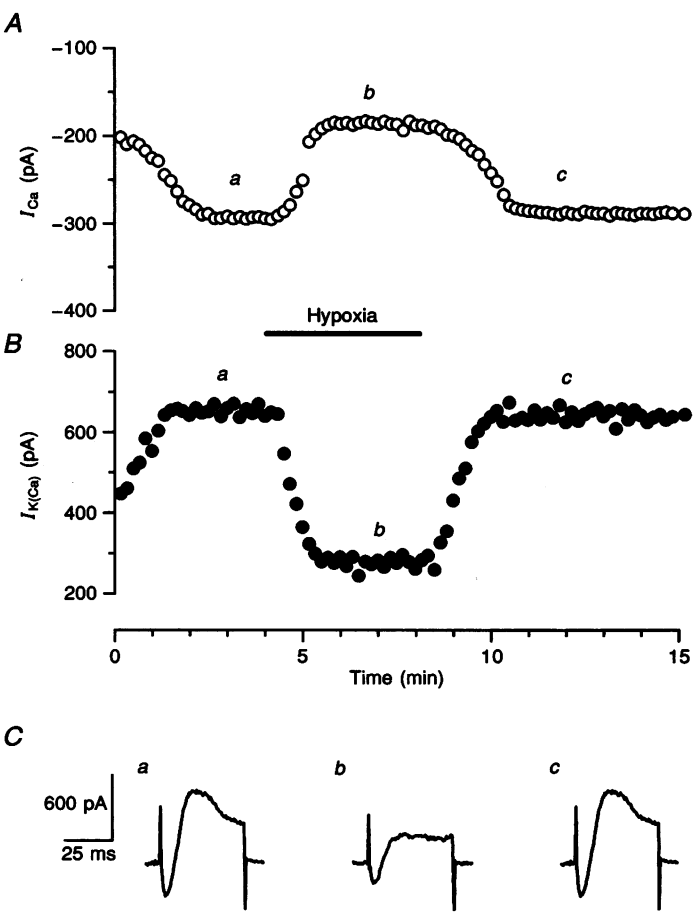
Whole-cell current was elicited by depolarizing pulses (50 ms duration) from the holding potential of −60 mV to 0 mV at a frequency of 0·067 Hz. The recording pipettes contained K^+-based solution; the bath contained PSS. *A* and *B* are time courses of the peak values of inward (○) and outward (●) membrane current components, respectively. The points under *a*–*c* on the graphs correspond with the actual whole-cell current recordings (*a*–*c*) in *C*. The bar under hypoxia in this and the subsequent graphs indicates a period when the oxygenated superfusing solution was switched to solution gassed with N_2 (hypoxic solution).

Effect of hypoxia on potassium current

As shown above, hypoxia inhibited both the inward and outward component of the whole-cell current. Its net effect on the outward current, however, could not be derived from these data. For this reason, K^+ current was investigated under experimental conditions preventing contamination of the whole-cell current by Ca^{2+} current. There are two principal approaches to isolate K^+ current, either using Ca^{2+}-free PSS or blocking the calcium channels

In the first approach, membrane current was elicited by depolarization as in the previous experiment. Introducing the Ca^{2+}-free PSS resulted in a transient (1–2 min) decrease of the outward current (data not shown). After this initial attenuation, the outward current increased and stabilized at a new level exceeding that in PSS (compare traces *a* and *b*, Fig. 2*A*) within 8 min. The dramatic change in K^+ current kinetics suggested that under these experimental conditions the outward current might be due to the efflux of K^+ not only through potassium channels but also through calcium channels (Yamamoto *et al.* 1989*a*). For this reason, the second approach was used to isolate potassium current.

In the second approach, Co^{2+} was used to block calcium channels. Although Co^{2+} itself could have a small direct effect on K^+ channels (Yamamoto *et al.* 1989*b*), the use of organic Ca^{2+} channel blockers is known to be associated with voltage- and use-dependent effects, which might complicate



the analysis even more (Lee & Tsien, 1983). In PSS, 2 mM $CaCl_2$ was equimolarly replaced by $CoCl_2$. In such a Co^{2+} bath solution the inward Ca^{2+} current disappeared and the amplitude of the outward K^+ current was markedly decreased (compare traces *c* and *d* in Fig. 2*B*). The average amplitude of this Co^{2+}-resistant potassium current was $40 \pm 6\%$ ($n = 7$) of the amplitude of the outward current in PSS. After stabilization at a new level, the amplitude of the residual potassium current was unaffected by exposure to hypoxia (Fig. 2*D*, filled circles).

As mentioned above, only one type of potassium current, namely $I_{K(Ca)}$, dominates in these cells, in which case the Co^{2+}-resistant potassium current represents the $I_{K(Ca)}$ available at the concentration of Ca^{2+} in the pipette solution, which did not exceed 10 nM. To confirm this statement, the concentration of Ca^{2+} in the pipette solution was adjusted to 100 nM, using a Ca–EGTA buffering system. Depolarizing pulses from the holding potential of −60 mV to the test potential of 0 mV of 50 ms duration were applied with a frequency of 0·067 Hz. The duration of 50 ms was used

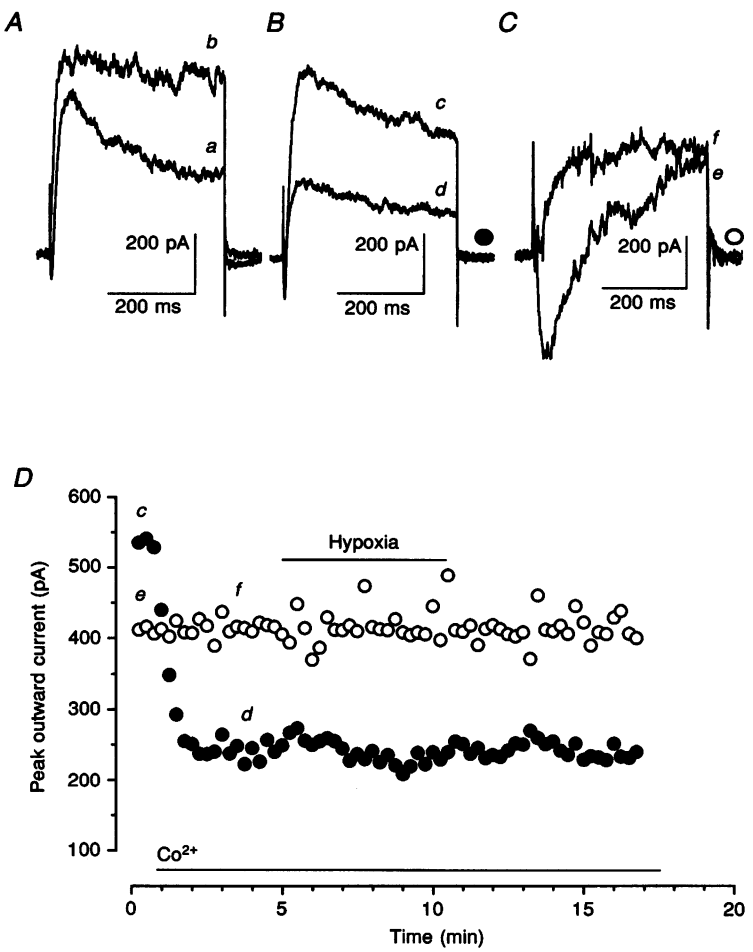


Figure 2. Hypoxia does not inhibit the Ca^{2+} influx-independent potassium current in taenia caeci myocytes

Potassium current was elicited by depolarizing pulses (400 ms duration or 50 ms when concentration of Ca^{2+} in the pipette solution was 100 nM) from the holding potential of −60 mV to 0 mV at a frequency of 0·067 Hz, using K^+-based pipette solution, containing 0·2 mM EGTA and K^+-based pipette solution in which $[Ca^{2+}]$ adjusted by the Ca–EGTA buffering system was 100 nM. *A*, typical traces of whole-cell current in PSS external solution (*a*) and in Ca^{2+}-free PSS (*b*), showing dramatic change in the kinetics of the potassium current. *B*, typical traces of whole-cell current in PSS external solution (*c*) or Co^{2+}-containing external solution (*d*), and K^+-based pipette solution containing 0·2 mM EGTA, showing reduction of the potassium current in Co^{2+}-containing external solution. *C*, traces of whole-cell current in PSS external solution (*e*) or Co^{2+}-containing external solution (*f*) and K^+-based pipette solution containing 100 nM Ca^{2+}, showing large potassium current in Co^{2+}-containing external solution. *D*, time course of the peak values of outward potassium current for K^+-based pipette solution (●) and for pipette solution containing 100 nM Ca^{2+} (○), showing lack of the inhibitory effect of hypoxia on residual potassium current. The points under *c*, *d*, *e* and *f* in the graph correspond with the actual current recordings (*c*, *d*, *e* and *f*) in *B* and *C*. The Co^{2+} bar indicates the period of superfusion with Co^{2+}-containing external solution.

since more prolonged depolarizations elicited contraction of the cells. Under these conditions, Co^{2+}-resistant potassium current was more pronounced and even slightly exceeded the outward current (compare traces *e* and *f* in Fig. 2*C*). This apparent contradiction was due to a considerable overlap of I_{Ca} and potassium current at the end of the 50 ms voltage step. Large fluctuations were also observed under these conditions (compare Fig. 2*C* and *B*). Hypoxia, as in the previous case, did not influence the residual potassium current (Fig. 2*D*, open circles). Similar results were obtained on all cells tested ($n = 6$).

Effect of hypoxia on spontaneous transient outward currents

Spontaneous transient outward currents (STOCs) depend on the presence of extracellular Ca^{2+}. They were proposed to reflect the synchronized opening of Ca^{2+}-activated K^+ channels caused by cyclic release of Ca^{2+} from intracellular stores inducing transient elevation of intracellular calcium concentration (Ohya, Kitamura & Kuriyama, 1987). This occurs at a depolarized level of membrane potential and with moderate EGTA concentration in the pipette solution (Benham & Bolton, 1986). When the membrane potential of the taenia caeci cell was held at −20 mV, we observed STOCs (Fig. 3*A*). The amplitude of STOCs was significantly reduced by hypoxia, but recovered within 2 min after reintroduction of normoxic solution into the bath (Fig. 3*A*). Under normoxic conditions, the amplitude distribution at −20 mV yielded one large (*c*) and two small (*d* and *e*) peaks (Fig. 3*B*). Peak *c*, representing the holding current at −20 mV, had a mean amplitude value of 39·5 ± 10·8 pA. The peaks *d* (68 pA) and *e* (141 pA) originated from STOCs. Under hypoxic conditions, the amplitude distribution at −20 mV yielded only one large peak (*f*) with the mean amplitude value of 38·7 ± 6·9 pA (Fig. 3*C*). These results indicate that hypoxia suppressed the peaks (representing STOCs) and significantly decreased the fluctuations of the holding current, but did not affect the amplitude of the holding current. Comparable results were obtained on all cells tested ($n = 7$).

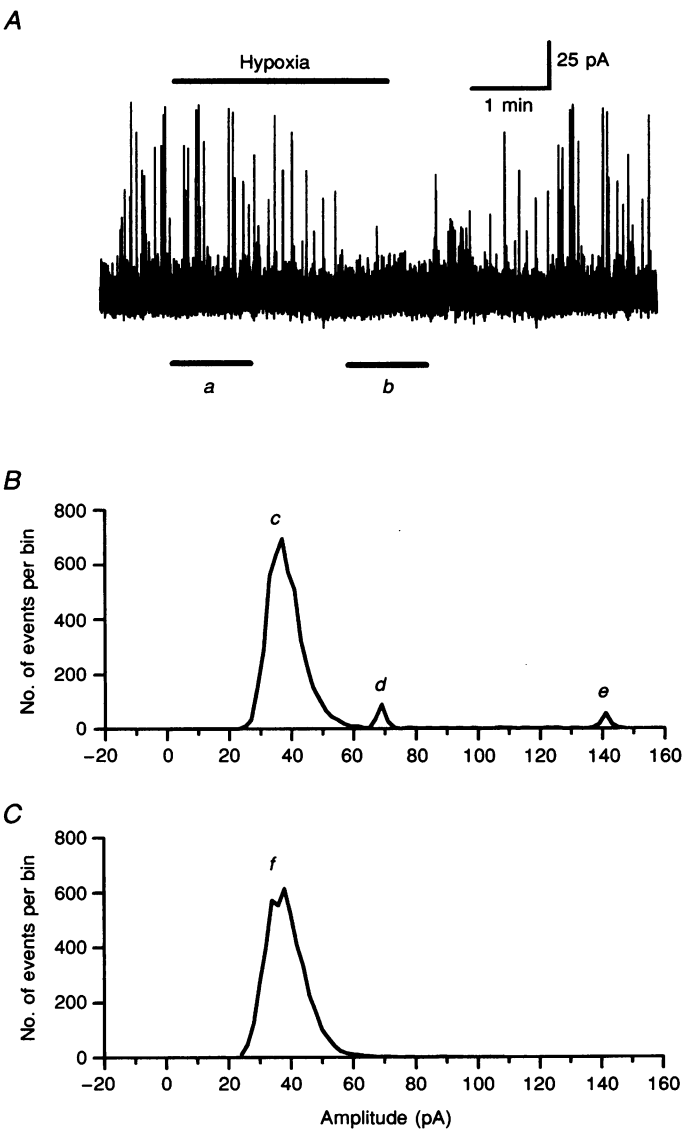


Figure 3. Hypoxia reversibly inhibits the STOCs in taenia caeci smooth muscle cells

A, the whole-cell current recorded from a cell held at −20 mV. The recording pipette contained K^+-based solution; the bath contained PSS. The bars *a* and *b* indicate the time intervals (1 min) used for creating amplitude histograms under normoxic and hypoxic conditions. The current was sampled at time intervals of 5 ms. *B*, amplitude histogram under normoxic conditions. The data were binned at 2 pA intervals. Peak *c*, representing the holding current, was fitted with a one-mode Gaussian function (mean, 39·5 pA; S.D., 10·8 pA). The peaks *d* (68 pA) and *e* (141 pA) reflect the amplitude distributions of STOCs. *C*, amplitude histogram under hypoxic conditions, showing only one peak (*f*), fitted with a one-mode Gaussian function (mean, 38·7 pA; S.D., 6·9 pA), representing the holding current.

Influence of hypoxia on calcium current

To record the voltage-operated Ca^{2+} channel current (I_{Ca}), cells bathed in PSS were dialysed with Cs^+-based pipette solution to block the outward potassium current ($I_{K(Ca)}$). The effect of hypoxia on I_{Ca} elicited by the depolarization step (from −60 mV to 0 mV, 100 ms duration, 0·067 Hz) was investigated after stabilization of the Ca^{2+} current amplitude. As shown in Fig. 1*A* and *B*, the I_{Ca} amplitudes apparently correlated with the changing of P_{O_2} in the bath solution. The decrease of P_{O_2} from 150 mmHg (normoxia) to 20 mmHg (hypoxia) induced I_{Ca} inhibition (Fig. 4*B*, point *b*). At a P_{O_2} of 20 mmHg, the mean inhibition of I_{Ca} was 30 ± 5% ($n = 10$). The increase of P_{O_2} from 20 to 150 mmHg (reoxygenation) led to I_{Ca} recovery (Fig. 4*B*, point *c*).

Slowly decaying inward tail currents (probably representing Cl^- current) were observed upon repolarization to the holding potential (Fig. 4*C*). The inward tail current was blocked by high EGTA (5 mM) in the pipette solution (data not shown), indicating its calcium dependency. This current might be activated by Ca^{2+} influx through L-type calcium channels. Ba^{2+} was therefore used as charge carrier instead of Ca^{2+} in further investigating the influence of hypoxia on calcium channels.

Effect of hypoxia on barium current

Inward current was elicited by depolarization from the holding potential of −60 mV to the test potential of 0 mV for 200 ms at 0·067 Hz. On establishing the whole-cell configuration, I_{Ca} progressively increased and reached a stable level (Fig. 5*A*, point *a*). When superfusion with PSS was replaced by superfusion with PSS in which Ca^{2+} was replaced by Ba^{2+}, the mixed inward current of Ca^{2+} and Ba^{2+} through calcium channels decreased transiently (Fig. 5*A*, point *b*). This phenomenon may be due to the anomalous mole fraction effect described for Ca^{2+} channels (Hess & Tsien, 1984). After this transient reduction, the amplitude of the inward current started to increase. At a new stable level, the amplitude of pure barium current (I_{Ba}) exceeded that of pure I_{Ca} (Fig. 5, point *c*). The tail current was found to be present only with Ca^{2+} but not with Ba^{2+} as a charge carrier (compare traces *a* and *c* in Fig. 5*B*). The hypoxic bath solution induced inhibition of I_{Ba} (Fig. 5*A* and *B*, point *d*), while removal of hypoxic conditions led to I_{Ba} recovery (Fig. 5*A*).

Effect of hypoxia on barium current at various membrane potentials

When Ba^{2+} was used as charge carrier, the error due to contamination of the tail current was minimal. This observation

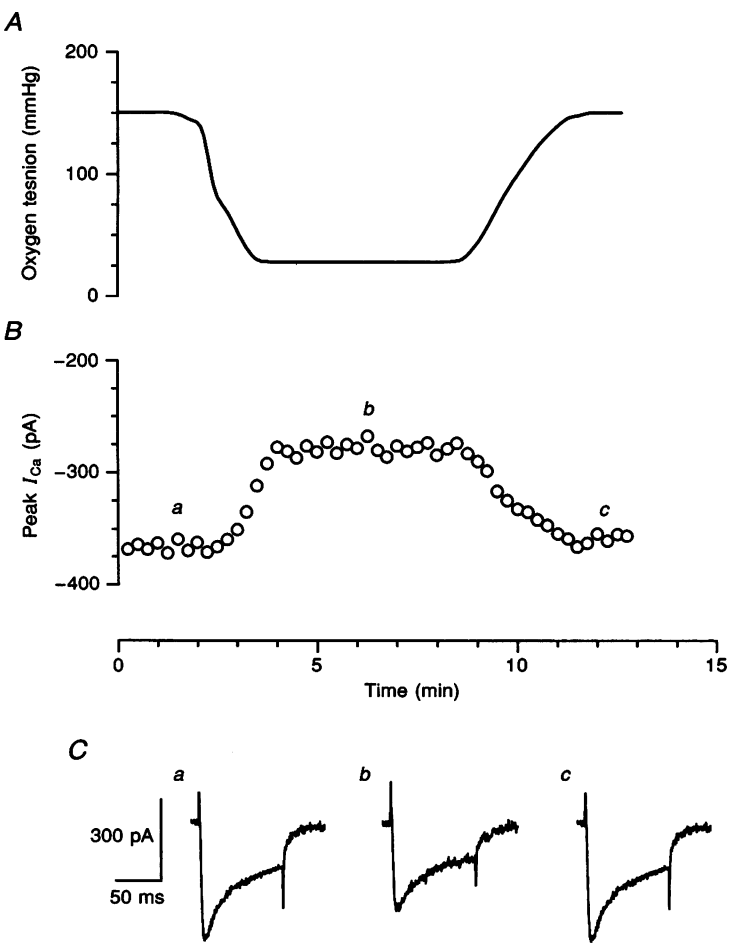


Figure 4. Hypoxia reversibly inhibits Ca^{2+} current in the guinea-pig taenia caeci smooth muscle cells

I_{Ca} was recorded in response to depolarizing pulses of 100 ms duration from the holding potential of −60 mV to 0 mV at a frequency of 0·067 Hz. The recording pipettes contained Cs^+-based solution; the bath contained PSS. *A*, time course of the change of P_{O_2} in the bath solution. *B*, time course of the change in I_{Ca} amplitude. The points under *a*–*c* in the graph correspond with the actual I_{Ca} recordings (*a*–*c*) in *C*. *C*, typical traces of I_{Ca} during normoxia (P_{O_2} = 150 mmHg; *a*), hypoxia (P_{O_2} = 20 mmHg; *b*), and reoxygenation (P_{O_2} = 150 mmHg; *c*), showing the reversible reduction in peak I_{Ca} elicited by hypoxia. In *A* and *B*, the superfusing solution was switched to hypoxic solution at 2 min and to normoxic one at 8·5 min.

allowed us to investigate the influence of hypoxia on I_{Ba} in a wide range of membrane potentials. The normoxic current–voltage (I–V) relationship was determined when the I_{Ba} elicited by depolarizing pulses to 0 mV from a holding potential of −60 mV had reached a steady-state value. Further, the cell was exposed to hypoxic solution, and when the I_{Ba} had reached a new steady-state value, the hypoxic I–V relationship was determined. The normalized I–V relationships of peak I_{Ba} averaged from six cells under normoxia (open circles) and under hypoxia (open triangles) are shown in Fig. 6*A*. Hypoxia suppressed I_{Ba} at the peak of the I–V relationship (+10 mV) by 40 ± 7%, without shifting the peak along the voltage axis. Hypoxia did not alter the reversal potential (+65 ± 6 mV) as well.

Figure 6*B* shows the effect of hypoxia on the steady-state activation of I_{Ba}. Dividing the currents in Fig. 6*A* in the range between −50 and + 20 mV by the reversal potential and normalizing the chord conductance at different voltages to that at +20 mV resulted in the steady-state activation curve which was fitted with the Boltzmann function given by the equation:

$$\sigma/\sigma_{max} = 1/[1 + \exp(-(V_{test} - V_{½})/s)]. \quad (1)$$

In normoxia, half-activation ($V_{½}$) was −3·5 ± 1·4 mV and the slope factor (s) was 6·6 ± 0·5 mV. $\chi^2 = 1{\cdot}8 \times 10^{-4}$. In hypoxia, $V_{½}$ was −2·2 ± 1·3 mV and s was 8·2 ± 0·7 mV. $\chi^2 = 2{\cdot}3 \times 10^{-4}$. These results indicate that hypoxia increased s and did not affect significantly the membrane potential for $V_{½}$.

The normoxic steady-state inactivation was determined when the I_{Ba} elicited by depolarizing pulses to 0 mV from a holding potential of −60 mV had reached a steady-state value. In the next step, the cell was exposed to hypoxic solution, and when the I_{Ba} had reached a new steady-state value, the hypoxic steady-state inactivation was determined. Figure 6*C* shows the effect of hypoxia on averaged ($n = 6$) and normalized steady-state inactivation (the availability curve), which was determined by measuring I_{Ba} at +10 mV from a holding potential of −70 mV, after applying pre-pulses (V_{cond}) lasting for 5 s and ranging from −60 to −10 mV. The curve was fitted with the Boltzmann function given by the equation:

$$I/I_{max} = 1/[1 + \exp((V_{cond} - V_{½})/s)]. \quad (2)$$

In the normoxic condition, $V_{½}$ and s were −33·8 ± 3 mV and −8·1 ± 0·4 mV, respectively. $\chi^2 = 1{\cdot}5 \times 10^{-5}$. In the hypoxic condition, $V_{½}$ and s were −34·4 ± 3 mV and −8·0 ± 0·3 mV. $\chi^2 = 5{\cdot}5 \times 10^{-5}$. These results indicate that hypoxia did not significantly affect the availability curve.

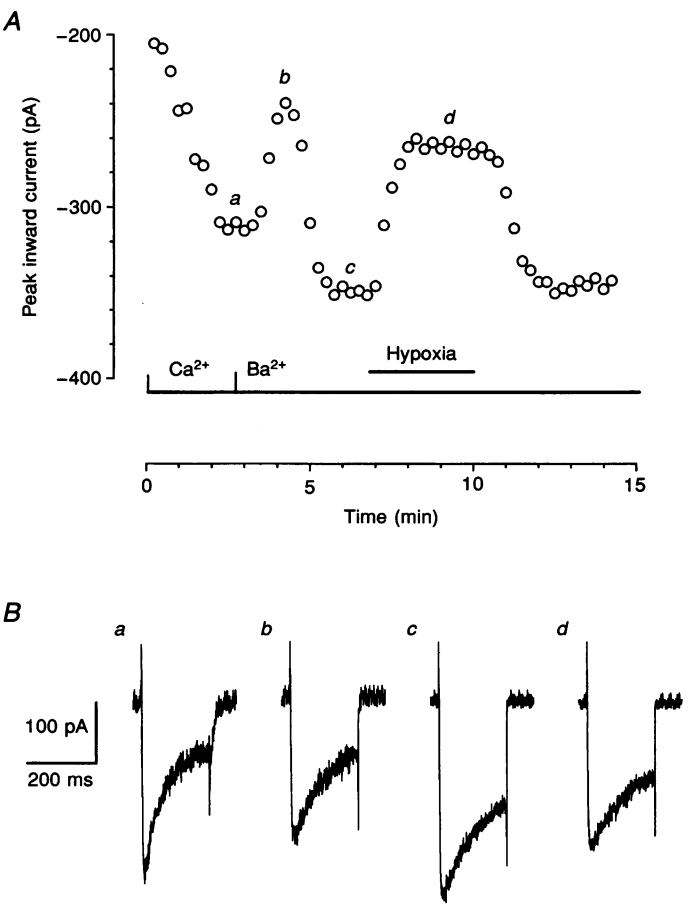


Figure 5. Influence of hypoxia on barium current of single smooth muscle cells isolated from guinea-pig taenia caeci

Inward current was recorded in response to depolarizing pulses of 200 ms duration from the holding potential of −60 mV to 0 mV at a frequency of 0·067 Hz. The recording pipettes contained Cs^+-based solution; the bath contained PSS or PSS in which Ca^{2+} had been replaced by Ba^{2+}. *A*, time course of the peak values of inward current: pure I_{Ca} (*a*), mixed I_{Ca} with I_{Ba} (*b*) and pure I_{Ba} (*c*), all in normoxia; and pure I_{Ba} during hypoxia (*d*). The points under *a*–*d* in the graph correspond with the actual current recordings (*a*–*d*) in *B*. *B*, typical traces of pure I_{Ca} (*a*), mixed I_{Ca} with I_{Ba} (*b*) and pure I_{Ba} (*c*) all in normoxia ($P_{O_2} = 150$ mmHg); and I_{Ba} during hypoxia ($P_{O_2} = 20$ mmHg; *d*), showing the reduction in peak I_{Ba} elicited by hypoxia.

Membrane potential dependence of hypoxia induced inhibition of I_{Ba} was calculated using the equation:

Percentage inhibition of I_{Ba} =

$$[(I-V)_{normoxia} - (I-V)_{hypoxia}]/(I-V)_{normoxia}. \quad (3)$$

Figure 6*D* shows that the inhibitory effect of hypoxia is strongly dependent on membrane potential with the maximum at + 10 mV.

Influence of ATP, high EGTA and GDPβS on hypoxia-induced suppression of calcium channel

Under the conditions used, when the cells were dialysed with a solution containing 2 mM ATP, hypoxia inhibited both I_{Ca} and I_{Ba}. This observation suggests that the intracellular concentration of ATP should not be regarded an important factor in the inhibitory effect of hypoxia on I_{Ca} and I_{Ba}. To confirm this suggestion we examined the influence of hypoxia on Ca^{2+} channels in the absence of ATP in the pipette solution.

As shown above, I_{Ca} and I_{Ba} reached the steady-state value over the first 3 min and 6 min, respectively, after establishing the whole-cell configuration. We assumed therefore that EGTA (mol. wt, 380), ATP (mol. wt, 505), and GDPβS (mol. wt, 477) had already equilibrated between the pipette solution and the cell interior at this time. First, the tail current was suppressed in high-EGTA pipette solution over

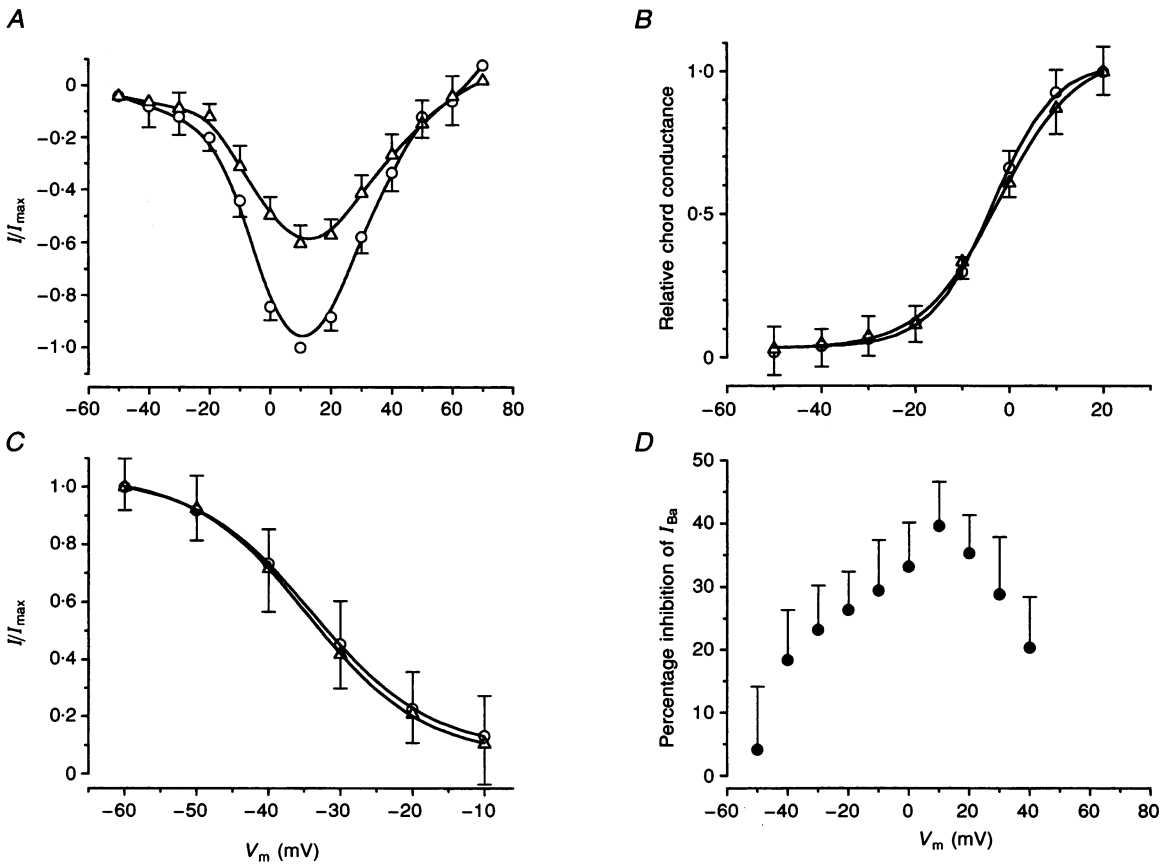


Figure 6. Potential dependency of hypoxia-induced inhibition of I_{Ba} in taenia caeci smooth muscle cells

A, averaged current–voltage (*I–V*) relationship of normalized peak I_{Ba} for cells exposed in normoxic solution (○) and after superfusion of hypoxic solution (△), showing the inhibitory effect of hypoxia on I_{Ba} in a wide range of test potentials and without shifting the maximum of the *I–V* curve along the voltage axis. Peak values were normalized to the values obtained at +10 mV in normoxic conditions. The data are means from six cells. For clarity error bars (S.D.) in this and the subsequent graphs of this figure are shown in one direction only. Curves were fitted to the data plot using B-spline connection. Cells were held at −60 mV and stepped for 20 ms to test potentials ranging from −50 to +70 mV. *B*, steady-state activation of I_{Ba} during normoxia (○) and hypoxia (△), with data points obtained from *A*. The curves represent the fitted Boltzmann eqn (1). *C*, averaged steady-state inactivation of I_{Ba} for cells exposed in normoxic solution (○) and after superfusion of hypoxic solution (△). The data are means from six cells. The curves were fitted to the data plot using the Boltzmann eqn (2). I_{Ba} was activated by stepping to +10 mV, from the preceding conditioning pulse of 5 s duration of various voltage (−60 to −10 mV). Peak currents of each cell were normalized to the value obtained at −60 mV conditioning voltage. *D*, inhibition of I_{Ba}–voltage relationship for cells suspended in hypoxic solution, with data points obtained from *A*.

the first 2 min after establishing whole-cell configuration, indicating that EGTA was equilibrated with the cytoplasm. Since the relative aqueous diffusion coefficients for EGTA, ATP and GDPβS scaled by the cube root of molecular weights were $D_{EGTA} : D_{ATP} : D_{GDP\beta S} = 1 : 0{\cdot}91 : 0{\cdot}93$, it seems that ATP and GDPβS do exchange adequately with the cytoplasm over the 2 or 3 min after gaining whole-cell access. Second, the omission of ATP from the pipette solution did dramatically increase the rate of run-down of I_{Ca}, suggesting that access of ATP to the cytoplasm was not restricted. The mean rates of I_{Ca} run-down for 10 min were $15{\cdot}4 \pm 3{\cdot}2\%$ ($n = 30$) with ATP in the pipette solution and $34{\cdot}2 \pm 5{\cdot}1\%$ ($n = 10$) without ATP in the pipette solution.

The mechanism underlying the gradual run-down of I_{Ca} is poorly understood. The preliminary experiments carried out with or without ATP in the pipette solution showed that the access (series) resistance (R_a) tended to increase during the experiments with a time course similar to that of gradual run-down of I_{Ca}. This observation suggests that a gradual increase of R_a may contribute to the mechanism of I_{Ca} run-down due to (1) increase in the overlap of the whole-cell capacitance transient and I_{Ca}; (2) increase in temporal error (membrane current changes and the command potentials are filtered by the time constant of the access resistance and the membrane capacitance); and (3) increase in voltage error ($I_{Ca}R_a$). On-line leak subtraction was implemented with the *P/N* protocol in order to avoid the first error. This procedure decreased the mean rate of I_{Ca} run-down from $34{\cdot}2 \pm 5{\cdot}1\%$ to $13{\cdot}6 \pm 3{\cdot}1\%$ per 10 min ($n = 10$). To resolve the effects of hypoxia on I_{Ca} or I_{Ba} from the run-down, the time course of changes in peak amplitude of I_{Ca} or I_{Ba} just prior to the introduction of hypoxia was fitted by a straight line and extrapolated to the period after the introduction of hypoxia. The difference between the line and the recorded amplitude at each time was taken as the effect of hypoxia.

The I_{Ca} and I_{Ba} were elicited as described earlier. The potency of the inhibitory effect of hypoxia on both I_{Ca} and I_{Ba} did not change in the absence of ATP in the pipette solution (Fig. 7).

There are reports indicating that hypoxia can modulate cytosolic Ca^{2+} in smooth muscle cells (Aalkjær & Lombard, 1995; Ureña, Franco-Obregón & Lópes-Barneo, 1996). On the other hand, cytosolic Ca^{2+} can inhibit Ca^{2+} channels via the mechanism of Ca^{2+} inactivation. To examine this possibility, the concentration of intracellular free Ca^{2+} was kept low by including 5 mM EGTA in the pipette solution. The inhibitory effect of hypoxia on I_{Ca} and I_{Ba} persisted under these experimental conditions (Fig. 7).

To determine the possible involvement of GTP-binding proteins in the hypoxia-induced inhibition of Ca^{2+} channels in taenia caeci, GDPβS was included in the pipette solution

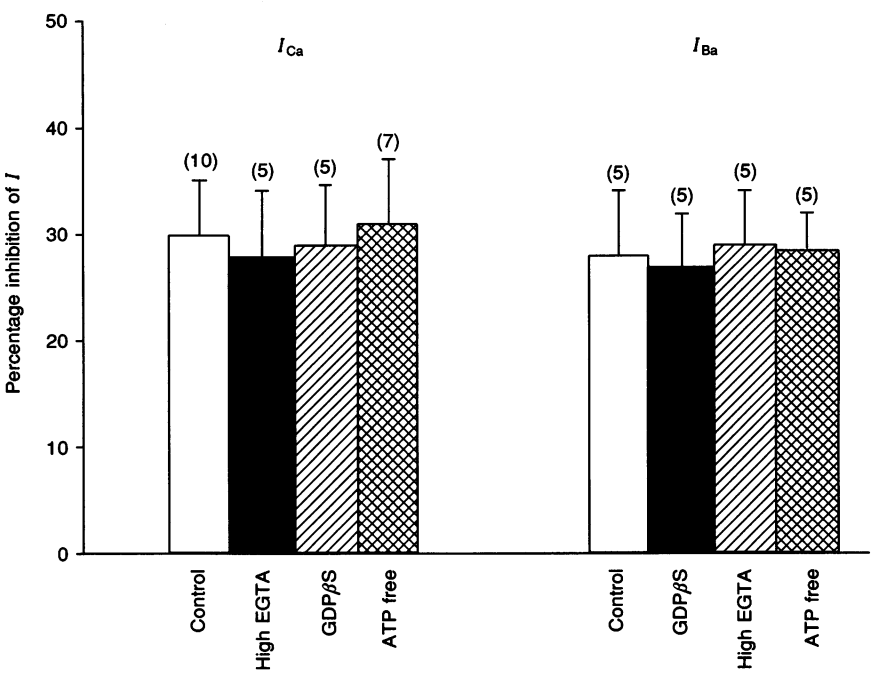


Figure 7. Effects of EGTA, GDPβS and ATP on hypoxia-induced inhibition of calcium current (I_{Ca}) and barium current (I_{Ba})

I_{Ca} and I_{Ba} were evoked by depolarization of 200 ms duration from −60 to 0 mV at a frequency of 0·067 Hz. The recording pipettes contained Cs^+-based solution. Hypoxia ($P_{O_2} = 20$ mmHg) reduced the amplitude of both I_{Ca} and I_{Ba} (□). This inhibitory effect of hypoxia did not change significantly when the concentration of EGTA in the pipette solution was increased from 0·2 to 5 mM (■), by inclusion of 1 mM GDPβS (▨) and exclusion of ATP (▩) from the pipette solution. Each column shows the mean + S.D. with the number of cells used shown in parentheses.

in some experiments. The intensity of the decrease of I_{Ca} and I_{Ba} by hypoxia remained unaffected by 1 mM GDPβS treatment (Fig. 7).

Influence of hypoxia on high K^+-induced contraction of taenia caeci

Hypoxia inhibited the L-type Ca^{2+} channels of taenia caeci smooth muscle cells. Therefore we expected that part of the hypoxia-induced relaxation of taenia caeci would be due to decreased Ca^{2+} influx through Ca^{2+} channels. To confirm this suggestion the influence of hypoxia on high K^+-induced contraction was investigated. When the taenia was exposed to a high K^+ solution (80 mM K^+), after a transient phasic contraction, the muscle developed a sustained contraction, which was fully relaxed by nifedipine (0·1 μM). This indicates that the sustained contraction was due to opening of the L-type voltage-operated calcium channels (Fig. 8*A*). The mean half-life ($t_{1/2}$) of the nifedipine-induced relaxation was 150 ± 12 s ($n = 8$).

The effect of hypoxia on the sustained high K^+ contraction differed from that of nifedipine. It was composed of three distinct phases. The initial rapid relaxation was followed by a small contraction and a late slowly developing relaxation. The $t_{1/2}$ of the rapid relaxation and the $t_{1/2}$ of the slow relaxation were 65 ± 12 s and 200 ± 18 s, respectively ($n = 10$). The contractile phase was present in 60% of recordings ($n = 10$) and was absent in taenia ($n = 8$) treated with atropine (1–10 μM). Neither the initial rapid nor the prolonged slowly developing relaxations were changed by guanethidine (10 μM) or atropine (10 μM) treatment.

The rapid phase of hypoxia-induced relaxation resembled that induced by nifedipine (compare Fig. 8*A* and *B*). For close examination, the rapid phase of hypoxia- and nifedipine-induced relaxations were normalized according to the amplitudes of their application points and they were overlapped (Fig. 8*C*). Their kinetics appeared to be similar.

DISCUSSION

This paper showed that superfusion of isolated taenia caeci myocytes with a solution in which the P_{O_2} was reduced to 20 mmHg led to reversible inhibition of Ca^{2+} current. Several lines of evidence from our present experiments suggest that hypoxia can act directly on Ca^{2+} channels.

First, the time course of change in peak amplitude of I_{Ca} was similar to the time course of change of P_{O_2} in the bath solution. Second, the inhibitory effect of hypoxia on I_{Ca} did not result from alterations in the intracellular Ca^{2+} concentration because responses to hypoxia at different

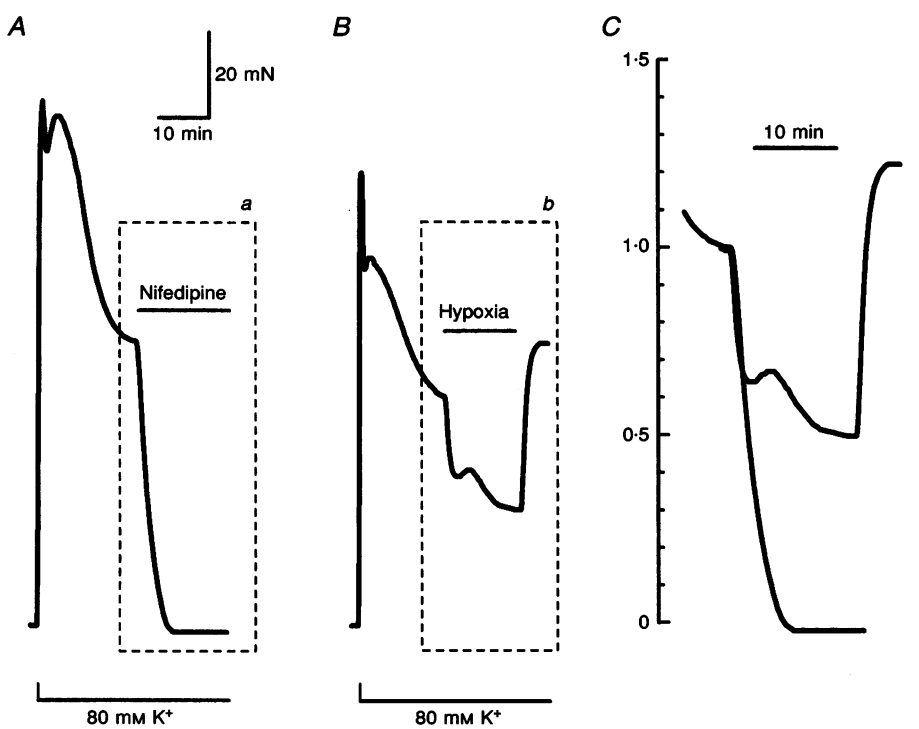


Figure 8. Effects of hypoxia on high K^+-induced contraction of guinea-pig taenia caeci

A, nifedipine (0·1 μM) fully relaxed the high K^+ (80 mM)-induced contraction, showing its dihydropyridine-sensitive origin. *B*, response of the high K^+-induced contraction to hypoxia shows three distinct components: rapid relaxation, small transient contraction and slow relaxation. *C*, the contraction recordings inside the dashed rectangles in *A* and *B* were scaled to equal levels and overlapped demonstrating similar kinetics of the rapid phase of hypoxia- and nifedipine-induced relaxations. The bars indicate superfusion with high-K^+ solution, the presence of nifedipine and duration of hypoxia.

Ca^{2+} buffering by EGTA in the pipette solution did not differ significantly. Moreover, the current reduction was the same whether the charge carrier was calcium or barium. Third, it is unlikely that inhibition of ATP synthesis under hypoxic conditions might be a major factor underlying the observed decrease of Ca^{2+} and Ba^{2+} currents because omission of ATP from the pipette solution did not affect significantly the hypoxia-induced decrease in I_{Ca} and I_{Ba}.

Molecular mechanisms underlying oxygen sensing by plasma membrane channels of mammalian tissues are not fully understood. The recent evidence suggests that oxygen is sensed by means of a haeme protein and this process, along with subsequent signal transduction, is shared by most if not all cells (for review see Bunn & Poyton, 1996).

Taenia caeci myocytes may respond to hypoxia by a similar mechanism. In this case the oxygen-sensing domain or signal transduction mechanism should be located in the electrical field of the plasma membrane because the inhibition of I_{Ba} by hypoxia is a voltage-sensitive process. The voltage-sensitive inhibition of the current amplitude might be due to the changed activation characteristics of the I_{Ba}. Besides, since internal application of 1 mM GDPβS did not affect significantly the hypoxia-induced decrease in I_{Ca} and I_{Ba}, activation of GTP binding proteins is unlikely to be involved in oxygen-sensing mechanism.

Our study demonstrated that in the PSS, hypoxia led to reversible inhibition of the outward potassium current ($I_{K(Ca)}$). Hypoxia, however, does not affect the residual potassium current in Co^{2+}-containing calcium-deficient bath solution. We believe that this Ca^{2+} influx-independent residual potassium current is carried through large-conductance voltage-activated and Ca^{2+}-sensitive K^+ channels. This suggestion is based on the work of Hu *et al.* (1989). They showed that in cell-attached patches of smooth muscle cells of guinea-pig taenia caeci, there are two types of K^+ channels differing in their conductance (147 and 63 pS). Openings of the 63 pS channel are rare, whereas openings of the 147 pS channel are frequent. The latter is known to be regulated by both voltage and cytosolic Ca^{2+} (maxi-K^+ channel), and pass almost exclusively the whole-cell outward potassium current during pulsed depolarization. Under the conditions of the present experiment, when 2 mM Ca^{2+} was replaced by Co^{2+} in the bath solution and 0·2 mM EGTA was included in the pipette solution, the concentration of the intracellular free Ca^{2+} may be sufficient for the activation of these channels to give rise to significant whole-cell current upon depolarization. Noack, Daitmer & Lammel (1992) and Ogata, Inoue, Nakano, Ito & Kitamura (1996) came to a similar conclusion. Our experiments carried out under the condition of elevated concentration of Ca^{2+} in the pipette solution also support this suggestion. Thus the residual whole-cell outward potassium current generated upon depolarization is most likely to have resulted from the activation of these channels. If this suggestion is correct then hypoxia has no direct action on the large-conductance voltage-activated and Ca^{2+}-sensitive K^+ channel. In the PSS, the reduction of the whole-cell outward potassium current ($I_{K(Ca)}$) upon hypoxia may have been due to decreased Ca^{2+} influx through the calcium channels.

The activity of STOCs was significantly inhibited by hypoxia. As in the case of $I_{K(Ca)}$, STOCs are due to the outflux of potassium ions through the same large-conductance voltage-activated and Ca^{2+}-sensitive K^+ channels (Yamamoto *et al.* 1989*a*). There is every reason to suppose that the inhibitory effect of hypoxia on STOCs may also have been due to decreasing Ca^{2+} influx through I_{Ca}. The observation that hypoxia decreased the fluctuations of the holding current without affecting its amplitude suggests that there is an additional Ca^{2+}-dependent ionic conductance in the taenia caeci myocytes. The reversal potential of this additional current should be more positive then −20 mV under our experimental conditions.

In the light of the data presented above we suggest that the oxygen-sensing mechanism may be responsible for the rapid phase of relaxation evoked by hypoxia in taenia caeci. First, hypoxia inhibited I_{Ca} in isolated taenia caeci myocytes. Second, the relative amplitude of this relaxation (~30%) was approximately equal to the inhibition of I_{Ca} amplitude. Third, the nifedipine-induced relaxation reflecting the inhibition of I_{Ca} matched the rapid phase of hypoxia-induced relaxation. The last comparison makes sense only in the case where the responses of the taenia caeci to hypoxia and to nifedipine are not limited by diffusion of O_2 and nifedipine in the tissue. We considered the outer layer (100 μm thickness) of this tissue to estimate the diffusion of O_2 and nifedipine into the taenia caeci. Because the mean cross-sectional area of the taenia caeci is 0·39 mm^2 (1 mm × 0·4 mm; Karaki, Suzuki, Urakawa, Ishida & Shibata, 1982), this layer contains ~60% of the total number of the cells in this muscle. The probability of O_2 and nifedipine being displaced by a distance x during time t may be estimated from the Fürth equation:

$$x^2 = 2Dt, \quad (4)$$

where D is a diffusion coefficient.

D_{O_2} in the taenia caeci is unknown, therefore the D_{O_2} measured by Buerk & Goldstick (1992) in the aortic wall ($9{\cdot}5 \times 10^{-6}$ cm^2 s^{-1}) was used to estimate the oxygen diffusion in the taenia caeci. Substitution of this value in eqn (4) yields the estimated time, t, of ~5 s. This value is one order of magnitude smaller than the $t_{½}$ of the rapid relaxation. Oxygen consumption in the taenia caeci was 0·45 μmol O_2 min^{-1} g^{-1} (Ishida & Paul, 1990) but only 0·079 μmol O_2 min^{-1} g^{-1} in the carotid artery (Krisanda & Paul, 1984). Because in a tissue O_2 diffusion always matches O_2 demand (O_2 consumption), D_{O_2} in the taenia caeci would exceed that in the aortic wall. No data on the diffusion of nifedipine in the taenia caeci have come to our attention in the literature. Therefore the ethanol diffusion coefficient ($9{\cdot}6 \times 10^{-6}$ cm^2 s^{-1} in 2% calcium alginate gel; Estapé,

Gódia & Solá, 1992) was used to estimate the cotransport of nifedipine (a lipophilic drug) with ethanol (the solvent) in the extracellular volume of the tissue. Then the apparent diffusion coefficient for the nifedipine–ethanol complex (1 molecule nifedipine per 10 molecules ethanol) scaled by the cube root of the molecular weights was $\sim 3{\cdot}7 \times 10^{-6}$ cm^2 s^{-1}. Substitution of this value in eqn (4) yields the estimated time, $t \approx 14$ s, which is smaller than that of $t_{½}$ of the nifedipine-induced relaxation. These estimations show that the responses of the taenia caeci to hypoxia and nifedipine were probably not limited by the transport of O_2 and nifedipine in this tissue.

Since the small transient contraction which followed the rapid phase of relaxation was prevented by atropine, it was presumably caused by release of acetylcholine from nerve terminals upon hypoxia.

On the basis of the present experiments, it is impossible to explain the mechanism underlying the slow phase of the hypoxia-induced relaxation. Nevertheless, we believe that this mechanism is of a myogenic origin. First of all, neither atropine nor guanethidine affected the slow phase of the hypoxia-induced relaxation. Moreover, α_1-adrenergic and non-adrenergic inhibitory mechanisms cannot operate under the condition of high K^+-evoked depolarization. In PSS (in taenia caeci myocytes, potassium equilibrium potential, $E_K \approx -89$ mV), the inhibitory response of the guinea-pig taenia caeci mediated by an α_1-adrenergic and non-adrenergic mechanism was shown to be due to an increase of potassium permeability of smooth muscle cell membrane accompanied by membrane hyperpolarization (Banks *et al.* 1979; Bauer & Rusko, 1982; Bridgewater, Cunnane & Brading, 1995). Yet in high-K^+ solution ($E_K \approx -20$ mV), the inhibitory mediator-induced potassium permeability would lead to the maintenance of high K^+-evoked depolarization. This, however, does not rule out the possibility of the transmitter being released from nerve endings upon hypoxia.

Ishida & Paul (1990) suggested that the overall response to hypoxia of the high K^+-contracted taenia caeci was due to an inhibition of energy metabolism, rather than to an oxygen-sensing step in excitation–contraction coupling. In contradistinction, our present study demonstrated that the first rapid phase of hypoxia-induced relaxation was mainly due to an inhibition of I_{Ca}. Nevertheless, we cannot exclude the possibility that the energy-limitation mechanism may have contributed to the slow phase of hypoxia-induced relaxation of high K^+ contraction.

In conclusion, our study indicates that the initial rapid phase of hypoxia-induced relaxation was mainly due to the suppression of Ca^{2+} influx elicited by inhibition of L-type Ca^{2+} channels. The inhibition of $I_{K(Ca)}$ was a secondary phenomenon, due to the reduced I_{Ca}.

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