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Vasoactivity of trimetazidine on guinea-pig isolated ductus arteriosus

D. Garnier & M.J. Roulet

Laboratoire d'Electrophysiologie et Pharmacologie Cellulaires, Faculté des Sciences, 37200, Tours, France

1 The effect of trimetazidine (TMZ), an anti-anginal drug, on the mechanical response of the guinea-pig ductus arteriosus placed under conditions of mild hypoxia ($P_{O_2} \cong 75$ mmHg) was investigated.

2 When the P_{O_2} of the bathing solution was 75 mmHg, TMZ caused a dose-dependent increase in tension. The median effective dose (ED_{50}) for the drug was 8×10^{-5} M.

3 TMZ-induced increase in tension was not significantly affected by pretreatment of the preparation with adrenoceptor blocking agents, or indomethacin.

4 The amplitude of the P_{O_2}-dependent tension was significantly augmented by exposure of the strip to TMZ 10^{-4} M, whereas neither the resting tone (low P_{O_2}), nor the oxygen-induced contraction (high P_{O_2}) were altered.

5 This ability of TMZ to increase the tension response during hypoxia was dependent on the external calcium concentration.

6 Under low P_{O_2} conditions, a contractile activity of 10^{-4} M TMZ was unmasked in preparations perfused with 18 mM K^+-PSS medium. This response to TMZ disappeared after the removal of calcium from the bath.

7 At the maximally effective dose of 10^{-3} M, and during low P_{O_2}, the TMZ-induced contractile response changed to a relaxation response when the external K^+ concentration was raised more than five fold.

8 The possibility that TMZ stimulates the mechanism by which oxygen normally controls the concentration of free intracellular calcium in the ductus arteriosus is proposed.

Introduction

Trimetazidine (TMZ), 1-(2,3,4-trimethoxybenzyl)-piperazine dichloride, is an anti-anginal drug used in clinical treatment of ischaemic diseases. A series of studies on isolated cardiac muscle has shown that TMZ exerts no appreciable effect on the normal contraction of the guinea-pig atria (Sugimoto *et al.*, 1970). From experiments carried out under abnormal conditions, i.e. in the presence of nicotine or in the absence of K^+ ions in the bathing medium, Nagata *et al.* (1971) demonstrated the antinicotinic effect of the drug and its inhibitory action on the outflow of cellular potassium ions. In addition, recent investigations have demonstrated a vasodilator activity of TMZ *in vivo* (Taira *et al.*, 1980; Asano *et al.*, 1983) as well as *in vitro* (Toda *et al.*, 1982). From experiments performed on canine isolated veins and arteries, Toda *et al.* (1982) showed that TMZ attenuated preferentially the responses of veins to transmural stimulation and to noradrenaline; the authors attributed the anti-anginal activity of the drug to a decrease in the load on the heart.

The present study was designed to characterize the vasoactivity of TMZ on isolated ductus arteriosus in an attempt to examine the proposed anti-hypoxic activity of the drug. Owing to its specific reactivity with oxygen, the ductus was used as a pharmacological model of hypoxia. Earlier work on the guinea-pig isolated ductus arteriosus has shown the crucial role of oxygen in triggering a contractile response (Kovalcik, 1963; Fay & Jobsis, 1972). Moreover, it has been established that the surrounding P_{O_2} regulates the force of contraction developed by an intact preparation or strip from the guinea-pig ductus arteriosus (Fay, 1971; Ikeda *et al.*, 1973; Roulet & Coburn, 1981). Data from Roulet & Coburn (1981) support the view that an oxygen-sensitive decrease in ductus cell membrane permeability to K^+ ions can account for the membrane depolarization recorded during an

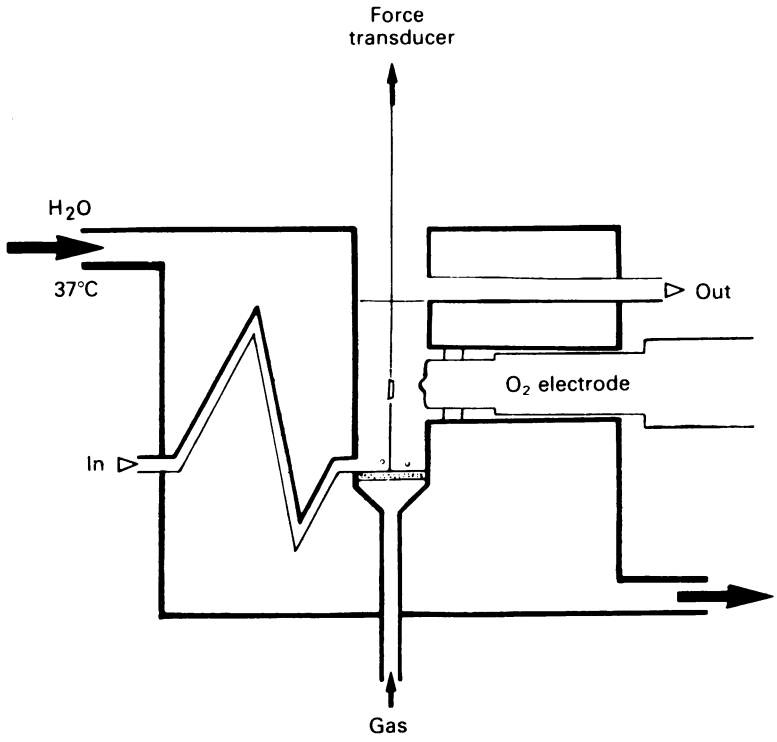


Figure 1 Experimental set-up used for measuring muscle tension of strips of ductus arteriosus from the guinea-pig. Both the fluid reservoir (not shown) and the organ bath were aerated with the same appropriate mixture of N_2 and/or O_2 containing 5% CO_2 (v/v). The preparation is attached to the bottom of the organ bath and the force transducer.

oxygen-induced contraction. This peculiar preparation therefore represents a useful model for the investigation of the mechanism underlying the anti-hypoxic activity of a drug.

Methods

Strips of ductus arteriosus from near term foetal guinea-pigs were prepared as described previously (Roulet & Coburn 1981) and mounted in a 4 ml water-jacketed organ-bath for isometric tension recordings (Figure 1). The organ-bath was perfused at 4 ml min^{-1} (Gilson, Minipuls II) with physiological saline solution (PSS) aerated with a calibrated mixture of 5% CO_2 in N_2 (low P_{O_2}) and/or in O_2. The oxygen tension in the bathing fluid (P_{O_2}) was monitored with a Clark-type oxygen electrode connected to a gas analyser (Corning, 161). Each strip was set up in the organ bath, attached to an isometric force transducer (Myograph F60, Biosystems), left to equilibrate for 30 min under zero tension in a low P_{O_2} environment and then made to contract maximally by being exposed to a perfusion of oxygen-saturated PSS (high P_{O_2}), before the experiment was started. In the low P_{O_2} conditions the resting tension of the strips ranged from 150–200 mg. Experiments were performed at 37 ± 0.1°C. Isometric tension and P_{O_2} were simultaneously recorded on a Beckman R612 polygraph.

Solutions and drugs

The physiological saline solution (PSS) had the following composition (mM): Na^+ 137, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 123, $H_2PO_4^-$ 1.2, HCO_3^- 26 and glucose 11. K^+ and Na^+ ions were substituted for each other in equimolar quantities in K-free and high-K solution, respectively. Ca^{2+} ions were simply omitted in Ca-free PSS, and reduced or increased in experiments with variable calcium levels as indicated in the Results. The pH of these solutions when equilibrated with CO_2 ranged from 7.33–7.38 at 37°C.

The following drugs were used: (±)-propranolol-HCl, yohimbine, tetrodotoxin, indomethacin, ouabain octahydrate (strophantin-G) (Sigma); phentolamine-HCl (Ciba-Geigy) and prazosin (kindly supplied by Pfizer). Stock solutions of indomethacin were prepared in ethanol at 10 mg ml^{-1}. TMZ was dissolved

in PSS at a concentration of 8×10^{-2} M and serially diluted with PSS (occasionally TMZ was prepared at 4×10^{-1} M). When the effect of TMZ was tested, the perfusion was stopped 5 min before injecting a quantity of 50 µl directly into the organ bath (cumulative additions of TMZ did not exceed 0.3 ml during the dose-response experiments). The preparation was exposed to the drug for a mean time of 10 min, the perfusion was re-started to wash out the drug usually at a rate of 6 ml min^{-1} for 10–15 min, followed by 4 ml min^{-1}.

Analysis of results

Nomenclature: low $Po_2 < 20$ mmHg, high $Po_2 > 350$ mmHg also referred to as normoxia, hypoxia in the Po_2 range from 40 to 100 mmHg (mild hypoxia refers to the specific Po_2 of 75 ± 5 mmHg). The resting tension during low Po_2 was regarded as the baseline level from which the amplitude of contraction was estimated, and the maximal increase in tension due to high Po_2 was taken as 100% (unless otherwise stated). Data were expressed as the mean ± s.e.mean response from the indicated number of experiments.

Results

Mechanical response to trimetazidine during mild hypoxia

Under our experimental conditions, a Po_2 set at 75 ± 5 mmHg ($n = 8$) caused the ductus strip to develop and sustain an active tone whose amplitude was $30 \pm 3\%$ ($n = 8$) of the maximal response induced by high Po_2. Addition of TMZ to the bath elicited a concentration-dependent increase in tension with a maximal response at 10^{-3} M. A typical time course of the effect of cumulative administration of TMZ is shown in Figure 2a. Data from 8 similar experiments

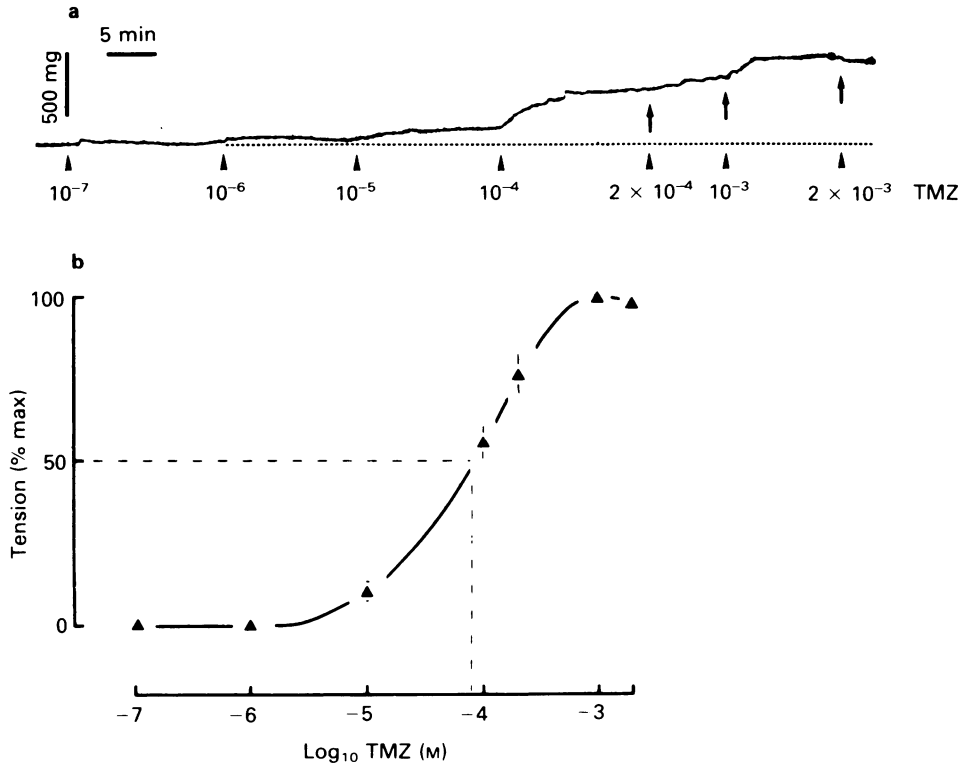


Figure 2 (a) Representative tracing illustrating the effect of increasing doses of trimetazidine (TMZ) on the mechanical tension of the ductal strip during mild hypoxia ($Po_2 = 75 \pm 5$ mm Hg). Note the slight contractile response induced by 10^{-6} M TMZ, and the relaxant activity of the drug at 2×10^{-3} M. (b) Dose-response relationship for the contraction of the ductal strip to TMZ compiled from experiments similar to the one shown above. Values plotted are the mean, with vertical lines showing s.e.mean, from 5 to 8 strips. The ED_{50} for the drug was 8×10^{-5} M. The amplitude of the response produced by 10^{-3} M TMZ was normalized as 100%, the mild hypoxia-induced tension was the reference level.

were used to plot the dose-effect curve shown in Figure 2b (the median effective dose (ED_{50}) for the drug was estimated as being 8×10^{-5} M). TMZ-induced tension was expressed as a percentage of the maximal response to the drug. The tone established under mild hypoxia was regarded as the baseline level. TMZ at a concentration of 10^{-6} M caused either an increase (4–8%, 2 strips) or a decrease (5–8%, 2 strips) in tension, or the preparation could be completely unresponsive to this concentration of TMZ (8 strips). The maximal effect obtained with 10^{-3} M TMZ was normally unaltered or slightly depressed by increasing the dose to 2×10^{-3} M, but it was consistently augmented with a high Po_2. Briefly, under conditions of mild hypoxia, the initial steady state tension that amounted to $30 \pm 3\%$ of the oxygen-induced contraction increased up to $86 \pm 6\%$ in the presence of a maximally active dose of TMZ, and was further enhanced to $95 \pm 3\%$ on exposure to high Po_2 ($n = 8$).

The effect of TMZ (10^{-4} M) under mild hypoxic conditions was not significantly altered following treatment of the preparation with tetrodotoxin (3×10^{-7} M), or with any of the adrenoceptor blocking drugs: phentolamine (10^{-6} M), yohimbine (3×10^{-7} M), prazosin (2×10^{-7} M) and propranolol (3×10^{-7} M). The addition of indomethacin (2×10^{-6} M) to the bath during mild hypoxia increased the baseline level of tension, but did not prevent the response to TMZ.

The importance of calcium ions for the TMZ-induced response was evaluated. Control contractions caused by TMZ 10^{-4} M and 10^{-3} M were recorded during mild hypoxia in normal PSS solution. Calcium ions were then removed for about 20 min before further addition of TMZ. In the absence of Ca^{2+} there was no mechanical response to the drug at any of the concentrations tested. Figure 3 shows the relationship between the amplitude of contraction developed in the presence of 10^{-4} M TMZ and the concentration of external calcium. The data indicate that the response to TMZ could result from a stimulation of the influx of Ca^{2+} ions into the cells.

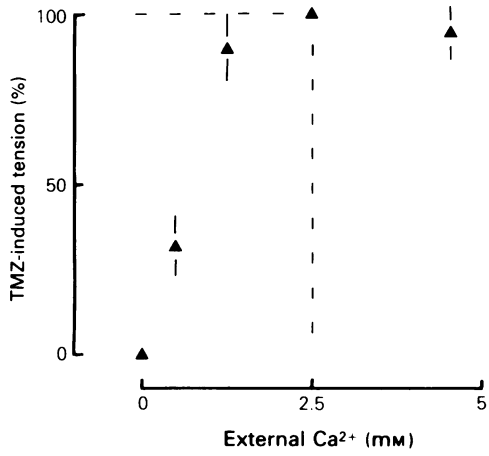


Figure 3 The amplitude of the trimetazidine (TMZ)-induced increase in tension in relation to the external Ca^{2+}. In the absence of Ca^{2+}, TMZ was ineffective in producing a response. The amplitude of contraction evoked during mild hypoxia in control PSS (2.5 mM Ca^{2+}) was normalized as 100%. The concentration of TMZ was 10^{-4} M throughout. The data represent the mean of 3 to 5 determinations and vertical lines show s.e.mean.

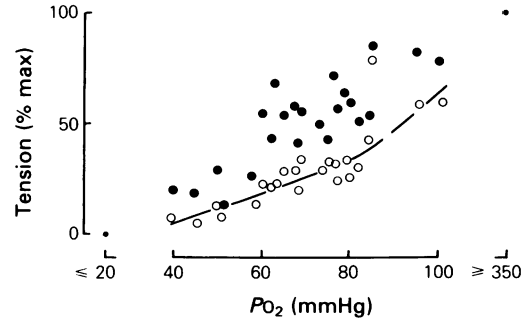


Figure 4 The relationship between the tension of the ductal strip and the concentration of oxygen (Po_2), and the effect of trimetazidine (TMZ) on this tension. The contractions elicited at Po_2 values between 40 and 100 mm Hg (○) were enhanced by TMZ 10^{-4} M (●), whereas the resting tone (◉) and the high Po_2-induced contraction (◉) were unaltered. The dashed line was drawn by eye to join the data points (○) obtained from separate experiments in control conditions. The amplitude of the oxygen-induced contraction evoked in the absence of the drug was normalized as 100%, the resting tone during low Po_2 was the reference level.

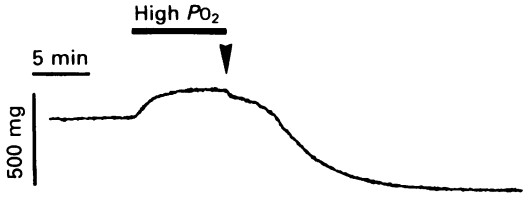


Figure 5 Trace showing the low Po_2-induced relaxation in the presence of 10^{-3} M trimetazidine (TMZ). The tension trace to the left resulted from the activity of 10^{-3} M TMZ under hypoxia ($Po_2 = 50$ mmHg). Increasing Po_2 to a high level (solid horizontal bar) enhanced the amplitude of tension. Application of low Po_2 ($\simeq$50 mmHg (arrow) reversed the tension incompletely. Note that the relaxation was delayed. The dashed line at the bottom right of the record indicates the control resting level of the preparation.

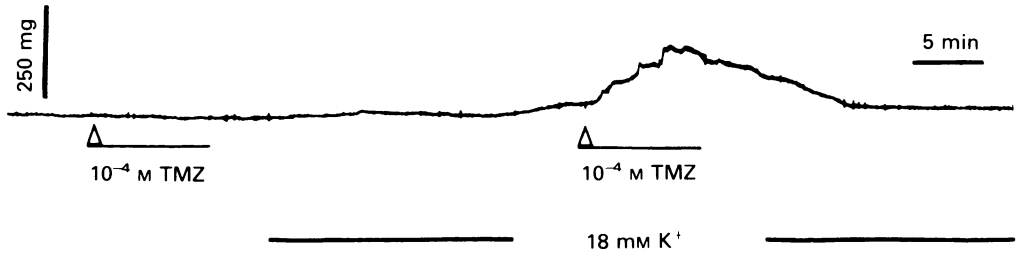
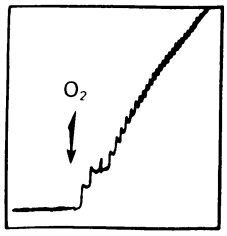


Figure 6 The vasoactivity of 10^{-4} M trimetazidine (TMZ) during low Po_2. The trace shows that TMZ, 10^{-4} M, elicited a contraction under low Po_2 conditions only after the addition of 18 mM K^+ PSS (solid horizontal bar), i.e. in the K-depolarized preparation TMZ produced a significant and reversible increase in tension. The horizontal lines headed with an open triangle indicate the 8 min exposure of the strip to TMZ.

The activity of trimetazidine and its dependence on the concentration of oxygen

It may be important to establish the relationship between the activity of TMZ and the degree of hypoxia imposed on the preparation. The TMZ response was assessed at a single dose of 10^{-4} M during low Po_2, hypoxia and high Po_2. Figure 4 shows that preparations exposed to TMZ developed a large contraction when the Po_2 ranged from 40 to 100 mm Hg (hypoxia), whereas at a low or high Po_2 TMZ induced no change in the initial tension. The leftward shift of the Po_2-response curve without enhancement of the minimal and maximal tension induced by TMZ (Figure 4), favours the hypothesis that the activity of the drug is related to the concentration of oxygen. Moreover, it was repeatedly observed that the contraction produced by TMZ at the maximally effective dose of 10^{-3} M during hypoxia could be partially reversed by exposure of the preparation to low Po_2 (Figure 5). The low Po_2-induced relaxation was initiated after a substantial delay as if there was some kind of 'protective' effect of the drug against the withdrawal of oxygen from the tissue.

The effect of TMZ could also be influenced by the contractile state of the ductus, independently of the presence of oxygen in the medium. Therefore, in order



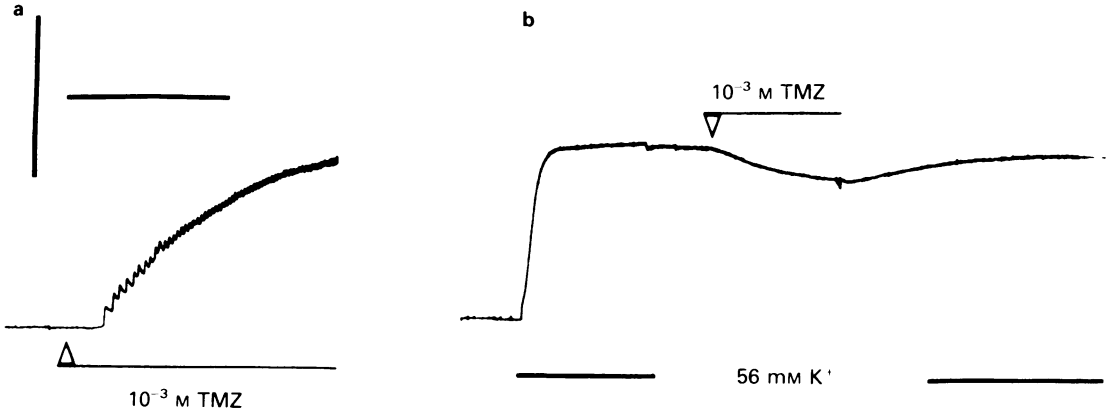


Figure 7 Effect of 10^{-3} M trimetazidine (TMZ) during low Po_2. (a) In normal PSS solution, TMZ elicited spontaneous mechanical activity. (b) After the ductal strip had been contracted by the addition of 56 mM K^+ TMZ initiated a reversible relaxation. For comparison with (a), the inset shows the tension developed in response to high Po_2 (O_2) during control PSS perfusion. The records were from the same preparation. The horizontal lines headed with an open triangle indicate the exposure to TMZ. Time and tension calibrations are: horizontal line 5 and 10 min, vertical line 250 and 500 mg, for (a) (plus inset) and (b), respectively.

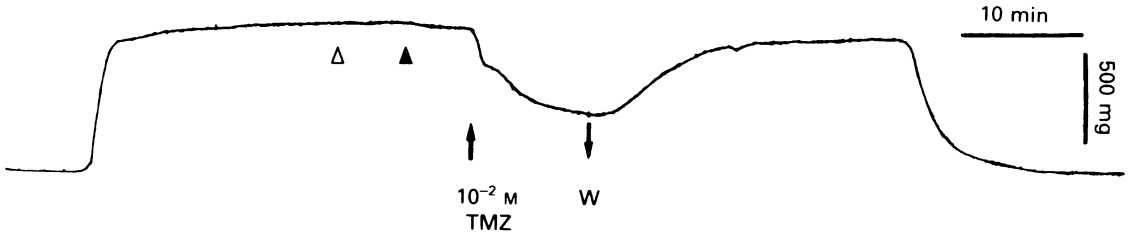


Figure 8 Trace showing the relaxation response induced by high concentrations of trimetazidine (TMZ) during high Po_2. The trace represents the mechanical response to high Po_2 (horizontal solid bar) and shows the effect of cumulative additions of TMZ on the steady state tension. TMZ at 10^{-4} M (Δ) and 10^{-3} M (▲) did not affect the oxygen-induced contraction. However, addition of TMZ at a final concentration of 10^{-2} M (arrow) resulted in a relaxation which was reversed after washing-out (W) the drug.

to discriminate between the need for oxygen as such, and the need for the mechanical and/or electrophysiological manifestation of oxygen, we investigated the activity of the drug in the virtual absence of oxygen, that is, during low Po_2 conditions (for O_2- and K-induced contraction and depolarization, see Roulet & Coburn, 1981). As shown in Figure 6, after equilibration at low Po_2, the preparation became responsive to TMZ 10^{-4} M following perfusion with 18 mM K^+-containing PSS. In normal conditions, the contractile effect of TMZ can be completely inhibited by removal of calcium from the 18 mM K^+ medium. Hence, this result is consistent with the hypothesis that membrane depolarization mediates the activity of TMZ in the ductus. If this is the case, one should expect the activity of TMZ to be affected by the cell membrane potential. As we could not get any more information using 10^{-4} M TMZ, we investigated the effect of 10^{-3} M TMZ during low Po_2 and with increasing concentrations of external K^+. The results (not shown here) demonstrated that the activity of TMZ is related to the K^+ concentration in the bathing medium: the contractile response changed into a relaxation response when the K^+ concentration was higher than 29 mM. Figure 7 shows an example of the effects of TMZ at normal and high K^+ concentration. In PSS, the contractile response to the drug was delayed and proceeded with a steplike mechanical activity (Figure 7a) similar to the spontaneous activity triggered by the addition of oxygen in control condi-

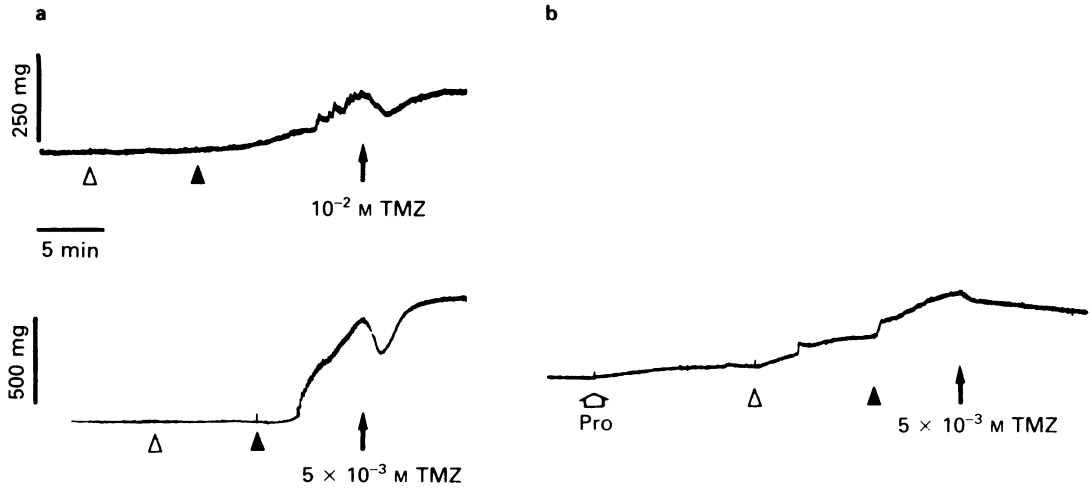


Figure 9 Different effects of trimetazidine (TMZ) during low Po_2 (a) and mild hypoxia (b). (a) Shows that TMZ induces only a delayed contractile response at doses as high as 10^{-3} M (▲) during low Po_2. Whereas during mild hypoxia (b) TMZ, 10^{-4} M, (Δ) and 10^{-3} M (▲) induced a contractile response. Higher doses of TMZ (a) 10^{-2} M and (a and b) 5 × 10^{-3} M induced a relaxation response which was transient during low Po_2 (a) and more prolonged during mild hypoxia (b). Propranol (Pro; 3 × 10^{-7} M) did not prevent the activity of TMZ during mild hypoxia (b).

tions (inset of Figure 7). In high K^+ medium, the preparation developed a tension which was significantly and reversibly suppressed by the addition of TMZ (Figure 7b). At an external K^+ concentration of 29 mM, administration of TMZ induced no change in tension. The preparation, after equilibration at low Po_2 either in K-free solution or in the presence of ouabain (5×10^{-6} or 10^{-5} M), developed a contractile response to TMZ suggesting that the Na-pump is not involved in the vasoactivity of the drug.

Effect of different conditions on the activity of trimetazidine

The data presented so far have shown the potentiator role of TMZ (up to 10^{-3} M) on the increased tension, induced by hypoxia, in the isolated ductus arteriosus. Although, TMZ behaved as a powerful vasodilator drug on maximally oxygen-contracted ductus with effective doses above 10^{-3} M (Figure 8), in concentrations ranging from 2×10^{-3} to 10^{-2} M, it reversibly suppressed the oxygen-induced contraction. In addition to this clear relaxant activity a depressant effect of TMZ at 2×10^{-3} M has been seen during mild hypoxia (see Figure 2). Figure 9b illustrates the effect of increasing doses of TMZ on a preparation exposed to hypoxia: at first, as already mentioned, the drug increased the developed tension. This was followed by a depression of the response (note that the presence of propranolol did not affect the dilator activity of the drug). Remarkable behaviour was observed on a strip equilibrated at low Po_2 (Figure 9a); elevated doses of TMZ ($> 10^{-3}$ M) triggered a transient relaxation but a single application of such high doses during low Po_2 or during mild hypoxia promoted a slowly developing tension.

Discussion

The current study is the first characterization of the anti-hypoxic activity of trimetazidine on isolated vascular tissue. The bath Po_2 has been regarded as the sole determinant of the tone of the preparation as suggested from the Po_2 vs tension relationship (Fay & Jobsis, 1972; Roulet & Coburn, 1981). A plot of normalized amplitude of contraction against surrounding Po_2, in control conditions and following a 10 min treatment with TMZ, demonstrated that the drug caused similar changes to oxygen, thereby amplifying the effect of oxygen on hypoxic tissue. The inability of TMZ to contract preparations exposed to low Po_2 strengthens the idea that the drug acts by potentiating the activity of a mechanism specific for oxygen. However, under low Po_2 conditions, doses of TMZ (10^{-4} M) which were ineffective in normal conditions could produce a contraction in a 18 mM K^+-depolarized preparation (a 14 mM depolarization was recorded under similar conditions by Roulet & Coburn, 1981). Therefore, in the virtual absence of oxygen, the K-induced membrane depolarization could trigger the response to TMZ, just like hypoxia. The susceptibility of the response to TMZ to both hypoxia and K^+ ions could be explained by the fact that both oxygen and K^+ ions cause the ducti smooth muscle cells to depolarize (Roulet & Coburn, 1981). In fact, depolarization of the membrane appears to be a prerequisite for the activity of TMZ at 10^{-4} M. The response to TMZ was not affected by pretreatment of the preparation with α-and β-adrenoceptor blocking agents or indomethacin, suggesting that adrenoceptors and prostaglandin synthesis are not involved in the response.

There is firm evidence that the contraction of smooth muscle results from an increase in free intracellular calcium ions available to contractile proteins (Filo *et al.*, 1965; Endo *et al.*, 1977). This Ca^{2+} may originate from intra- and/or extra-cellular sources (Somlyo & Somlyo, 1968). Incubation of strips, either hypoxia- or K^+-stimulated strips, in Ca^{2+}-free medium inhibited their reactivity to TMZ suggesting that TMZ-induced responses are associated with mobilization of an extracellular pool of calcium.

Under low Po_2 conditions, the administration of high doses of TMZ (10^{-3} M), elicited a delayed contraction, in agreement with the proposal that the drug's activity is dependent upon tissue oxygen concentration and/or the level of membrane depolarization (for example, TMZ at 10^{-4} M, ineffective during low Po_2, became active during hypoxia). In connection with this, it is interesting to note the different effects of the drug produced by varying the external K^+ concentration. At a single dose of 10^{-3} M and in low Po_2 medium, TMZ caused responses which appeared to be linked to the cell membrane potential. A probable explanation for the dual vasoactivity of the drug could be an altered membrane permeability to K^+ ions. This follows from the findings of: (1) a steplike increase in tension evoked by addition of TMZ in low Po_2 and control PSS, and (2) a TMZ-induced increase in tension followed by relaxation when the external K^+ concentration was raised during low Po_2. Similarly, repeated mechanical activity was evoked in normally quiescent vascular muscle after application of drugs which are known to lower P_K (Droogmans *et al.*, 1977; Harder & Sperelakis, 1978). Regarding the second point, the alternative that TMZ reduces the P_K accords with our results if one assumes that: (a) chloride ions contribute to the cell membrane conductance, as has been shown in vascular tissue (Whalstrom, 1973; Casteels *et al.*, 1977), and (b) the equilibrium potential for Cl^- remains constant, that is, more positive and then more negative than E_K, as

K^+ ions are increased in the bath. Assuming this is true, reducing P_K would change the membrane potential and thereby modulate the influx of calcium. It has indeed been shown that K-induced depolarization activates vascular muscle contraction by stimulating transmembrane influx (Breemen *et al.*, 1972).

Our findings that TMZ induces a relaxation response under conditions of normoxia, are consistent with those of Toda *et al.* (1982) who showed TMZ to have a dilator effect on prostaglandin- or 12–15 mM K^+-contracted vascular strips. However, on a 30 mM K^+-stimulated coronary artery strip, these authors noted that TMZ became ineffective at the usual doses. This supports our suggestion that some mechanism implicated in the activity of TMZ could be dependent on the membrane potential.

From our experimental data, it may be argued that the site of action of TMZ resides somewhere along the sequence of events coupling oxygen excitation to contraction. The activity of the drug is likely to be the result of an amplification of the step involving a reduction in P_K. Electrophysiological work in progress will enable us either to confirm or contradict this statement.

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