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Inhibition of calcium release from the sarcoplasmic reticulum of rabbit aorta by hydralazine

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1 The mechanism of hydralazine-induced vasorelaxation was investigated in rabbit isolated aorta, by determining its ability to interfere with force development under a variety of conditions.

2 Hydralazine relaxed phenylephrine-contracted aorta with half maximal relaxation at 17 μM and maximal relaxation above 100 μM. At 200 μM, hydralazine had little effect on contractions induced by 25 mM or 50 mM K^+.

3 Hydralazine was equally effective at inhibiting contractile responses to phenylephrine in the absence or presence of extracellular Ca^{2+}. Responses to phenylephrine in Ca^{2+}-free solution were blocked to the same degree whether hydralazine was applied during filling of the sarcoplasmic reticulum (SR) Ca^{2+} stores or after filling was complete. Caffeine-induced contractions were less sensitive to block by hydralazine.

4 Thapsigargin, cyclopiazonic acid, ryanodine, nifedipine and diltiazem all failed to block the inhibitory effect of hydralazine on tonic contractions to phenylephrine in the presence of extracellular Ca^{2+}. However, when cyclopiazonic acid was applied either with diltiazem or ryanodine, substantial inhibition of the hydralazine response was observed.

5 We propose that tonic contractions to phenylephrine are largely maintained by Ca^{2+} cycling through the SR, with Ca^{2+} entering the smooth muscle cell being sequestered by the SR eventually to leak out through IP_3-activated channels close to the contractile proteins. Sequestration of Ca^{2+} would employ two pathways, one sensitive to inhibitors of the SR Ca^{2+}-ATPase and the other to Ca antagonists. We further suggest that, in the presence of extracellular Ca^{2+} and phenylephrine, the leakage of Ca^{2+} through IP_3-activated channels is significantly reduced only if both routes for SR Ca^{2+} accumulation are blocked or the Ca^{2+}-ATPase is blocked while the SR is made leaky with ryanodine.

6 We conclude that the main action of hydralazine is to block the IP_3-dependent release of Ca^{2+} from the sarcoplasmic reticulum. Thus conditions that diminish the contribution of IP_3-induced Ca^{2+} release to tension can inhibit the hydralazine-induced vasorelaxation.

Keywords: Hydralazine; vasodilatation; rabbit aorta; smooth muscle; calcium release; calcium stores; sarcoplasmic reticulum; cyclopiazonic acid; ryanodine

Introduction

The antihypertensive drug, hydralazine, has been in clinical use for over 40 years, but its mechanism of action is still poorly understood. It acts directly on the vasculature and inhibits responses to a wide variety of vasoconstrictor agents (Kirpekar & Lewis, 1957). Hydralazine can relax vascular muscle *in vitro* after the endothelial cell layer has been removed, implying that its main action is at the level of the smooth muscle cell. Its effects are not correlated with an increase in tissue guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Diamond & Janis, 1978; 1980; Yen *et al.*, 1989), so it acts through a mechanism distinct from the nitrovasodilator drugs. Several studies conclude that hydralazine interferes with Ca^{2+} handling by the smooth muscle cell. For example, using the Ca^{2+} indicator aequorin, DeFeo & Morgan (1989) showed that the hydralazine-induced relaxation of ferret aorta was accompanied by a fall in the intracellular Ca^{2+} concentration. Furthermore, hydralazine did not alter the relationship between the intracellular Ca^{2+} concentration and the force developed by aortic strips (DeFeo & Morgan, 1989). Thus hydralazine appears to relax smooth muscle solely by reducing the intracellular Ca^{2+} concentration, and not by chaning the Ca^{2+} sensitivity of the contractile proteins. Further support for this conclusion is provided by the finding that hydralazine had no effect on the contraction of chemically skinned renal arteries, brought about by the addition of Ca-calmodulin and ATP (Kreye *et al.*, 1983).

Potential mechanisms that would lead to a fall in the intracellular Ca^{2+} concentration include inhibition of Ca^{2+} influx or Ca^{2+} release from intracellular stores, or stimulation of Ca^{2+} efflux or Ca^{2+} accumulation into the stores. It has been proposed that hydralazine inhibits Ca^{2+} influx, based on the observation in rabbit aorta that it inhibits K^+-stimulated contractures and the net uptake of $^{45}Ca^{2+}$ during these contractures (McLean *et al.*, 1978b). However, most of the studies that show a significant inhibition of K^+-contractures employed high concentrations (>0.1 mM) of hydralazine (Barron *et al.*, 1977; McLean *et al.*, 1978a,b; Orallo *et al.*, 1991), well above the levels that would be reached in plasma after a therapeutic dose (Talseth, 1976). There seems to be general agreement from studies employing low concentrations of hydralazine, that the drug is more effective at inhibiting contractures induced by receptor stimulation (McLean *et al.*, 1978a; Lipe & Moulds, 1981; Khayyal *et al.*, 1981; Higashio & Kuroda, 1988a; Yen *et al.*, 1989). Moreover, Lipe & Moulds (1981) noted that hydralazine was most potent at inhibiting contractions of human vascular muscle that showed the least requirement for extracellular Ca^{2+}. Consistent with this, contractions induced by noradrenaline in Ca^{2+}-free solutions are sensitive to block by hydralazine (McLean *et al.*, 1978a; Ebeigbe & Aloamaka, 1985; Higashio & Kuroda, 1988b; Orallo *et al.*, 1991). Thus hydralazine may relax vascular muscle by interfering with the intracellular storage of Ca^{2+}, rather than by inhibiting Ca^{2+} influx.

The aim of this study was to clarify the cellular mech-

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anisms by which hydralazine induces vasodilatation. We have examined the possible contributions of an effect on Ca^{2+} influx, mediated either by direct modulation of Ca channels or through membrane hyperpolarization, and an effect on intracellular Ca^{2+} storage by the sarcoplasmic reticulum (SR). We conclude that the main action of hydralazine is to inhibit the agonist-induced release of Ca^{2+} from the SR.

Methods

Male New Zealand white rabbits (2–3 kg) were killed by a lethal i.v. injection of sodium pentobarbitone (80 mg kg^{-1}; Ceva, Watford, Hertfordshire) and exsanguinated. The descending thoracic aorta was excised, placed in physiological salt solution then freed of connective tissue. The endothelial cell layer was removed by gently rubbing the intimal surface. Although initial experiments indicated that removal of the endothelium was successful, we did not routinely check for its absence. The vessel was cut into rings (1–2 mm wide) or transverse strips (1–2 mm wide, 0.8–1 cm long), which were mounted in a chamber (~0.5 ml volume) for isometric tension recording with a Harvard Apparatus (South Natick, MA, U.S.A.), model 60-2998 transducer. Tissues were placed under an optimal basal tension of 1.25 g and continuously perfused at 1 ml min^{-1} with physiological salt solution at 35 to 37°C. They were allowed to equilibrate for 1 h before experiments commenced.

Experimental protocols

To determine the relationship between hydralazine concentration and the induced smooth muscle relaxation, tissues were precontracted by changing the perfusion solution to one containing phenylephrine (1 μM). Once tension had reached a stable level, the tissue was perfused with hydralazine in the continued presence of phenylephrine, and the resulting relaxation was measured as a percentage of the phenylephrine-induced tone. A similar procedure was followed to investigate the effectiveness of hydralazine on K^+-contracted tissues. Potential inhibitors of the hydralazine-induced relaxation were tested against tissues precontracted with phenylephrine, with the inhibitors perfused for up to 1 h before hydralazine was added. In this case the relaxation response to hydralazine was measured as a percentage of the phenylephrine-induced tension recorded in the presence of the inhibitor, immediately before hydralazine was applied. After obtaining a steady response to hydralazine, the tissue was relaxed to baseline by perfusion with the normal physiological salt solution.

Contractions due to the release of Ca^{2+} from intracellular stores were induced by exposing the tissue either to caffeine (10 mM) or phenylephrine (1 μM) in the absence of extracellular Ca^{2+}. Tissues were perfused with Ca^{2+}-free solution containing 1 mM EGTA (ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N',N'-tetraacetic acid) for various times before, during and after a brief application of caffeine or phenylephrine. They were then perfused with normal physiological salt solution for 10 min to refill the stores, and the cycle was repeated until reproducible responses were obtained.

Results are expressed as mean ± s.e.mean. Statistical comparisons among groups of data were made using the GraphPAD Instat software to perform one way analysis of variance (ANOVA), with P values corrected by the Bonferroni method. When comparing only 2 groups of data, an unpaired t test was used.

Drugs and solutions

The physiological salt solution had the following composition (mM): NaCl 112, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, NaH_2PO_4 0.5, KH_2PO_4 0.5, $NaHCO_3$ 15, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 5, glucose 10, phenol red 0.02 and it was constantly bubbled with a 95% O_2, 5% CO_2 gas mixture (pH 7.2). The Ca^{2+}-free solutions were prepared by omitting $CaCl_2$ and adding 1 mM EGTA, with the pH readjusted to 7.2 with NaOH after equilibration with 95% O_2, 5% CO_2. Solutions with varying concentrations of K^+ were prepared by equimolar substitution of KCl for NaCl. They also contained 1 μM phentolamine to prevent smooth muscle stimulation by noradrenaline released from nerve terminals in the tissue.

Phenylephrine hydrochloride, hydralazine hydrochloride and diltiazem hydrochloride, all from Sigma Chemical Co. Ltd., Poole, Dorset, ryanodine from Calbiochem Novabiochem, Nottingham and phentolamine mesylate from Ciba Laboratories, Horsham, Sussex were prepared as 10–100 mM stock solutions in distilled water. Stock solutions of cyclopiazonic acid (100 mM; Calbiochem), thapsigargin (1 mM; Sigma) and nifedipine (10 mM; Sigma) were prepared by dissolving them in 100% dimethylsulphoxide. In each case, serial dilutions were made with the appropriate experimental solution. Dimethylsulphoxide, at the highest concentration achieved during experiments (0.1%), had no effect itself on the hydralazine response. Caffeine (Sigma) was dissolved directly in the experimental solution.

Hydralazine stability in solution

Hydralazine has been reported to degrade with a half-life of 30 min in oxygenated salt solutions at pH 7.4 (Worcel, 1978). We therefore diluted hydralazine into the experimental solution just before applying it to tissue. However, responses to hydralazine usually took at least 30 min to develop. We therefore performed control experiments on four muscle strips, prepared from adjacent segments of the same rabbit aorta, to determine the extent to which degradation of the drug might have influenced our results. The strips were contracted to a steady level of tension with 1 μM phenylephrine before perfusing hydralazine (50 μM) until a maximum response was reached. The hydralazine perfusing one strip was exchanged every 5 min with freshly prepared solution and induced a relaxation of 62%. The other three strips were challenged consecutively with the same hydralazine solution. Perfusion of the first of these strips began as soon as the hydralazine was prepared, and continued with the same solution until the maximum relaxation (55%) was reached. The second and third strips were perfused with the same solution, beginning 1.3 and 3.3 h later, and this resulted in relaxations of 69% and 60%, respectively. While there was some variation among the responses to hydralazine, this did not correlate with the length of time for which the hydralazine had been present in the oxygenated solution. Thus degradation of hydralazine did not appear to be significant in the present experiments.

Results

When applied to isolated aorta in the presence of physiological saline, phenylephrine (1 μM) caused a slowly developing contraction, which eventually reached a steady level of tension that was maintained for several hours. When applied during this sustained phase of tension, hydralazine induced a concentration-dependent relaxation as shown in Figure 1a. The concentration producing 50% relaxation (IC_{50}) was 17 μM, with the maximum relaxation achieved at around 200 μM hydralazine. However, even in the presence of a maximal concentration of hydralazine, aortic strips rarely relaxed completely, but retained around 20% of the initial phenylephrine-induced tone. The response to hydralazine was slow in onset, taking up to 1 h to develop fully even at the highest concentrations. In addition, the hydralazine-induced relaxations were slow to reverse; in many tissues, recovery was incomplete even after 3 h of washing. For this reason, hydralazine was tested only once on each tissue, with the

effects of various conditions determined by comparing the mean responses of several tissues. The relaxation response to hydralazine was frequently, but not always, preceded by a transient contraction, which was pronounced in some tissues (see Figure 4). We have not investigated this response further.

As can be seen in Figure 1b, hydralazine was much less effective in relaxing tissues when they were precontracted with high K^+ solutions. At 200 μM, hydralazine produced only 20 ± 6% ($n = 3$) inhibition of 25 mM K^+-induced contractures and 21 ± 5% ($n = 3$) inhibition of 50 mM K^+-induced contractures. Thus the response to hydralazine was the same regardless of the degree to which the extracellular K^+ concentration was elevated to cause contraction.

Role of stored Ca in the response to hydralazine

Rabbit aorta contracts in the absence of Ca^{2+} when exposed to agents that release Ca^{2+} from intracellular stores. Possible actions of hydralazine on Ca^{2+} stores were evaluated by testing its effects on responses to caffeine, which directly releases Ca^{2+} from intracellular stores (Herrmann-Frank *et al.*, 1991), and phenylephrine, in Ca^{2+}-free solution containing 1 mM EGTA. The caffeine- and phenylephrine-releasable stores have been shown to overlap completely in the rabbit aorta (Leijten & van Breemen, 1984). Consistent with this, responses to 10 mM caffeine in Ca^{2+}-free conditions were blocked completely by prior exposure to 1 μM phenylephrine ($n = 2$) and by 90% after previous exposure to caffeine ($n = 2$). Similarly, contractions to 1 μM phenylephrine were suppressed by 81 ± 6% ($n = 4$) or 63 ± 2% ($n = 4$) when preceded by exposure to phenylephrine or caffeine, respectively. Contractions induced by phenylephrine were never completely blocked. Even after 1 h continuous exposure to phenylephrine in Ca^{2+}-free solution, 18 ± 2% ($n = 4$) of the initial tension remained, suggesting a Ca^{2+}-independent component.

As illustrated in Figure 2, 100 μM hydralazine reduced responses to 2 min applications of 10 mM caffeine in Ca^{2+}-free solution by 44 ± 7% ($n = 5$). However, hydralazine was more effective ($P < 0.01$) in inhibiting contractions to Ca^{2+} released from intracellular stores when they were triggered by receptor activation with phenylephrine. When phenylephrine (1 μM) was substituted for caffeine in the above protocol, 100 μM hydralazine reduced the resulting contractions by 71 ± 4% ($n = 6$), which is similar to the inhibition of phenylephrine contractions observed in the presence of extracellular Ca^{2+}. Figure 3 shows results of experiments designed to test whether this effect of hydralazine was due to an action

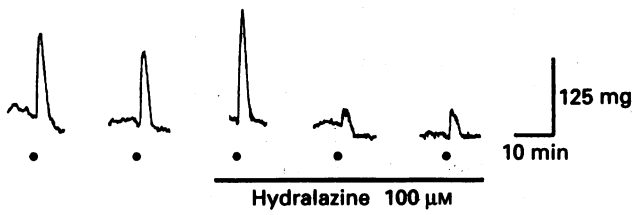

Figure 2 The effect of hydralazine on contractile responses to caffeine. The tissue was perfused with Ca^{2+}-free solution containing 1 mM EGTA for 10 min before, during and for 5 min after 2 min applications of 10 mM caffeine (●). Normal Ca^{2+} solution was then reintroduced for 10 min to refill the sarcoplasmic reticulum = Ca^{2+} stores. This cycle was repeated several times before and after the addition of 100 μM hydralazine, which is indicated by the bar. In this preparation, the response to caffeine was reduced by 65% in the presence of hydralazine. The mean inhibition in 5 preparations was 44 ± 7%.

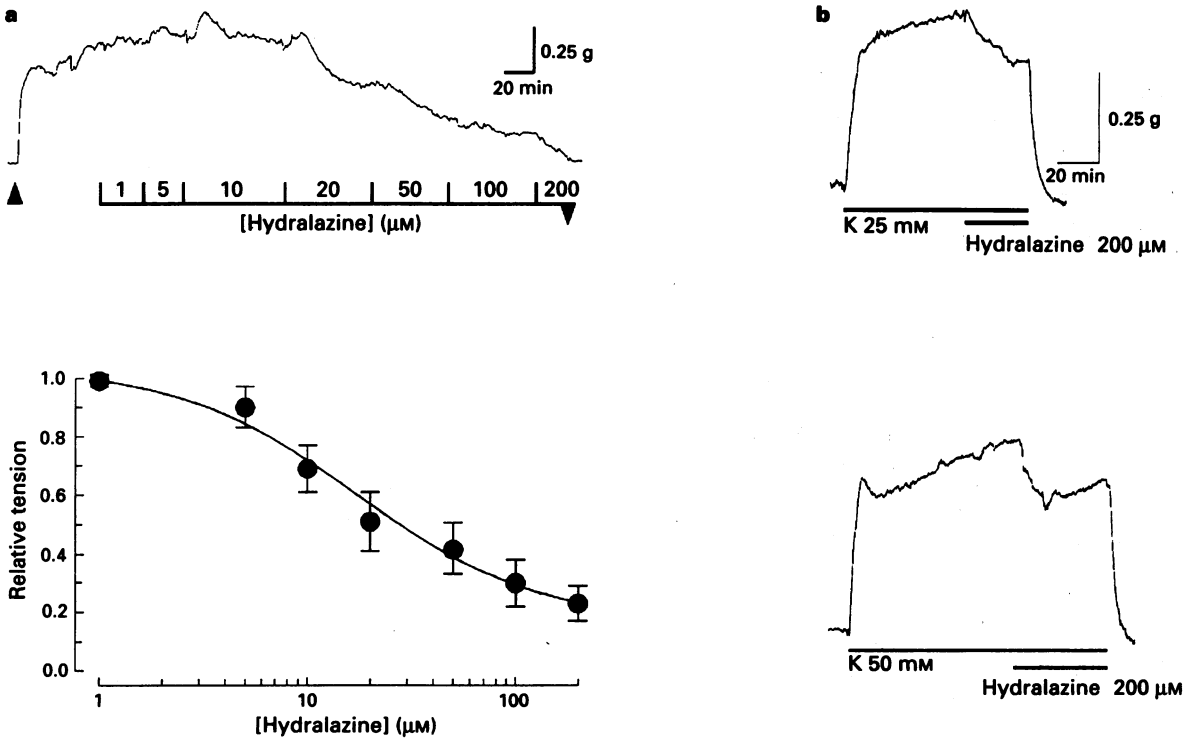

Figure 1 Concentration dependence of hydralazine-induced relaxation of precontracted rabbit aorta. (a) Top panel, increasing concentrations of hydralazine, cumulatively applied to a ring of aorta that was precontracted with 1 μM phenylephrine, caused increasing relaxation. The application and removal of phenylephrine is indicated by the arrowheads. Bottom panel, relationship between hydralazine concentration and the loss of phenylephrine-induced tension. Points represent means with s.e.mean of 6–8 observations. The curve shows the best fit to a standard hyperbolic equation, from which the concentration of hydralazine giving a half maximal response (IC_{50}) was estimated as 17 μM. (b) At 200 μM, hydralazine produced only a small relaxation when tissues were precontracted with 25 mM K^+ (top panel) or 50 mM K^+ (bottom panel).

on the release or refilling of the Ca^{2+} stores. In Figure 3a, extracellular Ca^{2+} was depleted by exposing the tissue to Ca^{2+}-free solution for 10 min, followed by a 5 min application of phenylephrine (1 μM), a 30 min wash in Ca^{2+}-free solution and a 10 min exposure to normal physiological saline in order to refill the intracellular Ca^{2+} stores. Once the responses to phenylephrine were reproducible (usually 4–6 cycles), 100 μM hydralazine was added for 25 min immediately before the next refilling period in normal saline, and throughout the filling period. This should have allowed development of hydralazine block before the stores were refilled. The subsequent response to phenylephrine was reduced by 69 ± 3% ($n = 3$), which is similar to the inhibition observed using the shorter protocol. In Figure 3b, hydralazine exposure was restricted to the period after the stores had been filled. To enable a similar duration of exposure to hydralazine, the protocol was adjusted so that tissues were perfused with Ca^{2+}-free solution for 30 min prior to the phenylephrine contraction and 10 min afterwards. In this situation, hydralazine (100 μM) inhibited the phenylephrine contraction by 68 ± 2% ($n = 5$). Since this value does not differ from that observed when hydralazine was applied before the store-refilling period, or when applied in the presence of extracellular Ca^{2+}, a major part of the action of hydralazine must be to inhibit the phenylephrine-induced release of Ca^{2+} from intracellular stores or the action of released Ca^{2+}.

Effects of modulators of sarcoplasmic reticulum function

To investigate further the role of SR Ca^{2+} stores in the action of hydralazine, we examined how its effects were influenced by a variety of agents known to alter SR function. These experiments were carried out in the presence of extracellular Ca^{2+}, using the normal physiological saline. Muscle contraction was initiated and maintained by applying 1 μM phenylephrine. Once a steady level of tension was achieved, hydralazine was applied at 100 μM, a concentration shown to produce near maximal relaxation under control conditions (Figure 1a). This resulted in a 76 ± 3% ($n = 11$) reduction of the sustained level of tension (Figure 4a). Drugs that modulate SR function were perfused across phenylephrine-contracted tissues for 1 h before adding 100 μM hydralazine, and the response to hydralazine was measured as the percentage loss of tension relative to that recorded immediately before hydralazine application. An example of such an experiment, examining the effect of ryanodine on the hydralazine response, is shown in Figure 4b. The results obtained with a number of different modulators of SR function are summarised in Figure 5.

At the concentration used (10 μM), ryanodine opens Ca^{2+}-permeable channels in the SR membrane, making the SR leaky to Ca^{2+} (Hwang & van Breemen, 1987). Consistent with this, we found that 10 μM ryanodine blocked transient contractions to phenylephrine in Ca^{2+}-free solution by 71 ± 2% ($n = 3$). Thus when ryanodine was applied to phenylephrine-contracted aorta in the presence of extracellular Ca^{2+}, an increase in tension of 46 ± 5% ($n = 3$) was observed, presumably reflecting an elevated cytoplasmic Ca^{2+} concentration. However, pretreatment with ryanodine failed to prevent the response to hydralazine (Figure 4b and 5a). Thapsigargin and cyclopiazonic acid are both thought to inhibit directly the Ca^{2+}-ATPase in the SR membrane, thereby limiting Ca^{2+} uptake (Seidler *et al.*, 1989; Thastrup, 1990). Thapsigargin at 1 μM, and cyclopiazonic acid at 30 μM, have previously been shown to substantially block enzyme activity and responses of rabbit aorta to phenylephrine in Ca^{2+}-free media (Luo *et al.*, 1993). In our hands, 30 μM cyclopiazonic acid suppressed phenylephrine-induced contractions in Ca^{2+}-free solution by 78 ± 3% ($n = 3$). In normal saline, both thapsigargin (1 μM) and cyclopiazonic acid (30 μM) had variable effects on phenylephrine-induced tension. No effect was observed in two out of six preparations exposed to thapsigargin and in two out of eight preparations exposed to cyclopiazonic acid. In three preparations, thapsigargin caused relaxation of 26 ± 10%, but it increased tension by 29% in another. Cyclopiazonic acid caused relaxation in two aortic strips with a mean of 12%, while increasing tension in the four remaining strips by 60 ± 26%.The reason for this variability is unclear. It may

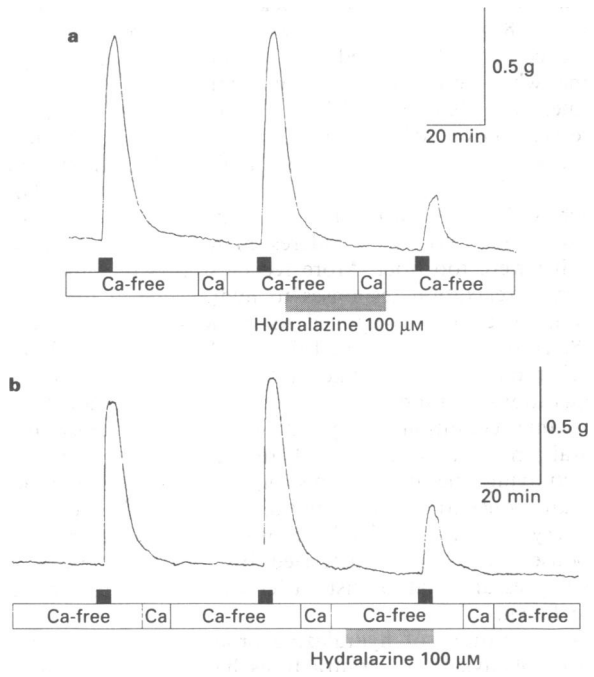

Figure 3 The effect of hydralazine 100 μM on contractions induced by phenylephrine (1 μM) in Ca^{2+}-free solution, containing EGTA (1 mM). Transient contractions to phenylephrine, applied for 5 min (indicated by short black bars) following 10 min (a) or 30 min (b) exposure to Ca^{2+}-free solution, were reproducible, provided stores were refilled between applications by 10 min exposure to Ca^{2+}-containing physiological solution. (a) Phenylephrine application was followed by 30 min in Ca^{2+}-free solution before restoring the extracellular Ca^{2+} for 10 min. Hydralazine was applied for the last 25 min in Ca^{2+}-free solution and during store refilling (shaded bar). (b) Phenylephrine application was followed by 10 min in Ca^{2+}-free solution before refilling the stores. Hydralazine was applied with the phenylephrine, in addition to 25 min before the phenylephrine application.

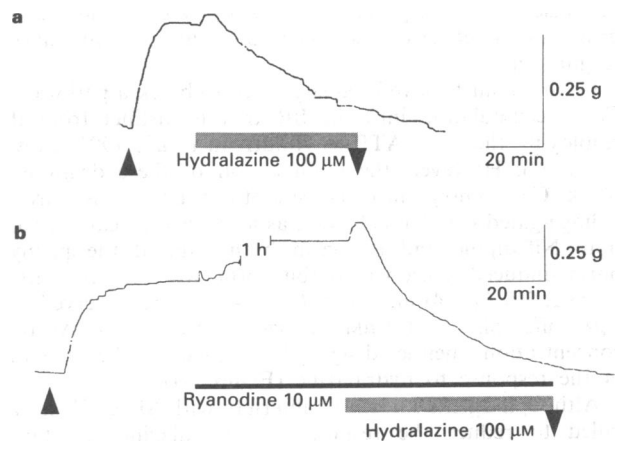

Figure 4 The effects of 100 μM hydralazine on tonic contractions to phenylephrine, applied in the absence (a) or presence (b) of ryanodine (10 μM). The application and removal of phenylephrine (1 μM) are shown by the arrowheads. Hydralazine and ryanodine exposures are indicated by bars. Ryanodine was continuously perfused during the 1 h gap indicated in the record in (b).

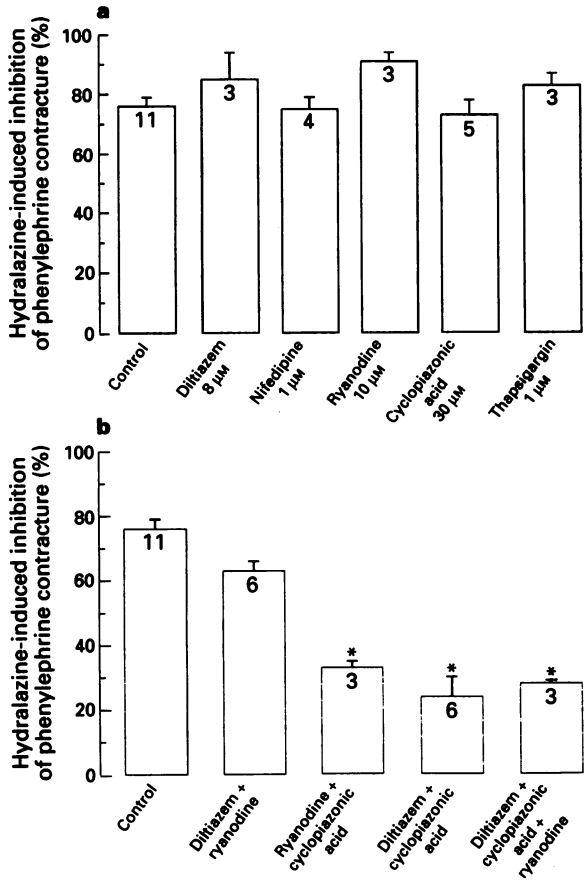

Figure 5 Effects of inhibitors of Ca^{2+} influx and sarcoplasmic reticulum function on the response to 100 μM hydralazine. Control columns show the percentage reduction by hydralazine of tonic phenylephrine-induced contractions in normal physiological saline. (a) Responses to hydralazine following exposure of the tissue to diltiazem (8 μM), nifedipine (1 μM), ryanodine (10 μM), cyclopiazonic acid (30 μM) or thaspsigargin (1 μM). (b) Responses to hydralazine following exposure to combinations of these drugs, applied together. *$P < 0.001$ as compared with control, from ANOVA with Bonferroni correction.

reflect inconsistency in the degree of endothelium removed from different tissues, since thapsigargin has been shown to relax vascular muscle through an action on the endothelium (Matsuyama *et al.*, 1993). Nevertheless, despite the variable responses to thapsigargin and cyclopiazonic acid, neither drug had any effect on the relaxation induced by hydralazine (Figure 5a).

Ca antagonists have been suggested to block a pathway for Ca^{2+} accumulation into the SR that is distinct from that employing the Ca^{2+}-ATPase (Bourreau *et al.*, 1991; Low *et al.*, 1992). However, the main action of these drugs is to block Ca^{2+} entry into the smooth muscle cells through voltage-gated Ca channels, and as a result they cause relaxation. Nifedipine and diltiazem both reduced the phenylephrine-induced tension in rabbit aorta. Maximal relaxations of 23 ± 5% ($n = 4$) and 18 ± 9% ($n = 3$) were observed with 1 μM nifedipine and 8 μM diltiazem, respectively. At these concentrations, neither drug had any effect on the amplitude of the response to hydralazine (Figure 5a).

Although agents known to interfere with SR Ca^{2+} storage failed to reduce the response to hydralazine, substantial block was observed when pairs of these drugs, acting through independent mechanisms, were applied together (Figure 5b). The relaxation elicited by 100 μM hydralazine was reduced to only 24 ± 6% ($n = 6$) after exposing aortic strips simultaneously to both cyclopiazonic acid and diltiazem for 1 h, which caused a 22 ± 13% ($n = 3$) loss of the phenylephrine-induced tone before hydralazine was added. A similar reduction in the hydralazine response was observed if thapsigargin was substituted for cyclopiazonic acid or nifedipine was substituted for diltiazem (not shown). The response to hydralazine was also substantially attenuated in the combined presence of cyclopiazonic acid and ryanodine, which had little effect on the phenylephrine-induced contraction before hydralazine was added (2 ± 16% relaxation; $n = 4$). This combination caused an 82 ± 3% ($n = 4$) inhibition of phenylephrine-induced contractions in Ca^{2+}-free solution, slightly more than either drug alone. Diltiazem and ryanodine together reduced tension by 41 ± 8% ($n = 3$), but this combination had only a small inhibitory effect on the response to hydralazine. The most potent combination appeared to be cyclopiazonic acid with diltiazem, and no additional block of the hydralazine response was observed upon adding ryanodine (Figure 5b). When ryanodine, cyclopiazonic acid and diltiazem were applied simultaneously, responses to phenylephrine in Ca^{2+}-free solution were blocked by 79 ± 5% ($n = 4$). When applied to tissues contracted with phenylephrine in the presence of normal Ca^{2+}, an initial 47 ± 5% ($n = 3$) loss of tension was observed followed by a gradual increase, such that after 60 min tension was 10 ± 3% higher than before the drugs were added.

Discussion

It has been suggested that different arterial preparations vary in responsiveness to hydralazine (Khayyal *et al.*, 1981; Kreye, 1984). In the rabbit renal artery, the IC_{50} for hydralazine inhibition of noradrenaline contractures was around 0.1 μM (Khayyal *et al.*, 1981). This contrasts with studies on the rabbit aorta, which suggest that hydralazine has little effect below mM concentrations (Kirpekar & Lewis, 1957; McLean *et al.*, 1978a). In our hands, however, the rabbit aorta proved to be consistently relaxed by μM concentrations of hydralazine when tested against tonic contractions to phenylephrine. The IC_{50} was 17 μM, with a maximal response observed above 100 μM. The difference between the hydralazine sensitivities reported here and in the earlier studies may be related to the slow onset of action of hydralazine, which took up to 1 h to produce its maximal effect. Responsiveness could have been underestimated if exposures to hydralazine were too brief. More recent studies on rabbit aorta observed relaxation responses to hydralazine at μM concentrations when it was applied for at least 1 h (Cook *et al.*, 1988; Higashio & Kuroda, 1988a,b). Furthermore, Higashio & Kuroda (1988a) found that noradrenaline-contracted preparations of rabbit renal artery and aorta, studied under the same conditions, respond with similar sensitivity to hydralazine. Rabbit aorta is therefore an appropriate model for studying the mechanisms of action of therapeutically relevant concentrations of hydralazine. The slow onset and recovery from hydralazine block is consistent with the clinical response, which is characterized by a gradual lowering of blood pressure that persists after blood levels of the drug have fallen.

Concentrations of hydralazine producing maximal inhibition of phenylephrine contractures had little effect on tonic contractions induced by elevating the extracellular K^+ concentration. This agrees with several studies employing a variety of arterial preparations (McLean *et al.*, 1978a, Khayyal *et al.*, 1981; Lipe & Moulds, 1981; Hermsmeyer *et al.*, 1983; Higashio & Kuroda, 1988a; Yen *et al.*, 1989). Reports claiming similar potency in blocking K^+-induced and agonist-induced contractures (Ebeigbe & Aloamaka, 1985; Orallo *et al.*, 1991) applied hydralazine for only a few minutes or used excessive concentrations, so the conclusions are uncertain. Overall, the evidence indicates that hydralazine primarily blocks a step in the pathway linking the activation of membrane receptors to contraction. Since K^+-induced tension mainly reflects Ca^{2+} influx through voltage-gated Ca chan-

nels, it is unlikely that Ca-channel blockade contributes significantly to hydralazine-induced vasodilatation. This conclusion is supported by the finding that Ca antagonists were without effect on the response to hydralazine. The Ca antagonist, D600, similarly failed to prevent hydralazine-induced relaxations in rat tail artery (Hermsmeyer *et al.*, 1983). Also, in agreement with a previous study on dihydralazine (Thirstrip & Nielsen-Kudsk, 1992), we conclude that hydralazine does not act as a K channel opener in rabbit aorta. Vasodilators that open K-channels, for example cromakalim, characteristically block contractions to low (<35 mM) but not high (>50 mM) concentrations of K^+ (reviewed in Gurney, 1994). Hydralazine was equally ineffective in inhibiting high K^+ contractures regardless of the K^+ concentration used.

Hydralazine blocked phenylephrine-induced contractions equally well in the presence or absence of extracellular Ca^{2+}. Thus it may interfere with Ca^{2+} handling by intracellular stores. The suppression of caffeine-induced contractions by hydralazine supports such a mechanism. Caffeine activates Ca^{2+} permeable channels in the SR of vascular muscle, with mediate Ca^{2+}-induced release of Ca^{2+} (Herrmann-Frank *et al.*, 1991). These are distinct from the channels that mediate SR Ca^{2+} release when the cell is stimulated by agonists such as phenylephrine. By acting on specific membrane receptors, agonists stimulate the formation of inositol 1,4,5-trisphosphate (IP_3), which binds to and opens specific IP_3-receptor channels in the SR membrane (Ehrlich & Watras, 1988). The caffeine-sensitive and IP_3-sensitive Ca^{2+} stores overlap completely in rabbit aorta (Leijten & van Breemen, 1984). Thus caffeine and IP_3 release the same pool of Ca^{2+}, but the Ca^{2+} leaves the SR through different channels. Since hydralazine reduced contractions to phenylephrine in Ca^{2+}-free solution more effectively than caffeine contractions, it may preferentially block a step in the IP_3-dependent pathway. An action on Ca^{2+} release is further supported by the finding that hydralazine exerted its full effect on phenylephrine contractions in Ca^{2+}-free solution, even when it was applied after the stores had been pre-filled with Ca^{2+}. Whether or not hydralazine has additional effects on Ca^{2+} accumulation into the stores could not easily be determined. The slow reversibility of hydralazine block meant that, even when tissue exposure to the drug was restricted to the store-filling period, its action would have persisted during the subsequent test with phenylephrine.

Agents known to deplete the SR of Ca^{2+} would be expected to block the action of a drug that interferes with the release of stored Ca^{2+}. That cyclopiazonic acid, thapsigargin and ryanodine can deplete the SR of Ca^{2+} is suggested by the fact that they cause pronounced inhibition of phenylephrine-induced contractions in Ca^{2+}-free solution. However, when applied by themselves, all of these agents failed to influence the hydralazine-induced relaxation. We were surprised to find though, that when cyclopiazonic acid was present together with either ryanodine or diltiazem, strong inhibition of the hydralazine response developed. The simplest explanation for this finding is that depletion of the SR is not readily achieved in the presence of extracellular Ca^{2+} and phenylephrine stimulation, but requires interference with more than one route of Ca^{2+} exchange with the SR. The results are consistent with an inhibitory action of hydralazine on IP_3-dependent Ca^{2+} release. This interpretation requires, however, that the maintained phenylephrine-induced contraction largely reflects the sustained, IP_3-dependent release of SR Ca^{2+}, which is balanced by uptake through two separate pathways, one requiring the SR Ca^{2+}-ATPase and the other blockable by Ca antagonists.

There is substantial evidence to support an SR Ca^{2+} cycling mechanism in the maintenance of tension in rabbit aorta. Although tonic tension clearly depends on Ca^{2+} influx (Lodge & van Breemen, 1985), Ca^{2+} entering the smooth muscle cell may first be accumulated into the peripheral SR (van Breemen, 1977). Receptor stimulation, by enhancing IP_3 production, induces a permeable SR, through which Ca^{2+} could cycle to be released in the vicinity of the myofilaments. This might well be important for maintaining tension in rabbit aorta, where IP_3 is produced continuously during exposure to α-adrenoceptor agonists for many minutes (Coburn *et al.*, 1988; Kajikuri & Kuriyama, 1990). During sustained tension, the cytoplasmic Ca^{2+} concentration would then depend on the relative rates of SR uptake and release, as well as on Ca^{2+} influx and efflux across the sarcolemma.

Separate pathways, sensitive either to cyclopiazonic acid or Ca antagonists, have been proposed for Ca^{2+} uptake into smooth muscle SR (Bourreau *et al.*, 1991; Low *et al.*, 1992). Direct evidence for a low affinity pathway, distinct from the Ca^{2+}-ATPase, has been presented for porcine aortic smooth muscle (Missiaen *et al.*, 1991). The high affinity Ca^{2+}-ATPase would provide the main route for Ca^{2+} uptake (Missiaen *et al.*, 1991). Thus, inhibiting its activity with cyclopiazonic acid prevented the stores from refilling after they had been discharged by phenylephrine in Ca^{2+}-free solution. Nevertheless, the low affinity uptake may persist after blockade of the Ca^{2+}-ATPase when intracellular Ca^{2+} levels are elevated, for example when the tissue is stimulated with phenylephrine in the presence of extracellular Ca^{2+}. Under these conditions, the low affinity pathway may be able to maintain Ca^{2+} cycling through the SR.

When cyclopiazonic acid and diltiazem were present together, both SR uptake pathways would have been blocked, leading to store depletion and a diminished contribution of IP_3-dependent Ca^{2+} release to tension. This can explain why the response to hydralazine was suppressed by cyclopiazonic acid and diltiazem, only when they were present together. Similarly, although ryanodine would have made the SR leaky, with no other blockers present Ca^{2+} reuptake could have maintained SR Ca^{2+} levels and IP_3-dependent Ca^{2+} release at near normal levels. The block of the hydralazine response observed when ryanodine was present with cyclopiazonic acid could reflect depletion of the SR, due to Ca^{2+} leaking out faster then it was accumulated. In contrast, the high affinity Ca^{2+}-ATPase may be able to maintain SR filling when diltiazem and ryanodine are present together, explaining why this combination only slightly reduced the hydralazine response.

The fact that diltiazem and cyclopiazonic acid were just as effective in inhibiting the hydralazine response as all three SR modulators together, suggests that blocking both routes for Ca^{2+} sequestration maximally depletes the stores even in the presence of extracellular Ca^{2+} and phenylephrine. The residual response to hydralazine observed in these conditions may reflect incomplete emptying of the SR stores. Consistent with this, phenylephrine contractions in Ca^{2+} free solution were never completely abolished, even with cyclopiazonic acid, ryanodine and diltiazem all present together. On the other hand, the contraction remaining under these conditions may not be due to released Ca^{2+}, since even after prolonged (>1 h) exposure to phenylephrine in Ca^{2+}-free/1 mM EGTA solution, some tension remained. Such apparently Ca^{2+}-independent actions of α-agonists have been seen before (Bolton, 1979) and may be mediated by protein kinase C (Khalil *et al.*, 1992). Thus the residual response to hydralazine after store depletion could represent a separate action, perhaps on the pathways involved in activation of the contractile proteins. While previous work argues against such an action (Kreye *et al.*, 1983; DeFeo & Morgan, 1989), it cannot be ruled out.

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