

Flosequinan, a vasodilator with a novel mechanism of action

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1 The mechanism of action of flosequinan was investigated in ferret aortic smooth muscle by the simultaneous measurement of aequorin luminescence and isometric force.

2 The control calcium-force curve was obtained by plotting the calibrated aequorin luminescence against the force from potassium-depolarized muscles. Flosequinan relaxed potassium-depolarized muscles by causing parallel changes in $[Ca^{2+}]_i$ and force with no shift in the control $[Ca^{2+}]_i$ -force relationship.

3 The $[Ca^{2+}]_i$ -force relationship in the presence of a maximally effective concentration of phenylephrine was significantly shifted to the left of that for the control, potassium-depolarized muscle. Flosequinan relaxed the phenylephrine-contracted muscle by causing a large decrease in force with only a minimal decrease in $[Ca^{2+}]_i$, resulting in an apparent rightward shift of the $[Ca^{2+}]_i$ -force relationship, toward the control curve.

4 In comparison, sodium nitroprusside caused relaxation of either the potassium- or phenylephrine-induced contraction solely by a decrease in $[Ca^{2+}]_i$, with no shift in either calcium-force relationship.

5 Milrinone caused no significant rightward shift of the calcium-force relationship during phenylephrine- or potassium-induced contractions, but when milrinone was added in the absence of vasoconstrictors, relaxation was obtained with no significant decrease in $[Ca^{2+}]_i$.

6 Flosequinan appears to differ in mechanism of action from both nitroprusside and milrinone. It relaxes depolarization-mediated contractions solely by decreasing $[Ca^{2+}]_i$ but also appears to be capable of reversing the apparent calcium sensitizing action of phenylephrine.

Keywords: Vasodilator; calcium; aequorin; flosequinan; vascular smooth muscle; milrinone; nitroprusside

Introduction

The mechanisms of action of vasodilators are varied. Vasodilators which are capable of increasing guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels, such as sodium nitroprusside, have been shown to decrease cytoplasmic ionized calcium levels ($[Ca^{2+}]_i$) (Morgan & Morgan, 1984b; Karaki *et al.*, 1988). Other vasodilators, typically those which are capable of increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels, such as forskolin, isoprenaline and papaverine, are also capable of significantly uncoupling $[Ca^{2+}]_i$ from force production (Morgan & Morgan, 1984b; Takuwa *et al.*, 1988; Nishimura & van Breemen, 1989). The purpose of the present study was to investigate the mechanism of action of flosequinan, a novel arteriovenous dilator agent which is currently under clinical trial for use in both hypertension and heart failure (Cowley *et al.*, 1987; Kessler & Packer, 1987; Sim *et al.*, 1988; Dupont *et al.*, 1989). Its mechanism of action is currently unknown and what little is known suggests that it may have a novel mechanism of action. It has been reported to increase cyclic GMP levels; however, the magnitude of the increase in cyclic GMP has been stated to be inadequate to explain the magnitude of the relaxation observed (Allcock *et al.*, 1988). Recent work also suggests that it may have non-specific phosphodiesterase activity which could result in elevations of cyclic AMP as well; however, this effect has an IC_{10} of 1.0×10^{-4} M (Frodsham *et al.*, 1989).

The purpose of the present study was to investigate the mechanism of action of flosequinan in an *in vitro* preparation by determining its effects on $[Ca^{2+}]_i$ levels and on $[Ca^{2+}]_i$ -force relationships.

Methods

Male ferrets (10 to 12 week old) were killed with an overdose of chloroform. All procedures were approved by the Institu-

tional Committee on Animal Research. The descending thoracic aorta was quickly removed to a physiological saline solution oxygenated with 95% O_2 , 5% CO_2 . The aorta was cut into strips measuring 5×1 mm with the long axis parallel to the circular fibres. The endothelium was removed by gentle rubbing of the intimal surface of all preparations. Experiments were performed at $37^\circ C$ in a physiological saline solution containing (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.2 and dextrose 11.5. Solutions containing elevated potassium concentrations were obtained by equimolar replacement of sodium chloride with potassium chloride.

All experiments were performed at 1.4 times the slack length, which we have previously found to be the optimal length for force production in the ferret aorta. In the organ bath experiments 1 h was allowed for equilibration after stretching and warming the muscle to $37^\circ C$. Force was recorded with a Gould UC2 transducer and stored on a magnetic FM tape deck.

Aequorin was loaded into ferret aorta strips by a method previously described (Morgan & Morgan, 1982). The method consisted of incubating the muscle in a series of ethylenedis-oxyethylenetriacetate (EGTA) and adenosine triphosphate (ATP) containing solutions at $2^\circ C$ for 30–90 min. The first solution (containing 10 mM EGTA) was designed to make the cell membrane hyperpermeable; the second solution contained 0.5 mg ml^{-1} aequorin; and the third solution contained 0.1 mM EGTA and 10 mM $MgCl_2$ and is thought to re-seal the membrane. The muscles were allowed to equilibrate overnight, until basal light was absolutely stable. The light emitted by the aequorin contained in the muscle was detected with an EMI 9635QA photomultiplier tube specially selected for low dark current and is reported in nanoamperes of anode current. Calibration of aequorin light signals was performed in the manner of Allen & Blinks (1979). The muscle cells were lysed with 0.5% Triton X-100 in 50 mM $CaCl_2$ and maximal luminescence (L_{max}) was determined. Light levels (L) during the experiments were expressed as fractional luminescence (L/L_{max}) which was then converted to

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absolute Ca^{2+} concentrations by the use of an *in vitro* calibration curve (Jiang & Morgan, 1987). A value of $[\text{Mg}^{2+}]_i = 0.5 \text{ mM}$ was assumed in these studies. This assumption was based on NMR measurements of $[\text{Mg}^{2+}]_i$ in smooth muscle cells (Kushmerick *et al.*, 1986).

Aequorin was obtained from the laboratory of Dr J.R. Blinks, Mayo Clinic (Rochester, MN, U.S.A.). The following drugs were used: flosequinan (Boots Pharmaceuticals, U.K.), sodium nitroprusside (Sigma, U.S.A.), milrinone (Sterling Winthrop, U.S.A.), (–)-phenylephrine HCl (Sigma, U.S.A.) and Triton X-100 (Fisher, U.S.A.).

Results are given as means $\pm$ s.e.mean. Statistical significance was accepted where the *P* value was less than 0.05 by Student's *t* test. Unless indicated otherwise, the comparisons were unpaired. All quantitative data points were taken from steady state measurements.

Results

Concentration-response relationships of flosequinan in ferret aorta

The effect of a range of concentrations of flosequinan, added cumulatively, in relaxing either 24 mM potassium (K)-induced contractions or phenylephrine (10^{-5} M)-induced contractions is shown in Figure 1. In both cases, unreasonably high concentrations of flosequinan would have been necessary to demonstrate a plateau in the concentration-response curve. It was noticed, however, that flosequinan, at $3 \times 10^{-3} \text{ M}$, was capable of decreasing the phenylephrine-induced force from 22 mN to 6.4 mN, whereas the same concentration of flosequinan could only reduce the K-induced force from 18 mN to 11 mN in this tissue. In both cases, the vasodilator actions began between 10^{-4} and 10^{-6} M . All of the calcium-force studies were performed with 10^{-3} M flosequinan so as to produce a response large enough to be reliably measured. Therapeutic blood concentrations have been estimated to be

10^{-5} M (Packer *et al.*, 1988), but comparisons between the *in vitro* bathing medium concentration and the *in vivo* blood levels may be misleading and may not reflect the concentration at the receptor level.

Effect of flosequinan on $[\text{Ca}^{2+}]_i$

Resting $[\text{Ca}^{2+}]_i$ in the ferret aorta at 37°C was $233 \pm 10 \text{ nM}$ (mean $\pm$ s.e.mean, $n = 28$ strips, 17 animals). Resting intrinsic tone measured as the active steady state tone generated on warming the muscle from 2°C to 37°C was $16.2 \pm 0.9 \text{ mN}$ ($n = 12$ strips, 9 animals). The addition of a physiological saline solution in which 24 mM KCl replaced an equal amount of NaCl (24 mM K), resulted in an increase in $[\text{Ca}^{2+}]_i$ to a steady state level of $318 \pm 22 \text{ nM}$ ($n = 14$ strips, 14 animals) and an increase in force of $14.1 \pm 1.0 \text{ mN}$ ($n = 17$ strips, 14 animals). The addition of 10^{-3} M flosequinan to a 24 mM K-contracted preparation resulted in a decrease in $[\text{Ca}^{2+}]_i$ and force (Figure 2). (In a group of 12 experiments (9 animals), where zero force was defined as the level of resting force at body temperature, a mean value of $207 \pm 16 \text{ nM}$ ($n = 9$ strips, 9 animals) and $-2.5 \pm 1.2 \text{ mN}$ ($n = 12$ strips, 9 animals) were obtained for $[\text{Ca}^{2+}]_i$ and force, respectively in the presence of 10^{-3} M flosequinan and 24 mM K.)

As previously reported (Morgan & Morgan, 1984a), the addition of the α -adrenoceptor agonist, phenylephrine, to the ferret aorta results in an initial large spike-like increase in $[\text{Ca}^{2+}]_i$ during the development of the contraction, but during tone maintenance, $[\text{Ca}^{2+}]_i$ returns to near-basal levels (Figure 3). The addition of flosequinan to a phenylephrine-contracted preparation resulted in a decrease in contractile force [from $22.4 \pm 1.2 \text{ mN}$ ($n = 15$ strips, 11 animals) to $6.3 \pm 2.1 \text{ mN}$ ($n = 5$ strips, 5 animals) where zero force is defined as the level of resting tone at 37°C] and a small, but significant decrease ($P < 0.05$ by a paired *t* test for $n = 5$ strips, 5 animals), in $[\text{Ca}^{2+}]_i$ levels from $291 \pm 18 \text{ nM}$ ($n = 15$ strips, 11 animals) to $240 \pm 27 \text{ nM}$ ($n = 5$ strips, 5 animals). It can be seen from Figure 3, that typically, during the action of flosequinan in the presence of phenylephrine, both $[\text{Ca}^{2+}]_i$ and force return to approximately baseline levels.

Flosequinan was also found to decrease intrinsic tone. As mentioned above, the ferret aorta generated an average of 16 mN of force on warming from 2°C to 37°C . This tone is stable and persists in the presence of blockade of neurotransmitters with phentolamine and tetrodotoxin. However, it is sensitive to blockade with the relatively selective protein kinase-C inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydro-chloride (H-7) (Ruzicky & Morgan, 1989). The addition of 10^{-3} M flosequinan to the ferret aorta at body temperature in the absence of vasoconstrictors results in a significant drop in tone of $4.4 \pm 0.1 \text{ mN}$ ($n = 5$ animals, 5 strips)

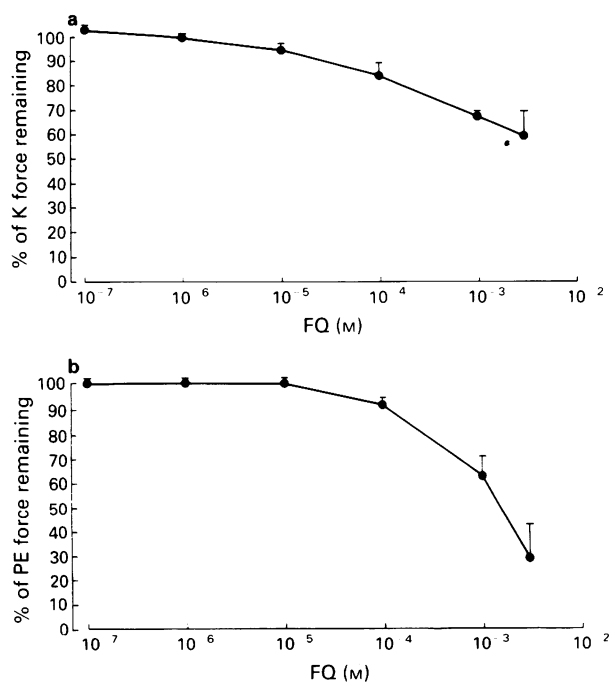


Figure 1 (a) Effect of flosequinan (FQ) on potassium (24 mM K)-induced contractile force in aortic strips. (b) Effect of flosequinan on phenylephrine (PE) (10^{-5} M)-induced contractile force in aortic strips. In both panels, each point shows the mean of 7 strips from 6 ferrets; s.e. shown by vertical bars. The control potassium contraction had a mean force of $18 \pm 1.9 \text{ mN}$ and the control phenylephrine contraction had a mean force of $22 \pm 1.9 \text{ mN}$.

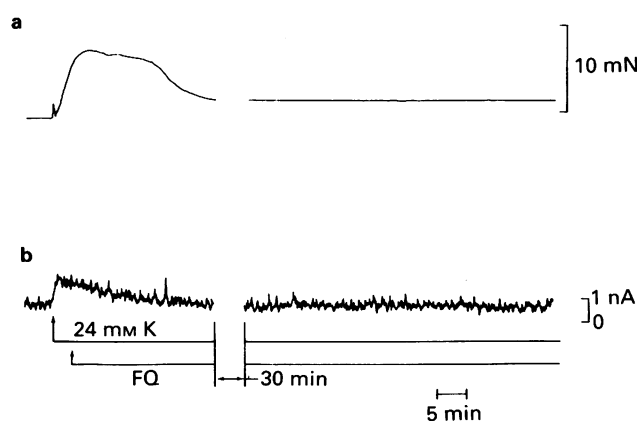


Figure 2 Trace (a): force; (b): luminescence. Effect of flosequinan (FQ) (10^{-3} M) in relaxing a potassium-induced contraction. Zero light is determined by dark current from the photomultiplier tube with the shutter closed.

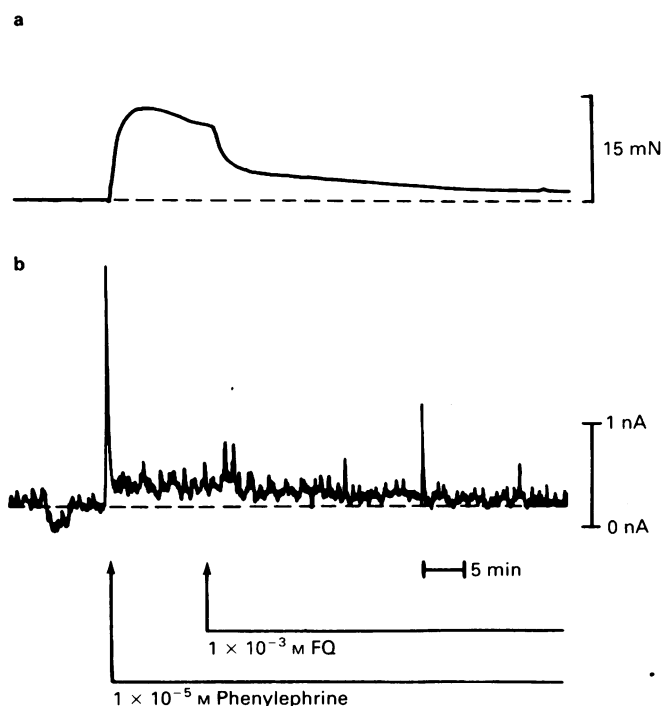


Figure 3 Trace (a): force; (b): luminescence. Effect of flosequinan (FQ) in relaxing a phenylephrine-induced contraction. Zero light is determined with the shutter closed. The closing of the shutter is indicated by the asterisk. The dashed lines mark the resting levels of force and luminescence.

and $[Ca^{2+}]_i$ to 220 ± 26 nM ($n = 5$ animals, 5 strips). Both the decrease in $[Ca^{2+}]_i$ and force were statistically significant ($P < 0.05$ by a paired Student's t test). A typical data trace is shown in Figure 4.

Effect of flosequinan on $[Ca^{2+}]_i$ -force relationships

In Figure 5, steady state $[Ca^{2+}]_i$ levels have been calibrated and plotted against the corresponding steady state force level. All forces have been normalized as a percentage of the force in response to an initial challenge to 22 mM K performed at the beginning of each experiment. The right hand curve (K curve) was obtained either by increasing levels (15 mM or 24 mM) of potassium depolarization or decreasing the extracellular $[Ca^{2+}]$ surrounding the potassium-depolarized (24 mM K) muscle. The extracellular $[Ca^{2+}]$ was decreased by using a nominally Ca^{2+} -free solution containing 2 mM EGTA. It has previously been reported that the removal of extracellular Ca^{2+} from the depolarized ferret aorta results in a decrease in $[Ca^{2+}]_i$ and force below baseline levels (Ruzicky & Morgan, 1989). The K curve probably reflects the true sensitivity of the contractile apparatus to calcium since no pharmacological manipulations were performed.

The phenylephrine curve was obtained in the presence of 10^{-5} M phenylephrine and varying (2.5, 0.5 or nominally Ca-free plus 2 mM EGTA) extracellular $[Ca^{2+}]$. This concentration of phenylephrine has previously been found to be maximally effective with respect to force production in ferret aorta (Ruzicky & Morgan, 1989). As previously reported (Morgan & Morgan, 1984a), and confirmed quantitatively here, phenylephrine appears to increase the Ca^{2+} sensitivity of the contractile apparatus.

The effect of flosequinan on a potassium-depolarized muscle as well as the resting muscle was to cause parallel changes in $[Ca^{2+}]_i$ and force such that flosequinan did not appear to cause a shift in the potassium-generated $[Ca^{2+}]_i$ -force relationship. The effect of flosequinan on the phenylephrine-contracted muscle is also shown in Figure 5. Flosequinan

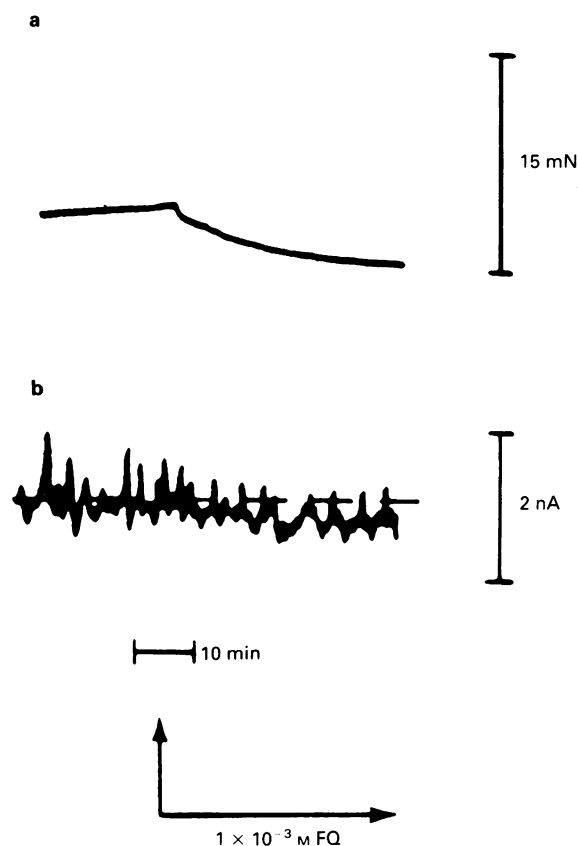


Figure 4 Trace (a): force; (b): luminescence. Effect of flosequinan (FQ) in relaxing intrinsic tone (the tone developed on warming from 2°C to 37°C). Zero light is the light level with the shutter closed. Dashed line marks the position of resting luminescence.

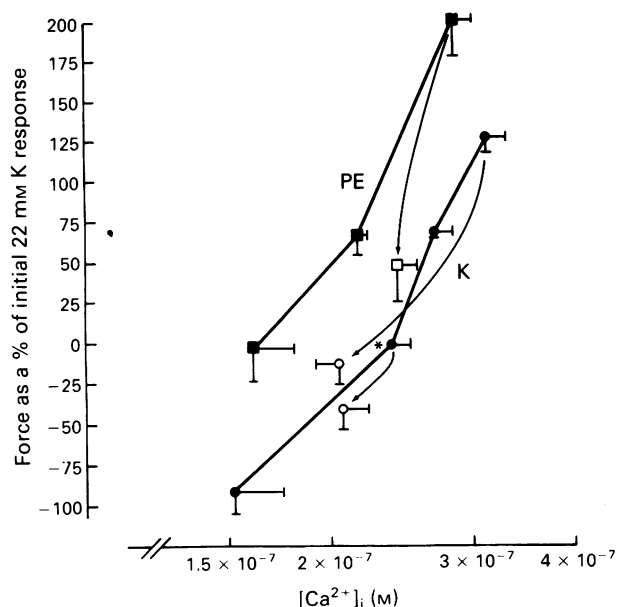


Figure 5 Relationship between $[Ca^{2+}]_i$ and force. Asterisk shows the position of the resting muscle. Presence of flosequinan (FQ) (10^{-3} M) shown by (○) and (□). Arrows connect the control to the appropriate flosequinan-induced relaxation. Potassium curve (K) (●) generated by increasing the [K] in the bathing medium or decreasing $[Ca^{2+}]$ in the bathing medium surrounding the depolarized muscle. Phenylephrine curve (PE) (■) obtained by removing extracellular Ca from the bathing medium in the presence of 10^{-5} M PE. 100% force is the level produced by an initial challenge to 22 mM K and to which data for each individual strip were normalized. The response to 22 mM K was 12.0 ± 1.2 mN ($n = 21$ strips) for the experiments for the K curve, 12.7 ± 1.1 mN ($n = 15$ strips) for the experiments for the PE curve and 11.6 ± 0.9 ($n = 15$ strips) for the experiments for the FQ points. Data points represent 5–16 experiments from 5–14 animals.

caused a small, but statistically significant (see above) decrease in $[Ca^{2+}]_i$, but it caused a much larger decrease in contractile force and it appears that flosequinan shifts the phenylephrine $[Ca^{2+}]_i$ -force relationship to the right.

Comparison with other vasodilators

The effect of sodium nitroprusside (10^{-5} M) on $[Ca^{2+}]_i$ -force relationships during either phenylephrine (10^{-5} M)- or potassium (24 mM K)-induced contractions is shown in Figure 6. In both cases, parallel decreases in $[Ca^{2+}]_i$ and force were observed as the muscle relaxed. In both cases, the magnitude of the decrease in $[Ca^{2+}]_i$ appeared to be sufficient to explain the magnitude of the decrease in force and there was no evidence for a shift in the $[Ca^{2+}]_i$ -force relationship. Thus, sodium nitroprusside differs from flosequinan in that even at 10^{-5} M it was not capable of reversing the calcium sensitizing actions of phenylephrine.

The effects of milrinone were also determined for comparison. Milrinone has been reported to increase cyclic AMP levels by inhibiting phosphodiesterase activity (Opie, 1986). As is shown in Figure 7, milrinone had a very different effect from that of flosequinan on basal tone in that it decreased intrinsic tone in the absence of a significant ($P > 0.05$) drop in $[Ca^{2+}]_i$. The effect of a range of concentrations of milrinone (10^{-3} - 10^{-6} M) displayed an unusual concentration-response relationship. With increasing concentrations, basal tone decreased progressively, but basal $[Ca^{2+}]_i$ increased. We have previously reported similar actions of other agents capable of increasing cyclic AMP levels, such as forskolin, papaverine and isoprenaline (Morgan & Morgan, 1984b); but this effect was not seen even at 10^{-3} M flosequinan. This action of uncoupling $[Ca^{2+}]_i$ from force was apparently lost when milrinone was added to the depolarized muscle. Although milrinone caused relaxation of the potassium-induced contraction, it apparently did this in the absence of a shift in the $[Ca^{2+}]_i$ -force relationship (Figure 7). In this regard, it differs from the previously described actions of other phosphodiesterase inhibitors and stimulants of adenylate cyclase (Morgan & Morgan, 1984b; DeFeo & Morgan, 1989); thus, it

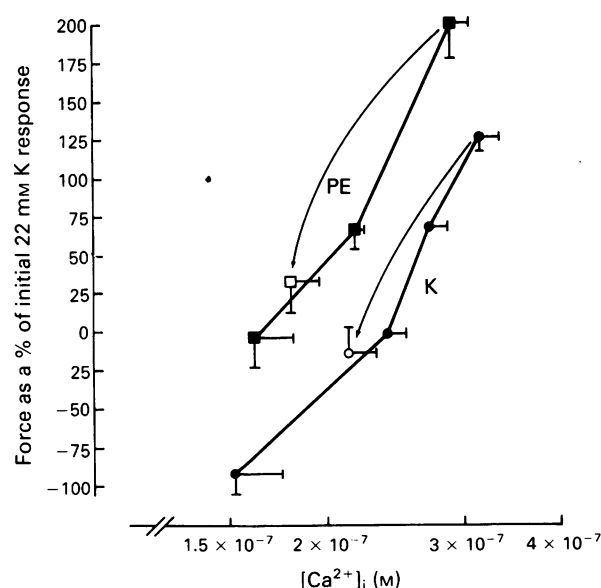


Figure 6 Relationship between intracellular ionized calcium levels and contractile force during relaxation of the muscle by sodium nitroprusside. Control potassium (●) and phenylephrine-induced (■) calcium-force relationships shown; (○) and (□) demonstrate the effect of 10^{-5} M sodium nitroprusside. The appropriate control contraction is connected to the nitroprusside-induced relaxation by the arrows. 100% force was 10.1 ± 1.8 mN ($n = 11$ strips) for the nitroprusside experiments. Data points represent 5-16 experiments from 5-14 animals.

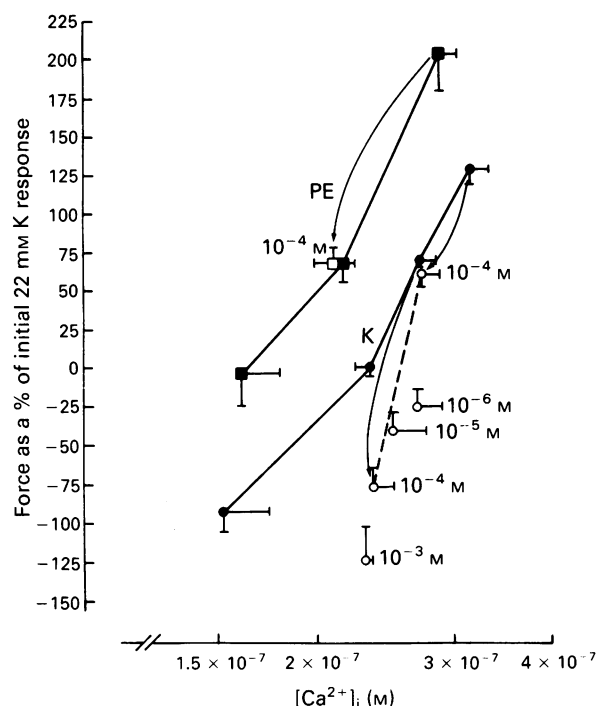


Figure 7 Effect of milrinone on the calcium-force relationship: (●) K curve; (■) phenylephrine curve; effect of milrinone: (□) and (○). All milrinone points are labelled with the concentration of milrinone used. The appropriate control contraction is connected to the milrinone-induced relaxation by the arrows. 100% force was 9.8 ± 0.1 mN ($n = 19$ strips). Data points represent 5-28 experiments from 5-17 animals.

appears that milrinone has a complex mechanism of action. Milrinone also relaxed the phenylephrine-induced contraction in the absence of a shift in the phenylephrine-generated $[Ca^{2+}]_i$ -force curve. In this regard, it also differed in mechanism of action from flosequinan.

Discussion

The main conclusion of this study is that flosequinan differs qualitatively in its mechanism of action both from agents that increase cyclic GMP and those which increase cyclic AMP. Although sodium nitroprusside has been previously reported to decrease intracellular ionized calcium levels in vascular smooth muscle cells (Morgan & Morgan, 1984b; Karaki *et al.*, 1988), the possibility of an additional effect involving change in sensitivity of the contractile apparatus to Ca^{2+} has not previously been investigated. In the present study we have determined quantitatively the effect of maximally effective concentrations of sodium nitroprusside on $[Ca^{2+}]_i$ -force relationships. We found no effect of this agent on the $[Ca^{2+}]_i$ -force relationship either when the muscle is potassium-depolarized or when the Ca^{2+} sensitivity is increased by the presence of phenylephrine. Thus, sodium nitroprusside causes relaxation in this muscle solely by decreasing $[Ca^{2+}]_i$ levels.

Milrinone has been referred to as an 'inodilator' because of its actions as both a positive inotropic agent on cardiac muscle as well as its vasodilator actions (Opie, 1986). Both of these actions are due at least in part, to the ability of this agent to inhibit phosphodiesterase. In a ferret model, studies of Falotico *et al.* (1989) have suggested that flosequinan might have similar properties. When applied to the resting muscle, milrinone is capable of causing a decrease in intrinsic tone in the absence of any decrease in $[Ca^{2+}]_i$. At the lower concentrations, an increase in $[Ca^{2+}]_i$ is actually observed. We have previously reported similar results in ferret aorta with another agent capable of increasing cyclic AMP levels, forskolin

(DeFeo & Morgan, 1989). We have also seen similar results in ferret portal vein with papaverine (Morgan & Morgan, 1984b) and adenosine (Bradley & Morgan, 1985), two additional agents capable of increasing cyclic AMP levels. The mechanism of this apparent uncoupling of Ca^{2+} from force production is unknown. Possible mechanisms include the phosphorylation of myosin light chain kinase by cyclic-A dependent kinase (Adelstein *et al.*, 1978; Silver & DiSalvo, 1979) or the phosphorylation of the myosin light chains by cyclic A-kinase (Walsh *et al.*, 1981). Flosequinan has been reported to be capable of inhibiting a non-specific phosphodiesterase in cardiac muscle with an IC_{50} of 1.0×10^{-4} M (Frodsham *et al.*, 1989) which could result in an increased cyclic AMP level in vascular smooth muscle as well. However, in the intact cell, even at the concentration of 10^{-3} M used in this study, we saw no evidence of a rightward shift in the potassium-generated $[\text{Ca}^{2+}]_i$ -force curve including the values from the resting muscles.

Milrinone appears to be capable of vasodilator actions separate from an increase in cyclic AMP levels since it did not cause a shift in the $[\text{Ca}^{2+}]_i$ -force relationship in the potassium-depolarized muscle. This is different from the previously reported actions of forskolin, adenosine, isoprenaline and papaverine in ferret aorta and portal vein (Morgan & Morgan, 1984b; Bradley & Morgan, 1985; DeFeo & Morgan, 1989). Milrinone also did not shift the phenylephrine $[\text{Ca}^{2+}]_i$ -force relationship, but the action of agents capable of

increasing cyclic AMP levels on this relationship has not been determined.

Flosequinan appears to fit the pattern of neither a vasodilator which increases cyclic GMP levels nor a vasodilator which increases cyclic AMP levels. Thus, the question arises as to the actual mechanism of vasodilatation produced by this compound. In the case of relaxation of potassium-contracted muscles, the relaxation can be explained solely on the basis of the decrease in $[\text{Ca}^{2+}]_i$, since no shift in the $[\text{Ca}^{2+}]_i$ -force relationship was observed under these conditions. Surprisingly, flosequinan did appear to be capable of shifting the phenylephrine-induced $[\text{Ca}^{2+}]_i$ -force relationship to the right. Thus, flosequinan appeared to be reversing the action of phenylephrine in sensitizing the contractile apparatus to Ca^{2+} . We and others have previously discussed the possibility that phenylephrine responses might involve the activation of protein kinase-C (Ruzycky & Morgan, 1989; Jiang & Morgan, 1987; Exton, 1985; Danthuluri & Deth, 1986). Although not all data are consistent with this interpretation, if tone maintenance during phenylephrine-induced contractions depends on C-kinase activation, this raises the interesting possibility that flosequinan might exert at least part of its vasodilator actions through an antagonism of protein kinase-C.

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