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Analysis of relaxation and repolarization mechanisms of nicorandil in rat mesenteric artery

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1 The mechanism by which nicorandil causes relaxation of rat isolated small mesenteric arteries mounted on a Mulvany myograph was investigated by use of a combination of putatively mechanism-specific antagonists.

2 In arteries precontracted by the thromboxane-mimetic, U46619, the EC_{50} for cromakalim and levcromakalim-induced relaxation curves were raised by 36 and 17 fold by glibenclamide (3 μM) while the EC_{50} for nicorandil-induced relaxation was unaffected by either glibenclamide or methylene blue (10 μM). A combination of these antagonists raised the EC_{50} for nicorandil by 8 fold.

3 In U46619-contracted arteries, nifedipine (100 nM) did not affect the cromakalim relaxation curve but it raised the EC_{50} for nicorandil by 5 fold. The combination of methylene blue, glibenclamide and nifedipine further inhibited the maximum relaxation to nicorandil.

4 In separate experiments, membrane potential (Em) and force responses were measured simultaneously. In arteries depolarized and contracted by U46619 both nicorandil and cromakalim repolarized (ΔE_m, 35 mV) and relaxed (100%) the vessels. Glibenclamide (3 μM) did not alter the relaxation-concentration curve to nicorandil but reduced the maximum repolarization to Δ10.8 mV. In contrast the Em and relaxation-response curves to cromakalim were shifted to the right by glibenclamide by 30–100 fold.

5 In unstimulated arteries, nicorandil (but not cromakalim) -induced hyperpolarization was significantly antagonized by methylene blue (10 μM) (6.6 fold rightward shift) or nifedipine (100 nM) (2.6 fold). In depolarized arteries (U46619), nifedipine but not methylene blue inhibited the nicorandil-induced hyperpolarization.

6 In arteries precontracted to 50% tissue maximum by either KCl or U46619, nifedipine (100 nM) relaxed the artery but failed to repolarize the Em. Presumably voltage-operated calcium channels (VOCC) were blocked preventing contraction but the artery remained depolarized, presumably through non VOCC mechanisms.

7 These data suggest that nicorandil may relax small arteries through 3 parallel pathways, (i) NO-donor mediated stimulation of guanylate cyclase and increase in cyclic GMP, (ii) K^+_{ATP} channel opening, and (iii) nifedipine-sensitive VOCC inhibition. Em data suggest that nicorandil-induced repolarization is caused principally through opening K^+_{ATP} channels. Blockade of this hyperpolarization by glibenclamide is not sufficient to alter the relaxation, indicating dissociation of nicorandil-induced changes in membrane potential and relaxation.

8 These results highlight the 'chameleon' actions of nicorandil where there is no apparent association of Em repolarization with relaxation, in contrast to the parallel responses for cromakalim.

Keywords: Nicorandil; cromakalim; levcromakalim; nifedipine; mesenteric artery; K^+_{ATP} channel, nitric oxide donor; membrane potential

Introduction

The anti-anginal agent, nicorandil, is considered to relax vascular smooth muscle primarily *via* two mechanisms. It elevates guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels by stimulating soluble guanylate cyclase (GC) in a similar function to nitroglycerin esters (Endoh & Taira, 1983; Holzmann *et al.*, 1983; Taira, 1987; 1989) and it may also increase membrane K^+ conductance (gK^+) leading to cellular hyperpolarization and the sequential closure of voltage-operated Ca^{2+}-channels. These events would result in vascular smooth muscle relaxation (Furukawa *et al.*, 1981; Taira, 1987; 1989).

Interestingly, vasorelaxation to nicorandil is highly tissue-dependent; a characteristic different from nitroglycerin esters (Greenberg *et al.*, 1991). For example, dog isolated mesenteric arteries are relaxed mainly *via* the guanylate cyclase mechanism (Endoh & Taira, 1983; Greenberg *et al.*, 1991) whereas, those from the rabbit are *via* dual mechanisms (Meisheri *et al.*, 1991; Magnon *et al.*, 1994). In rat, nicorandil dilates the basilar artery (Ksoll *et al.*, 1991) and increases coronary blood flow in rat perfused heart (Borg *et al.*, 1991) mainly *via* activation of K^+ channels, whereas in rat aorta the vasorelaxant effects of nicorandil are predominantly due to guanylate cyclase activation (Cavero *et al.*, 1988; Borg *et al.*, 1991).

While there is some information on the actions of nicorandil in rat large isolated arteries, there is no detail on the mesenteric small resistance arteries. Here we have explored the relaxation mechanisms of nicorandil, and the K^+ channel openers, cromakalim and its isomer, levcromakalim. We have used the absence or presence of selective inhibitors, namely methylene blue and glibenclamide to unravel the primary relaxant mechanisms of nicorandil in this setting. We also examined the effect of nifedipine alone and in combination with these antagonists to determine the role of voltage-operated calcium channels on the vasorelaxant response to nicorandil. In separate experiments, we measured membrane potential to aid the interpretation of drug action. Our findings suggest that nifedipine may inhibit the intracellular mechanism of nicorandil and may point to a possible adverse clinical response if given with L type voltage-operated calcium channel antagonists.

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Methods

Tissue preparation

Male Sprague Dawley rats aged between 6–7 weeks, weighing 200–300 g, were anaesthetized with carbon dioxide (80% CO_2, 20% O_2) and killed by exanguination. A loop of intestine with attached mesentery was removed and placed in cool Krebs solution (composition in mM: Na^+ 144, K^{2+} 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HPO4$^-$ 1.2, Cl^- 129, SO_4^- 1.2, HCO_3^- 25, glucose 11, EDTA 0.026) saturated with 5% CO_2 in O_2. Under a dissecting microscope, a 2nd order branch of mesenteric small artery was carefully dissected free of the fat and connective tissue, and 2 mm long segments were mounted on 40 μm diameter wires in a 6 ml bath in a Mulvany-Halpern style myograph (JP Trading, Aarhus, Denmark). After a 30 min equilibration period, the preparations were stretched radially using a normalization protocol as described previously (Mulvany & Halpern, 1977; Angus *et al.*, 1988). The vessel was set at a passive tension equivalent to that generated at 0.9 times the diameter when distended by a transmural pressure of 100 mmHg (D_{100}) which developed maximum force when activated by K^+ depolarization in this artery (Mulvany & Warshaw, 1979).

After normalization, the preparation was allowed to equilibrate for at least 30 min. The vessels were then activated with a potassium depolarizing solution, KPSS (Krebs with K^+ 120 mM substituted for sodium) and this response was used as an estimate of maximum response (E_{max}) in the tissue. This response was used as an internal standard contraction for subsequent responses.

Electrophysiology

In experiments where the intracellular membrane potential was monitored, the vessel was mounted as described above. A conventional glass electrode (1 mm diameter blanks, World Precision Instruments Inc., New Haven, U.S.A.) filled with 0.5 M KCl (tip resistance approximately 100 MΩ) was used to impale a single smooth muscle cell. The microelectrode was positioned by a Burleigh Inchworm motor deiven by a 6000 series controller (Burleigh, U.S.A.). The electrode was advanced in 0.5 μm steps until a stable impalement was achieved.

The myograph containing the vessel (15 ml volume) was part of 30 ml recirculating system which contained another organ bath, where the Krebs solution was warmed and oxygenated. Drugs could also be added to this bath. This design allowed cumulative steady state concentration-effect curves to be constructed. (Garland & McPherson, 1992).

We accepted membrane potential recordings which showed: (1) an abrupt change in potential upon impalement of cells; (2) a stable recording for at least 10 min prior to experimental procedures; (3) reversibility of the effects of vasoactive agents; (5) similar electrode resistance before and after impalement even if the electrode came out of the cell and was reinserted during the experiment.

Protocol

Concentration-relaxation curve Arteries were precontracted with either KCl (80 mM) or the thromboxane-mimetic, U46619 (100 nM) to give contractile responses approximately 50% of the E_{max} to K^+ 120 mM. After reaching a steady contraction, cumulative concentration-relaxation curves were generated to nicorandil, cromakalim, levcromakalim or glyceryl trinitrate (GTN). Arteries were equilibrated with vehicle (ethanol), glibenclamide (G, 3 μM), methylene blue (MB, 10 μM), N^{ω}-nitro-L-arginine (L-NOARG, 100 μM) or nifedipine (100 nM) alone, or in various combinations i.e. G+MB, G+MB+nifedipine, G+L-NOARG, G+nifedipine) for at least 30 min before reapplying the next relaxant concentration-response curve. Only one relaxant drug was tested on each vessel. Each vessel was used to obtain 3 concentration-response curves, (1) control, (2) with one antagonist and (3) with a combination of antagonists. We also examined 3 consecutive curves of the one relaxant drug as a vehicle time control for comparisons with other data.

Membrane potential (Em) After confirming stable impalement, arteries were equilibrated with vehicle (ethanol) or antagonists as for the protocol outlined above. In these experiments, changes in membrane potential and active force were recorded simultaneously in the presence of cumulative concentration of nicorandil and cromakalim, before and after treatment with antagonists. In some experiments, the vessels were precontracted and depolarized with U46619.

Data analysis

Vasorelaxant responses to all agents are represented as a percentage of the precontracted force to U46619 (100 nM taken as 100%). Responses were calculated as mean ± s.e.mean with n being the number of the vessels in the group. A maximum of 2 arteries were taken from a single rat. The relaxation sensitivity for each vasodilator agent was calculated as mean pEC_{50} ± s.e.mean ($pEC_{50} = -\log EC_{50}$) determined from the average of individual computer logistic-fitted concentration-normalized relaxation curves (Lew, 1995). A conservative Bonferroni multiple comparison test was used to compare multiple pEC_{50} values. Values of $P<0.05$ were considered to be significant. Shifts in location of sensitivity (pEC_{50}) for the vasodilator drugs in the presence of one or more antagonists were compared with the control curves (vehicle, antagonist absent) and were calculated as concentration-ratios [antilog (pEC_{50} control − pEC_{50} test)]. To ensure that the level of precontraction force was similar in the absence or presence of antagonists we arbitrarily selected a variation of ±10% of the precontraction in the presence of antagonists compared with the control (vehicle) precontraction. Relaxation data generated from precontractions outside the 10% were not used in the final analyses.

Drugs

Drugs used and their source were: nicorandil (Chugai Chemical, Inc., Tokyo, Japan), cromakalim (Sapphire Bioscience, Australia) levcromakalim (SmithKline, Beecham, Australia), methylene blue (Sigma, U.S.A.), glibenclamide (Hoechst, Australia) nitroglycerin (David Bull, Australia), N^{ω}-nitro-L-arginine (L-NOARG Sigma, U.S.A.), nifedipine (Bayer, Germany), KCl (BDH chemicals, Australia). U46619 (1,5,5)-hydroxy-11α, 9α-(epoxymethano) prosta-5Z, 13E-dienoic acid, (Upjohn, U.S.A.). Glibenclamide, nifedipine, cromakalim and levcromakalim were initially dissolved in absolute ethanol to obtain a concentrated solution (10 mM), with subsequent dilutions made in distilled water. L-NOARG was dissolved in 0.1 M $NaHCO_3$ with sonication and then diluted in distilled water. All other compounds were dissolved in distilled water. Control experiments were performed with the vehicles of glibenclamide, nifedipine, cromakalim and levcromakalim.

Results

Relaxation curves: K^+ versus U46619 precontraction

The mesenteric arteries had normalized (stretched to D_{100}) diamaters of 326 ± 12 μm. When precontracted with the depolarizing solution, 80 mM KCl, they reached a steady force of 54.7 ± 2.5% ($n=26$) of the E_{max} contraction to KPSS. Arteries precontracted with U46619 100 nM ($n=24$) reached a similar submaximal force of 52.4 ± 1.9% of KPSS. While the level of force was similar for the two precontraction agents, the relaxation-response curves for nicorandil, cromakalim and levcromakalim were dependent on the nature of the contraction agent. Levcromakalim, cromakalim and nicorandil fully re-

laxed the arteries when they were contracted with U46619. While the pEC_{50} values for levcromakalim and cromakalim were similar; 6.45 ± 0.12 and 6.34 ± 0.08, nicorandil was approximately 100 fold less potent than the other 2 agonists (pEC_{50}; 4.66 ± 0.05) (Figure 1, Table 1). In contrast, arteries precontracted with 80 mM K^+ were poorly relaxed by all 3 agonists and at the higher concentrations used (100 μM or 300 μM), the arteries were still contracted to approximately half of their precontraction force. The potencies of cromakalim and levcromakalim were significantly lower in K^+ compared with U46619 contracted vessels.

Mechanism of relaxation

U46619 precontraction The average concentration-relaxation curve for nicorandil was not significantly affected in range or sensitivity (pEC_{50}) by pretreatment with 3 μM glibenclamide, 100 μM L-NOARG or by 10 μM methylene blue (Figure 1, Table 1). In contrast, 10 μM methylene blue shifted GTN-induced relaxation curves to the right (40 fold). However, the nicorandil relaxation curve was significantly shifted to the right 8 fold by the combination of glibenclamide (3 μM) and methylene blue (10 μM) (Figure 1) but not by the combination of glibenclamide and L-NOARG (concentration-ratio <1). The pretreatment test drugs did not significantly affect the precontraction level of the arteries to U46619 (data not shown).

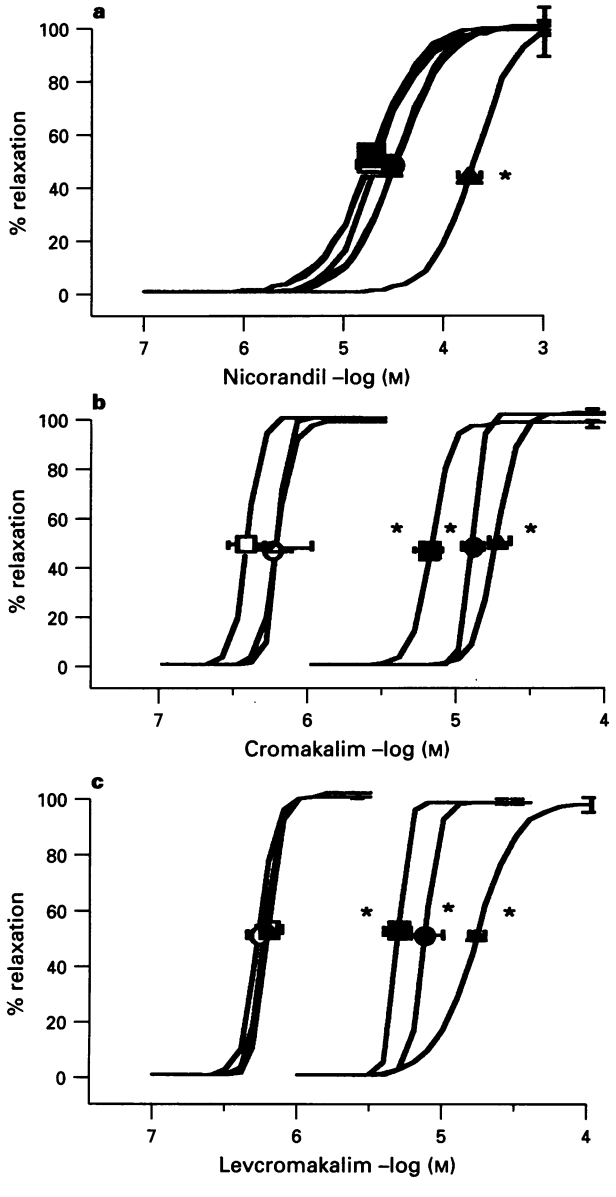

Figure 1 Logistic fitted concentration-relaxation curves for nicorandil (a), cromakalim (b) and levcromakalim (c) in rat small mesenteric arteries contracted by U46619 100 nM. Symbols represent, control (○); pretreatment with glibenclamide (●), 3 μM; methylene blue (Δ), 10 μM; L-NOARG (□), 100 μM; L-NOARG and glibenclamide (N+G, ■); glibenclamide and methylene blue (▲), respectively. Horizontal-bar represents s.e.mean of mean pEC_{50}, and vertical bar represents s.e.mean of the E_{max} ($n=6-15$). Relaxation is calculated as % decrease in precontracted force. *pEC_{50} significantly different from control: $P<0.05$.

Both the K^+_{ATP} channel opening drugs, cromakalim and levcromakalim had similar potency (pEC_{50}) in relaxing the mesenteric arteries contracted by U46619 (6.45 ± 0.12 and 6.34 ± 0.12). Glibenclamide (3 μM) alone caused significant antagonism of both relaxant drugs displacing the curves in parallel by a concentration ratio of 36 for cromakalim and 17 for levcromakalim (Figure 1). For both agonists, the combination of glibenclamide and methylene blue caused greater shifts (54 fold and 32 fold) while methylene blue alone was without significant effect on the pEC_{50} for either agonist (Figure 1). The NO synthase inhibitor, L-NOARG, had no significant effect when used alone on both relaxant agonists and did not enhance the shift when combined with glibenclamide as compared with glibenclamide alone (Figure 1).

K^+ precontraction In the arteries precontracted by K^+, the concentration-response curves to nicorandil, cromakalim and levcromakalim were poor with shallow slope and only partial relaxation (data not shown). These curves therefore provided little opportunity for quantitative analyses of mechanism. The control relaxation curves for nicorandil could be fitted to a logistic equation and the pEC_{50} was not significantly different in K^+ or U46619-precontracted arteries. As with the U46619-contracted arteries, the combination of methylene blue and glibenclamide significantly raised the pEC_{50}, of the nicorandil curve (concentration-ratio 3.8), the only combination of antagonists to do so.

Concentration-relaxation curves for cromakalim and levcromakalim were further depressed in range by glibenclamide but the poor quality of the curves and the level of sensitivities of the relaxation curves did not allow a quantitative measure of pEC_{50} (data not shown).

Nifedipine

In arteries contracted by U46619, the concentration-response curve for nicorandil was shifted to the right 5 fold by nifedipine (100 nM) which was similar to the shift for the combination of methylene blue and glibenclamide (8 fold). After pretreatment with the three drugs, methylene blue, glibenclamide and nifedipine, the nicorandil relaxation curve was strongly inhibited with a marked reduction in the maximal relaxation (Figure 2). After pretreatment with 100 nM nifedipine, more than 10 times higher concentration of U46619 (1–3 μM) was needed to maintain the same precontraction level as in control preparations. Sodium nitroprusside (10 μM) relaxed both the nifedipine-pretreated vessel and an artery pretreated with nifedipine vehicle with the same sensitivity ($pEC_{50} = 7.32 \pm 0.06$ and 7.43 ± 0.11) and range (100%) of relaxations. On the other hand, the relaxation curve for cromakalim was not significantly affected by nifedipine (100 nM) (1.7 fold shift), and the combination of glibenclamide and nifedipine did not shift the curve any further compared with vessels pretreated with glibenclamide alone (Figure 2).

We performed similar experiments with 1 μM verapamil ($n=5$), a phenylalkylamine Ca^{2+} antagonist. The EC_{50} for the relaxation curves for nicorandil were raised 4.3 fold by verapamil ($pEC_{50} = 4.03 \pm 0.03$), while the curve for cromakalim was not affected (data not shown in figure). Both nifedipine (see Figure 3) and verapamil abolished KCl-induced contractions.

Table 1 pEC_{50} values for the concentration-relaxation curves for various dilator agents in rat mesenteric arteries in the absence or presence of L-NOARG, methylene blue (MB), glibenclamide (GL), nifedipine (Nif), or various combinations

Vasorelaxant: *Contraction:*	*Nicorandil* U	*Cromakalim* U	*Levcromakalim* U	*Nicorandil* KCl	*GTN* U
Control	4.66±0.05(15)	6.45±0.12 (10)	6.34±0.08(8)	4.43±0.19(12)	6.92±0.14(8)
GL	4.96±0.10(8) <1	4.89±0.07(10) 36.3*	5.11±0.12(7) 1.7*	4.19±0.05(7) 1.7	
MB	4.64±0.09(6) 1.04	6.23±0.23(7) 1.7	6.21±0.08(6) 1.3	4.47±0.11(6) <1	5.31±0.17(8) 40.7*
L-NOARG	4.73±0.14(6) <1	6.50±0.13(6) <1	6.21±0.08(6) 1.34	4.51±0.21(6) <1	
Nif	3.97±0.06(8) 4.9*	6.12±0.11(6) 1.7			
L-NOARG+GL	4.71±0.09(6) <1	5.12±0.09(6) 21*	5.31±0.09(6) 10.7*	4.62±0.24(6) <1	
MB+GL	3.74±0.13(8) 8.3*	4.72±0.07(7) 54*	4.83±0.05(6) 32.3*	3.85±0.05(6) 3.8*	
GL+Nif		4.95±0.10(6) 32*			
MB+GL+Nif	3.62±0.03(11) 11.0*				

pEC_{50} values are $-\log M$ (EC_{50} mean±s.e.mean). Values in parentheses are *n*, number of arteries. Underlined values represent shift in EC_{50} from control to test calculated as concentration-ratio = antilog (control, pEC_{50} − test pEC_{50}) where < represents a leftward shift and sensitization. *Significant difference from control, $P<0.05$.

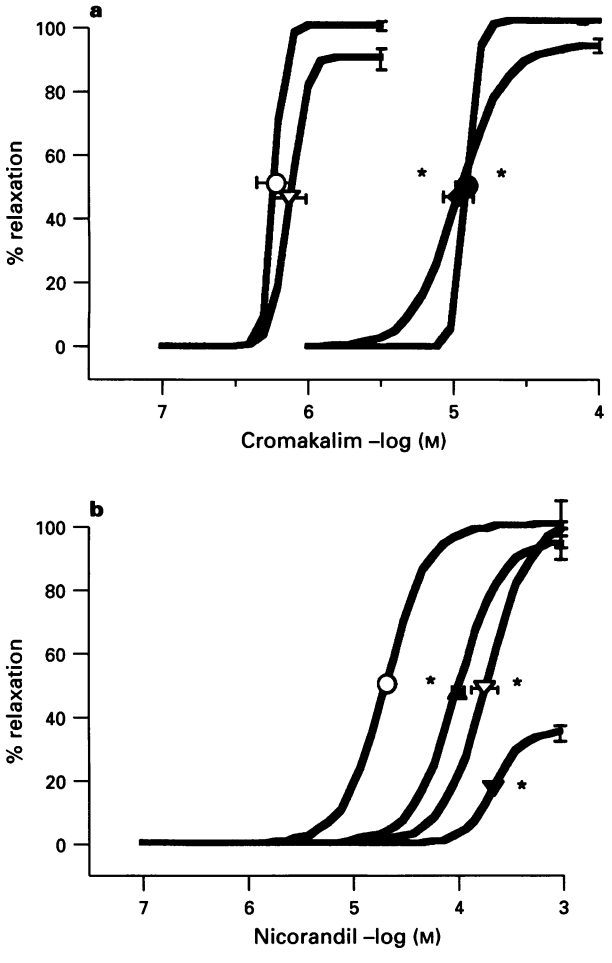

Figure 2 Logistic fitted concentration-relaxation curves for cromakalim (a) and nicorandil (b) in rat small mesenteric arteries contracted by U46619 (100 nM or 1 μM). Symbols represent: control (○); pretreatment with 3 μM glibenclamide (●); glibenclamide and 10 μM methylene blue (▲); 100 nM nifedipine (▽); nifedipine and glibenclamide (◆); glibenclamide, methylene blue and nifedipine (N+M+G, ▼). *pEC_{50} significantly different from control, $P<0.05$.

Role of membrane potential

Nifedipine, K^+ and U46619 precontraction In the arteries precontracted and depolarized by KCl, nifedipine (100 nM) did not repolarize the membrane potential but nifedipine completely abolished the force (Figure 3). The vessel was pretreated by 100 nM prazosin to remove the possible effects of KCl-induced noradrenaline release from sympathetic nerves. For the U46619-induced contraction, nifedipine again did not significantly affect the membrane potential, while the force was reduced to about 75%. Both the Em and remaining force returned to resting level after drug removal (Figure 3).

Uncontracted arteries In uncontracted resting arteries, cromakalim (10−1000 nM) and nicorandil (1−1000 μM) caused concentration-dependent hyperpolarization from −53 to −75 mV (Figure 4a, b) with no measured fall in force. In the presence of nifedipine (100 nM) a second concentration-response curve to cromakalim was unaffected while the Em pEC_{50} for nicorandil was raised 2.1 fold, $P<0.05$ (two tailed paired, *t* test) (Figure 4b). Similar findings occurred in arteries precontracted and depolarized to −35 mV by U46619 (100 nM). In the presence of nifedipine (100 nM), nicorandil hyperpolarization curves were again less sensitive (2.6 fold, $P<0.05$) in contrast to the complete lack of effect on the hyperpolarization curves to cromakalim (Figure 4c, d).

Methylene blue and hyperpolarization Methylene blue (10 μM MB) alone slightly depolarized the Em by 2.3±0.6 mV ($n=8$). The cromakalim concentration-hyperpolarization curves were completely unaffected by MB (10 μM) pretreatment in arteries either contracted and depolarized (Figure 5c, right) or in the unstimulated state (Figure 5a, right). Similarly nicorandil concentration-hyperpolarization curves were unaltered by MB when the arteries were precontracted and depolarized (Figure 5c, left). However, the nicorandil-induced hyperpolarization curves in the resting arteries were significantly shifted to the right by 6.6 fold (pEC_{50} 3.98±0.13) and the maximum Em hyperpolarization reduced to −67.6±0.88 mV (Figure 5a, left). The combination of MB plus nifedipine raised the pEC_{50} further (3.68±0.10, $n=5$) but this was not significantly different from MB alone (data not shown).

Glibenclamide Glibenclamide (3 μM) strongly inhibited the concentration-hyperpolarization curve for cromakalim, by 13 fold ($pEC_{50}=5.84\pm0.08$, $E_{max}=-63.1\pm0.73$ mV) and for nicorandil by 18 fold ($pEC_{50}=3.31\pm0.07$, $E_{max}=-63.5\pm0.7$) in uncontracted arteries (Figure 5a). We also examined the effect of MB, combined with nifedipine and glibenclamide on nicorandil-induced hyperpolarization curves, but the triple treatment did not shift the curve further from that caused by glibenclamide alone (data not shown). In U46619 precontracted and depolarized arteries, glibenclamide also inhibited the hyperpolarization curve for cromakalim by 37 fold ($pEC_{50}=5.20\pm0.11$, $E_{max}=-60.5\pm3.57$ mV) and reduced the maximum level of repolarization for nicorandil (1 mM) ($E_{max}=-47.2\pm1.35$ mV). The combination of MB and glibenclamide did not shift the curve further from the curve for glibenclamide treatment alone (Figure 5b).

The dissociation between membrane repolarization and

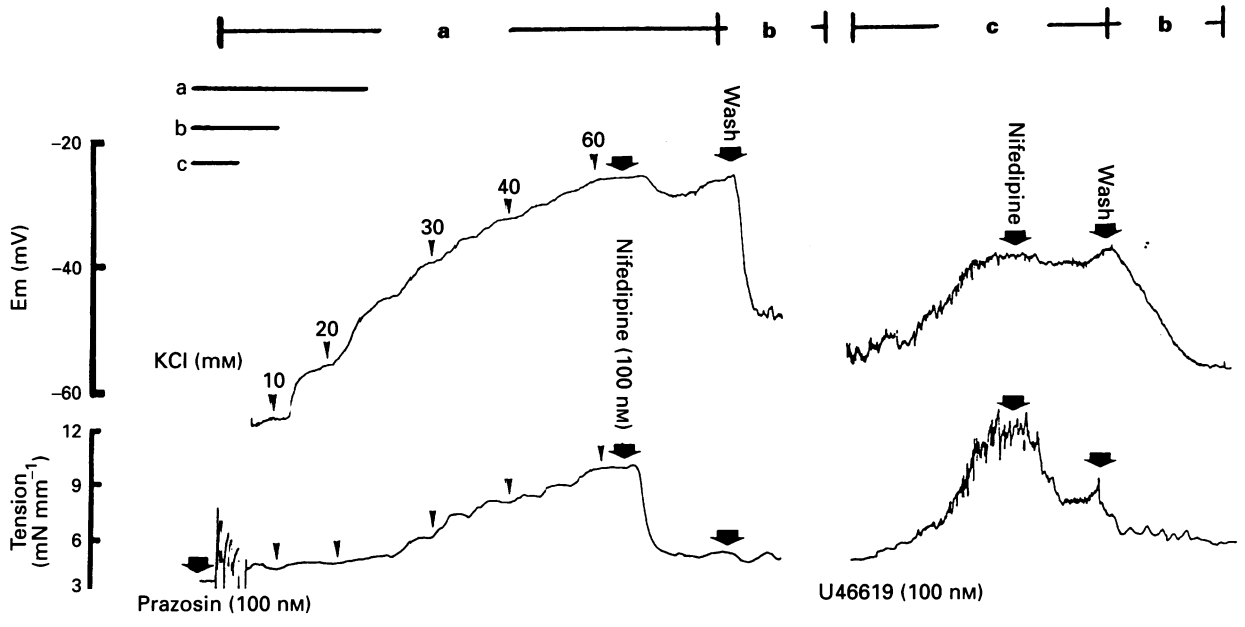

Figure 3 Original chart records of simultaneous recordings of membrane potential (Em) and tension in a rat mesenteric artery initially precontracted by increasing concentrations of KCl in the presence of prazosin (100 nM) (left) and U46619 (100 nM) (right). Both contractile agents increased tension and depolarized membrane potential to steady levels before nifedipine 100 nM was added. Wash is removal of all drugs from the circulating Krebs solution. a,b and c (topline) represent time periods with different chart speeds, and the corresponding bar lengths (left) represent 10 min.

relaxation was further demonstrated by the effect of glibenclamide on the nicorandil and cromakalim concentration-response curve in U46619 contracted arteries (Figure 5b, c). Glibenclamide (3 μM) caused a significant increase in the pEC_{50} for cromakalim (right shift) in both Em and relaxation curves but despite the marked reduction of the nicorandil-induced repolarization (only 10.8 mV), it had no significant effect on the concentration-relaxation curves to nicorandil (Figure 5c).

Discussion

The goal of this work was to unravel the mechanism(s) of vascular relaxation by nicorandil. The principle finding is that nicorandil relaxes blood vessels by a combination of mechanisms that are not sufficiently accounted for by the opening of K^+_{ATP} channels or by NO donor stimulation of guanylate cyclase. We propose that a third mechanism may be involved that is inhibited by drugs classified as L-type voltage-operated calcium channels. In addition we show that hyperpolarization is not an obligatory phenomenon for either nicorandil or nifedipine to relax these vessels. Importantly, we have measured membrane potential responses to nicorandil in precontracted arteries to assign properly the importance of hyperpolarization in the overall mechanism of relaxation.

To achieve our aim of understanding the action of nicorandil we used relatively specific blocking drugs in appropriate concentration that would shift the concentration-response curves of known, reportedly single mechanism vasorelaxant drugs by large (>10 fold) concentration-ratios. We used methylene blue at 10 μM which was shown to shift the GTN relaxation curve 40 fold and glibenclamide (3 μM) a known vascular K^+_{ATP} channel antagonist which shifted cromakalim and levcromakalim relaxation curves by 36 and 17 fold respectively.

In the majority of experiments in the rat small mesenteric artery we used the constrictor agent, U46619, to precontract the artery to a submaximal steady level of force. When arteries were submaximally contracted by K^+ depolarization, the relaxation curves to the K^+_{ATP} channel openers, cromakalim and levcromakalim, were markedly insensitive and gave a maximum of only 50% relaxation compared with arteries precontracted with U46619, reinforcing the notion that cromakalim and levcromakalim are largely dependent upon opening the K^+_{ATP} channel to relax this artery. K^+ depolarization will open voltage-operated L type Ca^{2+} channels, close K^+_{ATP} channels, and inhibit K^+_{ATP} channel-mediated relaxations. This environment is not useful if trying to apportion the action of nicorandil to mechanisms blocked or attenuated by the precontraction stimulus. Thus, by using K^+ precontraction the K^+_{ATP} channel is negated, making it inappropriate to test the role of K^+_{ATP} openers in this environment, and we preferred to analyse nicorandil actions in U46619 precontracted vessels compared with K^+ depolarized vessels.

The principle of the assay design was to use one relatively selective blocking drug; repeat the nicorandil concentration-relaxation curve before adding a second drug. If nicorandil had two actions in parallel and with similar potency, then it could be argued that the two blocking agents would be required before substantial right shift or depression of the nicorandil curve would be observed. In this rat artery neither glibenclamide, nor methylene blue alone caused any significant right shift in the relaxation curve to nicorandil. Evidence that each of the two blocking agents were used in appropriate concentration was shown by their antagonism of cromakalim, (and levcromakalim) and glyceryl trinitrate concentration-relaxation curves. Surprisingly therefore when both methylene blue and glibenclamide were given together the shift rose to 8 fold, still substantially less than 54 fold seen with cromakalim.

Nifedipine

Relaxation The L-type voltage operated calcium-channel antagonist, nifedipine, was tested to explore whether it altered the nicorandil concentration-relaxation curve. To precontract the arteries with U46619 to a similar level of force in the presence of, as in the absence of nifedipine, a 10 fold higher concentration of U46619 was required. This indicates that much of the Ca^{2+} required for contraction by U46619 is probably from intracellular sources in the presence of nifedipine. Nevertheless, both sodium nitroprusside relaxation

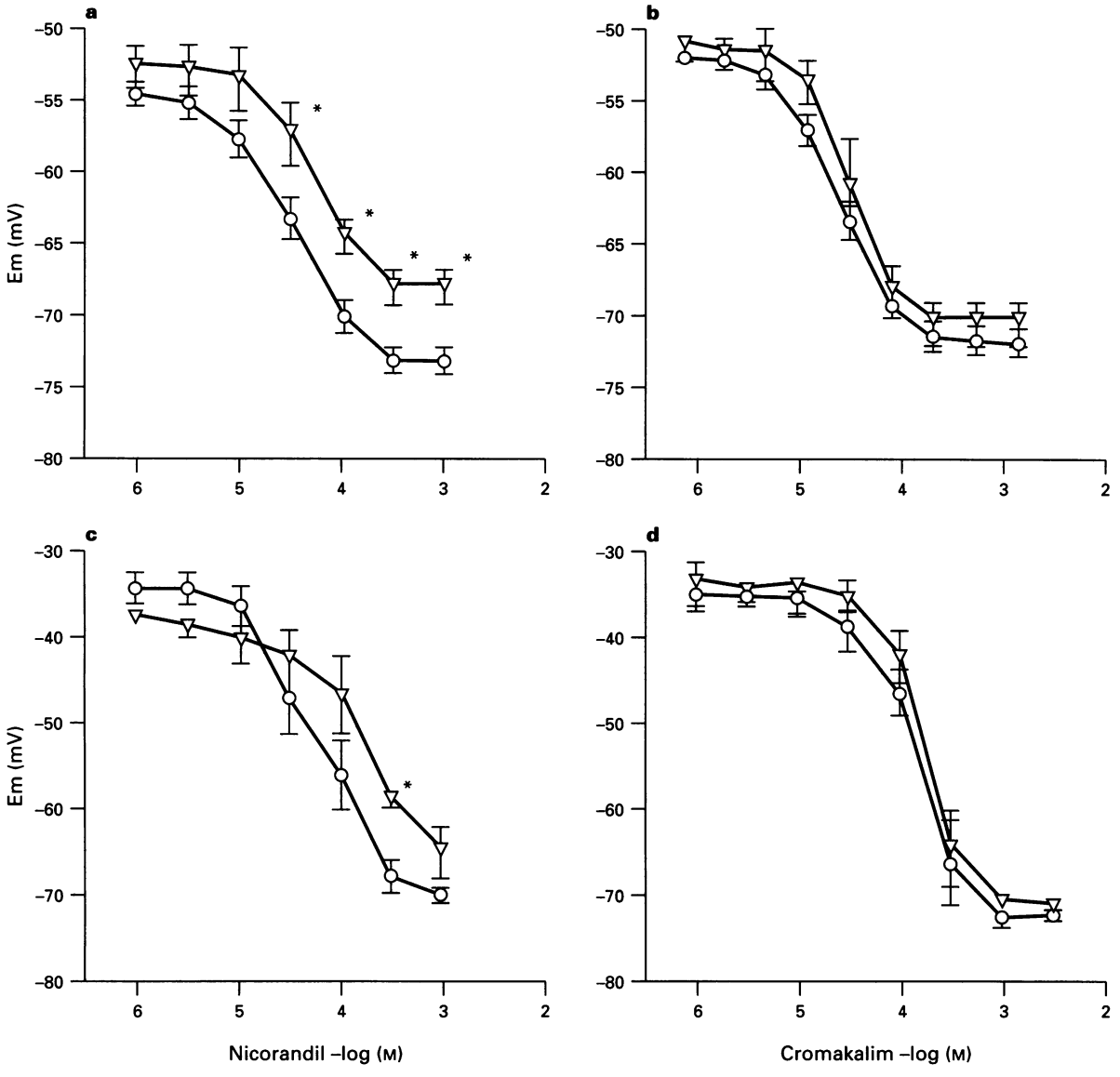

Figure 4 Effects of nifedipine 100 nM on nicorandil (a,c, $n=6-10$) and cromakalim (b,d, $n=6-7$) concentration-hyperpolarization curves. (a,b) are from arteries without precontraction (resting conditions) and (c,d) are from arteries precontracted and depolarized by U46619 (100 nM in the absence of 1 μM in presence of nifedipine). Symbols represent control (○) and nifedipine-treatment (▽). *Mean Em values significantly different from control values at that concentration, $P<0.05$.

curves and cromakalim relaxation curves were not affected significantly in range or sensitivity by nifedipine (100 nM). Thus, in this artery the NO donor and K^+_{ATP} channel opening mechanisms appeared to be unaffected by and thus separate from L-type voltage operated channel modulation. In contrast, nicorandil relaxation curves were significantly shifted to the right in location by nifedipine (100 nM) alone and further by the addition of glibenclamide and methylene blue. This latter triple treatment also severely attenuated the range of relaxation. Therefore, we tentatively speculate that L-type voltage operated calcium channels (VOCC) are important in the mechanism of action of nicorandil. To confirm that this action of nifedipine was a class effect we tested the phenylalkylamine Ca^{2+} antagonist, verapamil. Like nifedipine, verapamil lowered the pEC_{50} for nicorandil but not cromakalim.

Membrane potential We measured membrane potential to allow further insight into the mechanism of drug action. Nifedipine (100 nM) relaxed the artery precontracted and depolarized by U46619 without causing repolarization. This dissociation of force and Em was reported for nifedipine and verapamil in 5-hydroxytryptamine and KCl (20 mM) activated rabbit basilar artery (Clark & Garland, 1993). Their explanation is that calcium entry through the voltage-operated calcium channel (VOCC) is obligatory for the contraction but this current is not a significant component of the depolarization.

It is generally considered (Nelson & Quayle, 1995) that K^+_{ATP} channel openers, by repolarizing the membrane, would close VOCCs and thereby aid relaxation and fall in Em. It follows then that in the presence of VOCC antagonists such as verapamil or nifedipine, the K^+_{ATP} channel openers would be less effective. This was not the case in our vessel as nifedipine did not alter the concentration-relaxation or Em repolarization response curve to cromakalim when the vessel was contracted and depolarized by U46619. Thus cromakalim may relax this vessel through mechanisms other than closure of VOCC.

In contrast to these data, nifedipine shifted to the right the concentration-hyperpolarization curves to nicorandil in the presence or absence of U46619. This small antagonism by nifedipine is unlikely to be at the K^+_{ATP} channel given the cromakalim data and points to another difference between nicorandil and cromakalim.

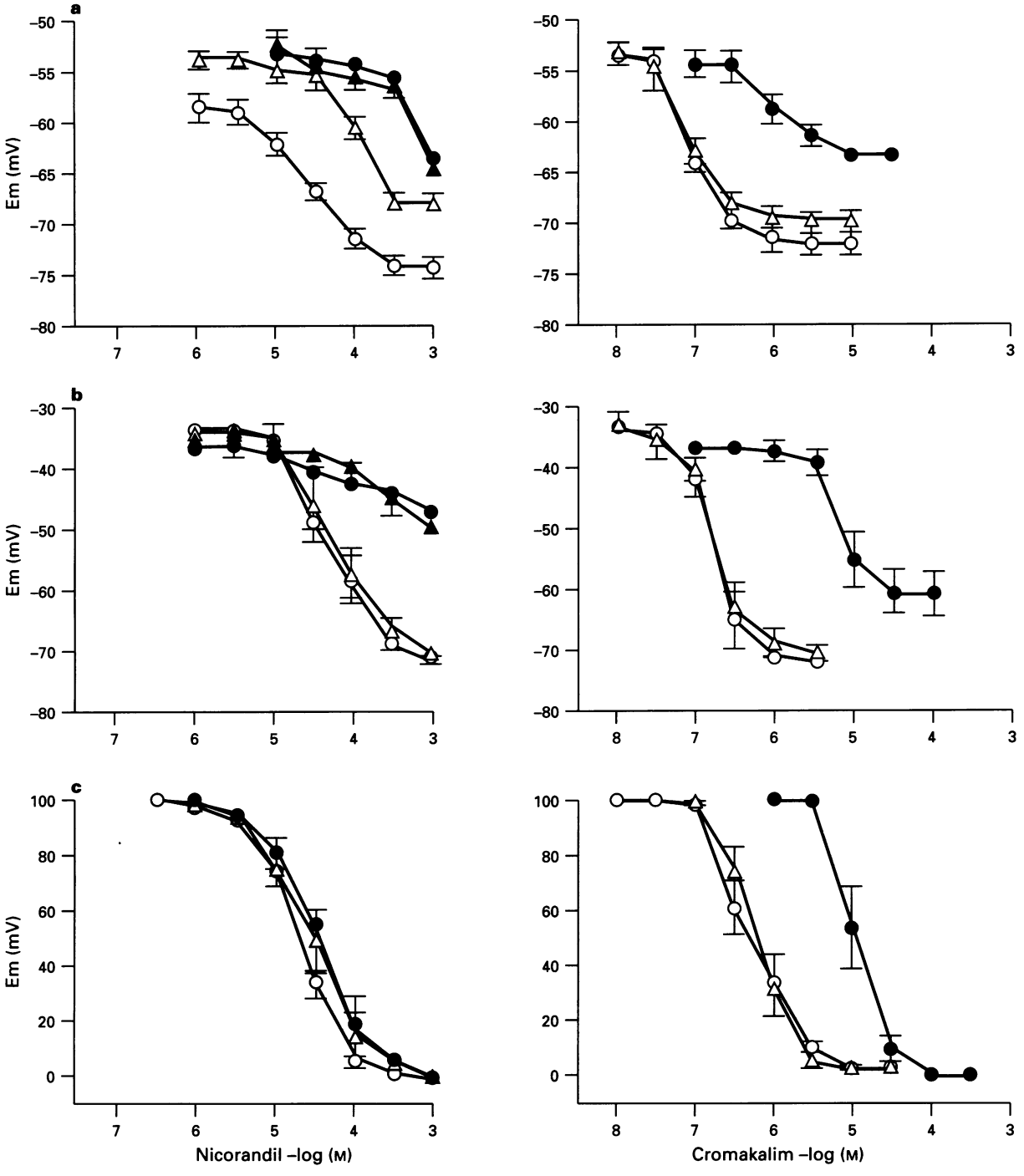

Figure 5 Effects of methylene blue (10 μM) and glibenclamide (3 μM) alone and in combination on nicorandil- and cromakalim-induced hyperpolarization (Em, mV) in resting arteries (a). In U46619 (100 nM) depolarized and contracted arteries, simultaneous measurements of repolarization (Em, b) and falls in isometric force (% force, c) are shown. Symbols represent, control (○); pretreatment with glibenclamide 3 μM (●); methylene blue 10 μM (Δ); and combination of glibenclamide and methylene blue (▲). Values are mean ± s.e.mean for $n = 5-12$ arteries. % force is relaxation expressed as % of precontraction force to U46619 (100%).

Methylene blue

Although methylene blue (MB) has numerous actions, at 10 μM it appeared to behave as a selective inhibitor of guanylate cyclase since it did not affect Em or force effects of cromakalim. The finding that MB inhibited hyperpolarization curves to nicorandil only when the vessel was relaxed and not depolarized is consistent with the scenario that NO donor-induced stimulation of guanylate cyclase can lead to hyperpolarization but this mechanism does not contribute significantly to relaxing a precontracted artery. Similarly, Garland & McPherson (1992) reported that the hyperpolarization to NO directly applied to uncontracted rat mesenteric arteries was inhibited by oxyhaemoglobin, depolarization or glibenclamide. The precise reason why depolarization reduces NO donor-induced hyperpolarization remains unknown.

Glibenclamide

At 3 μM glibenclamide was very effective as a K^+_{ATP} channel antagonist as shown by the rightward shift in the cromakalim-induced Em hyperpolarization in resting, and repolarization and relaxation in precontracted arteries. At first glance, glibenclamide behaved very similarly against nicorandil-induced

Em hyperpolarization and repolarization. But unlike cromakalim, this K^+_{ATP} channel antagonist of Em responses did not translate into an antagonist of relaxation. Thus like MB again we have evidence that antagonism of hyperpolatization is not sufficient to prevent the relaxation from nicorandil.

Overview

Nicorandil relaxes precontracted rat small arteries by at least 3 mechanisms: (i) NO-donor mediated stimulation of guanylate cyclase and increase in cyclic GMP, (ii) K^+_{ATP} channel opening, and (iii) nifedipine-sensitive Ca^{2+} inhibition. For relaxation, all three mechanisms appear to be important in parallel since antagonism of one action is not sufficient to inhibit significantly the responses to nicorandil.

The membrane potential studies have confirmed, (i) the role of methylene blue-sensitive NO donor hyperpolarization; (ii) the glibenclamide-sensitive K^+_{ATP} channel opening hyperpolarization; and (iii) the nifedipine-sensitive hyperpolarization. But in depolarized and precontracted arteries only the latter two mechanisms are evident. Our experiments have demonstrated however, that there is a distinct separation of membrane repolarization and relaxation events for nicorandil. Thus glibenclamide alone will prevent the repolarization of the artery to nicorandil but not affect the relaxation.

Finally, the VOCC mechanism appears to be partly responsible for nicorandil's activity shared with nifedipine and verapamil that contributes to the relaxation. This does not appear to be important for membrane repolarization as we found again no change in depolarized Em when relaxation to nifedipine occurs in contracted arteries. If these findings showing that nifedipine and verapamil can inhibit the relaxation to nicorandil in rat small arteries can be extrapolated to human arteries, then there may be concern for combination therapy of L type voltage-operated calcium channel inhibitors with nicorandil for the treatment of ischaemia heart disease.

In conclusion, these studies suggest that the mechanism of the relaxation of nicorandil resides with three actions: on guanylate cyclase, K^+_{ATP} channels and VOCC. These actions do not appear to be sequential but rather to be independent. By our analysis, a combination of each locus-selective antagonist was necessary to inhibit nicorandil-induced relaxation. Membrane repolarization was dissociated from relaxation making nicorandil a 'chameleon' vasodilator agent.

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