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# Potassium channel activation and relaxation by nicorandil in rat small mesenteric arteries

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**1** We used whole-cell patch clamp to investigate the currents activated by nicorandil in smooth muscle cells isolated from rat small mesenteric arteries, and studied the relaxant effect of nicorandil using myography.

**2** Nicorandil (300 $\mu$M) activated currents with near-linear current-voltage relationships and reversal potentials near to the equilibrium potential for $K^+$.

**3** The nicorandil-activated current was blocked by glibenclamide (10 $\mu$M), but unaffected by iberiotoxin (100 nM) and the guanylyl cyclase inhibitor LY 83583 (1 $\mu$M). During current activation by nicorandil, openings of channels with a unitary conductance of 31 pS were detected.

**4** One hundred $\mu$M nicorandil had no effect on currents through $Ca^{2+}$ channels recorded in response to depolarizing voltage steps using 10 mM $Ba^{2+}$ as a charge carrier. A small reduction in current amplitude was seen in 300 $\mu$M nicorandil, though this was not statistically significant.

**5** In arterial rings contracted with 20 mM $K^+$ Krebs solution containing 200 nM BAYK 8644, nicorandil produced a concentration-dependent relaxation with mean $pD_2 = 4.77 \pm 0.06$. Glibenclamide (10 $\mu$M) shifted the curve to the right ($pD_2 = 4.32 \pm 0.05$), as did 60 mM $K^+$. LY 83583 caused a dose-dependent inhibition of the relaxant effect of nicorandil, while LY 83583 and glibenclamide together produced greater inhibition than either alone.

**6** Metabolic inhibition with carbonyl cyanide m-chlorophenyl hydrazone (30 nM), or by reduction of extracellular glucose to 0.5 mM, increased the potency of nicorandil.

**7** We conclude that nicorandil activates $K_{ATP}$ channels in these vessels and also acts through guanylyl cyclase to cause vasorelaxation, and that the potency of nicorandil is increased during metabolic inhibition.

**Keywords:** Nicorandil; glibenclamide; mesenteric artery; $K_{ATP}$ channels; potassium current; metabolic inhibition

## Introduction

The vasodilator nicorandil is a nicotinamide derivative used clinically for the treatment of angina (Frampton *et al*., 1992; Goldschmidt *et al*., 1996). Nicorandil has been proposed to have at least two mechanisms of action: opening $K^+$ channels and also having a nitrovasodilator action, activating guanylyl cyclase (GC) and so increasing guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Holzmann *et al*., 1992; Frampton *et al*., 1992). $K^+$ channel activation causes membrane hyperpolarization, so reducing $Ca^{2+}$ entry through voltage-dependent $Ca^{2+}$ channels and possibly also inositol trisphosphate-dependent $Ca^{2+}$ release (Nelson *et al*., 1990; Nelson & Quayle, 1995). Cyclic GMP is thought to cause vasorelaxation by activating cyclic GMP-dependent protein kinase (PKG) to decrease intracellular $Ca^{2+}$ *via* a number of mechanisms such as cell membrane extrusion and $Ca^{2+}$ uptake into sarcoplasmic reticulum (Schmidt *et al*., 1993). The contribution of these two pathways to vasorelaxation appears to vary according to the tissue under study and the concentration of nicorandil used, the relative importance of the $K^+$ channel opening mechanism being greater in small vessels and at lower concentrations of nicorandil (Kukovetz *et al*., 1991; Holzmann *et al*., 1992; Akai *et al*., 1995). Clinically, in contrast to other nitrovasodilators, tolerance does not develop on continued application of nicorandil, and this lack of tolerance seems to be due its $K^+$ channel opening action (Frampton *et al*., 1992).

$K^+$ current activation by nicorandil has been measured directly using patch clamp of smooth muscle cells cultured from porcine coronary artery (Miyoshi *et al*., 1992; Wakatsuki *et al*., 1992) and cells isolated from rat and rabbit portal vein (Kajioka *et al*., 1990; Kamouchi & Kitamura, 1994), but not so far in cells from small arteries. In both portal vein and coronary artery cells, nicorandil has been reported to activate channels that are inhibited by intracellular ATP ($K_{ATP}$ channels); in rat portal vein cells the channel activated by nicorandil has been reported to also be activated by intracellular $Ca^{2+}$ (Kajioka *et al*., 1990), while in rabbit portal vein cells the channel is activated by intracellular nucleoside diphosphates as well as inhibited by ATP (Kamouchi & Kitamura, 1994). The sensitivity of nicorandil-activated currents in vascular smooth muscle to the sulphonylurea glibenclamide, which appears to be selective for $K_{ATP}$ channels in this tissue at concentrations up to 10 $\mu$M has not been investigated, though glibenclamide inhibition of nicorandil-activated currents has been reported in cells from the pig proximal urethra (Teramoto & Brading, 1997). The $K_{ATP}$ channel opening action of nicorandil, in common with other $K_{ATP}$ channel openers, is usually thought to occur as a direct action on the channel (see Quayle *et al*., 1997 for review). It is also possible, however, that channel activation occurs through activation of GC as a result of the nitrovasodilator action of nicorandil, since nitric oxide has been reported to cause glibenclamide-sensitive hyperpolarization of rat and rabbit mesenteric arteries (Garland & McPherson, 1992; Murphy & Brayden, 1995) and isosorbide dinitrate, atrial natriuretic

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peptide, and 8-bromo cyclic GMP can activate $K_{ATP}$ channels in cultured rat aortic cells (Kubo *et al*., 1994). Finally, on the basis of experiments using myography and membrane potential recording in rat mesenteric artery, Fujiwara & Angus (1996) have recently suggested that nicorandil might have a third mechanism of action, causing inhibition of nifedipine-sensitive $Ca^{2+}$ channels directly.

In the present study we have used patch clamp recording from cells freshly isolated from small branches of rat mesenteric artery to study the $K^+$ currents activated by nicorandil. We find that nicorandil activates a current whose properties are consistent with activation of $K_{ATP}$ channels, and that inhibition of guanylyl cyclase does not affect $K^+$ current activation by nicorandil. We also recorded currents through $Ca^{2+}$ channels, and found no evidence that they were significantly inhibited by nicorandil. In myograph recordings from ring segments of arterial branches equivalent to those used to isolate cells, relaxations in response to nicorandil were inhibited by both glibenclamide and an inhibitor of GC. Further, the relaxant action of nicorandil was enhanced by metabolic inhibition.

Some of these results have previously been presented to the British Pharmacological Society (Davie *et al*., 1997).

## Methods

Male adult Wistar rats were killed by cervical dislocation. The mesenteric vascular bed was removed and placed in ice cold Krebs solution (composition in mM: KCl 5, NaCl 135, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4 with NaOH). Second and third order arteries (diameter $377 \pm 12$ $\mu$m) were carefully freed from surrounding adipose and connective tissue, and were either used to provide isolated smooth muscle cells for patch clamp, or cut into ring segments for myograph recording.

### *Patch clamp experiments*

For patch clamp recording, cells were isolated enzymatically using papain and collagenase as described by Holland *et al*. (1996). Patch pipettes were pulled from thin-walled borosilicate glass tubing (o.d. 1.5 mm, Clark Electromedical), coated with dental wax (Kemdent), and fire polished. Their resistance was in the range 2–7 MΩ, and seals were in the order of 10 GΩ. Currents were recorded with an Axopatch 1B or 200A amplifier at a bandwidth of 10 kHz, and stored on DAT tape, or onto the hard disk of a microcomputer after digitization with an Axon Instruments A-D interface. Programs developed using the AxoBASIC library were used to apply command potentials and to analyse current records (Davies, 1993).

All currents were measured using the whole-cell patch clamp configuration. The bathing solution for $K^+$ currents contained (mM): KCl 140, $CaCl_2$ 0.1, $MgCl_2$ 1, HEPES-NaOH 10, glucose 10, pH 7.4, and the pipette solution contained (mM) KCl 107, KOH 33, EGTA 5, $MgCl_2$ 1, $Na_2ATP$ 0.1, ADP 0.1, glucose 10, pH 7.2. For $Ca^{2+}$ currents, the bathing solution contained (in mM): NaCl 130, $BaCl_2$ 10, KCl 5.4, glucose 10, EGTA 0.1, $MgCl_2$ 1, HEPES-NaOH 10, pH 7.4, and the internal pipette solution contained (in mM): CsCl 130, $MgCl_2$ 1, EGTA 5, glucose 10, $Mg_2ATP$ 2, GTP 0.5, pH 7.2. Patch clamp experiments were done at room temperature, 18–24°C.

### *Myograph experiments*

The vessels were cut into 2–4 mm rings and two rings were mounted in a small vessel wire myograph (Mulvany & Halpern, 1976; Mulvany *et al*., 1980). The rings were placed in a 10 ml bath that was gassed with $O_2$ and maintained at 37°C. All chemicals were added directly to the bath. All the solutions contained 20 $\mu$M $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) to eliminate endogenous nitric oxide activity. The vessels were contracted using 20 mM $K^+$ Krebs solution (in mM: KCl 20, NaCl 125, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4 with NaOH) with 200 nM of the calcium channel agonist BAYK 8644 (20K/BAYK). This solution was used because it elicited a very stable contraction while keeping the $K^+$ equilibrium potential ($E_K$) sufficiently negative that $K^+$ channel activation will cause hyperpolarization and relaxation. The maximal tone achieved was approximately 10 mN, and all measurements were calculated as the reduction in tone. Once a stable contraction was achieved ($\approx$30 min) cumulative concentration-response curves to nicorandil (1–300 $\mu$M) were measured. The mechanism of action of nicorandil was investigated using glibenclamide, a $K_{ATP}$ channel blocker, and 6-anilino-5, 8-quinolinedione (LY 83583), an inhibitor of guanylyl cyclase. For these experiments, maximal tone was achieved with 20K/BAYK and 10 $\mu$M glibenclamide was applied prior to measurement of the cumulative nicorandil concentration-response curve. Similar curves were repeated in the presence of different concentrations of LY 83583, which was added before the application of BAYK into the bath. In some experiments we used a 60 mM $K^+$ solution, made by equimolar substitution of KCl for NaCl in the 20 mM $K^+$ solution described above. Where used, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added to the bath to give a final concentration of 30 nM. To inhibit glycolysis, 2-deoxyglucose (2-DOG) was substituted for 4.5 mM of the glucose in the extracellular 20K solution, so reducing the glucose concentration to 0.5 mM.

### *Data analysis*

The decrease in tone (relaxation) was measured from the maximal contraction generated with 20K/BAYK. The difference between the maximum tone and the baseline just prior to the contraction was calculated and used to convert the values to a percentage relaxation. All values are given as mean $\pm$ s.e.mean. Individual concentration-response curves were fitted with the expression

$$y = M[1 + (k/x)^H]^{-1} \quad (1)$$

where $y$ is the % relaxation, $x$ the nicorandil concentration, $M$ the maximum response, $k$ the $EC_{50}$ for nicorandil, and $H$ the Hill coefficient, using the least squares algorithm in Sigmaplot (Jandel Scientific). $EC_{50}$ values were obtained as the concentration at which half maximal reduction in tone occurred, and $pD_2$s calculated as $-\log EC_{50}$ (M). $pD_2$ values are given throughout as mean $\pm$ s.e.mean. Statistical significance was assessed using Student's *t*-test for simple comparisons, and analysis of variance with Duncan's *post hoc* test for multiple comparisons.

### *Drugs*

Nicorandil was a gift from Chugai Pharmaceuticals and iberiotoxin (IbTX) was a gift from Zeneca. Sources of other drugs were as follows: glibenclamide (Sigma), CCCP (Sigma), 2-DOG (Sigma), LY 83583 (Lilly), BAYK 8644 (RBI), L-

NAME (Sigma). Nicorandil (100 mM), glibenclamide (10 mM), LY 83583 (10 mM) and BAYK 8644 (200 $\mu$M) were made up as stock solutions in DMSO and diluted into the final experimental solution on the day of use. DMSO alone was without effect at the concentrations used (up to 0.3%). L-NAME was made up as a 20 mM stock solution in water.

## Results

### *$K^+$ current activation by nicorandil*

We used whole-cell patch-clamp to investigate the activation of $K^+$ currents by nicorandil in smooth muscle cells isolated from small mesenteric arteries of the rat. To study the voltage-dependence and reversal potential of the current induced by nicorandil, voltage ramps were applied in the absence and presence of 300 $\mu$M nicorandil. Figure 1a shows the current-voltage relations obtained from a cell bathed in 140 mM $K^+$ using voltage ramps from $-50$ to $+50$ mV. Nicorandil induced a large increase in both inward and outward current, and the reversal potential for the nicorandil-activated current was $-2$ mV in this cell. The mean value from similar experiments on nine cells was $+0.4\pm1.1$ mV, close to the calculated $K^+$ equilibrium potential of 0 mV. The current-voltage relation of the nicorandil-activated current was near linear, consistent with the underlying channels showing little or no voltage-dependence. Equivalent current-voltage relations measured from a cell bathed in 60 mM external $K^+$ using ramps from $-50$ to $+30$ mV are shown in Figure 1b. In this cell the nicorandil-induced current reversed at $-21$ mV, close to the predicted $E_K$ of $-20$ mV. Figure 1c shows current-voltage relations measured in a cell bathed in the physiological external $[K^+]$ of 6 mM. Here the nicorandil-activated current reversed at $-70$ mV, again quite close to the calculated $E_K$ of $-79$ mV. A further four cells studied in 60 mM $[K^+]_o$ and a second cell in 6 mM $[K^+]_o$ gave similar results.

### *Effects of the $K^+$ channel blockers IbTX and glibenclamide*

To study the effect of $K^+$ channel blockers on the nicorandil-induced current, we measured the current activated by nicorandil in cells bathed in 140 mM $[K^+]_o$ and held at $-60$ mV, so that the electrochemical driving force on $K^+$ is inward and opening $K^+$ channels leads to an inward current. Figure 2a shows such an experiment. Switching the external solution superfusing the cell from 6 mM $K^+$ to 140 mM $K^+$ (arrow) led to the development of a background inward current, and the addition of nicorandil (300 $\mu$M) activated a substantial further inward current. Application of the selective blocker of large conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$) iberiotoxin (IbTX, 100 nM) did not affect the nicorandil-activated current (Figure 2a). IbTX has a dissociation constant of about 2 nM on $BK_{Ca}$ channels incoporated into neutral planar bilayers (Candia *et al*., 1992), and the IbTX used in the present study was effective in inhibiting single $BK_{Ca}$ channels of acutely dissociated cerebral arterial smooth muscle cells (Holland *et al*., 1996).

To assess the contribution of $K_{ATP}$ channel activation, we investigated the effect of 10 $\mu$M glibenclamide on the nicorandil-induced current. Glibenclamide inhibits $K_{ATP}$ currents induced by pinacidil in smooth muscle cells from the rabbit mesenteric artery with a $K_i$ of 101 nM, and is quite selective for $K_{ATP}$ channels at concentrations up to 10 $\mu$M (Quayle *et al*., 1997). Figure 2a shows that glibenclamide rapidly inhibited the current activated by nicorandil. The results of similar experiments using IbTX or glibenclamide on

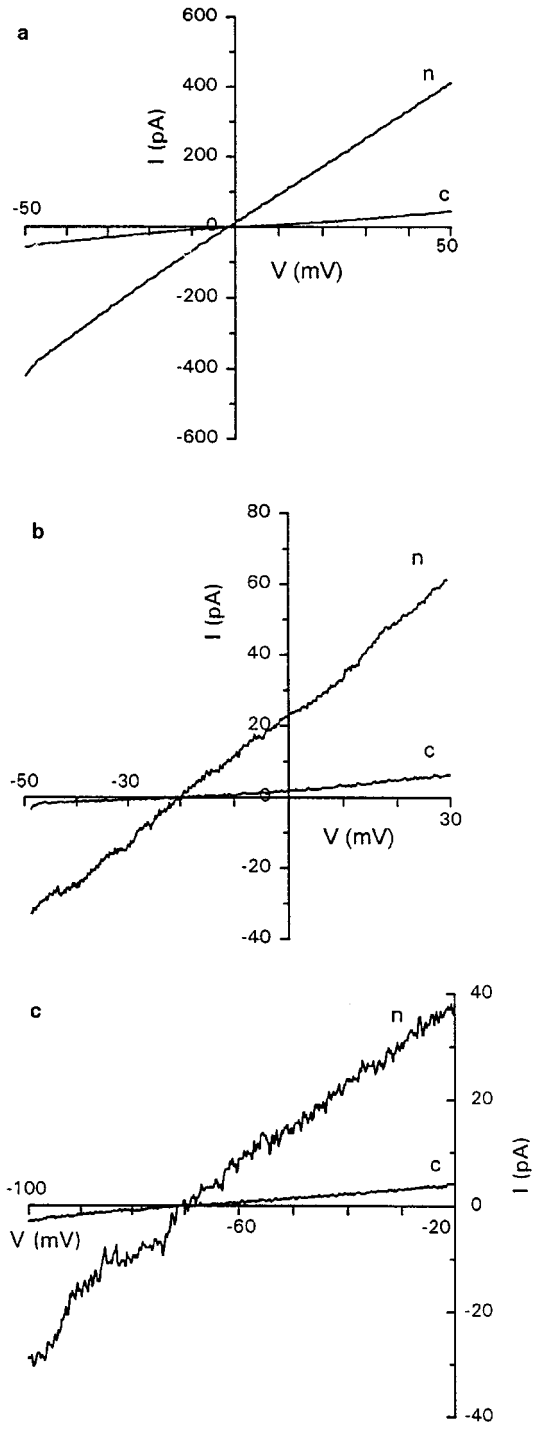



**Figure 1** Activation of whole-cell $K^+$ currents by nicorandil. (a) Averaged current recorded in response to a linear voltage ramp from $-50$ to $+50$ mV from a cell in 140 mM $[K^+]_o$ under control conditions (c) and in the presence of nicorandil (n) (300 $\mu$M) as indicated. Each trace is the average of the response to 20 identical ramps. (b) Averaged current recorded in response to a linear voltage ramp from $-50$ to 30 mV from a cell bathed in 60 mM $[K^+]_o$ under control conditions and in the presence of nicorandil (300 $\mu$M). Each trace is an average of 20 identical ramps. (c) Averaged current recorded in response to a linear voltage ramp from $-100$ to 0 mV from a cell bathed in 6 mM $[K^+]_o$ under control conditions and in the presence of nicorandil (300 $\mu$M) as indicated. Each trace is the average of the response to 20 identical ramps.

a number of cells are summarized in Figure 2b, which shows that 10 $\mu$M glibenclamide completely inhibited the nicorandil-activated current, whereas IbTX was without significant effect.

### *The effect of the guanylyl cyclase inhibitor LY 83583*

We investigated the possibility, discussed in the Introduction, that activation of GC might contribute to $K_{ATP}$ channel activation by nicorandil by using the selective inhibitor of GC 6-anilino-5, 8-quinolinedione (LY 83583) (Mülsch *et al.*, 1988), which has been shown to act very rapidly on isolated smooth muscle cells, inhibiting sodium nitroprusside activation of $Ca^{2+}$-activated $K^+$ channels of tracheal cells within 30 s (Yamakage *et al.*, 1996). As shown in Figure 3a, the superfusion of 1 $\mu$M LY 83583 onto a cell, in the presence of nicorandil, had no effect on the inward $K^+$ current activated by nicorandil. The results obtained in similar experiments on a number of further cells are summarized in Figure 3b, and show

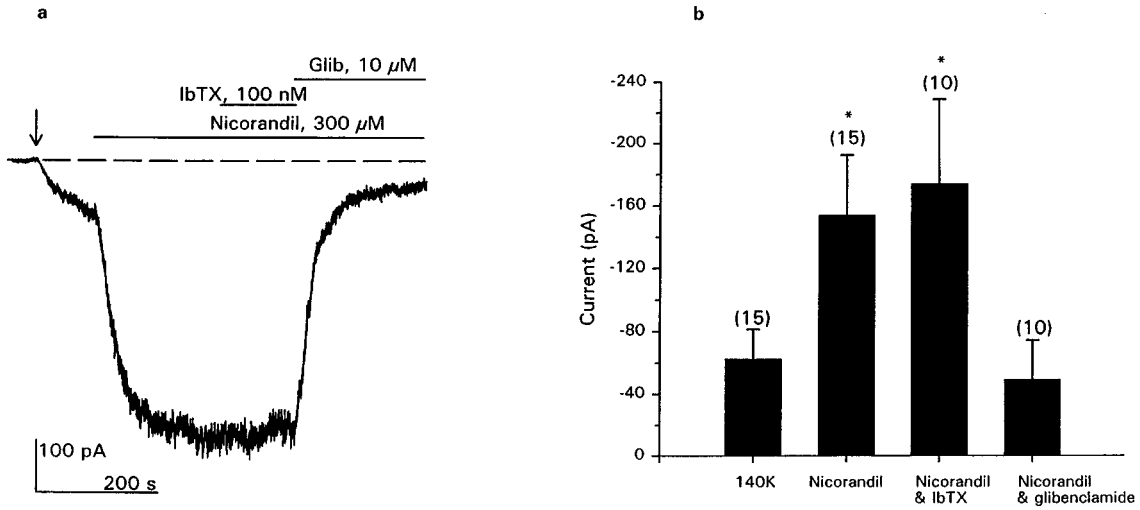



**Figure 2** Block of nicorandil-activated currents by glibenclamide. (a) Recording from a cell held at −60 mV. The solution perfusing the outside of the cell was changed from 6 mM $K^+$ solution to 140 mM $K^+$ solution where indicated by the arrow. Nicorandil (300 $\mu$M), iberiotoxin (100 nM) and glibenclamide (10 $\mu$M) were applied where indicated. The record was filtered at 2 kHz for display. (b) The effect of nicorandil (300 $\mu$M) alone and together with iberiotoxin (100 nM) or glibenclamide (10 $\mu$M) on currents measured at −60 mV from cells bathed in 140 mM $K^+$ solution. The bars show mean currents ± s.e.mean, and the number of cells is given in brackets. *Indicates currents significantly different ($P<0.05$) from the inward current measured in 140 mM $K^+$ in the absence of nicorandil, shown by the first bar.

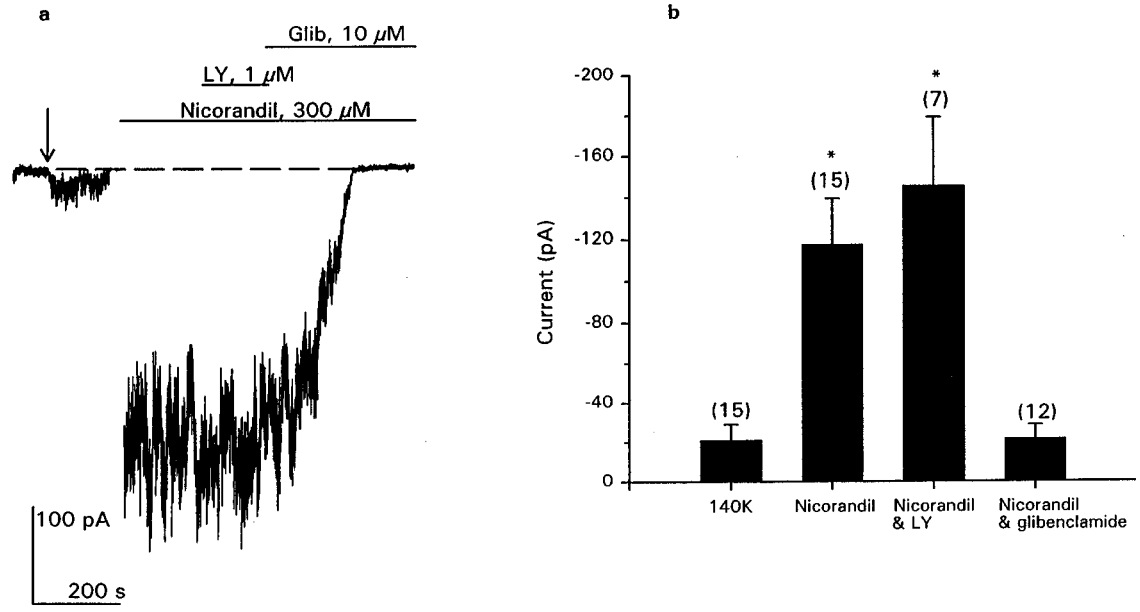



**Figure 3** LY 83583 does not affect nicorandil-activated currents. (a) Recording from a cell held at −60 mV. The solution perfusing the outside of the cell was changed from 6 mM $K^+$ solution to 140 mM $K^+$ solution where indicated by the arrow. Nicorandil (300 $\mu$M), LY 83583 (1 $\mu$M), and glibenclamide (10 $\mu$M) were applied as indicated. The break in the record at the beginning of the application of nicorandil occurred because of a solution exchange artefact. The record was filtered at 2 kHz for display. (b) The effect of nicorandil (300 $\mu$M) alone and together with LY 83583 (1 $\mu$M) or glibenclamide (10 $\mu$M) on currents measured at −60 mV from cells bathed in 140 mM $K^+$ solution. The bars show mean currents ± s.e.mean, and the number of cells is given in brackets. *Indicates currents significantly different ($P<0.05$) from the inward current measured in 140 mM $K^+$ in the absence of nicorandil, shown by the first bar.

that LY 83583 did not significantly affect the current activated by nicorandil. These findings imply that GC plays no role in the activation of $K_{ATP}$ current by nicorandil under our experimental conditions.

### *Single channels activated by nicorandil*

The low level of background channel activity in arterial smooth muscle cells sometimes makes it possible to resolve single channel events in whole cell recordings (Dart & Standen, 1993; 1995). We were able to use this approach to study the channels activated by nicorandil in mesenteric arterial cells. Figure 4a shows a series of records made during the initial phase of current activation by nicorandil, in 140 mM $[K^+]_o$ at $-60$ mV. Openings of a channel with a unitary current of about $-1.8$ pA were detected, and the channel activity increased as the response to nicorandil developed. Figure 4b shows a histogram of current amplitude made during the onset of the response to nicorandil from the same cell, showing peaks corresponding to current levels with either zero, one or two channels open. In experiments like that of Figure 4 on six cells, the mean unitary current was $-1.88 \pm 0.04$ pA. Assuming a reversal potential of 0 mV, this corresponds to a single channel conductance of 31 pS in symmetrical 140 mM $K^+$ solutions.

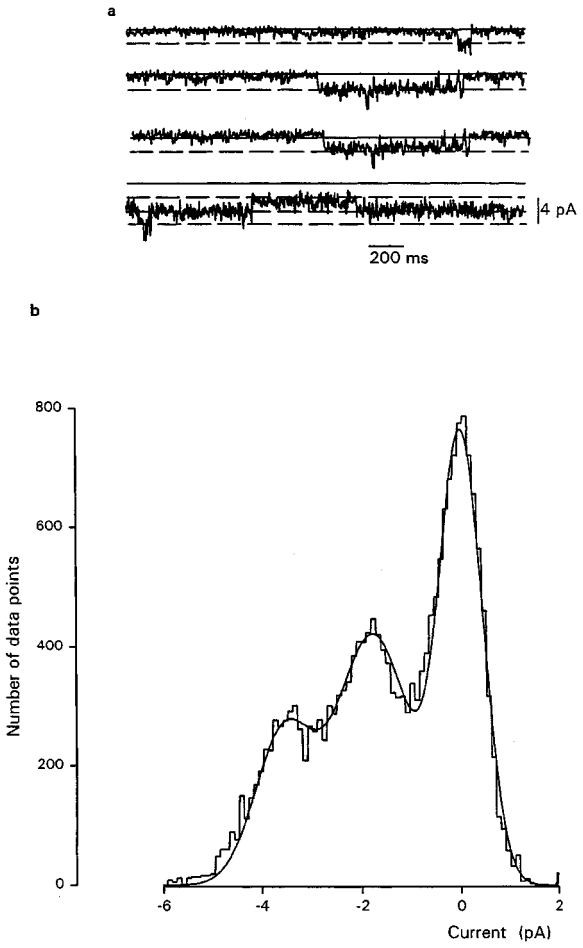



**Figure 4** Single channels activated by nicorandil in whole-cell recording. (a) Segments of currents recorded early during activation by nicorandil. The records have been filtered at 200 Hz and digitized at 1 kHz. The solid line indicates the zero current level and the dashed lines the level corresponding to one or two (bottom record) channels open. Holding potential, $-60$ mV; $[K^+]_o$ 140 mM. (b) Histogram of current amplitude formed from recordings made during the onset of the response to nicorandil from the same cell as in (a). The histogram has been fitted with the sum of three Gaussian curves with mean and s.d. values (pA) of: 0, 0.44; $-1.79$, 0.73; $-3.58$. 0.59 respectively.

### *Nicorandil has little effect on Ca currents*

Since it has been suggested that direct inhibition of L-type $Ca^{2+}$ channels may contribute to the relaxant action of nicorandil in rat mesenteric artery (Fujiwara & Angus, 1996) we investigated this possibility directly by measuring the effect of nicorandil on currents through $Ca^{2+}$ channels in isolated cells. Currents were measured in response to 100 ms voltage steps from a holding potential of $-60$ mV, using 10 mM $Ba^{2+}$ as the charge carrier. The internal solution contained 130 mM CsCl to minimize outward current through potassium channels. Figure 5a shows mean current-voltage relationships measured immediately after establishing whole-cell access in response to a series of steps to voltages ranging from $-100$ mV to $+40$ mV in a total of 14 cells either in the absence or presence of 100 $\mu$M nicorandil. Currents were normalized according to the cell capacitance, and mean currents in the absence and presence of nicorandil were not significantly different at any membrane potential.

$Ca^{2+}$ currents in response to 100 ms steps to $+10$ mV were also measured from cells before, during and after the application of nicorandil at either 100 or 300 $\mu$M. Figure 5b shows mean current values measured from four cells immediately before application of nicorandil, after 5 min exposure to 100 $\mu$M nicorandil, and 5 min after return to nicorandil-free solution, while Figure 5c shows results from equivalent experiments on four cells using 300 $\mu$M nicorandil. Example of current recordings are shown above the histogram in each case. One hundred $\mu$M nicorandil did not affect peak $Ca^{2+}$ currents (Figure 5b), and while Figure 5c suggests that 300 $\mu$M nicorandil may have caused a small reversible reduction in $Ca^{2+}$ current, this effect did not reach statistical significance. Our results therefore do not provide evidence for $Ca^{2+}$ channel inhibition by nicorandil at concentrations up to 100 $\mu$M.

### *Inhibition of nicorandil relaxations by glibenclamide and LY 83583*

In order to assess the relative contribution of $K_{ATP}$ channel opening and guanylyl cyclase activation to vasorelaxation by nicorandil in rat mesenteric arterial branches, we used a small-vessel myograph to measure concentration-response curves for relaxations of arterial rings, precontracted with 20K/BAYK (see Methods), to nicorandil in the presence of the $K_{ATP}$ channel inhibitor glibenclamide or the GC inhibitor LY 83583.

Figure 6a shows the concentration-response curve for the relaxant effects of nicorandil in our experiments in the absence and presence of glibenclamide (10 $\mu$M). Nicorandil produced a concentration-dependent vasorelaxation, with a maximum at 300 $\mu$M of $71.0 \pm 3.5\%$ of the 20K/BAYK contraction, a $pD_2$ of $4.77 \pm 0.06$, and a Hill coefficient of $0.94 \pm 0.05$ ($n = 21$). Glibenclamide (10 $\mu$M) shifted the concentration effect curve for nicorandil to the right, consistent with the involvement of $K_{ATP}$ channels in the action of nicorandil ($pD_2 = 4.32 \pm 0.05$, $n = 6$). $pD_2$ values for nicorandil under different conditions are given in Table 1. Further evidence for the involvement of $K^+$ channel activation comes from experiments in which nicorandil relaxations of contractions to 60 mM $K^+$ external solution, rather than 20K/BAYK, were studied. Under these

conditions, $E_K$ becomes much less negative ($\approx -23$ mV) so that opening $K^+$ channels will no longer cause hyperpolarization and relaxation. As expected, nicorandil was less potent against 60 mM $K^+$ contractions, with a $pD_2$ of $4.36 \pm 0.06$ ($n=8$), close to that measured for its effect on 20K/BAYK contractions in the presence of glibenclamide (Figure 6b, Table 1), confirming that $K^+$ channel opening contributes significantly to its action.

We also measured the effects of nicorandil in the presence of the GC inhibitor, LY 83583. Figure 7a shows curves measured in the absence of LY 83583, and in its presence at 10 nM, 100 nM, 1 $\mu$M and 10 $\mu$M. LY 83583 produced a dose-

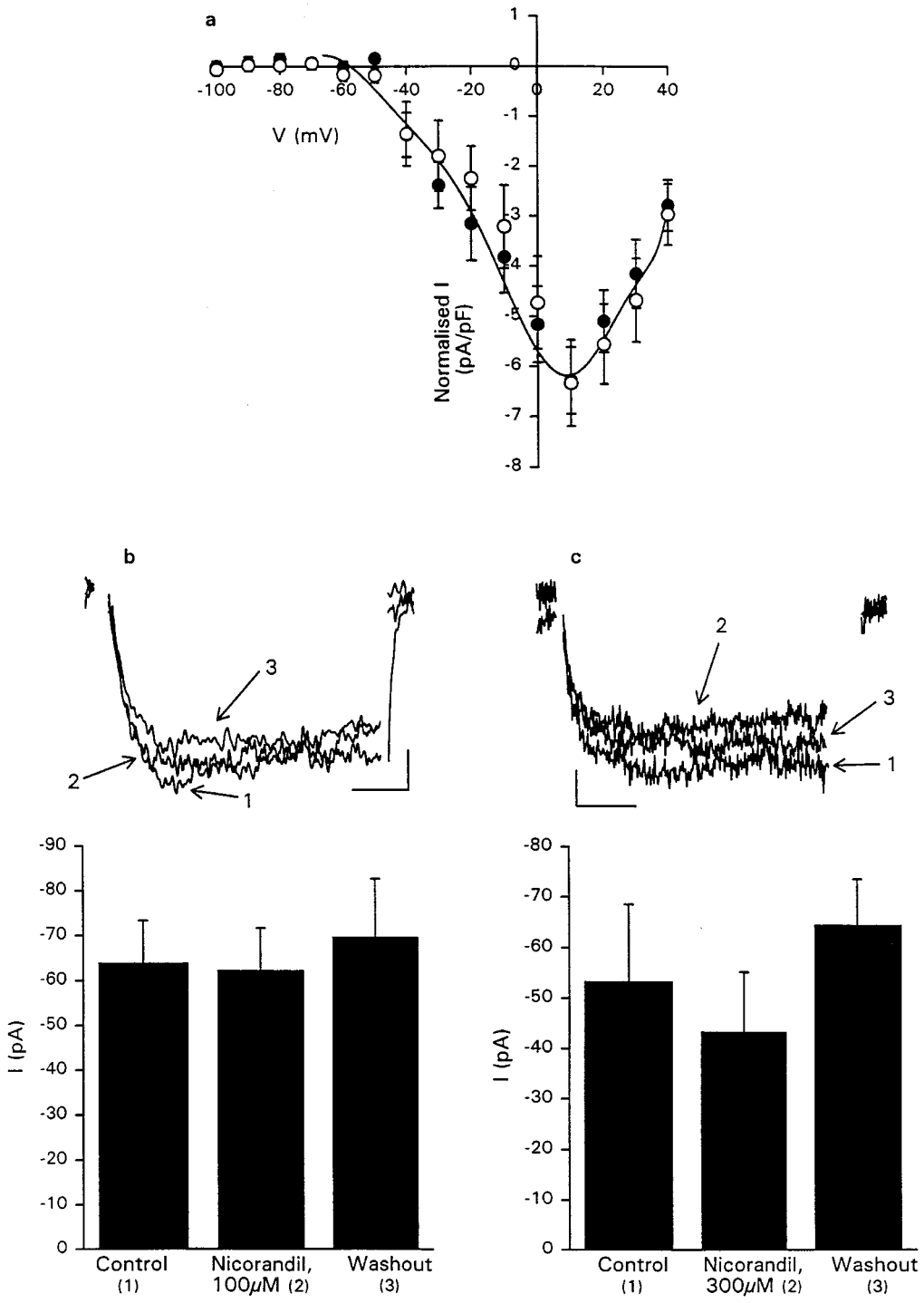



**Figure 5** The effect of nicorandil on $Ca^{2+}$ channel currents. (a) Mean ($\pm$s.e.mean) current-voltage relations for seven cells bathed in 10 mM $Ba^{2+}$ solution without nicorandil (○) and seven cells in 10 mM $Ba^{2+}$ solution with 100 $\mu$M nicorandil (●). Currents were recorded in response to a series of voltage steps applied immediately after whole-cell recording was established. (b) The histogram shows peak inward $Ca^{2+}$ channel current (mean$\pm$s.e.mean, $n=4$ cells) measured in response to 100 ms voltage steps to +10 mV from a holding potential of −60 mV before the addition of nicorandil (1), after 5 min in 100 $\mu$M nicorandil (2) and 5 min after return to nicorandil-free solution (3) as indicated. The traces above the histogram show examples of current recordings from one cell under the conditions 1, 2 and 3 as indicated above. The scale bars represent 20 pA and 20 ms. (c) Peak inward $Ca^{2+}$ channel current (mean$\pm$s.e.mean, $n=4$ cells) and recordings measured as described in (b) above, except that nicorandil was applied at 300 $\mu$M. The scale bars represent 20 pA and 20 ms.

dependent rightward shift of the nicorandil response curve, suggesting that activation of guanylyl cyclase also makes an important contribution to the action of nicorandil. The relaxing effect of nicorandil against contractions produced by 60 mM $K^+$, where the effects of $K^+$ channel opening should be abolished, were also substantially inhibited by 1 $\mu$M LY 83583 (Figure 6b).

In the presence of both 10 $\mu$M glibenclamide and 1 $\mu$M LY 83583, a greater rightward shift of the relaxation curve for nicorandil was observed than with either agent alone, as is shown in Figure 7b. Under these conditions the $pD_2$ value for nicorandil was further decreased to $3.5 \pm 0.1$ ($n=5$), suggesting that the effects of glibenclamide (10 $\mu$M) and LY 83583 (1 $\mu$M) on relaxations to nicorandil are cumulative, consistent with these agents inhibiting two separate mechanisms involved in relaxation.

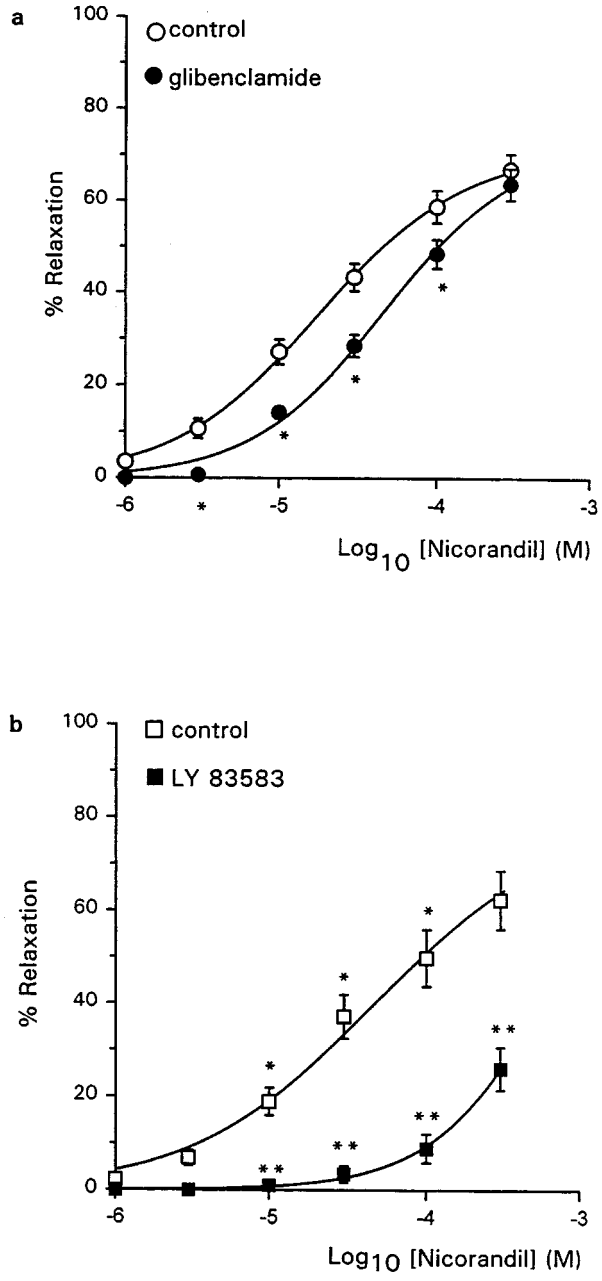



**Figure 6** Relaxation of mesenteric arterial rings by nicorandil. (a) Concentration-response curves for the relaxation of mesenteric arterial rings by nicorandil under control conditions ($n=21$), and in the presence of 10 $\mu$M glibenclamide ($n=6$). Rings were contracted with 20K/BAY K as described in the text, and the points show mean $\pm$ s.e.mean. *Indicates responses significantly different ($P<0.05$) from those under control conditions. The curves are drawn using equation (1). (b) Concentration-response curves for nicorandil relaxation of tone produced by 60 mM $K^+$ solution, in the absence ($n=6$) and presence ($n=6$) of 1 $\mu$M LY 83583. **Indicates $P<0.01$ significance.

## *Effects of metabolic inhibition on nicorandil relaxations*

$K_{ATP}$ channels in vascular smooth muscle respond to the metabolic state of the cell, and may be activated in whole-cell patch clamp experiments by metabolic inhibitors or by hypoxia (Zhang & Bolton, 1995; Dart & Standen, 1995). Quast & Cook (1989) proposed that the action of potassium channel openers may also be enhanced under such conditions. Indeed, the vasorelaxant action of the $K_{ATP}$ channel opener levcromakalim, though not pinacidil, appears to be enhanced under such conditions, since levcromakalim was more potent in causing vasodilation of the rabbit ear artery in hypoxia or when oxidative metabolism was impaired (Randall & Griffith, 1993). We therefore, examined the effects of inhibition of oxidative phosphorylation and of glycolysis on the relaxant action of nicorandil.

To inhibit oxidative metabolism, we used carbonyl cyanide m-chlorophenyl hydrazone (CCCP) which uncouples electron transfer in the respiratory chain from ATP generation. In these experiments, maximal tone was obtained with 20K/BAYK and CCCP was then added to achieve a bath concentration of 30 nM. CCCP induced a slight reduction in the tone, averaging $13.3 \pm 1.9\%$, to a new stable level. Figure 8a shows that nicorandil was more potent at causing vasorelaxation in the presence of CCCP, its concentration-effect curve being shifted to the left ($pD_2 = 4.88 \pm 0.04$, $n=8$). Table 2 gives the $pD_2$ values used for statistical comparison. The maximal relaxation obtained with nicorandil was also somewhat increased in the presence of CCCP. In the presence of CCCP, glibenclamide (10 $\mu$M) caused a rightward shift, decreasing the $pD_2$ to $4.4 \pm 0.04$, a value not significantly different from that seen with glibenclamide in the absence of CCCP (see Table 1). This is consistent with the extra potency of nicorandil in the presence of CCCP resulting from its action on $K_{ATP}$ channels.

We also investigated the effect of inhibiting glycolysis by reducing the glucose concentration to 0.5 mM, and replacing 4.5 mM of the extracellular glucose with its non-metabolizable analogue, 2-deoxyglucose (2-DOG). In rat coronary arteries such inhibition has been reported to cause vasodilatation which can be reversed with glibenclamide (Conway *et al.*, 1993). We found that a reduction in glucose to 0.5 mM alone caused a significant increase in the potency of nicorandil ($pD_2 = 4.94 \pm 0.06$, $n=10$), while addition of 2-DOG had no further effect (Figure 8b, Table 2). In addition, when CCCP and 2-DOG (30 nM and 4.5 mM respectively) were applied together to inhibit both oxidative phosphorylation and glycolysis, the

**Table 1** $pD_2$ values for nicorandil relaxation of 20K/BAYK contractions under control conditions and in the presence of glibenclamide (10 $\mu$M) or LY 83583 (1 $\mu$M), and of contractions to 60 mM $K^+$

| | *$pD_2$ nucorandil* (M) | n |
|---|---|---|
| Control (20K/BAYK) | $4.77 \pm 0.06$ | 21 |
| Glibenclamide | $4.32 \pm 0.05$** | 6 |
| LY 83583 (1 $\mu$M) | $4.16 \pm 0.15$* | 5 |
| Glib + LY 83583 | $3.93 \pm 0.06$** | 5 |
| 60 mM $K^+$ | $4.30 \pm 0.22$* | 6 |

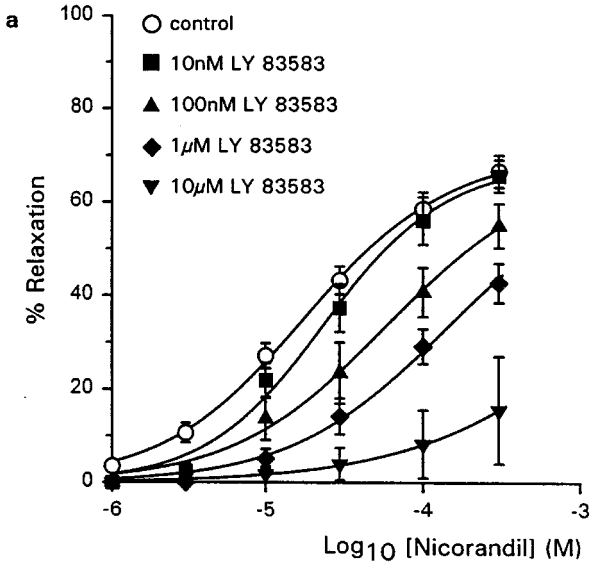
*$P<0.05$ or **$P<0.01$ compared to control. Values are mean $\pm$ s.e.mean.

effect of nicorandil was equivalent to that seen in the presence of 30 nM CCCP alone (results not shown).

## Discussion

### *$K^+$ channel activation by nicorandil*

Our results suggest that the current activated by nicorandil flows through a $K^+$ channel that shows little voltage-dependence, is inhibited by the sulphonylurea glibenclamide, and has a unitary conductance in symmetrical 140 mM $K^+$ solutions of 31 pS. These properties are consistent with activation of a channel of the $K_{ATP}$ family. Nicorandil has also been shown to activate ATP sensitive $K^+$ channels in cultured porcine coronary arterial cells and cells isolated from rat and rabbit portal vein. The unitary conductance is similar to that of the channel activated by nicorandil in cultured porcine coronary arterial cells (30 pS, Miyoshi *et al.*, 1992; Wakatsuki *et al.*, 1992), and of the channel activated by



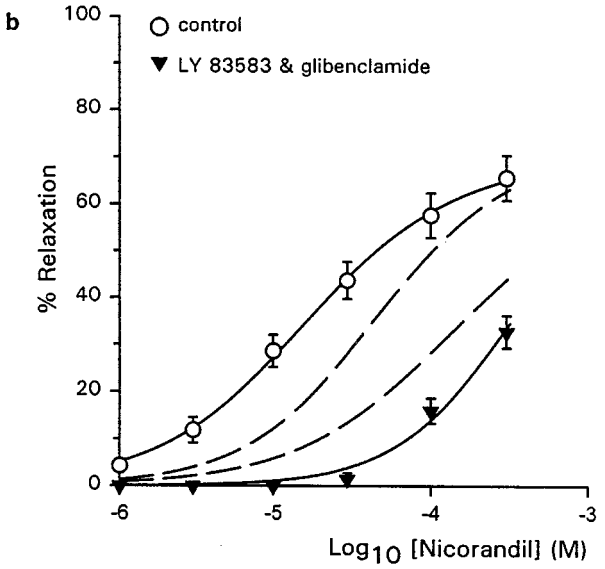
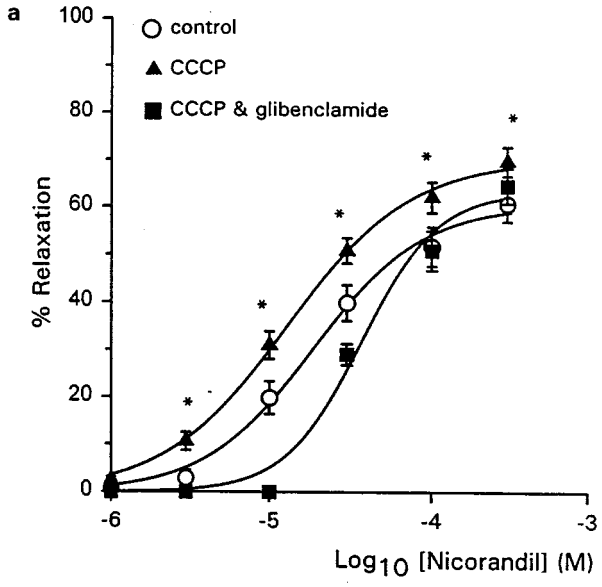




**Figure 7** The inhibitory effect of LY 83583 on nicorandil relaxations. (a) Concentration-response curves for relaxations to nicorandil under control conditions as in Figure 6a, and in the presence of LY 83583 at 10 nM ($n=6$), 100 nM ($n=4$), 1 μM ($n=6$), and 10 μM ($n=4$). Rings were contracted with 20K/BAY K. The curves were drawn using equation (1). (b) Concentration-response curve in the presence of both 10 μM glibenclamide and 1 μM LY 83583 ($n=5$). The control values are as in Figure 6a. The broken lines show the corresponding curves (from Figures 6a and 7a) in the presence of glibenclamide and LY 83593 alone.



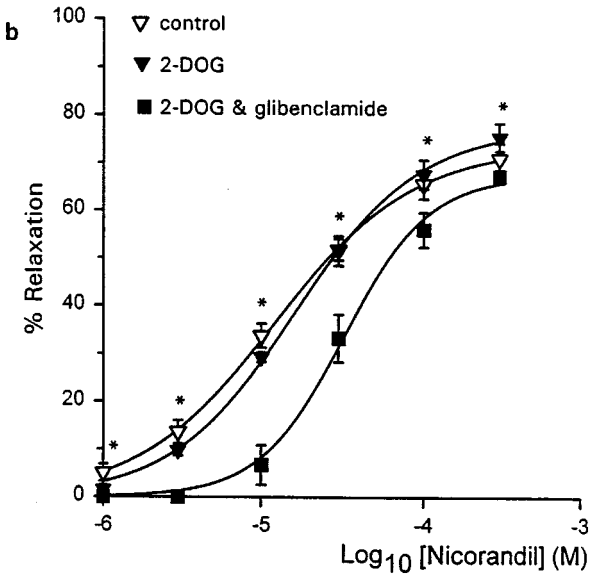




**Figure 8** Effects of metabolic inhibition on nicorandil relaxations. (a) Concentration-response curves for nicorandil under control conditions ($n=9$), and in the presence of 30 nM carbonyl cyanide m-chlorophenylhydrazone (CCCP, $n=9$), and of CCCP and glibenclamide ($n=5$). *Indicates responses significantly different ($P<0.05$) from those under control conditions. (b) Concentration-response curves for nicorandil in low-glucose solution ($n=10$). *Indicates responses significantly different ($P<0.05$) from those under the control (normal glucose) conditions shown in Figure 7a. Concentration-response curves in low-glucose solution with 4.5 nM 2-deoxyglucose ($n=6$), and in low glucose with 2-deoxyglucose and 10 μM glibenclamide ($n=6$) are also shown.

**Table 2** $pD_2$ values for nicorandil relaxations of 20K/BAYK contractions in the absence and presence of CCCP (30 nM) and in low glucose or low glucose + 2-deoxyglucose

| | *$pD_2$ nicorandil* (M) | n |
|---|---|---|
| Control | 4.68 ± 0.09 | 9 |
| CCCP | 4.88 ± 0.04* | 8 |
| 0.5 mM glucose | 4.94 ± 0.06* | 10 |
| 0.5 glu + 2-DOG | 4.78 ± 0.01 | 6 |
| CCCP + 2-DOG | 5.07 ± 0.04* | 4 |

*$P < 0.05$ compared to control. Values are mean ± s.e.mean.

adenosine or hypoxia in cells freshly isolated from the same tissue (35 pS, Dart & Standen, 1993; 1995). In rabbit portal vein, the channel activated may have a slightly lower conductance of 26 pS (Kamouchi & Kitamura, 1994), though a higher conductance channel has also been reported to be activated by another $K_{ATP}$ channel opener, pinacidil, in this tissue (50 pS, Kajioka *et al*., 1991). In cells from pig urethra, Teramoto & Brading (1997) found that nicorandil activated a glibenclamide-sensitive channel with a somewhat larger conductance, 43 pS in symmetrical high $[K^+]$. The channels activated by nicorandil and other $K_{ATP}$ channel openers have often been found to also be susceptible to activation by intracellular nucleoside diphosphates (Kamouchi & Kitamura, 1994; Teramoto & Brading, 1997). In rat mesenteric arterial cells, Zhang & Bolton (1995) found that pinacidil or intracellular GDP activated a glibenclamide-sensitive channel with a conductance of 20 pS in 60 mM $[K^+]_o$/130 mM $[K^+]_i$ solution. It is possible that the 31 pS channel activated by nicorandil in our experiments is the same channel, with the difference in conductance being explained by the higher $[K^+]$ used in our work. The properties of $K_{ATP}$ channels that have been reported to be activated by nicorandil and other $K_{ATP}$ channel openers in different smooth muscle tissues are quite diverse, however; in some cases the channels activated have been reported to show $Ca^{2+}$-sensitivity (Kajioka *et al*., 1990). This may correspond to differences in the isoforms of $K_{ATP}$ channel expressed (see Quayle *et al*., 1997 for review). Nicorandil has recently been shown to activate cloned $K_{ATP}$ channels formed by the expression in heterologous systems of the sulphonylurea receptor SUR2B, which has been proposed to be the smooth muscle isoform (Isomoto *et al*., 1996), in combination with either of the pore-forming subunits Kir6.1 or Kir6.2 (Yamada *et al*., 1997; Shindo *et al*., 1998).

$K_{ATP}$ channel openers are generally considered to activate channels by a direct interaction with the channel protein, probably leading to a reduction in sensitivity to inhibition by ATP, though other mechanisms may also be involved (reviewed by Quayle *et al*., 1997). Nicorandil is unusual, however, since it also has a nitrate action, raising the possibility that activation of GC and the resulting increase in cyclic GMP might underlie or contribute to channel activation. Our results suggest that this is not the case in rat mesenteric small arteries at high concentrations of nicorandil, since $K^+$ current activation by 300 μM nicorandil was unaffected by inhibition of GC with LY 83583 (Figure 3). Similarly, in pig urethra, Teramoto & Brading (1997) found that methylene blue did not affect channel activation by nicorandil. Relaxations to nicorandil at concentrations below 300 μM, however, were almost completely inhibited by a high concentration of LY 83583 (10 μM, Figure 7a). Further, relaxation to the lowest effective concentration of nicorandil, 3 μM, was almost completely inhibited by either glibenclamide or 1 μM LY 83583. These results raise the possibility that stimulation of cyclic GMP might contribute to activation of $K_{ATP}$ channels at lower concentrations of nicorandil. As discussed in the Introduction, stimulation of guanylyl cyclase has been shown to activate $K_{ATP}$ channels of cultured vascular smooth muscle cells, an effect that might occur either through activation of protein kinase G by cyclic GMP, or by way of cross-activation of protein kinase A (Kubo *et al*., 1994; Quayle *et al*., 1997).

### *Mechanisms of relaxation by nicorandil*

Our studies of the relaxations of rings of small mesenteric arteries are consistent with nicorandil acting *via* two pathways to cause relaxation; activation of $K_{ATP}$ channels to cause hyperpolarization, and activation of guanylyl cyclase to increase cyclic GMP and so reduce intracellular $Ca^{2+}$ by the mechanisms discussed in the Introduction and above. The $pD_2$ for nicorandil was increased either by $K_{ATP}$ channel inhibition with glibenclamide or by inhibition of GC with LY 83583. $K_{ATP}$ channel activation can also be dissociated from hyperpolarization and so relaxation by using 60 mM $[K^+]$ solution, causing an equivalent increase in $pD_2$ to that seen in the presence of 10 μM glibenclamide. Under these conditions it is likely that the remaining vasorelaxant properties of nicorandil occur through activation of GC, but do not involve $K^+$ channels. Our results also show that LY 83583 can act as a competitive antagonist of nicorandil relaxation, the $pD_2$s increasing with increasing concentration of LY 83583 (Table 1).

Our findings of two pathways for relaxation by nicorandil are consistent with those of Holzmann *et al*. (1992) using bovine coronary arteries. It appears that the relative contribution of the two pathways varies between tissues, and according to the size of the vessel studied (Yoneyama *et al*., 1990; Holzmann *et al*., 1992; Akai *et al*., 1995). Our results differ in some respects from those obtained recently by Fujiwara & Angus (1996), using the same preparation, rings of rat small mesenteric arteries. They found that neither glibenclamide nor methylene blue alone shifted the concentration-response curve for relaxation by nicorandil, though both antagonists in combination were effective. Glibenclamide, however, was an effective blocker of the membrane hyperpolarization caused by nicorandil, implying a dissociation between hyperpolarization and relaxation under their experimental conditions. The main difference between our experiments and those of Fujiwara & Angus appears to be the means used to produce arterial contraction, since we use a combination of depolarization with 20 mM $K^+$ solution ($E_K \approx -52$ mV) and activation of L-type $Ca^{2+}$ channels with the agonist BAYK 8644, which we found to produce a very stable contraction, while their experiments used the thromboxane $A_2$ agonist U46619. Using 20K/BAYK it is likely that entry of extracellular $Ca^{2+}$ through voltage-activated $Ca^{2+}$ channels plays the dominant role in inducing contraction, whereas U46619, which increases inositol trisphosphate, causes release of intracellular calcium, and may also change the $Ca^{2+}$ sensitivity of the contractile proteins as well as causing depolarization to activate $Ca^{2+}$ channels (Yamagishi *et al*., 1994). Under these conditions it may be that either $K^+$ channel opening or guanylyl cyclase activation is sufficient to cause relaxation, so that inhibition of both pathways is necessary to shift the concentration-response curve.

Fujiwara & Angus (1996) also found that the nicorandil relaxations of contractions to U46619 were inhibited by nifedipine, and suggested that direct inhibition of $Ca^{2+}$ channels might provide a third mechanism for the action of

nicorandil. We addressed this possibility directly, by investigating the effect of nicorandil on $Ca^{2+}$ channel currents measured under voltage clamp. We did not find evidence for $Ca^{2+}$ channel inhibition by nicorandil. The experiments of Fujiwara & Angus were complicated by the need to raise the concentration of U46619 10 fold in the presence of nifedipine to obtain an equivalent background contraction to that in its absence. If this, combined with the presence of nifedipine, were to alter the balance of the means by which U46619 causes contraction in the direction of mechanisms other than activation of voltage-sensitive $Ca^{2+}$ channels, this could provide an alternative explanation for the reduced effectiveness of nicorandil.

### *The effect of metabolic inhibition on vasorelaxation by nicorandil*

The $K_{ATP}$ channels activated by nicorandil and by other $K^+$ channel openers are characterized by their sensitivity to cellular metabolic state, increasing their open probability under conditions where metabolism is compromised, such as in hypoxia or in the presence of metabolic inhibitors (von Beckerath *et al*., 1991; Dart & Standen, 1995; Zhang & Bolton, 1995). This suggests that $K_{ATP}$ opening drugs might be more effective under conditions of metabolic compromise, where background channel activity is raised. Evidence that this is the case for levcromakalim has been obtained by Randall & Griffith (1993) and Randall *et al*. (1994), who showed that hypoxia, inhibition of oxidative phosphorylation, or adenosine increased the potency of the drug in causing vasorelaxation in the rabbit ear artery, though the potency of pinacidil was unaffected. In porcine coronary arteries, we have recently shown that nicorandil relaxations were enhanced by metabolic inhibition, decreased pH, or adenosine receptor activation, so in this respect the $K^+$ channel opening action of nicorandil appears to resemble that of levcromakalim rather than pinacidil (Davie & Standen, 1998). Our present results suggest that this is also so in rat small mesenteric arteries, since both inhibition of oxidative phosphorylation with CCCP and removal of glucose potentiated nicorandil-induced relaxations. The increase in the potency of nicorandil was abolished by glibenclamide, suggesting that it results from the $K_{ATP}$ channel opening action of the drug. The synergism between the effects of metabolic compromise and nicorandil in causing vasorelaxation may be of clinical relevance in that nicorandil may act as a more effective vasodilator in ischaemic than in adequately perfused tissue.

In conclusion, our results suggest that the functional vasodilator effects of nicorandil in the rat mesenteric small artery occurs *via* two mechanisms; direct activation of 31 pS $K_{ATP}$ channels, and activation of guanylyl cyclase. It is also possible that stimulation of guanylyl cyclase contributes to $K_{ATP}$ channel activation at low concentrations of nicorandil. We did not find evidence for inhibition of $Ca^{2+}$ channels by nicorandil. Further, metabolic inhibition increased the relaxant potency of nicorandil.

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