Effects of nicorandil as compared to mixtures of sodium nitroprusside and levcromakalim in isolated rat aorta

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1 The contribution of the relaxant mechanisms of nicorandil (NIC) were analysed by comparing its effects with those of sodium nitroprusside (SNP), levcromakalim (LEM) and mixtures (1:10, 1:30 and 1:100) of SNP:LEM in isolated endothelium-denuded rat aorta.

2 In rings precontracted with KCl (25 mM), the relative inhibitory potency of the soluble guanylate cyclase inhibitor ODQ and the K_{ATP} channel inhibitor glibenclamide (GLI) on SNP:LEM mixtures showed a good correlation with the relative proportion of SNP and LEM in the mixtures. Furthermore, the degree of the inhibition by ODQ and GLI of the effects of the 1:30 SNP:LEM mixture varied as a function of the relative potency of SNP and LEM in KCl-, noradrenaline- (NA) or NA plus nifedipine-treated arteries.

3 The inhibitory effects of ODQ, GLI and ODQ plus GLI on NIC-induced relaxation was similar to that for the 1:30 SNP:LEM mixture in NA plus nifedipine-contracted arteries, but the inhibition of GLI or ODQ plus GLI was smaller in KCl-contracted arteries.

4 In conclusion, the relative importance of activation of the cyclic GMP pathway and K_{ATP} channel opening in mixtures of SNP and LEM could be predicted by the proportion of the drugs in the mixtures and by the relative potency of SNP vs LEM in different experimental conditions. Furthermore, the present results suggest that besides these two mechanisms, a third ODQ- and GLI-insensitive mechanism, possibly involving Ca^{2+} channel blockade, also participates in the relaxant effects of NIC in KCl-induced contractions.

Keywords: Nicorandil; levcromakalim; nitroprusside; rat aorta; drug mixtures

Abbreviations: DMSO, dimethylsulphoxide; E_{max} , maximal relaxant effect; GLI, glibenclamide; IC_{30} , drug concentration producing 30% relaxation; k , factor which represents the slope of the concentration-response curve; LEM, levcromakalim; K_{ATP} , ATP-dependent K^+ channels; NA, noradrenaline; NIC, nicorandil; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; pD_2 , drug concentration exhibiting 50% of the E_{max} expressed as negative log molar; SNP, sodium nitroprusside

Introduction

Nicorandil (NIC) is an antianginal agent which causes vascular smooth muscle relaxation *via* two main mechanisms: it increases membrane K^+ conductance through the opening of ATP-dependent K^+ channels (K_{ATP}) leading to hyperpolarization and it releases nitric oxide (NO) elevating cyclic GMP levels by stimulating soluble guanylate cyclase (Furukawa *et al.*, 1981; Hester, 1985; Hamilton & Weston, 1989). Thus, NIC shares properties of K^+ channel openers and NO donors.

The relative contribution of these two mechanisms to the NIC-induced vasorelaxation varies depending on the experimental protocol and preparation studied. For example, in dog isolated mesenteric artery (Greenberg *et al.*, 1991), rat aorta contracted with endothelin-1 (Borg *et al.*, 1991) or noradrenaline (NA), (Magnon *et al.*, 1994), and rabbit and piglet pulmonary arteries contracted by NA (Magnon *et al.*, 1994; Pérez-Vizcaino *et al.*, 1998), the main mechanism of action of NIC appears to be due to stimulation of soluble guanylate cyclase. However, nicorandil increases blood flow in rat perfused heart (Borg *et al.*, 1991) and relaxes basilar arteries contracted with 5-HT (Ksoll *et al.*, 1991), mainly by K^+ channel activation and acts *via* both mechanisms in pig coronary (Borg *et al.*, 1991) and rabbit and piglet mesenteric arteries (Meisheri

et al., 1991; Magnon *et al.*, 1994; Pérez-Vizcaino *et al.*, 1998). Besides these two well known vasodilator mechanisms, an additional action of NIC involving the blockade of nifedipine-sensitive Ca^{2+} channels has been recently suggested (Fujiwara & Angus, 1996). The explanation for the varying contribution of each mechanism to the relaxant effect of NIC in different experimental conditions, i.e. the so called 'chameleon' effect of NIC (Fujiwara & Angus, 1996), remains elusive.

In the present study we have analysed the contribution of the different relaxant mechanisms of NIC by comparing its effects with those of the NO donor sodium nitroprusside (SNP), the K^+ channel opener levcromakalim (LEM) and several mixtures with different proportions (1:10, 1:30 and 1:100) of SNP:LEM in endothelium-denuded rat aorta. For this purpose we have used the specific inhibitors of K_{ATP} channels, soluble guanylate cyclase, and L-type Ca^{2+} channels, glibenclamide (GLI), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and nifedipine, respectively.

Methods

Tissue preparation

Male Wistar rats (300–350 g), were killed by a blow on the head and then exsanguinated. The descending thoracic aorta

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was rapidly dissected and placed in Krebs' solution of the following composition (in mM): NaCl 118, KCl 5, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 2, KH₂PO₄ 1.2 and glucose 11 at pH 7.4. After excess fat and connective tissue had been removed, the aorta was cut into rings (2–3 mm) and the endothelium was mechanically removed by gently rubbing the intimal surface of the rings with a metal rod. The rings were suspended horizontally by means of two parallel L-shaped stainless steel holders inserted into the lumen in 5 ml organ baths filled with Krebs' solution and bubbled with a 95% O₂–5% CO₂ gas mixture and maintained at 37°C. One holder served as anchor and the other was attached to an isometric force-displacement transducer coupled to a signal amplifier (Model PRE 206-4, Cibertec, Madrid) and connected to a computer *via* an A/D interface. Contractile tension was recorded by a REGXPC computer program (Cibertec, Madrid) as previously described (Pérez-Vizcaino *et al.*, 1993; 1998). Each ring was stretched to a resting tension of 2 g and allowed to equilibrate for 60–90 min. During this period tissues were restretched and washed every 30 min with warm Krebs' solution. The procedure of endothelium removal was tested by the lack of relaxant effects of acetylcholine (10^{−6} M) in rings precontracted with NA (10^{−6} M).

Experimental procedures

After equilibration, aortic rings were contracted by KCl (25 mM) and when the contractile response reached a stable tension, they were treated with vehicle (dimethylsulphoxide, DMSO), GLI (3 × 10^{−6} M), ODQ (10^{−6} M) or both inhibitors for 15 min. Thereafter, concentration-response curves to NIC (10^{−8}–3 × 10^{−4} M), SNP (10^{−10}–3 × 10^{−6} M), LEM (10^{−8}–3 × 10^{−5} M) or the 1:10, 1:30 and 1:100 mixtures of SNP:LEM were constructed by cumulative addition of the drugs. The proportions of the mixtures SNP:LEM were chosen because in preliminary experiments SNP was about 30 times more potent than LEM.

In another set of experiments, the aortic rings were contracted with NA (10^{−6} M) and subsequently treated with vehicle, nifedipine (10^{−7} M), GLI (3 × 10^{−6} M), ODQ (10^{−6} M) or combinations of these inhibitors for 30 min. Cumulative concentration-response curves were then constructed for SNP, LEM, the 1:30 SNP:LEM mixture or NIC.

Drugs

The following drugs were used: sodium nitroprusside, glibenclamide, acetylcholine chloride and noradrenaline (Sigma Chemical, Madrid, Spain), nicorandil (Merck Farma y Quimica, Barcelona, Spain), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Tocris Cookson, Bristol, U.K.), levromakalim (Smith Kline Beecham Pharmaceuticals) and nifedipine (Bayer AG, Leverkusen, Germany). All drugs were dissolved in distilled deionized water to prepare stock solutions (except GLI and ODQ which were dissolved in DMSO, and nifedipine in ethanol), and further dilutions were made in Krebs' solution. Ascorbic acid (10^{−4} M) was present in the stock solution of noradrenaline to prevent oxidation. The 1:10, 1:30 and 1:100 (mol:mol) mixtures of SNP:LEM were prepared by mixing the stock solutions of both drugs in the appropriate proportions just prior to use and further dilutions were made in Krebs' solution.

Analysis of the results

Results are expressed as means ± s.e. mean of measurements in *n* arteries. Individual cumulative concentration-response curves were fitted to the following logistic equation:

$$E = \frac{E_{\max}}{1 + 10^{-k \cdot ([\text{drug}] - \text{pD}_2)}}$$

where *E* is the relaxant effect of the drug, *E*_{max} is the maximal relaxant effect (an index of the efficacy of the vasodilator) expressed as a percentage of the initial contractile response, *k* is a factor which represents the slope of the curve, and *pD*₂ is the drug concentration exhibiting 50% of the *E*_{max} expressed as negative log molar (an index of the potency of the vasodilator). The results obtained with the SNP:LEM mixtures were expressed and calculated considering only the concentration of LEM. Statistically significant differences were calculated by a two way ANOVA analysis followed by a Newman Keuls test. *P* < 0.05 was considered statistically significant. The rightward shift of the concentration-response curves to the vasodilators induced by ODQ, GLI, nifedipine or their combinations was expressed as log dose-ratio. Log dose ratios were used to evaluate the potency of the inhibitors instead of the more common *pK*_B values (*pK*_B = −Log [inhibitor] + Log (dose ratio − 1)) because they allow determination of the inhibition produced by combinations of inhibitors. The log dose ratio values were calculated by subtracting the log concentrations of the vasodilator agents inducing a 30% relaxation (logIC₃₀) in the presence and absence of the inhibitors. The level of 30% relaxation was chosen because ODQ reduced the maximal relaxant effect of SNP to less than 40%.

Results

Relaxant effects of SNP, LEM, 1:10, 1:30 and 1:100 SNP:LEM mixtures and NIC on 25 mM KCl-induced contractions

SNP, LEM and NIC relaxed in a concentration-dependent manner (*pD*₂ values of 8.45 ± 0.13, *n* = 9, 7.12 ± 0.06, *n* = 8 and 5.10 ± 0.11, *n* = 10 respectively) the contractile responses induced by 25 mM KCl (1308 ± 40 mg, *n* = 162) with a similar efficacy (*E*_{max} values of 96 ± 6, 91 ± 3 and 99 ± 4%, respectively)

Figure 1 Concentration-response curves to sodium nitroprusside (SNP), levromakalim (LEM), nicorandil (NIC) and SNP:LEM mixtures in proportions 1:10, 1:30 and 1:100 in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM). The concentration of the mixtures are expressed as concentrations of LEM. Each symbol represents the mean ± s.e. mean of 7–10 experiments.

(Figure 1). The slope (k) of the concentration-response curve to LEM was significantly steeper than that to SNP (6.3 ± 1.4 vs 2.9 ± 0.4 , respectively, $P < 0.05$) and the slope of the curve to NIC was in between of both values (3.6 ± 0.3). The 1:10, 1:30 and 1:100 SNP:LEM mixtures also induced monophasic relaxant effects on 25 mM KCl-induced contractions. The pD_2 values, expressed in terms of LEM, were 7.75 ± 0.16 , $n = 8$, 7.43 ± 0.04 , $n = 8$ and 7.15 ± 0.04 , $n = 7$, respectively, and the E_{max} values were 100 ± 2 , 100 ± 5 and $93 \pm 5\%$, respectively.

The slope of these curves increased as the proportion of LEM increased in the mixture (3.1 ± 0.3 , 4.5 ± 0.3 and 5.2 ± 0.8 , respectively).

ODQ (10^{-6} M), GLI (3×10^{-6} M) or their combination, when added after the 25 mM KCl-induced contractions reached steady-state, had no effect on arterial tone (2.3 ± 1.2 , 2.9 ± 1.2 and $4.4 \pm 1.7\%$ relaxation, respectively) as compared to vehicle (DMSO, $3.5 \pm 0.8\%$). ODQ strongly shifted rightwards the curve to SNP ($pD_2 = 7.06 \pm 0.18$, $n = 6$), reduced the

Figure 2 Concentration-response curves to sodium nitroprusside (SNP), levromakalim (LEM), nicorandil (NIC) and SNP:LEM mixtures in proportions 1:10, 1:30 and 1:100 in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM) in the absence (control) or in the presence of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M) or both inhibitors. The concentration of the mixtures are expressed as concentrations of LEM. Each symbol represents the mean $\pm$ s.e. mean of 5–10 experiments.

$E_{\max}$ ($36 \pm 7\%$) and had no effect on the slope, whereas GLI, either in the absence or presence of ODQ, had no significant effect on any parameter (Figure 2A, $n=6$). In contrast, GLI produced a strong rightward shift of the curve to LEM ($pD_2=5.52 \pm 0.15$, $n=8$) whereas ODQ, either in the absence ($n=6$) or presence of ODQ ($n=9$), had no effect (Figure 2B). Neither ODQ nor GLI had any significant effect on the $E_{\max}$ or the slopes of the curves to LEM.

Both ODQ and GLI significantly shifted rightwards the concentration-response curves for the SNP:LEM mixtures (Figure 2C–E $n=6-10$). ODQ reduced the $E_{\max}$ ($87 \pm 3\%$) and increased the slope (5.5 ± 1.0) of the 1:10 mixture. The curves for the 1:30 and 1:100 mixtures appeared to be biphasic in the presence of ODQ, with a high affinity component which accounted for about 70–80% of the $E_{\max}$. GLI had no effect on the $E_{\max}$ but significantly reduced the slopes of the concentration-response of the 1:10, 1:30 and 1:100 mixtures (1.7 ± 0.2 , 2.2 ± 0.3 and 2.7 ± 0.6 , respectively). The inhibitory potency of ODQ and GLI decreased and increased, respectively, as the proportion of LEM increased in the mixture (Figure 3). The combination of ODQ plus GLI produced a

greater inhibition on the SNP:LEM mixtures ($n=5-8$) than any of the inhibitors alone (Figures 2 and 3). ODQ produced a significant rightward shift of the curve to NIC ($pD_2=4.53 \pm 0.12$, $n=6$, Figure 2F), increased its slope (5.3 ± 0.8) but it had no significant effect on the $E_{\max}$ value ($91 \pm 5\%$). GLI had no effect on the pD_2 or $E_{\max}$ values of the curve to NIC but significantly reduced its slope (1.7 ± 0.1 , $n=5$). Moreover, the combination of ODQ plus GLI produced a greater shift of the concentration-response curves to NIC than ODQ alone (Figures 2 and 3).

Relaxant effects of SNP, LEM, 1:30 SNP:LEM mixture and NIC on NA-induced contractions

NA (10^{-6} M) induced sustained contractile responses which averaged 1717 ± 52 mg ($n=164$) which was partially relaxed by 10^{-7} M nifedipine ($24 \pm 1\%$, $n=84$). SNP was six times less potent in relaxing NA- ($pD_2=7.65 \pm 0.14$, $n=7$) than 25 mM KCl-induced contractions, whereas LEM was equipotent in relaxing both contractile responses (Figure 4, pD_2 for NA-induced contractions = 7.03 ± 0.06 , $n=8$). No differences were found in the slope or $E_{\max}$ values in SNP- or LEM-induced relaxant response in NA- as compared to KCl-contracted arteries (not shown). Nifedipine did not modify the effect of SNP ($pD_2=7.72 \pm 0.11$, $n=8$) but shifted rightwards the curve to LEM ($pD_2=6.29 \pm 0.09$) and reduced its slope (2.8 ± 0.4 , Figure 4). ODQ and GLI produced rightward shifts of the curves to SNP and LEM, respectively, which were similar in KCl-, NA- and NA plus nifedipine-contracted arteries (Figure 4).

The 1:30 SNP:LEM mixture was three times less potent in relaxing NA-contracted vessels ($pD_2=6.96 \pm 0.14$, $n=8$) than 25 mM KCl-induced contractions and nifedipine did not produce any further change ($pD_2=7.10 \pm 0.16$, $n=9$, Figure 5). NIC was equipotent in relaxing the contractile responses in the three experimental conditions (pD_2 in NA- and NA plus nifedipine-contracted arteries 5.18 ± 0.04 , $n=7$, and 4.97 ± 0.16 , $n=7$, respectively, Figure 5). No differences were found in the slope or $E_{\max}$ values in the curves to the 1:30 mixture (4.9 ± 1.0 and $94 \pm 4\%$, respectively) or NIC (3.1 ± 0.5 and $98 \pm 3\%$, respectively) in NA- as compared to KCl-contracted arteries whereas nifedipine tended to reduce the slope of both the curves (3.0 ± 0.3 and 2.0 ± 0.4 , respectively, $P<0.05$) but had no effect on the $E_{\max}$ values.

In ODQ-treated arteries, either in the absence or presence of nifedipine, the curves for the 1:30 mixture were shifted to the right and appeared to be biphasic, the high affinity component accounting for 60–70% of the $E_{\max}$ (Figure 5A and B, respectively). GLI had no significant effect on the $E_{\max}$ values but reduced the slope (4.1 ± 0.6 , not significant and 2.2 ± 0.2 , $P<0.05$, in nifedipine-untreated and treated arteries, respectively) and shifted the curve to the 1:30 mixture to the right ($pD_2=6.15 \pm 0.11$ and 6.52 ± 0.14 , respectively, $P<0.05$ nifedipine-treated vs untreated arteries). ODQ shifted rightwards to a similar extent the curve to NIC in the absence and presence of nifedipine ($pD_2=4.54 \pm 0.07$ and 4.55 ± 0.15 , respectively) and reduced the $E_{\max}$ ($87 \pm 4\%$ not significant and 86 ± 4 , $P<0.05$, respectively) but did not significantly change the slopes of the curves (Figure 5C and D). GLI produced a weak rightward shift of the curves to NIC which was only significant in the absence of nifedipine ($pD_2=4.78 \pm 0.13$), reduced the slopes (1.9 ± 0.3 , $P<0.05$ and 1.5 ± 0.2 , $P>0.05$, in the absence and presence of nifedipine, respectively) and reduced the $E_{\max}$ only in the presence of nifedipine ($83 \pm 7\%$). However, despite the statistical differences, as can be observed in Figure 5C and D, the behaviour of

Figure 3 Inhibitory effects (expressed as log [dose ratio]) of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M) or both inhibitors on the relaxant effects induced by sodium nitroprusside (SNP), levcromakalim (LEM), nicorandil (NIC) and SNP:LEM mixtures in proportions 1:10, 1:30 and 1:100 in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM). The log dose ratios were calculated from Figure 2 at the level of 30% relaxation. * $P<0.05$ vs 0.

ODQ and GLI on NIC-induced relaxations were quite similar in untreated and nifedipine-treated arteries.

The combination of ODQ plus GLI induced a shift of the concentration-response to the relaxant effects of the 1:30 SNP:LEM mixture and NIC which was significantly greater than that induced by any of the inhibitors alone (Figure 5). The combination was equally potent to shift the curve to the SNP:LEM mixture in KCl-, NA- or NA plus nifedipine-treated arteries whereas it was significantly more potent in inhibiting NIC-induced relaxations in NA- or NA plus nifedipine- than in KCl-contracted arteries (Figure 5). Thus, GLI and GLI plus ODQ produced a significantly greater inhibition on the effects of the 1:30 mixture than on those of NIC in KCl contracted arteries, but no differences were observed in the potencies of the antagonists in NA or NA plus nifedipine contracted arteries (Figure 6).

Figure 7 shows the correlation between the relative potency of ODQ vs GLI to inhibit the relaxant effects of the 1:1, 1:10, 1:30, 1:100 and 1:1000 SNP:LEM mixtures and the relative potency of SNP vs LEM to relax KCl-contracted arteries. The figure also shows the correlation between the relative potency of ODQ vs GLI to inhibit the relaxant effects of the 1:30 SNP:LEM mixture and the relative potency of SNP vs LEM in KCl-, NA- and NA plus nifedipine-treated arteries. Both correlations followed a similar trend so that the data with different proportions and different experimental conditions were pooled for the regression analysis yielding a regression

line (slope = 1.1 ± 0.1 and y intercept = -0.6 ± 0.1) with a very good correlation ($r = 0.98$, $P < 0.01$). In contrast, there was no correlation between the relative potency of ODQ vs GLI to inhibit the relaxant effects of NIC and the relative potency of SNP vs LEM to relax KCl-, NA- and NA plus nifedipine-treated arteries.

Discussion

In the present study we have analysed the mechanism of NIC-induced relaxation in isolated endothelium-denuded rat aorta under several experimental conditions. Nicorandil is known to relax vascular smooth muscle mainly by releasing NO and increasing the intracellular concentrations of cyclic GMP and by opening K_{ATP} channels (Furukawa *et al.*, 1981; Hester, 1985; Hamilton & Weston, 1989). The contribution of guanylate cyclase activation and K_{ATP} channel opening in the relaxant effects of NIC has been analysed by means of their specific inhibitors ODQ and GLI, respectively and by comparison with the specific stimulator of guanylate cyclase SNP, the specific K_{ATP} channel opener LEM and mixtures of both drugs. The analysis of the mechanisms of NIC-induced relaxation is complicated by several factors: (a) the possibility that the pharmacological tools employed are not specific enough; (b) the ability of a given mechanism to induce a relaxant response may vary in the different experimental

Figure 4 Concentration-response curves to sodium nitroprusside (SNP) and levromakalim (LEM) in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM), NA (10^{-6} M) or the combination of NA plus 10^{-7} M nifedipine (NA+NIF) in the absence or in the presence of ODQ (10^{-6} M) or glibenclamide (GLI, 3×10^{-6} M). Each symbol represents the mean $\pm$ s.e. mean of 6–9 experiments.

conditions; (c) the possible interactions between the different mechanisms; and (d) finally, the existence of other 'unexpected' mechanisms.

Specificity of SNP, LEM, ODQ and GLI

Inhibitors of guanylate cyclase such as methylene blue have been widely used to analyse the possible involvement of cyclic GMP in drug-induced relaxation. However, this drug is relatively unspecific and conclusions drawn may be inadequate (Gryglewski *et al.*, 1992). Thus, we have employed ODQ, a much more specific tool to inhibit guanylate cyclase. ODQ has been reported to be both a competitive and a non competitive

inhibitor of guanylate cyclase (Schrammel *et al.*, 1996). Likewise, in our experiments ODQ produced a rightward shift of the concentration-response to SNP (typical of a competitive antagonist) but also reduced the maximal relaxant effect of SNP (typical of a non competitive antagonist). Furthermore, ODQ did not interfere with LEM-induced relaxation. In functional studies, GLI behaves as an apparent competitive antagonist of LEM (Pérez-Vizcaino *et al.*, 1993; Edwards & Weston, 1993). Likewise, in the present study, GLI produced a parallel shift of the concentration-response curves to LEM but had no effect on SNP-induced relaxation. Furthermore, ODQ did not modify the inhibitory action of GLI on LEM-induced relaxation and, conversely, GLI did not affect the inhibitory

Figure 5 Concentration-response curves to the 1:30 sodium nitroprusside:levromakalim (SNP:LEM) mixture and nicorandil (NIC) in endothelium-denuded rat aortic rings pre-contracted with NA (10^{-6} M) or the combination of NA plus 10^{-7} M nifedipine (NA+NIF) in the absence (control) or in the presence of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M) or both inhibitors. The concentration of the mixtures are expressed as concentrations of LEM. Each symbol represents the mean $\pm$ s.e.mean of 6–10 experiments.

Figure 6 Inhibitory effects (expressed as log [dose ratio]) of ODQ (10^{-6} M), glibenclamide (GLI, 3×10^{-6} M), nifedipine (NIF, 10^{-7} M) or combinations of these inhibitors on the relaxant effects induced by nicorandil (NIC) and the 1:30 SNP:LEM mixture in endothelium-denuded rat aortic rings pre-contracted with KCl (25 mM), or NA (10^{-6} M). The log dose ratios were calculated from data in Figures 2 and 5 at the level of 30% relaxation. * $P < 0.05$ SNP:LEM vs NIC.

Figure 7 Correlation between the relative potency of ODQ vs glibenclamide (GLI) to inhibit the relaxant effects of SNP:LEM mixtures and nicorandil (NIC) and the relative potency of SNP vs LEM in different experimental conditions. Ordinate: logarithm of the ratio of the potency of ODQ and GLI expressed as apparent K_B . The apparent K_B were calculated at the level of 30% relaxation. Abscissa: logarithm of the ratio of the potency of SNP and LEM expressed as IC_{30} . The relative potencies (IC_{30} (SNP)/ IC_{30} (LEM)) were 0.038, 0.125 and 0.021 for KCl, NA and NA plus nifedipine, respectively, and these values were corrected for the proportion factor in the mixtures, for the data with NIC these values were artificially set to those of the 1:30 SNP:LEM mixture. The data for the 1:10, 1:30 and 1:100 SNP:LEM mixtures were calculated from previous figures and the data for the 1:1 and 1:1000 are not shown. Since open circles and squares followed a similar trend they were pooled for the regression analysis. Note that data for the 1:30 SNP:LEM mixture fell into the line, while those for NIC did not follow any correlation. Since GLI had no inhibitory action on NIC-induced relaxation in KCl-stimulated arteries, the $\text{Log } (K_B \text{ (ODQ)}/K_B \text{ (GLI)})$ cannot be calculated but has to be smaller than -3 .

action of ODQ on SNP-induced relaxation. In spite of the differences in potency of SNP and LEM under different experimental conditions (arteries treated with KCl, NA or NA plus nifedipine) no apparent changes in the mechanism of action of these drugs were observed since ODQ and GLI produced similar inhibitory actions. In summary, in our experimental conditions, both the agonists (SNP and LEM) and the antagonists (ODQ and GLI) appeared to be very specific tools.

Effects of the SNP:LEM mixtures

The concentration-response curves to the SNP:LEM mixtures in the absence of inhibitors were monophasic. This can be explained because both drugs produced a nearly full relaxant effect and the combination of both drugs did not produce a maximal relaxation greater than that induced by the individual drugs. It could be expected that the contribution of K_{ATP} channel opening would increase and that the contribution of the cyclic GMP pathway in the relaxant effects of the mixtures would decrease with increasing proportions of LEM in the mixture. In fact, the potency of GLI and ODQ to inhibit the relaxant effects of the mixtures increased and decreased, respectively, as the proportion of LEM increased in the mixture (Figure 3). Additionally, since the concentration-response curves to LEM were steeper than those to SNP, the slopes of the curves to the mixtures increased as the proportion of LEM increased (Figure 1). Furthermore, due to this difference in the slope observed for the individual drugs, the contribution of cyclic GMP should predominate at low concentrations, while the contribution of K_{ATP} channel opening should be more important at high concentrations of the mixtures. Indeed, GLI produced a greater inhibition at high concentrations, decreasing the slopes of the curves, whereas ODQ produced a greater inhibition at low concentrations of the mixture (Figure 2). The biphasic nature of the curves to the SNP:LEM mixtures in the presence of ODQ may be explained because this drug reduced the maximal relaxant effect of the SNP component.

We hypothesized that under the different experimental conditions (i.e. KCl-, NA- and NA plus nifedipine-treated arteries) the differences in the relative potency of SNP compared to LEM (26.3, 8.0 and 46.8, respectively, when calculated at 30% relaxation) should affect the relative contribution of cyclic GMP and K_{ATP} channel opening of the relaxant effects of the 1:30 SNP:LEM mixture. As shown in Figure 7, when the relative potency of SNP vs LEM is varied by using mixtures with different proportions of SNP:LEM, the relative potency of the inhibitors ODQ vs GLI changed accordingly with a very good correlation. When the relative potency of ODQ vs GLI to inhibit the effect of 1:30 SNP:LEM mixture was plotted against the relative potency of SNP and LEM under the different experimental conditions (KCl-, NA- and NA plus nifedipine-treated arteries), the points fell in the same regression line as for the different mixtures in KCl-contracted arteries. Thus, the relative importance of a mechanism contributing to the final effect of drug mixtures depends not only on the relative proportion of the individual drugs in the mixture but also on the relative potency of the two drugs, and this relative potency may vary depending on the tissue and experimental conditions. By extrapolation, for a given drug exerting its effects through two independent pathways (e.g. NIC), which can be considered as a drug mixture in a fixed proportion, the relative contribution of a single mechanism should depend on the relative potency of that component.

Effects of NIC

The concentration-response curves to NIC were monophasic showing a slope which was intermediate between that of SNP and LEM. The relaxant effects of NIC on 25 mM KCl-induced contractions were inhibited by ODQ indicating that activation of the cyclic GMP pathway is an important mechanism by which NIC induces its relaxant effect in the rat aorta. In contrast, GLI had a weak effect on NIC-induced relaxation which was only observed at high concentrations of NIC, i.e. it reduced the slope of the curve. Thus, as occurred for SNP:LEM mixtures, the contribution of K_{ATP} channel opening is more important at high concentrations of NIC. However, the combination of ODQ and GLI induced a greater inhibitory action than ODQ alone, indicating that K_{ATP} channel opening may also contribute when the cyclic GMP pathway is already inhibited. Since the two pathways (K_{ATP} channels and cyclic GMP) may act simultaneously in NIC-induced relaxation and interactions between them might occur, the relative contribution of each mechanism would be unpredictable based only on the effects of SNP and LEM. Therefore, the effects of NIC were compared with those of SNP:LEM mixtures in different proportions. Based on the inhibition induced by ODQ, NIC behaved as the 1:30 SNP:LEM mixture, and since at this proportion SNP and LEM are approximately equipotent, the relaxant effects of NIC might be attributed equally to the cyclic GMP pathway and K_{ATP} channel opening. Based on the effects of GLI, NIC behaved as SNP, so that the effects of NIC might be attributed solely to the stimulation of cyclic GMP pathway. Finally, the inhibition induced by the combination of ODQ plus GLI was similar for LEM and NIC which could be interpreted if NIC exerted its relaxant effect only by activation of K_{ATP} channels. This apparent paradox can be explained by a third ODQ- and GLI-insensitive mechanism of action of NIC. In fact, it has been recently reported that in rat mesenteric arteries stimulated by the thromboxane A_2 mimetic U46619, nifedipine inhibited the relaxant action of NIC, whereas it had no effect on cromakalim-induced relaxation suggesting an additional action of NIC involving the blockade of nifedipine-sensitive Ca^{2+} channels (Fujiwara & Angus, 1996). We could not test this on KCl-induced contractions because at 10^{-7} M nifedipine fully relaxed 25 mM KCl-induced contractions. However, in our experiments, nifedipine did not modify the relaxant effects of NIC on NA-induced contractions, whereas it reduced the relaxant effect of LEM, i.e. opposite results than those reported by Fujiwara & Angus (1996). Nevertheless, these results do not exclude that NIC may relax rat aorta contracted by 25 mM KCl-induced contractions through Ca^{2+} channel blockade since this mechanism is much more effective in relaxing KCl- than NA-induced contractions. The discrepancy between our results and those of Fujiwara & Angus (1996) may be explained because agonist-induced contractions are more sensitive to the blockade of Ca^{2+} channels in resistance arteries than in the aorta (Cauvin *et al.*, 1983). Therefore, Ca^{2+} channel blockade might participate in NIC-induced relaxant effects on KCl- but not on NA-induced contractions in the rat aorta. According to this proposal, the

combination of ODQ plus GLI produced a similar inhibition of the relaxation induced by NIC and the 1:30 SNP:LEM mixture on NA-induced contractions indicating that the third relaxant mechanism of NIC is absent on NA-induced contractions. As discussed above, Ca^{2+} channel blockade modifies the relative contribution of the cyclic GMP pathway and K_{ATP} channel opening to the effects of the 1:30 SNP:LEM mixture. However, nifedipine did not modify the inhibitory action of ODQ or GLI on NIC-induced relaxation which can be explained assuming that NIC is already exerting a nifedipine-like effect. Thus, in the presence of nifedipine the relative contribution of the cyclic GMP pathway and K_{ATP} channel opening was similar for NIC and for the 1:30 mixture. In the present study, the lack of correlation (Figure 7) between the relative inhibitory action of ODQ vs GLI on NIC-induced relaxations and the relative potency of SNP and LEM under the different experimental conditions may be explained by the third, ODQ- and GLI-insensitive mechanism of relaxation. In a previous paper we suggested that the relative contribution of the mechanisms involved in the relaxant effects of NIC under different experimental conditions (piglet mesenteric and pulmonary arteries) could only be partly explained by the relative efficacy and potency of SNP vs LEM (Pérez-Vizcáino *et al.*, 1998). In these experiments, it can be observed that the experimental conditions which were relatively less sensitive to the Ca^{2+} channel blocker verapamil (pulmonary arteries or arteries stimulated by U46619) were more sensitive to inhibition by the combination of ODQ plus GLI suggesting that a third mechanism involving Ca^{2+} channel blockade may also participate in the relaxant effects of NIC. Nevertheless, the evidences for the Ca^{2+} channel blocking properties of NIC in the present paper and that of Fujiwara & Angus (1996) are only indirect so that this conclusion has to be taken with caution.

In conclusion, mixtures of SNP and LEM produced relaxant effects in isolated rat aorta through the activation of the cyclic GMP pathway and K_{ATP} channel opening. The relative importance of each mechanism, as determined by the inhibitory action of ODQ and GLI, could be predicted by the proportion of the drugs and by their relative potency. Both mechanisms were also involved in the relaxant effects of NIC. However, our results suggest that a third, ODQ- and GLI-insensitive mechanism, possibly involving Ca^{2+} channel blockade, may also participate in the relaxant effects of NIC. Therefore, NIC induces a relaxant effect on KCl-induced contractions which is mediated by activation of the cyclic GMP pathway, K_{ATP} channel opening and possibly Ca^{2+} channel blockade. NIC relaxes NA-induced contractions by activation of the cyclic GMP pathway and K_{ATP} channel opening; the possible Ca^{2+} channel blockade of NIC, although not contributing to its relaxant effects, may alter the relative potency of activation of the cyclic GMP pathway and K_{ATP} channel opening.

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