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Nicorandil directly and cyclic GMP-dependently opens K$^+$ channels in human bypass grafts

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ABSTRACT

As we previously demonstrated the role of different K$^+$ channels in the action of nicorandil on human saphenous vein (HSV) and human internal mammary artery (HIMA), this study aimed to analyse the contribution of the cGMP pathway in nicorandil-induced vasorelaxation and to determine the involvement of cGMP in the K$^+$ channel-activating effect of nicorandil. An inhibitor of soluble guanylate cyclase (GC), ODQ, significantly inhibited nicorandil-induced relaxation, while ODQ plus glibenclamide, a selective ATP-sensitive K$^+$ (K$_{ATP}$) channel inhibitor, produced a further inhibition of both vessels. In HSV, ODQ in combination with 4-aminopyridine, a blocker of voltage-gated K$^+$ (K$_V$) channels, did not modify the concentration-response to nicorandil compared with ODQ, whereas in HIMA, ODQ plus iberiotoxin, a selective blocker of large-conductance Ca^{2+}-activated K$^+$ (BK$_{Ca}$) channels, produced greater inhibition than ODQ alone. We showed that the cGMP pathway plays a significant role in the vasorelaxant effect of nicorandil on HSV and HIMA. It seems that nicorandil directly opens K$_{ATP}$ channels in both vessels and BK$_{Ca}$ channels in HIMA, although it is possible that stimulation of GC contributes to K$_{ATP}$ channels activation in HIMA. Contrary, the activation of K$_V$ channels in HSV is probably due to GC activation and increased levels of cGMP.

1. Introduction

Nicorandil (N-[2-hydroxyethyl])-nicotinamide nitrate) is an antianginal agent with a distinctive dual mechanism of action. The drug exerts its pharmacodynamic effect through the opening of K$^+$ channels and by increasing cyclic guanosine monophosphate (cGMP) levels by activating guanylate cyclase (GC) via its nitrate (1). The opening of K$^+$ channels in vascular smooth muscle cells leads to K$^+$ efflux and membrane hyperpolarisation, which inhibits calcium influx and promotes relaxation (2,3). Cyclic GMP activates the cGMP-dependent protein kinase (PKG), which initiates phosphorylation events that lead to vascular relaxation (4).

There is some evidence that plasmalemmal K$^+$ channels play a significant role in PKG-induced vasorelaxation (5). For example, large-conductance Ca^{2+}-activated K$^+$ (BK$_{Ca}$) channels and voltage-gated K$^+$ (K$_V$) channels have been found to be activated via the cGMP pathway in human umbilical (6) and human pulmonary (7) arteries. The properties of nicorandil as a hybrid compound raise the possibility that activation of GC and the resulting increase in cGMP might contribute to K$^+$ channel activation (8).

Recently, we have demonstrated that different K$^+$ channel subtypes are involved in the nicorandil-induced relaxation of human bypass grafts. In particular, we have shown that: i) nicorandil endothelium-independently relaxed human saphenous vein (HSV)

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and human internal mammary artery (HIMA), the most commonly used conduit vessels for coronary artery bypass surgery; ii) ATP-sensitive K^+ (K_{ATP}) channels and 4-aminopyridine (4-AP)-sensitive K^+ channels located in the smooth muscle of HSV mediated the relaxation induced by nicorandil and iii) K_{ATP} and BK_{Ca} channels are probably involved in the action of nicorandil on HIMA (9). Until now, it has not been clear whether the nicorandil-induced activation of these K^+ channels in HSV and HIMA occurs directly or dependently of cGMP. Previous studies have demonstrated that the K^+ channel-dependent effect of nicorandil is independent of cGMP, acting on the channel (10,11). In contrast, some authors suggested the possible involvement of cGMP on nicorandil-induced activation of K_{ATP} (8) and BK_{Ca} (12) channels. Thus, the principal aim of the present study was to analyse the contribution of the cGMP pathway to the nicorandil-induced vasorelaxation of HSV and HIMA and to determine the role of cGMP as a second messenger in the K^+ channel-activating effect of nicorandil.

2. Materials and methods

2.1. Tissue preparation

Discarded segments of HSV (n = 32) and HIMA (n = 39) were obtained from 53 male patients (mean age ± S.E.; 63 ± 6 years) undergoing coronary artery bypass surgery. All patients were informed in detail about the aims of investigation and gave their written consent for the excision of remaining tissue. The experiments involving human vessels were approved by the Ethics Committee of Institute for Cardiovascular Diseases "Dedinje" and carried out in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice. After excision, the vessel segments were immediately placed in cold (4 °C) Krebs-Ringer-bicarbonate solution, and transported to the laboratory for study.

Excess fat and connective tissue were dissected and the HSV and HIMA segments were cut into 3-mm rings. One to two rings were obtained from each vessel segment. The endothelium was removed mechanically by gently rubbing the intimal surface with a stainless steel wire. Denudation of endothelium was verified by the inability of veins and arteries to relax after treatment with acetylcholine (1 μM) (13).

The rings were mounted between two stainless-steel triangles in a 10 ml organ bath filled with Krebs-Ringer-bicarbonate solution, maintained at 37 °C and aerated with 95% O_2–5% CO_2. The preparation was stretched to a resting force of 2 g (14,15) and equilibrated for 60 min, with frequent washing and force adjustment.

2.2. Experimental protocol

After equilibration, HSV and HIMA rings were contracted with phenylephrine (10 μM). The concentration of phenylephrine was elected on the basis of previous publications (16,17). When the contractile response by this vasoconstrictor agent reached a stable plateau, increasing cumulative concentrations of nicorandil (0.001 μM–300 μM) were added to the bath. Increasing concentrations of nicorandil were added after the relaxation evoked by the previous concentration reached its plateau or after 20 min if no response was obtained. To determine the role of the cGMP pathway in nicorandil-induced relaxation, the rings were treated with (1) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), an inhibitor of soluble GC, and (2) different K^+ channel blockers for minimum 20 min before the next concentration-response curve to nicorandil was assessed. Control rings had no blocking drugs added before titration with nicorandil.

2.3. Treatment of data and statistics

The relaxation produced by each concentration of nicorandil was measured and expressed as a percentage of the maximum possible relaxation (i.e., relaxation back to the baseline tension). The concentration of nicorandil producing 50% of the maximum response (EC_{50}) was calculated from each concentration–relaxation curve using a non-linear least squares fit with a logistic function and presented as pD_2 ($pD_2 = -\log EC_{50}$).

The results are expressed as the mean ± standard deviation (SD). The value of n indicates the number of experiments. Significant difference between means of different groups was determined by the unpaired Student's t-test, and a P value <0.05 was considered statistically significant. All calculations were performed using the computer program Origin (version 8; OriginLab Corporation, Northampton, MA, USA).

2.4. Drugs

The following drugs were used: nicorandil, phenylephrine hydrochloride, acetylcholine iodide, ODQ, glibenclamide, 4-AP and iberiotoxin (Sigma–Aldrich Inc., St. Louis, MO, USA). Nicorandil was dissolved in distilled water prior to being used. Glibenclamide and ODQ were dissolved in dimethyl sulfoxide. Previous experiments established that the solvents used had no effects on the preparations at the concentrations applied (data not shown). The drugs were added directly to the bath, and the concentrations given are the calculated final concentrations in the bath solution.

Krebs-Ringer-bicarbonate solution had the following composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 11.1 glucose; pH 7.4.

3. Results

3.1. Effects of nicorandil on HSV and HIMA without endothelium pre-contracted by phenylephrine

Nicorandil (0.001 μM–300 μM) induced a concentration-dependent relaxation of denuded HSV and HIMA rings with pD_2 values of 5.87 ± 0.10 (maximal response 100 ± 3%, n = 8) (Fig. 1A) and 5.60 ± 0.09 (maximal response 100 ± 4%, n = 8) (Fig. 1B), respectively. The difference between the pD_2 values was not statistically significant ($P > 0.05$).

A time-matched control was used throughout the experiments, and any observed changes in tension were used for the subsequent adjustment of the drug-induced relaxation. The following method was used: the percentage relaxation in control conditions was compared with that in the presence of nicorandil at the same time-point after phenylephrine application.

3.2. Effects of ODQ and the combination of ODQ and K^+ channel blockers on the relaxation response of HSV and HIMA to nicorandil

In control rings, to which ODQ or a combination of ODQ plus K^+ channel blockers were not added, the relaxation induced by nicorandil was similar in initial and subsequent concentration-response curves. No significant differences with respect to either pD_2 or maximal response were found between the relaxation curves.

To examine the role of soluble GC in nicorandil-induced relaxation, the effects of ODQ (10 μM), an inhibitor of soluble GC, were investigated. Pre-incubation with ODQ caused a significant inhibition of the nicorandil-stimulated relaxation of HSV (Table 1 and Fig. 1A) and partially antagonised the nicorandil-induced relaxation of HIMA (Table 2 and Fig. 1B).
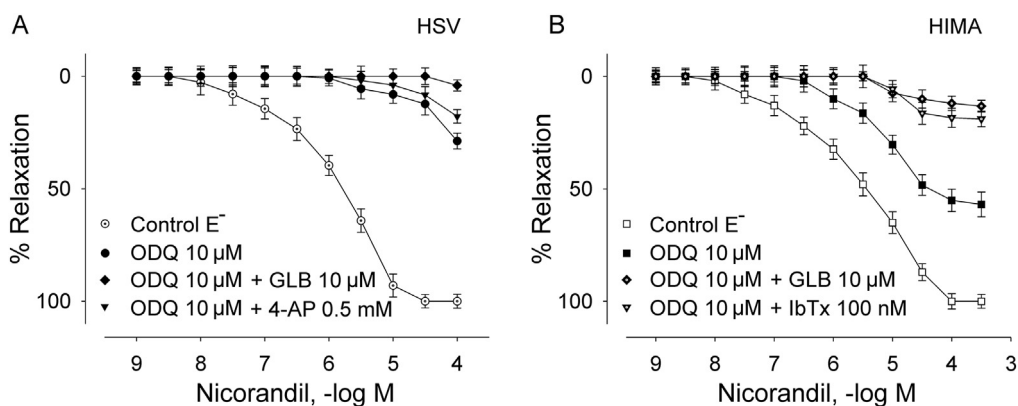
Fig. 1. Relaxant responses to nicorandil in human saphenous vein (HSV) (A) and human internal mammary artery (HIMA) (B) pre-contracted with phenylephrine (10 μM) in the absence and presence of ODQ and K^+ channel blockers. Concentration-response curves to nicorandil in the absence and presence of ODQ (10 μM) (A, B), ODQ (10 μM) plus glibenclamide (GLB, 10 μM) (A, B), ODQ (10 μM) plus 4-aminopyridine (4-AP, 0.5 mM) (A) or ODQ (10 μM) plus iberiotoxin (IbTx, 100 nM) (B). Endothelium was removed mechanically by rubbing with a steel wire. Responses are expressed as a percentage of the maximum possible relaxation, i.e., the return of vessel tension to the pre-phenylephrine level. Each point represents the mean ± SD (n = 6–8).

To determine the role of cGMP as a second messenger in the K^+ channel-activating effect of nicorandil, we used combinations of ODQ and K^+ channel blockers (glibenclamide, 4-AP, iberiotoxin) that had previously been shown to significantly inhibit the nicorandil-induced relaxation of HSV and HIMA [9].

The combination of ODQ plus glibenclamide (10 μM), a selective K_{ATP} channel inhibitor, produced a greater inhibition of nicorandil-induced relaxation of both HSV (Table 1 and Fig. 1A) and HIMA (Table 2 and Fig. 1B) than did ODQ alone.

In HSV, pre-incubation with ODQ in combination with 4-AP (0.5 mM), a widely used blocker of K_V channels, did not significantly modify the concentration-response to nicorandil compared with ODQ alone (Table 1 and Fig. 1A).

In HIMA, the combination of ODQ plus iberiotoxin (100 nM), a selective blocker of BK_{Ca} channels, produced inhibition of nicorandil-induced relaxation, which was significantly greater than that induced by ODQ alone (Table 2 and Fig. 1B).

Glibenclamide (10 μM), ODQ (10 μM) and 4-AP (0.5 mM) had no apparent effect on the resting tension or the phenylephrine-induced contraction. The presence of iberiotoxin (100 nM) caused an increase in resting tension (data not shown). Changes in tension were recorded, and 100% relaxation was considered when the tension returned to baseline level [18].

4. Discussion

Nicorandil induces vasodilation via two main mechanisms of action, activation of K^+ channels, acting as a potassium channel opener, and increasing cGMP levels by stimulation of GC, like a nitrate (Fig. 2). The relative contribution of each of these vasodilator mechanisms of action depends on the experimental protocol and the preparations studied [11]. To analyse the involvement of cGMP in nicorandil-induced relaxation of the isolated, endothelium-denuded HSV and HIMA grafts, we used ODQ (10 μM), an inhibitor of soluble GC. It has been shown that ODQ inhibits NO-stimulated soluble GC activity and does not affect the activity of either particulate GC or adenylate cyclise [19]. Since the IC_{50} value of ODQ for the NO-stimulated enzyme ranges from 0.2 to 0.7 μM, it can be expected that ODQ, in concentration of 10 μM, achieves virtually complete inhibition of NO-stimulated soluble GC [20]. Our results show that the relaxant effect of nicorandil on phenylephrine-induced contraction of the HSV was significantly inhibited by ODQ (E_{max} reduced from 100 ± 2% to 29 ± 3%). This observation is in agreement with results reported by Wang et al. [21] indicating that activation of the cGMP pathway is an important mechanism by which nicorandil induced its relaxant effect on HSV. In HIMA, ODQ produced partial inhibition (E_{max} reduced from 100 ± 3% to 57 ± 6%) suggesting that the cGMP pathway is, at least in part, involved in the action of nicorandil on this blood vessel. The differing nicorandil responses of HSV and HIMA in the presence of ODQ could be the consequence of the well-documented tissue-specific action of nicorandil. Additionally, it is well known that the vasodilation caused by nitrates is essentially predominant in the venous circulation [22,23].

As we have previously shown the roles of different K^+ channel subtypes (K_{ATP}, BK_{Ca} and 4-AP-sensitive K^+ channels) in the action

Table 1
Effects of ODQ and combinations of ODQ and K^+ channel blockers on the nicorandil-induced relaxation of HSV.

	n	pD_2	E_{max} (%)
Control E⁻	6	5.88 ± 0.19	100 ± 2
ODQ 10 μM	6	nc	29 ± 3**
ODQ 10 μM	7	nc	29 ± 2
ODQ 10 μM + GLB 10 μM	7	nc	4 ± 1##
ODQ 10 μM	7	nc	28 ± 4
ODQ 10 μM + 4-AP 0.5 mM	7	nc	18 ± 2

The results are expressed as mean ± SD. n, number of experiments; $pD_2 = -\log EC_{50}$; E_{max}, maximal relaxation; E⁻, without endothelium; GLB, glibenclamide; 4-AP, 4-aminopyridine; nc, not calculated (because of the low efficacy). ** P < 0.01 vs. control group, ## P < 0.01 vs. ODQ-treated group.

Table 2
Effects of ODQ and combinations of ODQ and K^+ channel blockers on the nicorandil-induced relaxation of HIMA.

	n	pD_2	E_{max} (%)
Control E⁻	7	5.58 ± 0.18	100 ± 3
ODQ 10 μM	7	4.13 ± 0.17**	57 ± 6**
ODQ 10 μM	7	4.14 ± 0.13	55 ± 3
ODQ 10 μM + GLB 10 μM	7	nc	13 ± 2##
ODQ 10 μM	8	4.09 ± 0.21	57 ± 4
ODQ 10 μM + IbTx 100 nM	8	nc	19 ± 3##

The results are expressed as mean ± SD. n, number of experiments; $pD_2 = -\log EC_{50}$; E_{max}, maximal relaxation; E⁻, without endothelium; GLB, glibenclamide; IbTx, iberiotoxin; nc, not calculated (because of the low efficacy). ** P < 0.01 vs. control group, ## P < 0.01 vs. ODQ-treated group.
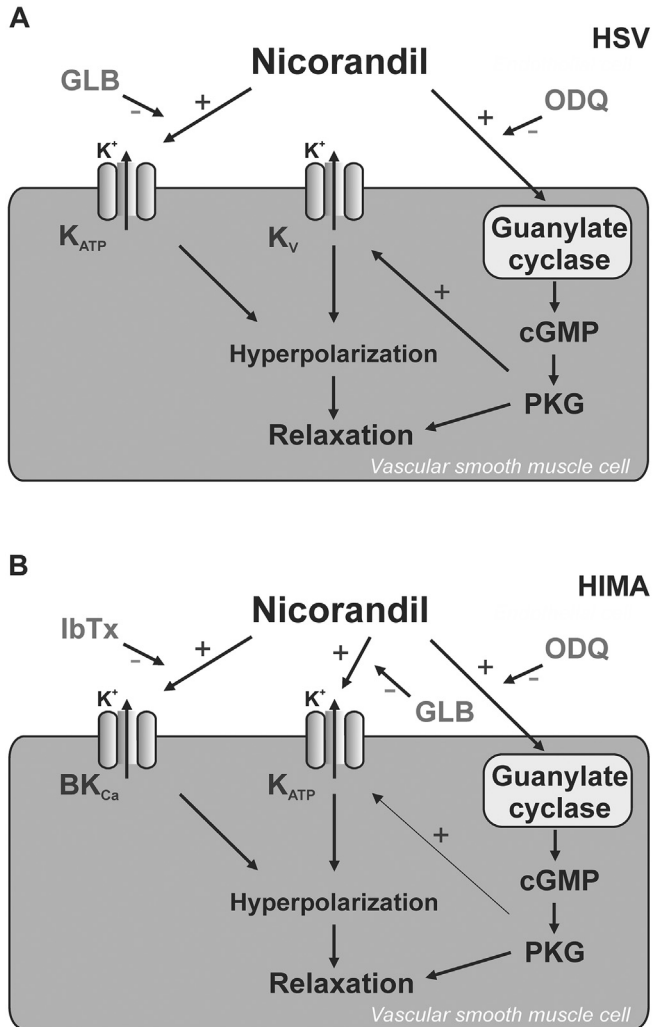
A   **HSV**

B   **HIMA**

Fig. 2. Mechanism of vasorelaxant effect of nicorandil on human saphenous vein (HSV) (A) and human internal mammary artery (HIMA) (B). This figure suggests that nicorandil exerts its vasorelaxant effect via both mechanisms, i.e., K^+ channel opening and guanylate cyclase stimulation. Opening of ATP-sensitive K^+ (K_{ATP}) channels on both vessels, as well as large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel opening in HIMA, most likely occurs as a result of direct activation by nicorandil, although there is a possibility that stimulation of guanylate cyclase contributes to the K_{ATP} channels activation in HIMA. In contrast, activation of voltage-gated K^+ (K_V) channels in HSV is most likely due to guanylate cyclase activation and an increase of cyclic guanosine monophosphate (cGMP) levels. IbTx, iberiotoxin; GLB, glibenclamide; PKG, cGMP-dependent protein kinase.

of nicorandil on HSV and HIMA (9), we further sought to determine whether the drug opens these channels directly or in a cGMP-dependent manner.

K_{ATP} channels have several physiological roles and respond to changes in the cellular metabolic state, as well as to a number of endogenous vasodilators that act mostly through the stimulation of cAMP-dependent protein kinase (PKA). Functional modulation of K_{ATP} channels by cGMP, presumably through activation of PKG, was first demonstrated in vascular smooth muscle cells (24), and later in pancreatic β-cells (25), neuronal (26) and cardiac (27) cells. To investigate the involvement of cGMP in the nicorandil-stimulated activation of K_{ATP} channels in HSV and HIMA, we used a combination of ODQ and glibenclamide (10 μM). Glibenclamide is known as one of the specific blockers of K_{ATP} channels at concentrations up to 10 μM, although when used in a high concentration (>30 μM), it may block some other types of K^+ channels (8,28,29). Earlier studies with nicorandil demonstrated that K_{ATP} channel opening

and GC stimulation were independent pathways that induced the relaxation of isolated vessels (10,11,30). In contrast, Davie et al. (8) suggested the possible involvement of cGMP in the activation of K_{ATP} channels by nicorandil. We showed that nicorandil is likely to exert a direct stimulatory action on smooth muscle K_{ATP} channels, as glibenclamide further reduced the relaxation of HSV and HIMA in the presence of ODQ. Additionally, by comparing the maximal inhibitory effect ($100 - E_{max}$) induced by the combination of ODQ and glibenclamide in HSV rings (96%), with total effect of blockers used individually (71% and 36% (9), respectively), we confirmed that in HSV these two pathways act in parallel. However, the same comparison made in HIMA (maximal inhibition of nicorandil-induced relaxation in the presence of ODQ and glibenclamide in combination with 87% vs. ODQ alone with 43%, and glibenclamide alone with 71% (9)) may indicate existence of some interaction. It may imply possible contribution of cGMP pathway in nicorandil-induced K_{ATP} channels activation in HIMA.

On the other hand, since the combination of ODQ and glibenclamide was not sufficient to produce complete inhibition of nicorandil-induced relaxation of HIMA, it may suggest the possible involvement of some complementary mechanism of action in this vessel. According to our previous findings, the complementary mechanism of action could be the activation of BK_{Ca} channels (9). To verify the possibility that BK_{Ca} channels are directly activated by nicorandil, we used ODQ in combination with iberiotoxin (100 nM) (31). Iberiotoxin is a selective blocker of BK_{Ca} channels which does not block small-conductance (SK_{Ca}) and intermediate-conductance (IK_{Ca}) Ca^{2+}-activated K^+ channels, nor other types of K^+ channels (32,33).

It is well known that BK_{Ca} channels are primarily activated either by an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or by membrane depolarisation (34). In addition, BK_{Ca} channels are regulated by various protein kinases, including PKG (35). These channels have been found to be a downstream effector of the soluble GC/cGMP pathway and to modulate the vasodilator response to both exogenous nitrovasodilators and endogenous receptor-mediated NO release in various arteries, such as human coronary (36) and human pulmonary (7) arteries. However, some authors showed that NO donors could activate BK_{Ca} channels independently of the soluble GC/cGMP pathway (21,37,38), which is in agreement with the results of the present study. Namely, we showed that the use of iberiotoxin in addition to ODQ caused a further reduction in the relaxation of HIMA (total block of nicorandil effect was 81%), which seemed additive relative to either agent alone (blocking effects of iberiotoxin (34%) (9) plus ODQ (43%)). This result suggests that nicorandil may open BK_{Ca} through a cGMP-independent mechanism, i.e. these two pathways of nicorandil action act in parallel. Consistent with these data, in single smooth muscle cells isolated from the rat mesenteric artery, an ODQ-insensitive increased activity of BK_{Ca} channels has been reported (39).

K_V channels are considered the major K^+ channels controlling resting membrane potential and $[Ca^{2+}]_i$ in vascular smooth muscle cells (40,41). These channels appear to be activated by a few intracellular signalling pathways (42). Although there are numerous data to support a physiological role for PKA activation of K_V channels, data supporting a similar role for PKG are less abundant (43). For example, Sobey and Faraci (44) demonstrated that the dilatation of basilar arteries by sodium nitroprusside and 8-bromo cGMP was selectively inhibited by 4-AP, suggesting the participation of Kv channel activation in these cGMP-dependent responses.

Previously, we showed that 4-AP-sensitive K^+ channels are involved in the nicorandil-induced relaxation of HSV (9). To further investigate the mechanism by which nicorandil activates K_V

channels, we tested the effects of nicorandil in the presence of 4-AP (0.5 mM) plus ODQ. 4-AP, in concentration of 0.5 mM (45), is able to block K_V channels, but has no effect on BK_{Ca} or inward rectifier K^+ (Kir) channels (32), nor does it affect the function of K_{ATP} channels (46). By comparing the effects of ODQ and a combination of ODQ and 4-AP on nicorandil-induced relaxation, it appears that the maximal inhibition is similar. Further, attenuation of relaxation made by ODQ and 4-AP when used in combination (82%) is less than additive attenuation made by both agents when used alone (71% and 57% (9), respectively). These results indicate existence of interaction between these pathways, suggesting that nicorandil probably activates K_V channels via a cGMP-dependent signalling mechanism. This is consistent with results previously obtained on human umbilical (6), pulmonary (7) and basilar (44) arteries.

K_V channels represent a diverse family, of which the K_V1 (Shaker-related) family is the most prominently expressed in the vasculature (47). Similar to BK_{Ca}, K_V channels are heteromultimeric proteins composed of transmembrane pore-forming α and cytosolic β subunits. Analysis of the protein sequence of K_V channels known to be present in vascular smooth muscle cells indicates that the $K_V1.5$ and the $K_V\beta1$ subfamily have consensus PKG phosphorylation sites that may be responsible for the activation of K_V channels by this kinase (43). In HSV, the expression of $K_V1.3$ and $K_V1.5$ channels was demonstrated (48,49). However, we have previously shown that margatoxin, a selective inhibitor of $K_V1.3$ channels, did not modify nicorandil-induced relaxation of HSV (9). This result raised the possibility that the activation of $K_V1.5$ through a cGMP-dependent pathway is at least partially involved in the relaxation of HSV induced by nicorandil.

In conclusion, we have demonstrated that the cGMP pathway plays a significant role in the vasorelaxant effect of nicorandil on HSV and HIMA. Furthermore, it seems that nicorandil directly opens K_{ATP} channels in both vessels and BK_{Ca} channels in HIMA, although there is a possibility that stimulation of GC contributes to the K_{ATP} channels activation in HIMA. In contrast, the activation of K_V channels in HSV is most likely due to the activation of soluble GC and an increase in cGMP levels.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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