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Effects of large conductance Ca^{2+}-activated K^+ channels on nitroglycerin-mediated vasorelaxation in humans

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Abstract

Nitric oxide (NO)-induced vasorelaxation and the regulation of endothelial superoxide anion levels is partly mediated by vascular large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels. Nitroglycerin acts through the release of NO and its effect is modulated by changes in endothelial superoxide levels. This study examines the effect of BK_{Ca} channel blockade on nitroglycerin-induced vasorelaxation in human arterial and venous vascular segments and whether responses to BK_{Ca} channel blockade are influenced by the development of venous nitroglycerin tolerance. Dose–relaxation curves to nitroglycerin (10^{-10}–10^{-4} M) were obtained in segments of the saphenous vein and the left mammary artery. Studies were performed with and without pre-incubation with the BK_{Ca} channel blocker iberiotoxin (10^{-7} M) and venous tolerance to nitroglycerin were induced by a 24-h i.v. infusion (0.5 μg/kg/min). Iberiotoxin reduced the vasorelaxant effect of nitroglycerin (E_{max}) by 60% in endothelium-intact arteries and 13% in endothelium-denuded arteries ($P<0.05$). Development of nitroglycerin tolerance did not affect the response to iberiotoxin in the venous vascular segments ($P>0.05$) and (compared to arterial segments) veins were less sensitive to BK_{Ca} channel blockade (30% reduction in E_{max}) or endothelial removal. The results suggest that primarily arterial effects of nitroglycerin are significantly inhibited by changes in the activity of the endothelial BK_{Ca} channels. Although endothelial BK_{Ca} are likely regulators of mechanisms underlying arterial tolerance development to nitroglycerin, they do not appear to play a role in human venous nitroglycerin tolerance development.

Keywords: Nitroglycerin; Iberiotoxin; Nitrate tolerance; K^+ channel

1. Introduction

Nitroglycerin induces vasorelaxation by releasing nitric oxide (NO), which relaxes the vascular smooth muscle cells through an increase in cyclic guanosine monophosphate (cGMP)-levels. Recent animal studies show that this response is partly mediated by activation of vascular large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (Bang et al., 1999; Carrier et al., 1997; Waldron and Cole, 1999). The contribution of the BK_{Ca} channel system to NO-mediated vasodilation varies between different animal species and different vascular beds (Feletou and Vanhoutte, 2000; Waldron and Cole, 1999). Currently, the interactions between BK_{Ca} channels and nitroglycerin-mediated vasorelaxant responses in humans are not clear.

The usefulness of organic nitrates like nitroglycerin is limited by the development of tolerance during continuous therapy. The exact mechanisms of in vivo nitrate tolerance are likely to be multifactorial. One of the major mechanisms, however, relates to a nitroglycerin-mediated endothelium-dependent increased vascular production and bioavailability of superoxide anion (O^{2-}) (Münzel et al., 1995). In arterial vascular segments, O^{2-} is produced by endothelial membrane-bound oxidases and can directly inactivate NO formed by nitroglycerin biotransformation. Since the activity of the membrane-bound oxides are regulated by K^+ channels (and the membrane potential) (Münzel et al., 1999a), changes in the activity of K^+ channels may be involved in the development of arterial tolerance to nitroglycerin. The findings that both endothelial removal and administration of hydralazine, which acts as a BK_{Ca} channel opener (Bang et al., 1998), prevents nitroglycerin tolerance development in rabbit arteries [(Münzel et al., 1996), are in

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line with this assumption. It is, however, not clear to what degree the vascular endothelium and BK_{Ca} channels participate in the development of venous tolerance to nitroglycerin.

The aim of the present study was, first to examine the contribution of BK_{Ca} channels to the arterial and venous vasorelaxant effects of nitroglycerin in humans, and next to investigate whether such an interaction is influenced by the development of nitrate tolerance.

2. Materials and methods

2.1. Patients and experimental design

The study population consisted of two groups. All study subjects gave informed consent. The study was approved by the Ethics Committee and followed the principles of the Helsinki Declaration.

2.1.1. Group 1

To investigate the effect of BK_{Ca} channel modulation on nitroglycerin-mediated arterial vasorelaxation, 15 patients (Table 1) with stable angina pectoris undergoing coronary bypass surgery were investigated. During the operation, the discarded segments of the left internal mammary artery were collected and placed in ice-cold Krebs solution for immediate myographic examination of nitroglycerin responsiveness. None of the patients received Ca^{2+}/K^{+} channel modulating agents like pinacidil and nicorandil or long-acting nitrates within the last 5 days before the operation. Beta-blockers were used in seven patients and angiotensin converting enzyme inhibitors in four patients (Table 1).

2.1.2. Group 2

This group included 12 patients with chronic lower limp ischemia awaiting infrainguinal arterial reconstruction using the in situ saphenous vein bypass technique. Baseline demographics are shown in Table 1. None of the patients used calcium antagonists, or K^{+} channel openers and any use of long-acting nitrates were suspended at least 5 days prior to surgery. Using a randomized double-blind parallel-group design, each patient was randomized (sealed envelopes) to receive either intravenous nitroglycerin (0.5 μg/kg/min) ($n=6$, nitroglycerin group) or corresponding placebo (0.9% saline) ($n=6$, control group) for 24 h immediately before surgery. Blood pressure and heart rate were measured during the start of the infusion (0.2 μg/kg/min) and every 15 min until an infusion rate of 0.5 μg/kg/min was reached. This dose of nitroglycerin is five times higher than doses previously shown to augment venous volume and to dilate the human radial artery in vivo (Boesgaard et al., 1994). The infusion rate was diminished if the systolic blood pressure was reduced by more than 40 mm Hg or reached a level lower than 100 mm Hg. During surgery, a segment of the greater saphenous vein was detained and placed in ice-cold Krebs solution for immediate myographic examination.

Table 1
Baseline characteristics of patients included in the study

	Saphenous vein		Left internal mammary artery
	Control ($n=6$)	Nitroglycerin ($n=6$)	($n=15$)
Age (mean ± S.E.M.)	77 ± 4	70 ± 3	65 ± 3
Diabetes mellitus	3	1	1
Hypertension	1	1	–
ABPI	0.35	0.40	–
ACE-inhibitors	2	1	4
Beta-blockers	1	0	7
Nitrates	0	0	0
Ca^{2+} channel antagonists	0	0	1
Digoxin	0	0	1
Diuretics	2	1	3

ACE: angiotensin-converting enzyme. IDDM: insulin-dependent diabetes mellitus. ABPI: the ankle-brachial pressure index. NTG: nitroglycerin.

2.2. Vascular reactivity studies

Four vascular ring segments (length: 2.0 mm; outer diameter: 500–800 μm) were prepared from each vascular segment, and the endothelium was gently removed from two of the segments. By inserting two fine stainless threads (outer diameter: 40 μm) into the lumen, the segments were mounted in a precision myograph (Multi Myograph model 610 M), and connected to a highly isometric strain-gauge transducer, allowing recordings of isometric tension changes. Endothelium-denuded and endothelium-intact vessel segments from the same patient were studied simultaneously. During investigations, the segments were suspended in a 5 ml organ bath containing Krebs solution (in mM: NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.15, $NaHCO_3$ 24.9, KH_2PO_4 1.15 and glucose 5.5), which was constantly aerated (O_2 95% and CO_2 5%), and kept at pH = 7.4. Via the amplifier in the myograph, transducer signals were displayed on an eight-channel recorder (MacLab/8s). This method has been described previously (Bang et al., 1999).

Arterial and venous segments were both stretched to a resting tension of 1 g (optimal contraction in response to K^{+}-depolarization) and allowed to equilibrate for 60 min. Indomethacin 3×10^{-6} M (inhibits prostaglandin synthesis) was added in all experiments and segments were precontracted with norepinephrine 3×10^{-7} M. The presence or absence of a functionally intact endothelium was examined by recording the endothelium-dependent vasodilatory response to acetylcholine (10^{-6} M). Following a washout period of 45 min, the segments were precontracted with prostaglandin $F_{2\alpha}$ 10^{-6} M to produce submaximal tension in both the artery and vein. At a level of stable contraction, each segment was exposed to increasing concentrations of nitroglycerin (10^{-10}–10^{-4} M). In order to study the effect of selective BK_{Ca} channel blockade on

nitroglycerin responsiveness, the vasorelaxant effect of nitroglycerin was examined with or without pre-incubation with iberiotoxin 10^{-7} M for 20 min. Using this experimental set-up, the effect of iberiotoxin on nitroglycerin-mediated vasorelaxation was examined in endothelium-intact and endothelium-denuded human venous (control group) and arterial segments not previously exposed to nitroglycerin, and in venous segments (nitroglycerin group) exposed to nitroglycerin for 24 h.

2.3. Drugs

Acetylcholine, iberiotoxin and indomethacin were purchased from Sigma. Norepinephrine and nitroglycerin from SAD, Denmark, and prostaglandin $F_{2\alpha}$ (Dinoprost) from Upjohn. Acetylcholine, prostaglandin $F_{2\alpha}$, nitroglycerin and iberiotoxin were all prepared in 0.9% saline, while indomethacin was dissolved in a $NaHCO_3$ buffer. All chemicals were adjusted to pH 7.4.

2.4. Statistics

All data are presented as mean ± S.E.M. Nitroglycerin-induced vasorelaxation is expressed as the percentage of relaxation relative to the level of precontraction (before nitroglycerin administration). Values of E_{max} and EC_{50} (given as $-\log EC_{50}$) are estimated by fitting the concentration–relaxation curve to the three-parameter logistic equation (Hill equation) by nonlinear regression analysis. Comparisons between experimental groups were done by paired or unpaired Student's *t*-tests as appropriate. Comparisons between more than two E_{max} values were performed using analysis of variance with post hoc Bonferroni analysis. Statistical significance was assumed at $P<0.05$.

3. Results

3.1. Effect of endothelial removal and BK_{Ca} channel blockade on arterial and venous nitroglycerin responses

Nitroglycerin produced concentration-dependent relaxation of human endothelium-intact left internal mammary artery preparations ($-\log EC_{50}$ 6.8 ± 0.1, E_{max} 76 ± 2%) (Fig. 1A). This response was markedly inhibited by iberiotoxin which reduced E_{max} by approximately 60% (iberiotoxin; $-\log EC_{50}$ 6.8 ± 0.1, E_{max} 31 ± 1%, $P<0.05$)

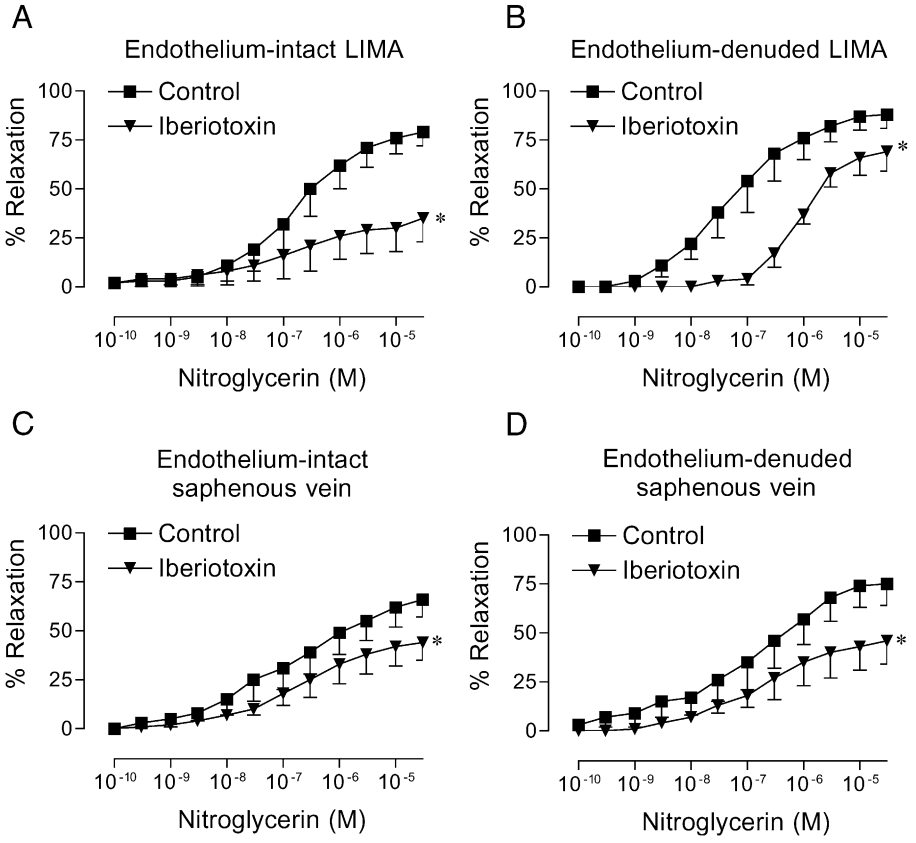

Fig. 1. Effect of iberiotoxin on nitroglycerin dose–relaxation curves in endothelium-intact and endothelium-denuded arteries and veins. LIMA: left internal mammary artery. *: E_{max} significantly different from control group ($P<0.05$).

(Fig. 1A). In endothelium-denuded arteries, nitroglycerin produced a concentration-dependent vasorelaxation with an expected (3-fold) higher potency than in endothelium-intact vessels ($-\log EC_{50}$ 7.3 ± 0.1, E_{max} $83 \pm 2\%$). However, iberiotoxin only reduced E_{max} by 13% in these endothelium-denuded arteries ($-\log EC_{50}$ 6.0 ± 0.1, E_{max} $72 \pm 1\%$, $P < 0.05$) (Fig. 1B), suggesting an endothelium-dependent interaction between nitroglycerin-induced vasorelaxation and the BK_{Ca} channel.

Saphenous vein segments were less sensitive than mammary artery to nitroglycerin. In contrast to the findings in arterial segments, the response to nitroglycerin were similar in endothelium-intact ($-\log EC_{50}$ 6.8 ± 0.2, E_{max} $64 \pm 3\%$) and endothelium-denuded veins ($-\log EC_{50}$ 6.7 ± 0.1, E_{max} $74 \pm 3\%$) ($P > 0.05$) (Fig. 1C and D). Endothelial denudation also did not affect the inhibitory response of iberiotoxin on E_{max}. During iberiotoxin treatment, the maximal response to nitroglycerin was reduced by 30% in the endothelium-intact veins (E_{max} $45 \pm 2\%$, $-\log EC_{50}$ 6.6 ± 0.1) and by 36% ($P > 0.05$) in the endothelium-denuded veins (E_{max} $47 \pm 2\%$, $-\log EC_{50}$ 6.6 ± 0.1) (Fig. 1C and D). The effect of iberiotoxin on nitroglycerin responsiveness (E_{max}) was diminished by a factor of 2.4 in endothelium-intact veins when compared to endothelium-intact arteries ($P < 0.05$).

Thus, endothelial removal and/or BK_{Ca} channel blockade with iberiotoxin predominantly affects the vasorelaxant response to nitroglycerin in the arterial vascular bed.

3.2. Effect of endothelial removal and BK_{Ca} channel inhibition on nitroglycerin responses in human nitroglycerin tolerant veins

Compared with segments from the control group, segments from the nitroglycerin group were less responsive to nitroglycerin compatible with the development of venous tolerance to nitroglycerin (Fig. 2). In endothelium-intact segments, E_{max} was reduced by 31% from $64 \pm 3\%$ (control) to $44 \pm 1\%$ (nitroglycerin group) and the dose–relaxation curve was significantly right-shifted with a 2.4-fold increase in EC_{50} in the nitroglycerin group ($-\log EC_{50}$ 6.8 ± 0.2 vs. 6.4 ± 0.1). A similar pattern was seen in endothelium-denuded vessels (nitroglycerin group; $-\log EC_{50}$ 6.2 ± 0.1, E_{max} $42 \pm 1\%$ vs. controls; $-\log EC_{50}$ 6.7 ± 0.1, E_{max} $74 \pm 3\%$) ($P < 0.05$), suggesting that in vivo induced tolerance can be detected ex vivo in both intact- and endothelium-denuded human veins after a 24-h intravenous infusion of nitroglycerin (Fig. 2).

As seen in the nontolerant control vessels, iberiotoxin significantly reduced the vasorelaxant effect of nitroglycerin in both endothelium-intact and endothelium-denuded tolerant vessels (Fig. 2). The response (an approximately 35% decrease in E_{max} and right-shift of the curve) was not different from that seen in control vessels and did not differ between intact ($-\log EC_{50}$ 6.8 ± 0.1, E_{max} $28 \pm 1\%$) and endothelium-denuded veins ($-\log EC_{50}$ 6.9 ± 0.1, E_{max} $26 \pm 1\%$) ($P > 0.05$). The results suggest that neither the venous endothelium nor BK_{Ca} channels contribute significantly to venous tolerance development to nitroglycerin.

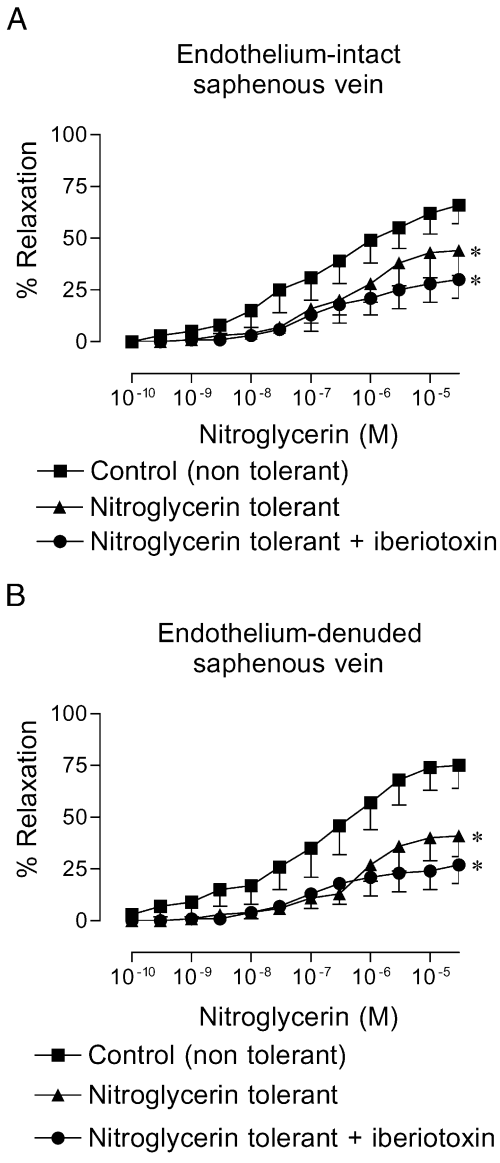

Fig. 2. Effect of iberiotoxin on nitroglycerin tolerance in (A) endothelium-intact and (B) endothelium-denuded veins. *: E_{max} significantly different from control group ($P < 0.05$).

4. Discussion

This is the first study to investigate the role of the endothelium and BK_{Ca} channels on nitroglycerin responsiveness in human arteries and veins. The main findings are (1) that inhibition of vascular BK_{Ca} channels differently affects arterial and venous vasorelaxant effect of nitroglycerin and (2) that neither the vascular endothelium nor BK_{Ca} channels are likely mediators of venous tolerance development to nitroglycerin in humans.

4.1. BK_{Ca} channels, NO and superoxide anion interactions

BK_{Ca} constitute a subgroup among many different types of K^+ channels and serve as a negative feedback mechanism limiting the depolarizing and Ca^{2+} increasing effects of vasoconstrictors. Opening of BK_{Ca} channels allow K^+ flux out of the cell leading to a change in the membrane-potential in a hyperpolarizing direction and induce vasorelaxation. Recent data suggest that BK_{Ca} channels may be activated via the NO–cGMP pathway and that BK_{Ca} channels modulate the vasodilator response to both exogenous nitrovasodilators and endogenous receptor-mediated NO release in isolated arteries (Khan et al., 1998; Bychkov et al., 1998). We and others have recently reported that the vasorelaxant effects of nitroglycerin in rat and porcine coronary arteries are specifically inhibited by iberiotoxin (Bang et al., 1999) and that the inorganic nitrovasodilator sodium nitroprusside opens BK_{Ca} channels in isolated human coronary arteries (Bychkov et al., 1998). The present data extend these observations and show that an interaction between BK_{Ca} channels and nitroglycerin may markedly affect the response to nitroglycerin in the human mammary artery. Interestingly, the effect of iberiotoxin on the maximum nitroglycerin response is significantly attenuated by arterial endothelial removal. This is in accordance with findings in the rat coronary artery (Bang et al., 1999) and implies that the effect of iberiotoxin on arterial nitroglycerin responses may be partly mediated through BK_{Ca} channels located in the vascular endothelium. Since membrane hyperpolarizing agents attenuate and membrane depolarizing agents augment endothelial vascular superoxide anion (O^{2-}) production [(Münzel et al., 1999a), it is likely that the activity of BK_{Ca} channels may affect the vascular response to nitroglycerin-derived NO through an effect on endothelial O^{2-} formation elicited by changes in the membrane potential [(Münzel et al., 1999a).

To the best of our knowledge, data on the effect of BK_{Ca} channel blockade on NO-mediated venorelaxation has not previously been reported. The present results show that iberiotoxin slightly inhibits the effect of nitroglycerin, but this effect is less pronounced than in the arterial vascular segments. The reason for this difference is not clear but the effects of NO on vascular K^+ channels are complex and depend on several factors including differences in the population of channels expressed in various vascular segments/cells and different effector pathways (e.g. cGMP-dependent/independent pathways) in different vessels (Feletou and Vanhoutte, 2000). In further contrast to the arterial findings, the effect of iberiotoxin in the saphenous vein was completely endothelium-independent. Although speculative, the relative absence of venous vasoconstrictor forces and consequently less need for endothelial BK_{Ca} opening may theoretically explain the lack of changes in the venous iberiotoxin response after endothelial removal.

4.2. Venous nitroglycerin tolerance

Tolerance to nitroglycerin in the isolated human saphenous vein developed within a 24-h period of intravenous nitroglycerin. This is in line with previous in vivo human studies (Jurt et al., 2001; Boesgaard et al., 1994) and confirm data from the only previous study on isolated human veins (Sage et al., 2000). Venous tolerance, in that study (Sage et al., 2000), was seen using an approximately 5-fold lower nitroglycerin dose than in the present study.

It is important to note that although the venous vascular bed is extremely prone to nitroglycerin tolerance development, the dominant hypotheses of the underlying mechanisms of tolerance development are almost completely based on arterial studies.

In a combination of several elegant animal experiments (Kurz et al., 1999; Münzel et al., 1995, 1996, 1999b) in vivo manipulation of superoxide anion levels were shown to affect tolerance development during ex vivo examinations of animal arterial vascular segments. Hydralazine, a BK_{Ca} channel opening agent (Bang et al., 1998), and another hyperpolarizing agent, pinacidil, inhibits activation of the superoxide generating oxidases in rabbits [(Münzel et al., 1996). These effects can be blocked by pretreatment with potassium chloride, suggesting that the activity of the arterial endothelial oxidases are regulated by K^+ channels and the membrane potential. The importance of the endothelium in arterial nitroglycerin tolerance development is also stressed by the finding that tolerance is reversed by endothelial removal [(Münzel et al., 1995; Laursen et al., 1996).

In light of this, we examined whether human venous nitroglycerin tolerance development may be linked to changes in the activity of vascular BK_{Ca} channels. The findings of the present investigation show that venous nitroglycerin tolerance does not modify the response to BK_{Ca} channel blockade with iberiotoxin. In addition, endothelial removal does not affect the response to nitroglycerin in tolerant human veins. Thus, in humans, neither endothelial removal nor changes in BK_{Ca} activity influences venous tolerance development the way it has been demonstrated in animal arterial vascular segments. Instead, the present experiments suggest that other mechanisms may be responsible for human venous tolerance. Sage et al. (2000) reported that venous tolerance is primarily associated with an impaired nitroglycerin bioconversion. This observation underscores the fact that the mechanisms of tolerance, the degree of tolerance and the extent of tolerance may vary between different vascular beds and areas. The present findings should provide impetus to future investigations on human venous tolerance.

In summary, the results of this study show that the vasorelaxant effect of nitroglycerin in humans is significantly inhibited by changes in the activity of the vascular BK_{Ca} channel. This effect is partly endothelium-dependent in the mammary artery and more pronounced in the arterial vascular segments than in the saphenous vein. Although

BK_{Ca} channels are likely regulators of experimental arterial tolerance development to nitroglycerin, they do not appear to play a role in human venous nitroglycerin tolerance development.

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