Differential Antivasoconstrictor Effects of Levcromakalim and Rilmakalim on the Isolated Human Mammary Artery and Saphenous Vein

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Abstract. It is well established that spasm of an arterial and venous graft conduit may occur during harvesting or after coronary artery bypass grafting (CABG). The antivasoconstrictor effect of levcromakalim and rilmakalim, K⁺ channel openers (KCOs), was studied in isolated human internal mammary artery (HIMA) and human saphenous vein (HSV) prepared for CABG. HIMA and HSV rings were contracted by electrical field stimulation (EFS, 20 Hz) or with exogenous noradrenaline (NA). Levcromakalim induced a concentration-dependent and equipotent inhibition of contraction of HIMA and HSV preconstricted by EFS and exogenously applied NA, while rilmakalim produced a stronger inhibition of EFS- than NA-evoked contractions. Glibenclamide, a selective ATP-sensitive K⁺ channel (K₃ATP channel) blocker, significantly antagonized levcromakalim-induced inhibition of EFS- and NA-evoked contractions, as well as rilmakalim-induced inhibition of EFS-evoked contractions on HIMA and HSV. However, glibenclamide failed to antagonize rilmakalim-induced inhibition of NA-evoked contractions. The results suggest that the antivasoconstrictor effect of levcromakalim occurs postsynaptically by the opening K₃ATP channels in the vascular smooth muscle cells. They also suggest that the effect of rilmakalim on EFS-evoked contractions involves K₃ATP channels located pre-synaptically. However, the mechanism by which rilmakalim inhibits NA-evoked contraction seems to be K₃ATP channel independent and warrants further elucidation.

Keywords: ATP-sensitive K⁺ channel, levcromakalim, rilmakalim, electrical field stimulation, human vascular bypass graft

Introduction

The human internal mammary artery (HIMA) and human saphenous vein (HSV) are the vessels of choice for coronary artery bypass grafting (CABG). Spasm of arterial and venous graft conduit may occur during harvesting or immediately following CABG (1 – 3). The mechanism of graft spasm is not yet elucidated, although physical (mechanical manipulation, temperature changes), and humoral factors (circulating vasoconstrictor substances) have been evoked (4, 5). Vasodilators such as papaverine, calcium antagonists, and long-acting nitrates are usually added locally before grafting. However they seem to be more effective in suppression than prevention of graft spasm (6).

K⁺ channel openers (KCOs), a new class of vasodilators (pinacidil, levcromakalim, rilmakalim, diazoxide, etc.), was found to exert their effects on vascular smooth muscles by opening ATP-sensitive K⁺ channels (K₃ATP channels) that produce hyperpolarization of the vascular cell membrane leading to vasorelaxation (7, 8). K₃ATP channels have been identified in other tissues such as cardiac and skeletal muscle, smooth muscle, neurons,
and pancreatic β cells where they couple the metabolic state of a cell to its electrical activity (9–11). In addition, KCOs were found to modulate vascular tone by reducing neurotransmitter release from presynaptic nerve terminals (12–14). We have provided evidence that the antivasoconstrictor action of pinacidil on the neurogenic contractions of the rabbit portal vein is associated with presynaptically located K<sub>ATP</sub> channels (15).

Taking into consideration that the search for drugs capable of modifying blood flow through human vascular grafts is warranted, the present study was designed to examine the antivasoconstrictor effects of levermakalim and rilmakalim on the HIMA and HSV, as well as the possible contribution of pre- and post-synaptically located K<sub>ATP</sub> channels. The HIMA and HSV were contracted by exogenously applied noradrenaline (NA) through the involvement of postsynaptic α-adrenoceptors and by endogenously released NA during electrical field stimulation (EFS) that involves presynaptic mechanisms.

Materials and Methods

The HIMA (n = 71) and HSV (n = 66) segments were collected from patients undergoing CABG suffering from coronary artery disease. The patients were informed in detail about the purpose of the investigation and had given the written consent for the excision of the preparations by their free will. The vessels were excised within 10 min of clamping the blood flow and placed in cold (4°C) Krebs-Ringer-bicarbonate solution. After excision, the vessels were immediately transported to the laboratory.

Vascular preparations

The HIMA and HSV segments were dissected free from connective tissue. After removal of endothelium, rings (3 mm) were mounted between two stainless-steel triangles in an organ bath containing 15 ml Krebs-Ringer-bicarbonate solution (37°C, pH of 7.4), aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. One of the triangles was attached to a displacement unit, allowing fine adjustment of tension, and the other was connected to the isometric transducer (K30; Hugo Sachs, Freiburg, Germany).

The preparations were allowed to equilibrate for 60 min. During this period, the vessels were washed with a fresh buffer solution every 15 min.

Experimental procedure

Contraction of the HIMA and HSV evoked by EFS: Intramural nerves were stimulated using two platinum wire electrodes. The repetitive transmural EFS was carried out at 20 Hz with square wave pulses of 0.3-ms duration and supramaximal voltage. Trains of pulses of 3-s duration were delivered at 2-min intervals from a Grass S44 electronic stimulator (15).

The preparations were allowed to stabilize for at least 30 min (until twitch responses became consistent) before addition of drugs. Concentration-response curves were constructed by addition of KCOs directly into the bathing solution in a cumulative way, taking the amplitude of the response measured immediately before addition of a drug as a control contraction (100%). Higher concentration of KCO was added only when the previous concentration had produced an equilibrium response.

In separate experiments, after twitch responses became consistent, the potassium channel antagonist (glibenclamide) was added into the bathing solution at least 20 min before exposure to KCO. Addition of glibenclamide (1 μM) did not modify the basal contractions of HSV (n = 4) and HIMA (n = 4) evoked by EFS. The amplitude of the response measured immediately before addition of KCOs was taken as the control (100%).

The confirmation that EFS-induced contraction is mediated by neurotransmitter release from sympathetic nerves was obtained by addition of tetrodotoxin or phentolamine to the bathing solution, 20 min before applying EFS.

Contraction of the HIMA and HSV evoked by exogenous NA: In a separate series of experiments, the vascular preparations were challenged repeatedly, for 2 min at 60-min (HSV) and 90-min (HIMA) intervals, with NA (10 μM) to produce contractions similar in shape (maximal amplitude and slope) to those evoked by EFS. After achieving the maximal amplitude of the phasic contraction induced by exogenous NA, the vascular preparation was washed to prevent the development of sustained (tonic) contraction. KCOs were added into the medium for 10 min, and the preparation was rechallenged to NA. The control exposure to NA was taken as 100% response. The antivasoconstrictor effect of levermakalim and rilmakalim was then investigated in the presence of glibenclamide added to the preparation 20 min prior to KCOs. Glibenclamide alone did not modify the basal contractions of HSV or HIMA (n = 4 for both) evoked by exogenous NA.

Treatment of data and statistics

The results are expressed as the means ± standard error of the mean (S.E.M.); n refers to the number of experiments. The least squares method was used for calculating linear regression. The concentration of rilmakalim or levermakalim producing 50% of the maximum response (EC<sub>50</sub>) was determined graphically.
for each curve by linear interpolation. The EC<sub>50</sub> values are presented as pEC<sub>50</sub> (−log EC<sub>50</sub>). Statistical difference between means was determined by Student’s t-test, and a P value < 0.05 was considered statistically significant.

**Drugs**

The following drugs were used: rilmakalim (gift from Dr. I. Kocic, Gdansk); levromakalim (gift from Dr. T. Hamilton, SmithKline Beecham Pharmaceuticals, Betchwort, UK); noradrenaline hydrochloride, tetrodotoxin, phentolamine hydrochloride, and glibenclamide, (Sigma Chemical Co., St. Louis, MO, USA). Levromakalim was dissolved in 70% v/v ethanol with further dilution in distilled water before use. Rilmakalim was dissolved in ethanol and dimethyl sulphoxide (1:1) and further diluted with distilled water. Glibenclamide was dissolved in polyethylene glycol. Noradrenaline was dissolved in dilute acid solution (0.1 N HCl). Previous experiments showed that the solvents used had no effects on preparations at the concentrations applied.

All drugs were added directly to the bath in a volume of 100 µL and the concentrations given are the calculated final concentrations in the bath solution.

**Results**

**Effects of tetrodotoxin and α-adrenoceptor blockade on EFS induced contractions**

Tetrodotoxin (1 µM) abolished the EFS evoked contraction of HSV (n = 4) and HIMA (n = 4). Phentolamine (1 µM) reduced the EFS evoked contraction of HSV (88 ± 3%, n = 4), and HIMA (87 ± 2%, n = 5). Data are not shown.

**Effects of levromakalim and rilmakalim on EFS evoked contractions and exogenous NA-induced contractions**

Figure 1 shows the antivasoconstrictor effects of levromakalim and rilmakalim on EFS (20 Hz) evoked contractions and exogenous NA (10 µM)-induced contractions in the HSV (Fig. 1: A and B) and HIMA.
Levcromakalim (1 nM – 30 μM) induced a concentration-dependent inhibition of both neurogenic contractions, and contractions evoked by exogenous NA of the HSV with pEC\textsubscript{50} values of 6.4 ± 0.1 (maximal response 89 ± 2%, n = 7) and 6.3 ± 0.1 (maximal response 86 ± 2%, n = 6), respectively (Fig. 1A). The difference between the pEC\textsubscript{50} values was not statistically significant (P>0.05). Rilmakalim (0.3 nM – 30 μM) induced a concentration-dependent inhibition of neurogenic contractions and contractions evoked by exogenous NA of the HSV with pEC\textsubscript{50} values of 6.8 ± 0.1 (maximal response, 80 ± 2%, n = 6) and 6.1 ± 0.1 (maximal response, 73 ± 2%, n = 6), respectively (Fig. 1B). The difference between the pEC\textsubscript{50} values was statistically significant (P<0.05).

Figures 1C and 1D show the effect of levcromakalim and rilmakalim on neurogenic and exogenous NA-induced contractions in the HIMA. Levcromakalim (0.1 nM – 30 μM) produced a concentration-dependent inhibition of neurogenic contractions (maximal response, 77 ± 2%, n = 7) and contractions evoked by exogenous NA (maximal response, 71 ± 2%, n = 8) of the HIMA (Fig. 1C). The pEC\textsubscript{50} values were not statistically significant (6.2 ± 0.3 and 5.8 ± 0.3, respectively; P>0.05) (Fig. 1C). The difference between the pEC\textsubscript{50} values of rilmakalim (1 nM – 100 μM) effects on the neurogenic (maximal response, 79 ± 2%, n = 8) contractions and contractions evoked by exogenous NA (maximal response, 63 ± 3%, n = 8) in the HIMA was statistically significant (6.9 ± 0.1 and 6.0 ± 0.1, respectively; P<0.05, Fig. 1D).

Effects of glibenclamide, a selective K\textsubscript{ATP} antagonist on the inhibition of neurogenic contractions and contractions evoked by exogenous NA produced by KCOs

Figure 2 illustrates the effect of glibenclamide on the inhibition of neurogenic contractions and contractions evoked by exogenous NA produced by levcromakalim and rilmakalim in the human saphenous vein (A, B) and in the human internal mammary artery (C, D). The contractions were evoked by electrical field stimulation (ES, 20 Hz ; left bars) or by exogenous noradrenaline (NA, 10 μM; right bars). The amplitude of the contraction measured just before addition of drugs was taken as the control contraction (100%). Effects are expressed as a percentage of the control contraction. Each bar represents the mean of 5 – 7 experiments. **P<0.01, *P>0.05.
Glibenclamide (1 μM) significantly antagonized the inhibitory action of levromakalim (10 μM) on the neurogenic contractions (% of control contraction: 11 ± 1% in the absence vs 74 ± 1% in the presence of glibenclamide, P<0.01, n = 5) and on the contractions evoked by exogenous NA (% of control contraction: 14 ± 2% in the absence vs 70 ± 2% in the presence of glibenclamide, P<0.01, n = 5) of the HSV (Fig. 2A).

The inhibitory action of rilmakalim (10 μM) on the neurogenic contractions of HSV was significantly antagonized by 1 μM of glibenclamide (% of control contraction: 22 ± 2% in the absence vs 85 ± 2% in the presence of glibenclamide, P<0.05, n = 7, Fig. 2B). In addition, glibenclamide (1 μM) did not antagonize the inhibitory action of 0.1 μM of rilmakalim (% of control contraction: 74 ± 2% in the absence vs 76 ± 3% in the presence of glibenclamide, P>0.05, n = 4) or 1 μM of rilmakalim (% of control contraction: 44 ± 2% in the absence vs 46 ± 2% in the presence of glibenclamide, P>0.05, n = 5) on the NA-evoked contractions (data not shown).

In the HIMA, glibenclamide (1 μM) antagonized the inhibitory action of levromakalim and rilmakalim on the contractions evoked by EFS and exogenous NA similarly as in the HSV (Fig. 2: C and D). The inhibitory action of levromakalim (10 μM) was antagonized by glibenclamide both on the neurogenic contractions (% of control contraction: 23 ± 2% in the absence vs 85 ± 2% in the presence of glibenclamide, P<0.01, n = 6) and on the contractions evoked by exogenous NA (% of control contraction: 30 ± 2% in the absence vs 82 ± 2% in the presence of glibenclamide, P<0.01, n = 5), as shown in Fig. 2C.

Glibenclamide (1 μM) significantly antagonized the inhibitory action of rilmakalim (10 μM) on the contractions evoked by exogenous NA (% of control contraction: 39 ± 2% in the absence vs 41 ± 3% in the presence of glibenclamide, P>0.05, n = 7, Fig. 2D). Glibenclamide (1 μM) did not antagonize the inhibitory action of 0.1 μM of rilmakalim (% of control contraction: 82 ± 2% in the absence vs 85 ± 2% in the presence of glibenclamide, P>0.05, n = 4) or 1 μM of rilmakalim (% of control contraction: 53 ± 2% in the absence vs 55 ± 2% in the presence of glibenclamide, P>0.05, n = 4) on the NA-evoked contractions (data not shown).

**Discussion**

It is now well established that EFS of perivascular nerve terminals on isolated blood vessels releases neurotransmitters and co-transmitters such as noradrenaline, adenosine 5′-triphosphate, and acetylcholine to induce vascular contractions (16, 17). To validate the effect of transmural EFS on the isolated blood vessels, we applied tetrodotoxin in 1 μM concentration that selectively paralyzes nerve endings (18). The confirmation of the neurogenic nature of the contraction induced by EFS in our experiments was obtained by 100% inhibition of EFS-induced contraction of the HIMA and HSV by tetrodotoxin. In order to further define the site of action/mechanisms by which EFS induces contraction of human grafts, we used phentolamine. A significant reduction of EFS-induced contraction by phentolamine (>80%) indicates that NA released from perivascular sympathetic nerve endings of vascular preparations acts on vascular, postjunctional α-adrenoceptors.

Levromakalim concentration-dependently inhibited both the neurogenic contraction and the contraction evoked by exogenous NA on HIMA and HSV. The fact that there was no significant difference in the inhibition of the neurogenic and NA-induced contraction suggests that the antivasoconstrictor effect of levromakalim occurs postsynaptically and that it involves the activation of K⁺ channels located on the vascular tissue. This finding is in line with results of Standen et al. (7) and Nelson and Quayle (8) who reported that KCOs inhibit the NA-induced vasoconstrictor response on various vascular smooth muscle preparations by opening K⁺ channels located on the postsynaptic membrane. However, we observed that rilmakalim produced a more potent inhibition of the neurogenic contraction of the HIMA and HSV than the contractions induced by exogenously applied NA. Differences in the antivasoconstrictor effects of levromakalim and rilmakalim on the contractions of the HSV and HIMA evoked by EFS and exogenous NA suggest that KCOs may have different sites/mechanisms of antivasoconstrictor action. This assumption is in line with our previous study on the antivasoconstrictor effect of pinacidil and levromakalim on the rabbit portal vein (15).

To further evaluate this possibility, glibenclamide, a Kₐ,ATP channel blocker, was introduced. The concentration of glibenclamide we used (1 μM) was reported to be highly selective for vascular, pancreatic, and neuronal Kₐ,ATP channels (19). In higher concentrations, glibencl-
Levcromakalim was found to modulate other ion channels (20), such as chloride channels (30 μM) and L-type Ca\(^{2+}\) channels (10 μM), as well as the kinetics of calcium release from the sarcoplasmic reticulum (concentrations >1 μM; refs. 14, 20). In our experiments, glibenclamide did not modify the basal contractions evoked by EFS, suggesting that at 1 μM concentration glibenclamide does not interfere with neurotransmitter release. This is in line with earlier reports showing that glibenclamide in much higher concentrations (at least 30 μM) modulate neurotransmitter release (13, 20). In the present study glibenclamide antagonized the levomakalim-induced inhibition of contractions on the HIMA and HSV evoked by EFS as well as the contraction evoked by exogenous NA. This further confirms that the antivasoconstrictor effect of levomakalim is mediated by the opening of vascular K\(_{ATP}\) channels. Moreover, the concentration of glibenclamide we used minimizes the possibility that the antagonistic effect of glibenclamide on the antivasoconstrictor effect of levomakalim involves mechanisms other than K\(_{ATP}\) blockade. This result corresponds well with the reports of Edwards and Weston (21) and with our previous reports on KCOs and the HIMA (22) and the rabbit portal vein (15).

Glibenclamide used in the same concentration also antagonized the antivasoconstrictor effect of rilmakalim on the neurogenic contractions. K\(_{ATP}\) channels have been identified in noradrenergic nerve terminals of the rat cortex (14, 23, 24), GABAergic terminals of rat substantia nigra (25–27) and on the mouse cholinergic motor nerve endings (28). These channels seem to be involved in the control of transmitter release when the energy content of the nerve terminal is low as in ischemia, hypoxia, or hypoglycemia (28, 12). Moreover, Takata et al. (29) demonstrated a linear correlation between evoked NA release, intracellular ATP concentration, and \(^{86}\)Rb efflux, supporting a role for K\(_{ATP}\) channels in the modulation of transmitter release. Therefore, it seems reasonable to propose that glibenclamide-sensitive K\(_{ATP}\) channels, located presynaptically, could be involved in the antivasoconstrictor effect of rilmakalim on neurogenically but not NA-induced contraction of the HSV and HIMA.

Nonetheless glibenclamide did not modify the effect of rilmakalim (10 μM) on the contraction induced by exogenously applied NA. The failure of glibenclamide to antagonize rilmakalim-induced inhibition of NA-evoked contractions was additionally confirmed in two other series of experiments using lower concentrations of rilmakalim (0.1 and 1 μM). These results imply that the antivasoconstrictor effect of rilmakalim on NA-evoked contraction of HIMA and HSV involves vascular K\(_{ATP}\) channel independent mechanisms. Recent studies by other authors have shown that KCOs may inhibit smooth muscle tone by interaction with intracellular Ca\(^{2+}\) stores (20, 30, 31), by inhibition of inositol-1,4,5-triphosphate (IP\(_3\)) syntheses (32), by reducing Ca\(^{2+}\) sensitivity of the contractile proteins (33), or by inhibition of Ca\(^{2+}\) influx independent of K\(^{+}\) channel opening (34). Moreover, Langheinrich et al. (35) provided evidence that rilmakalim induces Ca\(^{2+}\)-transients in microvascular endothelial cells. We have also shown that pinacidil (≥10 μM) relaxes HIMA rings bathed by a medium containing 100 mM K\(^+\) with a maximum response >80% (22). Although the mechanism by which rilmakalim prevents NA evoked contraction of HIMA and HSV is beyond the scope of this paper and necessitates further elucidation, we may speculate that modulation of the intracellular signaling pathways by rilmakalim may be involved.

Our results show that levomakalim and rilmakalim, are potent antivasoconstrictor agents on the HSV and HIMA and that they can be considered as potential drugs in prevention of the bypass graft spasm. They also suggest that the site of action and the mechanisms underlying the antivasoconstrictor action of levomakalim and rilmakalim are not the same. The effect of levomakalim occurs postsynaptically by the opening K\(_{ATP}\) channels in the vascular smooth muscle cells. However, it seems that the effect of rilmakalim on EFS-evoked contractions involves K\(_{ATP}\) channels located pre-synaptically. The exact mechanism/s by which rilmakalim antagonizes NA-evoked contractions of HIMA and HSV warrants further elucidation.

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