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Effects of chronic oral administration of levcromakalim on in vitro contractile responses of arterial smooth muscle

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Abstract

It has been shown that oral administration of 0.038–0.15 mg/kg levcromakalim elicits a dose-related antihypertensive response in spontaneously hypertensive rats (Clapham et al., Arzneim. Forsch. 41 (1991) 385). In the present study, we examined the effects of long term administration of a high dose of levcromakalim on in vitro vascular contractility. Levcromakalim (2.25 mg/kg/day) was administered to the rats for 2 weeks and the thoracic aorta was then isolated. The levcromakalim treatment markedly reduced the relaxant effect of levcromakalim itself on norepinephrine-induced contraction. Relaxant effects of sodium nitroprusside and 8-bromo-cGMP were also attenuated by the levcromakalim treatment, although the relaxant effects of verapamil and forskolin were unchanged. The levcromakalim treatment decreased the threshold concentration for KCl and norepinephrine to induce contraction. The chronic levcromakalim treatment did not affect the cGMP production due to 3-isobutyl-1-methylxanthine and/or sodium nitroprusside. The aorta isolated from spontaneous hypertensive rats did not exhibit spontaneous activity in normal solution. After treatment with levcromakalim, however, the aorta showed spontaneous rhythmic contractions. Verapamil (10 μM) completely suppressed the spontaneous activity and decreased the basal tension below the original level. Similar to the effects of chronic treatment with levcromakalim, high-K^+ solution (15.4 mM) augmented the contractile response to norepinephrine in the aorta of normotensive rats and induced rhythmic contractions in the aorta of spontaneously hypertensive rats. These results suggest that chronic treatment with a high dose of levcromakalim attenuates not only the effects of levcromakalim itself but also the cGMP-mediated relaxation, possibly by desensitizing the K^+ channel.

Keywords: Levcromakalim; Sodium nitroprusside; Aorta, rat; Chronic treatment; Tolerance

1. Introduction

Levcromakalim (BRL 38227), the (−)-enantiomer of cromakalim (BRL 34915), is one of the K^+ channel openers, a new class of vasodilating agents (Hof et al., 1988; Hamilton and Weston, 1989). The mode of action of these agents is to activate ATP-sensitive K^+ channels, leading to hyperpolarization of the membrane (Standen et al., 1989; Edwards and Weston, 1990; Russell et al., 1992) and subsequent inhibition of Ca^{2+} entry through voltage-operated Ca^{2+} channels (Hamilton et al., 1986; Cook, 1988; Nakao et al., 1988). In addition, hyperpolarization of the membrane also reduces agonist-induced accumulation of inositol 1,4,5-trisphosphate, and consequently the Ca^{2+} mobilization from intracellular stores (Ito et al., 1991; Quast, 1993).

Long-term administration of drugs such as a nitrovasodilator, nitroglycerin, causes tolerance in vascular smooth muscle (Needleman and Johnson, 1973; Keith et al., 1982; Flaherty, 1989; Newman et al., 1990). The inhibitory effect of cromakalim on uterine contraction in vivo also decreases after repeated administrations (Downing et al., 1989; Downing and Hollingsworth, 1992). However, the effects of chronic levcromakalim treatment on vascular smooth muscle contractility have not been reported upon. In the present study, we examined the effects of chronic administration of a high dose of levcromakalim on in vitro contractility of the rat aorta.

2. Materials and methods

2.1. Treatment with levcromakalim

Male Wistar rats aged 8 weeks were divided into three groups; untreated group, vehicle control group and levcro-

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makalim-treated group. Ethanol (1.75% solution, 3 ml/kg) and levcromakalim (0.15 or 0.75 mg/kg) were given orally to vehicle control and levcromakalim-treated groups, respectively, 3 times a day at 9:00, 15:00 and 21:00 every day for 2 weeks. Approximately 12 h after the last administration, the rats were killed by stunning at the neck and bleeding. The thoracic aorta was then quickly removed and used for measurement of muscle tension and cGMP content. Some experiments were conducted using 8-week-old spontaneously hypertensive rats obtained from Charles River Japan (Yokohama, Japan).

2.2. Measurement of muscle tension

The isolated aorta was cut into rings (2–3 mm wide) and placed in a normal physiological salt solution (PSS) which contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, glucose 5.5 and ethylene diamine tetraacetic acid 0.01. The endothelium was removed by gently rubbing the intimal surface of the aortic ring with the tip of the forceps moistened with PSS. The high-K^+ (72.7 mM) solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37°C to maintain the pH at 7.4. Muscle tension was recorded isometrically with a force displacement transducer. Each muscle ring was attached to a holder under a resting tension of 10 mN. After equilibration for 30 min in a 2 ml muscle bath, each strip was repeatedly exposed to high-K^+ solution until responses became stable (60–90 min). At the end of experiments, the muscle rings were blotted with filter paper and weighed on an analytical balance. The contractile responses to KCl and norepinephrine were expressed as mN/mg tissue wet weight.

2.3. Measurement of cGMP levels

The thoracic aorta was cut open longitudinally and further cut transversely into 4 segments. Each segment weighed about 10 mg. After equilibration in PSS at 37°C for 1 h, the strips were exposed to test agents for 5 min. Some strips were used as untreated controls. Subsequent to an incubation, the muscle strips were frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. After centrifugation at 1400 × g twice, trichloroacetic acid in the supernatant was removed by washing with

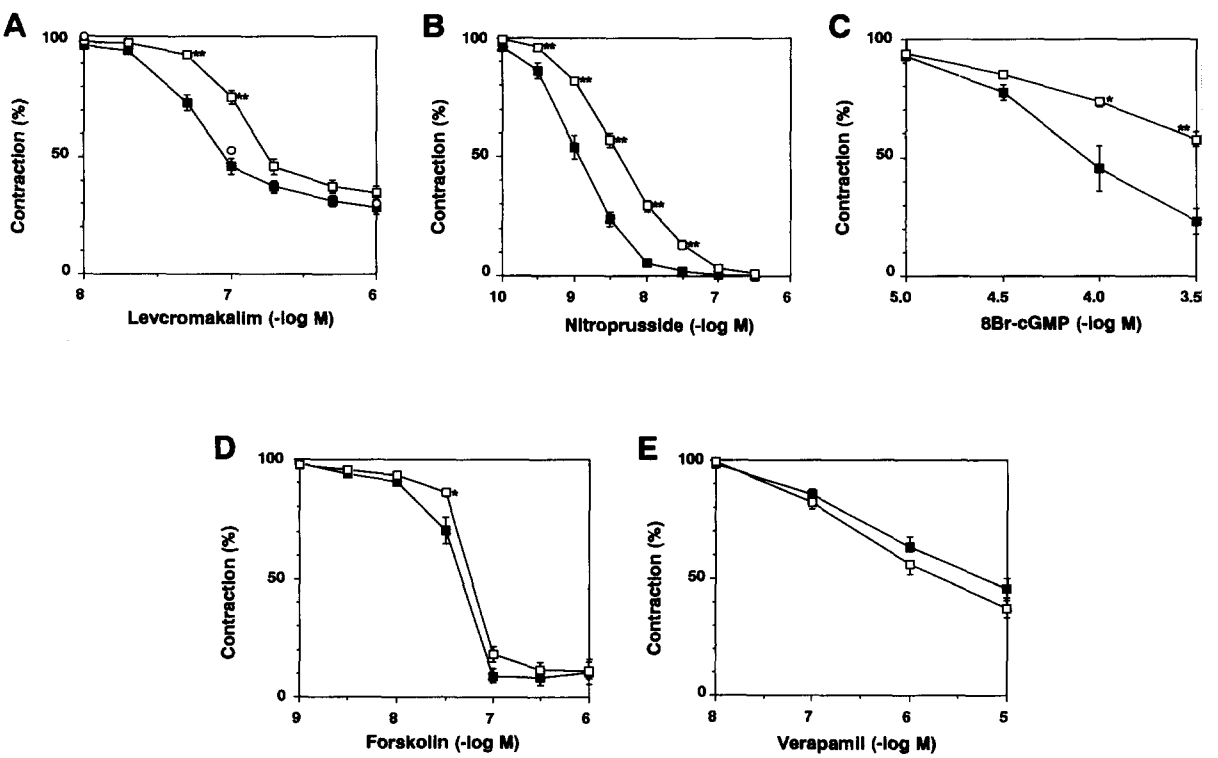

Fig. 1. Effects of chronic treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks) on relaxation of norepinephrine-induced contraction induced by levcromakalim (10 nM to 1 μM, $n = 12$) (A), sodium nitroprusside (0.1–300 nM, $n = 10$) (B), 8-bromo-cGMP (10–300 μM, $n = 6$) (C), forskolin (1 nM to 1 μM) (D) or verapamil (10 nM to 10 μM, $n = 6$) (E). After the norepinephrine (100 nM)-induced contraction reached steady state, these inhibitors were added cumulatively. ■: Aorta isolated from vehicle (1.75% ethanol, 3 ml/kg × 3 for 2 weeks)-treated rat. □: Aorta isolated from levcromakalim (0.75 mg/kg × 3 for 2 weeks)-treated rat. Data from aorta pretreated with levcromakalim (0.15 mg/kg × 3 for 2 weeks) were also shown by ○ ($n = 4$). Results are expressed as means ± S.E. *,** Significantly different from control with $P < 0.05$ and $P < 0.01$, respectively (ANOVA for panel A and Student's t-test for panel B, C, D and E).

water-saturated ether, and cGMP was assayed using an enzyme immunoassay kit (Cayman Chemical, MI, USA). Tissue cGMP levels were expressed as pmol/g wet weight.

2.4. Chemicals

Chemicals used were levcromakalim (donated by Smith Kline Beecham Pharmaceutical Co., Surrey, UK), norepinephrine bitartrate, sodium nitroprusside (Wako Pure Chemical, Tokyo, Japan), forskolin (donated from Nihon-Kayaku Co., Tokyo, Japan), carbachol hydrochloride, 8-bromo-cGMP, 3-isobutyl-1-methylxanthine and verapamil hydrochloride (Sigma Chemicals, St. Louis, MO, USA). Levcromakalim was dissolved in 70% ethanol at a concentration of 10 mM and diluted to the desired concentration in distilled water. Other drugs were dissolved and diluted in distilled water.

2.5. Statistics

The results of the experiments are expressed as means ± S.E.M. Student's *t*-test and analysis of variance (ANOVA, when comparison involved more than two groups) were used for the statistical analysis of the data. The P values less than 0.05 were considered to be significant.

3. Results

3.1. Effect of chronic levcromakalim treatment on relaxation induced by levcromakalim, sodium nitroprusside, 8-bromo-cGMP, forskolin and verapamil

The effects of chronic treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks) on in vitro responses to various vasodilators were studied. As shown in Fig. 1A, the concentration-response curve for levcromakalim on norepinephrine (100 nM)-induced contraction was shifted to the right by the levcromakalim treatment (IC_{50} in $-\log$ M were 7.0 ± 0.05, $n = 12$, for vehicle control and 6.7 ± 0.05, $n = 12$, for levcromakalim-treated; $P < 0.05$). Treatment with a low dose of levcromakalim (0.15 mg/kg, 3 times a day for 2 weeks) did not change the concentration-response curve for levcromakalim. In addition, the concentration-response curves for levcromakalim in the vehicle control rat aorta were not different from those for the untreated rat aorta (see Fig. 3A).

Treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks) also markedly shifted the concentration-response curve for sodium nitroprusside to the right (IC_{50}: 9.0 ± 0.07, $n = 10$, for vehicle control and 8.4 ± 0.05, $n = 10$, for levcromakalim-treated; $P < 0.01$) (Fig. 1B). Furthermore, the relaxing effect of the membrane permeable analogue of cGMP, 8-bromo-cGMP, was also significantly reduced by the levcromakalim treatment (Fig. 1C). In contrast, the relaxant effect of forskolin was scarcely affected by the levcromakalim treatment (IC_{50}: 7.4 ± 0.05, $n = 6$, for vehicle control and 7.2 ± 0.02 for levcromakalim-treated, $n = 6$) (Fig. 1D). The concentration-response curve for verapamil (10 nM to 10 μM) was also unaltered by levcromakalim (IC_{50}: 5.6 ± 0.2, $n = 6$, for vehicle control and 5.8 ± 0.2 for levcromakalim-treated, $n = 6$) (Fig. 1E).

The effects of chronic treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks) on the in vitro response to levcromakalim were also studied in aorta isolated from spontaneous hypertensive rats. The concentration-response curve for levcromakalim on the norepinephrine (100 nM)-induced contraction was shifted to the right by levcromakalim (IC_{50} in $-\log$ M were 7.3 ± 0.03, $n = 4$, for vehicle control and 6.9 ± 0.08, $n = 4$, for levcromakalim-treated; $P < 0.01$).

3.2. Effect of chronic levcromakalim treatment on contractions induced by high-K^+ and norepinephrine

The effects of long-term treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks) on the in vitro contractile response to various stimulants were studied. Fig. 2 shows that the concentration-response curves for KCl (5.4–65.4 mM) (EC_{50}: 1.5 ± 0.01, $n = 6$) and norepinephrine (0.1–300 nM) (EC_{50}: 8.2 ± 0.04, $n = 10$) were shifted to the left by levcromakalim (EC_{50}: 1.7 ± 0.03, $n = 6$, $P < 0.01$ for KCl and 8.8 ± 0.06, $n = 10$, $P < 0.01$ for norepinephrine). However, the maximum contractions (expressed as mN/mg tissue wet weight) induced by KCl and norepinephrine did not differ between vehicle control and levcromakalim-treated preparations.

3.3. Contractile responses in aorta isolated from spontaneous hypertensive rats

In the aorta isolated from spontaneous hypertensive rats treated with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks), spontaneous rhythmic contractions were observed. Addition of 10 μM verapamil completely suppressed these contractions and decreased the resting tension (Fig. 3A). In contrast, the aorta isolated from the vehicle control spontaneous hypertensive rats did not exhibit any rhythmic activity and 10 μM verapamil did not affect the resting tension (data not shown). Depolarization of the vehicle control spontaneous hypertensive rats aorta by the addition of 15.4 mM KCl (addition of 10 mM KCl to normal solution) induced spontaneous contractions and elevated the resting tone. Verapamil (10 μM) inhibited these contractions and reduced the resting tension (Fig. 3B).

3.4. Effect of KCl depolarization on responses to levcromakalim, sodium nitroprusside, 8-bromo-cGMP, forskolin, verapamil and norepinephrine

The aorta isolated from the untreated rats was pretreated with 15.4 mM KCl before the application of norepineph-

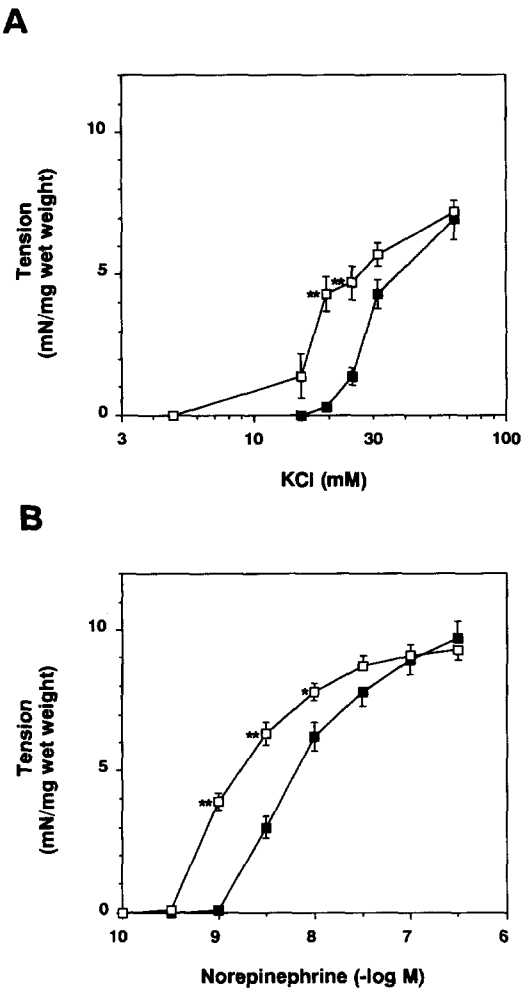

Fig. 2. Effects of chronic treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks) on contraction induced by cumulative addition of high-K^+ (5.4–65.4 mM, $n = 6$) (A) or norepinephrine (0.1–300 nM, $n = 10$) (B). ■: Aorta isolated from vehicle-treated rat. The effects of 15.4 mM KCl on the contractile effect of norepinephrine is shown in panel B. □: Aorta isolated from levcromakalim-treated rat. Results are expressed as means ± S.E. (mN/mg wet weight). *,** Significantly different from control with $P < 0.05$ and $P < 0.01$, respectively (Student's t-test).

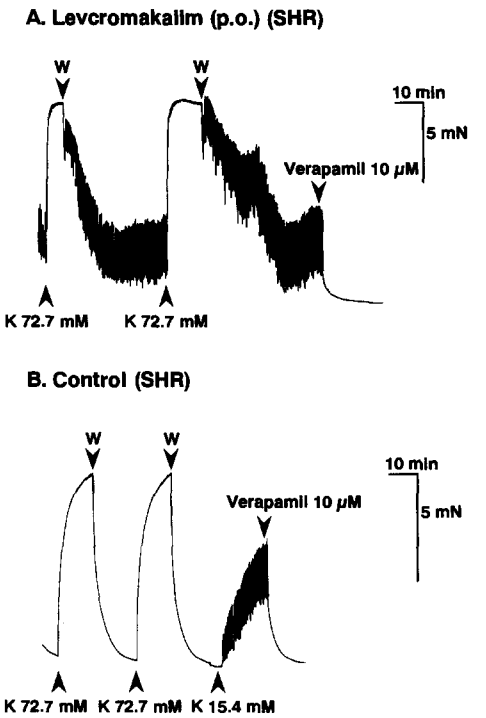

Fig. 3. Effects of verapamil (10 μM) on resting tension of aorta isolated from spontaneous hypertensive rats after chronic treatment with levcromakalim (0.75 mg/kg, 3 times a day for 2 weeks). After observation of the response to 72.7 mM KCl, 10 μM verapamil was added (A: spontaneous hypertensive rat aorta after levcromakalim for 2 weeks, B: spontaneous hypertensive rats aorta in the presence of 15.4 mM KCl).

rine (100 nM). KCl (15.4 mM) did not induce contraction. As shown in Fig. 4A, the concentration-response curve for the inhibitory effect of levcromakalim on norepinephrine-induced contraction was shifted to the right by 15.4 mM KCl. Similarly, the concentration-response curves for sodium nitroprusside and 8-bromo-cGMP were shifted to the right in the presence of 15.4 mM KCl (Fig. 3B,C). In contrast, the relaxant effects of forskolin (1 nM to 1 μM) and verapamil (10 nM to 10 μM) were unchanged by the depolarization with 15.4 mM K^+ (Fig. 4D,E).

The concentration-response relationship for the contractile effects of norepinephrine (0.1–300 nM) was shifted to the left in the presence of 15.4 mM KCl (EC_{50}: 8.2 ± 0.07, $n = 6$ for control and 9.0 ± 0.06, $n = 5$, $P < 0.01$ for KCl treatment) (Fig. 4F).

3.5. Effect of levcromakalim treatment on cGMP production increased by 3-isobutyl-1-methylxanthine and/or sodium nitroprusside

As shown in Table 1, an inhibitor of phosphodiesterase, 3-isobutyl-1-methylxanthine (1 mM), increased the cGMP

Table 1
cGMP content in vehicle control and levcromakalim-treated muscles

Treatment	n	cGMP (pmol/g wet weight)	
		Control	Levcromakalim-treated
Control	4	108 ± 7	89 ± 9
3-Isobutyl-1-methylxanthine (1 mM)	4	420 ± 31 [a]	504 ± 93 [a]
3-Isobutyl-1-methylxanthine (1 mM) + sodium nitroprusside (30 nM)	4	2311 ± 425 [b]	2031 ± 298 [b]

Muscle strips were treated with 3-isobutyl-1-methylxanthine (1 mM) or 3-isobutyl-1-methylxanthine plus sodium nitroprusside (30 nM) for 5 min. [a] Significantly different ($P < 0.01$) from control. [b] Significantly different ($P < 0.01$) from 3-isobutyl-1-methylxanthine.

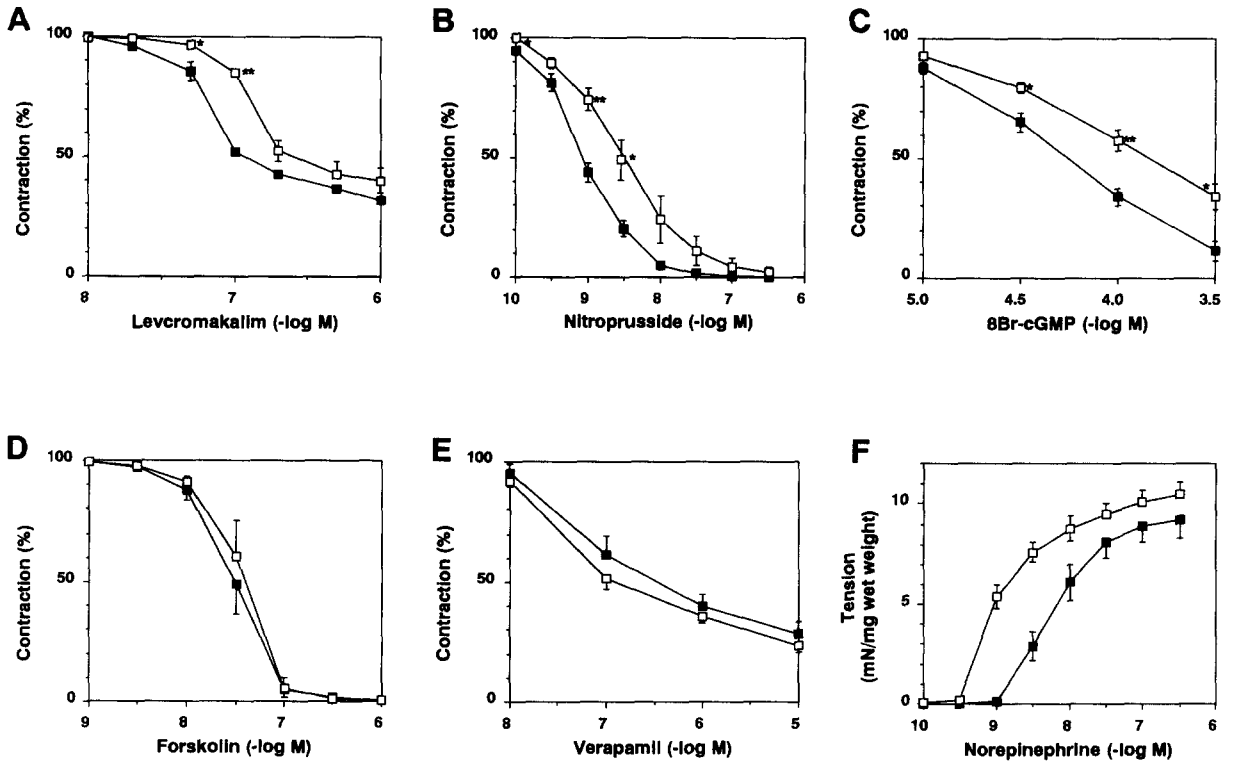

Fig. 4. Effects of 15.4 mM KCl on relaxation of norepinephrine-induced contraction induced by levcromakalim (10 nM to 1 μM) (A), sodium nitroprusside (0.1–300 nM) (B), 8-bromo-cGMP (10–300 μM) (C), forskolin (1 nM to 1 μM) (D) or verapamil (10 nM to 10 μM) (E). The effect of 15.4 mM KCl on norepinephrine-induced contraction is also shown in panel F. The tissues were not pretreated (■) or pretreated with (□) 15.4 mM KCl for 20 min. Results are expressed as means ± S.E. of 4–6 experiments. *,** Significantly different from control with $P < 0.05$ and $P < 0.01$, respectively (Student's t-test).

content 3.9-fold in vehicle control tissue and 5.6-fold in levcromakalim-treated tissue (0.75 mg/kg, 3 times a day for 2 weeks). In the presence of 3-isobutyl-1-methylxanthine, sodium nitroprusside (30 nM) increased the cGMP content 21-fold in control tissue and 22-fold in levcromakalim-treated tissues.

4. Discussion

It has been shown that, in spontaneously hypertensive rats, oral administration of 0.038–0.15 mg/kg levcromakalim elicits a dose-related antihypertensive response (Clapham et al., 1991). The present study revealed that in vivo administration of a high dose of levcromakalim (2.25 mg/kg/day) for 2 weeks attenuated the in vitro vasodilator response to levcromakalim. Since levcromakalim acts selectively on the ATP-sensitive K^+ channel, it is reasonably to assume that the reduced relaxant effect of levcromakalim in the levcromakalim-treated aorta is caused by desensitization of ATP-sensitive K^+ channel activity. We found that levcromakalim treatment also attenuated the vasodilating effects of sodium nitroprusside and 8-bromo-cGMP. Moreover, the vasoconstrictor responses to KCl and norepinephrine were increased after the levcromakalim treatment. Thus levcromakalim does not simply decrease the sensitivity to levcromakalim but also decreases the effects of cGMP-related vasodilators and non-selectively augments the contractility of vascular smooth muscle. It is possible that the reduced activity of vasodilators after the levcromakalim treatment is due to the decreased blood pressure. However, this possibility is not likely since chronic treatment with verapamil (15 mg/kg/day) for 4 weeks did not change the relaxant effects of several vasodilators, such as levcromakalim, sodium nitroprusside, nicorandil and verapamil itself (unpublished observations). It is also possible that the cross-tolerance to sodium nitroprusside is due to the decreased activity of cGMP production system. However, 3-isobutyl-1-methylxanthine and/or sodium nitroprusside similarly increased the cGMP content in control and levcromakalim-treated tissues. Thus it seems likely that levcromakalim and the cGMP share a common pathway for relaxation and the chronic treatment desensitizes this pathway.

Since the Ca^{2+}-activated K^+ channel is activated by cGMP (Fujino et al., 1991), it is possible that chronic hyperpolarization due to chronic levcromakalim treatment inactivates not only the ATP-sensitive K^+ channel but also other types of K^+ channels, such as Ca^{2+}-activated K^+ channels. Inactivation of this channel may counteract the relaxation induced by cGMP-dependent mechanisms.

It is known that the membrane excitability of vascular

smooth muscle cells is increased in spontaneous hypertensive rats and that oscillation of the membrane potential is generated without stimulation or by small depolarizations, while the vascular smooth muscle cells in normotensive rats are quiescent (Sunano and Shimamura, 1991). Thus the cytoplasmic Ca^{2+} level in vascular smooth muscle is elevated in spontaneous hypertensive rats (Sugiyama et al., 1986; Erne and Hermsmeyer, 1989) and spontaneous Ca^{2+} oscillations are generated (Sada et al., 1990). Such an increased vascular reactivity as seen in spontaneous hypertensive rats could be due to the membrane depolarization of vascular smooth muscle cells (Cheng, 1984; Stekiel et al., 1986) or enhancement of voltage-dependent Ca^{2+} channel activity (Rusch and Hermesmeyer, 1988). Therefore, if the membrane is depolarized after the chronic levcromakalim treatment, the reactivity of vascular smooth muscle would be increased as compared with that in normotensive rats. Under our experimental conditions, the aorta isolated from spontaneous hypertensive rats did not exhibit spontaneous activity in normal solution. However, after levcromakalim, the aorta showed spontaneous rhythmic contractions. Inhibition of voltage-dependent Ca^{2+} channels by verapamil completely suppressed this spontaneous activity and decreased the basal tension below the original level. Furthermore, 15.4 mM KCl decreased the threshold concentration of norepinephrine to induce contraction as did levcromakalim treatment. These results support the possibility that vascular smooth muscle cells are depolarized after levcromakalim treatment.

In contrast to the effects of cGMP-related compounds, the relaxation induced by forskolin, which is mediated by an increase in cAMP, was unaffected by the chronic treatment with levcromakalim. It has been suggested that either cAMP or cGMP inhibits vascular smooth muscle contraction by at least four common mechanisms; (1) inhibition of Ca^{2+} channels by hyperpolarization following the activation of Ca^{2+}-activated K^+ channels in vascular smooth muscle (Fujino et al., 1991; Sadoshima et al., 1988), (2) direct inhibition of L-type Ca^{2+} channels (Ishikawa et al., 1993), (3) decrease in Ca^{2+} release from the sarcoplasmic reticulum (Karaki et al., 1988; Abe and Karaki, 1989) and (4) decrease in Ca^{2+} sensitivity of contractile elements (Karaki et al., 1988; Abe and Karaki, 1989). Furthermore, cGMP has been suggested to activate the membrane Ca^{2+} pump in vascular smooth muscle cells (Furukawa et al., 1988). The various effects of chronic treatment with levcromakalim on the relaxations due to sodium nitroprusside, 8-bromo-cGMP and forskolin suggest that the mechanisms mentioned above may contribute differently to the cAMP- and cGMP-induced relaxations in rat aorta. To confirm this possibility, we examined the effects of these vasodilators in the presence of 15.4 mM KCl. The results indicated that the relaxant effects of cGMP-related compounds but not cAMP-related compounds were attenuated in the presence of 15.4 mM KCl. This suggests that the relaxant effects of cGMP-related compounds, but not the effects of cAMP-related compounds depend on the opening of K^+ channels.

In summary, in vivo chronic treatment with a high dose of levcromakalim decreased not only the relaxant effect of levcromakalim itself but also the cGMP-related relaxation without changing cGMP generation. Prolonged opening of K^+ channels may decrease the sensitivity of K^+ channels to levcromakalim and cGMP-dependent vasodilators. Desensitization of K^+ channels may also depolarize the membrane and augment the contractile activity in response to various stimulants.

Acknowledgements

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