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ORIGINAL ARTICLE

Short- and long-term inhibition of cardiac inward-rectifier potassium channel current by an antiarrhythmic drug bepridil

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Abstract Bepridil is an effective antiarrhythmic drug on supraventricular and ventricular arrhythmias, and inhibitor of calmodulin. Recent investigations have been elucidating that bepridil exerts antiarrhythmic effects through its acute and chronic application for patients. The aim of this study was to identify the efficacy and the potential mechanism of bepridil on the inward-rectifier potassium channel in neonatal rat cardiomyocytes in acute- and long-term conditions. Bepridil inhibited inward-rectifier potassium current (I_{K1}) as a short-term effect with IC_{50} of 17 μM. Bepridil also reduced I_{K1} of neonatal cardiomyocytes when applied for 24 h in the culture medium with IC_{50} of 2.7 μM. Both a calmodulin inhibitor (W-7) and an inhibitor of calmodulin-kinase II (KN93) reduced I_{K1} when applied for 24 h as a long-term effect in the same fashion, suggesting that the long-term application of bepridil inhibits I_{K1} more potently than that of the short-term application through the inhibition of calmodulin kinase II pathway in cardiomyocytes.

Keywords Bepridil · Inward-rectifier K^+ channel · Calmodulin · W-7 · KN93

Introduction

Bepridil is an effective drug for a wide range of supraventricular and ventricular tachyarrhythmias. Although bepridil has originally been recognized as a class IV antiarrhythmic agent, an improved understanding of the pharmacological effects of this drug has reinforced the characteristics; bepridil is referred to as a multichannel blocker nowadays. In view point of pharmacodynamics, bepridil is a highly lipophilic drug (log $P = 5.49$, pKa = 9.16 at 37 °C) of which protein binding is approximately 99 % [1], and therapeutic plasma concentration may range from 0.5 to 5.0 μM [2]. In electropharmacological investigations, reported short-term effects of bepridil have included the blocking of various types of ion channels and transporters, such as inward-rectifier potassium current (I_{K1}) [3], transient outward potassium current (I_{to}) [3], rapid component of delayed rectifier potassium current (I_{Kr}), slow component of delayed rectifier potassium current (I_{Ks}), ultra-rapid component of delayed rectifier potassium current (I_{Kur}) [4, 5], muscarinic acetylcholine-activated K^+ current ($I_{K,ACh}$), Na^+-activated K^+ current ($I_{K,Na}$), sarcolemmal ATP-sensitive K^+ current ($I_{K,ATP}$) [6–8], voltage-gated sodium channel current (I_{Na}), L-type calcium channel current ($I_{Ca,L}$), T-type calcium channel current ($I_{Ca,T}$) [2] and Na^+–Ca^{2+} exchanger current [9–11]. More recently, long-term effects of bepridil on ionic currents have been recognized [12, 13]. Also several clinical researches have demonstrated that bepridil could be effective for treatment of persistent atrial fibrillation (AFib) and for the maintenance of normal sinus rhythm [14]. Remarkably, bepridil terminated AFib in 2 weeks after starting the administration in this clinical study, which suggests that bepridil has a long-term effect to reverse atrial electrical remodeling. Also several in vivo animal studies demonstrated that a long-term administration of bepridil prevented the shortening of the effective refractory period in the atrium when high-frequent electrical pacing was applied [15, 16]. These results also suggest that bepridil has a long-term antiarrhythmic effect besides its inhibitory action on various ionic channels, although the underlying

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mechanisms remain unclear. We hypothesized that bepridil might exert long-term electrophysiological effects on cardiomyocytes preferable as an antiarrhythmic drug. Because the inward-rectifier K^+ channel is a major K^+ channel responsible for the remodeling in the atrium with persistent AFib, we investigated a long-term effect of bepridil focusing on I_{K1} in comparison with its short-term effect on the same ionic channel by use of rat neonatal cardiomyocytes.

Materials and methods

The experimental protocol was approved in advance by the Ethics Review Committee for Animal Experimentation of Oita University School of Medicine.

Cell preparation

Neonatal rat cardiomyocytes (NRCMs) were prepared from 1–3-day-old Wistar rats as described previously [17, 18]. Isolated cardiomyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 95 % O_2-5 % CO_2 incubator. Bepridil hydrochloride was a kind gift from Daiichi-Sankyo Company (Tokyo, Japan). Bepridil was prepared as 1 mM stock solution in distilled water. W-7, a calmodulin inhibitor, and KN93, an inhibitor of Ca^{2+}/calmodulin-dependent kinase type II, purchased from Wako Pure Chemical Industries (Osaka, Japan), were dissolved in distilled water as 10 and 1 mM stock, respectively.

Electrophysiological recording

I_{K1} in NRCMs were recorded by whole-cell patch clamp using an EPC-9 amplifier controlled by Pulse ver. 8 software (HEKA Eletronik, Lambrecht, Germany). Patch pipettes were pulled from 75-mm plain capillary tubes (Drummond Scientific Co., Broomall, PA, USA) by Model P-97 (Sutter Instrument Co., Novato, CA, USA), and were heat-polished subsequently to achieve the pipette resistance at 2–4 MΩ when filled with the pipette solution. Series resistance was compensated by at least 80 % and was continually monitored throughout the experiment. The I_{K1} current was elicited by 1000 ms depolarizing steps from a holding potential of −40 mV to potentials ranging from −120 to +30 mV in 10-mV increments. All the current measurements were done at room temperature (20–23 °C). For the current recording, the chamber was filled with bath solution contained (mM) NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10, glucose 10 (pH 7.4 by NaOH). To suppress potential interference of I_{to}, I_{Ks} and $I_{Ca.L}$ to the measurement of I_{K1}, 4-aminopyridine (2 mM), chromanol 293B (10 μM) and $CdCl_2$ (0.3 mM) were added to the bath solution. The patch electrodes were filled with pipette solution consists of (mM) KCl 140, $MgCl_2$ 1, EGTA 10, HEPES 10, and Mg-ATP 5 (pH 7.2 by KOH).

Statistical analysis

All data are presented mean ± SEM. Statistical analysis was performed by one-way ANOVA with Student–Newman–Keuls or Fisher LSD. IC_{50} values were estimated using non-linear least square curve-fitting programs in Sigma plot software ver. 10 (SPSS, Chicago, IL, USA). Differences were considered significant when p values were less than 0.05 if nothing else is mentioned.

Results

Acute effect of bepridil on I_{K1}

We first examined the acute effect of bepridil on I_{K1} in NRCMs. The cells were superfused with normal Tyrode's solution and the membrane potential was held at −80 mV followed by a short prepulse (−40 mV, 300 ms) to establish a block of the voltage-gated sodium (Na^+) channel and the transient outward potassium (K^+) channel. To begin with, we applied Ba^{2+} into the bath solution to confirm that the evoked currents were mainly composed of I_{K1} (Fig. 1b). Figure 1a illustrates typical acute effects of bepridil (1, 5, 10 μM) on I_{K1} as sequentially applied on the same cardiomyocyte, demonstrating a dose-dependent inhibition of I_{K1} by acute application of bepridil. After application of bepridil in 5 min, the terminal current of I_{K1} was decreased by 15 % (1 μM), by 24 % (5 μM) and by 32 % (10 μM) as assessed at −120 mV in this cardiomyocyte. The acute effects of bepridil on I_{K1} were plotted against the membrane potentials imposed (I–V relationship), and summarized in Fig. 1b: bepridil of 1, 5, 10 and 30 μM inhibited I_{K1} by 16.4, 31, 39.6 and 53.6 % at −100 mV, respectively. Slope conductance of I_{K1} at −100 mV was decreased by bepridil in a dose-dependent manner; bepridil at concentrations of 1, 5, 10 and 30 μM decreased the conductance to 0.56 ± 0.03, 0.52 ± 0.06, 0.46 ± 0.05, 0.36 ± 0.02 nS/pF, respectively, from the control value of 0.68 ± 0.06 nS/pF (Fig. 1c). Slope conductance of I_{K1} at +20 mV was also decreased by bepridil in a dose-dependent manner; bepridil at 1, 5, 10 and 30 μM decreased the conductance to 0.031 ± 0.01, 0.028 ± 0.004, 0.004 ± 0.003, 0.006 ± 0.001 nS/pF, respectively, from the control value of 0.062 ± 0.004 nS/pF (Fig. 1d).

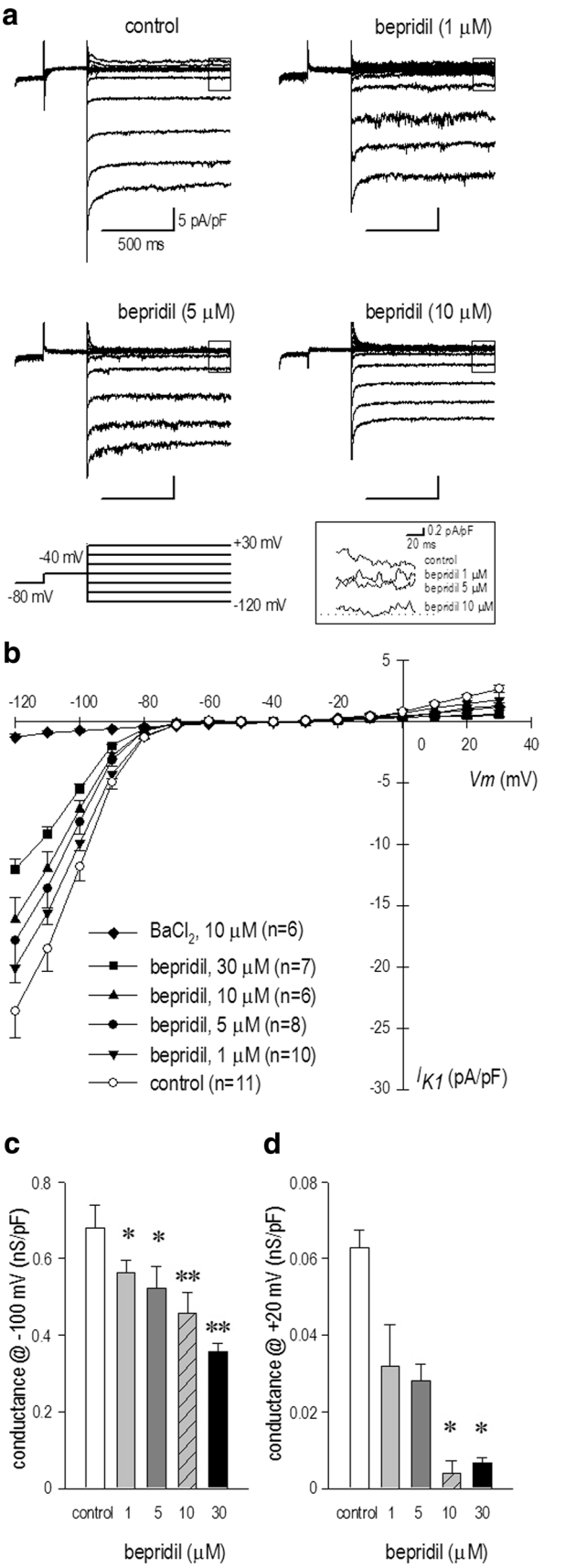

Fig. 1 Acute effect of bepridil on inward rectifier potassium current (I_{K1}). **a** Current traces of I_{K1} in the control and during the acute (5 min) application of bepridil (1, 5, 10 μM) are shown. Outward I_{K1} traces at the potentials of +20 mV at the terminal phase indicated by *box* are shown in an *inset*. **b** I–V relationships constructed by using group data in control and during the application of 1, 5, 10 and 30 μM bepridil in 5 min. The current density of I_{K1} at −100 mV was reduced to −9.9 ± 0.6 mV at 1 μM ($p = 0.027$), −8.2 ± 1 mV at 5 μM ($p = 0.003$), −7.1 ± 0.7 mV at 10 μM ($p = 0.001$) and −5.5 ± 0.4 mV at 30 μM ($p = 0.001$ vs. control). **c**, **d** The slope conductance in the control and during the acute effect of 1, 5, 10 and 30 μM bepridil at −100 mV (**c**) and +20 mV (**d**). *$p < 0.05$, **$p < 0.01$ compared with control

Acute effect of W-7 and KN93 on I_{K1}

Because bepridil is a potent inhibitor of calmodulin, we next studied the impact of acute calmodulin inhibition on I_{K1}. Representative traces in the absence or presence of a calmodulin inhibitor (W-7) or an inhibitor of calmodulin kinase type II (KN93) are illustrated in Fig. 2a. The acute application of W-7 (10, 20 μM) was without effect on I_{K1} (Fig. 2a, b), and so was KN93 (10 μM). There was no significant change in the slope conductance of I_{K1} at −100 and +20 mV by blocking calmodulin with W-7 or by blocking calmodulin kinase type II with KN93 (Fig. 2c, d).

Long-term effect of bepridil on I_{K1}

We then assessed the action of bepridil as applied in the culture medium for 24 h on I_{K1} in NRCMs. Figure 3a indicates representative samples of I_{K1} recorded with or without actions of bepridil (1, 5, 10 μM) for 24 h, demonstrating a long-term inhibitory effect of bepridil on I_{K1}. Note that cardiomyocytes were incubated with bepridil-free culture medium for 1 h prior to the electrophysiological study, and that the bath solution was without bepridil in this patch-clamp study. The I–V relationships revealed a dose-dependent long-term reduction of I_{K1} by bepridil for 24 h; I_{K1} was reduced by 27 % (0.3 μM), 34 % (1 μM), 61 % (5 μM) and 84 % (10 μM) as assessed at −120 mV (Fig. 3b). Slope conductances of I_{K1} at −100 and +20 mV were also dose-dependently decreased by incubation with bepridil for 24 h (Fig. 3c, d). Importantly, reduction ratios of outward components and inward components of I_{K1} were nearly comparable (Fig. 3c, d).

Long-term effect of W-7 and KN93 on I_{K1}

Impact of long-term inhibition of calmodulin and calmodulin kinase type II on I_{K1} was accordingly assessed under the same experiment condition. As shown in Fig. 4a, representative traces of I_{K1} demonstrated long-term inhibitory effects of W-7 and KN93 which did not exert any acute

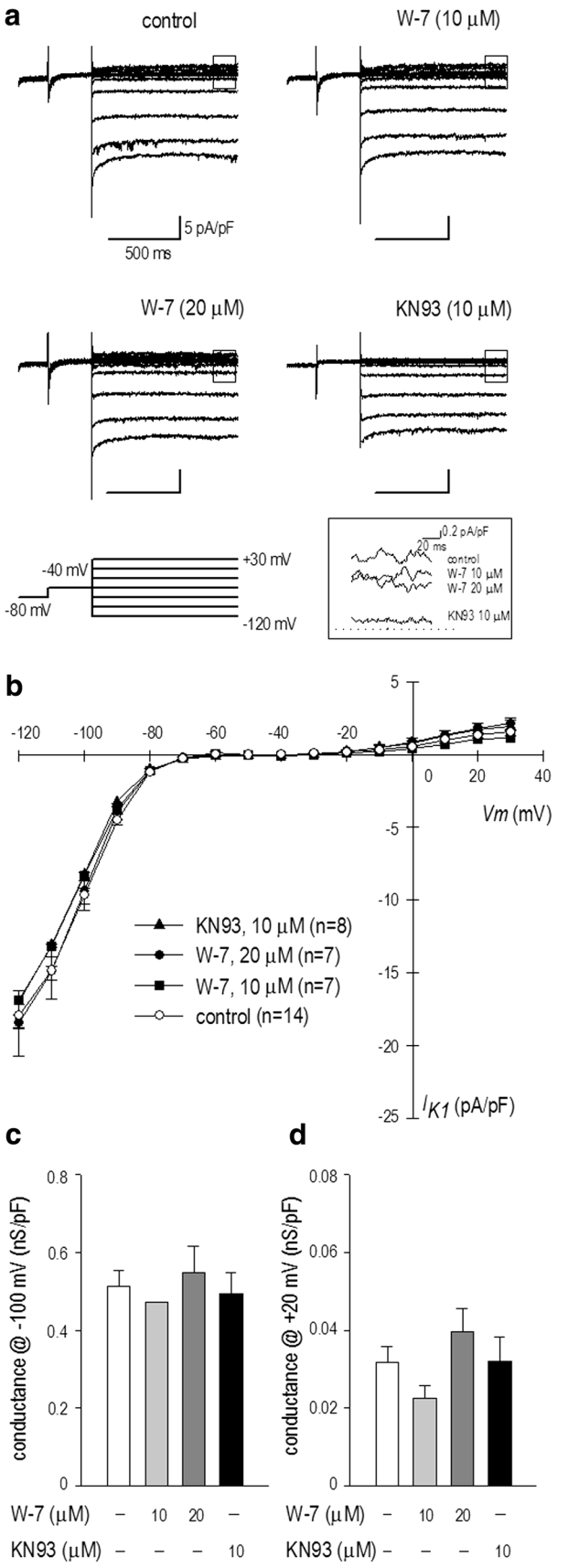

Fig. 2 Acute effect of calmodulin inhibition on I_{K1}. **a** Current traces of I_{K1} in the control and during the acute (5 min) application of W-7, a calmodulin inhibitor, and KN93, an inhibitor of calmodulin kinase type II, are shown. Outward I_{K1} traces at the potentials of +20 mV at the terminal phase indicated by *box* are shown in an *inset*. **b** *I–V* relationships constructed by using group data in control and during the acute application of W-7 (10, 20 μM) and KN93 (10 μM). **c**, **d** The slope conductance in the control and during the acute effect of W-7 (10, 20 μM) and KN93 (10 μM) at −100 mV (**c**) and +20 mV (**d**)

inhibitory effect on I_{K1} (Fig. 2). In the presence of 10 and 20 μM W-7 in the culture medium, I_{K1} was reduce by 36 and 42 %, respectively, as assessed at −120 mV. Slope conductance of I_{K1} was also reduced by 10 μM W-7 (by 35 %) and by 20 μM W-7 (by 42 %) at −100 mV (Fig. 4c, d). A inhibitor of calmodulin kinase type II (KN93, 10 μM) which does not have any inhibitory effect on calmodulin per se, also significantly reduced I_{K1} by 71 % at −100 mV by a long-term application for 24 h in the culture medium (Fig. 4a, b). Note that reduction ratios of I_{K1} by 10 μM W-7 (35 %), 1 μM bepridil (33 %), and 10 μM W-7 with 1 μM bepridil (33 %) were all nearly comparable (Fig. 4c).

Comparison of short-term and long-term effects of bepridil on I_{K1}

Concentration-dependent short-term (5 min) and long-term (24 h) inhibitory effects of bepridil on I_{K1} were assessed and compared in Fig. 5. Bepridil reduced I_{K1} by a short-term effect with IC_{50} of 17 μM, whereas by a long-term effect with IC_{50} of 2.7 μM.

Discussion

In the present study, we found that an antiarrhythmic drug bepridil not only inhibited I_{K1} as a short-term effect (5 min) but also reduced I_{K1} density as a long-term effect (24 h) in NRCMs. The drug's potency to inhibit I_{K1} was approximately 6 times greater in a long-term effect than that in a short-term effect. A calmodulin inhibitor (W-7) and an inhibitor of calmodulin kinase type II (KN93), both of which have no direct effect on I_{K1}, reduced I_{K1} density as a long-term effect. Reductions of I_{K1} by bepridil and by bepridil with W-7 were nearly comparable when applied for 24 h, which suggests that a long-term inhibitory effect of bepridil on I_{K1} depends upon the modulation of calmodulin activity in cardiomyocytes. A multichannel blocker bepridil may exert its antiarrhythmic effects not only through blocking ionic currents acutely but also through regulating calmodulin signals so as to possibly modulate cellular potassium channel expression as a long-term action.

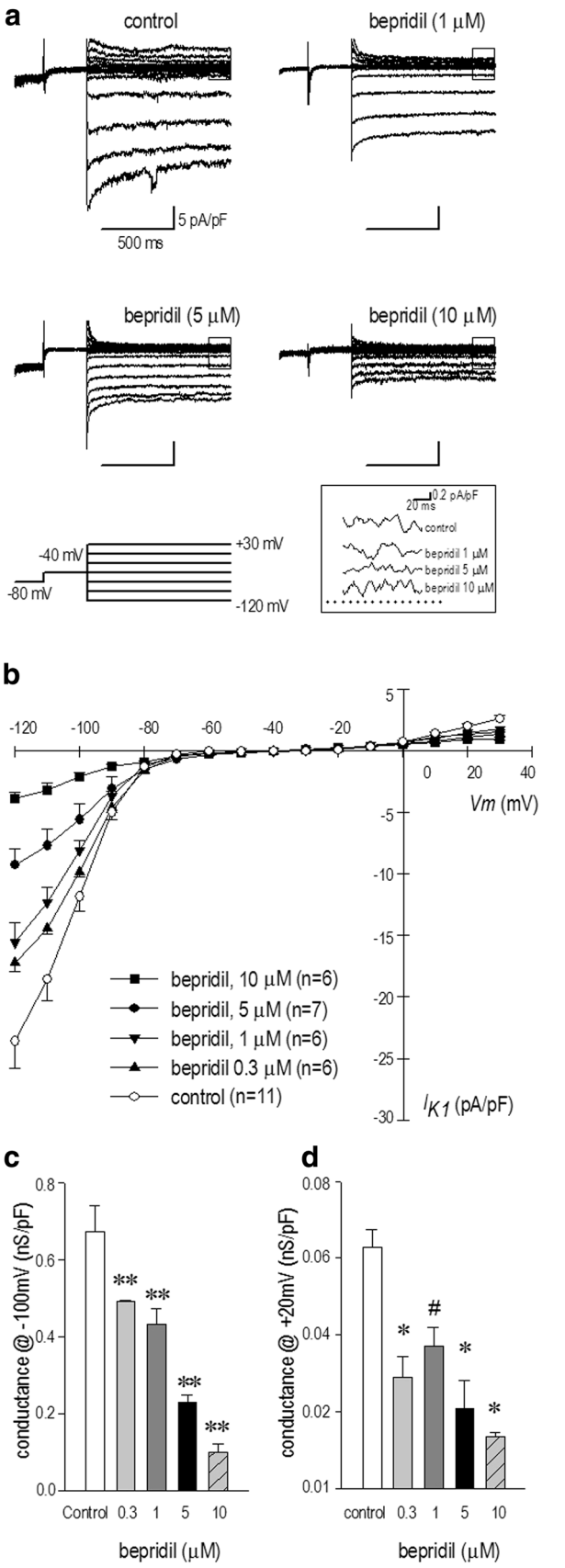

Fig. 3 Long-term effect of bepridil on I_{K1}. **a** Current traces of I_{K1} in the control (vehicle for 24 h) and after the long-term (24 h) treatment with bepridil (1, 5, 10 μM) are shown. Outward I_{K1} traces at the potentials of +20 mV at the terminal phase indicated by *box* are shown in an *inset*. During the records of I_{K1}, bepridil was exclude from the bath solution. **b** I–V relationships constructed by using group data in control and after the treatment with 0.3, 1, 5, and 10 μM bepridil. The current density of I_{K1} at −100 mV was reduced to −9.8 ± 0.4 mV at 0.3 μM ($p = 0.173$), −8.1 ± 0.8 mV at 1 μM ($p = 0.036$), −5.6 ± 1.3 mV at 5 μM ($p < 0.001$) and −2.1 ± 0.4 mV at 10 μM ($p < 0.001$ vs. control). **c**, **d** The slope conductance in the control and after the long-term treatment with 0.3, 1, 5, and 10 μM bepridil at −100 mV (**c**) and +20 mV (**d**). $^{\#}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control

Bepridil decreased I_{K1} as a short-term effect

Bepridil inhibited I_{K1} in NRCMs as a short-term effect in a dose-dependent manner with IC_{50} of 17 μM (Fig. 5). A previous study reported that bepridil blocked I_{K1} in sheep Purkinje fibers by the two-microelectrode technique with IC_{50} of <1.8 μM [3]. Electropharmacology of bepridil revisited by our study indicates that the potency of the drug on I_{K1} channel might depend upon species differences. On the other hand, the two-electrode voltage-clamp technique reportedly faces limits in two important application areas: the penetration by a second electrode in small cells often caused damage that resulted in leakage of cellular contents and a large electrical conductance across the membrane, and in some preparations the cells were commonly out of sight and it was difficult to drive the second electrode into the same cell [19]. Particularly the first limitation could be interfering the accurate measurement of non-voltage gated ionic currents such as I_{K1}. Consequently, patch-clamp study may offer some advantages over microelectrode techniques for this type of electropharmacological evaluation of the drug on I_{K1}.

Bepridil is therefore relatively less potent in inhibition of I_{K1} as a short-term effect (IC_{50} of 17 μM) in comparison with its effect on other ionic channels. While bepridil inhibits other cardiac ionic channels or transporters relatively potently (IC_{50}): L-type Ca^{2+} channel ($I_{Ca.L}$) (1.6 μM) [7], T-type Ca^{2+} channel ($I_{Ca.T}$) (0.4–10.6 μM) [2], voltage-gated Na^{+} channel (I_{Na}) (4–96 μM) [7, 12], delayed rectifier K^{+} channels (6.6 μM for ultrarapid component (I_{Kur}) [5], 13.2 μM for rapid component (I_{Kr}) [4], 6.2 μM for slow component (I_{Ks}) [4]), Na^{+}-activated K^{+} channel ($I_{K,Na}$) (2.2 μM) [6], transient outward current (I_{to}) (~3 μM) [3], ATP-sensitive K^{+} channel ($I_{K,ATP}$) (6.6–10.0 μM) [6], and Na^{+}–Ca^{2+} exchanger (8.1 μM) [11].

Bepridil decreased I_{K1} as a long-term effect

Recent investigations have shown that chronic administration of some antiarrhythmic drugs results in various long-term

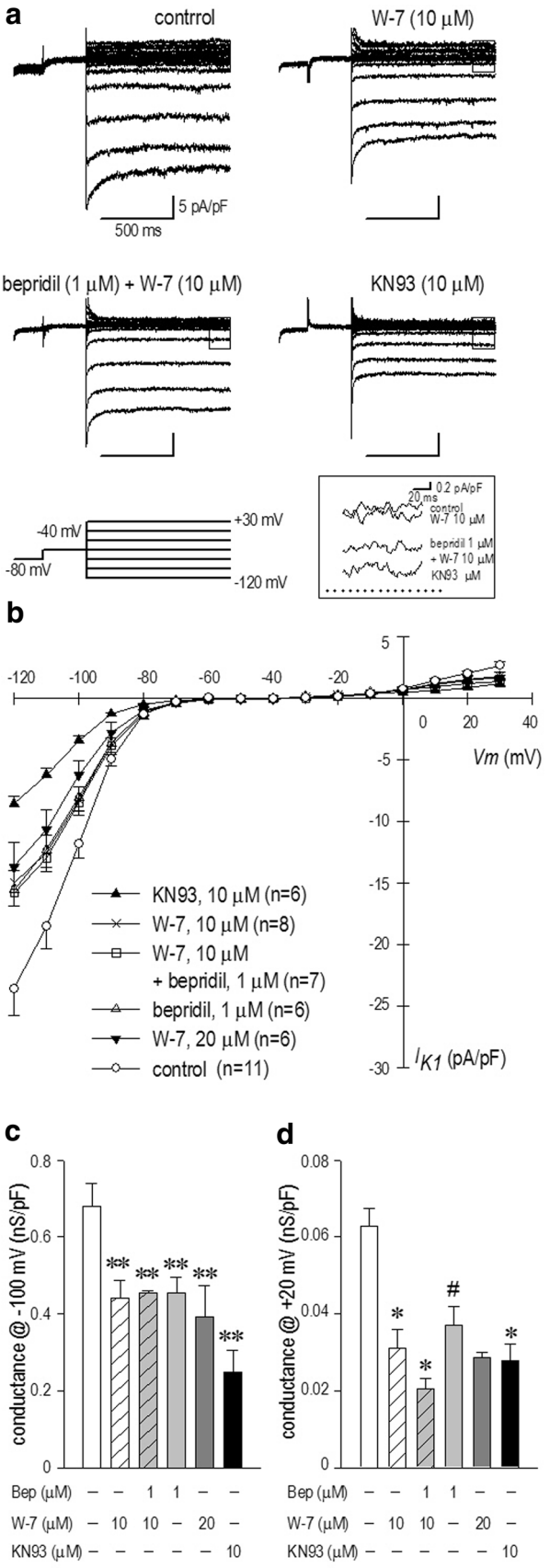

Fig. 4 Long-term effect of bepridil on I_{K1}. **a** Current traces of I_{K1} in the control (vehicle for 24 h) and after the long-term (24 h) treatment with bepridil (1, 5, 10 μM) are shown. Outward I_{K1} traces at the potentials of +20 mV at the terminal phase indicated by *box* are shown in an *inset*. During the records of I_{K1}, bepridil was exclude from the bath solution **b** I–V relationships constructed by using group data in control and after the treatment with 0.3, 1, 5, and 10 μM bepridil. The current density of I_{K1} at −100 mV was reduced to −9.8 ± 0.4 mV at 0.3 μM ($p = 0.173$), −8.1 ± 0.8 mV at 1 μM ($p = 0.036$), −5.6 ± 1.3 mV at 5 μM ($p < 0.001$) and −2.1 ± 0.4 mV at 10 μM ($p < 0.001$ vs. control). **c**, **d** The slope conductance in the control and after the long-term treatment with 0.3, 1, 5, and 10 μM bepridil at −100 mV (**c**) and +20 mV (**d**). $^{\#}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control

effects besides blocking channels/receptors as a short-term effect. Class I antiarrhythmic drugs, which have definite inhibitory action on the voltage-gated Na^+ channel, resulted in up-regulation of cardiac Na^+ channel expression [20]. Also recent studies indicate that bepridil upregulated I_{Na} density and $K_v1.5$ channel expression in a dose-dependent manner apart from its acute blocking effects [12, 13]. However, to our knowledge, there are no published studies regarding the long-term effect of bepridil on the I_{K1} channel. In this context, our present study first demonstrates that bepridil significantly decreases I_{K1} by a long-term effect in a dose-dependent manner in cardiomyocytes. Importantly, the IC_{50} value of long-term I_{K1} inhibition by bepridil was 2.7 μM, suggesting that bepridil inhibits I_{K1} by a long-term effect with greater potency than that by a short-term effect (IC_{50} value of 17 μM).

To investigate the cellular mechanism, we observed I_{K1} after inhibition of calmodulin or calmodulin kinase type II in the same fashion, because bepridil is a potent calmodulin inhibitor [21–23]. This study clearly demonstrated that a calmodulin inhibitor (W-7) and an inhibitor of calmodulin kinase type II (KN93) greatly reduced I_{K1} as a long-term effect. Because the long-term I_{K1} inhibition by 10 μM W-7 was comparable to the long-term inhibition by 1 μM bepridil, and because long-term application of 10 μM W-7 with 1 μM bepridil reduced I_{K1} as much as either of 10 μM W-7 alone or 1 μM bepridil alone, it is conceivable that bepridil down-regulated I_{K1} via the calmodulin-dependent pathway. We have previously reported that a long-term application of bepridil upregulated I_{Na} through the inhibition of calmodulin action [12]. Taken together, it is suggested that an antiarrhythmic drug bepridil is a modulator of ion channel expression in cardiomyocytes depending upon the Ca^{2+}—calmodulin/calmodulin kinase II pathway.

Impact of I_{K1} block by bepridil

I_{K1} plays a critical role in shaping action potentials in ventricular cardiomyocytes, especially the final phase of

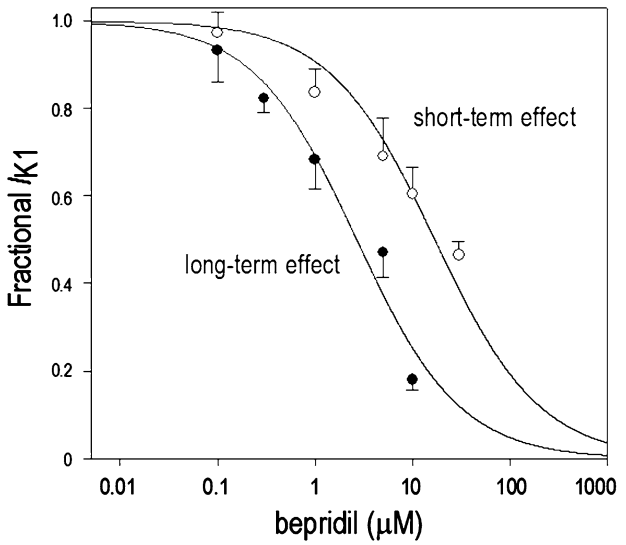

Fig. 5 Comparison of the short-term (5 min) and long-term (24 h) inhibitory effect of bepridil on I_{K1}. Fractional changes in I_{K1} at −100 mV were plotted against the concentration for bepridil (*open circles* for the short-term effect and *closed circles* for the long-term effect). Each plot indicates mean ± SE. *Solid lines* were drawn by fitting the Hill plot equation to the experimental data, demonstrating the half maximal inhibitory concentration (IC_{50}) of 17 μM for the short-term effect and 2.7 μM for the long-term effect of bepridil on I_{K1}

repolarization, and stabilizing repolarized resting membrane potential [24]. Reduction in the outward potassium currents prolongs action potential duration (APD) and effective refractory period (ERP), which may result in the destabilization and early termination of reentrant-based arrhythmias [25]. Standing on these viewpoints, we speculate that bepridil decreases I_{K1} both in short- and long-term application to prolong APD and promote early termination of ventricular arrhythmias. Bepridil is also known to suppress supraventricular arrhythmias potently. Indeed, a nominative effect of bepridil in a canine model of rapid electrical pacing for 2 weeks has been reported; bepridil suppressed the shortening of atrial effective refractory period (AERF) in the first week and further restored AERP to the pre-pacing level in the second week [16]. In this article, a beneficial effect of bepridil was considered in the context of bepridil-induced AERP shortening, although the underlying mechanism was not identified. The long-term inhibitory effect of I_{K1} by bepridil may account for the mechanisms of AERP prolongation in the pathological condition of the heart such as in rapid electrical pacing animal models.

Kir2.1, the major isoform of I_{K1} present in the heart, is mainly expressed in the ventricular tissue [26]. However, recent studies have recognized that Kir2.1/I_{K1} increases in the atrial tissue obtained from patients with chronic atrial fibrillation (AFib) and in animal models of AFib [27–30]. Although AFib is a complex arrhythmias with multiple mechanisms [31, 32], I_{K1} is a particularly important mechanistic determinant of AFib-supporting reentry. Enhanced I_{K1} may shorten APD and AERP by accelerating the membrane repolarization, and may also increase Na^+ current availability which results in acceleration and stabilization of reentrant rotor [33, 34] in the atrium. Hence, the long-term application of bepridil would be counteracting the upregulation of I_{K1} in the atrium caused by AFib, and accordingly beneficial for the termination of chronic AFib. Currently, bepridil is one of the pharmacological options for paroxysmal AFib and persistent AFib as well. J-BAF Study has demonstrated that bepridil effectively converted to sinus rhythm in patients with persistent AFib [14]. A long-term inhibitory effect of I_{K1} in cardiomyocytes by bepridil, presented in this study, may account for the active mechanism of this drug for AFib treatment.

Limitations

There are several limitations in the present study. Although we have obtained IC_{50} values of bepridil to inhibit I_{K1}, a simple comparison of drug concentrations between short-term and long-term effects may not be appropriate. Because bepridil is a highly lipophilic drug, assessment of drug efficacy on I_{K1} by an in vitro experiment without serum proteins and/or serum lipids may not represent the effect of bepridil in in vivo application. Furthermore, a caution would be needed to extrapolate these neonatal rat heart experiments to human therapeutics. Although our study has identified plausible mechanism for the down-regulation of I_{K1} as a long-term effect of bepridil, there is a need for additional evidence on the levels of channel transcription and translation to reveal the molecular pharmacological actions of bepridil.

Conclusion

In summary, our study revealed that the long-term application of bepridil inhibits I_{K1} more potently than that of the short-term effect through the inhibition of calmodulin kinase II pathway in cardiomyocytes, which may explain the hitherto unknown pharmacological mechanism of bepridil when applied for a long-term period to exert its relevant antiarrhythmic actions for the treatment of chronic AFib patients.

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Compliance with ethical standards

Conflict of interest None.

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