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A Novel Action of the Antianginal Drug Bepredil: Induction of Internal Ca^{2+} Release and External Ca^{2+} Influx in Madin–Darby Canine Kidney (MDCK) Epithelial Cells

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ABSTRACT. The effect of the antianginal drug bepridil on Ca^{2+} signaling in Madin–Darby canine kidney (MDCK) cells was investigated by using fura-2 as a Ca^{2+} probe. Bepridil at 10–50 μM evoked a significant rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a dose-dependent manner. The $[Ca^{2+}]_i$ rise consisted of an immediate initial rise and a slow decay. Removal of external Ca^{2+} partly inhibited the Ca^{2+} signals by reducing both the initial rise and the decay phase, suggesting that bepridil activated both external Ca^{2+} influx and internal Ca^{2+} release. In Ca^{2+}-free medium, pretreatment with 50 μM bepridil nearly abolished the Ca^{2+} release induced by thapsigargin (1 μM), an endoplasmic reticulum Ca^{2+} pump inhibitor, and vice versa, pretreatment with thapsigargin inhibited most of the bepridil-induced Ca^{2+} release, suggesting that the thapsigargin-sensitive Ca^{2+} store was the main source of bepridil-induced Ca^{2+} release. Bepridil (50 μM) induced considerable Mn^{2+} quench of fura-2 fluorescence at an excitation wavelength of 360 nm, which was partly inhibited by La^{3+} (0.1 mM). Consistently, La^{3+} (0.1 mM) pretreatment significantly inhibited the bepridil-induced $[Ca^{2+}]_i$ rise. Addition of 3 mM Ca^{2+} induced a significant $[Ca^{2+}]_i$ rise after prior incubation with 10–50 μM bepridil in Ca^{2+}-free medium, suggesting that bepridil induced dose-dependent capacitative Ca^{2+} entry. However, 50 μM bepridil inhibited 1 μM thapsigargin-induced capacitative Ca^{2+} entry by 38%. Pretreatment with aristolochic acid (40 μM) so as to inhibit phospholipase A_2 inhibited 50 μM bepridil-induced internal Ca^{2+} release by 42%, but inhibition of phospholipase C with U73122 (2 μM) or inhibition of phospholipase D with propranolol (0.1 mM) had little effect, suggesting that bepridil induced internal Ca^{2+} release in an inositol 1,4,5-trisphosphate-independent manner that could be modulated by phospholipase A_2-coupled events. This is the first report providing evidence that bepridil, currently used as an antianginal drug, induced a rise in $[Ca^{2+}]_i$ in a non-excitable cell line. BIOCHEM PHARMACOL **59**;6:639–646, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. bepridil; capacitative Ca^{2+} entry; fura-2; MDCK cells; Ca^{2+} signaling

Bepridil is an antianginal agent with multiple therapeutic actions [1, 2]. At the cellular level, it has been found to affect a variety of targets. For example, bepridil inhibits the activities of many transmembrane proteins such as voltage-gated Ca^{2+} channels in cardiac cells [3, 4], receptor-operated Ca^{2+} channels in vascular smooth muscle cells [5], voltage-gated K^+ channels in the CA1 pyramidal neurons [6], outward rectifying K^+ channels in tobacco protoplasts [7], Na^+ channels in guinea-pig ventricular myocytes [8, 9], Na^+-K^+-ATPase in dog kidney cells [10], Na^+/Ca^{2+} exchangers in cardiac cells [11, 12] and platelets [13], and *N*-methyl-D-aspartic acid receptors in rat hippocampal neurons [14]. Additionally, due to its lipophilicity, bepridil can cross the plasma membrane and interfere with intracellular events. For example, bepridil has been shown to antagonize calmodulin-dependent events [15–17].

The effect of bepridil on Ca^{2+} signaling in non-excitable cells has not been investigated previously. In this study, we examined the effect of bepridil on Ca^{2+} handling in MDCK† cells. We previously showed that in this non-excitable epithelial cell which does not possess voltage-gated Ca^{2+} channels [18], IP_3-dependent agonists such as ATP [19] and bradykinin [20] cause a rise in $[Ca^{2+}]_i$ by depleting Ca^{2+} from the ER Ca^{2+} store followed by capacitative Ca^{2+} entry [21]. Also, thapsigargin [22] and 2,5-di-*tert*-butylhydroquinone [23] increase $[Ca^{2+}]_i$ by inhibiting the ER Ca^{2+} pump without increasing IP_3 levels,

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† *Abbreviations:* $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; ER, endoplasmic reticulum; fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester; IP_3, inositol 1,4,5-trisphosphate; MDCK cells, Madin–Darby canine kidney cells; and U73122, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione.

resulting in Ca^{2+} release from the ER followed by capacitative Ca^{2+} entry. Thus, we use MDCK cells as a model for investigating drug effects on Ca^{2+} homeostasis in non-excitable cells.

Using fura-2 as a Ca^{2+}-sensitive fluorescent probe, we found in this study that in contrast to its inhibition of voltage-gated Ca^{2+} channels in excitable cells, bepridil induced a considerable rise in $[Ca^{2+}]_i$ in MDCK cells. We established the dose–response relationships both in the presence and absence of external Ca^{2+} and determined the underlying mechanisms of the bepridil-induced $[Ca^{2+}]_i$ rise. The effect of bepridil on thapsigargin-induced capacitative Ca^{2+} entry was also examined.

MATERIALS AND METHODS

Cell Culture

MDCK cells obtained from the American Type Culture Collection (CRL-6253) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° in 5% CO_2-containing humidified air.

Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; HEPES 10; glucose 5. Ca^{2+}-free medium contained no Ca^{2+} plus 1 mM EGTA (calculated $[Ca^{2+}]_i < 0.1$ nM). The experimental solution contained ≤1% of solvent (ethanol) which did not affect $[Ca^{2+}]_i$ ($N = 3$).

Optical Measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/mL) were loaded with 2 μM fura-2/AM for 30 min at 25° in DMEM. Cells were washed and resuspended in Ca^{2+} medium and subsequently washed every hour during experiments to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°) with continuous stirring; the cuvette contained 1 mL of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Japan) by continuously recording the excitation signals at 340 and 380 nm and the emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding Triton X-100 (0.1%) and EGTA (20 mM) sequentially at the end of an experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$, as described previously [24]. Mn^{2+} quench experiments were performed in Ca^{2+} medium containing 50 μM $MnCl_2$ by continuously recording the excitation signal at 360 nm and the emission signal at 510 nm at 1-sec intervals. Our previous studies showed that trypsinized cells prepared by our protocol respond to stimulation with ATP [19], bradykinin [20], or thapsigargin [22] in a similar manner to cells attached to coverslips. We decided to use trypsinized cells because this procedure is easier and less time-consuming.

Chemical Reagents

The reagents for cell culture were from GIBCO. Fura-2/AM was from Molecular Probes. U73122 and aristolochic acid were from Biomol. Bepridil was from RBI. The other reagents were from Sigma.

Statistical Analysis

All values are reported as means ± SE of 5–6 experiments. Statistical comparisons were determined by using Student's paired *t*-test, and significance was accepted when $P < 0.05$.

RESULTS

Bepridil-Induced $[Ca^{2+}]_i$ Rise in MDCK Cells

Bepridil elicited a rise in $[Ca^{2+}]_i$ at a concentration range of 10–50 μM in the presence of external Ca^{2+} (Fig. 1A). Over a time period of 5 min, the $[Ca^{2+}]_i$ rise consisted of an initial rise, a gradual decay, and an elevated phase. For example, at a dose of 50 μM, bepridil induced a nearly immediate rise in $[Ca^{2+}]_i$ which peaked 50 ± 3 sec later at a height of 585 ± 14 nM (*trace a*; $N = 6$; $P < 0.05$), followed by a gradual decay which remained at a height of about 300 nM at the time point of 250 sec. The rise of the Ca^{2+} signal was slower in response to lower doses of bepridil. At a dose of 1 μM, bepridil did not alter $[Ca^{2+}]_i$ (*trace d*). The $[Ca^{2+}]_i$ rise was not caused by the vehicle (≤1% ethanol), because addition of 1% ethanol had no effect on $[Ca^{2+}]_i$ ($N = 3$; not shown). At a dose of 100 μM, bepridil induced an immediate and persistent rise in $[Ca^{2+}]_i$, most likely reflecting cell membrane damage, and thus the result was not shown.

Internal Ca^{2+} Release and External Ca^{2+} Influx to Bepridil-Induced $[Ca^{2+}]_i$ Rise

In order to investigate the source(s) of the bepridil-induced $[Ca^{2+}]_i$ rise, the experiments in Fig. 1B were carried out. Removal of external Ca^{2+} (no added Ca^{2+} plus 1 mM EGTA) considerably decreased the Ca^{2+} signals induced by 10–50 μM bepridil. The dose–response plots of the bepridil-induced $[Ca^{2+}]_i$ rise both in the presence and absence of external Ca^{2+} are illustrated in Fig. 1C. The y axis represents the net maximum $[Ca^{2+}]_i$ with baseline subtracted. Ca^{2+} removal reduced the $[Ca^{2+}]_i$ rise induced by 10, 20, and 50 μM bepridil by 83 ± 7, 71 ± 6, and 40 ± 6%, respectively ($N = 5–6$; $P < 0.05$).

Thapsigargin-Sensitive Ca^{2+} Store as the Internal Ca^{2+} Source of Bepridil Response

We sought to explore the internal Ca^{2+} source of the bepridil-induced Ca^{2+} response. Figure 2A shows that in

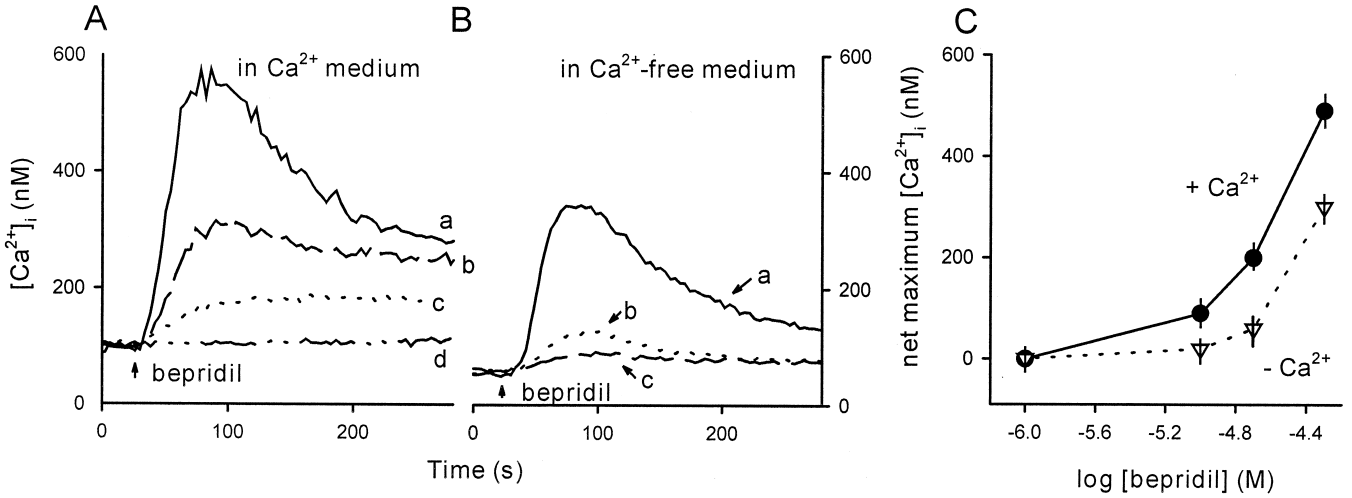

FIG. 1. (A) Dose-dependent effects of bepridil on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. The concentration of bepridil was 50 μM in *trace a*, 20 μM in *trace b*, 10 μM in *trace c*, and 1 μM in *trace d*. Bepridil was added at 30 sec. The experiments were performed in Ca^{2+} medium. Traces are typical of 5–6 experiments. (B) Similar to (A), except that the experiments were performed in Ca^{2+}-free medium (no added Ca^{2+} plus 1 mM EGTA). (C) Dose–response plots of bepridil-induced Ca^{2+} signals in the presence (*filled circles*) or absence (*open triangles*) of external Ca^{2+}. The y axis is the net peak height of the $[Ca^{2+}]_i$ rise with baseline (50–100 nM) subtracted. The data are means ± SE of 5–6 experiments. *$P < 0.05$ between filled circles and open triangles.

the absence of external Ca^{2+} and after the 50-μM bepridil-induced rise in $[Ca^{2+}]_i$ had decayed to a plateau, addition of 1 μM thapsigargin, an ER Ca^{2+} pump inhibitor [25], only induced a $[Ca^{2+}]_i$ rise with a height less than 50 nM. Vice versa, pretreatment with 1 μM thapsigargin in Ca^{2+}-free medium nearly abolished the bepridil-induced $[Ca^{2+}]_i$ rise (Fig. 3C). These data imply that the thapsigargin-sensitive Ca^{2+} store is the source of bepridil-induced internal Ca^{2+} release.

Mechanisms of Bepridil-Induced Ca^{2+} Influx

We went on to investigate how bepridil induces Ca^{2+} influx. We applied Mn^{2+} quench experiments to directly measure Ca^{2+} influx. Mn^{2+} enters cells through similar pathways as Ca^{2+}, but quenches fura-2 fluorescence at all excitation wavelengths [26]. Therefore, Mn^{2+} quench of fura-2 fluorescence reflects Ca^{2+} influx. Fluorescence intensity was continuously recorded at 1-sec intervals at the Ca^{2+}-insensitive excitation wavelength of 360 nm and emission wavelength of 510 nm in Ca^{2+} medium containing 50 μM $MnCl_2$. Bepridil (50 μM) induced an immediate and gradual decrease in the 360-nm signal compared to control without bepridil addition (Fig. 2B; *trace c* vs *trace a*). This result provided evidence that bepridil induced considerable Ca^{2+} influx.

Lathanides are the most potent of the Ca^{2+} entry blockers we have examined in MDCK cells to this point [19, 22, 23, 27–29]; thus, we next examined how La^{3+} affects the bepridil-induced Mn^{2+} quench of fura-2 fluorescence. Figure 2B (*trace b*) shows that La^{3+} (0.1 mM) added 10 sec prior to bepridil (50 μM) significantly inhibited the bepridil-induced Mn^{2+} quench of fura-2 fluorescence by 25 ± 3% in terms of the area under the curve ($N = 6$; $P <$ 0.05). Higher doses of La^{3+} occasionally caused cell aggregation and thus were not tested in this experiment. We also investigated the effect of La^{3+} on the bepridil-induced $[Ca^{2+}]_i$ rise. Figure 2C shows that, in Ca^{2+} medium, pretreatment with La^{3+} (0.1 mM) for 10 sec inhibited the bepridil-induced $[Ca^{2+}]_i$ peak by 46 ± 6% (*trace b* vs *trace a*; 211 ± 12 nM vs 310 ± 21 nM; $N = 6$; $P < 0.05$).

Effects of Bepridil on Capacitative Ca^{2+} Entry

Our previous reports have shown that depletion of internal Ca^{2+} in MDCK cells often triggers capacitative Ca^{2+} entry [19, 20, 22, 23, 27–29]. Thus, we investigated whether capacitative Ca^{2+} entry contributes to bepridil-induced Ca^{2+} influx. Capacitative Ca^{2+} entry was measured by addition of 3 mM Ca^{2+} to cells pretreated with bepridil in Ca^{2+}-free medium. Figure 3A shows that after depletion of the ER Ca^{2+} store for 520 sec with bepridil (20 μM), addition of Ca^{2+} induced a rise in $[Ca^{2+}]_i$ with a net maximum height of 201 ± 10 nM (*trace a*; $N = 6$), which was 10-fold higher than control without bepridil pretreatment (20 ± 7 nM; *trace b*; $N = 6$; $P < 0.05$). Figure 3B shows the relationship between the dose of bepridil (10–50 μM) and the magnitude of bepridil-induced capacitative Ca^{2+} entry. We next examined whether bepridil could affect thapsigargin-induced capacitative Ca^{2+} entry. Figure 3C shows that 1 μM thapsigargin induced significant capacitative Ca^{2+} entry with a peak height of 402 ± 11 nM (*trace a*) which was inhibited by 38 ± 4% in the area under the curve by adding bepridil (50 μM) 110 sec prior to Ca^{2+} (*trace b*; $N = 6$; $P < 0.05$).

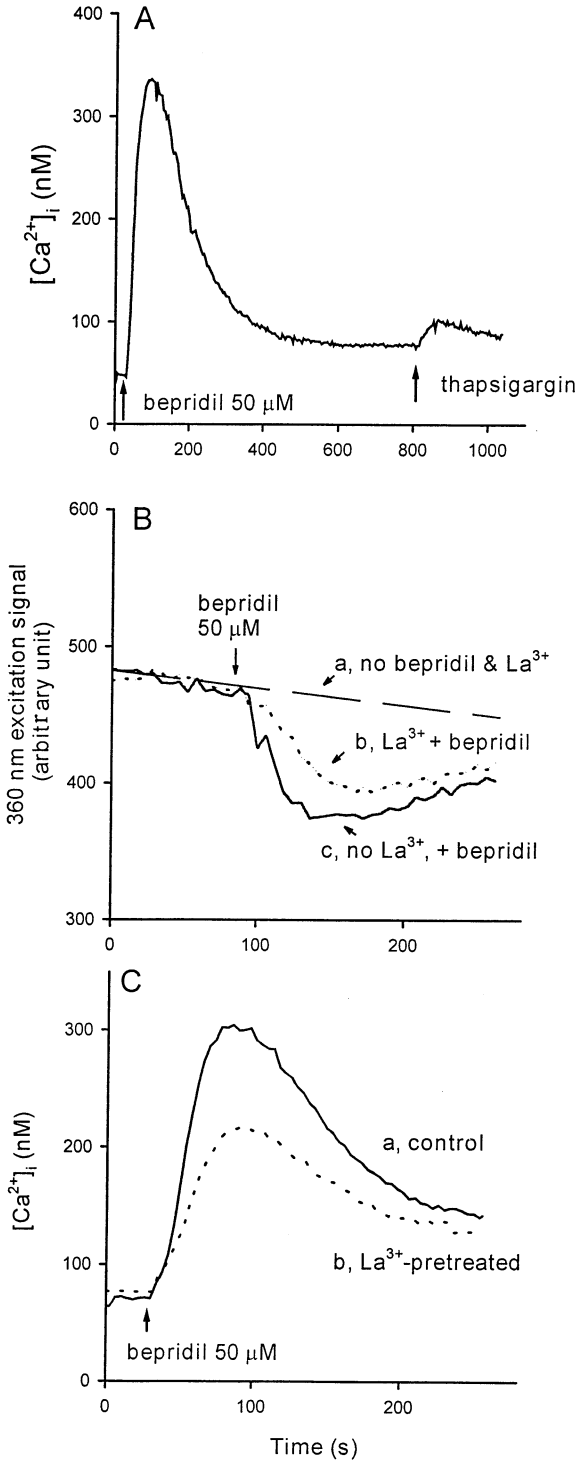

FIG. 2. (A) In Ca^{2+}-free medium, bepridil was added at 30 sec followed by thapsigargin (1 μM) at 800 sec. (B) Ca^{2+} influx detected by Mn^{2+} quench measurements. The experiments were performed in Ca^{2+} medium plus 50 μM $MnCl_2$. The excitation signal at 360 nm and the emission signal at 510 nm were continuously recorded at 1-sec intervals. *Trace a*: control without addition of bepridil. *Trace c*: bepridil (50 μM) was added at 100 sec. *Trace b*: La^{3+} (0.1 mM) was added 10 sec prior to bepridil. Traces are typical of 5–6 experiments. (C) *Trace a*: bepridil (50 μM)-induced $[Ca^{2+}]_i$ rise in Ca^{2+} medium. Bepridil was added at 30 sec. *Trace b*: similar to *trace a* except that La^{3+} (0.1 mM) was added 10 sec prior to bepridil.

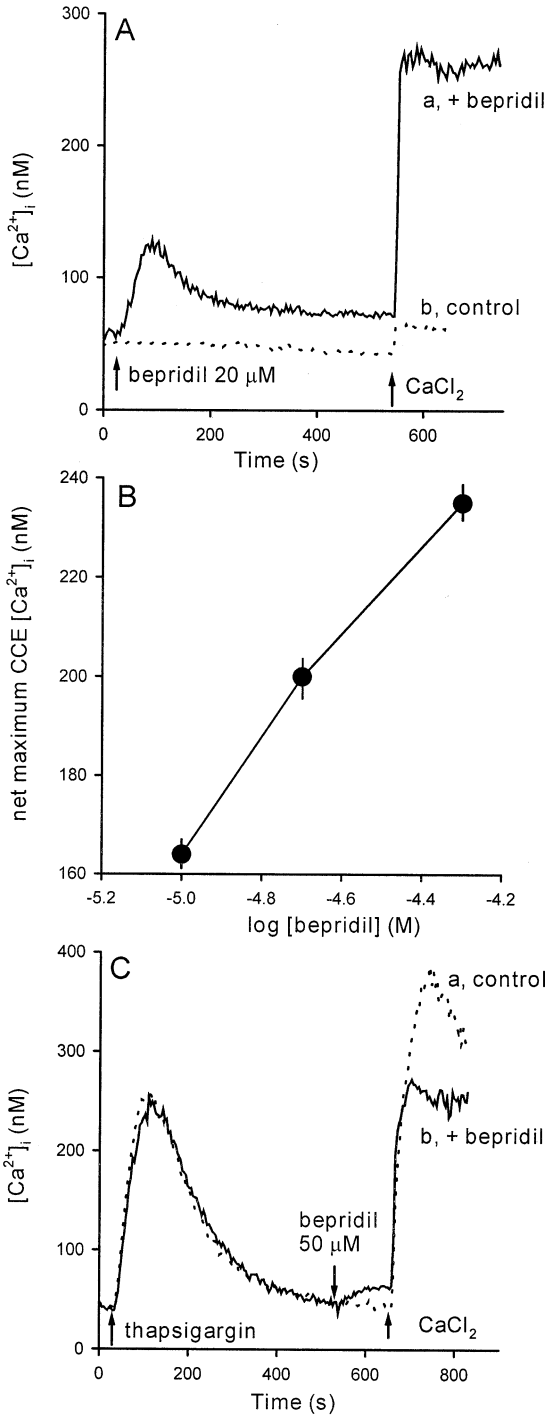

FIG. 3. Effects of bepridil on capacitative Ca^{2+} entry. Capacitative Ca^{2+} entry was induced by depleting internal Ca^{2+} stores in Ca^{2+}-free medium followed by addition of 3 mM $CaCl_2$. (A) Capacitative Ca^{2+} entry induced by bepridil. *Trace a*: bepridil (20 μM) was added at 30 sec followed by $CaCl_2$ at 530 sec. *Trace b*: $CaCl_2$ was added at 530 sec without bepridil preincubation. (B) Dose–response plot of bepridil-induced capacitative Ca^{2+} entry. The x axis represents doses of bepridil (10, 20, and 50 μM) and the y axis the net change in maximum $[Ca^{2+}]_i$ induced by capacitative Ca^{2+} entry (CCE). Data are means ± SE of 5–6 experiments. (C) *Trace a*: thapsigargin (1 μM) was added at 30 sec followed by $CaCl_2$ at 650 sec. *Trace b*: similar to *trace a* except that 50 μM bepridil was added 110 sec prior to $CaCl_2$. Traces are typical of 5–6 experiments.

Effects of Inhibition of Phospholipase C, A_2, or D on Bepridil-Induced Internal Ca^{2+} Release

To understand the mechanism by which bepridil releases internal Ca^{2+}, we examined the effect of inhibition of phospholipase C, A_2, or D on bepridil-induced Ca^{2+} release. We have previously shown that ATP induces immediate internal Ca^{2+} release in an IP_3-dependent manner [27]. Figure 4A shows that ATP (10 μM) induced a sharp $[Ca^{2+}]_i$ rise with a net peak height of 301 ± 21 nM (*trace a*; N = 6). Incubation with U73122 (2 μM), a phospholipase C inhibitor [30], for 210 sec induced a small $[Ca^{2+}]_i$ transient, consistent with our previous report [27], but nearly completely prevented subsequently applied ATP (10 μM) from increasing $[Ca^{2+}]_i$ (*trace b*; N = 6; $P < 0.05$), suggesting that U73122 pretreatment effectively inhibited phospholipase C-dependent IP_3 formation. After U73122 pretreatment for 260 sec, application of bepridil (50 μM) induced a $[Ca^{2+}]_i$ rise with a net peak height indistinguishable from control (*trace c*, bepridil effect without U73122 pretreatment; N = 6; $P > 0.05$). Figure 4B shows that pretreatment with aristolochic acid (40 μM), a phospholipase A_2 inhibitor [31], for 260 sec did not alter resting $[Ca^{2+}]_i$, but did inhibit the 50-μM bepridil-induced $[Ca^{2+}]_i$ rise by 42 ± 4% in net peak height (*dashed trace* vs *trace c* in Fig. 4A; 41 ± 8 nM vs 71 ± 10 nM; N = 6; $P < 0.05$). Pretreatment with propranolol (0.1 mM), a phospholipase D inhibitor [32], for 260 sec induced a small $[Ca^{2+}]_i$ transient without significantly altering the bepridil (50 μM)-induced $[Ca^{2+}]_i$ rise (*solid trace* vs *trace c* in Fig. 4A; net $[Ca^{2+}]_i$ change = 68 ± 5 nM vs 71 ± 10 nM; N = 6; $P > 0.05$).

Direct Induction of Ca^{2+} Influx by Bepridil?

Although bepridil induced Mn^{2+} quench of fura-2 fluorescence and capacitative Ca^{2+} entry, it remains obscure whether bepridil-induced Ca^{2+} influx is via capacitative Ca^{2+} entry, which depends on Ca^{2+} store depletion, or by directly opening a plasmalemmal Ca^{2+} channel, or is a contribution of both. We investigated whether bepridil could directly open a plasmalemmal Ca^{2+} channel by first pretreating cells with thapsigargin in Ca^{2+} medium for 40 min to completely deplete internal Ca^{2+} stores and then adding bepridil to see if bepridil could cause a rise in $[Ca^{2+}]_i$. Figure 4C shows that bepridil (50 μM) only induced a very small rise in $[Ca^{2+}]_i$ with a net maximum height of 31 ± 3 nM, which was only 6% of the $[Ca^{2+}]_i$ peak induced by bepridil without thapsigargin pretreatment (Fig. 1A). The ER Ca^{2+} stores had been completely depleted by thapsigargin pretreatment in this experiment, because we previously found that adding 2,5-di-*tert*-butylhydroquinone, another ER Ca^{2+} pump inhibitor [33], subsequently to thapsigargin pretreatment did not increase $[Ca^{2+}]_i$ [23].

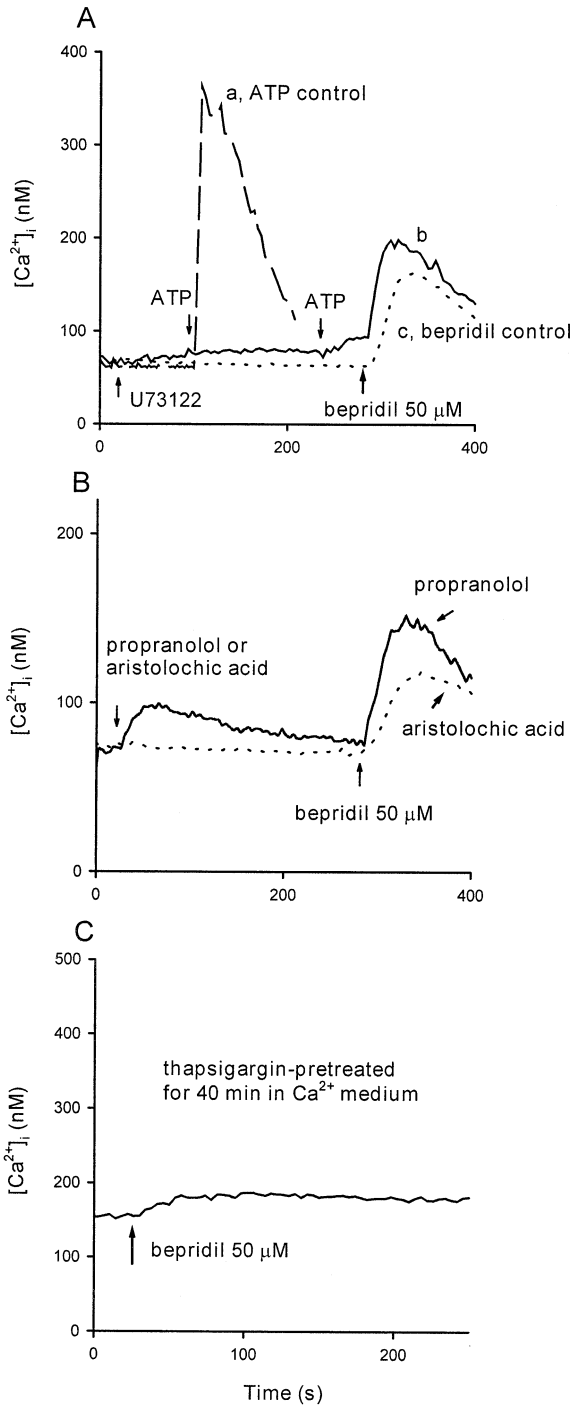

FIG. 4. (A) *Trace a*: ATP (10 μM) was added at 100 sec. *Trace b*: U73122 (2 μM) was added at 30 sec followed by ATP (10 μM) at 230 sec and bepridil (50 μM) at 280 sec, respectively. *Trace c*: control effect of bepridil (50 μM) without pretreatment with U73122 and ATP. The experiments were performed in Ca^{2+}-free medium. (B) *Solid trace*: propranolol (0.1 mM) was added at 30 sec followed by bepridil (50 μM) at 280 sec. *Dashed trace*: aristolochic acid (40 μM) was added at 30 sec followed by bepridil (50 μM) at 280 sec. The control bepridil effect is shown in (A). The experiments were performed in Ca^{2+}-free medium. (C) Cells were incubated with thapsigargin (1 μM) for 40 min in Ca^{2+} medium before fluorescence measurements were started. Bepridil (50 μM) was added at 30 sec. Traces are typical of 5–6 experiments.

DISCUSSION

In the present study, we have found that bepridil elicited a considerable $[Ca^{2+}]_i$ rise in MDCK cells. At doses between 10–50 μM, bepridil induced both external Ca^{2+} influx and internal Ca^{2+} release, because the Ca^{2+} signals were reduced by 40–83% by external Ca^{2+} removal, while Mn^{2+} quench measurements directly demonstrated that bepridil (50 μM) induced Ca^{2+} influx. Ca^{2+} removal reduced both the rising and decay phases, suggesting that external Ca^{2+} influx contributes to the $[Ca^{2+}]_i$ rise throughout the whole course of measurement (~5 min). The inhibition of the bepridil-induced $[Ca^{2+}]_i$ rise by Ca^{2+} removal is not due to depletion of internal Ca^{2+} stores, because cells were incubated in Ca^{2+}-free medium for less than 1 min before experiments were started.

To our knowledge, this is the first study carefully investigating the effect of bepridil on Ca^{2+} signaling in a non-excitable cell line and providing evidence that this antianginal drug induces considerable rises in $[Ca^{2+}]_i$. Previous studies on bepredil have mostly focused on its effects on cardiovascular systems due to its antianginal effects. For example, bepridil has been reported to inhibit voltage-gated Ca^{2+} channels in cardiac cells [3, 4], receptor-operated Ca^{2+} channels in vascular smooth muscle cells [5], Na^+ channels in guinea-pig ventricular myocytes [8, 9], and Na^+/Ca^{2+} exchangers in cardiac cells [11, 12]. Studies of the action of bepredil on $[Ca^{2+}]_i$ in cardiovascular systems and cells from other origin, such as ours, may help lead to an understanding of the adverse reactions to bepredil reported in controlled clinical trials conducted in patients with stable angina, such as dizziness, nausea, headache, asthenia, diarrhea, dyspepsia, nervousness, polymorphous ventricular tachycardia, and ventricular arrhythmia.

Our findings suggest that the thapsigargin-sensitive ER Ca^{2+} store is the main source of bepridil-induced internal Ca^{2+} release, because in Ca^{2+}-free medium, pretreatment with 50 μM bepridil almost completely depleted the Ca^{2+} store sensitive to 1 μM thapsigargin, and vice versa, pretreatment with thapsigargin in Ca^{2+}-free medium nearly prevented bepridil from releasing more Ca^{2+}. The question arises as to how bepridil releases Ca^{2+} from the thapsigargin-sensitive store. So far, we have found that the two major mechanisms by which Ca^{2+} could be released from the ER in MDCK cells are mediated by IP_3 formation, as in the case of the ATP- or bradykinin-induced $[Ca^{2+}]_i$ rise [19, 20], and by direct inhibition of the ER Ca^{2+} pump, as in the case of the thapsigargin- or 2,5-di-*tert*-butylhydroquinone-induced $[Ca^{2+}]_i$ rise [22, 23]. We first investigated whether the internal Ca^{2+} release induced by bepridil was mediated by a rise in IP_3 levels. We used U73122, a phospholipase C inhibitor, to suppress IP_3 formation. Our results imply that bepridil-induced internal Ca^{2+} release might not need a preceding rise in intracellular IP_3 levels, because under the circumstances where IP_3 production was abolished by U73122 pretreatment (evidenced by the observation that subsequently added ATP failed to induce a significant rise in $[Ca^{2+}]_i$, bepridil still induced a $[Ca^{2+}]_i$ rise indistinguishable from control. It is also unlikely that phospholipase D plays a considerable role, because pretreatment with propranolol (0.1 mM) in an attempt to inhibit this enzyme did not have an effect. However, bepridil-induced internal Ca^{2+} release might be modulated by phospholipase A_2, because inhibition of this enzyme with aristolochic acid (40 μM) inhibited the bepridil response by 39% in peak height without depleting internal Ca^{2+} stores. It remains unclear how bepridil releases Ca^{2+} from the thapsigargin-sensitive Ca^{2+} store.

We also examined the mechanisms via which bepridil induces Ca^{2+} entry. Bepridil (10–50 μM) induced significant capacitative Ca^{2+} entry, consistent with the observation that 50 μM bepridil induced significant Mn^{2+} quench of fura-2 fluorescence. It seems that the bepridil-induced Ca^{2+} influx is mainly contributed by capacitative Ca^{2+} entry rather than by direct opening of a plasmalemmal Ca^{2+} channel, because 50 μM bepridil only induced a very small $[Ca^{2+}]_i$ rise after cells had been depleted with internal Ca^{2+} by pretreatment with 1 μM thapsigargin for 40 min in Ca^{2+} medium. Using the same protocol, we previously found that while ATP and 2,5-di-*tert*-butylhydroquinone induced significant capacitative Ca^{2+} entry, they did not induce $[Ca^{2+}]_i$ rises in Ca^{2+} medium after cells had been depleted with internal Ca^{2+} with thapsigargin for 30 min, suggesting that they did not directly open a plasmalemmal Ca^{2+} channel [19, 23].

Bepridil per se induced capacitative Ca^{2+} entry, but inhibited 38% of thapsigargin-induced capacitative Ca^{2+} entry. One possible interpretation for bepridil-induced inhibition of capacitative Ca^{2+} entry is that bepridil depolarizes the cell membrane by inhibiting K^+ channels, leading to a decrease in the driving force for Ca^{2+} entry. This hypothesis is unlikely, because depolarization of cells by inhibiting K^+ channels with tetraethylammonium (TEA; 20 mM) plus charybdotoxin (10 μM) did not alter thapsigargin-induced capacitative Ca^{2+} entry (not shown). In Ca^{2+}-free medium, the 1 μM thapsigargin-induced $[Ca^{2+}]_i$ rise had completely returned to baseline by the time point of 500 sec (Fig. 3C), while the $[Ca^{2+}]_i$ rise induced by bepridil (50 μM) still remained elevated above baseline by ~25 nM at the time point of 800 sec (Fig. 2A). We have found similar phenomena in the Ca^{2+} release induced by econazole and SKF 96365 [28, 29]. One possible explanation is that bepridil inhibited the plasmalemmal Ca^{2+} pump, leading to a delayed Ca^{2+} efflux.

The Ca^{2+} influx activated by 50 μM bepridil, which is mainly mediated by capacitative Ca^{2+} entry, could be separated into two components on the basis of sensitivity to La^{3+} (0.1 mM), because La^{3+} partly inhibited bepridil-induced Mn^{2+} quench of fura-2 fluorescence. Consistently, La^{3+} partly reduced the bepridil-induced $[Ca^{2+}]_i$ rise in fura-2 measurements. We have previously shown that MDCK cells have two Ca^{2+} influx pathways which could be distinguished by sensitivity to La^{3+} (0.1 mM). For example, an La^{3+}-sensitive Ca^{2+} influx and an La^{3+}-

insensitive Ca^{2+} influx contributed equally to the Ca^{2+} entry induced by econazole and SKF 96365 [28, 29].

Because bepridil was found to inhibit calmodulin-coupled intracellular events [15–17] and calmodulin antagonists such as calmidazolium and fendiline have been found to induce a rise in $[Ca^{2+}]_i$ in *Dictyostelium discoideum* [34], one might argue that bepridil induces a $[Ca^{2+}]_i$ rise in MDCK cells by inhibiting calmodulin. Our data suggest that bepridil's effect on $[Ca^{2+}]_i$ is not necessarily associated with inhibition of calmodulin, because although some calmodulin inhibitors such as W-7, fendiline, calmidazolium, and clotrimazolium induced a rise in $[Ca^{2+}]_i$ in MDCK cells, trifluoperazine (5–50 μM), phenoxybenzamine (100–200 μM), and fluphenazine-*N*-chloroethane (2–100 μM) did not.*

Collectively, we have characterized the $[Ca^{2+}]_i$ rise induced by bepridil in MDCK cells and have investigated the underlying mechanism. We found several effects of bepridil: 1) induction of a $[Ca^{2+}]_i$ rise in a dose-dependent manner by activating both external Ca^{2+} influx and internal Ca^{2+} release; 2) release of internal Ca^{2+} from the thapsigargin-sensitive internal Ca^{2+} store; 3) activation of Ca^{2+} influx via capacitative Ca^{2+} entry which could be separated into two pathways by sensitivity to La^{3+} (0.1 mM); 4) inhibition of thapsigargin (1 μM)-induced capacitative Ca^{2+} entry; and 5) mobilization of internal Ca^{2+} release in an IP_3-independent manner which could be regulated by phospholipase A_2. To our knowledge, this is the first study reporting that bepridil, conventionally deemed as a blocker of both voltage-gated and receptor-coupled Ca^{2+} entry, induced a rise in $[Ca^{2+}]_i$ in a non-excitable epithelial cell line.

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