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EFFECT OF METHYLATED BEPRIDIL ON SLOW ACTION POTENTIALS IN CARDIAC MUSCLE AND VASCULAR SMOOTH MUSCLE

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Abstract—The anti-anginal agent bepridil blocks slow Ca^{2+} channels in a variety of tissues. Since bepridil accumulates inside cells, the possibility exists that bepridil acts, at least partially, from inside the cell. To test this possibility, we examined the effects of a quaternary ammonium analog of bepridil, methylated bepridil, which presumably would enter the cells less readily, on the Ca^{2+}-dependent slow action potentials of guinea pig papillary muscles (in 25 mM $[K^+]_o$) and rabbit pulmonary arteries (in tetraethylammonium chloride). In cardiac muscle, methylated bepridil had little effect on the slow action potentials at low stimulation frequencies (0.5 Hz), but at higher frequencies (1.0 and 2.0 Hz) the slow action potentials were depressed and/or the muscle was unable to follow each stimulation. These effects are similar to those obtained with bepridil, but bepridil was more potent than methylated bepridil. In vascular smooth muscle cells, methylated bepridil inhibited the slow action potentials at a somewhat lower dose than bepridil. We conclude that, in cardiac muscle, bepridil probably has two sites of action, one outside the cell (presumably on or associated with the slow Ca^{2+} channel) and a second site inside the cell. On the other hand, in vascular smooth muscle cells, bepridil may act only on an external site.

The anti-anginal agent, bepridil, has been classified as a Ca^{2+} antagonist. Bepridil blocks the Ca^{2+}-dependent slow action potentials (APs) of guinea pig myocardium [1], vascular smooth muscle [2, 3], and skeletal muscle [4], and depresses the slow inward current in voltage-clamped frog atrial fibers [5]. Thus, bepridil acts as a slow channel blocker.

Bepridil readily enters myocardial cells and vascular smooth muscle cells, and actually accumulates in these cells [6–8]. Thus, the possibility exists that bepridil may exert its slow-channel blocking effects at least partially from inside the cell. To test this possibility, we examined the effects of a methylated analog of bepridil, which is charged and presumably would enter the cell less readily.

METHODS

Guinea pig papillary muscles. Guinea pigs (~300 g, either sex) were stunned by a blow to the head, and the heart was removed immediately. Papillary muscles were rapidly dissected from the right ventricle in oxygenated physiological saline solution (PSS) at room temperature. Immediately after excision, the preparations were pinned to the Sylgard resin (Dow Corning) base of a chamber (0.6 ml volume). PSS flowed through the chamber at a rate of 1.75 ml/min.

The composition of the normal PSS solution was (mM): 114.3 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.7 NaH_2PO_4, 0.76 $MgCl_2$, 2.0 $CaCl_2$, and 11.0 glucose. Elevated K^+ solutions (25 mM) were prepared by equimolar substitution of KCl for NaCl. The temperature was maintained at 37.0 ± 0.5°, and the perfusing solution was gassed with 95% O_2 and 5% CO_2.

For point stimulation, one stimulating electrode was a thin glass tube (0.6 mm outer diameter), filled with PSS solution, pressed directly against the papillary muscle so as to reduce the voltage required for stimulation. The other electrode was a platinum plate placed 2–3 mm from the preparation. For field stimulation, two platinum plates were used to stimulate the muscles. No differences were noted between point and field stimulation in the effects of bepridil or methylated bepridil. Square wave pulses, 1–3 msec in duration, were applied at a frequency of 0.5 to 2.0 Hz (Grass S88 stimulator). Action potentials were recorded by microelectrodes (resistances of 18–40 MΩ) filled with 3 M KCl. Ag:AgCl half-cells were used. The electrodes were connected to a Dagan 8500 microelectrode preamplifier. An RC differentiator was used for differentiation of the APs. Action potentials and their first derivatives were displayed on a Tektronix 5111 storage oscilloscope and then photographed (Grass C4 kymograph camera). Action potentials were also stored on a computer (Apple IIe) for subsequent data analysis.

Slow APs were induced in the high K^+ solution

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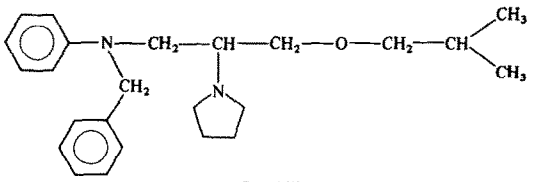
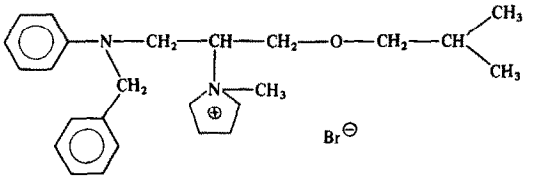


Bepridil



Methylated bepridil
(CERM 11888)

Fig. 1. Structures of bepridil and its quaternary ammonium derivative, methylated bepridil (CERM 11888).

by the addition of 10^{-6} M isoproterenol-HCl to the solution.

Bepridil (Wallace Laboratories) was dissolved in ethanol/H_2O, and methylated bepridil (CERM 11888, Riom Laboratories – C.E.R.M.) was dissolved in either H_2O or ethanol/H_2O to give a final stock concentration of 10^{-2} M, and kept at 4° until used. The final concentration of ethanol was $\leq 0.03\%$, a concentration that has no effect on slow APs [1]. Furthermore, no differences were noted between the effects of methylated bepridil dissolved in ethanol/H_2O and that dissolved in H_2O alone. The structures of bepridil and methylated bepridil are given in Fig. 1.

Rabbit pulmonary artery. Male New Zealand white rabbits (2–3 kg) were stunned by a blow to the back of the head. The main pulmonary artery was excised, and segments of the artery were cut open to form strips (2 × 3 mm). An arterial strip was pinned to the Sylgard resin (Dow Corning Corp.) base of a Lucite tissue chamber (0.5 ml volume). The muscle was superfused at 37° with the PSS at a rate of approximately 2 ml/min.

The PSS had the following composition (in mM): 115 NaCl, 5.0 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 1.7 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose. The solution was equilibrated with a mixture of 95% O_2 and 5% CO_2 (pH of 7.3–7.4). Hypertonic solutions were prepared by adding 234 mM sucrose to the PSS. Tetraethylammonium chloride (TEA, Eastman

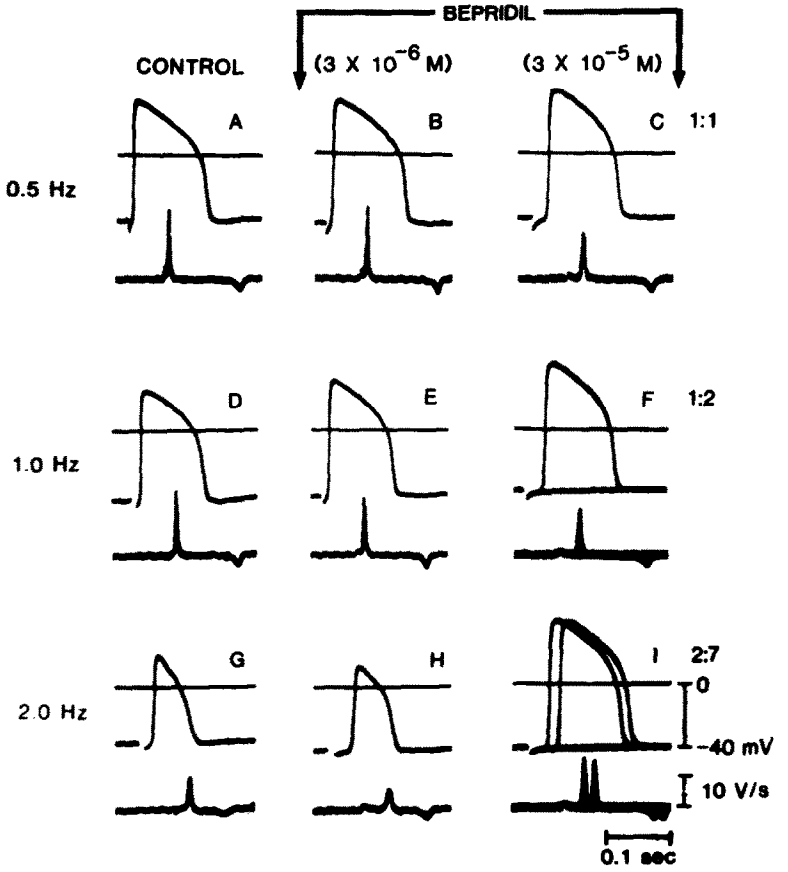


Fig. 2. Frequency- and dose-dependent effects of bepridil on slow APs in isolated guinea pig papillary muscle. The lower trace is dV/dt. A,D,G: Control slow APs induced by 10^{-6} M isoproterenol (in 25 mM $[K^+]_o$) at 0.5 Hz (A), 1.0 Hz (D), and 2.0 Hz (G). B,E,H: Superfusion of the muscle for at least 30 min with 3 × 10^{-6} M bepridil. C,F,I: Superfusion of the muscle, for at least 30 min, with 3 × 10^{-5} M bepridil.

Kodak Co.) was added to give a final concentration of 5 or 10 mM.

Electrical activity was recorded intracellularly from the intimal surface of the arterial strips. The microelectrodes, made of Kwik-fil glass capillaries (WPI), were filled with 3 M KCl and had resistances of 30–60 MΩ. The microelectrodes were positioned with a Zeiss sliding micromanipulator. The recording preamplifier was a WPI (model M4A) electrometer, and the reversible half-cells were Ag:AgCl. The arterial strips were stimulated extracellularly (Grass S4 stimulator) by rectangular current pulses (10–30 msec in duration) applied through platinum plate electrodes (field stimulation). Responses were displayed on a Tektronix (Type 565) oscilloscope and photographed with a Grass (model C4) kymograph camera.

Methylated bepridil was dissolved in H_2O to prepare a stock solution, as described above.

RESULTS

Induction of cardiac slow action potentials. In the normal (adult) vertebrate ventricular action potential, a large fast inward Na^+ current is responsible for the electrogenesis of the early spike component, while a slow small inward current contributes to the maintenance of the plateau component. To examine the properties of the slow inward current, it is convenient to inactivate the fast Na^+ channels with TTX or elevated $[K^+]_o$. Slow responses (accompanied by contractions) can then be elicited upon electrical stimulation following the addition of certain agents which increase the slow inward current or decrease the outward current.

After the recording of fast APs in normal Tyrode's (4.7 mM K^+) solution, the fast Na^+ channels were voltage in activated by partial depolarization (to about −40 mV) with an elevated (25 mM) K^+ solution. This resulted in a loss of excitability and mechanical activity, despite intense (typically 10–20 times threshold) stimulation.

Addition of 10^{-6} M isoproterenol–HCl allowed a slowly rising, overshooting, electrical response to be elicited upon electrical stimulation within 2 min. In contrast with the normal fast APs, slow APs rise slowly (~5–25 V/sec vs. ~100–250 V/sec for fast APs); however, they overshoot and are similar in shape to the plateau component of the normal fast AP.

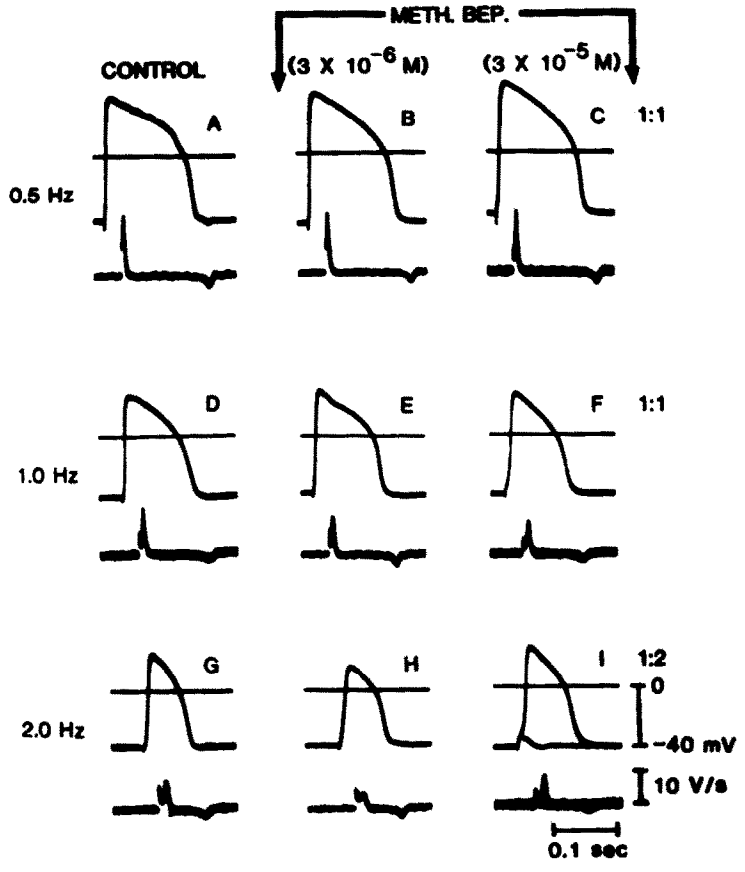


Fig. 3. Frequency- and dose-dependent effects of methylated bepridil (CERM 11888) on slow APs in isolated guinea pig papillary muscle. A,D,G: Control slow APs induced by 10^{-6} M isoproterenol (in 25 mM $[K^+]_o$) at 0.5 Hz (A), 1.0 Hz (D), and 2.0 Hz (G). B,E,H: Superfusion of the muscle, for at least 30 min, with 3×10^{-6} M methylated bepridil. C,F,I: Superfusion of the muscle, for at least 30 min, with 3×10^{-5} M methylated bepridil.

Effects of methylated bepridil on cardiac slow action potentials. Following induction of slow APs with isoproterenol, subsequent superfusion of six muscles with 10^{-6} M–3×10^{-5} M bepridil for 30–45 min had only a small effect on the slow APs at 0.5 Hz (Fig. 2). In each instance while bepridil had only a small effect on cardiac slow APs at 0.5 Hz, at higher frequencies there was a much more pronounced effect. Thus, following superfusion with 3×10^{-6} M bepridil, at stimulating frequencies of 1.0 Hz and 2.0 Hz there was some inhibition of the slow APs (depressed $\dot{V}_{max}$). In 3×10^{-5} M bepridil, at these higher frequencies, the muscle could no longer respond to each stimulation. Thus, there was only one AP for every two stimuli, as noted to the right of panel F (1:2). At 2.0 Hz (I), the muscle responded with two APs for every seven stimuli (2:7); however, these APs were larger than the control APs at 2.0 Hz, presumably due to the very low effective frequency.

As can be seen in Fig. 3, the effects of methylated bepridil were quite similar to those of bepridil (Fig. 2). Doses of both drugs ranging from 10^{-6} M to 3×10^{-5} M were used. The effects of both compounds were quite similar, i.e. a dose-dependent and frequency-dependent depression of $\dot{V}_{max}$ and eventual inability to follow each stimulation, though bepridil tended to be more potent. Thus for example, at 3×10^{-5} M the muscle was generally unable to follow each stimulation at a lower stimulation frequency for bepridil than methylated bepridil for there was no effect on the slow APs at 0.5 Hz (C), while at 1.0 Hz (F) this dose of methylated bepridil substantially inhibited the slow APs, and at 2.0 Hz (I) the muscle responded to every two stimuli with one AP (1:2). The effects of methylated bepridil generally disappeared following 10–20 min of washout of the drug (Fig. 4) at concentrations $\leq 10^{-5}$ M, though following prolonged exposure to 3×10^{-5} M methylated bepridil the effects were less readily reversible upon washout. The effects of bepridil were much less readily reversed upon washout (i.e. they either did not reverse at all or took 1 hr or more of washout to reverse).

Effects of methylated bepridil on TEA-induced action potentials in rabbit pulmonary artery. The vascular smooth muscle (VSM) cells of the rabbit pulmonary artery are normally electrically quiescent. However, upon addition of 5–10 mM tetraethylammonium (TEA), Ca^{2+}-dependent slow APs can be elicited in response to electrical stimulation [9]. Figure 5 illustrates a typical inhibitory effect of methylated bepridil on the TEA-induced slow APs. Superfusion with methylated bepridil (10^{-7}–3×10^{-6} m) in four experiments resulted in a dose-dependent decrease in the action potential amplitude (Fig. 6). (Bepridil was not quite as potent an inhibitor in this

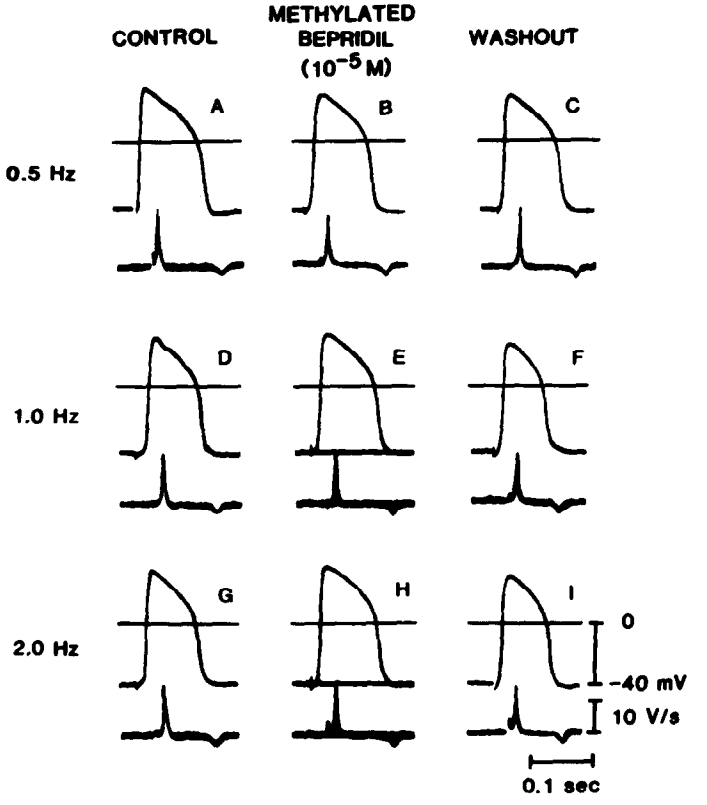


Fig. 4. Washout of the effect of methylated bepridil (CERM 11888) on slow APs induced by 10^{-6} M isoproterenol (in 25 mM $[K^+]_0$). A,B,C: Control slow APs induced by 10^{-6} M isoproterenol (in 25 mM $[K^+]_0$). B,E,H: Frequency-dependent inhibition of slow APs by 10^{-5} M methylated bepridil. C,F,I: Washout of the drug for 10–20 min.

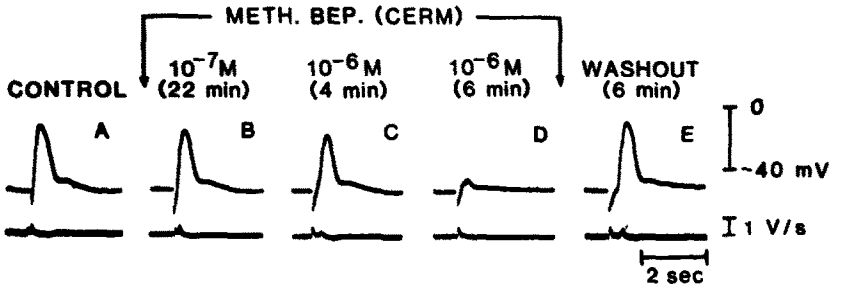


Fig. 5. Effect of methylated bepridil (CERM 11888) on slow APs in rabbit pulmonary artery. A: Control slow AP induced by 5 mM TEA. B: Superfusion of the muscle with 10^{-7} M methylated bepridil for 22 min slightly inhibited the slow APs. C,D: Superfusion of the muscle with 10^{-6} M methylated bepridil for 4 min inhibited (C) and at 6 min abolished (D) the slow APs. E: Washout of the drug for 6 min resulted in recovery of the slow APs.

preparation.*) The APs were depressed within 5–10 min in the presence of 10^{-6} M methylated bepridil and were abolished almost completely by 3×10^{-6} M methylated bepridil. The inhibitory action of methylated bepridil could be reversed partially by increasing the stimulus strength or $[Ca^{2+}]_o$ (not shown). The time required for recovery of the APs upon washout of the drug depended upon its concentration, i.e. recovery was rapid (<10 min) at low concentrations ($<3 \times 10^{-6}$ M; Fig. 5), but was much slower (>50 min) at higher concentrations.

Effects of methylated bepridil (ROGERS). A second methylated analog of bepridil was synthesized.† This compound—methylated bepridil (ROGERS)—was used in five experiments (see below). A stock solution of this compound was made as described for methylated bepridil (CERM 11888) above, using ethanol/H_2O as the solvent.

The breakdown product of methylated bepridil (ROGERS) was extracted from solution (stored at 4° for 1 week), with 500 μl of $CHCl_3$ followed by 2×500 μl of chloroform/methanol (2:1). The organic phases were combined and dried under N_2. The product was recrystallized from ethanol/H_2O (1:1).

While both methylated bepridils (ROGERS and CERM 118888) had no effect on cardiac muscle at 0.5 Hz when used fresh (i.e. within 3 days), if the ROGERS compound was stored for a week or more, it became quite potent at 0.5 Hz (Fig. 7). On the other hand, in seven attempts the CERM compound did not become active at 0.5 Hz, even after storage for up to 7 weeks.

* J. M. Ousterhout and N. Sperelakis, unpublished results.

† T. B. Rogers and J. C. Biswas, unpublished results.

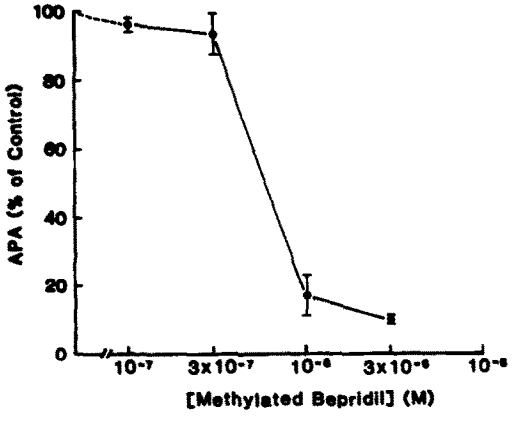


Fig. 6. Dose-dependent effects of methylated bepridil (CERM 11888) on slow TEA-induced APs of rabbit pulmonary artery. Each point represents the mean ±S.E.M. of four values.

DISCUSSION

In the original paper on the cardiac electrophysiology of bepridil [1], it was hypothesized that bepridil might have two sites of action, one external and one internal—since contractions were depressed more readily than Ca^{2+}-dependent slow APs, and the effects of bepridil on contraction were not readily washed out. One might alternatively hypothesize two external sites of action—one on the slow channels and secondly on some other sarcolemmal transport system (e.g. Ca^{2+}-ATPase). Later results from our laboratory and others [6–8], indicated that bepridil readily enters cardiac muscle and vascular smooth muscle cells (and actually attains apparent internal concentrations much higher than the extracellular concentration); such uptakes allow for the possibility of an internal site of action. Contrary to bepridil, since methylated bepridil is a quarternary ammonium compound, it presumably does not readily enter cells, a hypothesis which is supported by the relative (compared to bepridil) rapidity of washout. Thus, we consider that methylated bepridil acts largely, if not entirely, from outside the cell.

Several possible internal sites of action for bepridil have been proposed. Bepridil has been shown to block Ca^{2+} uptake into and Na^+-induced Ca^{2+} release from mitochondria [10, 11], and to bind tightly to actin [8]. It also affects adenylate cyclase [12] and the Ca^{2+}-ATPase of sarcoplasmic reticulum [13]. One internal site of action that has been described for bepridil is an inhibition of the calcium-regulating protein, calmodulin [14–16]. This may be an important mechanism for Ca^{2+}-channel blockade since results from our laboratory have shown that

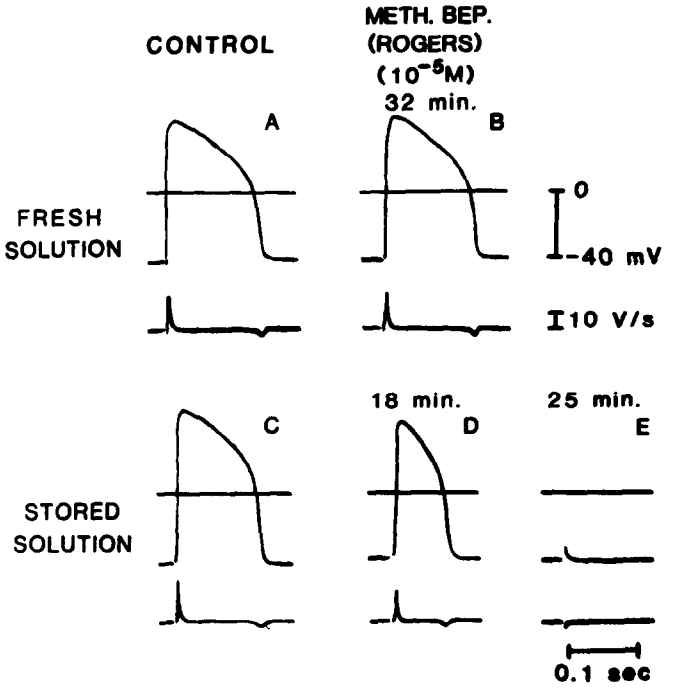


Fig. 7. Effects of fresh and stored solutions of methylated bepridil (ROGERS) on slow APs in isolated guinea pig papillary muscle. A: Control slow AP induced by 10^{-6}M isoproterenol (in 25 mM $[K^+]_0$). B: Superfusion of the muscle with a fresh solution of 10^{-5} M methylated bepridil (ROGERS) for 32 min at 0.5 Hz. C: Control slow AP. D,E: Superfusion of the muscle with methylated bepridil (ROGERS), the stock solution of which had been stored for 14 days, for 18 min inhibited (D) and, after 25 min, abolished (E) the slow APs.

other calmodulin inhibitors (trifluoperazine, calmidazolium) block cardiac slow action potentials [17, 18]. However, the results of the present study, in which methylated bepridil inhibited the Ca^{2+}-dependent slow action potentials in rabbit pulmonary artery and isolated guinea pig papillary muscles much like bepridil, suggest that bepridil also has a major external site of action, presumably on the Ca^{2+} channels or an associated regulatory protein.

In vascular smooth muscle, it may be that the external site of action for bepridil is the only site, since the potencies of methylated bepridil (CERM 11888) and bepridil are similar in depressing contractions of rabbit aortic rings*, and, if anything, methylated bepridil is more potent than bepridil in inhibiting slow APs in this tissue. On the other hand, it appears likely that bepridil has at least one, internal site of action in cardiac muscle, since bepridil was more potent than methylated bepridil in depressing slow APs (Fig. 2 and 3) and contractions* of guinea pig papillary muscles. In addition, it has been found that bepridil could nearly abolish contractions with only a moderate effect on the slow APs, suggesting that there may be a second mechanism for the contractility depression of bepridil in addition to effects on the slow channels [1]. Thus, one could hypothesize that bepridil actually has three sites of action in cardiac muscle: (a) one external affecting the slow Ca^{2+} channels, (b) one internal affecting the slow Ca^{2+} channels, and (c) one internal (or external) affecting contraction without affecting the slow Ca^{2+} channels. An alternative possibility is that methylated bepridil and bepridil act at the same site(s), but that in cardiac muscle they have different potencies, while in VSM the potencies are similar or reversed.

Methylated bepridil (ROGERS) was found to have somewhat different actions than methylated bepridil (CERM 11888). While, like the CERM compound, the ROGERS compound has no effect when a fresh stock solution was used (at 0.5 Hz), unlike the CERM compound there was a block of slow APs at this frequency when the ROGERS stock solution had been stored for a week or longer (Table 1, Fig. 7). This indicated that some chemical transformation had taken place in the ROGERS compound. Storage of the CERM stock solution for up to 7 weeks did not induce slow channel blocking activity at 0.5 Hz. Thus, four different compounds (at least) were used in these experiments. These four compounds [bepridil, methylated bepridil (CERM 11888), methylated bepridil (ROGERS), and the breakdown product of methylated bepridil (ROGERS)] are indeed distinct compounds, as determined on the basis of differing physical properties. The four compounds have different melting points, solubilities in H_2O, and can be separated chromatographically.† The very high melting point of the breakdown product of methylated bepridil (ROGERS) suggested that it was still quite polar. Further testing using the method of Reiss [19] determined that the breakdown

* N. Busch and J. C. Lamar, unpublished results.
† J. C. Biswas and T. B. Rogers, unpublished results.

Table 1. Comparison of effects of fresh and stored methylated bepridil solutions on slow action potentials in guinea pig papillary muscles stimulated at 0.5 Hz

Methylated bepridil* (10^{-5} M)	$\dot{V}_{max}$	APA (% of control)	APD_{50}
CERM 11888			
Fresh	101 ± 8	100 ± 4	94 ± 5
Stored	95 ± 10	94 ± 4	90 ± 6
Rogers compound			
Fresh	92 ± 4	98 ± 2	93 ± 4
Stored	13 ± 10†	19 ± 13†	16 ± 11†

Fresh solutions were those that were used within 3 days of preparation of the stock solution. Stored solutions were those that had been stored (as stock solution at 4°) for at least 7 days prior to use. APA = action potential amplitude; APD_{50} = AP duration at 50% repolarization; $\dot{V}_{max}$ = maximum rate of rise of the AP. Stimulation frequency 0.5 Hz; see text for effects at higher rates. Values are means ±S.E.M., N = 5–7.

* Methylated bepridil (CERM 11888) was prepared at RIOM Laboratories. Methylated bepridil (ROGERS) was prepared by J. C. Biswas and T. B. Rogers.

† $P < 0.05$.

product was still a quarternary ammonium compound. The exact structures of methylated bepridil (ROGERS) and its breakdown product are not known at this time. However, from the results obtained, it is likely that methylated bepridil (ROGERS) is methylated on the phenyl nitrogen, rather than on the same nitrogen as methylated bepridil (CERM 11888). These results suggest that there may be analogs of bepridil which are more potent cardioactive agents than bepridil.

It is of interest that methylated bepridil (CERM 11888) had no slow channel blocking effect in cardiac muscle at low stimulation rates, and only a modest effect at high rates, but was quite potent in vascular smooth muscle even at the very low stimulation rates employed. The frequency-dependent actions of bepridil and methylated bepridil suggest a slowed recovery from channel inactivation. The differences between vascular smooth muscle and cardiac muscle would seem to indicate that there is some difference in the bepridil binding sites of these two tissues, and if the binding site is part of the slow Ca^{2+} channel complex, it may then reflect a difference in the slow channels from these two tissues. However, the high sensitivity of VSM to bepridil could also be due to fewer functional Ca^{2+} channels (reflected in a lower $\dot{V}_{max}$ of VSM compared to cardiac slow APs). The clinical implications of this differential sensitivity are profound, since the major effect of methylated bepridil *in vivo* is to increase coronary flow, without substantially depressing contractility.* Thus, methylated analogs of bepridil seem to have great promise both clinically and in basic research on slow Ca^{2+} channels.

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* N. Busch and J. C. Lamar, unpublished results.

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