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Antipsychotic Pimozide Is a Potent Ca^{2+} Channel Blocker in Heart

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SUMMARY

The diphenylbutylpiperidine (DPBP) antipsychotic pimozide was identified as a potent new Ca^{2+} channel antagonist in heart. In whole-cell patch-clamp experiments, pimozide blocked Ca^{2+} current through L type channels of rat ventricular myocytes, in a voltage-dependent manner. At holding potentials positive to −40 mV, approximately 90% of current was blocked by 200 nM pimozide. Nearly half of this block, 48 ± 5% (mean ± SE, n = 5), was relieved by 5-min hyperpolarization to −100 mV. In quin2-loaded myocytes, pimozide blocked 50 mM KCl-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) half maximally at a concentration of 75 ± 15 nM (n = 5). Two other DPBPs, penfluridol and fluspirilene, also blocked the KCl-induced response at similar concentrations. The contractile force of cardiac tissue was also depressed by pimozide. One micromolar pimozide reduced twitch tension in rat papillary muscles by an average of 36 ± 8% (n = 3). These results demonstrate that the DPBPs comprise a potent new class of Ca^{2+} antagonists in heart, which will be useful in studying cardiac Ca^{2+} channels. They also suggest that these agents may have therapeutic value outside the field of psychiatry.

Excitable neural, endocrine, and muscle cells express one or more of several distinct types of voltage-gated Ca^{2+} channels. These Ca^{2+} channels are differentially sensitive to the major groups of organic Ca^{2+} antagonists (1–5). Several years ago, it was proposed that DPBP antipsychotics comprise a potent new class of Ca^{2+} channel blocker. This claim was based on the observation that these agents inhibited both the specific binding of the dihydropyridine Ca^{2+} channel ligand [^{3}H]nitrendipine to neuronal membranes and depolarization-stimulated contractions in smooth muscle (6). More direct evidence has been obtained in voltage-clamp studies on two separate types of excitable cells. The DPBP fluspirilene at nanomolar concentrations blocked Ca^{2+} current through high threshold, slowly inactivating, L type Ca^{2+} channels in rat skeletal muscle myoballs (7). We found that the antipsychotic pimozide potently blocked Ca^{2+} current through similar channels in the GH_4C_1 pituitary cell line (8).

Ca^{2+} channels play a fundamental role in regulating cardiac excitation and contraction (9–11). They are the primary targets of organic Ca^{2+} antagonists, which are proven therapeutic agents in the treatment of arrhythmias and coronary artery disease (12, 13). The aim of the present study was to characterize the block of L type Ca^{2+} channels in the myocardium by the DPBP pimozide. The effects of pimozide and two other DPBPs on Ca^{2+} currents, $[Ca^{2+}]_i$, and muscle contraction were studied. Some of these results have been published in an abstract (14).

Materials and Methods

Myocyte isolation. The method for myocyte isolation was similar to that described previously (15). Briefly, rats were anesthetized with ether, the thorax was opened, and the heart along with a 5-mm section of the aortic arch was quickly excised. The heart was mounted on a plastic cannula inserted into the aorta and was perfused with Joklik tissue culture medium (GIBCO) containing collagenase (Worthington, Type I and II) and various concentrations of calcium. After dissociation, cells were suspended in modified Krebs-Henseleit solution containing (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES, and bovine serum albumin 0.1%, with the pH adjusted to 7.4 using NaOH.

Ca^{2+} currents. For patch-clamp experiments, myocytes were transferred to protein-free saline containing (in mM): 140 NaCl, 4 CsCl, 10 glucose, 2 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES, with the pH adjusted to 7.36 using NaOH. Na^+ channel currents were eliminated by the addition of 15 μM tetrodotoxin to the bath. Patch electrodes were filled with a solution designed to eliminate all K^+ channel currents and to minimize Ca^{2+} channel rundown. The composition was (in mM): 100 Cs-aspartate, 36 CsCl, 7.5 EGTA, 2.93 $CaCl_2$, 1 $MgCl_2$, 3 Mg-ATP, 5 Na_2-phospho-

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ABBREVIATIONS: DPBP, diphenylbutylpiperidine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

creatine, and 10 HEPES, with the pH adjusted to 7.2 using CsOH. The pipette solution resulted in a free Ca^{2+} concentration of 100 nM and minimized calcium current rundown.

Patch electrodes with resistances of 3–5 MΩ were fabricated from Clay Adams Accu-fill 90 glass micropipettes, and whole-cell Ca^{2+} currents were recorded following the procedure of Hamill *et al.* (16), using a List EPC-7 patch-clamp amplifier. Cells were placed in a recording chamber (2-ml volume) and approached with fire-polished pipettes containing the internal solution. After a gigaohm seal was formed, the patch was ruptured to give a whole-cell clamp. Series resistance was compensated to provide the fastest possible capacity transient without ringing. Ca^{2+} current amplitudes in myocytes were usually less than 1.5 nA. With a 3-MΩ patch electrode, an estimated 4.5-mV steady state series resistance error would occur in the absence of compensation. Pulse generation, data acquisition, analysis, and *P/N* on-line linear leak subtraction were done with a Dell System 200 computer (Dell Computer Company, Austin, TX), using the pCLAMP software package (Axon Instruments, Burlingame, CA).

Stock solutions of DPBPs were prepared by dissolving the drug in dimethyl sulfoxide. The final concentration of the diluent in the recording chamber never exceeded 0.05%, which by itself had no effect on Ca^{2+} currents. Pimozide was applied by direct addition to the bath or by gravity perfusion of the chamber at 3–5 ml/min.

$[Ca^{2+}]_i$ measurements. Myocytes were loaded with the fluorescent indicator quin2 (Calbiochem), as described (15). Quin2-loaded cells were washed several times, centrifuged at 50 × *g* for 1 min, and resuspended in fresh Krebs-Henseleit solution. Only cell suspensions consisting of >75% rod-shaped cells were used for experiments.

For fluorescence measurements, aliquots of quin2-loaded myocyte suspensions were added to a quartz cuvette, at a concentration of 10^6 cells/ml (final volume was 2 ml), and placed in a Perkin-Elmer LS-5 spectrofluorimeter equipped with a magnetic stirrer and thermostated cell holder. The cells were gently stirred at 37° with continuous oxygenation. Monochromator wavelength settings were 339 nm for excitation (3-nm slit) and 490 nm for emission (10-nm slit).

To calculate $[Ca^{2+}]_i$, the following equation was used: $[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F)$, where the dissociation constant (K_d) equals 115 nM (17). The maximal fluorescence value (F_{max}) was obtained by adding the Ca^{2+} ionophore ionomycin (5 μM) or digitonin (70 μM) and 20 mM $CaCl_2$ to the cell suspension. The minimum signal (F_{min}) was obtained by adding 20 mM $MnCl_2$ to quench quin2 fluorescence.

Tension measurements. Papillary muscles (200- to 500-μm in diameter) were dissected from the right ventricle of male Sprague-Dawley rats and attached at one end to the bottom of the recording chamber and at the other to a piezoelectric force transducer (Model 405; Cambridge Technology, Cambridge, MA), as previously described (18). Muscles were superfused with a Tyrode's solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 0.1% bovine serum albumin, and 5 HEPES, with the pH adjusted to 7.4 using NaOH. Contractions were elicited by field stimulation at 2 times the threshold voltage, with a frequency of 2 Hz. The resting muscle lengths were adjusted to give 100% maximal twitch tension and were stimulated at 2 Hz for 1 hr before test conditions were imposed.

Results

In whole-cell patch-clamp experiments, pimozide potently blocked current through L type Ca^{2+} channels. Fig. 1 shows the result of 1 of 12 experiments in which cells were exposed to pimozide at concentrations ranging from 0.1 to 1 μM, while Ca^{2+} currents elicited at 0.1 Hz were recorded. During the 2 min before the addition of 250 nM pimozide, only a small gradual decrease of current amplitude was observed (rundown of Ca^{2+} current). Upon exposure to the drug, the current was rapidly reduced, with a time course clearly distinguishable from that of rundown. Fig.1, *inset*, shows the individual Ca^{2+} current records obtained immediately before and 60, 100, and 360 sec after exposure to pimozide (Fig. 1, *inset, traces 1, 2, 3,* and *4*, respectively). In three experiments, 250 nM pimozide blocked 87% of Ca^{2+} current measured 200 sec after addition of the drug. Table 1 summarizes the effect of five different concentrations of pimozide on peak Ca^{2+} current.

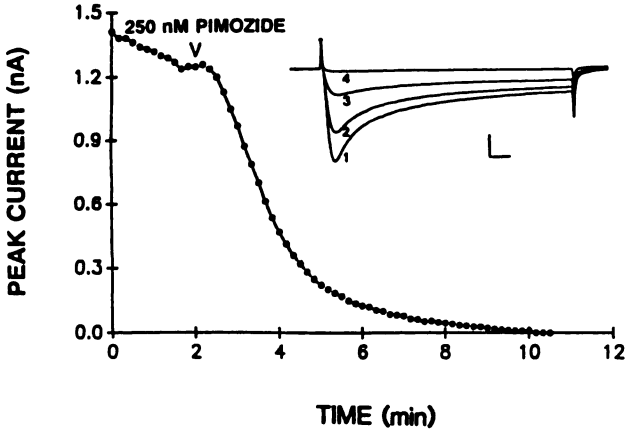

Fig. 1. Blockade of rat myocyte Ca^{2+} current (I_{Ca}) by pimozide. Ca^{2+} currents were activated by 40-mV depolarizing steps, delivered at 0.1 Hz, from a holding potential of −35 mV. Peak Ca^{2+} current amplitudes measured over a 10.5-min period are plotted. *Arrowhead*, addition of pimozide. *Inset*, Ca^{2+} current records from the same myocyte immediately before and 60, 160, and 360 sec after exposure to pimozide (*traces 1, 2, 3,* and *4*, respectively). *Scale bars*, vertical, 300 pA; horizontal, 20 msec.

TABLE 1

Effect of pimozide on peak Ca^{2+} current

Experimental protocol was as described in Fig. 1. For determination of percentage of block, peak Ca^{2+} currents were measured immediately before and after a 200-sec exposure to the drug. Holding potentials were −40 to −30 mV in all experiments.

Pimozide concentration	Decrease in peak current
nM	%
1000 (n = 4)	>97
500 (n = 2)	96
250 (n = 3)	87
200 (n = 2)	88
100 (n = 1)	70

Blockade of Ca^{2+} currents by pimozide was voltage dependent, being more pronounced at depolarized holding potentials. Fig. 2 shows results from one of five similar experiments in which current-voltage relationships were determined at each of six different holding potentials, ranging from −100 to 0 mV, before and after exposure to pimozide. In the experiments, current-voltage relationships were first obtained in control saline from a common holding potential of −40 mV subsequent to 5-min pulse-free intervals at −100, −80, −60, −40, −20, and 0 mV. Myocytes were then exposed to pimozide while test pulses from −40 mV were applied at 0.1 Hz. After Ca^{2+} currents were recorded for 600 sec in this manner to ensure that steady state block was reached, current-voltage relationships were again obtained at each of the six holding potentials in sequence, beginning with 0 mV. Recovery from block was monitored at progressively negative holding potentials.

Fig. 2A, *upper left*, shows voltage steps and associated currents in control solution after a 5-min period at −20 mV. When the membrane potential was held at −20 mV for 5 min after exposure to 200 nM pimozide, the subsequent voltage-elicited

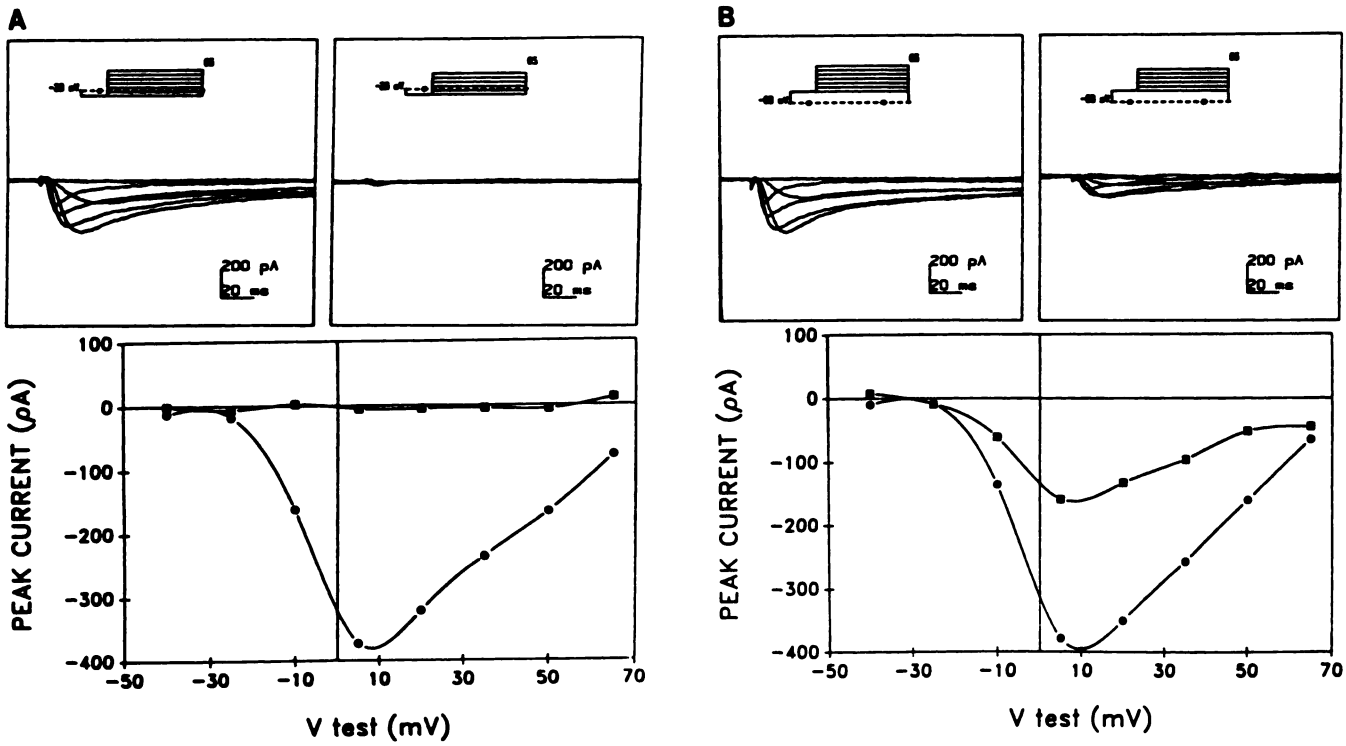

Fig. 2. Voltage dependence and reversibility of pimozide block. All current-voltage (*I-V*) relationships were obtained from a common potential (−40 mV), subsequent to 5-min pulse-free intervals at six different holding potentials (0, −20, −40, −60, −80, and −100 mV), before (*upper left*) and after the addition (*upper right*) of 200 nM pimozide. When changed to saline containing pimozide, Ca^{2+} currents were recorded for 600 sec, as described in the legend to Fig. 1, to guard against rundown artifacts and to ensure that steady state block was attained. Current records and corresponding *I-V* relationships at −20 (A) and −80 mV (B), before (●) and after (■) pimozide, are shown. For *I-V* relationships, peak current is plotted as a function of test potential (*lower*). *Asterisks* on voltage protocols, period of displayed currents. *Dashed line*, 5-min prepulse holding potential.

currents were completely blocked at every test potential (Fig. 2A, *upper right*). The corresponding *I-V* relationships before and after pimozide are plotted in Fig. 2A, *lower*. In Fig. 2B, when the same myocyte was held for 5 min at a more negative potential (−80 mV) before test pulses were given, partial recovery of Ca^{2+} current occurred (*upper left*, before pimozide; *upper right*, in the presence of pimozide). In this experiment, 40% of the maximum current was restored at a holding potential of −80 mV, as indicated in the *I-V* relationship (Fig. 2B, *lower*).

Similar findings were obtained in each of the five experiments where inhibition and recovery were studied at the six various holding potentials. These results are summarized in Fig. 3. At a pimozide concentration of 200 nM, block was maximal at holding potentials positive to −40 mV. The fraction of blocked channels decreased continuously at more hyperpolarized holding potentials. After 5 min at −100 mV, 48 ± 5% (n = 5) of the maximum peak Ca^{2+} current was restored.

Quin2-loaded myocytes. Depolarization of enzymatically dissociated myocytes with saline containing 50 mM KCl produces a sustained rise in $[Ca^{2+}]_i$ as measured by quin2 fluorescence. Seventy to 80% of this increase is blocked by conventional organic Ca^{2+} antagonists for L type channels (19). We used quin2-loaded myocytes to determine the potency of pimozide and two other DPBPs as antagonists of cardiac Ca^{2+} channels. Pimozide antagonized KCl-stimulated increases in $[Ca^{2+}]_i$ with characteristics similar to those of other organic Ca^{2+} channel blockers. In the results shown in Fig. 4A, depolarizing myocytes, when external KCl was raised from 5 to 50 mM by addition of 45 mM KCl to the cuvette, increased

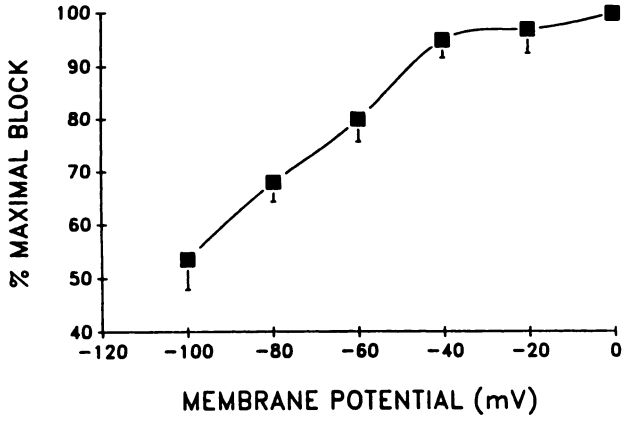

Fig. 3. Voltage dependence of block of maximum peak current. Experimental protocol is as described in the legend to Fig. 2. Block at each holding potential is expressed as a percentage of the maximum block achieved at a holding potential of 0 mV. In each experiment, >90% of the maximum peak current was blocked after 5 min at this potential. Results are mean ± standard error of five experiments.

$[Ca^{2+}]_i$ from 185 to 450 nM. Addition of 1 μM pimozide inhibited this increase by 80%. When cells from the same heart were exposed to pimozide before depolarization, the response to KCl was blunted and $[Ca^{2+}]_i$ increased from 165 to only 210 nM (Fig. 4B).

The reduction of depolarization-induced increases in $[Ca^{2+}]_i$ by pimozide was concentration dependent and half-maximal at 75 ± 15 nM (n = 5) (Fig. 5A). As with other organic Ca^{2+} antagonists, complete inhibition of the KCl-stimulated rise in

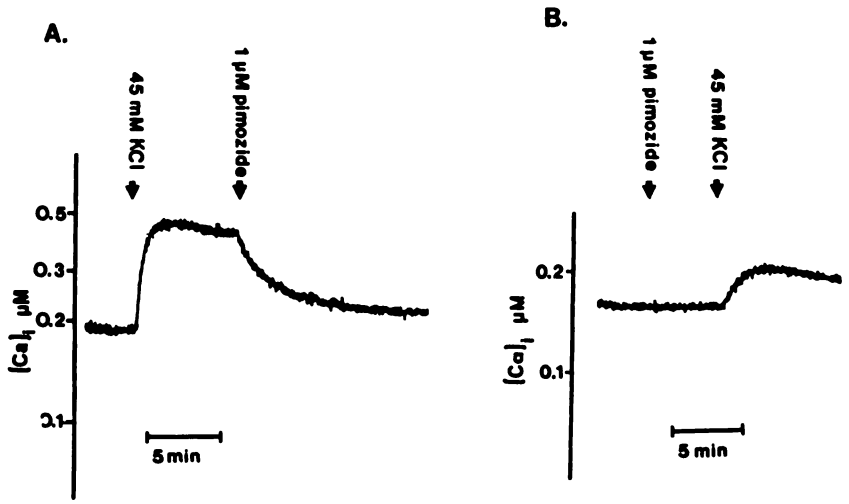

Fig. 4. Inhibition of depolarization-stimulated increases in $[Ca^{2+}]_i$ by pimozide. Quin2 was used to continuously monitor $[Ca^{2+}]_i$ in suspensions of enzymatically dissociated rat ventricular myocytes. A, KCl and pimozide were added sequentially as shown from 100-fold concentrated stock solutions, yielding the indicated final concentrations; B, similar experiments in which pimozide was added before KCl.

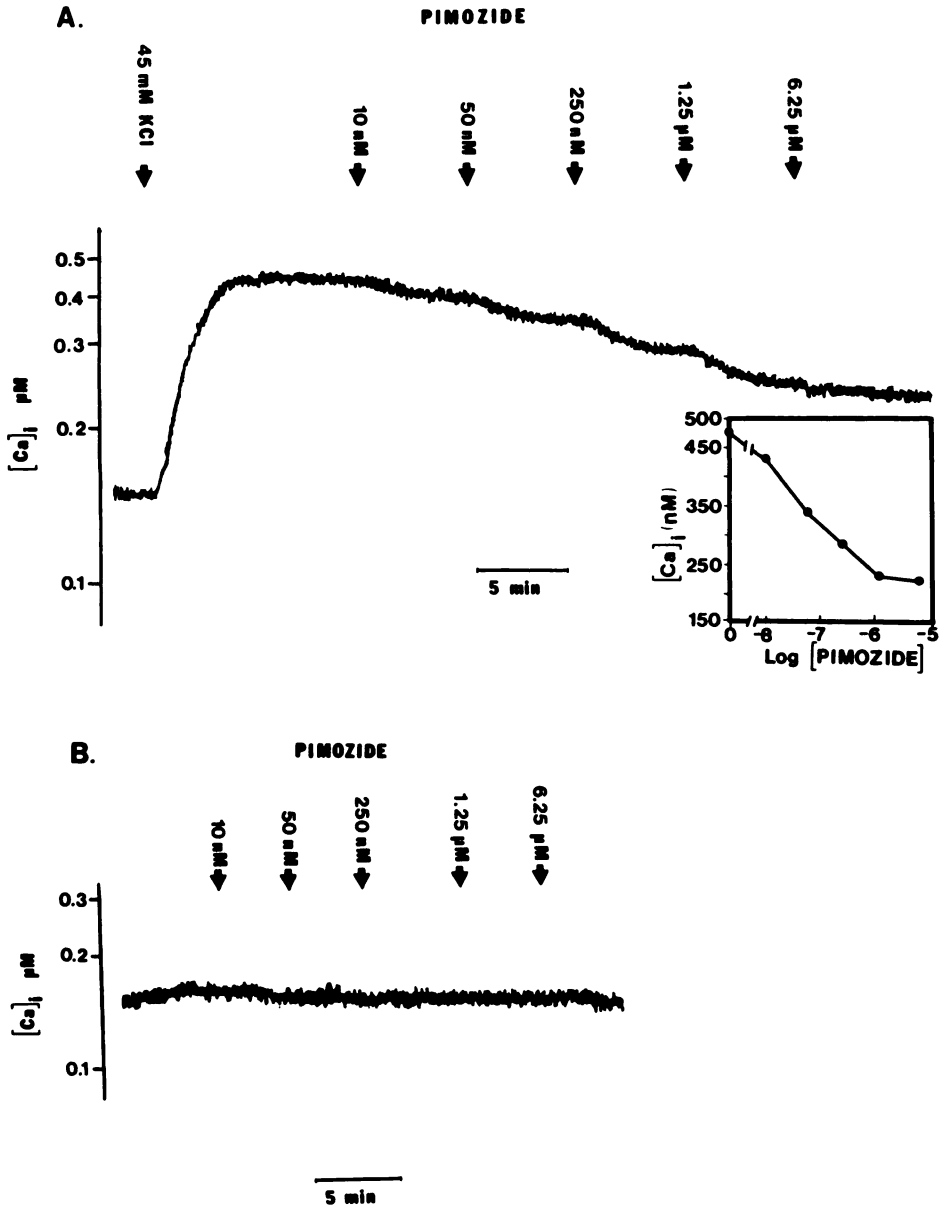

Fig. 5. Dose-response relationship for pimozide effect on $[Ca^{2+}]_i$ in control and KCl-depolarized myocytes. Quin2 was used to continuously monitor $[Ca^{2+}]_i$ in suspensions of enzymatically dissociated rat ventricular myocytes. Myocytes incubated in depolarizing (A) or control (B) saline were exposed to increasing concentrations of pimozide as shown, by addition from concentrated stock solutions. *Inset*, inhibition curve derived from data in A.

$[Ca^{2+}]_i$ by pimozide was never observed in healthy cells with a normal resting $[Ca^{2+}]_i$ of 100–200 nM. In five experiments, maximally effective concentrations of pimozide inhibited KCl responses by an average of 73 ± 6%. Once the maximum effect was achieved, addition of the potent dihydropyridine Ca^{2+} antagonist nimodipine did not reduce $[Ca^{2+}]_i$ further (data not shown). Pimozide did not decrease $[Ca^{2+}]_i$ of myocytes whose Ca^{2+} channels had not been activated by KCl-induced depolarization (Fig. 5B).

Other DPBPs were also found to block KCl-stimulated increases in $[Ca^{2+}]_i$ in cardiac myocytes, with a potency similar to that of pimozide. In each of three experiments, fluspirilene and penfluridol, at maximally effective concentrations of 1.25 μM, inhibited depolarization-dependent increases in $[Ca^{2+}]_i$ by 70–80%. The IC_{50} values for these two drugs were approximately 40 and 70 nM, respectively.

To assess the action of pimozide on cardiac contractility, we studied its effect on twitch tension in isolated rat papillary muscles electrically stimulated at 2 Hz. At concentrations ranging from 1 to 10 μM, pimozide caused a marked reduction in contractile force. One micromolar pimozide inhibited twitch tension by 36 ± 8% ($n = 3$).

Discussion

Whole-cell patch-clamp recordings demonstrated that pimozide potently blocks L type Ca^{2+} channels in rat ventricular myocytes. The drug resembled other major classes of organic Ca^{2+} antagonists in that block was voltage dependent, being more pronounced at depolarized holding potentials (20, 21). The restoration of approximately 50% of blocked channels to the conducting pool with 5 min of hyperpolarization to −100 mV also indicates that pimozide acts by a reversible blocking action rather than by a toxic effect or acceleration of a rundown process. Shorter periods of hyperpolarization were less effective at restoring current. The inability to more completely restore current indicates that, at these pimozide concentrations, many of the channels remained blocked even at hyperpolarized potentials. It is unlikely that Ca^{2+} channel "rundown" masked reversal of block, because rundown in these experiments was found to be less than 10% over a 30-min period immediately before addition of drug. Reversal of block of cardiac L type channels after holding for several minutes at relatively hyperpolarized potentials has been reported for other organic antagonists including nisoldipine and diltiazem (20, 22).

The DPBP fluspirilene has been reported to block L type channels in rat skeletal muscle myoballs with very little voltage dependence (7). However, these researchers allowed only 2 min at hyperpolarized potentials to reverse block. More recently, fluspirilene has been reported to block L type channels in GH_3 pituitary cells in a voltage-dependent manner (23). Within the framework of the modulated receptor hypothesis (24), the binding of any of the Ca^{2+} antagonists to their receptor is influenced by the state of the channel, which is controlled by the membrane voltage. Pimozide resembles other antagonists in that it appears to bind preferentially to inactivated channels, which are prevalent at depolarized potentials.

In this regard, blockade of myocyte Ca^{2+} currents by pimozide resembles that reported for the dihydropyridine Ca^{2+} antagonist nisoldipine in cardiac Purkinje fibers, wherein inhibition increased markedly upon change of the holding potential from −70 to −45 mV (20). In terms of the modulated receptor scheme, it was concluded that nisoldipine preferentially bound and stabilized inactivated channels, and this became an absorbing state. Consequently, a large fraction of channels became unavailable for activation, possibly due to a hyperpolarizing shift in the steady state inactivation curve (h_∞ curve). The observed slow recovery from block is believed to result from restricted diffusion of drug molecules from the receptor via a hydrophobic pathway. Presumably, the charged form of pimozide ($pK_a = 7.32$) would contribute disproportionately to this effect.

Consistent with their blockade of Ca^{2+} current, the DPBPs inhibit depolarization-stimulated increases of $[Ca^{2+}]_i$ in quin2-loaded myocytes. The effective concentrations were similar to those that block KCl-stimulated hormone secretion from GH_4C_1 cells and contractures of vas deferens smooth muscle (6, 8). It has been reported that the KCl-induced increase in $[Ca^{2+}]_i$ is mostly due to Ca^{2+} influx through L type channels (19). These results indicate a similarity among L type Ca^{2+} channels in muscle and endocrine cells with respect to sensitivity to the DPBPs. They also illustrate that, among the major organic antagonists, only the dihydropyridines are more potent than the DPBPs at blocking L type Ca^{2+} channels. Approximately 25% of the increase of $[Ca^{2+}]_i$ in KCl-depolarized myocytes is resistant to block by organic antagonists, including pimozide, and can be attributed to the voltage-dependent Na^+-Ca^{2+} exchange (19).

Blockade of cardiac myocyte Ca^{2+} channels by DPBPs should produce a negative inotropic effect. Pimozide attenuated contractions in papillary muscles but at somewhat higher concentrations than observed in our patch-clamp and quin2 experiments. Presumably, this reflects the decreased potency of the drug in well polarized cells. The action potential duration of rat papillary muscle is less than 100 msec. At the experimental stimulation frequency of 2 Hz, the fiber spends at least 80% of the time near its resting potential, where pimozide is not as effective an antagonist. It is also possible that the diffusion barrier in the intact preparation may reduce drug accessibility.

In blocking Ca^{2+} channels of heart and smooth muscle, the DPBPs resemble Ca^{2+} antagonists such as diltiazem, verapamil, and nifedipine, which are used clinically to treat hypertension, arrhythmias, and angina pectoris (12, 13, 25). It remains to be seen whether members of the DPBP class will find utility in cardiovascular pharmacology. In this regard, a second population of Ca^{2+} channels has been identified in pituitary and heart, the low threshold, rapidly inactivating T channel (26–28). Ca^{2+} movement through these T channels in rabbit sinoatrial cells contributes to the pacemaker potential, suggesting that they may act in regulating the heart rate (10). T channels in heart and pituitary are relatively insensitive to the major organic antagonists (1, 27). We found that pimozide is distinctive in its ability to block this second population of Ca^{2+} channels in GH_4C_1 pituitary cells at low concentrations (29). It will be interesting to determine the effect of DPBPs on T channel current and rhythmicity in the heart.

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