Quaternary Diltiazem Can Act from Both Sides of the Membrane in Ventricular Myocytes

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Received January 25, 1993 Accepted February 1, 1993

ABSTRACT—A quaternary derivative of diltiazem (quat-DTZ) was tested to determine whether diltiazem approaches L-type Ca$^{2+}$ channels from the outside or inside of the cell membrane. In single ventricular myocytes, both extra- and intracellular application of quat-DTZ effectively blocked the L-type Ca$^{2+}$ channel current, whereas D890 was effective only when applied intracellularly. These results strongly suggest that diltiazem binds to the channel from the outside as well as the inside of the membrane in a manner different from that of phenylalkylamines.

Keywords: Quaternary diltiazem, Ca$^{2+}$ channel (L-type), Ventricular myocyte

Three major types of Ca$^{2+}$ antagonists, i.e., 1,4-dihydropyridines (1,4-DHP), phenylalkylamines and 1,5-benzothiazepines (1), are thought to have separate binding sites on the $\alpha_1$-subunit of the L-type Ca$^{2+}$ channel. Recently, the binding sites for 1,4-DHP and phenylalkylamines have been identified on the basis of electrophysiological and biochemical studies (2). Permanently charged quaternary ammonium ion derivatives of 1,4-DHP and phenylalkylamines, which are considered not to permeate the cell membrane, have proved to be useful tools for electrophysiological studies to test whether the binding sites are located on the extracellular or intracellular surface of the cell membrane. The results obtained using quaternary derivatives of phenylalkylamine (D890) (3) and 1,4-DHP (SDZ-270-180) (4) have indicated that the binding site for phenylalkylamines is located on the intracellular surface of the membrane, whereas the 1,4-DHP-binding site lies close to the extracellular surface. However, there is little information on the binding site for 1,5-benzothiazepines. Diltiazem has been reported to be active, like D600, from the inside of the cell membrane (5). Nevertheless, the result did not determine the binding site, because diltiazem is considered to be permeable through the cell membrane.

In the present study, we used a quaternary ammonium ion derivative of diltiazem (quat-DTZ, Fig. 1A) to determine the side of the cell membrane from which diltiazem has access to the L-type Ca$^{2+}$ channel. First, we examined the binding properties of quat-DTZ in comparison with those of diltiazem, and then we tested the effects of extra- and intracellular application of quat-DTZ on L-type Ca$^{2+}$ channel currents ($I_{Ca(L)}$) in ventricular myocytes.

Crude T-tubule membranes were prepared from back muscles of male New Zealand white rabbits (1.5 kg) according to the method described by Ikeda et al. (6). Radioligand binding studies with d-cis-$[^3]H$]diltiazem (86.4 Ci/mmol, NEN Research Products, Boston, MA, USA) and (+)-$[^3]H$]PN200-110 (87.1 Ci/mmol, Amer sham International plc., Buckinghamshire, England) were carried out in 50 mM Tris-HCl (pH 7.4) as described previously (6).

Ventricular myocytes were isolated from male Hartley guinea pigs (300–400 g) using collagenase (collagenase-S-1, Nitta Gelatin Co., Osaka) according to the methods described by Cavalié et al. (7). The recordings were made with the whole-cell patch-clamp technique (8) (DAGAN-8900 amplifier with a 100-MΩ head stage). The external solution was composed of: 120 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM glucose, 5 mM HEPES and 30 mM tetraethylammonium chloride, (pH 7.4). The tip resistance of the fire-polished glass pipette was 2–4 MΩ when filled with pipette solution composed of: 80 mM KCl, 20 mM tetraethylammonium chloride, 40 mM CsOH, 10 mM ethyleneglycol-bis-$N,N',N''$-tetraacetic acid, 10 mM HEPES and 4 mM MgATP (pH 7.4). All the experiments were performed at room temperature.

To determine the onset and recovery of the blocking action of drugs, we used the voltage protocol described by Kass et al. (4) with a small modification. The protocol
was based on the idea that the blocking action of diltiazem on the slow inward current in cardiac cells is dependent on the holding potential (9, 10). The protocol was as follows: \( I_{\text{Ca,L}} \) was activated by a test pulse to 0 mV for 200 msec at 0.2 Hz. The voltage-dependent Na\(^+\) current and T-type current were suppressed by applying a prepulse to -40 mV for 150 msec. The holding potential \((V_h)\) was kept at 90 mV for 5 min until \( I_{\text{Ca,L}} \) became stable, and the external solution including or without the drug was perfused for another 3 min. Then the test pulse was stopped, and \( V_h \) was changed to -40 mV. After 10 sec, the test pulse was started again and changes in \( I_{\text{Ca,L}} \) were estimated for 3 min. The recovery from the block was confirmed by changing \( V_h \) from -40 mV to -90 mV. In the case of intracellular drug application, drugs were dissolved in pipette solution and the influences of the drugs on \( I_{\text{Ca,L}} \) were observed according to the protocol described above.

Diltiazem and quat-DTZ were supplied by Tanabe Seiyaku Co., Ltd., Saitama. D890 was a gift from Knoll AG, Ludwigshafen, Germany. Bonferroni's multiple t-test or Student's t-test was employed to assess the statistical significance of differences. Data are represented as the mean±S.E.M.

Figure 1B shows the inhibition of \( d\)-cis-[\( ^3\)H]diltiazem binding by diltiazem and quat-DTZ. At 25°C, the \( K_i \) value and \( B_{\text{max}} \) for diltiazem were 0.19±0.01 \( \mu \text{M} \), 5.53±0.43 pmol/mg protein, respectively. \( K_i \) values for diltiazem and quat-DTZ were calculated to be 0.16±0.01, 6.75±0.39 (\( \mu \text{M} \), \( n=4 \)), respectively, using the following equation: \( K_i = IC_{50}/(1+L/K_d) \), where \( L \) is the concentration of radioligand (30 nM). The Hill coefficients for both drugs were close to unity. Both diltiazem and quat-DTZ augmented (\(+\))[\( ^3\)H]PN200-110 binding to 177.1±5.6% and 193.5±3.2% (\( n=4 \)) of the control value, respectively, at the maximum effect at 37°C.

These results suggest that quat-DTZ binds to an identical site with diltiazem in a competitive manner and exerts a conformational change on the 1,4-DHP binding site similar to that exerted by diltiazem.

Figures 2A and 2B show the effects of quat-DTZ applied extra- and intracellularly on \( I_{\text{Ca,L}} \) in single ventricular myocytes. The \( I_{\text{Ca,L}} \) amplitudes during the test period at \( V_h -40 \text{ mV} \) were normalized with \( I_{\text{Ca,L}} \) amplitudes at \( V_h -90 \text{ mV} \) just before the change in \( V_h \) as 100%. Under control conditions without the drug, \( I_{\text{Ca,L}} \) at the end of the 3-min test period was 86.8±2.0% (\( n=6 \)). Extracellularly applied quat-DTZ exerted a concentration-dependent blocking action on \( I_{\text{Ca,L}} \) when \( V_h \) was changed from -90 mV to -40 mV. The blockade progressed in a use-dependent manner. Quat-DTZ at 10\(^{-3} \) M, 10\(^{-4} \) M and 10\(^{-5} \) M suppressed \( I_{\text{Ca,L}} \) to 71.8±7.1% (\( n=6 \)), 51.4±5.4% (\( n=6 \), \( P<0.05 \), vs. control) and 25.2±7.1% (\( n=5 \), \( P<0.01 \) vs. control), respectively. The effective concentration for quat-DTZ was 100 times as high as that for diltiazem (data not shown), the difference being close to that in the \( K_i \) values for the diltiazem-binding site described above. Intracellularly applied quat-DTZ exerted a concentration-dependent blocking action on \( I_{\text{Ca,L}} \) when \( V_h \) was changed from -90 mV to -40 mV. The blockade progressed in a use-dependent manner. Quat-DTZ at 5×10\(^{-4} \) M also effectively blocked \( I_{\text{Ca,L}} \) in a use-dependent manner to 22.2±6.6% (\( n=6 \), \( P<0.01 \) vs. control). Extra- and intracellular application of potassium iodide up to 10\(^{-3} \) M had no effect.
Figure 2C shows the effects of D890. Extracellular application of D890 at 10⁻⁴ M was almost completely ineffective (80.6±6.3%, n=5, not significant vs. control), whereas intracellular application at the same concentration suppressed ICa(L) in a use-dependent manner (45.2±5.9%, n=6, P<0.01 vs. extracellular application).

Thus we confirmed that D890 acts solely from the intracellular surface of the cell membrane, as Hescheler et al. reported (3). These results exclude the possibility of drug action through leakage of the drug from one side of the membrane to the other through the pipette-cell membrane interface.

Our results demonstrate that quat-DTZ exerts its effects from the outside as well as inside of the cell membrane. Assuming that quat-DTZ does not permeate the cell membrane, these results suggest two possibilities: one is that the binding site forquat-DTZ is located within the channel and thus accessible from both sides, and the other is that quat-DTZ has binding sites with close affinity on both the extracellular and intracellular surfaces of the membrane. In conclusion, the present results strongly suggest that diltiazem binds to the channel from the outside as well as inside of the membrane in a manner different from that of phenylalkylamines.

Acknowledgments
This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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