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CLONAZEPAM AND DILTIAZEM BOTH INHIBIT SODIUM-CALCIUM EXCHANGE OF MITOCHONDRIA, BUT ONLY DILTIAZEM INHIBITS THE SLOW ACTION POTENTIALS OF CARDIAC MUSCLES

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Clonazepam, up to concentrations of 5 x 10^{-5} M produced only 15% inhibition of contraction without effecting isoproterenol-induced slow action potentials (APs) of guinea pig papillary muscles. On the other hand, 10^{-6} M diltiazem completely inhibited both slow APs and contractions. Both clonazepam and diltiazem inhibited Na^+-induced Ca^{2+} release from isolated mitochondria. The half-maximum effect of clonazepam and diltiazem occurred at 7 and 8 x 10^{-6} M respectively. The results suggest that clonazepam more specifically inhibits the Na^+-induced Ca^{2+} release process of mitochondria.

The energy-dependent Ca^{2+} uptake and the Na^+-induced Ca^{2+} release are two distinct and well characterized ion transport processes of mitochondria in vitro. Yet the role of these Ca^{2+} transport processes in the cell is not clear. The Na^+-induced Ca^{2+} release process is very active in mitochondria of certain excitable tissues such as brain and heart, but is almost negligible in mitochondria of non-excitable tissues such as liver and kidney (1-3). It is possible that this process in cardiac muscle is operating in vivo since the apparent K_m for Na^+ of this process (4) is similar to the intracellular Na^+ concentrations (5). Since Na^+-induced Ca^{2+} release from mitochondria in vivo may function in concert with other Ca^{2+} transport processes, inhibition of this process with a specific inhibitor may help elucidate the role of this process in cellular functions.

We have shown previously that diltiazem inhibits Na^+-induced Ca^{2+} release from heart and brain mitochondria in vitro (6-8). However, diltiazem also inhibits the

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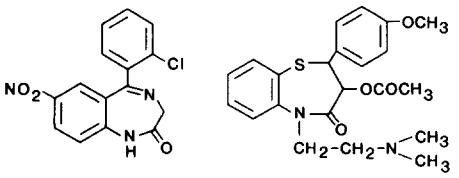

CLONAZEPAM DILTIAZEM

Fig. 1 Chemical Structure of Clonazepam and Diltiazem

voltage-dependent Ca^{2+} slow channels in the cell membrane (9). Therefore, obscuring may occur in experiments designed to inhibit the Na^+-induced Ca^{2+} release process of mitochondria in vivo with diltiazem and to correlate the changes in cellular functions. Since clonazepam, a benzodiazepine, is structurally analogus to diltiazem, a benzothiazepine (Fig. 1), we have investigated the effects of clonazepam to determine whether it can inhibit the Na^+-induced Ca^{2+} release from mitochondria without having any effect on the Ca^{2+} slow channels.

MATERIALS AND METHODS

All the chemicals were the purest grade commerically available. Clonazepam was supplied by Hoffman-LaRoche, Nestley, New Jersey, and d-cis-diltiazem was supplied by Marion Laboratories, Kansas City, Kansas. Clonazepam solution was prepared in ethanol and diltiazem in distilled water. The same concentrations of ethanol were used in the control experiment.

Various parameters of slow action potentials of guinea-pig papillary muscles induced by 10^{-6} M isoproterenol were measured in Tyrode solution as described earlier (10). The composition of normal Tyrode solution in mM was as follows: 141 Na^+, 5.4 K^+, 1.8 Ca^{2+}, 1.0 Mg^{2+}, 0.43 $H_2PO_4^-$, 25 HCO_3^-, 5.5 glucose and 124 Cl^-. The temperature was maintained at 37° C and the perfusion medium was gassed with 95% O_2 and 5% CO_2.

The isolation of mitochondria from guinea-pig heart and measurements of Ca^{2+} uptake and Na^+-induced Ca^{2+} release were carried out as described previously (6, 7).

Uptake of clonazepam in guinea-pig heart muscles was determined using ^3H-clonazepam (10^{-6} M), according to the procedure previously described (11).

RESULTS

Effect on Muscle Strips

Normal fast action potentials (APs) were observed in Tyrode solution containing 5.4 mM K^+ (Fig. 2A). Elevation of K^+ to 25 mM, completely abolished excitability of the cells due to the voltage-inactivation of the fast Na^+ channels (Fig. 2B). Isoproterenol (10^{-6} M) rapidly restored excitability in the form of slow APs (Fig. 2C).

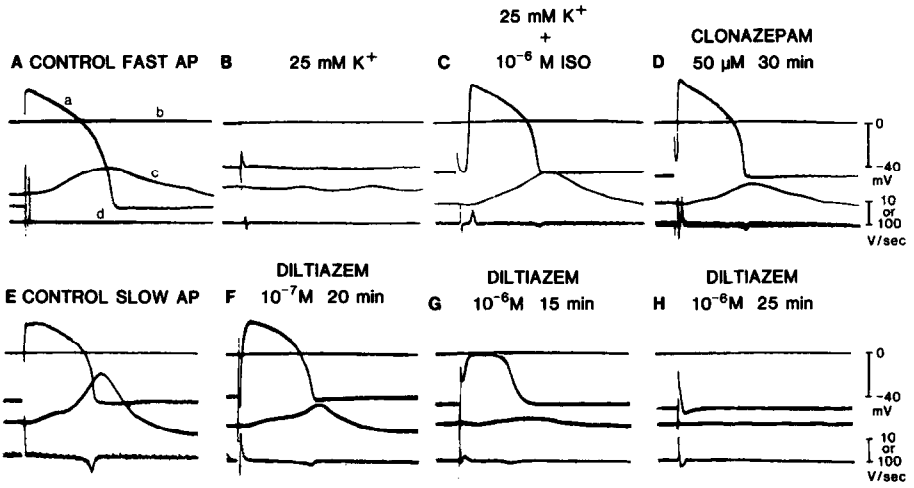

Fig. 2 Effect of Clonazepam and Diltiazem on Slow Action Potentials Induced by 10^{-6} M Isoproterenol. (a) Action potential (b) Zero potential level (c) Tension (d) $\frac{dv}{dt}$, scale 10 is for slow action potential and 100 for fast action potential.

The presence of clonazepam up to 5 x 10^{-5} M for 30 min had very little effect on the slow APs (Fig. 2D). However, 5 x 10^{-5} M clonazepam slightly depressed the contractions (Fig. 2D). The depressant effect of clonazepam on the contraction was reversed upon washout for 30 min (not shown here).

Diltiazem, on the other hand, severely depressed the slow APs at 10^{-7} M after 15 min exposure (Fig. 2F). Longer exposure (25 min) to 10^{-6} M diltiazem completely abolished the slow APs, concomitant with complete loss of contractions (Fig. 2G and H). The depressant effect of diltiazem was also reversed upon washout (not shown here).

Figure 3 summarizes the effects of clonazepam and diltiazem on various parameters of the isoproterenol-induced slow APs. Clonazepam, as shown in Fig. 3A, did not affect APD_{50}, AP amp, and $\dot{V}_{max}$. On the other hand, diltiazem at 10^{-6} M completely abolished the slow APs (Fig. 3B). The 50% inhibition of the AP duration at 50% of repolarization (APD_{50}), action potential amplitude (AP_{amp}), and maximum upstroke velocity ($\dot{V}_{max}$) occurred at about 7 x 10^{-7} M diltiazem. However, it was observed that concentrations of diltiazem below 10^{-7} M slightly increased APD_{50}.

Figure 4 shows the effect of clonazepam and diltiazem on contractile force developed by the muscles. Clonazepam, up to 5 x 10^{-6} M, produced no effects

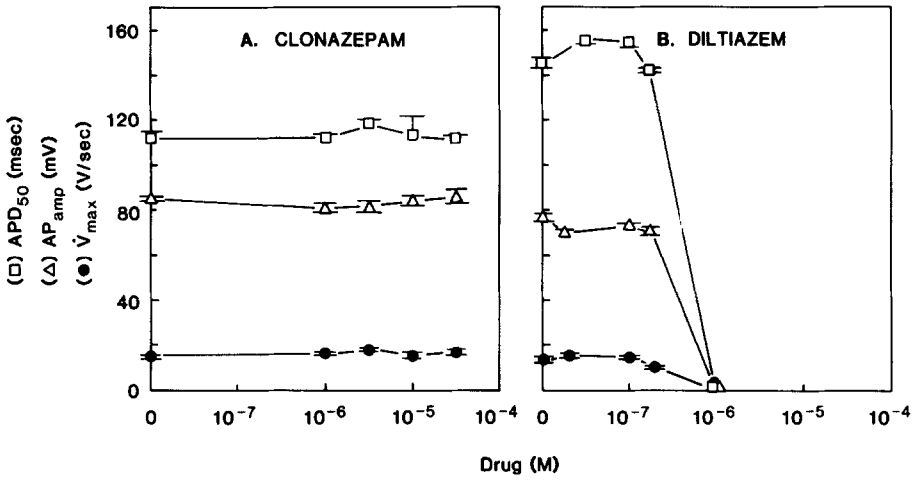

Fig. 3 Effect of Clonazepam on APD_{50}, AP_{amp} and $\dot{V}_{max}$ of Slow Action Potentials Induced by 10^{-6} M Isoproterenol. Each data point represent average of at least 4 determinations from 2-3 muscle strips. The horizontal bars represent S.E. of the mean. □—□ , ADP_{50} (msec); △—△ , AP_{amp} (mV); ●—●, $\dot{V}_{max}$ (V/sec).

although higher concentrations inhibited contractile force (Fig. 5). At 5 x 10^{-5} M, clonazepam inhibited contraction about 25%. Diltiazem, on the other hand, was much more effective in inhibiting the contractile force; 50% inhibition was observed at about 6 x 10^{-7} M (Fig. 5).

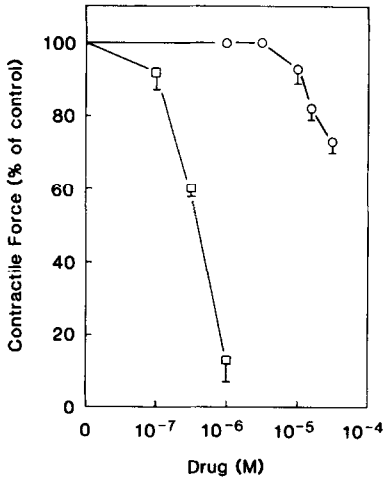

Fig. 4 Effect of Clonazepam and Diltiazem on Contractile Force Induced by 10^{-6} M Isoproterenol. Each data point represent an average of 3 determinations from 3 muscle strips. O—O, clonazepam; □ □ , diltiazem. The control tension was 0.2 - 0.3 g. The experiments were carried under exactly similar conditions as the experiments on slow action potentials.

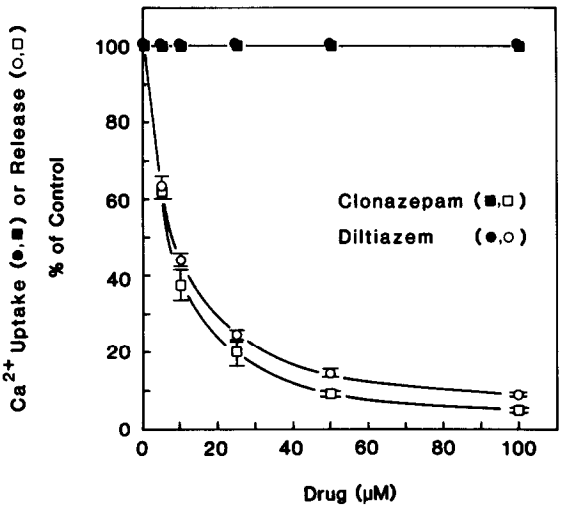

Fig. 5 Effect of clonazepam and diltiazem on Ca^{2+} uptake and Na^{+}-induced Ca^{2+} release of mitochondria isolated from guinea pig hearts. Each data point represents mean of 4 separate experiments. ■—■, rate of Ca^{2+} uptake in the presence of clonazepam (control 49 ± 3 nmoles Ca^{2+}/min/mg protein); ●—●, rate of Ca^{2+} uptake in the presence of diltiazem (control 43 ± 6 nmoles Ca^{2+}/min/mg protein); □—□, rate of Na^{+}-induced Ca^{2+} release in the presence of clonazepam (control 118 ± 2 nmoles/min/mg protein); ●—●, rate of Na^{+}-induced Ca^{2+} release in the presence of diltiazem (control 104 ± 8 nmoles Ca^{2+}/min/mg protein). The assay conditions were the same as in ref. 6 and 7.

Effects on Mitochondria

Figure 5 shows the effects of clonazepam and diltiazem on Ca^{2+} uptake and Na^{+}-induced Ca^{2+} release from isolated mitochondria. Clonazepam and diltiazem, up to 10^{-4} M, produced no effect on the rate of Ca^{2+} uptake into mitochondria. On the other hand, both drugs inhibited the rate of Na^{+}-induced Ca^{2+} release in a concentration-dependent manner. The 50% inhibition was observed at 7 and 8 x 10^{-6} M, clonazepam and diltiazem, respectively.

Uptake of Clonazepam in Cardiac Muscle

In preliminary studies, the tissue content of ^{3}H-clonazepam was found to increase with time of incubation. The ratio of drug tissue/medium was greater than 6.0 at 2 hr.

DISCUSSION

Diltiazem inhibited isoproterenol-induced slow APs and force of contraction of guinea pig papillary muscles. Clonazepam, which is structurally analogus to diltiazem,

exerted no significant effect on the slow APs, although it produced a small inhibition of contraction at 5×10^{-5} M. This small inhibition of contraction does not appear to be due to the inhibition of slow APs.

In vitro, both clonazepam and diltiazem inhibited Na^+-induced Ca^{2+} release from mitochondria with almost identical potency. At concentrations which inhibited Na^+-induced Ca^{2+} release from mitochondria, clonazepam had no effects on: (a) voltage-dependent Ca^{2+} slow channels in the sarcolemma, (b) Ca^{2+} uptake in mitochondria, (c) Ca^{2+} transport in sarcoplasmic reticulum (unpublished observations), and (d) Na^+/Ca^{2+} exchange in sarcolemma (8). Thus, clonazepam appears to be a specific inhibitor of Na^+-induced Ca^{2+} release from mitochondria.

Inhibition of Na^+-induced Ca^{2+} release from mitochondria may cause a decrease in cytosolic Ca^{2+}. This could explain the small inhibition of contraction of guinea-pig papillary muscle produced by clonazepam. A small inhibition of contraction due to the inhibition of Na^+-induced Ca^{2+} release in vivo would be consistent with the minor role played by mitochondria in the contractility of cardiac muscle under physiological conditions. Such an effect of clonazepam in vivo would depend on whether it can enter the cell. Preliminary studies indicate that clonazepam does accumulate in cardiac muscles. Thus, clonazepam appears to be a potential tool for the elucidation of the nature and the role of Na^+-induced Ca^{2+} release from mitochondria in cardiac muscles.

The mechanisms of the pharmacological effects of clonazepam on cardiac muscle are unclear. Certain benzodiazepines inhibit adenosine uptake and potentiate cardiac response to adenosine (12). Diazepam (Valium) increases coronary flow, probably due to its vasodilatory effects (13). Whether these effects of benzodiazepines are due to the inhibition of the Na^+-induced Ca^{2+} release from cardiac or vascular smooth muscle mitochondria is not known.

A peripheral type of benzodiazepine receptor has been identified and characterized by specific binding of [^{3}H]-RO5-4864 to cardiac membranes with a K_D of 33 nM (14-16). These benzodiazepine receptors were unaffected by clonazepam which suggests that peripheral benzodiazepine receptors may be distinct from the Na^+/Ca^{2+}

exchange carrier in mitochondria, although the available data cannot completely rule out the possibility that they are the same.

The primary action of clonazepam (Clonopin) is on the central nervous system, and is therapeutically used as an antiepileptic with sedative and antidepressant actions (17). However, the mechanisms of action of clonazepam on the central nervous system are not clear. Since brain mitochondria possess an active Na^+-induced Ca^{2+} release process (1), inhibition of this process, and resulting decreased intracellular Ca^{2+}, we postulate, may be one of the mechanisms for its pharmacological actions.

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