Negative Inotropic Effect of Diazepam in Isolated Guinea Pig Heart

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ABSTRACT. The inotropic effect of diazepam, a benzodiazepine derivative, and its mechanism of action were examined using guinea pig heart and single ventricular cell preparations. In Langendorff hearts and right ventricular free-wall preparations, diazepam (10 to 100 µM) produced a monophasic negative inotropic effect in a concentration dependent manner. Neither a central type (flumazenil 1 µM) nor a peripheral type (PK11195 10 µM) of benzodiazepine receptor antagonist antagonized the monophasic negative inotropic effects of diazepam. Diazepam (10 to 100 µM) shortened action potential duration of papillary muscle in a concentration dependent manner. In isolated single ventricular cells, diazepam (30 and 100 µM) inhibited the calcium current (I_{Ca}) in a concentration dependent manner. Diazepam produced a significant decrease in I_{Ca} elicited by first depolarizing pulses, however, the decrease of I_{Ca} was not augmented during a train of depolarizing pulses. Thus, diazepam appears to produce a tonic block of cardiac calcium channels and the mode of inhibition is clearly different from the use-dependent block of verapamil. From these results, it was concluded that diazepam produces a monophasic negative inotropic effect that is independent of the benzodiazepine receptor, and is probably mediated through an inhibition of I_{Ca} in guinea pig heart preparations.

KEY WORDS: calcium current, diazepam, heart, negative inotropic effect, patch clamp method.

FULL PAPER

Materials and Methods

This study was approved by the Animal Care and Use Committee of our school and was done in compliance with the “Guide Principle for the Care and Use of Laboratory Animals” approved by The Japanese Pharmacological Society.

Guinea pigs (250–500 g) of both sexes, anesthetized with pentobarbital, were used. Hearts were rapidly excised and transferred to a Langendorff setup. The aorta was cannulated in order to allow retrograde perfusion with Tyrode’s solution gassed with 95% O_{2} + 5% CO_{2} at 36°C, under a constant hydrostatic pressure of 50 cm H_{2}O. Contractile force was measured as the left ventricular pressure. A latex balloon filled with water was inserted in the left ventricle and connected to a pressure transducer (TP-400T, Nihon Kohden, Tokyo, Japan) to measure changes in force of contraction. The pressure was recorded continuously on a polygraph (RM-6100, Nihon Kohden). After equilibration for 15 min, the experiment started with exposure of the heart preparation to diazepam or one of the other drugs used.

The ventricular free-wall preparations were carefully dissected from the heart. The muscles were mounted under 1 g of resting tension in a water-jacketed organ bath containing 20 ml of Tyrode’s solution. The solution in the bath was gassed with 95% O_{2} + 5% CO_{2}, and its temperature was maintained at 35 ± 1°C. The muscles were stimulated by rectangular pulses of 3.3 Hz in frequency, 5 ms in duration.
and 1.5 times the threshold voltage (1.5 to 4 V). The field stimulation was delivered through a pair of platinum electrodes connected to an electronic stimulator (SEN-3301; Nihon Kohden, Tokyo, Japan). Isometric tension developed in the preparation was measured with a force transducer (TB-611T or TB-612T; Nihon Kohden) and recorded on a pen recorder (WI-641G; Nihon Kohden) after preamplification (AP-600G; Nihon Kohden). The preparations were allowed to equilibrate for at least 60 min before the experiments began.

The ventricular papillary muscles were carefully dissected from the heart. The preparations were stimulated at a rate of 0.5 Hz using a bipolar stainless steel electrode at the cut end during the equilibration period. Stimuli were rectangular pulses of 1 ms duration at 1.5 times the threshold voltage (1 to 4 V), delivered from an electronic stimulator (SEN-3301; Nihon Kohden). Transmembrane potentials were recorded from a microelectrode filled with 3 M KCl (resistance of 20–30 MΩ). The microelectrode was coupled through an Ag/AgCl junction to an amplifier (MEZ-7101; Nihon Kohden). An agar bridge containing 3 M KCl was used as a common reference electrode. The action potential was displayed on a dual beam oscilloscope (VC-11, Nihon Kohden) and photographed by a camera (RLG-6201; Nihon Kohden).

Single ventricular cells were isolated by enzymatic dispersion, as previously described [26]. Briefly, the heart was mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation with a normal HEPES-Tyrode solution (modified Kraftbrühe (KB) solution) [13, 16]. Ventricular tissue was cut into small pieces in the modified KB solution and gently shaken to isolate cells. Dispersed cells were stored in the KB solution at 4°C and used over the next 7–10 hr.

Whole-cell membrane currents were recorded by the patch clamp method [9]. Single ventricular cells were placed in a recording chamber (1-mL volume) attached to an inverted microscope (Olympus IMT-2, Tokyo, Japan) and superfused with the HEPES-Tyrode solution at a rate of 3 mL/min. The temperature of the external solution was kept constant at 36°C. Glass patch pipettes were filled with a pipette solution. The resistance of the patch pipette filled with the pipette solution was 2–3 MΩ. After the gigahm-seal between the tip of the electrode and the cell membrane was established, the membrane patch was disrupted by applying more negative pressure to make the whole-cell voltage-clamp mode.

The electrode was connected to a patch clamp amplifier (Nihon Kohden CEZ-2300, Tokyo, Japan). Command pulses were generated by a 12 bit digital-to-analog converter controlled by pClamp software (Axon Instruments, Inc., Foster City, CA, U.S.A.). The liquid junction potential between the internal solution and the bath solution of -8 mV was corrected. Current signals were digitized with a sampling interval of 2 kHz and stored on the hard disk of an IBM compatible computer.

The composition of Tyrode’s solution was (mM): NaCl 125, KCl 4, CaCl2 2.7, MgCl2 0.5, Na2HPO4 1.8, NaHCO3 25, glucose 5.5. The normal HEPES-Tyrode solution was composed of (mM): NaCl 143, KCl 1.4, CaCl2 1.8, MgCl2 0.5, NaH2PO4 0.33, glucose 5.5 and HEPES-NaOH buffer (pH 7.4) 5.0. The composition of modified KB solution was (mM): KOH 70.0, l-glutamic acid 50.0, KCI 40.0, taurine 20.0, KH2PO4 20.0, MgCl2 3.0, glucose 10.0 and HEPES-KOH buffer (pH 7.4) 10.0. The normal HEPES-Tyrode solution was used as an external solution to record the whole cell membrane current and the calcium current. The composition of the pipette solution was (mM): K-aspartate 110, KCl 20, MgCl2 1.0, ATP-K7 5.0, EGTA 10 and HEPES-KOH buffer (pH 7.4) 5.0. The free calcium concentration in the pipette solution was adjusted to pCa 8 according to the calculation by Fabiato and Fabiato [8] with the correction of Tsien and Rink [32].

The following drugs were used: diazepam (Yamanouchi Pharmaceuticals Co., Ltd., Tokyo, Japan), flumazenil (kindly supplied by Yamanouchi Pharmaceuticals), PK11195 (1-(2-chlorophenyl)-N-methyl-N-methylpropyl-3-isouquinoline carboxamide, Research Biochemical Inc., MA, U.S.A.) and verapamil HCl (Eisai, Tokyo, Japan). Diazepam, flumazenil and PK11195 were dissolved in dimethyl sulfoxide (Wako, Japan). Verapamil was dissolved in the HEPES-Tyrode solution.

All values are presented in terms of mean ± S.E.M. Analysis of variance (ANOVA) followed by Fisher’s test was used for statistical analysis of the data. P values of less than 0.05 were considered significant.

RESULTS

A typical effect of diazepam on the cardiac contractile force of a guinea pig Langendorff heart is shown in Fig. 1A. Diazepam at 30 and 100 µM produced monophasic inhibition of the cardiac contractility in a concentration dependent manner. The decrease in contractility was observed after perfusion of diazepam and lasted to the end of the perfusion. Thus, the effect of diazepam on the cardiac contractility in the isolated guinea pig heart was monophasic negative inotropicism. The negative inotropic effect of diazepam was rapidly reversed after the washout. The concentration-response relationships are shown in Fig. 1B. Concentration-dependent inhibition of contractile force was observed in the treatment with diazepam (3 to 100 µM). The EC50 value of diazepam for depression of contractile force was 42.2 µM. The influences of benzodiazepine receptor antagonists were examined in the same preparation. Diazepam 30 µM reduced the contractility of the Langendorff heart to 72.8 ± 6.5% of the control (n=13). PK11195, a peripheral-type benzodiazepine receptor antagonist [23], at a concentration
NEGATIVE INOTROPIC EFFECT OF DIAZEPAM

![Graph](image)

Fig. 1. Influences of diazepam on the contractile force of guinea-pig Langendorff heart. (A) Representative recordings of inotropic response of the heart to diazepam administered in stepwise increasing concentrations (30 and 100 µM). Perfusion of diazepam is shown by the bar above the original trace. Diazepam produced a concentration dependent negative inotropic effect. The negative inotropic effect of diazepam lasted to the end of perfusion and was reversed by washout. (B) Concentration response relationships of the inotropic response of the heart to diazepam. Significant inhibition of contractile force was observed by the treatment with diazepam (n=10). Each point represents mean ± S.E.M.

of 10 µM was applied to the preparation after steady state effects of diazepam (30 µM) were achieved. No antagonistic action was observed in the contractile response after the treatment of PK11195 (55.2 ± 7.5% of the control, NS). In another set of experiments, the influence of a central-type benzodiazepine receptor antagonist, flumazenil [14], was examined. Again, diazepam 30 µM inhibited the contraction to 56.0 ± 8.2% of the control (n=13). Administration of flumazenil 1 µM produced no influence on the reduction of contraction produced by diazepam (56.0 ± 4.1%, NS). The concentrations of flumazenil and PK11195 used in the present study were 112 times and 833 times higher than that of their dissociation constant (Kd, 8.9 nM for flumazenil, 12 nM for PK11195), respectively [29, 30].

In a right ventricular free-wall preparation, the developed tension was 117.6 ± 18.3 mg (n=20) after a 60 min equilbrium period. This value was taken as 100% in the calculation of the data. Cumulative applications of diazepam 10, 30, 50, 70, 90 and 110 µM inhibited the developed tension concentration dependently. After washout of diazepam and a 60 min stabilization period, flumazenil (1 µM) and PK11195 (10 µM) were applied to the preparation 30 min before eliciting a second concentration-response curve of diazepam. Neither flumazenil (n=10) nor PK11195 (n=10) altered the concentration-response curve of diazepam, significantly. Thus, both central- and peripheral-type benzodiazepine receptors did not play important roles on the negative inotropic action of diazepam in the isolated guinea pig heart preparations. The quantitative results are summarized in Fig. 2.

Figure 3 illustrates typical changes in action potential configuration before and after 30 min exposure to diazepam 100 µM in ventricular papillary muscle constantly driven at 0.5 Hz. The baseline characteristics of the action potentials were as follows: the resting membrane potential (RMP), –91.1 ± 2.1 mV; action potential amplitude (APA), 137.1 ± 1.4 mV; maximum rate of rise of action potential upstroke (Vmax), 191.7 ± 10.1 V/s; action potential duration (APD) at 20% repolarization level (APD20), 122.3 ± 5.0 ms; APD at 50% repolarization level (APD50), 190.0 ± 7.9 ms; APD at 90% repolarization level (APD90), 224.2 ± 9.2 ms (n=10). APD was shortened by the treatment (30 min) of diazepam and recovered partially after 30 min washout. Concentration related changes in APD produced by diazepam are shown in Fig. 3B. Diazepam, 10, 30, and 100 µM, produced decreases in APD20 to 120.8 ± 4.7 ms (NS), 109.3 ± 5.7 ms (NS), 94.7 ± 5.8 ms (p<0.05, n=10), respectively. Concentration dependent decreases in APD90 and APD90 were also observed by the treatment of diazepam 10 to 100 µM. Diazepam at a concentration of 100 µM insignificantly reduced RMP, APA and Vmax of the action potential to –88.3 ± 2.1 mV (NS), 134.7 ± 1.4 mV (NS) and 178.8 ± 11.3 V/s (NS), respectively.

Effects of diazepam on membrane currents were examined in guinea-pig ventricular myocytes by means of the whole-cell patch clamp method. A representative example of the membrane ionic currents of an isolated ventricular myocyte before and after application of 100 µM diazepam is shown in Fig. 4. Membrane currents were elicited by 300-ms depolarizing pulses (from –30 to 40 mV) and hyperpolarizing pulses (from -50 to -100 mV) from a holding potential of –40 mV at 0.1 Hz. Diazepam 100 µM decreased the peak inward current and increased slightly the late outward current produced by depolarizing pulses. On hyperpolarizing pulses, the steady state outward and inward currents were decreased by diazepam (100 µM) (Fig. 4A, B). These findings suggest that diazepam decreased the calcium current (Icalc) and the inward rectifier potassium current. However, we did not examine detailed effects of diazepam on the potassium currents in the present study. The amplitude of Icalc was obtained by subtracting the late current from the peak of the inward current produced by the depolarizing pulse to 0 mV from a holding potential of –40 mV at a duration of 300 ms. Diazepam, in concentrations of 30 and 100
µM, decreased the amplitude of ICa to 69.0 ± 6.7% (p<0.05, n=4) and 35.9 ± 2.9% (p<0.05, n=4) of the control (100%), respectively (Fig. 4C).

The mode of inhibition of ICa by diazepam was examined and compared with those of a calcium antagonist, verapamil. Diazepam inhibited ICa, and a steady state inhibition was achieved within 5 to 10 min. Use-independent block (tonic block) and use-dependent block (UDB) were evaluated 5 to 10 min after treatment with the drug by applying a train of 30 depolarizing pulses of 300 ms duration from a holding potential of –40 mV to 0 mV at 1 Hz after a 60 s pulse-free period. This depolarizing pulse produces the maximum amplitude of peak inward current. Diazepam (30 µM) depressed the peak ICa elicited by the first depolarizing pulse and no further decrease in ICa was observed with a successive train of pulses, as shown in Fig. 5A. Tonic block, UDB and total block of ICa by 30 µM diazepam was 17.0 ± 6.0%, 8.3 ± 1.8% and 24.6 ± 4.8% (n=5), respectively. Since a small reduction of ICa (7.1 ± 3.6%, n=5) was observed during a train of 30 depolarizing pulses in the

Fig. 2. Concentration-response curve for the effect of diazepam alone (Control; open circles), diazepam with flumazenil (triangles) and diazepam with PK11195 (closed circles) on the contraction of the ventricular free-wall strips of guinea-pig heart. Diazepam alone, applied in a cumulative manner, produced concentration dependent negative inotropic effects (n=20). Pretreatment of flumazenil 1 µM (n=10), a central-type benzodiazepine receptor antagonist, and PK11195 10 µM (n=10), a peripheral-type benzodiazepine receptor antagonist, did not affect the negative inotropic action of diazepam. Each point represents mean ± S.E.M.

Fig. 3. Effects of diazepam on action potential configuration in guinea pig papillary muscles. (A) Representative recordings of the action potential before, during and after the treatment of diazepam 100 µM. (B) Changes in action potential duration. APD_{20} (open circles): action potential duration (APD) at 20% repolarization level, APD_{50} (closed circles): APD at 50% repolarization level, APD_{90} (triangles): APD at 90% repolarization level. Each point represents mean ± S.E.M. n=10.
Fig. 4. Influences of diazepam on whole-cell transmembrane current by means of the patch clamp method in guinea pig single ventricular myocytes. (A) Actual traces of whole-cell currents in a single ventricular cell. Current traces induced by a depolarizing pulse (0 mV) from a holding potential of -40 mV (upper traces) and hyperpolarizing pulses (-50 mV to -100 mV) (lower traces). Control recordings are shown on the left, and the recordings after the treatment with diazepam 100 µM are on the right. 0 and - indicate 0 current level. Note that diazepam (100 µM) decreased the peak inward current (circles), increased the late outward current induced be depolarizing pulses (triangles, upper traces) and decreased the steady state outward and inward currents induced by hyperpolarizing pulses (triangles, lower traces). (B) Current-voltage relationships. Symbols used in this graph correspond to the symbols in (A). Open symbols represent control recordings, and closed symbols represent recordings after treatment with diazepam. Circles and triangles represent current-voltage relation for the peak inward current and the late steady state current at the end of a 300 ms test pulse, respectively. Similar pattern was observed in 3 other preparations. (C) Effect of diazepam on the calcium current (I_{Ca}). The amplitude of I_{Ca} was obtained by subtracting the late current from the peak of the initial inward current induced by depolarizing pulses to 0 mV from a holding potential of -40 mV over a duration of 300 ms. Diazepam (30 and 100 µM) produced concentration dependent inhibition of I_{Ca} (n=4). * p<0.05.
absence of any drug, UDB of diazepam appeared to be negligible. After the exposure to verapamil (0.1 µM), a significant tonic block and UDB of I_{Ca} were observed (Fig. 5B). Tonic block and UDB produced by 5 to 10 min exposure to verapamil were 53.1 ± 9.8% (n=6) and 82.7 ± 7.4%, respectively. The decline of the peak I_{Ca} during the delivery of repetitive depolarizing pulses followed a single exponential, and the onset rate of UDB at 1 Hz was calculated as 0.147 ± 0.022 pulse^{-1} in 6 cells.

DISCUSSION

Diazepam produced concentration dependent and monophasic negative inotropic effects by means of long
term continuous perfusion of certain concentrations of diazepam in Langendorff preparation of isolated guinea pig heart. Leeuwin et al. [20, 21], however, reported biphasic inotropic effects, a negative inotropic effect preceding a positive inotropic effect, of diazepam by a single bolus (10 sec) injection in rat Langendorff heart. A difference in the perfusion methods, continuous perfusion and bolus injection, might cause the discrepancy in the results. The monophasic negative inotropic effect of diazepam was also observed by the experiments with the isolated right ventricular free-wall preparation in our present experiment (Fig. 2). Consistent with our observation, Hernández [14] has reported a similar monophasic negative inotropic effect of diazepam in the isolated rat ventricular free-wall preparation.

Benzodiazepines produce their pharmacological effects through binding to specific receptors. These receptors are classified as central and peripheral types and have been shown to be present in peripheral tissues [22, 23]. In order to study the involvement of the benzodiazepine receptors located in the heart, influences of benzodiazepine antagonists on the reduction of contractile responses induced by diazepam were examined in Langendorff heart and right ventricular preparations. Neither the central type, flumazenil (1 μM), nor the peripheral type, PK11195 (10 μM), of benzodiazepine receptor antagonists influenced the negative inotropic effects of diazepam in both preparations. Hernández [14] and Leeuwin et al. [21] reported the antagonistic action of both receptor antagonists on the negative inotropic effect of diazepam in rat right ventricular strips within these concentrations. It has been demonstrated that flumazenil (0.3 μM) did not influence the shortening of intracellular action potential duration induced by diazepam in isolated guinea pig papillary muscle preparation [25]. Ruiz et al. [31] reported that diazepam-induced reduction of chronotropism in isolated rat atria was mediated neither by central nor peripheral-type benzodiazepine receptors and could be due to a nonspecific interaction with the cell membrane. They also showed in the same report that γ-aminobutyric acid (GABA) related compounds, such as GABA and picrotoxin, did not modify the effect of diazepam. Moreover, Nonaka et al. [27] reported that flumazenil (1 μM) did not prevent the decrease in the Ca²⁺ transient and beating rate induced by diazepam in cultured fetal mouse cardiac myocytes. Thus, the diazepam-induced negative inotropic effect may not be related to the known benzodiazepine receptors.

There are changes in several electrophysiological parameters behind the alteration of the contractile response of heart muscles. In the present experiment, diazepam shortened action potential duration without significant influences on RMP, APA and Vmax in guinea-pig papillary muscle preparations. This observation was consistent with a previous report by Mestre et al. [25]. Diazepam decreased the peak inward current produced by depolarizing pulses from -30 to 40 mV and the late outward and inward currents produced by hyperpolarizing pulses from -50 to -100 mV in guinea pig ventricular myocytes by means of the whole-cell patch clamp method. These findings suggest that diazepam decreases ICU and the inward rectifier potassium current. We also observed a dose related inhibition of ICU in the present study. The mode of inhibition of ICU by diazepam was examined and compared with those of a calcium antagonist, verapamil. Organic calcium channel blockers are reported to produce little tonic block and significant UDB of ICU, due to higher affinity for open calcium channel [17, 19]. Consistent with these reports verapamil showed tonic block and UDB of ICU in the present study. The onset rate of UDB produced by verapamil was almost the same in our previous report [10]. Diazepam, however, produced tonic block and negligible UDB of ICU, similar to general anesthetics [3, 10]. Thus, the mode of inhibition of ICU by diazepam is a tonic block. In other words, diazepam may block the calcium channel in a state-independent manner.

We have previously reported that diazepam increases calcium sensitivity of contractile protein in skinned heart muscles [11]. Potentiation of the contractile effect of histamine by diazepam through increase of the cyclic AMP level in a guinea pig heart preparation was also reported [12]. Other authors report that diazepam potentiates the positive inotropic effect induced by β-adrenoceptor agonists through enhancing cyclic AMP production in the rat ventricular strip [24]. These phenomenon directly relate to the positive inotropic effects induced by diazepam in vivo. However, some studies showed that diazepam itself possessed a negative inotropic effect in the heart [6, 14, 25]. Nonaka et al. [27] observed that diazepam decreased the calcium transient in cultured fetal mouse cardiac myocytes. They suggested in their discussion that the L-type calcium channel is important in the negative inotropism of diazepam. It has been reported that a calcium-entry blocker, flunarizine, strongly reduced the negative inotropic response to diazepam [21]. The negative inotropic response to diazepam may be associated with a site possessing affinity for calcium-entry blocking drugs of the type of flunarizine. Thus, the involvement of ICU mechanisms with diazepam-induced negative inotropic responses in the isolated heart was indicated from indirect evidence. Our present paper provides the direct evidence of ICU suppression by diazepam. The negative inotropic effect induced by diazepam can be partly explained by a suppression of the transsarcolemmal ICU in cardiac cells. We did not examine the influence of flumazenil and PK11195 on ICU in ventricular myocytes in the present experiment. However, it has been reported that flumazenil and PK11195 had no effect on ICU or the inhibitory effect of ICU produced by benzodiazepines in canine tracheal smooth muscle cells [33]. It can be suggested that both flumazenil and PK11195 failed to affect on ICU in cardiac cells.

The recommended therapeutic range for plasma concentration of diazepam is 500 to 700 ng/ml, about 1.5 to 2.5 μM, for control of seizure in status epilepticus in animals [2]. The concentrations of diazepam used in the present study are higher than these of the therapeutic range. The negative inotropic effect of diazepam will appear in the case of overdosing or the very early phase of intravenous injection of
diazepam. Diazepam could affect cardiac contractility, however, the data and their interpretation were rather contradictory. The sum of the two effects of diazepam, i.e., inhibition of $I_{Ca}$ and enhancement of the myofilament calcium sensitivity [11], influences the actual force of contraction. The relative contribution of these effects to inotropic response to diazepam may be dependent on the species and the cardiac preparation being studied. This could partly explain the contradictory data and interpretations so far reported.

In conclusion, diazepam produces a monophasic negative inotropic effect that is independent of benzodiazepine receptors, and is probably mediated through inhibition of $I_{Ca}$ in guinea pig heart preparations.

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