

Benzodiazepines Inhibit the Acetylcholine Receptor-Operated Potassium Current ($I_{K,ACH}$) by Different Mechanisms in Guinea-pig Atrial Myocytes

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ABSTRACT. The anticholinergic effects of 7 benzodiazepines, bromazepam, camazepam, chlordiazepoxide, diazepam, lorazepam, medazepam and triazolam, were compared by examining their inhibitory effects on the acetylcholine receptor-operated potassium current ($I_{K,ACH}$) in guinea-pig atrial myocytes. All of these benzodiazepines (0.3–300 μ M) inhibited carbachol (1 μ M)-induced $I_{K,ACH}$ in a concentration-dependent manner. The ascending order of IC_{50} values for carbachol-induced $I_{K,ACH}$ was as follows; medazepam, diazepam, camazepam, triazolam, bromazepam, lorazepam and chlordiazepoxide (>300 μ M). The compounds, except for bromazepam, also inhibited $I_{K,ACH}$ activated by an intracellular loading of 100 μ M guanosine 5'-[γ -thio]triphosphate (GTP γ S) in a concentration-dependent manner. The ascending order of IC_{50} values for GTP γ S-activated $I_{K,ACH}$ was as follows; medazepam, diazepam, camazepam, lorazepam, triazolam chlordiazepoxide (>300 μ M) and bromazepam (>300 μ M). To clarify the molecular mechanism of the inhibition, IC_{50} ratio, the ratio of IC_{50} for GTP γ S-activated $I_{K,ACH}$ to carbachol-induced $I_{K,ACH}$, was calculated. The IC_{50} ratio for camazepam, diazepam, lorazepam, medazepam and triazolam was close to unity, while it for chlordiazepoxide could not be calculated. These compounds would act on the GTP binding protein and/or potassium channel to achieve the anticholinergic effects in atrial myocytes. In contrast, since the IC_{50} ratio for bromazepam is presumably much higher than unity judging from the IC_{50} values (104.0 \pm 30.0 μ M for carbachol-induced $I_{K,ACH}$ and >300 μ M for GTP γ S-activated $I_{K,ACH}$), it would act on the muscarinic receptor. In summary, benzodiazepines had the anticholinergic effects on atrial myocytes through inhibiting $I_{K,ACH}$ by different molecular mechanisms.

KEY WORDS: acetylcholine receptor-operated potassium current, atrial myocyte, benzodiazepines, bromazepam, patch clamp method.

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Benzodiazepine derivatives are established therapeutic tools with relatively low incidence of adverse effects in human and veterinary medicine and are used as preanesthetics, tranquilizers, muscle relaxants, and anticonvulsant agents [6]. In veterinary clinical fields, benzodiazepine derivatives are also utilized for animal behavior disorders [17]. Virtually, no cardiac effect is observed after ingestion of therapeutic doses of diazepam in otherwise healthy patients [2]. While previous *in vitro* studies have suggested that diazepam could affect cardiac contractility, the data and their interpretation are rather contradictory. For example, both positive and negative inotropic actions, biphasic inotropic action, and no effect of diazepam have been reported in different species of animals using various ranges of concentrations [1, 5, 8, 23]. Our previous studies have provided one explanation for this contradiction, i.e., diazepam produced the negative inotropic effect in isolated guinea pig heart through inhibition of the calcium current [15], while at the same time, it increased calcium sensitivity of the cardiac muscle fiber in the same concentration ranges [13]. From these reports about diazepam, benzodiazepine derivatives

are presumed to affect the ionic currents in the heart. However, there is no data available determining the effects of benzodiazepines on cardiac ligand-gated currents.

The acetylcholine receptor-operated potassium current ($I_{K,ACH}$), a ligand-gated potassium current, has been known to play an important role in the repolarization of the action potential as well as maintenance of the resting potential in atrial cells [20]. In atrial cells with chronic atrial fibrillation, $I_{K,ACH}$ was constitutively active without muscarinic receptor stimulation [7]. The inhibition of $I_{K,ACH}$ appears to be one of the mechanisms for the termination and prevention of atrial flutter and fibrillation [4]. Thus, to explore the influence of benzodiazepine derivatives on $I_{K,ACH}$ is of great clinical significance. In the present study, influences of 7 benzodiazepine derivatives including diazepam on $I_{K,ACH}$ were examined by a whole-cell patch clamp method in guinea pig atrial myocytes. And mechanisms of the anticholinergic action of benzodiazepines were explored.

MATERIALS AND METHODS

This study was performed in compliance with the “Guiding Principles for the Care and Use of Laboratory Animals” approved by the Japanese Pharmacological Society and the Kitasato University. The methods for cell preparations and current recordings were the same as the previous ones [12, 16]. Briefly, guinea-pig (male, 250–750 g body

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weight) hearts were isolated under sodium pentobarbital (50 mg/kg i.p. injection) anesthesia and set on a modified Langendorff apparatus for isolation of single atrial myocytes by an enzymatic digestion with collagenase. Whole-cell patch clamp method was used for recording of $I_{K,ACH}$ as an outward current at a holding potential of -40 mV. $I_{K,ACH}$ was induced by a superfusion of $1 \mu\text{M}$ carbachol or by an intracellular application of $100 \mu\text{M}$ guanosine 5'-[γ -thio] triphosphate (GTP γ S), a nonhydrolysable guanosine 5'-triphosphate (GTP) analogue. The normal N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid (HEPES)-Tyrode solution (pH 7.4) and the standard pipette solution were used as superfusate and inner solution, respectively. The composition of HEPES-Tyrode solution was (mM): NaCl 143, KCl 5.4, CaCl_2 1.8, MgCl_2 0.5, NaH_2PO_4 0.33, glucose 5.5 and HEPES 5.0. The composition of the standard pipette solution was (mM): K-aspartate 110, KCl 20, MgCl_2 1.0, GTP 0.1, adenosine-5'-triphosphate (ATP)-K 5.0, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 10 and HEPES 5.0 (pH 7.4, free Ca^{2+} concentration, pCa 8).

All benzodiazepines, bromazepam, camazepam, chlordiazepoxide, diazepam, lorazepam, medazepam and triazolam, are obtained from the Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, the present company name is Astellas Pharm Inc.) and are dissolved in dimethyl sulfoxide (DMSO) as a stock solution. The final concentration of DMSO is less than 1% and this concentration of DMSO did not affect $I_{K,ACH}$ recording.

Data analysis: In the recordings of the $I_{K,ACH}$ current, the activated current is followed by a continuous decline by the desensitization [22]. Continuous current decline before benzodiazepine derivative treatment was assumed as quasi-steady state (QSS). We used QSS as a maximum current. All values are presented as mean \pm standard error of mean (S.E.M.). The concentrations required to produce 50% of the maximal inhibitory effect (IC_{50}) were calculated from concentration-response curves using Math Curve Fitter (SigmaPlot, Systat Software, Inc., San Jose, CA, U.S.A.) to solve nonlinear equations. For comparison of IC_{50} value, statistical analyses were performed using un-paired Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant. To elucidate the mechanisms for the inhibitory effect of benzodiazepines, the ratio of IC_{50} values for inhibition of the GTP γ S-activated $I_{K,ACH}$ to the carbachol-induced $I_{K,ACH}$ were calculated using the following equation [10]:

$$\text{IC}_{50} \text{ Ratio} = [\text{IC}_{50} \text{ for GTP}\gamma\text{S-activated current}] / [\text{IC}_{50} \text{ for carbachol-induced current}]$$

RESULTS

Effects of 7 benzodiazepines on the carbachol-induced $I_{K,ACH}$ in a single guinea pig atrial myocyte: Effects of 7 benzodiazepines (0.3 – $300 \mu\text{M}$), bromazepam, camazepam, chlordiazepoxide, diazepam, lorazepam, medazepam and triazolam, on the carbachol-induced $I_{K,ACH}$ were examined. The $I_{K,ACH}$ was induced by an extracellular application of

carbachol ($1 \mu\text{M}$) in the GTP ($100 \mu\text{M}$)-loaded atrial myocytes using the whole-cell mode of patch clamp method at a holding potential of -40 mV. After induction of $I_{K,ACH}$, benzodiazepines were added to the bath solution in the presence of carbachol. Concentration of benzodiazepines was increased in a stepwise fashion every 3 min. All of the benzodiazepines used in the present study inhibited the carbachol-induced $I_{K,ACH}$ effectively in a concentration-dependent manner (Figs. 1 and 3). The outward current reappeared after a wash-out of each drug. Inhibitory effect of the maximum concentration of chlordiazepoxide ($300 \mu\text{M}$) on the current was weak and did not attain 50% inhibition ($45.2 \pm 4.8\%$ inhibition, $n=8$). The IC_{50} values for the carbachol-induced $I_{K,ACH}$ are shown in Table 1. The ascending order of IC_{50} values for the carbachol-induced $I_{K,ACH}$ was as follows; medazepam ($12.9 \pm 2.4 \mu\text{M}$), diazepam ($54.8 \pm 10.7 \mu\text{M}$), camazepam ($85.6 \pm 7.5 \mu\text{M}$), triazolam ($93.1 \pm 21.8 \mu\text{M}$), bromazepam ($104.0 \pm 30.0 \mu\text{M}$), lorazepam ($134.3 \pm 4.6 \mu\text{M}$) and chlordiazepoxide ($>300 \mu\text{M}$; $45.2 \pm 4.8\%$ inhibition at $300 \mu\text{M}$).

Effects of 7 benzodiazepines on the GTP γ S-activated $I_{K,ACH}$ in a single guinea pig atrial myocyte: Effects of 7 benzodiazepines (0.3 – $300 \mu\text{M}$) on the the GTP γ S-activated $I_{K,ACH}$ were examined. In these experiments, the pipet solution containing $100 \mu\text{M}$ GTP γ S instead of GTP was used. Intracellular loading of GTP γ S in atrial myocytes gradually activated the outward current, i.e., $I_{K,ACH}$, at a holding potential of -40 mV. Camazepam, diazepam, lorazepam, medazepam and triazolam inhibited the GTP γ S-activated $I_{K,ACH}$ in a concentration-dependent manner (Figs. 2 and 3). Bromazepam produced only a slight inhibition of the current even at the highest concentration ($12.6 \pm 2.5\%$ inhibition, $n=6$, at $300 \mu\text{M}$, Figs. 2 and 3). Chlordiazepoxide $300 \mu\text{M}$ inhibited the current by $33.1 \pm 7.4\%$ ($n=6$). The IC_{50} values for the GTP γ S-activated $I_{K,ACH}$ are shown in Table 1. The ascending order of IC_{50} values for the GTP γ S-activated $I_{K,ACH}$ was as follows; medazepam ($20.7 \pm 3.6 \mu\text{M}$), diazepam ($75.9 \pm 9.1 \mu\text{M}$), camazepam ($81.6 \pm 5.9 \mu\text{M}$), lorazepam ($98.8 \pm 3.4 \mu\text{M}$), triazolam ($125.3 \pm 25.5 \mu\text{M}$), chlordiazepoxide ($>300 \mu\text{M}$; $33.1 \pm 7.4\%$ inhibition at $300 \mu\text{M}$) and bromazepam ($>300 \mu\text{M}$; $12.6 \pm 2.5\%$ inhibition at $300 \mu\text{M}$).

IC_{50} ratio of 7 benzodiazepines in guinea pig atrial myocytes: To elucidate the mechanisms for the inhibitory effect of benzodiazepines, IC_{50} ratio of 7 benzodiazepines was calculated (Table 1). The IC_{50} ratio for camazepam (0.95), diazepam (1.45), lorazepam (0.74), medazepam (1.60) and triazolam (1.35) was close to unity. Because bromazepam and chlordiazepoxide did not show the maximum inhibitory effects for GTP γ S-activated $I_{K,ACH}$ and/or carbachol-induced $I_{K,ACH}$, the IC_{50} ratio was not determined.

DISCUSSION

The anticholinergic effects of 7 benzodiazepines were compared by examining their inhibitory effects on $I_{K,ACH}$ in guinea-pig atrial myocytes. All benzodiazepines (0.3 – $300 \mu\text{M}$) used in the present study inhibited the carbachol-induced $I_{K,ACH}$ in a concentration-dependent manner, while the

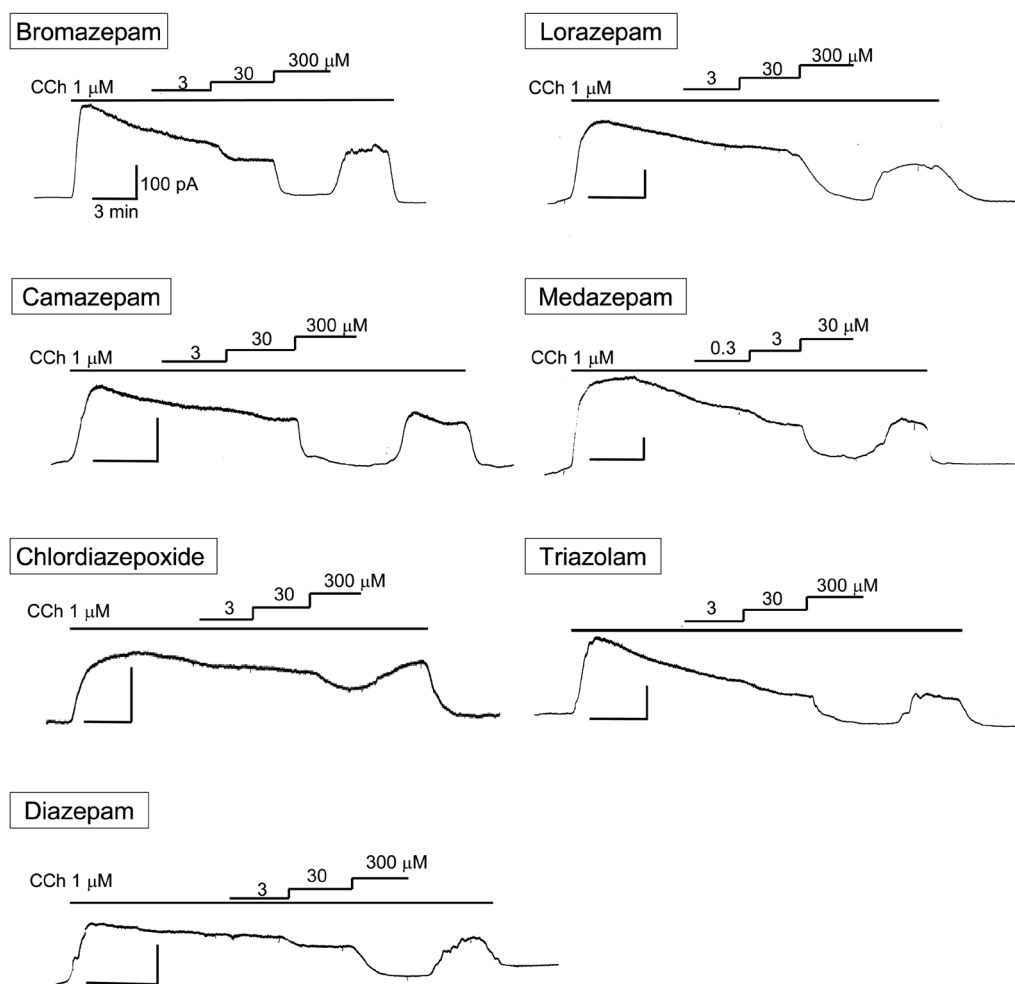


Fig. 1. Representative recording for the effects of 7 benzodiazepines, bromazepam, camazepam, chlordiazepoxide, diazepam, lorazepam, medazepam and triazolam, on the muscarinic acetylcholine receptor-operated potassium current ($I_{K,ACh}$) induced by an extracellular application of carbachol (CCh) ($1 \mu\text{M}$) in a guinea-pig single atrial myocyte. Whole-cell patch clamp method is used for recording of $I_{K,ACh}$ as an outward current at a holding potential of -40 mV . Applications of CCh and benzodiazepines are shown by the bars in each original current trace. The concentrations of benzodiazepines were increased in a stepwise fashion every three minutes. Each benzodiazepine compound inhibited the current in a concentration-dependent manner ($0.3\text{--}300 \mu\text{M}$).

Table 1. Inhibitory effects of 7 benzodiazepines on the carbachol-induced and guanosine 5'-[γ -thio]triphosphate (GTP γ S)-activated $I_{K,ACh}$ in a single guinea pig atrial myocyte

Drugs	IC ₅₀ value (μM)		IC ₅₀ ratio
	Carbachol-induced $I_{K,ACh}$	GTP γ S-activated $I_{K,ACh}$	
Bromazepam	104.0 ± 30.0	$>300^a)$	ND
Camazepam	85.6 ± 7.5	81.6 ± 5.9	0.95
Chlordiazepoxide	$>300^b)$	$>300^b)$	ND
Diazepam	54.8 ± 10.7	75.9 ± 9.1	1.45
Lorazepam	134.3 ± 4.6	98.8 ± 3.4	0.74
Medazepam	12.9 ± 2.4	20.7 ± 3.6	1.60
Triazolam	93.1 ± 21.8	125.3 ± 25.5	1.35

IC₅₀ values were determined by a mathematical curve fitting of concentration-response curves described in Fig. 3. IC₅₀ ratio was calculated by the following equation; IC₅₀ Ratio = [IC₅₀ for GTP γ S-activated current] / [IC₅₀ for carbachol-induced current]. a) Bromazepam ($300 \mu\text{M}$) inhibited GTP γ S-activated $I_{K,ACh}$ by $12.6 \pm 2.5\%$. b) Chlordiazepoxide ($300 \mu\text{M}$) inhibited carbachol-induced $I_{K,ACh}$ and GTP γ S-activated $I_{K,ACh}$ by $45.2 \pm 4.8\%$ and $33.1 \pm 7.4\%$, respectively. ND: Not determined.

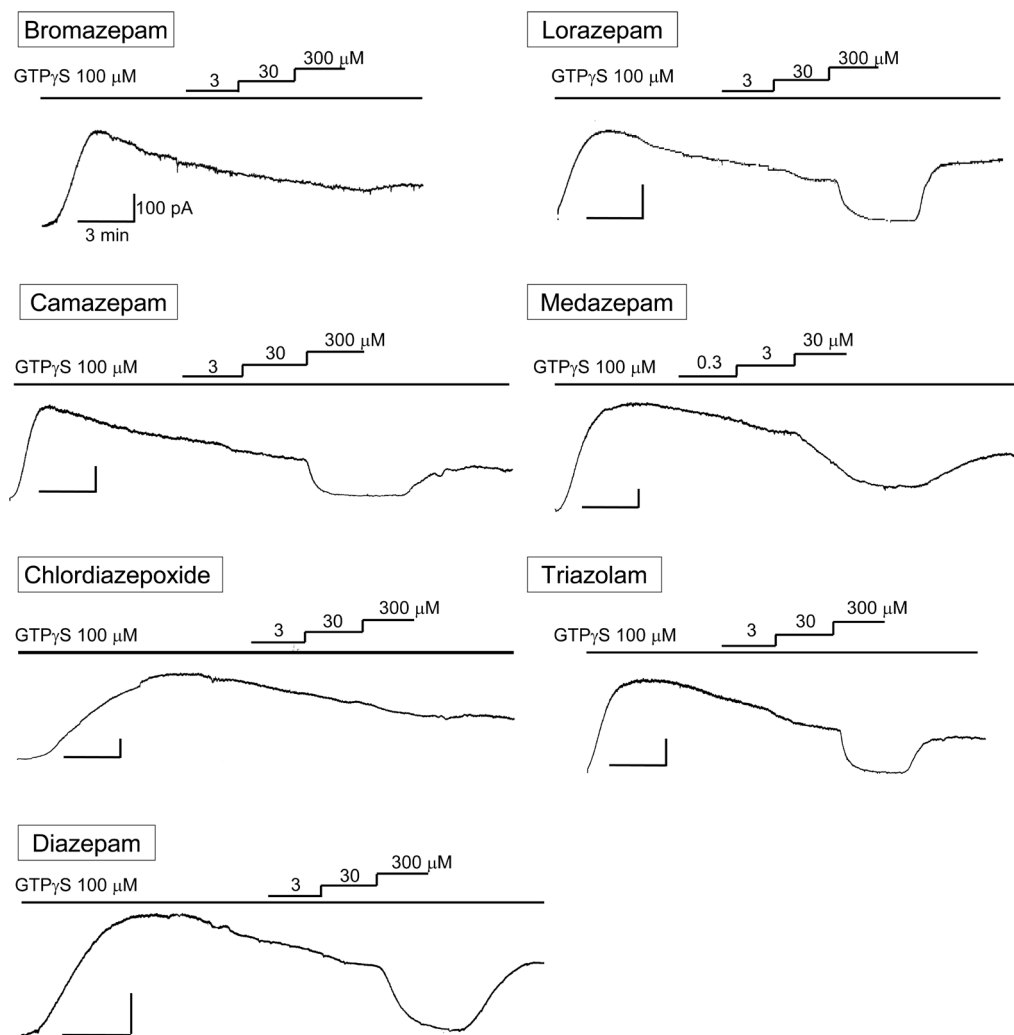


Fig. 2. Representative recording for the effects of 7 benzodiazepines, bromazepam, camazepam, chlordiazepoxide, diazepam, lorazepam, medazepam and triazolam, on $I_{K_{ACh}}$ activated by an intracellular loading of guanosine 5'-[γ -thio]triphosphate (GTP γ S) (100 μ M) in a guinea-pig single atrial myocyte. Whole-cell patch clamp method is used for recording of $I_{K_{ACh}}$ as an outward current at a holding potential of -40 mV. Applications of GTP γ S and benzodiazepines are shown by the bars each original current trace. The concentrations of benzodiazepines were increased in a stepwise fashion in every three minutes. Each benzodiazepine compound, except for bromazepam, inhibited the current in a concentration-dependent manner (0.3–300 μ M).

inhibitory effect of chlordiazepoxide seems weaker than the others judging from the IC_{50} values. The compounds, except for bromazepam, also inhibited the $I_{K_{ACh}}$ activated by an intracellular application of GTP γ S in a concentration-dependent manner. Interaction of acetylcholine receptor-operated potassium channel and G $\beta\gamma$ subunit of GTP binding proteins is important for activation of $I_{K_{ACh}}$ in cardiac myocytes [20, 21]. Many drugs, including antiarrhythmic drugs, anticancer chemotherapeutic drugs and antimalarial drugs, that produce anticholinergic actions in the heart have been reported to inhibit $I_{K_{ACh}}$ [10, 11, 14, 24, 26]. Molecular mechanisms by which several drugs inhibit $I_{K_{ACh}}$ have been proposed; some drugs block the muscarinic receptors and others inhibit the muscarinic potassium channel itself and/or GTP-binding

proteins [10, 11, 18, 19, 24]. These molecular mechanisms can be elucidated using data from the patch clamp method in atrial myocytes [3, 11, 24]. The $I_{K_{ACh}}$ is activated by an application of carbachol through binding to the muscarinic M_2 receptor in GTP-loaded cells. Moreover, intracellular loading of GTP γ S can directly activate the GTP-binding proteins and evoke antagonist-resistant, persistent activation of $I_{K_{ACh}}$ [3]. Thus, the muscarinic potassium channel opening through activation of GTP-binding proteins is a common pathway for induction of $I_{K_{ACh}}$. If one drug acts on the common pathway, inhibitory effects on carbachol-induced and GTP γ S-activated $I_{K_{ACh}}$ would appear in the same concentration range. To clarify the molecular mechanism of the inhibition, IC_{50} ratio, the ratio of IC_{50} for GTP γ S-activated

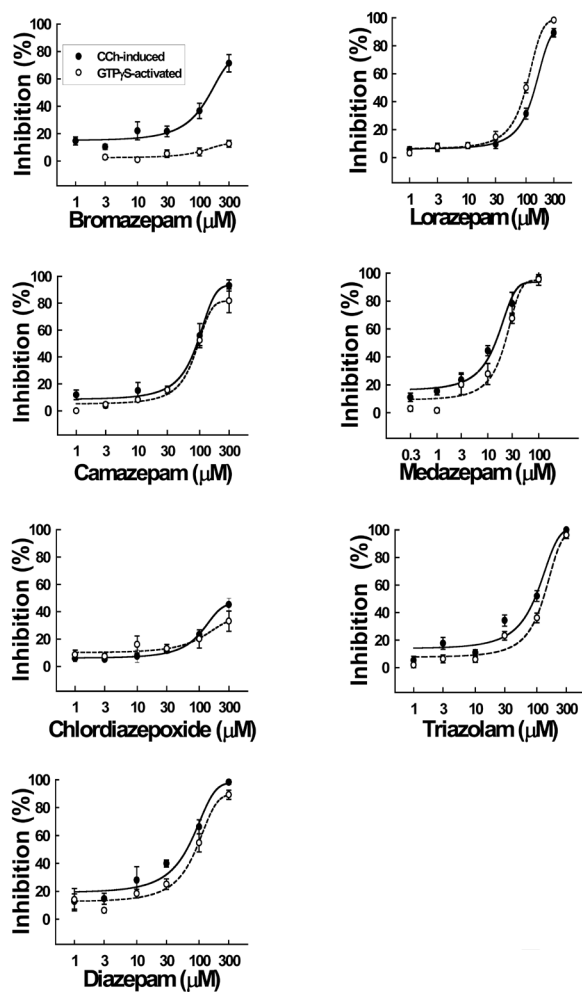


Fig. 3. Concentration-response curves for the inhibitory effects of 7 benzodiazepines on the CCh-induced (closed circle) and GTP γ S-activated (open circle) $I_{K,ACh}$ in a guinea-pig single atrial myocyte. Results are expressed as means \pm S.E.M. of four to fifteen myocytes. Each benzodiazepine compound, except for bromazepam, inhibited both currents in a concentration-dependent manner. Only slight inhibitory effect of bromazepam on the GTP γ S-activated current was observed.

$I_{K,ACh}$ to carbachol-induced $I_{K,ACh}$, has been proposed [10]. In the case of acting on the common pathway, the IC_{50} ratio for the drug would be close to unity. On the other hand, if the inhibitory action was caused through blockade of the muscarinic receptor binding, the IC_{50} ratio would be higher than unity. The IC_{50} value and IC_{50} ratio obtained from the present study are listed in Table 1. The IC_{50} ratio for medazepam was 1.60. However, there is no statistical difference between the IC_{50} for carbachol-induced $I_{K,ACh}$ and that for GTP γ S-activated $I_{K,ACh}$ (data not shown). Thus, we believe that the IC_{50} ratio (1.60) for medazepam would be close to unity. By the same reason, the IC_{50} ratio for diazepam (1.45) and triazolam (1.35) would also be close to unity. The reason why the IC_{50} ratio for camazepam (0.95) and lorazepam

(0.74) was lower than 1 cannot be explained at present. Since the inhibitory effect of drugs on the carbachol-induced $I_{K,ACh}$ should be occurred at lower concentration than that on the GTP γ S-activated $I_{K,ACh}$, the IC_{50} ratio is theoretically ≥ 1 . Thus, we believe that the IC_{50} ratio (0.74) would be close to unity considering the experimental errors. Based on these observations, the IC_{50} ratio for camazepam, diazepam, lorazepam, medazepam and triazolam was close to unity and these compounds would act on the GTP binding protein and/or potassium channel, i.e., the common pathway, to produce the anticholinergic effects in atrial myocytes. Although the inhibitory effects of chlordiazepoxide on the both currents were slight, the concentration-response curves on the currents were similar (Fig. 3). Therefore, chlordiazepoxide might act on the GTP binding protein and/or potassium channel in atrial myocytes similar to camazepam, diazepam, lorazepam, medazepam and triazolam. Because of a weak inhibitory effect of bromazepam on the GTP γ S-activated $I_{K,ACh}$ even at the highest concentration ($12.6 \pm 2.5\%$ inhibition at $300 \mu M$), the IC_{50} value was not determined in the present study. However, the IC_{50} ratio for bromazepam should be higher than unity judging from the IC_{50} values ($104.0 \pm 30.0 \mu M$ for carbachol-induced $I_{K,ACh}$ and $>300 \mu M$ for GTP γ S-activated $I_{K,ACh}$). So, it is presumed that bromazepam may act on the muscarinic receptor.

Clinical significance of the present study should be discussed. It has been reported that blood concentrations of benzodiazepines in human clinical medicine were as follows; bromazepam $0.08\text{--}0.2 \mu g/ml$, camazepam $0.1\text{--}0.6 \mu g/ml$, chlordiazepoxide $0.4\text{--}3 \mu g/ml$, diazepam $0.2\text{--}2 \mu g/ml$, lorazepam $0.001\text{--}0.02 \mu g/ml$, medazepam $0.1\text{--}0.5 \mu g/ml$, and triazolam $0.002\text{--}0.02 \mu g/ml$ [9, 25]. These concentrations were lower than those used in the present study. Because all benzodiazepines studied in the present study have the anticholinergic effect in the atrial myocytes at higher concentrations than the clinical setting, it is suggested that the effect was not so serious issue as a side effect in normal clinical situation. The present study also revealed that benzodiazepines have different molecular mechanisms depending on the compounds. Structure-action relationships among the benzodiazepines (1,4-benzodiazepine) are interesting. All compounds, except for bromazepam, have the same residues, phenyl and chloride, at position 5 and 7, respectively. However, bromazepam has pyridinyl at position 5 and bromide at position 7 as substitutions. These substitutions in bromazepam might limit to access to the intracellular machinery, the GTP binding protein and/or potassium channel. Thus, bromazepam has different molecular mechanisms to achieve the anticholinergic action.

In summary, 7 benzodiazepines have the anticholinergic action in atrial myocytes by inhibiting $I_{K,ACh}$. Camazepam, diazepam, lorazepam, medazepam and triazolam would act on the GTP binding protein and/or potassium channel. Bromazepam might preferentially act on the muscarinic receptor. The exact mechanism for chlordiazepoxide was not determined. It is suggested that the adverse effect of benzodiazepines in atrial myocytes may not be observed at the concentrations for clinical use.

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