Advance Access Publication 27 October 2004

eCAM 2004;1(3)277–284
doi:10.1093/ecam/neh044

Original Article

Comparative Electropharmacological Actions of Some Constituents from *Ginkgo biloba* Extract in Guinea-pig Ventricular Cardiomyocytes

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Effects of the constituents from *Ginkgo biloba* extract (GBE) on the action potentials and the ionic currents in guinea pig ventricular cardiomyocytes were investigated using whole-cell and current-clamp techniques. The constituents, ginkgolides A, B, C and quercetin, had depressant effects at 0.1–3 μM on the action potential configuration. Ginkgolide A (1–3 μM) prolonged the action potential (action potential duration: APD) at 75% and 90% repolarizations (APD_{75} and APD_{90}). However, ginkgolides B and C at low concentrations prolonged APD, but at higher concentrations (>1 μM) shortened APD. Quercetin at 3 μM prolonged the APD, but not at the lower concentrations. These constituents also inhibited the V_{max}. The resting potential was unaffected. In voltage-clamp experiments, ginkgolides A and B (0.1–3 μM) markedly and concentration-dependently increased the Ca^{2+} current (I_{Ca}) and the delayed rectifier K^+ current (I_K), and decreased the inwardly rectifying K^+ current (I_{K1}). On the other hand, ginkgolide C failed to affect the I_{Ca} but increased the I_K by 14.0 ± 2.3% ($n = 6$, $P < 0.05$) at 1 μM. Quercetin inhibited I_{Ca}, and enhanced I_K but decreased I_{K1}. These responses to the constituents were almost reversible (80–90% of control) after a 10- to 20-min washout. These results indicate that even at acute administrations, these constituents produce the effective actions on the APD and the underlying ionic currents in cardiomyocytes. Each constituent does not exhibit a uniform response, although GBE acts as a net.

Keywords: action potential – ionic currents – ginkgolide A – ginkgolide B – ginkgolide C – *Ginkgo biloba* extract – guinea pig ventricular cardiomyocytes – quercetin

Introduction

The extract from the leaves of *Ginkgo biloba* (GBE) plays a role in cellular physiological and pharmacological functions. GBE is used for treating impaired brain functions in elderly and peripheral arterial occlusive diseases (1,2). Pharmacological experiments have recently demonstrated that GBE (i) increases cerebral blood flow, decreases viscosity and antagonizes platelet activating factor receptors (3,4); (ii) increases tolerance for anoxia by preventing the decrease in ATP level (5); (iii) improves neurotransmitter disturbances (6,7); and (iv) prevents cell damage induced by free radicals (8–10).

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GBE contains many chemical constituents. The major constituents are terpenes such as bilobalide, ginkgolide A, ginkgolide B and ginkgolide C, and flavonoids such as quercetin, kaempferol and isorhamnetin (6,11). In the cardiovascular system, GBE does not affect heart rate and contractility, but produces a concentration-dependent increase in coronary flow (12,13). In our previous experiments, however, we have found that GBE prolongs the action potential duration (APD), whereas bilobalide, a main constituent, shortens the APD in cardiomyocytes (14). The alterations of the action potential configurations clinically would lead to the pathophysiological changes in the electrocardiogram (ECG). In addition, Tamago *et al*. (15) have shown that ginkgolide B is a PAF-antagonist and acts on the APD, consistent with our results.

The mechanisms of the therapeutic effectiveness of GBE are expected to be extremely complex, because of the large

number of its constituent substances (6,8). Unknown mechanisms of these constituents for the ionic channel currents still remain. The aim of the present experiments was to examine, by use of a patch-clamp technique, how these constituents of GBE, other than bilobalide, affect the action potentials and the underlying ionic currents. In addition, the cardiac electropharmacological actions of each constituent were compared.

Materials and Methods

All experiments were carried out according to the guidelines laid down by the Nara Medical University Animal Welfare committee, and also under the terms of the Declaration of Helsinki.

Cell Preparation

Cells were prepared from tissue taken from the ventricle muscle of guinea pig hearts, using methods similar to those described previously (14,16). Under sodium pentobarbital (30 mg/kg, i.p.) anesthesia, the chest was opened and the aorta was cannulated *in situ*. The heart was dissected out and perfused with normal Tyrode solution on the Langendorff apparatus. After a washout of blood, the heart was perfused with Ca^{2+}-free Tyrode solution, and spontaneous beating ceased. Then, the perfusate was switched to low-Ca^{2+} (30–60 μM) Tyrode solution containing 0.4 mg/ml collagenase (Type I, Sigma Chemical, St Louis, MO) for about 20 min. The heart was washed out by high-K^+ and low-Cl^- solution (KB solution), and was dissected with scissors. The temperature of all solutions was maintained at 36°C.

Current- and Voltage-clamp Experiments

Current-clamp and whole-cell voltage-clamp recordings were performed using an Axopatch patch-clamp amplifier (Axon Instruments, Burlingame, CA) and standard techniques. Patch pipettes from borosilicate glass capillaries were fabricated using a two-stage puller, and had a resistance of 5–7 MΩ. The series resistance error was <3–7 mV, and no compensation was used. The liquid junction potential between the pipette solution and the external solution (<10 mV) was corrected for all membrane potential recordings. Experiments were carried out at a temperature of 36.5°C. The data were stored and analyzed on an IBM-AT microcomputer, using the PCLAMP analysis program (Axon Instruments). Current traces were filtered using a cut-off frequency of 1 kHz for plotting. The I_{Ca} was measured as the difference between the peak current and the zero current, and the I_{K1} was the difference between the current at the end of a 1-s test pulse and the zero current. The I_K was measured at the peak of the outward tail current. All values are given as mean ± S.E.M. The differences of the mean values were analyzed by Student's *t*-test and ANOVA for paired data, and a *P*-value of <0.05 was considered significant.

Experimental Solutions

The composition of the modified Tyrode solution was: 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 5.0 mM glucose and 5.0 mM HEPES. The pH was adjusted to 7.4 with NaOH. The constituents of *Ginkgo biloba* extract, ginkgolides A, B and C, and quercetin (Tokiwa Phytochemical Co., Tokyo, Japan) were dissolved with DMSO. Bath solutions with the desired concentrations were made and superfused. The pipette solution (intracellular) contained: 110 mM K-aspartate, 20 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 5 mM Mg-ATP, 5 mM creatine phosphate and 5 mM HEPES (pH 7.2). The pipette solution for the measurement of I_{Ca} alone contained: 110 mM CsOH, 20 mM CsCl, 2 mM $MgCl_2$, 10 mM EGTA, 5 mM MgATP, 5 mM creatine phosphate, 100 mM aspartic acid and 5 mM HEPES (pH 7.2).

Results

Gingko biloba Effects on the Action Potentials

To examine the effects of each constituent on the action potential configuration, a current-clamp experiment was carried out. The isolated single cell was stimulated at 1 Hz. As shown in Fig. 1A, ginkgolide A (at >1 μM) markedly prolonged the APD; at 3 μM, 75% and 90% repolarizations of APD (APD_{75} and APD_{90}) were by +43.8 ± 3.6% (*n* = 8,

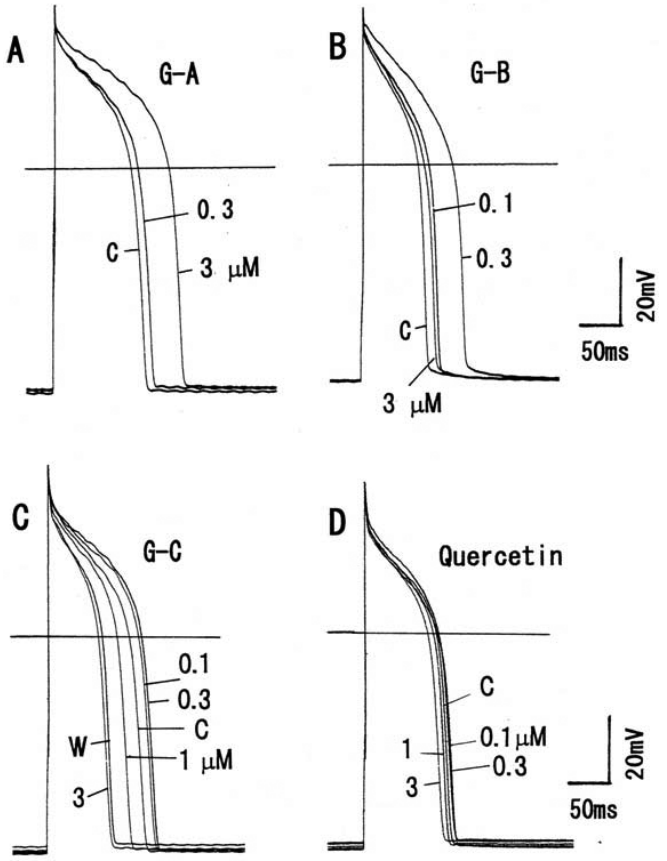

Figure 1. Modulation of the action potentials by the constituents of *Ginkgo biloba* extract in guinea pig ventricular cardiomyocytes. (**A**) Effects of ginkgolide A on the action potentials. (**B**) Action potentials in ginkgolide B. (**C**) Action potentials in ginkgolide C. (**D**) Quercetin on action potentials. Each constituent from 0.1–3 μM was cumulatively added to bath solution. Horizontal lines indicate 0 mV. Symbols used are control (C) and washout (W).

$P < 0.001$) and by $+40.9 \pm 4.2\%$ ($n = 8$, $P < 0.001$), respectively (Fig. 2A). Ginkgolide B at low concentrations (0.1–0.3 μM) markedly prolonged APD_{75} and APD_{90}, but at high concentrations (1–3 μM) it decreased both of the APDs (although the APDs were still enhanced in comparison with the control) (Fig. 1B). The APD_{75} and APD_{90} at 0.3 μM were $+63.6 \pm 4.8\%$ ($n = 9$, $P < 0.001$) and $+58.3 \pm 3.6\%$ ($n = 6$, $P < 0.001$), respectively, and at 3 μM were $+16.2 \pm 3.9\%$ ($n = 9$, $P < 0.05$) and $+15.0 \pm 3.7\%$ ($n = 9$, $P < 0.05$), respectively (Fig. 2B).

On the other hand, ginkgolide C at low concentrations (0.3 μM) prolonged APD, whereas at high concentrations (1–3 μM) it markedly shortened APD (Fig. 1C). The APD_{75} and APD_{90} at 0.3 μM were altered by $+15.5 \pm 3.7\%$ ($n = 8$, $P < 0.05$) and $+9.9 \pm 2.6\%$ ($n = 8$, $P < 0.05$), respectively, and at 3 μM they were altered by $-38.5 \pm 2.8\%$ ($n = 8$, $P < 0.001$) and $-37.2 \pm 3.5\%$ ($n = 8$, $P < 0.01$), respectively (Fig. 2C). Quercetin at 0.1–1 μM tended to prolong the APD, and at 3 μM prolonged the APD_{75} by $+11.9 \pm 3.4\%$ ($n = 8$, $P < 0.05$) and APD_{90} by $+17.9 \pm 3.0\%$ ($n = 8$, $P < 0.05$) (Figs 1D and 2D).

The maximum rate of deporization (V_{max}) significantly decreased by ~18–20% ($n = 8$–9) at higher concentrations (1–3 μM) of these constituents. Other action potential parameters were also unaffected in the presence of all the constituents. The modulation of the action potential configurations by the constituents is summarized in Fig. 3. A washout of the drugs for 15–20 min recovered to ~70–80% of the control value.

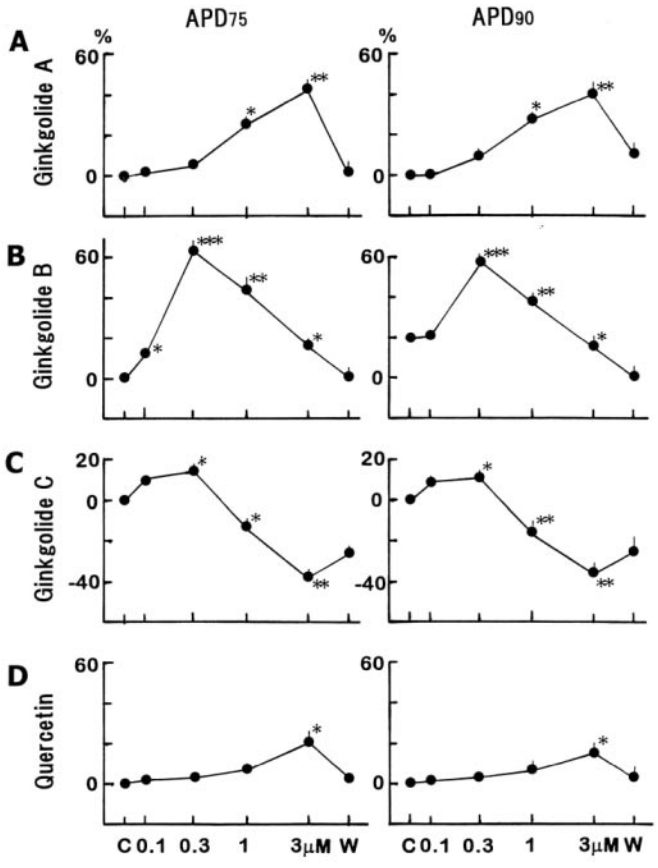

Figure 2. Changes in the action potential durations (75% and 90% repolarizations) in the presence of the constituents (0.1–3 μM). Maximum responses at different concentrations of each constituent are plotted. Values are represented as mean ± S.E.M. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, with respect to control value.

Constituents of *Gingko biloba* and Quercetin Exert Contrasting Effects on Ionic Currents

Whole-cell patch voltage-clamp experiments were performed to examine the effects of the constituents on the underlying ionic currents. Test pulses (1 s duration) were applied to −20 to 60 mV and −40 to −120 mV from a holding potential of −30 mV. The average capacitance was 86.1 ± 2.0 pF ($n = 44$). As shown in Fig. 4, ginkgolide A (1 μM) enhanced both the Ca^{2+} current (I_{Ca}), by 68.2 ± 2.8% ($n = 7$, $P < 0.001$), and the delayed rectifier K^+ current (I_K) by 66.3 ± 3.8% ($n = 7$, $P < 0.001$). Each constituent (0.1–3 μM) was cumulatively administrated to the bath solution. Ginkgolide B at 0.3 μM also increased the I_{Ca} at 10 mV by 58.1 ± 2.8% ($n = 6$, $P < 0.001$) (Fig. 5). Increasing the concentrations (1–3 μM) still enhanced the I_{Ca}, but the maximal response at 0.3 μM decreased. The I_K at 60 mV increased by 89.3 ± 3.8% ($n = 6$, $P < 0.001$) at 3 μM, but the inwardly rectifying K^+ current (I_{K1}) was not affected significantly. Ginkgolide C had a smaller or no effect on the I_{Ca}, but markedly increased the I_K by 59.2 ± 2.8% ($n = 7$, $P < 0.001$) at 3 μM (Fig. 6).

On the other hand, the application of quercetin (0.1–3 μM) inhibited the I_{Ca} (Fig. 7A and B). The I_{Ca} at 10 mV decreased by 34.9 ± 3.2% ($n = 8$, $P < 0.05$) at 0.3 μM and by 56.8 ± 3.3% ($n = 8$, $P < 0.05$) at 3 μM. The responses were produced in a concentration-dependent manner. Simultaneously, the I_K at 60 mV increased by 60.4 ± 2.7% ($n = 8$, $P < 0.001$) at 0.3 μM and by 89.7 ± 3.3% ($n = 8$, $P < 0.001$) at 3 μM. Quercetin simultaneously did not affect the I_{K1} to a significant extent (by ~10–15%) at low concentrations, but at 3 μM inhibited the I_{K1} by 12.4 ± 2.1% ($n = 8$, $P < 0.05$). The responses to the constituents for the I_{Ca} and I_K currents are summarized in Fig. 8. The responses to all the constituents were recovered to ~80–90% of the control value after a 20-min washout.

Discussion

The present experiments showed that: (i) ginkgolide A prolonged the APD, but ginkgolides B and C shortened APD; (ii) quercetin prolonged the APD; (iii) the constituents also inhibited the V_{max} and the RP was unaffected; (iv) ginkgolides A and B increased I_{Ca} and I_K, and decreased I_{K1}; (v) ginkgolide C failed to affect the I_{Ca} but increased the I_K; (vi) quercetin inhibited I_{Ca}, and enhanced I_K but decreased I_{K1}; and (vii) these responses were almost reversible after a washout. These results indicate that acute administrations of these constituents can produce the effective actions on cardiomyocytes. Each constituent never exhibits a uniform response.

In recent clinical and experimental experiments, GBE has been found to be effective against ischemic brain injury (11,17)

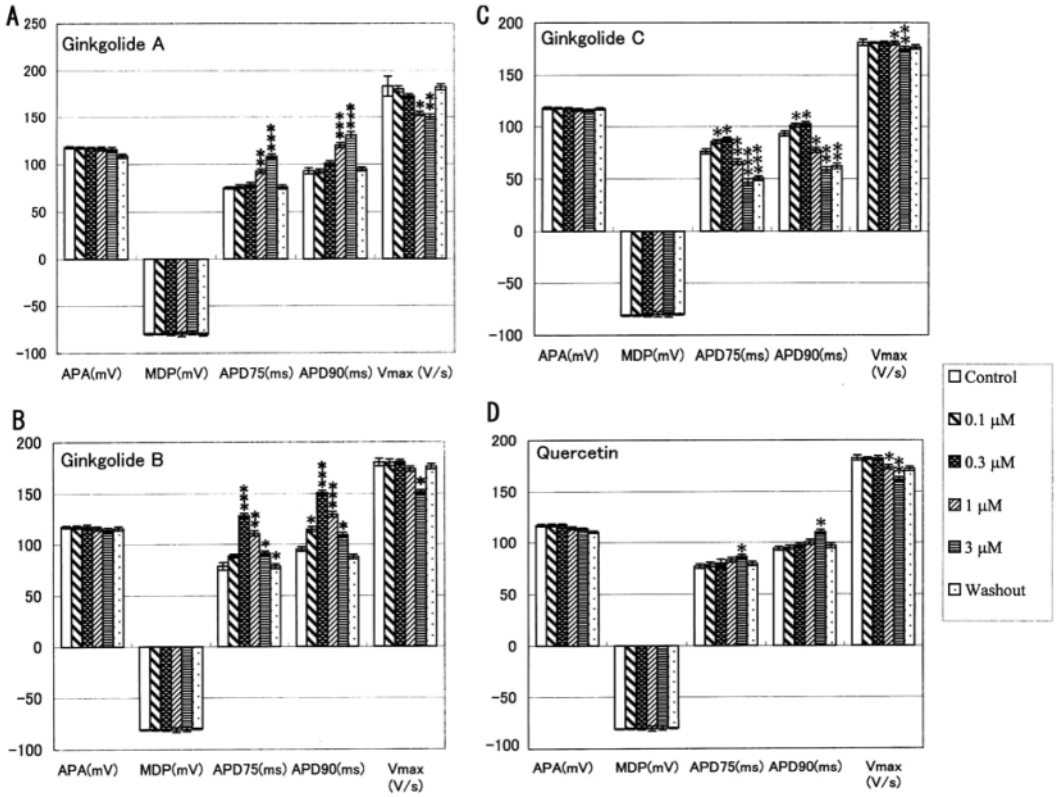

Figure 3. Summary of the effects on action potential parameters. (**A**) Ginkgolide A, (**B**) ginkgolide B, (**C**) ginkgolide C, (**D**) quercetin. APA, action potential amplitude; MDP, maximum diastolic potential; APD_{75}, 75% repolarization of the action potential duration; APD_{90}, 90% repolarization of the action potential duration; V_{max}, maximum rate of depolarization. Values are represented as mean ± S.E.M. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, with respect to control value.

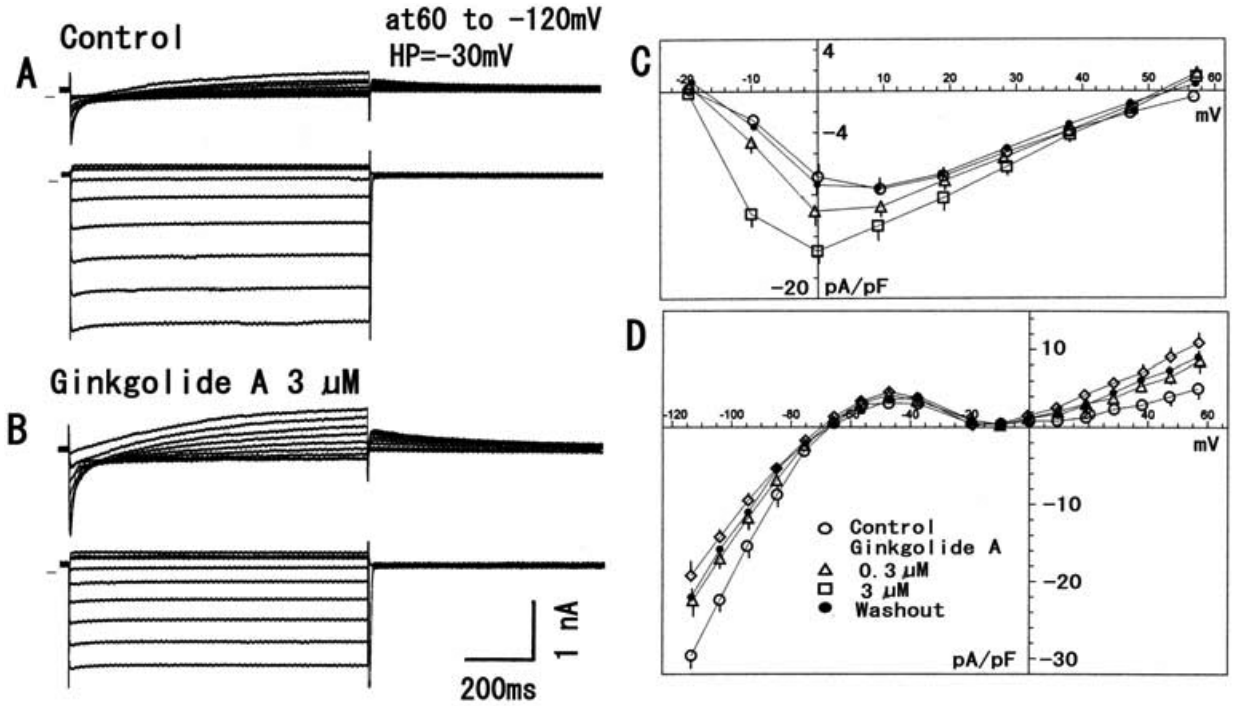

Figure 4. Modulation by ginkgolide A of the ionic currents. (**A**) Current traces in control. (**B**) Current traces in ginkgolide A (3 μM). Test pulses for 1 s were applied to −20 to 60 mV and −40 to −120 mV from a holding potential of −30 mV. Horizontal line before the current traces indicates zero current level. (**C**) Current–voltage relationship for Ca^{2+} current. (**D**) Current–voltage relationship for the delayed rectifier K^+ current and the inwardly rectifying K^+ current. Symbols used are control (open circles), 0.3 μM (triangles) and 1 μM (squares) of ginkgolide A, and washout (filled circles).

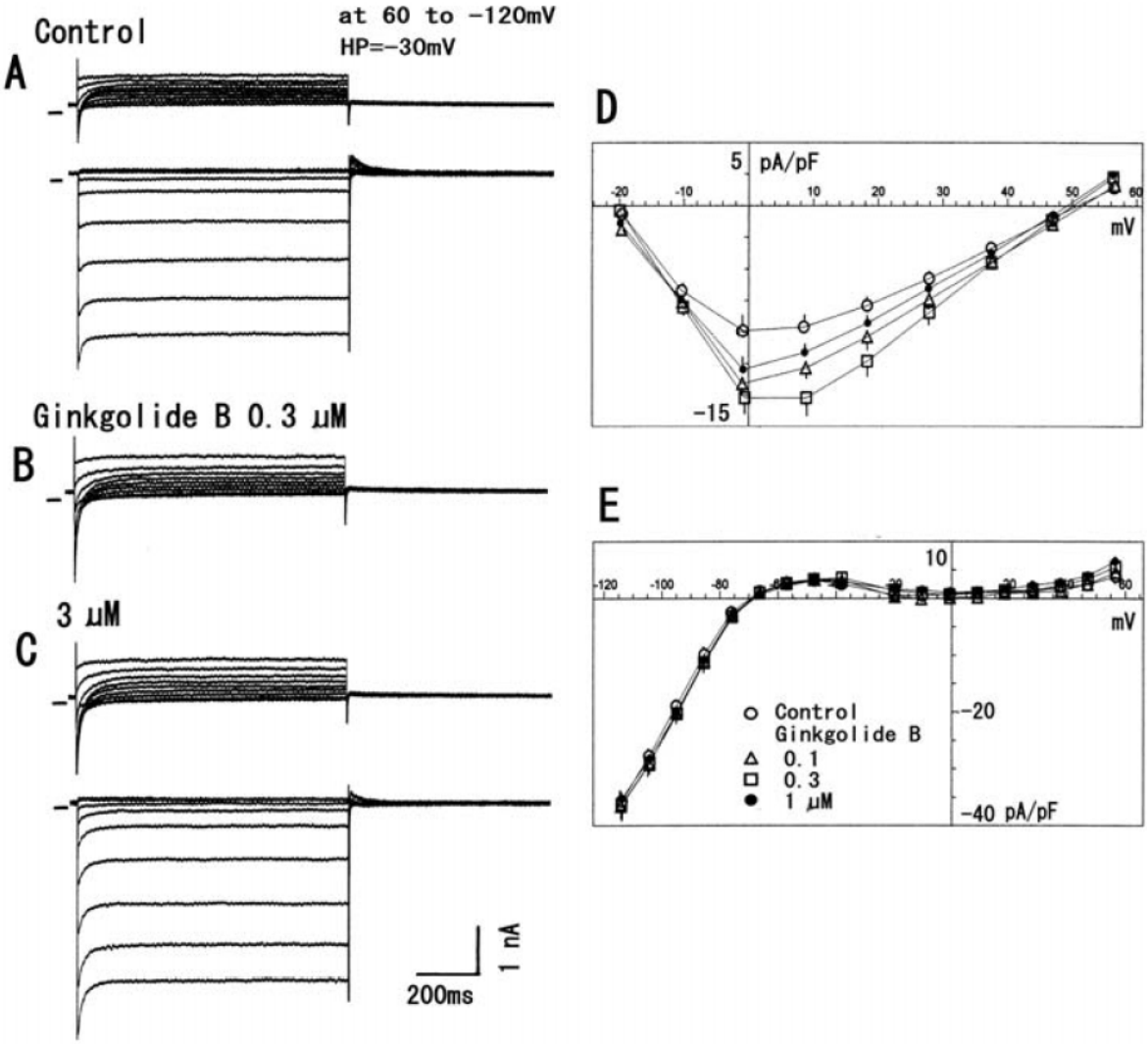

Figure 5. Modulation by ginkgolide B of the ionic currents. (**A**) Current traces in control. Test pulses for 1 s were applied to −20 to 60 mV and −40 to −120 mV from a holding potential of −30 mV. (**B**) Current traces in ginkgolide B (0.3 μM). Test pulses for 1 s were applied to −20 to 60 mV. Holding potential was −30 mV. (**C**) Current traces at 3 μM. Test pulses for 1 s were applied to −20 to 60 mV and −40 to −120 mV from a holding potential of −30 mV. Horizontal line before the current traces indicates zero current level. (**D**) Current–voltage relationship for Ca^{2+} current. (**E**) Current–voltage relationship for the delayed rectifier K^+ current and the inwardly rectifying K^+ current. Symbols used are control (open circles), 0.1 μM (triangles), 0.3 μM (squares) and 1 μM (filled circles) of ginkgolide B.

and cerebral disorders due to aging (18). The mechanisms of the beneficial effects of GBE are considered to be improvements in: (i) hemodynamic disorders (6,7,19); (ii) PAF-associated abnormalities (3,4,20,21); (iii) cell damage induced by free radicals (8–10); and (iv) a decrease in ATP level during anoxia (5). In fact, these effects have recently been recognized clinically by several double-blind studies with GBE versus placebo (22–25).

APD Regulation

In experiments for the cardiovascular system, GBE does not affect heart rate and contractility, but produces a concentration-dependent increase in coronary flow (12,13). In our laboratory, however, GBE caused a negative chronotropic effect in rabbit SA nodal cells (unpublished data). Also, GBE inhibited I_{Ca} and I_K, but bilobalide (a constituent) increased them in guinea pig ventricular cardiomyocytes (14). Simultaneously, GBE prolonged the APD, whereas bilobalide shortened it. In the present experiments, a major action of the constituents was the alteration of APD involved with the effects on the ionic channels. The APD means a period for the repolarization of the membrane, making T wave on ECG. Thus, the APD of cardiac myocytes is clinically reflected directly to the QT interval. The QT interval is a reflection of the repolarization of action potential, which is mainly responsible for the modulation of I_K. The APD prolongation increases the refractory period and simultaneously elevates the cellular Ca^{2+} concentration (26–28). Therefore, the constituents would play an important role as ionic channel modulators of

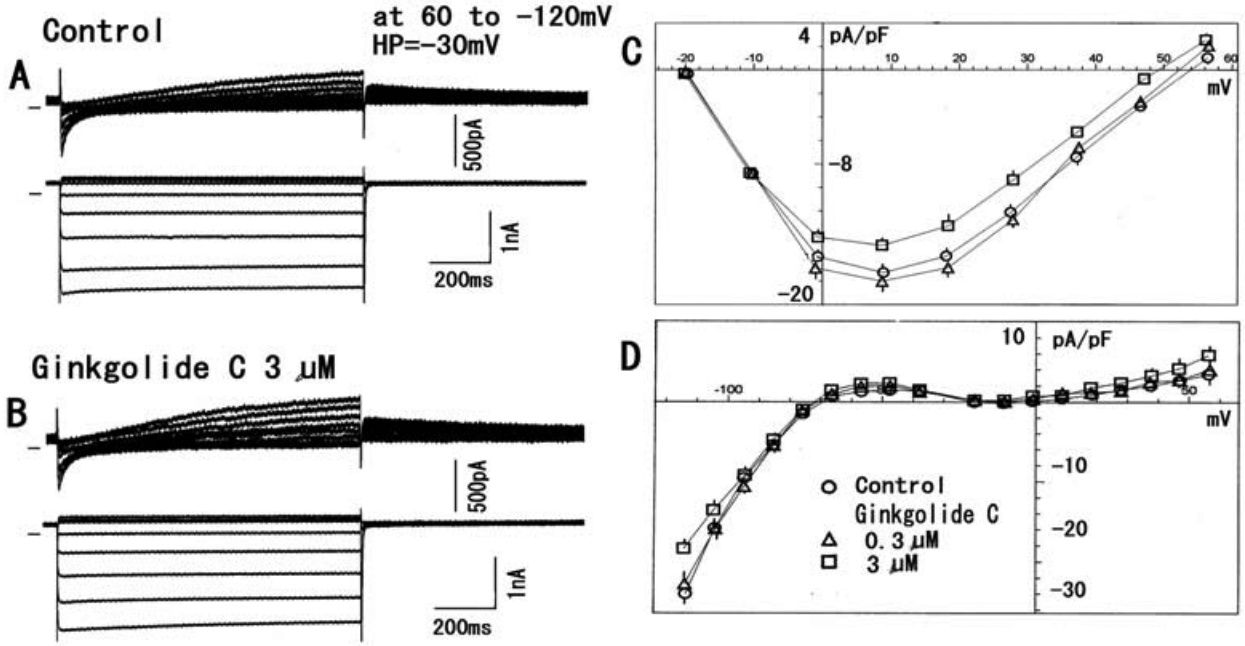

Figure 6. Modulation by ginkgolide C of the ionic currents in guinea pig ventricular cardiomyocytes. (**A**) Current traces in control. (**B**) Current traces in ginkgolide C (3 μM). Test pulses for 1 s were applied to −20 to 60 mV and −40 to −120 mV from a holding potential of −30 mV. Horizontal line before the current traces indicates zero current level. (**C**) Current–voltage relationship for the Ca^{2+} current. (**D**) Current–voltage relationship for the delayed rectifier K^+ current and the inwardly rectifying K^+ current. Symbols used are control (open circles), 0.3 μM (triangles) and 3 μM (squares) of ginkgolide C.

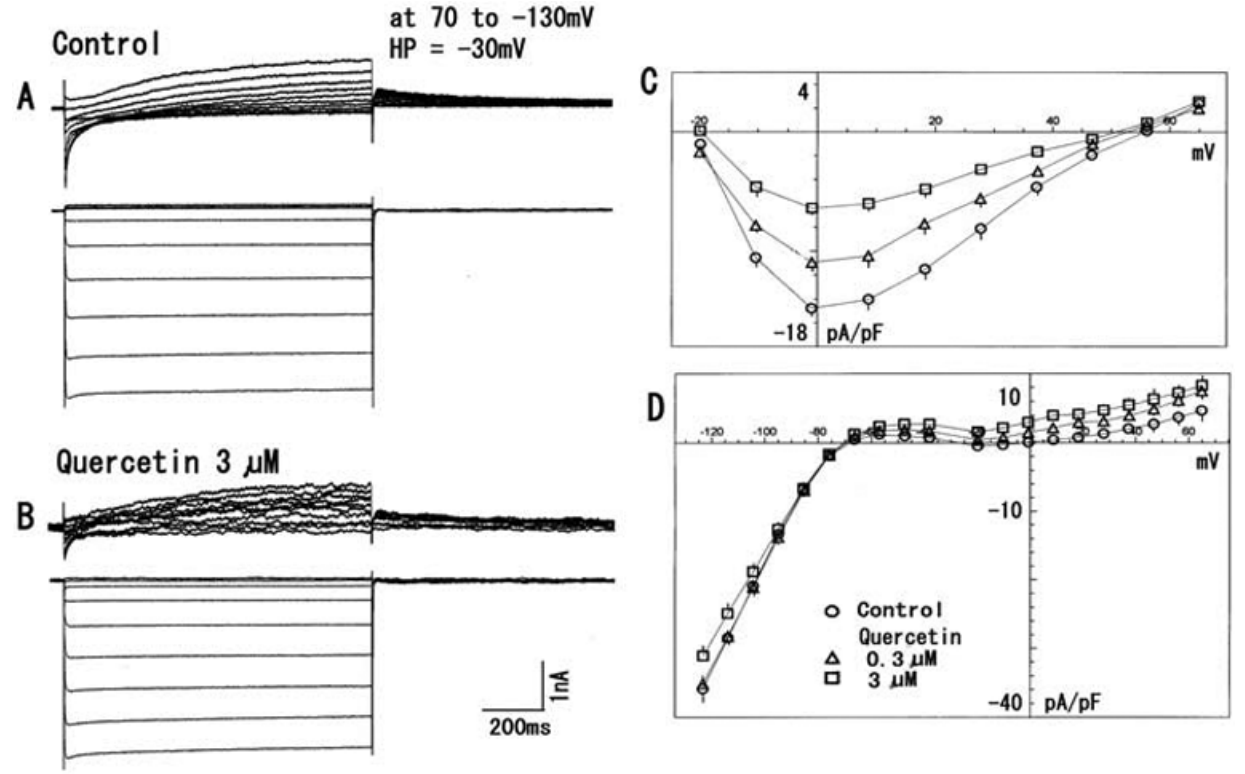

Figure 7. Modulation by quercetin of the ionic currents in guinea pig ventricular cardiomyocytes. (**A**) Current traces in control. (**B**) Current traces in quercetin (3 μM). Test pulses for 1 s were applied to −20 to 60 mV and −40 to −120 mV. Holding potential was −30 mV. Horizontal line before the current traces indicates zero current level. (**C**) Current–voltage relationship for Ca^{2+} current. (**D**) Current–voltage relationship for the delayed rectifier K^+ current and the inwardly rectifying K^+ current. Symbols used are control (open circles), 0.3 μM (triangles) and 3 μM (squares) of quercetin.

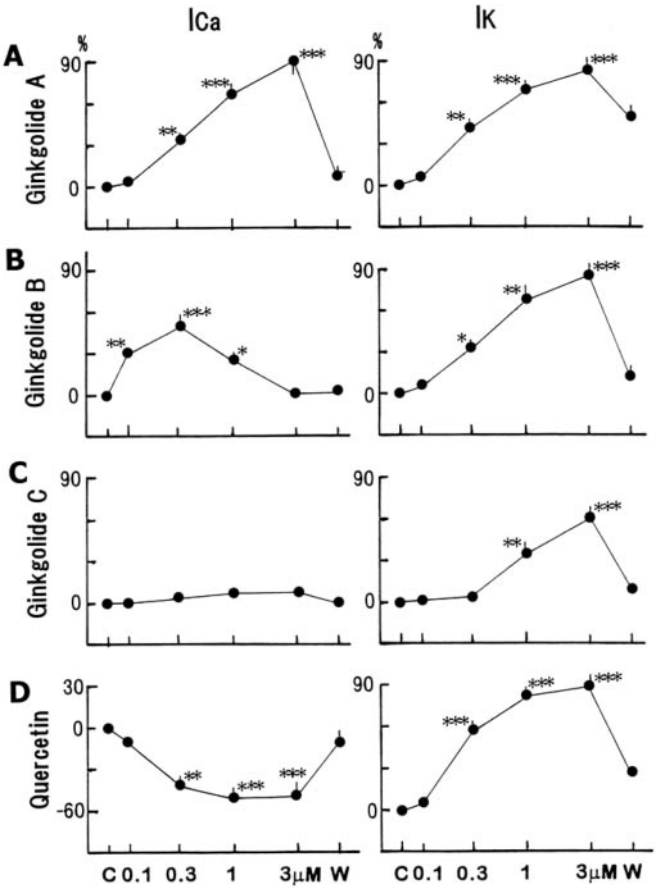

Figure 8. Concentration-dependent responses to the constituents on the Ca^{2+} and delayed rectifier K^+ currents. (**A**–**D**) Percentage changes in control and at 0.3 and 3 μM of ginkgolide A, B, C and quercetin. Maximum values for the currents at different concentrations of each constituent are plotted. Values are represented as means ± S.E.M. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, with respect to control value.

cadiomyocytes, although the effects of each constituent on the APD and the ionic currents were not exhibited in a uniform direction. Quercetin, a kind of flavonoid, had a smaller or no effect on the action potentials, but caused the APD prolongation only at high concentrations. Also, quercetin inhibited I_{Ca} and enhanced I_K. Quercetin has been reported to possess many pharmacological effects (29,30); the modification of eicosanoid synthesis, prevention of platelet aggregation, and vasorelaxation due to the inhibition of PK-C.

Modulation of the Ionic Currents

Furthermore, these constituents also acted on the V_{max}, although they did not affect other action potential parameters. In general, the V_{max} is used as an activator of the Na^+ channel current (I_{Na}). Thus, the constituents have an inhibitory action on the I_{Na}, resulting in an inhibition of the conduction velocity and a suppression of excitability. In addition, the constituents simultaneously modulated the ionic channel currents such as I_{Ca}, I_K, I_{K1} and I_{Na}. These effects might cause antiarrhythmic actions. In all the constituents, I_K enhancement was finally produced, indicative of a cell protection due to an APD shortening and a decline of cellular Ca^{2+} concentration.

The channel activity of single myocytes might be caused a run-down, especially Ca^{2+} channel and rapidly activating K^+ channel. The cells not causing run-down were chosen and used for the experiments. However, there may be a limitation to patch-clamp experiments. The responses to the constituents were considered to be due to the effects of constituents, because of the reversible response. Therefore, these constituents would exert many helpful and protective actions upon cardiac cells.

Clinical Uses and Summary

GBE can be administered to patients with mild to moderate symptoms of cerebral insufficiency (1,2). The half-life of GBE is 2–3 h (11). In pharmacokinetic analysis, the bioavailabilities of ginkgolides A and B are practically high, but that of ginkgolide C is very low (31). Of the GBE-containing flavonoids, quercetin has the highest percentage (8.91%). With regard to the distribution of radioactivity in the cardiovascular system, the tissues in areas such as vein, heart and aorta are relatively higher 3–7 h after oral administration (32). Quercetin possesses a vasodilating action (33,34). Also, the histamine-induced contraction of isolated guinea pig intestine was inhibited by a mixture of flavonoids (35). In our laboratory, GBE as a mixture exhibited a potent vasodilatory action (34). Therefore, these responses are most likely produced by the modulation of ionic channel currents that have been demonstrated in this study.

It has been reported that a single dose of GBE does not produce potent pharmacological activities, and repeated doses are needed to produce beneficial effects over a long period of administration (36–38). In the present *in vitro* experiments, however, acutely single administrations produced marked actions on the ionic channel currents and the action potentials. Since GBE is a mixture of its constituents, the total response to GBE would be a result of complex interactions with the constituents. Therefore, the constituents from GBE would play an important role in modulating the strong APD prolongation by interacting with each other. Although relatively higher concentrations were used in this study, because the pharmacological effects on the ionic channels were so remarkable, GBE and its constituents would be helpful in the treatment of cerebral disorders due to their effects on central nervous system (CNS) neurons. Further experiments are needed to elucidate the detailed mechanisms of the cardiac actions of *Ginkgo biloba*.

Acknowledgements

The author wishes to thank Tokiwa Phytochemical Co. Ltd. for providing the constituents of *Ginkgo biloba* extract (ginkgolides A, B and C, quercetin).

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Received July 27, 2004; accepted September 16, 2004