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Bradykinin B_2-receptor-mediated modulation of membrane currents in guinea-pig cardiomyocytes

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1 In order to define the electrophysiological mechanism(s) responsible for bradykinin (BK)-induced positive inotropic and chronotropic responses in isolated guinea-pig atria, effects of BK on the membrane currents were examined in isolated atrial cells using patch clamp techniques.

2 BK (0.1–1000 nM) increased the L-type Ca^{2+} current (I_{Ca}), which was recorded from enzymatically-dissociated atrial myocytes by the nystatin-perforated patch method, in a concentration-dependent fashion, and the calculated EC_{50} value for increasing I_{Ca} was 5.2 nM. In conventional ruptured patch experiments, BK inhibited the muscarinic acetylcholine receptor-operated K^+ current ($I_{K.ACh}$) that was activated by the muscarinic agonist carbachol (1 μM) with an EC_{50} value of 0.57 nM. Both the increase in I_{Ca} and the decrease in $I_{K.ACh}$ were blocked by HOE140, a selective bradykinin B_2 receptor antagonist.

3 The BK-induced inhibition of $I_{K.ACh}$ was significantly attenuated by staurosporine and calphostin C, protein kinase C inhibitors. In addition, the $I_{K.ACh}$ inhibition by BK was also attenuated by the tyrosine kinase inhibitor genistein or tyrphostin but not by daidzein, an inactive analogue of genistein. However, neither protein kinase C inhibitor nor tyrosine kinase inhibitor affected the BK-induced increase in I_{Ca}.

4 In the presence and absence of muscarinic stimulation, BK prolonged the action potential recorded from the atrial cells in the current clamp mode.

5 We conclude that BK increases I_{Ca} and decreases $I_{K.ACh}$ in atrial cells, resulting in positive inotropic and chronotropic responses in atrial preparations. Protein kinase C activation, and possibly tyrosine kinase activation, may be involved in the B_2-receptor-mediated $I_{K.ACh}$ inhibition.

Keywords: Bradykinin; Ca^{2+} current; K^+ current; tyrosine kinase; protein kinase C

Introduction

Bradykinin (BK) is a nonapeptide mediator implicated in inflammation, pain sensation, vasodilation, edema formation and smooth muscle contraction (Bhooka *et al*., 1992). BK has been shown to affect cardiac function directly or indirectly (Iven *et al*., 1980; Nakashima *et al*., 1982; Minshall *et al*., 1994; Tesfamariam *et al*., 1995). BK was reported to facilitate the evoked release of norepinephrine from the sympathetic nerve endings and thereby produce a positive inotropic response in rat atrial and ventricular muscles (Minshall *et al*., 1994) and guinea-pig atrial muscles (Iven *et al*., 1980). BK was also shown to produce direct positive inotropic and chronotropic effects in isolated guinea-pig atria (Iven *et al*., 1980; Nakashima *et al*., 1982; Tesfamariam *et al*., 1995). A recent radioligand binding study (Minshall *et al*., 1995) has demonstrated the existence of specific BK binding site on myocardial membranes from many animal species including guinea-pig. Therefore, it is conceivable that BK may produce a direct action on cardiomyocytes, at least, of guinea-pig. To our best knowledge, however, effects of BK on the membrane current system of cardiac cells have not been evaluated. The purpose of the present study was to evaluate the effects of BK on membrane currents of guinea-pig atrial cells, with special reference to the L-type calcium current (I_{Ca}) and the muscarinic acetylcholine receptor-operated potassium current ($I_{K.ACh}$) which play an important role in inotropic responses in atrial preparations. Since we found that BK increases I_{Ca} and decreases $I_{K.ACh}$ in atrial myocytes, we attempted to define the intracellular mechanisms of the BK receptor-mediated modulation of the membrane currents.

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Methods

Mechanical function study

All experiments were performed under the regulations of the Animal Research Committee of the School of Medicine, Chiba University. Guinea-pigs, weighing 250–350 g, were killed by a blow on the head and the hearts were removed rapidly. Left and right atria were dissected and mounted vertically in a 20 ml water-jacketed bath containing Krebs-Henseleit solution bubbled with 95%O_2+5%CO_2 at 30°C. The composition of the solution was (mM): NaCl 119, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 24.9 and glucose 10.0. The lower end of the left atrium was fixed on a hook and the other end was connected to a force transducer (Nihon Kohden TB-651T, Tokyo, Japan). The resting tension applied to the atrial preparation was adjusted to 0.5 g. The left atrial preparations were electrically stimulated by rectangular pulses at 0.5 Hz, 5 ms in the duration and 1.5 times the threshold voltage. The pulses were delivered from an electronic stimulator (Nihon Kohden S-7272B). Isometric tension developed in the preparation was recorded on a chart recorder (NEC San-ei 8K21, Tokyo, Japan) through a preamplifier (Nihon Kohden SEN 6104). The spontaneously beating right atrium was

suspended in a 20 ml water-jacketed bath containing the Krebs-Henseleit solution, as described above. Spontaneously beating rate was counted on a chart recording of its developed tension. The preparations were allowed to equilibrate for at least 60 min before the commencement of experiments.

Patch-clamp study

Single cells of the guinea-pig atrial tissues were obtained by an enzymatic dissociation method, as previously described (Tohse *et al*., 1992). In brief, the heart was removed from guinea-pigs anaesthetized with pentobarbital sodium and mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation with normal HEPES-Tyrode's solution. The perfused medium was then changed to a nominally Ca^{2+}-free Tyrode's solution and then changed to a solution containing 0.02% wt/vol collagenase (Wako. Osaka, Japan). After digestion, the heart was perfused with high-K^+/low-Cl^- solution (Kraft-brühe (KB) solution). Atrial tissue was cut in small pieces in the KB solution, and the cell suspension was stored in a refrigerator (4°C) for later use. The composition of the normal HEPES-Tyrode's solution was (in mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, and HEPES-NaOH buffer (pH 7.4) 5.0. The composition of KB solution was (in mM): KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 1.0 and HEPES-KOH buffer (pH 7.4) 10.

Whole-cell membrane currents were recorded by conventional ruptured patch or nystatin-perforated patch configuration of the patch clamp method (Hamill *et al*., 1981; Horn & Korn, 1992). The perforated whole-cell voltage clamp mode was used for the recording of Ca^{2+} current in order to avoid 'rundown' of I_{Ca} and to maintain intracellular milieu. On the other hand, the conventional ruptured whole cell voltage clamp mode was mainly used for recording of $I_{K.ACh}$ in order to apply some compounds into the pipette solution. Single atrial cells were placed in a recording chamber (1 ml volume) attached to an inverted microscope (Olympus IX70, Tokyo, Japan) and superfused with the HEPES-Tyrode's solution at a rate of 3 ml min^{-1}. The temperature of the external solution was kept constant at 36.0 ± 1.0°C. Glass patch pipettes with a diameter of 1.5 mm were filled with an internal solution. For the conventional ruptured whole-cell clamp experiments the internal solution containing (in mM) potassium aspartate 110, KCl 20, $MgCl_2$ 1.0, potassium ATP 5.0, potassium phosphocreatinine 5.0, sodium GTP 0.1, EGTA 10, and HEPES-KOH buffer (pH 7.4) 5.0 was used. The free Ca^{2+} concentration in the pipette solutions was adjusted to pCa 8 according to the calculation by Fabiato & Fabiato (1979) with the correction of Tsien & Rink (1980). For nystatin perforated whole-cell clamp experiments, the pipette solution contained (in mM): potassium aspartate 110, KCl 20, $MgCl_2$ 1.0, EGTA 1.0, HEPES-KOH buffer (pH 7.4) 5.0, and added nystatin. Nystatin was dissolved in methanol at a concentration of 10 mg ml^{-1} and added to the pipette solution in concentration of 300–500 μg ml^{-1} just before experiments. The resistance of the patch pipette filled with these pipette solution was 2–3 MΩ.

After the gigaohm seal between tip and cell membrane was formed, the membrane patch was disrupted by applying more negative pressure to make whole-cell voltage clamp mode in the conventional ruptured patch configuration. In the perforated patch configuration, the negative pressure was released to await gradual opening of nystatin-induced pores. All experiments were commenced after gaining stable electrical access. The electrode was connected to a patch clamp amplifier (Nihon Kohden CEZ-2400). Command pulses were generated, and data were acquired by an IBM compatible computer (Fujitsu FM-V, Tokyo, Japan) through a 12-bit AD/DA converter (Digidata 1200 interface, Axon Instruments, Foster City, CA, U.S.A.) controlled by pCLAMP software (Axon Instruments, Foster City, CA, U.S.A.). A liquid junction potential of −8 mV between the pipette solution and the bath solution was corrected.

The $I_{K.ACh}$ was activated by extracellular application of 1 μM carbachol or 10 μM adenosine in atrial cells held at −40 mV, as previously described (Watanabe *et al*., 1996). Effects of bradykinin on the preactivated $I_{K.ACh}$ were examined. To calculate per cent inhibition of $I_{K.ACh}$, the difference between the steady-state current in the solution containing 1 μM carbachol and the current level in the absence of any agonist was taken as 100%.

In part of the experiments, a ramp pulse protocol was used to record the quasi-steady-state membrane current. The membrane potential was held at −40 mV and depolarized first to +50 mV at a rate of 1.2 mV ms^{-1}. It was then repolarized or hyperpolarized to −100 mV with a slope of −1.2 mV ms^{-1}, during which time the change in the membrane current was automatically plotted against the membrane potential. The current-voltage relation was measured during the repolarized or hyperpolarized phase. The ramp voltage pulses were applied at appropriate timing.

The I_{Ca} was evoked by a 300 ms depolarizing pulse to 0 mV from a holding potential −40 mV. The test pulse was repeated at a frequency of once every 20 s. After the stabilization of the depolarization-induced currents, effects of bradykinin on the currents were examined. In a part of experiments, a current-voltage relationship was obtained by delivering 300 ms test pulses to various potentials from a holding potential of −40 mV at 0.1 Hz before and after application of bradykinin.

Current clamp experiments were also performed in the whole-cell recording mode at 36 ± 1°C. External and pipette solutions were the same as those used in the ruptured whole-cell voltage clamp experiments. The cells were stimulated by rectangular 2 ms currents through the pipette at a rate of 0.2 Hz. After stabilization of action potential configuration, effects of bradykinin on action potential were examined.

Drugs

The following compounds were used: bradykinin (Bachem California Inc., CA, U.S.A.), D-arginyl-[$Hyp^3$$Thr^5$,D-$Tic^7$, Oic^8]-bradykinin (HOE140, Peptide Institute Inc., Osaka, Japan), daidzein, tyrphostin 25, d-myo-inositol 1,4,5-triphosphate (IP_3), carbachol, glibenclamide (Sigma Chemical, St. Louice, MO, U.S.A.), atropine sulphate, adenosine, staurosporine, genistein, dl-propranolol hydrochloride, nystatin, calphostin C (Wako, Osaka, Japan), prazosin hydrochloride (Pfizer, Tokyo, Japan). Nystatin were dissolved as described above. Genistein, daidzein, tyrphostin 25, staurosporine and glibenclamide were dissolved in dimethylsulfoxide, and then diluted with suitable buffer solution just prior to experiments. The other compounds were dissolved in distilled water as a stock solution. It was confirmed that the concentration of dimethylsulfoxide used had no influence on the membrane currents and the action potential parameters.

Statistics

All results were expressed as means ± s.e.mean. Student's *t*-test was used for statistical analysis of the paired observations and ANOVA was used to test the difference among the groups. A

value of $P<0.05$ was considered significant. The concentration-effect data were fitted and the EC_{50} or IC_{50} values were obtained by use of Delta Graph Professional (Delta Point, Poloroid computing, Tokyo Japan).

Results

Positive inotropic and chronotropic effects of BK in guinea-pig atria

Effects of BK on developed tension (DT) were examined in atrial preparations treated with propranolol (1 μM), prazosin (1 μM) and atropine (1 μM). As shown in Figure 1, BK at 10 nM and higher concentrations produced an increase in DT in left atrial preparations stimulated at 0.5 Hz. The increase in DT after BK reached its peak level around 3–5 min and then gradually declined in spite of the continued presence of BK. BK in concentrations higher than 100 nM produced an increase in beating rate in isolated right atrial preparations. The increase in beating rate after BK also reached its peak level after several minutes and then gradually decreased. Thus, BK produced positive inotropic and chronotropic responses in the presence of the autonomic blockers.

Effects of BK on membrane currents in guinea-pig atrial cells

In order to evaluate changes of ionic currents underlying the positive inotropic and chronotropic responses, we evaluated the effects of BK on the membrane currents in guinea-pig atrial cells. In preliminary experiments using conventional ruptured patch configuration of the patch clamp method, BK insignificantly increased I_{Ca}. In eight cells BK (1 μM) changed I_{Ca}, elicited by a depolarizing pulse to 0 mV from a holding potential of -40 mV, from 7.24 ± 0.93 pA/pF to 8.67 ± 0.91 pA/pF ($n=10$). Therefore, we examined the effects of BK on I_{Ca} using nystatin-perforated patch configuration of the patch clamp technique. Membrane currents were elicited by test pulses to various potentials from a holding potential of -40 mV. As shown in Figure 2, BK at a concentration of 1 μM consistently increased the peak inward current elicited by depolarizing pulses. BK hardly affected the tail current recorded upon clamp back to the holding potential. A current-voltage relationship is shown in Figure 2. BK at a concentration of 1 μM significantly increased I_{Ca} from 6.46 ± 0.98 pA/pF to 9.51 ± 1.31 pA/pF at 0 mV without affecting its voltage dependence ($P<0.05$, $n=8$).

Time course change of the amplitude of I_{Ca} after 1 μM BK is shown in Figure 3. After the application of BK, I_{Ca} that was elicited by a 300 ms test pulse to 0 mV from a holding potential of -40 mV increased and reached its peak value within a few minutes. Then, I_{Ca} gradually declined toward the control level. The time course change of I_{Ca} was similar to that of DT after BK. BK at a concentration of 1 μM increased the amplitude of I_{Ca} at 0 mV by $28\pm5\%$ ($P<0.05$, $n=14$). A concentration-response curve for the stimulatory effect of BK on I_{Ca} is shown in Figure 3. The EC_{50} value of BK for increasing I_{Ca} was 5.2 nM. The increase in I_{Ca} after 1 μM BK was completely abolished by HOE140 (1 μM), a selective bradykinin B_2 receptor antagonist (Wirth *et al*., 1991) ($n=4$).

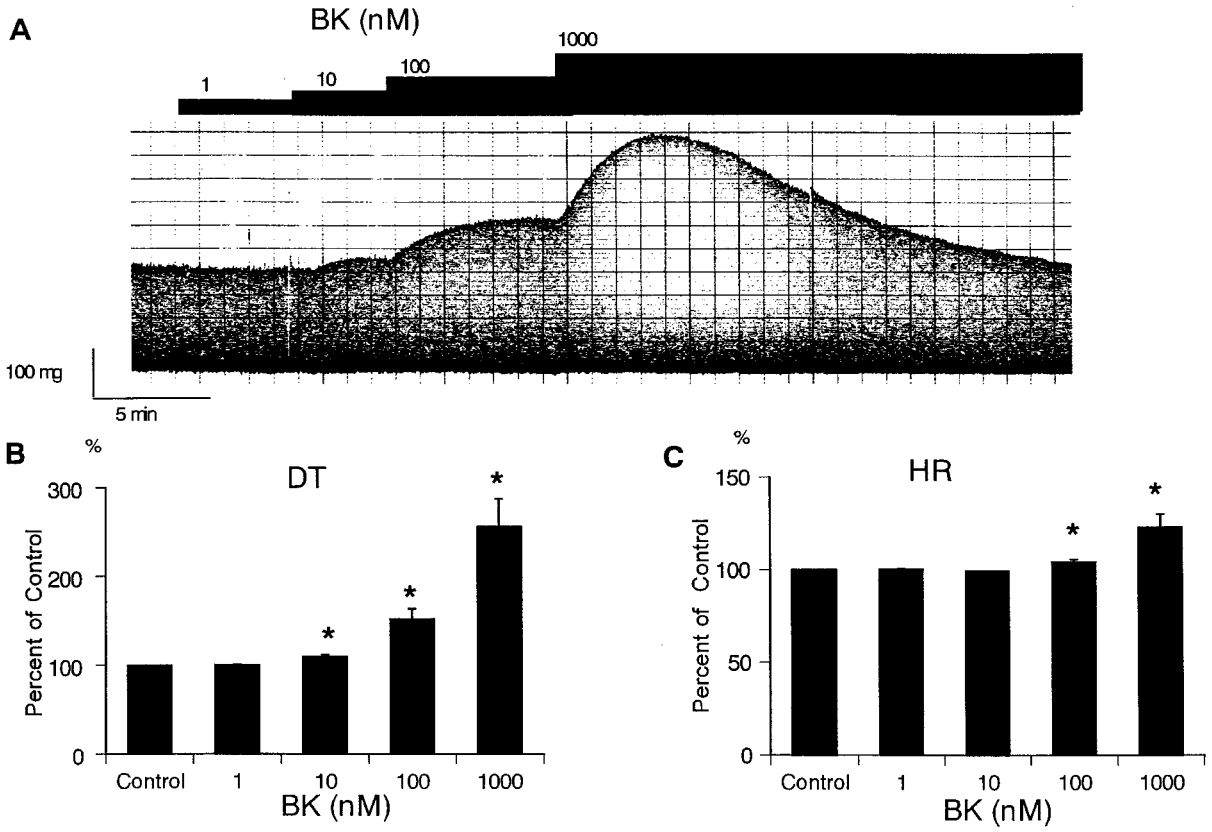

Figure 1 Effects of bradykinin (BK) on developed tension (DT) of left atrium (A,B) and spontaneously beating rate (heart rate, HR) of right atrium (C) of guinea-pigs. A representative change in DT after BK in a left atrial preparation is shown in (A). Responses to BK are expressed as percentage of control values in (B) and (C). Basal developed tension and heart rate were 211 ± 17 mg ($n=6$) and 143 ± 12 beats min^{-1} ($n=5$), respectively. All experiments were performed in the presence of 1 μM propranolol, 1 μM prazosin and 1 μM atropine. *represents a significant change from the control value at $P<0.05$.

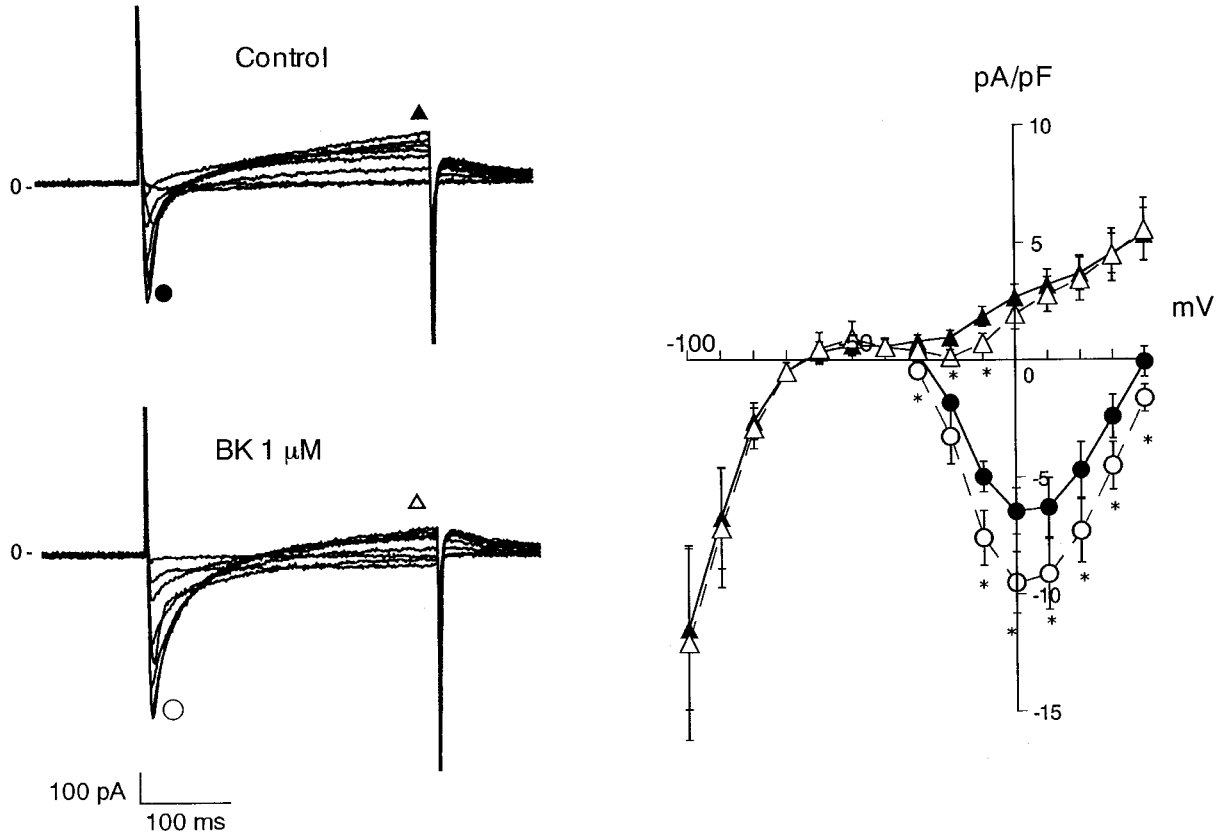

Figure 2 Effects of bradykinin (BK) on membrane currents recorded using nystatin-perforated patch configuration of the patch clamp technique. Actual current traces induced by depolarizing pulses from a holding potential of −40 mV before and after 1 μM BK in a single atrial cell are shown on left. Current-voltage relations for the peak current (circles) and the current at the end of 300 ms test pulse (triangles) in the absence (closed symbols) and presence (open symbols) of BK, obtained from eight cells, are shown on right. *represents a significant change from the control value at $P<0.05$.

In atrial cells held at −40 mV, extracellular application of 1 μM carbachol produced an outward current (Figure 4). Addition of 1 μM BK inhibited the outward current and little recovery was observed upon washout of BK. In order to evaluate the reversal potential of the outward current that was activated by carbachol and inhibited by BK, the quasi-steady-state membrane current was recorded by a ramp pulse protocol of 125 ms from +50 to −100 mV. Carbachol produced a marked increase in the quasi-steady-state outward current at potentials positive to −75 mV and then gradually declined, probably due to desensitization. Addition of BK markedly decreased the steady-state outward current at −40 mV (Figure 4A). However, the reversal potential of the quasi-steady state current elicited by the ramp pulse protocol was not changed and was close to the calculated equilibrium potential of K^+. BK at a concentration of 1 μM inhibited the carbachol (1 μM)-induced $I_{K.ACh}$ by 42.8±2.2% ($n=10$). In a part of experiments, effects of BK on $I_{K.ACh}$, which was recorded using nystatin-perforated patch method, were also evaluated. BK at a concentration of 1 μM inhibited the carbachol (1 μM)-induced $I_{K.ACh}$ by 45.2±10.8% ($n=3$), which was not significantly different from the data obtained in the experiments using the conventional ruptured patch method. In the presence of the B_2-receptor antagonist HOE140 (1 μM), BK failed to inhibit the carbachol-induced $I_{K.ACh}$ (Figure 4B). Although carbachol and adenosine act on different membrane receptors, i.e., muscarinic M_2-receptors and adenosine A_1-receptors, adenosine can also induce $I_{K.ACh}$ through the activation of pertussis toxin-sensitive GTP-binding protein in atrial cells (Kurachi *et al.*, 1986). BK produced almost identical inhibitory effect on the adenosine (10 μM)-induced $I_{K.ACh}$. BK at a concentration of 1 μM inhibited the adenosine-induced $I_{K.ACh}$ by 48.9±9.8% in four cells. In the presence of 10 μM glibenclamide, carbachol at a concentration of 1 μM still increased an outward current. Again BK inhibited the carbachol-induced $I_{K.ACh}$ by 41.9±6.2% in the glibenclamide-treated atrial cells ($n=4$). The concentration-response relationship of the inhibitory effects of BK on the carbachol-induced $I_{K.ACh}$ is summarized in Figure 4C. The maximal inhibition of about 40% was achieved with 100 nM BK, and the EC_{50} value of BK for inhibiting the $I_{K.ACh}$ was 0.57 nM.

Intracellular mechanism of bradykinin B_2 receptor-mediated modulation of Ca^{2+} and K^+ currents

Since BK has been reported to facilitate the phosphatidylinositol (PI) hydrolysis in cardiomyocytes (Minshall *et al.*, 1995), we examined whether production of inositol 1,4,5,-triphosphate (IP_3) or activation of protein kinase C (PKC) was involved in the inhibition of $I_{K.ACh}$. In order to test the involvement of IP_3 in the B_2-receptor-mediated inhibition of $I_{K.ACh}$, we used a pipette solution containing a high concentration (20 μM) of IP_3. We thought that the preactivation of IP_3 pathway may damp the BK-induced $I_{K.ACh}$ inhibition if IP_3 production is prerequisite for the $I_{K.ACh}$ inhibition. Intracellular perfusion of cells with IP_3 failed to affect the BK-induced $I_{K.ACh}$ inhibition. However, staurosporine (30 nM), a PKC inhibitor, significantly attenuated the BK-

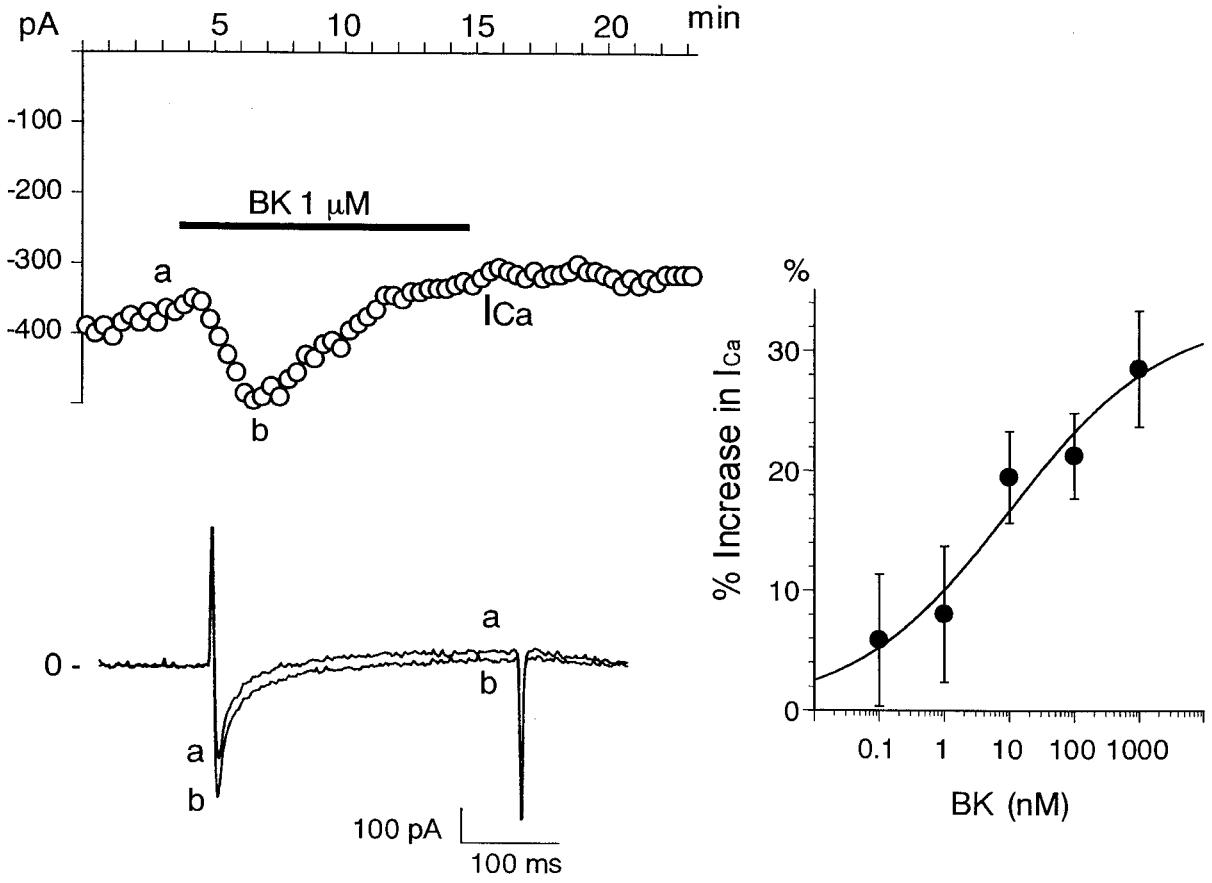

Figure 3 Effects of bradykinin (BK) on the L-type calcium current (I_{Ca}) in guinea-pig atrial cells. Time course change of I_{Ca}, which was elicited by a 300 ms depolarizing test pulse to 0 mV from a holding potential of −40 mV, after 1 μM BK is shown in the upper left and superimposed current tracings obtained at the time point of a and b are depicted in the lower left. A concentration-response curve for increasing effect of BK on I_{Ca} is shown in the right. Values are expressed as means ± s.e.mean of 5–14 experiments.

induced inhibition of $I_{K.ACh}$, as shown in Figure 5. Calphostin C (100 nM), another PKC inhibitor, also abolished the BK-induced inhibition of $I_{K.ACh}$ (Table 1). Recently it has been also reported that BK produces cellular responses via an increase in tyrosine phosphorylation in a variety of cells (Leeb-Lundberg & Song, 1991; Lee *et al.*, 1993; Coutant *et al.*, 1995). Therefore, we tested effects of tyrosine kinase (TK) inhibitors, genistein and tyrphostin, on the BK-induced inhibition of $I_{K.ACh}$ in atrial cells. In the presence of 30 μM genistein or 30 μM tyrphostin, BK (1 μM) failed to inhibit the carbachol-induced $I_{K.ACh}$ (Figure 5 and Table 1). However, daidzein, an inactive analogue of genistein, did not significantly affect the BK-induced inhibition of $I_{K.ACh}$ (Figure 5 and Table 1). These findings suggest that both PKC and TK may be involved in the BK-induced inhibition of $I_{K.ACh}$.

Influences of PKC and TK inhibitors on the BK-induced increase in I_{Ca} were also evaluated in isolated atrial cells. Staurosporine (30 nM) failed to inhibit the BK-induced increase in I_{Ca} which was measured using nystatin perforated patch-clamp techniques (Table 2). In addition, neither genistein (30 μM) nor tyrphostin (30 μM) attenuated the increase in I_{Ca} observed after the application of 1 μM BK.

Effects of BK on the action potential configuration in guinea-pig atrial cells

Figure 6 illustrates the effects of BK (1 μM) on the action potentials recorded in the current clamp mode from single atrial myocytes stimulated at 0.2 Hz. The baseline characteristics of action potentials were resting membrane potential, −72.7 ± 8.9 mV; action potential amplitude, 122.7 ± 2.0 mV; action potential duration at 50% repolarization (APD_{50}), 48.7 ± 2.7 ms; action potential duration at 90% repolarization (APD_{90}), 80.2 ± 2.8 ms ($n = 14$). BK at a concentration of 1μM *per se* increased APD_{50} by 40.6 ± 4.5% in eight cells ($P < 0.05$) (Figure 6). BK also partially reversed the carbachol-induced action potential shortening. Carbachol at a concentration of 0.1μM markedly shortened APD_{50} from 49.1 ± 4.9 ms to 24.4 ± 3.0 ms ($P < 0.05$, $n = 6$). Addition of 1μM BK reversed APD_{50} to 83.7 ± 11.4% of control (Figure 6). Thus, BK prolonged APD in the absence and presence of muscarinic receptor stimulation.

Discussion

Consistent with previous reports (Iven *et al.*, 1980; Nakashima *et al.*, 1982; Tesfamariam *et al.*, 1995), BK produced positive inotropic and chronotropic responses in isolated guinea-pig atria. BK was shown to enhance noradrenaline release from the sympathetic nerve endings through the activation of B_2 receptors (Minshall *et al.*, 1994; Vas-da-Silva *et al.*, 1996). However, these responses were observed even in the presence of autonomic blockers in this study. Recently it has been also reported that the BK binding sites are expressed in adult guinea-pig myocardial membranes (Minshall *et al.*, 1995).

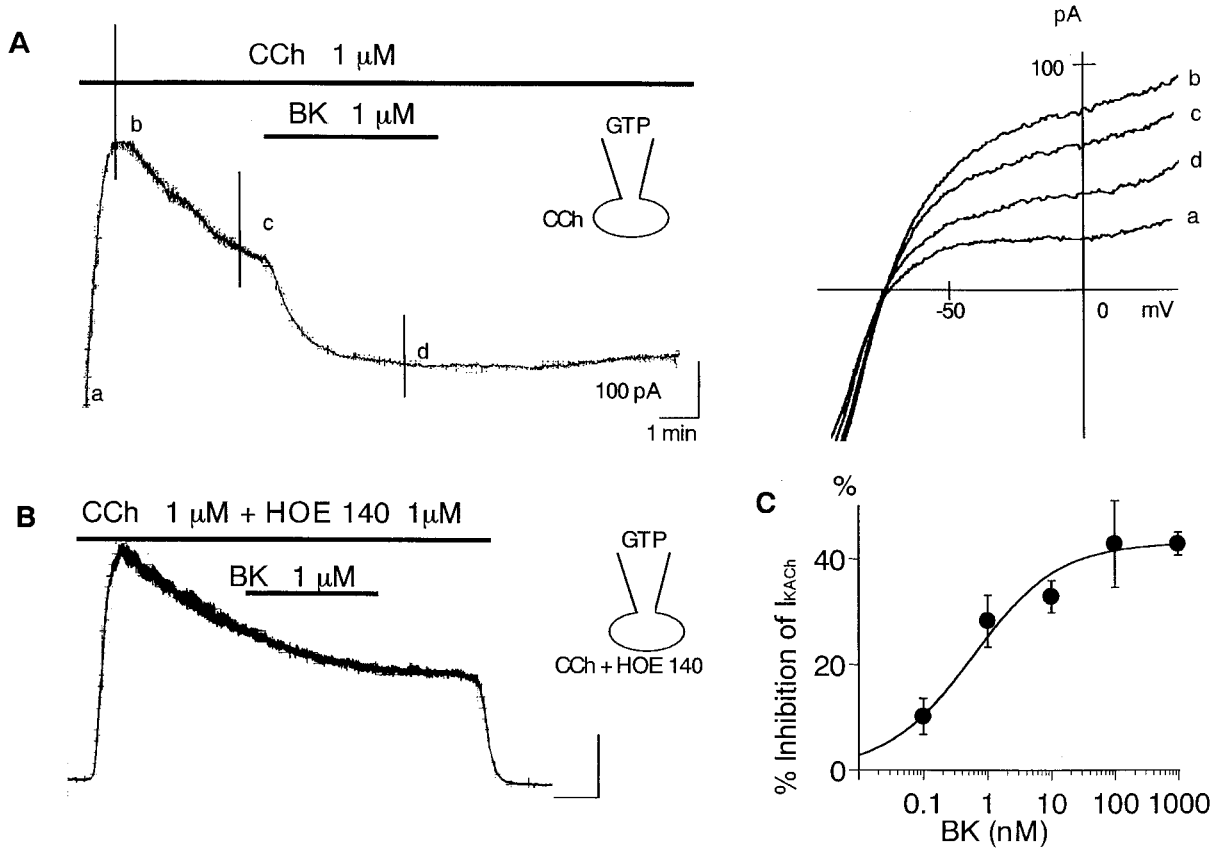

Figure 4 Effects of bradykinin (BK) on the muscarinic acetylcholine receptor-operated K^+ current ($I_{K.ACh}$) in guinea-pig atrial cells. Whole cell membrane currents were recorded using the conventional ruptured patch configuration of the patch clamp technique. $I_{K.ACh}$ was activated by extracellular application of 1 μM carbachol (CCh) in GTP (100 μM)-loaded atrial cells. The cells were held at -40 mV and ramp-voltage pulses were applied with appropriate timing (A). The actual tracings of the quasi-steady-state membrane current, which was obtained at the time points of a, b, c and d, are superimposed in the right of panel A. The BK-induced inhibition of $I_{K.ACh}$ was abolished by the B_2-receptor antagonist HOE140 (1 μM) (B). The horizontal line of time scale indicates the zero current level in (A) and (B). A concentration-response curve for the inhibitory effect of BK on the CCh (1 μM)-induced $I_{K.ACh}$ is shown in (C). Values are expressed as means $\pm$ s.e.mean of five to ten experiments.

Therefore, the positive inotropic and chronotropic responses could be ascribed to the direct action of BK on cardiac cells. It is well known that both I_{Ca} and $I_{K.ACh}$ are important for the contractility of atrial cells and automaticity of sinoatrial nodal cells (Pappano, 1995). Accordingly we thought it would be of importance to evaluate effects of BK on the membrane currents in isolated cardiac cells.

In guinea-pig atrial myocytes BK increased the I_{Ca} recorded in the perforated patch configuration, which was abolished by the B_2-receptor antagonist HOE140. However, in the conventional ruptured patch configuration BK failed to increase I_{Ca} significantly. These findings imply that the B_2-receptor-mediated production of some second messenger(s) in the cytoplasma appear to play an important role in the enhancement of I_{Ca}. In other words, the replacement of intracellular environment by artificial pipette solution might damp some intracellular constituent(s) responsible for the increase in I_{Ca}. The increase in I_{Ca} after BK was transient, which was consonant with the time course changes in DT and beating rate. It is not clear from the present study why the BK-induced increases in I_{Ca} and DT were transient. Desensitization of B_2-receptors and/or diminishment of some important intracellular modulator(s) of L-type Ca^{2+} channels might be involved. The increase in APD, observed in the current clamp experiments of this study, might at least in part stem from the increase in I_{Ca} after BK.

BK inhibited the carbachol- and adenosine-induced $I_{K.ACh}$ in a concentration-dependent manner. The EC_{50} value (0.57 nM) for inhibiting the carbachol (1μM)-induced $I_{K.ACh}$ was about ten times smaller than that for increasing I_{Ca} (5.2 nM), indicating BK modulates $I_{K.ACh}$ more potently than I_{Ca}. The inhibition of $I_{K.ACh}$ by BK was not observed in the presence of HOE140, indicating that the inhibitory effect would be also produced by B_2-receptor stimulation. A recent report indicated that acetylcholine can activate the ATP-sensitive K^+ current ($I_{K.ATP}$) in cat atrial cells (Wang & Lipsius, 1995). Therefore, we examined effects of BK on the carbachol-induced outward current in the presence of the ATP-sensitive K^+ channel blocker glibenclamide. BK still inhibited the outward current to a similar extent in glibenclamide-treated myocytes. In addition, BK also inhibited the adenosine-induced outward current. Therefore, the outward current that was suppressed by BK would be $I_{K.ACh}$. The inhibition of $I_{K.ACh}$ by BK was reflected in the action potential change observed in current clamp experiments. BK reversed the carbachol-induced action potential shortening. In the heart *in situ* parasympathetic nerve is tonically stimulated and the activated $I_{K.ACh}$ may play a role in the regulation of cardiac function such as the sinus node automaticity and the repolarization of atrial action potential. Therefore, it is conceivable that BK may influence the cardiac function by inhibiting $I_{K.ACh}$ in *in situ* heart.

It has been reported that BK stimulates not only phosphoinositide hydrolysis but also protein tyrosine phosphorylation in various types of cells (Minshall *et al*., 1995; Leeb-Lundberg & Song, 1991; Lee *et al*., 1993; Coutant *et al*., 1995). Therefore, we examined effects of several protein kinase C (PKC) and tyrosine kinase (TK) inhibitors on the BK-induced changes in the membrane currents of guinea-pig atrial cells. In the present study, staurosporine abolished the BK-induced inhibition of $I_{K.ACh}$. However, staurosporine is known to inhibit also protein kinase A and TK in its higher concentration (Nakano *et al*., 1987). Therefore, we examined the effects of calphostin C, a more specific PKC inhibitor (Kobayashi *et al*., 1989), on the BK-induced inhibition of $I_{K.ACh}$. Calphostin C also abolished the $I_{K.ACh}$ inhibition by BK, suggesting the involvement of PKC. Furthermore, genistein and tyrphostin, TK inhibitors, also suppressed the $I_{K.ACh}$ inhibition. Thus, not only serine/threonine phosphorylation but also tyrosine phosphorylation may play a role in the inhibition of $I_{K.ACh}$ by BK. Recently it has been demonstrated that stimulation of BK receptor, a G-protein-coupled receptor, activates the non-receptor protein tyrosine kinase PYK2 through the increase in phosphatidylinositol hydrolysis in PC12 cells (Lev *et al*., 1995). In addition, BK has been shown to activate many isoform of PKC including the atypical PKC through the activation of B_2 receptors (Tippmer *et al*., 1994) and to stimulate TK through the PKC-dependent

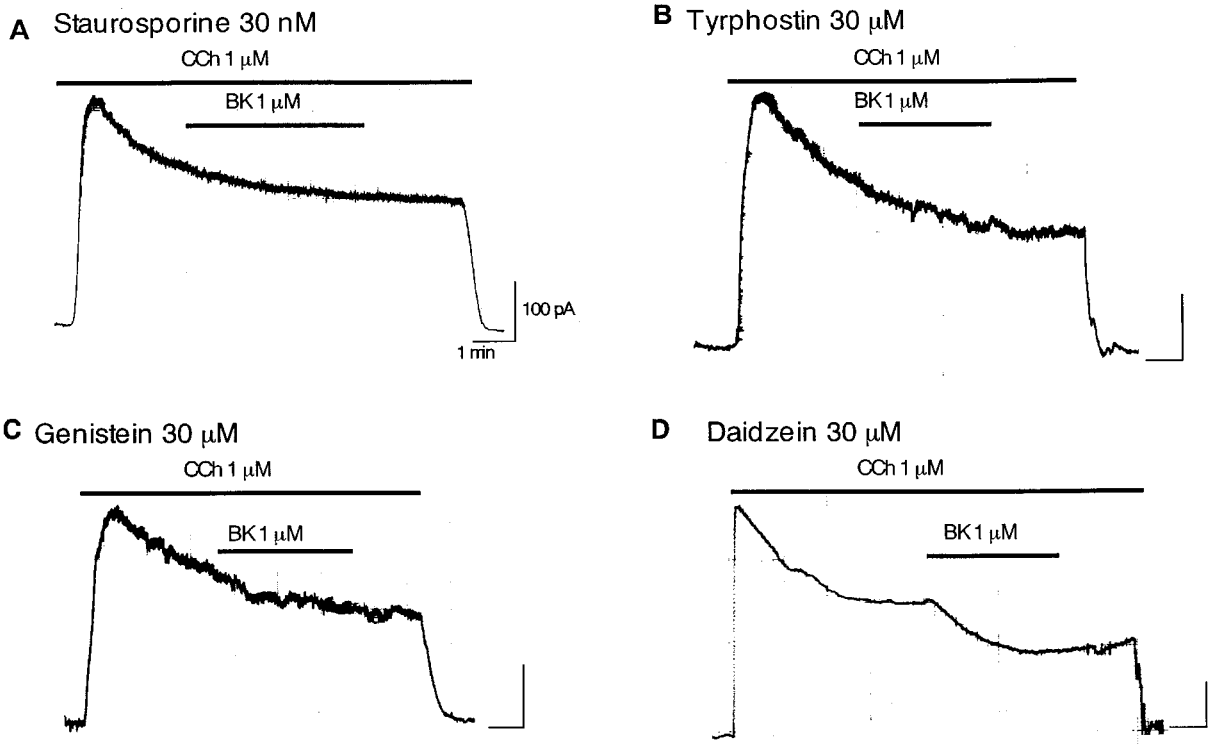

Figure 5 Influences of treatment with staurosporine (A), tyrphostin (B), genistein (C) and daidzein (D) on the inhibitory effects of bradykinin (BK) on the carbachol (CCh)-induced $I_{K.ACh}$. Atrial cells were held at −40 mV. Horizontal bars indicate time scale and zero current level.

Table 1 Effects of various treatments on the bradykinin (BK)-induced inhibition of the muscarinic acetylcholine receptor-operated K^+ current ($I_{K.ACh}$) in guinea-pig atrial cells

	None	*IP_3* (20 μM)	*Staurosporine* (30 nM)	*Calphostin C* (100 nM)	*Genistein* (30 μM)	*Tyrphostin* (30 μM)	*Daidzein* (30 μM)
% Inhibition of $I_{K.ACh}$	42.8±2.2	38.7±2.6	14.7±4.2*	3.1±3.1*	17.5±7.6*	14.6±8.2*	31.3±7.5

BK (1 μM)-induced inhibition of carbachol (1 μM)-induced $I_{K\ ACh}$ is expressed as mean±s.e.mean of five to ten experiments. *$P<0.05$ vs control inhibition (none). IP_3 was included in the pipette solution and other compounds were applied extracellularly.

Table 2 Effects of various treatments on the bradykinin (BK)-induced increase in the L-type Ca^{2+} current (I_{Ca}) in guinea-pig atrial cells

	None	*Staurosporine* (30 nM)	*Genistein* (30 μM)	*Tyrphostin* (30 μM)
% Increase in I_{Ca}	28.5±4.8	28.3±14.9	35.4±7.4	21.0±9.4

BK (1 μM)-induced increase in I_{Ca} is expressed as mean±s.e.mean of 5–14 experiments. *$P<0.05$ vs control increase in I_{Ca} by BK (none). These compounds were applied extracellularly.

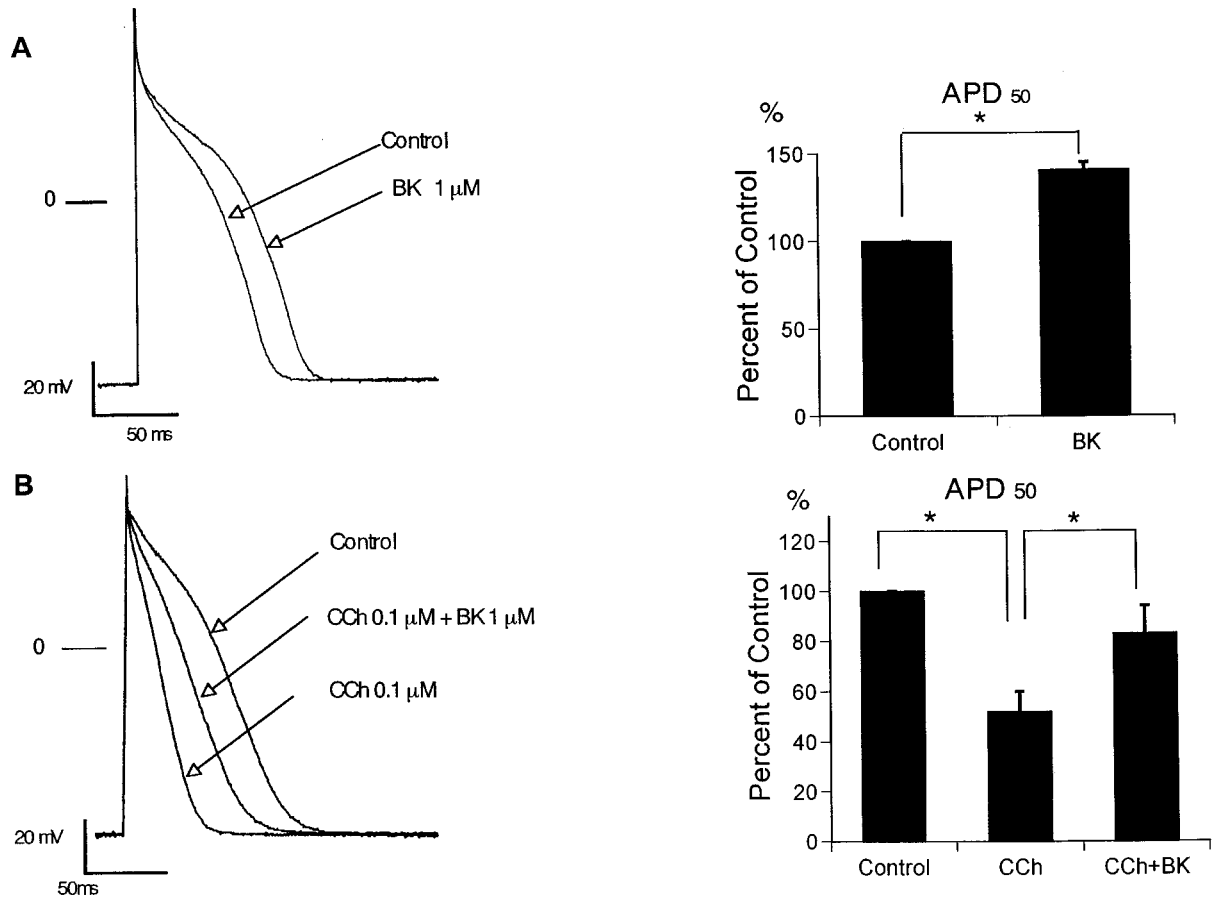

Figure 6 Effects of bradykinin (BK) on the action potential of atrial cells stimulated at 0.2 Hz in the absence (A) and presence of muscarinic receptor stimulation by carbachol (CCh)(B). Actual records of the action potentials are depicted on left part of each panel and summarized data of changes in action potential duration at 50% repolarization level (APD_{50}) are shown on right. Values are expressed as mean ± s.e.mean of eight (A) and six experiments (B). *represents a significant change at $P<0.05$.

mechanism in noncardiac cells (Coutant *et al.*, 1995). Therefore, PKC–TK pathway might be involved in the inhibition of $I_{K.ACh}$ by BK. However, we must be careful in concluding that tyrosine phosphorylation is crucial for the $I_{K.ACh}$ inhibition by BK because TK inhibitors including genistein may have other actions. It has been suggested that genistein may inhibit serine/threonine protein phosphatases and interact with ion channels possibly at a nucleotide binding site (Reenstra *et al.*, 1996; Yang *et al.*, 1997; Weinreich *et al.*, 1997). Further biochemical studies are needed to prove the involvement of TK in the regulation of $I_{K.ACh}$.

Recently, Sorota (1995) has reported that genistein inhibited the swelling-induced Cl^- current ($I_{Cl.swell}$) in canine atrial myocytes, suggesting that tyrosine phosphorylation is necessary for activation of $I_{Cl.swell}$. On the other hand, genistein has been shown to enhance the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in non-cardiac cells (Illek *et al.*, 1995) although some actions other than TK inhibition might be involved (Reenstra *et al.*, 1996; Yang *et al.*, 1997; Weinreich *et al.*, 1997). In terms of K^+ channel regulation by TK, recent several reports have indicated that delayed rectifier type K^+ channels having six transmembrane domains, such as Kv1.2 (RAK) and Kv1.5, are inhibited by the activation of TK (Lev *et al.*, 1995; Huang *et al.*, 1993; Holmes *et al.*, 1996). In addition, BK has been shown to inhibit Kv1.2 channel in non cardiac cells (Lev *et al.*, 1995; Huang *et al.*, 1993). Direct association of the Src tyrosine kinase with Kv1.5 channel at proline rich motif has been also suggested (Holmes *et al.*, 1996). The present study has demonstrated that TK inhibitors abolish the BK-induced inhibition of the muscarinic K^+ channel having putative two transmembrane domains in native cardiac cells.

In contrast to the modulation of $I_{K.ACh}$ by BK, neither PKC inhibitor nor TK inhibitor affected the increase in I_{Ca} after BK. Recently arginine vasopressin was reported to increase L-type Ca^{2+} current through the V_1-receptor-PKC activation pathway in guinea-pig ventricular cells (Hirano *et al.*, 1994). However, staurosporine could not abolish the BK-induced increase in I_{Ca} in the present study. One possible mechanism by which BK increases the L-type Ca^{2+} current may be that an increase in $[Ca^{2+}]_i$ might potentiate I_{Ca}, although we did not measure the $[Ca^{2+}]_i$ change after BK. A moderate increase in $[Ca^{2+}]_i$ can potentiate the L-type Ca^{2+} current through calmodulin-dependent or independent mechanism in cardiac cells (Gurney *et al.*, 1989; Tseng, 1988; Anderson *et al.*, 1994; Yamaoka & Seyama, 1996). The present findings that the enhancement of I_{Ca} could be observed in nystatin-perforated patch method but not in the conventional ruptured patch method with intracellular Ca^{2+} buffering capacity may also support this concept. However, we can not exclude other possibilities from this study and further experimentation is needed to clarify the precise mechanism(s).

Angiotensin-converting enzyme (ACE) inhibitors are recognized as an effective therapy for hypertension as well as heart failure, and are widely used in clinical settings (Jackson & Garrison, 1996). Inhibition of ACE, i.e., kininase II, are

expected to increase BK at least locally. BK was reported to provide cardiac protection during myocardial ischaemia (Goto *et al*., 1995; Brew *et al*., 1995; Wall *et al*., 1994). In addition, BK that is endogeneously generated in ischaemic myocardium may afford protection against life-threatening ischaemia-induced ventricular arrhythmias (Parratt, 1993; Vegh *et al*., 1994). Further studies are needed to evaluate BK effects on other cardiac ion channels including ATP-sensitive K^+ channel.

In conclusion, BK increases I_{Ca} and decreases $I_{K.ACh}$ in atrial cells, and thereby produces direct positive inotropic and chronotropic responses. Protein kinase C activation, and possibly tyrosine kinase activation, may be involved in the B_2 receptor-mediated $I_{K.ACh}$ inhibition.

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