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Diazepam, γ-aminobutyric acid, and progesterone open K^+ channels in myocytes from coronary arteries

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Abstract

Benzodiazepines enhance coronary blood flow and lower blood pressure, but the cellular basis of this action remains unclear. The present study now demonstrates a direct effect of diazepam, γ-aminobutyric acid (GABA), and progesterone on the large conductance, Ca^{2+}- and voltage-activated K^+ channel (BK_{Ca}) in single myocytes isolated from porcine coronary arteries. These GABA receptor agonists significantly increased whole-cell (perforated patch) K^+ currents and stimulated the activity of single BK_{Ca} channels in cell-attached patches dramatically. This effect is not mediated via cyclic AMP or cyclic GMP, but involves stimulation of Ca^{2+} influx in response to activation of a bicuculline-sensitive $GABA_A$-like receptor. We propose that localized, subsarcolemmal increases in Ca^{2+} levels open BK_{Ca} channels, thereby promoting K^+ efflux, membrane repolarization, and coronary relaxation. This transduction pathway can now account, at least in part, for the direct vasodilatory effects of diazepam, progesterone, and GABA.

Keywords: Diazepam; Progesterone; γ-Aminobutyric acid (GABA); K^+ channel; Coronary

1. Introduction

Benzodiazepines are among the most frequently prescribed medications and are commonly used to produce sedation-hypnosis, muscle relaxation, decreased anxiety and anticonvulsant activity. Most clinical effects of benzodiazepines result from action on the central nervous system; however, an interesting peripheral effect of these compounds is vasodilation (Hobbs et al., 1996). Diazepam-induced hypotension was reported nearly 40 years ago (Randall et al., 1961), and more recent studies have confirmed that diazepam and other benzodiazepines lower systemic vascular resistance and blood pressure (Nugent et al., 1982; Raza et al., 1987, 1989). Interestingly, diazepam-induced vasodilation occurs in the presence of autonomic blockade (Daniell, 1975; Cote et al., 1976), suggesting direct effects of diazepam on the vasculature. Because benzodiazepines inhibit contraction even in endothelium-denuded arteries (Gimeno et al., 1994), vascular smooth muscle appears to be a target of benzodiazepine action. Despite the clear evidence that benzodiazepines induce vascular relaxation, no clear understanding of the cellular/molecular basis of how these compounds modulate blood pressure and flow has emerged, nor has a cellular effector molecule that might mediate their direct relaxant effect on vascular smooth muscle been identified.

Diazepam increases coronary blood flow 30–65% (Abel et al., 1970), but no cellular transduction pathway has been suggested. In the present study, we have characterized the effects of diazepam on single myocytes isolated from porcine coronary arteries. The present whole-cell (perforated patch) and single-channel patch–clamp studies now provide direct evidence for a novel diazepam-stimulated effector molecule that could mediate its action on the vasculature: the large conductance, Ca^{2+}- and voltage-activated K^+ (BK_{Ca}) channel. This channel is highly expressed in both human and pig coronary arteries (Toro and Scornik, 1991; Gollasch et al., 1996), and its opening promotes K^+ efflux, membrane repolarization, and vasodilation (Bolton and Beech, 1992). In addition to diazepam, progesterone or γ-aminobutyric acid (GABA) also stimu-

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lated BK_{Ca} channel activity in these myocytes. These findings suggest that stimulation of a peripheral (i.e., vascular) GABA receptor, which has been identified in arterial smooth muscle (Krause et al., 1980), might mediate the vasodilatory effects of benzodiazepines. This novel cellular mechanism could account for the vascular relaxation produced by diazepam, GABA, or progesterone, and might also contribute to diazepam's ability to lower blood pressure (Randall et al., 1961), delay the onset of exercise-induced myocardial ischemia (Rossetti et al., 1994), and increase the latency to ventricular fibrillation following acute myocardial infarction (Pinto et al., 1991).

2. Materials and methods

2.1. Materials

Diazepam, GABA, progesterone, bicuculline, nifedipine, nystatin, amphotericin B, dithiothreitol, papain, and tetraethylammonium (TEA) were purchased from Sigma, St. Louis, MO. Albumin, [1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] (BAPTA), and (8*R*,9*S*,11*S*)-(−)-9-methoxy-9-meth-oxycarbonyl-8-methy 1,2,3,9,10 tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11ariazadibenzo(*a*,*g*)cycloocta(*cde*)-trinden-1-one (KT5823) were purchased from Calbiochem, San Diego, CA.

2.2. Methods

2.2.1. Cell isolation

Fresh porcine hearts were obtained from local abattoirs. The smooth muscle layer of the left anterior descending coronary artery was isolated by dissecting away the adventitia and removing the endothelium by rubbing the intimal surface of the artery, and single myocytes were isolated by a modification of a procedure described previously (White et al., 1995). Approximately 7 cm of the tunica media was cut into 1 mm strips and placed in test tubes containing a low Ca^{2+} dissociation medium of the following composition (mM): 110 NaCl, 5 KCl, 0.16 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 glucose, 0.49 EDTA, and 10 taurine at a pH of 6.9. Media strips were incubated at 37°C in 5 ml of the above solution with 2.5 mg dithiothreitol, 5 mg papain, and 0.2% bovine serum albumin. After 30 min of gentle shaking, the digested tissue was triturated and the enzyme activity was diluted by adding excess enzyme-free solution. The solution was then removed and centrifuged at low speed for 20 min. The pellet was then removed and stored at 4°C in the dissociation medium. Experiments were performed within 9 h of dissociation.

2.2.2. Patch–clamp studies

For perforated patch experiments, several drops of cell suspension were placed in a recording chamber (Warner Instruments) containing a standard bath solution of the following composition (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 30 glucose (pH 7.4; 22–25°C). To measure K^+ currents the tip of the patch pipette (1–3 MΩ) was filled with a solution containing (mM): 60 K_2SO_4, 30 KCl, 5 $MgCl_2$, 5 $CaCl_2$, 5 HEPES and 40 $MgSO_4$ (pH 7.4). Because divalent ions do not pass through pores in the perforated membrane, the high Ca^{2+} in the pipette solution does not enter the cell, thus obviating the need for artificial Ca^{2+} buffers. In contrast, the K^+ channels were always exposed to physiological levels of Ca^{2+} (bath solution) in these whole-cell recording experiments and not the 5 mM of the pipette solution. The remainder of the pipette was back-filled with the same solution to which 6 mg/ml amphotericin B or nystatin (diluted by sonication from a 50 mg/ml stock in dimethylsulphoxide) was added. Voltage–clamp and voltage pulse generation were controlled with an Axopatch 200 A patch–clamp amplifier (Axon Instruments), and data were acquired and analyzed with pCLAMP 6.0.4 (Axon Instruments). Voltage-activated currents were filtered at 1 kHz and digitized at 10 kHz. Leakage currents were algorithmically subtracted using short duration, small amplitude negative prepulses. Single K^+ channels were measured in cell-attached patches by filling the patch pipette (2–5 MΩ) with the standard bath solution minus glucose, and making a gigaohm seal on a single myocyte. Voltage across the patch was controlled by setting the cellular membrane potential to 0 mV using a high K^+ extracellular solution (mM): 140 KCl, 10 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, and 30 glucose (pH 7.4). Currents were filtered at 1 kHz and digitized at 10 kHz. Average channel activity in patches with multiple BK_{Ca} channels was measured as mean open probability (NPo), as described previously (White et al., 1995). In experiments recording K^+ channel activity of inside-out patches, the bathing solution exposed to the cytoplasmic surface of the membrane consisted of the following (mM): 60 K_2SO_4, 30 KCl, 2 $MgCl_2$, 0.16 $CaCl_2$, 10 HEPES, 5 ATP, 1 (BAPTA) and 10 glucose (pH 7.4; 22–25°C). The pipette solution was the same Ringer solution described above. For recording of single Ca^{2+} channels, we employed the standard bath solution described above and a pipette solution of the following composition (mM): 90 $BaCl_2$; 20 HEPES; 10 TEACl (pH 7.4). Both whole-cell and single BK_{Ca} channel activity were observed over a range of membrane potentials. For statistical purposes and to insure accuracy, we routinely adopt the common convention of calculating NPo for single BK_{Ca} channels at a potential where channel openings can be clearly differentiated from other channel species. For example, at +40 mV, there is no other ion channel expressed in these cells with an amplitude of 7–9 pA, making statistical analysis of BK_{Ca} channel activity more accurate. Moreover, recording single-channel activity at more depolarized potentials helps somewhat offset the artificial depression of activity we would otherwise encounter from recording channel activity at room tempera-

ture (22–25°C). We have recently published a more detailed discussion of the limitations and minor caveats of these recording conditions under these same experimental conditions (Han et al., 1999). Whole-cell current–voltage relationships are representative curves.

2.2.3. Statistics

All data were expressed as the mean value ± standard error (S.E.M.). Statistical significance between two groups was determined by Student's *t*-test for paired data. Comparison between multiple groups was made by a multiple measures analysis of variance test, with a post-hoc Tukey's test to determine significant differences among the data groups. *N* values represent the number of cells studied. A probability of less than 0.05 was considered to indicate a significant difference.

3. Results

3.1. Diazepam increases outward currents of myocytes isolated from coronary arteries

Initial studies were performed to characterize the effects of diazepam on ionic currents in single myocytes from porcine coronary arteries. Instead of traditional whole-cell recordings, which dialyze the cytoplasm and require exogenous Ca^{2+} buffers, we employed the perforated patch configuration to obtain whole-cell currents from "metabolically-intact" myocytes. Coronary myocytes exhibit prominent outward currents due to K^+ channel activity (White et al., 1995; Darkow et al., 1997). As illustrated in Fig. 1A, diazepam increased these outward currents substantially. For example, steady-state outward current was increased by an average of 76.8 ± 3% at +40 mV ($n = 4$; $p = 0.02$) 10 min after addition of 10 μM diazepam to the bath solution. Moreover, this effect of diazepam was observed at all voltages where outward current was elicited, and the increase in current was statistically significant at voltages of −20 mV ($p < 0.01$; $n = 4$) and at more depolarized potentials, although the effect was also nearly significant at −30 mV as well ($p = 0.052$). A complete current–voltage relationship for steady-state outward current in another coronary myocyte before and after 10 μM diazepam is illustrated in Fig. 1B. These results demonstrate that diazepam stimulates outward currents in coronary myocytes, and subsequent studies were done to identify the specific channel involved.

3.2. Diazepam stimulates the activity of BK_{Ca} channels in coronary myocytes

Our previous pharmacological studies on whole-cell currents from these same cells excluded significant involvement of K_{ATP} or delayed rectifier K^+ channels in these cells under normal conditions (White et al., 1995). In the present study, whole-cell currents were attenuated by either 1 mM TEA (Fig. 2A) or charybdotoxin (Fig. 2B), both of which exhibit selectivity for BK_{Ca} channels at these concentrations. TEA (1 mM) inhibited outward current at all voltages (e.g., 85 ± 11% at +40 mV; $n = 4$), as did charybdotoxin (30 nM; 48 ± 9% at +40 mV; $n = 3$). In addition, studies on cell-free (inside-out patches) revealed that at the molecular level membrane electrical activity was dominated by a single species of large-conductance channel (> 150 pS in symmetrical K^+ gradients). In cell-attached patches, the activity of this channel was limited but excision of the patch into the inside-out configuration dramatically enhanced channel opening when "intracellular" [Ca^{2+}] was increased to 100 μM (Fig. 2C). This increase in "intracellular" Ca^{2+} was employed only as a positive control, and was done simply to verify the

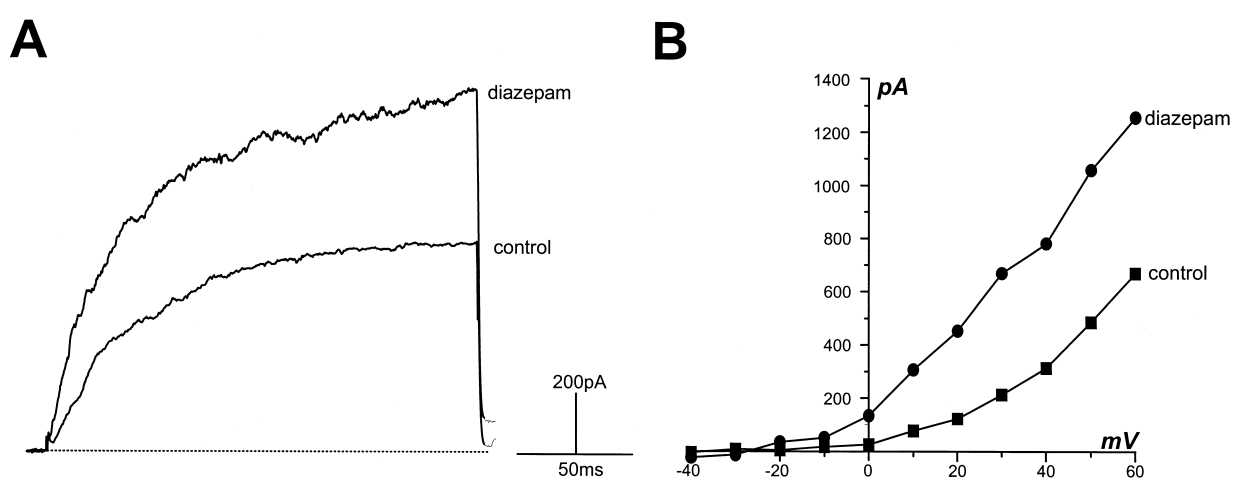

Fig. 1. Diazepam increases K^+ currents in single myocytes from coronary arteries. (A) Steady-state outward currents (+40 mV; holding potential −60 mV) before and after a 10-min exposure to 10 μM diazepam. Broken line indicates baseline current. (B) Complete current (pA)–voltage (mV) relationship for steady-state outward current of another cell before and 10 min after exposure to 10 μM diazepam (holding potential −60 mV).

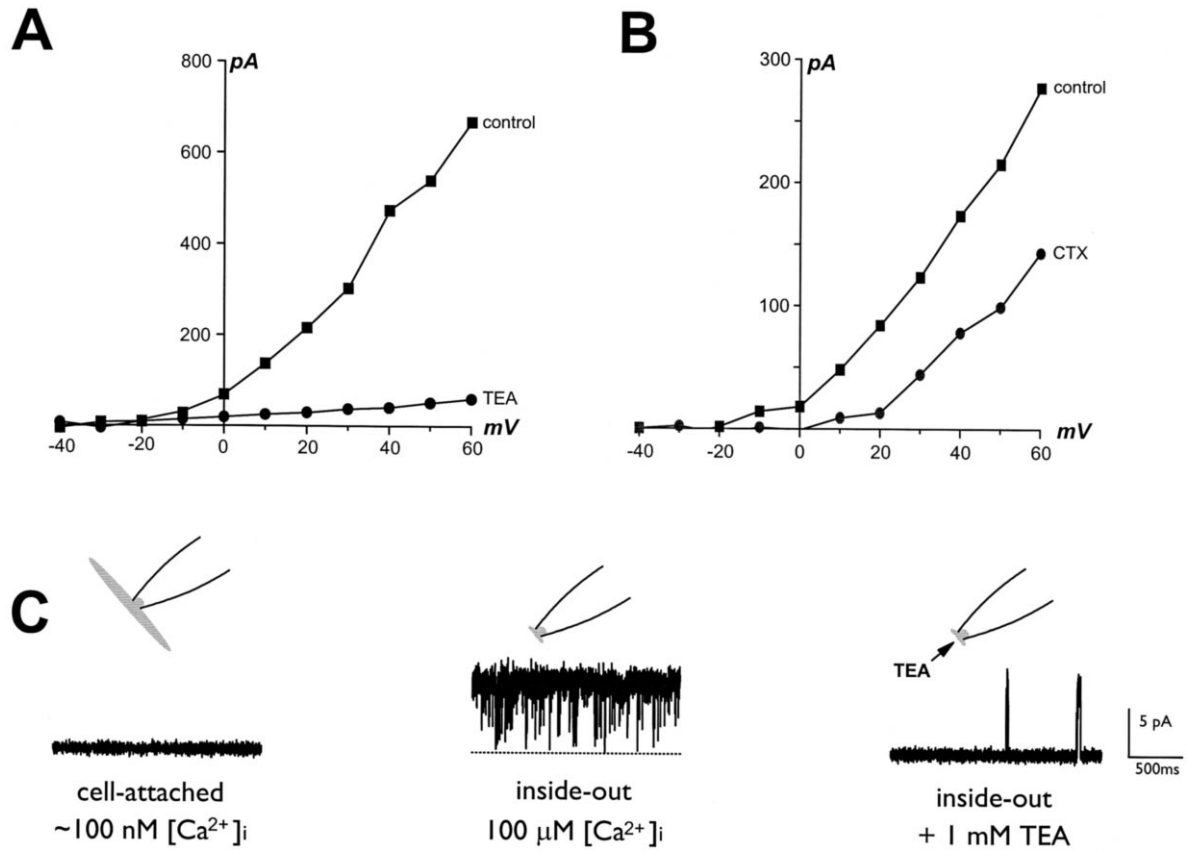

Fig. 2. Whole-cell currents from coronary myocytes are conducted primarily through BK_{Ca} channels. (A) Complete current (pA)–voltage (mV) relationship for steady-state outward currents recorded from a myocyte (holding potential −60 mV) before and 15 min after 1 mM TEA. (B) Complete current (pA)–voltage (mV) relationship for steady-state outward currents recorded from a myocyte (holding potential −60 mV) before and 15 min after 30 nM charybdotoxin (CTX). (C) Representative recordings from the same membrane patch (+40 mV) in the cell-attached configuration (left panel) and after excision into the inside-out configuration (100 μM $[Ca^{2+}]$). TEA (1 mM) reversed Ca^{2+}-stimulated channel activity (right panel). Channel openings are upward deflections from the baseline (closed) state (dashed line).

presence of BK_{Ca} channels in the patch. Furthermore, application of 1 mM TEA inhibited Ca^{2+}-induced activation of this channel. These experiments clearly identify the BK_{Ca} channel as the predominant K^+ channel expressed in pig coronary myocytes, and are consistent with previous studies on these same cells (Toro and Scornik, 1991; White et al., 1995) and human coronary arteries (Gollasch et al., 1996). No other channel expressed in these cells exhibits this specific conductance, amplitude, or Ca^{2+} sensitivity, thus making identification of the BK_{Ca} channels in these experiments unequivocal. These whole-cell studies strongly suggested diazepam increased outward currents by stimulating BK_{Ca} channels, and single-channel experiments were performed to identify the target of diazepam directly. Addition of diazepam to the pipette (extracellular) solution produced a dramatic stimulation of single-channel activity, as illustrated in Fig. 3. Channel open probability (NPo) was near 0 under control conditions; but when patches were exposed to 10 μM diazepam, NPo was 0.305 ± 0.12 (+40 mV; $n = 4$; $p < 0.04$). Stimulation of this channel promotes relaxation of vascular and other types of smooth muscle, and further implicates the BK_{Ca} channel as a likely effector molecule that could mediate diazepam-induced vascular relaxation.

3.3. Other GABA receptor agonists also open BK_{Ca} channels in coronary myocytes

The next series of experiments employed the BK_{Ca} channel as a sensitive molecular assay to further characterize the signal transduction mechanism of diazepam action in coronary myocytes. To our knowledge, no previous studies have demonstrated direct molecular evidence for a likely mechanism to explain benzodiazepine-induced coronary vasodilation; however, diazepam is known to bind and activate GABA receptors in the central nervous system. Therefore, we tested the possibility that a vascular GABA receptor might be involved in the response of coronary arteries to diazepam. Interestingly, application of GABA mimicked the effect of diazepam on outward currents (Fig. 4A). Perforated patch recordings demonstrated that addition of 500 nM GABA increased whole-cell currents over a range of membrane voltages. For example, at

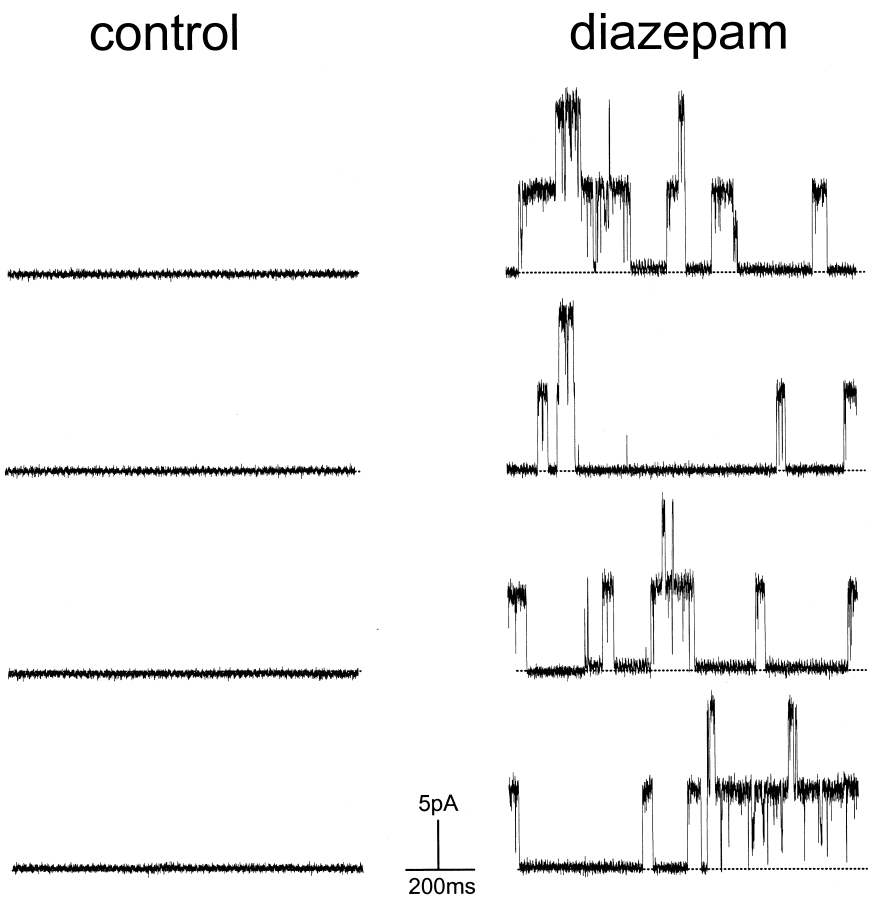

Fig. 3. Diazepam stimulates the activity of single BK_{Ca} channels in coronary myocytes. Control currents (left panel) are continuous 1-s recordings from a cell-attached patch at +40 mV without diazepam. Right panel consists of continuous 1-s recordings from another cell-attached patch at +40 mV with 10 μM diazepam included in the pipette solution. Channel openings are upward deflections from the baseline (closed) state (dashed line).

+40 mV GABA increased current amplitude from 341 ± 43 to 1026 ± 115 pA, for an average increase of 214 ± 30% ($n = 9$; $p < 0.0001$). In addition, GABA also stimulated the activity of single BK_{Ca} channels in cell-attached patches (Fig. 4B). On average, 500 nM GABA increased channel NPo from approximately 0 to 0.444 ± 0.12 (+40 mV, $n = 4$, $p < 0.04$). In addition to GABA, another GABA receptor agonist, β-progesterone, also stimulated outward currents in coronary myocytes (Fig. 4C). On average, 1 μM β-progesterone increased steady-state currents by 48 ± 8%, from 342 ± 53 to 495 ± 53 pA at +40 mV ($n = 4$; $p = 0.0001$). These findings now provide a physiological correlate for previous biochemical studies demonstrating expression of a GABA receptor in arterial smooth muscle (Krause et al., 1980).

3.4. Effect of GABA receptor agonists on BK_{Ca} channels is bicuculline-sensitive, but does not involve cyclic nucleotides

Subsequent experiments characterized the signaling mechanisms stimulated by GABA receptor agonists. Previous studies from our laboratory and others have demonstrated that the stimulatory effect of cyclic GMP-dependent phosphorylation on BK_{Ca} channel activity in arterial smooth muscle and other cells (White et al., 1993, 2000; Archer et al., 1994; Darkow et al., 1997). To test for possible involvement of cyclic nucleotides, single-channel studies were performed on cell-attached patches. As observed with diazepam and GABA, β-progesterone stimulated the activity of single BK_{Ca} channels (Fig. 4D). On average, 1 μM β-progesterone increased channel NPo by over two orders of magnitude (0.002 ± 0.001, control; 0.382 ± 0.080, β-PG; $n = 11$; $p = 0.0008$). Therefore, all three GABA agonists produced a similar stimulatory effect on single BK_{Ca} channels; however, this response does not appear to involve cyclic GMP-dependent phosphorylation. As illustrated by the channel activity plot (Fig. 4D), inhibition of the cyclic GMP-dependent protein kinase with 300 nM KT5823 had no effect on agonist-induced channel activity ($n = 4$). Because we have previously demonstrated that KT5823 is a powerful inhibitor of channel activity induced by either cyclic GMP- or cyclic AMP-producing agents (Han et al., 1999; White et al., 2000), these experiments strongly suggest that potential involvement of these second messengers is unlikely. In contrast, addition of

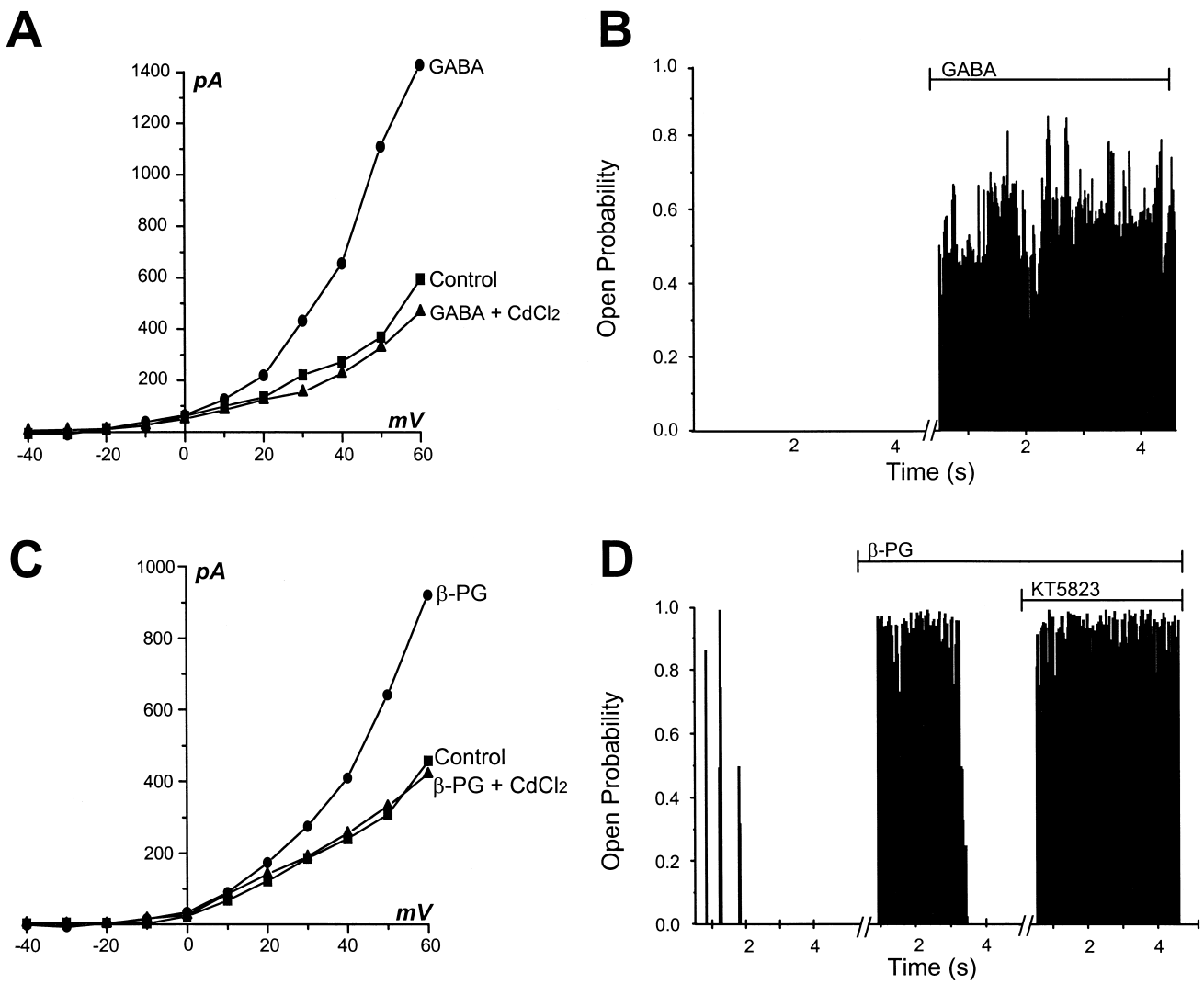

Fig. 4. GABA and progesterone stimulate BK_{Ca} channel activity by a mechanism involving Ca^{2+} influx, but not the cyclic GMP-dependent protein kinase. (A) Complete current (pA)–voltage (mV) relationship for steady-state outward current from a myocyte before and 10 min after exposure to 500 nM GABA, and then after subsequent exposure to 1 mM $CdCl_2$ (holding potential −60 mV). (B) Activity plot of BK_{Ca} channel open probability in a cell-attached patch immediately after obtaining a gigaohm seal, and 10 min later in the presence of 500 nM GABA. Channel open probability (NPo) was calculated for each of a series of 100 ms pulses to +40 mV, and plotted as a vertical bar on the activity histogram. Total recording time under each condition was 5 s, as indicated on the time axis. The breaks in the time axis represent the 10 min allowed for GABA to diffuse into the tip of the pipette and activate the channel. Period of drug exposure is indicated by the horizontal line above the histogram. (C) Complete current (pA)–voltage (mV) relationship for steady-state outward current from a myocyte before and 20 min after exposure to 1 μM β-progesterone (β-PG), and then after subsequent exposure to 1 mM $CdCl_2$ (holding potential −60 mV). (D) Activity plot of BK_{Ca} channel open probability in a cell-attached patch before and 20 min after 1 μM β-progesterone, and then in the presence of 300 nM KT5823. Channel activity (NPo) was plotted as in (B). The breaks in the time axis represent 20 min incubation periods with either β-progesterone or β-progesterone + KT5823.

1 μM bicuculline to the pipette solution prevented any stimulatory effect of β-progesterone even after 60 min ($n = 4$; data not shown).

3.5. Ca^{2+} channel antagonists inhibit the effect of diazepam or GABA receptor agonists on BK_{Ca} channels

Previous studies of non-muscle cells demonstrated that progesterone activates a bicuculline-sensitive GABA receptor to stimulate Ca^{2+} influx (Shi and Roldan, 1995), suggesting involvement of Ca^{2+} channels in response to GABA receptor agonists. We tested the hypothesis that a similar Ca^{2+}-dependent mechanism might exist in coronary smooth muscle. Interestingly, inhibiting Ca^{2+} channel activity with either 1 mM $CdCl_2$ (Fig. 4C; $n = 4$) or 100 nM nifedipine (data not shown, $n = 4$) abolished the stimulatory effect of β-progesterone on K^+ currents. In addition, Ca^{2+} antagonists completely reversed the stimulatory effect of 500 nM GABA ($n = 4$; $p < 0.001$; Fig. 4A); however, $CdCl_2$ did not reduce currents below control

levels. Similarly, pretreatment with $CdCl_2$ completely prevented the stimulatory effect of 500 nM GABA ($n = 3$, Fig. 5A). At the molecular level, addition of $CdCl_2$ to the extracellular solution inhibited the stimulatory effect of GABA ($n = 4$; Fig. 5B) or diazepam ($n = 3$; Fig. 5C) completely. As a positive control to verify the presence of BK_{Ca} channels in these experiments, patches were excised into an inside-out configuration where the "cytoplasmic" $[Ca^{2+}]$ was increased to 0.1 mM. As observed before (Fig. 2C), increasing $[Ca^{2+}]$ at the cytoplasmic surface of the patch stimulated BK_{Ca} channel activity dramatically (in the presence of extracellular cadmium). Therefore, $CdCl_2$ does not inhibit BK_{Ca} channel activity by binding directly to the channel complex, but most probably works indirectly via inhibition of Ca^{2+} influx. These findings strongly suggested that the response to GABA agonists involved opening of Ca^{2+} channels, and recordings from cell-attached patches suggested that GABA agonists do indeed increase Ca^{2+} channel activity. As illustrated in Fig. 6, application of 1 μM β-progesterone (30 min) stimulated the activity of single Ca^{2+} channels (current carried by 90 mM Ba^{2+}). Ca^{2+} channel activity increased from an NPo

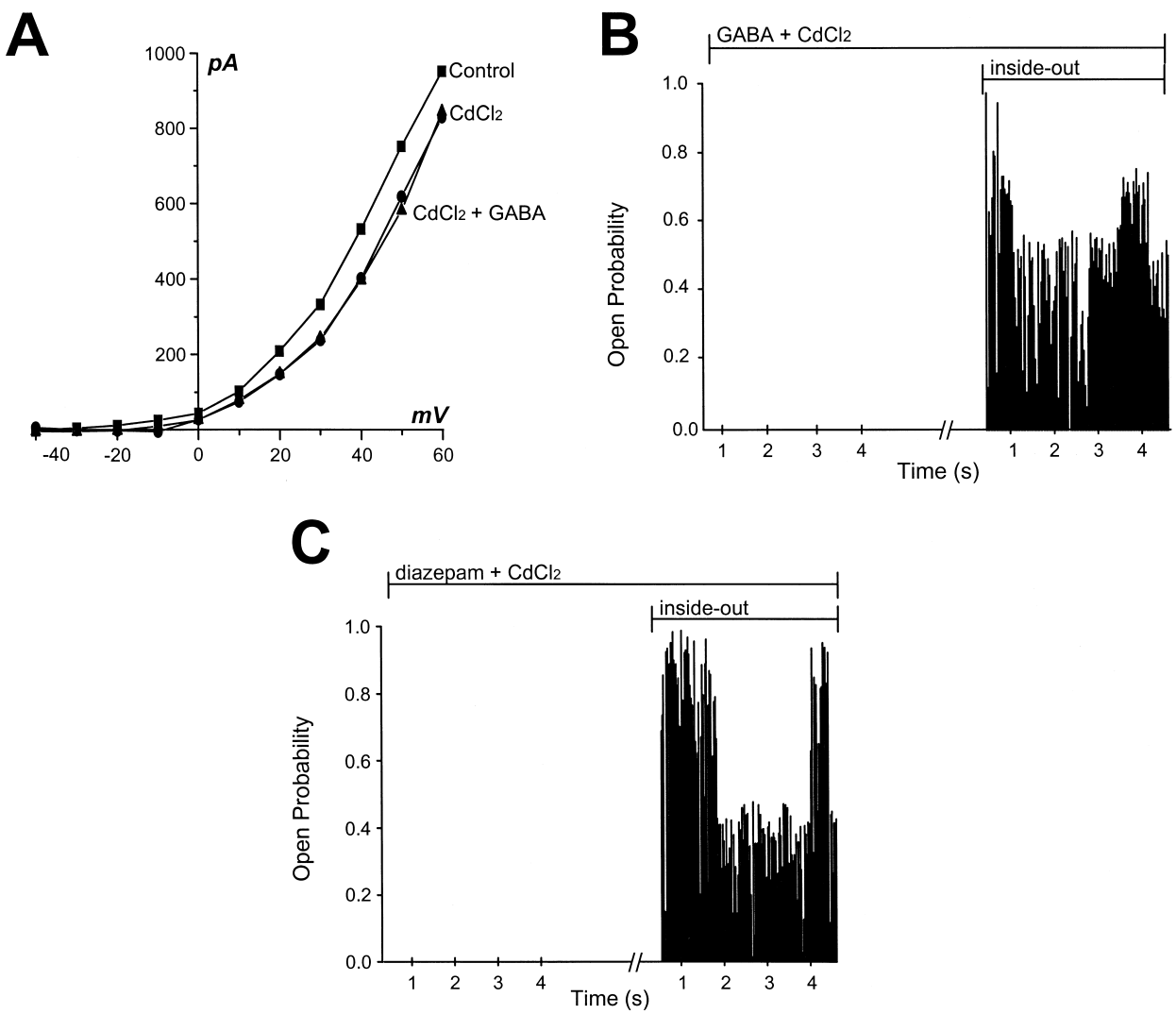

Fig. 5. $CdCl_2$ does not block BK_{Ca} channels directly, but inhibits the effect of GABA or diazepam. (A) Complete current (pA)–voltage (mV) relationship for steady-state outward current of a myocyte before and 10 min after exposure to 1 mM $CdCl_2$, and after subsequent exposure to 500 nM GABA in the presence of $CdCl_2$ (holding current −60 mV). (B) Activity plot of BK_{Ca} channel open probability in a cell-attached patch with 1 mM $CdCl_2$ and 500 nM GABA included in the pipette (extracellular) solution. After 20 min of no channel activity, patch was excised into an inside-out configuration exposing the cytoplasmic face of the patch to 0.1 mM $[Ca^{2+}]$. Total recording time under each condition was 5 s, as indicated on the time axis. The break in the time axis represents excision of the patch during which activity was not recorded. Periods of drug exposure and patch configuration are indicated by the horizontal lines above the histogram. (C) Activity plot of BK_{Ca} channel open probability in a cell-attached patch with 1 mM $CdCl_2$ and 10 μM diazepam included in the pipette (extracellular) solution. After 20 min of no channel activity, patch was excised into an inside-out configuration exposing the cytoplasmic face of the patch to 0.1 mM $[Ca^{2+}]$.

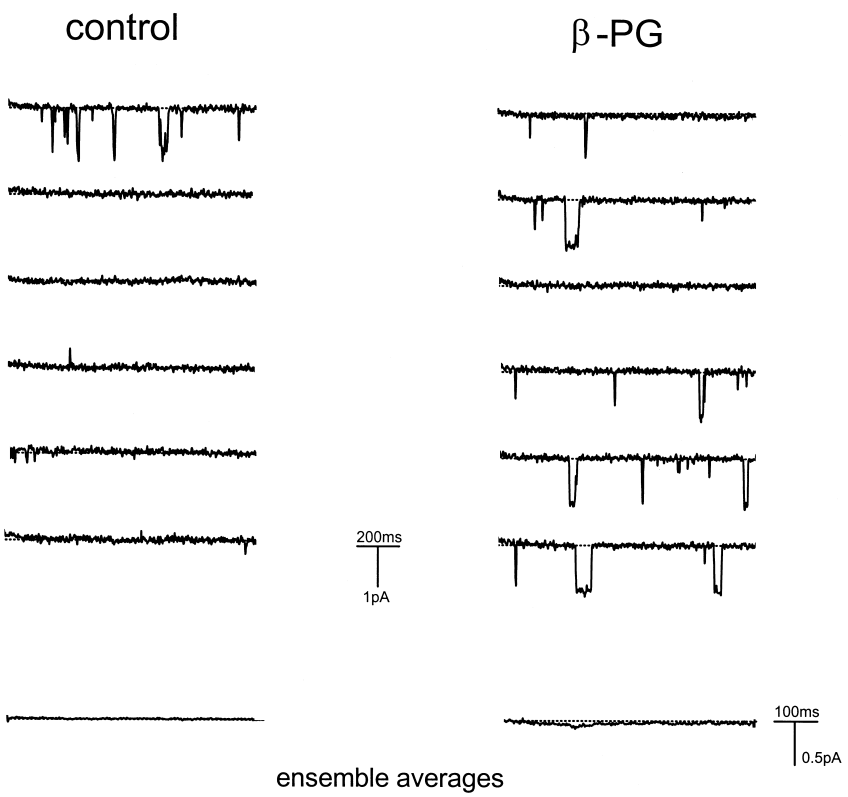

Fig. 6. Progesterone increases activity of single Ca^{2+} channels in coronary myocytes. Representative traces from the same cell-attached patch (+20 mV) before and 30 min after exposure to 1 μM β-progesterone. Channel openings are downward deflections from the baseline (closed) state (dashed lines). Current was carried by 90 mM Ba^{2+}. The ensemble average of 100 individual traces under each condition is illustrated below each set of traces.

of 0.001 ± 0.001 to 0.003 ± 0.003 ($n = 2$; +20 mV) after β-progesterone.

4. Discussion

Only two clinical effects of benzodiazepines appear to result from action outside the central nervous system: neuromuscular blockade at very high doses and coronary vasodilation at therapeutic (micromolar) doses (Hobbs et al., 1996); however, the cellular basis of benzodiazepine-induced relaxation of vascular smooth muscle remains speculative. It has been generally assumed that coronary vasodilation produced by diazepam was indirect, and possibly related to accumulation of adenosine due to inhibition of uptake or nucleoside transport (Barker and Clanachan, 1982). Surprisingly, no direct effects of diazepam on single coronary smooth muscle cells have been reported. In contrast, the present findings now demonstrate a molecular mechanism that could mediate a direct vasodilatory effect of benzodiazepines on the coronary circulation: opening of BK_{Ca} channels. These channels are highly expressed in coronary and other arteries (Gollasch et al., 1996), and are molecular targets for a number of important endogenous and exogenous vasodilators, such as nitric oxide/nitrovasodilators (Carrier et al., 1997), steroid hormones (White et al., 1995; Rosenfeld et al., 2000), catecholamines (Kume et al., 1994; Han et al., 1999; White et al., 2000), oxidants (Barlow and White, 1998), and arachidonic acid metabolites (Barlow et al., 2000). Furthermore, the stimulatory effect of diazepam, GABA, or progesterone on these channels appears to be mediated via a bicuculline-sensitive GABA receptor expressed in coronary myocytes. Interestingly, inhibition of Ca^{2+} influx with either nifedipine or $CdCl_2$ abolished the effect of GABA receptor agonists on BK_{Ca} channel activity (with neither agent inhibiting BK_{Ca} channels directly). Taken together, these findings have identified a novel molecular effector, the BK_{Ca} channel that could contribute to the vascular effects of benzodiazepines, GABA, or progesterone. Furthermore, pharmacological and direct patch–clamp evidence suggests that GABA receptor-stimulated Ca^{2+} influx may be the physiological trigger, which stimulates BK_{Ca} channel activity in response to these agents.

Forty years have passed since it was discovered that parenteral administration of GABA lowers blood pressure in animals and humans (Takahashi et al., 1955; Elliot and Hobbiger, 1959), suggesting the presence of functional GABA receptors in the cardiovascular system. More recent radioligand binding studies employing [^{3}H]muscimol, a high affinity probe for the $GABA_A$ receptor, have identified binding sites in cerebral blood vessels (Krause et al., 1980) and intestinal smooth muscle (Napoleone et al., 1991). Functional studies have demonstrated GABA-in-

duced relaxation of cerebral arteries in vitro (Fujiwara et al., 1975). Other studies report GABA- and muscimol-induced suppression of arterial smooth muscle cell contraction, and these responses were inhibited by bicuculline, a selective $GABA_A$ antagonist (Shirakawa et al., 1989). These biochemical and physiological studies argue strongly for $GABA_A$ receptor-mediated relaxation of vascular smooth muscle; however, the cellular basis for this response is incompletely understood. Findings from the present study provide direct evidence for a cellular effector that could mediate this response, as GABA increased both whole-cell K^+ current and single BK_{Ca} channel activity in single coronary myocytes. Although we did not measure effects of GABA receptor agonists on membrane potential, opening of BK_{Ca} channels would lead to membrane repolarization and relaxation of arterial smooth muscle. Interestingly, Shirakawa et al. (1989) reported that although 100 nM GABA inhibited 5-HT-induced contraction of arterial myocytes, even 10 μM GABA was ineffective at suppressing 40 mM K^+-induced contraction of these same cells. These results suggest that the inhibitory effect of GABA might involve opening of K^+ channels, as high extracellular K^+ concentrations functionally inhibit K^+ channel activity by diminishing the electrochemical driving force for K^+ efflux. These findings, therefore, are consistent with the present results indicating a stimulatory effect of GABA receptor agonists on BK_{Ca} channels. Both diazepam and the neurosteroid progesterone relax coronary and other arteries (Abel et al., 1970; Cote et al., 1976; Jiang et al., 1992). Intravenous diazepam reduces vascular resistance and blood pressure in dogs (Abel et al., 1970; Daniell, 1975) and humans (Raza et al., 1987; Rossetti et al., 1994), even in the presence of combined sympathetic and parasympathetic blockade (Cote et al., 1976). These findings indicate a direct action of diazepam on the vasculature. Other benzodiazepine agonists induce an endothelium-independent inhibition of aortic contraction in vitro (Gimeno et al., 1994). In addition to benzodiazepines, progesterone relaxes placental arteries (Omar et al., 1995), and promotes endothelium-independent relaxation of coronary arteries in vitro (Jiang et al., 1992). Thus, it is apparent that these GABA receptor agonists target vascular smooth muscle cells. Although both diazepam and progesterone activate GABA receptors, we first considered the possibility that the vasodilatory effect of diazepam might involve a peripheral-type benzodiazepine receptor that is expressed in aorta and other smooth muscle (French and Matlib, 1988; Cox et al., 1991); however, the peripheral-type benzodiazepine receptor is insensitive to GABA (Zisterer and Williams, 1997). Because GABA and progesterone produce the same effects as diazepam, it appears that significant involvement of a peripheral-type benzodiazepine receptor is unlikely. Further support for this conclusion is derived from previous studies, suggesting that peripheral-type benzodiazepine receptors are not involved in regulating contractility of smooth muscle (Schaufele et al., 1995). On the other hand, the fact that bicuculline inhibits the stimulatory effect of progesterone on BK_{Ca} channels in coronary myocytes argues strongly for involvement of a $GABA_A$ receptor. Considering our current cellular studies in light of previous biochemical and tissue studies, we believe the most likely conclusion is that diazepam, GABA, and progesterone stimulate BK_{Ca} channel activity in coronary myocytes via activation of a $GABA_A$-like receptor; however, specific identification of this receptor protein awaits further molecular studies.

The central $GABA_A$ receptor is a fast-acting ligand-gated chloride channel, but the receptor expressed in coronary smooth muscle does not appear to be of a similar nature. In the present study, both whole-cell and single-channel studies revealed that by far the most prominent effect of GABA receptor agonists was stimulation of K^+ channel activity. Furthermore, this response was attenuated by $CdCl_2$ or nifedipine, which inhibit Ca^{2+} (but not chloride) channel activity. Because these Ca^{2+} channel blockers did not inhibit K^+ currents directly (e.g., Fig. 5), we propose that the stimulatory effect of GABA receptor agonists on BK_{Ca} channel activity is actually indirect, i.e., due to GABA receptor agonist-stimulated Ca^{2+} influx. Interestingly, peripheral $GABA_A$ receptor-mediated Ca^{2+} influx has been described previously in sperm cells (Shi and Roldan, 1995). In this study, GABA, progesterone, and muscimol each triggered influx of extracellular Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels, as nifedipine or verapamil inhibited the response to these GABA receptor agonists. In addition, bicuculline blocked the response to progesterone in these cells, further implicating involvement of a $GABA_A$-like receptor. Furthermore, we observed that β-progesterone produced a small stimulation of single Ca^{2+} channel activity in coronary myocytes. At first consideration, it might seem counterintuitive that Ca^{2+} influx would promote vasodilation, but there is increasing evidence that localized increases in subsarcolemmal "Ca^{2+} sparks" do indeed relax vascular smooth muscle via hyperpolarization induced by BK_{Ca} channel activity (Jaggar et al., 2000). In fact, GABA receptor agonists elevate intracellular Ca^{2+} in other cells. In addition to the studies on sperm cells cited above, GABA and muscimol evoked Ca^{2+} transients in astrocytes by opening Ca^{2+} channels and/or by releasing Ca^{2+} from internal stores (Nilsson et al., 1993). On the other hand, benzodiazepines are reported to inhibit Ca^{2+} influx in synaptosomes (Taft and DeLorenzo, 1984), PC12 cells (Nakazawa et al., 1991), bladder smooth muscle (Schaufele et al., 1995), pituitary cells (Gershengorn et al., 1988), and airway smooth muscle (Raeburn et al., 1988); however, none of these studies established or excluded potential involvement of a peripheral $GABA_A$-like receptor. The present findings do not constitute conclusive proof that GABA receptor agonists increase Ca^{2+} spark activity in coronary smooth muscle. Verification of this mechanism will await further imaging studies. Nonetheless, our phar-

macological and patch–clamp evidence strongly suggests that GABA receptor-mediated Ca^{2+} influx appears to be the most likely cellular mechanism that could account for diazepam- and GABA receptor agonist-induced opening of BK_{Ca} channels in coronary smooth muscle.

Diazepam (10 μM) potentiates cyclic AMP-dependent effects on ventricular muscle (Martinez et al., 1995) and inhibits cyclic nucleotide phosphodiesterase activity in cardiac (Collado et al., 1998) and tracheal smooth (Fehri and Advenier, 1997) muscle. Previous studies have demonstrated that increases of either cyclic GMP or cyclic AMP relax porcine coronary arteries via stimulation of cyclic GMP-dependent protein kinase activity (Francis et al., 1988), and our laboratory has reported that the cyclic GMP-dependent protein kinase mediates the stimulatory effect of either nucleotide on BK_{Ca} channels in coronary myocytes (Han et al., 1999; White et al., 2000). Therefore, it was possible that the effects of GABA receptor agonists on BK_{Ca} channels could involve accumulation of cyclic nucleotides. However, our studies with 300 nM KT5823, which at this concentration is a highly selective inhibitor of the cyclic GMP-dependent protein kinase, make this possibility unlikely. GABA receptor agonist-induced stimulation of channel activity was unaffected by KT5823. Therefore, it is doubtful that GABA receptor agonists stimulate BK_{Ca} channel activity in coronary smooth muscle by enhancing synthesis of cyclic nucleotides or by inhibiting phosphodiesterase activity. Instead, this mechanism involves activation of BK_{Ca} channels, possibly via GABA receptor-stimulated Ca^{2+} influx.

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