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ORIGINAL ARTICLE

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β-Adrenoceptor stimulation activates large-conductance Ca^{2+}-activated K^+ channels in smooth muscle cells from basilar artery of guinea pig

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Abstract We studied the effect of isoproterenol on the Ca^{2+}-activated K^+ (BK) channel in smooth muscle cells isolated from the basilar artery of the guinea pig. Cells were studied in a whole-cell configuration to allow the clamping of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. Macroscopic BK channel currents were recorded during depolarizing test pulses from a holding potential (V_H) of 0 mV, which was used to inactivate the outward rectifier. The outward macroscopic current available from a V_H of 0 mV was highly sensitive to block by external tetraethylammonium·Cl (TEA) and charybdotoxin, and was greatly augmented by increasing $[Ca^{2+}]_i$ from 0.01 to 1.0 μM. With $[Ca^{2+}]_i$ between 0.1 and 1.0 μM, 0.4 μM isoproterenol increased this current by 58.6 ± 17.1%, whereas with $[Ca^{2+}]_i$ at 0.01 μM a sixfold smaller increase was observed. With $[Ca^{2+}]_i \geq 0.1$ μM, 100 μM dibutyryl -adenosine 3′:5: cyclic monophosphate (cAMP) and 1 μM forskolin increased this current by 58.5 ± 24.1% and 59.7 ± 10.3%, respectively. The increase with isoproterenol was blocked by 4.0 μM propranolol extracellularly, and by 10 U/ml protein kinase inhibitor intracellularly. Single-channel openings during depolarizing test pulses from a V_H of 0 mV recorded in the whole-cell configuration under the same conditions (outside-out-whole-cell recording) indicated a slope conductance of 260 pS. In conventional outside-out patches, this 260-pS channel was highly sensitive to block by external TEA, and in inside-out patches, its probability of opening was greatly augmented by increasing $[Ca^{2+}]_i$ from 0.01 to 1.0 μM. Outside-out-whole-cell recordings with $[Ca^{2+}]_i \geq 0.1$ μM indicated that 100 μM dibutyryl-cAMP increased the probability of opening of the 260-pS channel by 152 ± 115%. In inside-out patches, the catalytic subunit of protein kinase A increased the probability of opening, and this effect also depended on $[Ca^{2+}]_i$, with a 35-fold larger effect observed with 0.1–0.5 μM Ca^{2+} compared to 0.01 μM Ca^{2+}. We conclude that the BK channel in cerebrovascular smooth muscle cells can be activated by β-adrenoceptor stimulation, that the effect depends strongly on $[Ca^{2+}]_i$, and that the effect is mediated by cAMP-dependent protein kinase A with no important contribution from a direct G-protein or phosphorylation-independent mechanism. Our data indicate that the BK channel may participate in β-adrenoceptor-mediated relaxation of cerebral vessels, although the importance of this pathway in obtaining vasorelaxation remains to be determined.

Key words Ca^{2+}-activated K^+ channel · BK channel · Isoproterenol · β-Adrenoceptor · Protein kinase A · Smooth muscle cell · Basilar artery · Smooth muscle relaxation

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Introduction

In smooth muscle generally, β-adrenoceptor stimulation causes relaxation that is mediated adenosine 3′:5: cyclic monophosphate-(cAMP)-dependent protein kinase A (PKA), via the familiar G-protein-coupled cascade (see [30]). In isolated cerebral blood vessels as well, stimulation of β-adrenoceptors leads to formation of cAMP [26] and causes relaxation that is independent of the endothelium [25]. Relaxation of cerebral vessels also is observed with direct or indirect activation of PKA by dibutyryl-cAMP or forskolin [28, 36], as well as with inhibition of protein phosphatase by okadaic acid [15].

A number of phosphoprotein-dependent mechanisms have been identified in smooth muscle that may

be activated by PKA and contribute to relaxation, including phosphorylation of myosin light chain kinase and activation of Ca^{2+} sequestration or Ca^{2+} extrusion (see [30]). Another mechanism would involve reduction of Ca^{2+} influx. This last mechanism may be particularly important in cerebral smooth muscle, as compared to peripheral vascular smooth muscle, because in cerebral smooth muscle activator Ca^{2+} required for myogenic tone originates more from Ca^{2+} influx than from internal stores [1, 37]. Recently, Asano et al. [3, 4] showed greater Ca^{2+} influx at rest in cerebral compared to peripheral vascular smooth muscle cells, and Langton and Standen [19] presented direct evidence in cerebral smooth muscle for steady-state Ca^{2+} influx via voltage-dependent Ca^{2+} channels.

Although one might expect a decrease in Ca^{2+} current with β-adrenoceptor stimulation or activation of PKA, this generally has not been observed in patch-clamp experiments on smooth muscle (see [38]). Indeed, in basilar artery cells, we recently reported that both β-adrenoceptor stimulation [34] and activation of PKA with 8-bromo-cAMP increases availability of L- type Ca^{2+} channels [38]. To explain our findings we postulated that, even with an increase in availability, Ca^{2+} channels could be turned off indirectly by PKA by undergoing voltage-dependent deactivation. One mechanism proposed for deactivating Ca^{2+} channels in vascular smooth muscle involves adenosine 5′-triphosphate-(ATP)-sensitive K^+ channels [27], which are opened by activation of PKA [29]. Alternatively, deactivation of Ca^{2+} channels could be obtained by opening large-conductance Ca^{2+}-activated K^+(BK) channels, with PKA increasing the probability of opening of the type I, charybdotoxin-sensitive subtype (see [22]).

Available studies on β-adrenergic activation of BK channels in vascular [31] and nonvascular [2, 10, 11, 17, 18] smooth muscle are limited. Somewhat more information is available on PKA-dependent activation of this channel in inside-out patches from vascular [24, 31] and nonvascular smooth muscle cells [8, 17]. Most studies have concluded that β-adrenergic activation of BK channels occurs largely by way of a cAMP-dependent mechanism [17, 31], but this has been challenged recently [18, 32]. The BK channel has been identified in cerebrovascular smooth muscle and is believed to contribute to cerebrovascular tone [3, 4, 7, 39], but its activation via β-adrenoceptor stimulation or by PKA has not been shown. Here we report that β-adrenoceptor stimulation as well as direct activation of PKA in basilar artery smooth muscle cells increased the whole-cell macroscopic current due to BK channels, and increased the probability of opening of single BK channels recorded in a whole-cell mode, a recording configuration referred to as the "outside-out-whole-cell" mode. Also, the β-adrenergic effects that we observed were blocked completely by protein kinase inhibitor, giving direct evidence for involvement of a cAMP-dependent mechanism and suggesting no important role in basilar artery cells for a cAMP-independent mechanism [18, 32]. A preliminary account of this work has been presented [35].

Materials and methods

Voltage-clamp experiments

Smooth muscle cells were isolated from basilar arteries of guinea pigs (*Cavia porcellus*, Hartley strain, 200–400 g) as described previously [33, 38]. Experiments were carried out using a recording chamber fabricated from a U-shaped capillary tube (inside diameter, 1.5 mm) positioned inside a 35-mm Petri dish. Cells were placed in the dish and a seal was formed to one of them. The attached cell was transferred under water into the lumen of the tube by passing through a 300-μm, Sylgard-lined orifice in its side, after which the solution in the dish was drained to a level below that of the orifice in the tube. Inflow to the tube came from a small reservoir (1 ml) that allowed changing of the test solution. Flow through the tube was driven by negative pressure (siphon) and was turned on and off by an electrically operated valve. In tests with inside-out patches, concentration jumps with a time constant of 50–60 ms could be obtained. For whole-cell recordings, slower flow rates were used to obtain > 90% complete solution changes within 1 s. For some recordings of whole-cell macroscopic current (Fig. 1E), the pipette solution was changed by perfusion during the experiment, using commerically available equipment (Pipette Perfusion Kit, Adams and List, Westbury, N.Y., USA). Cells or membrane patches were voltage-clamped using conventional whole-cell, inside-out or outside-out configurations of the patch-clamp technique [12]. In addition, for the experiments of Figs. 5A and 7, single-channel currents were recorded in a whole-cell configuration (see Fig. 17 of [12]). We refer to this mode as "outside-out-whole-cell" recording, to distinguish it from the typical whole-cell mode which usually refers to recording of macroscopic currents, and to distinguish it from the typical outside-out mode that usually is performed on excised patches.

Macroscopic currents were amplified (List EPC-7), filtered (8-pole Bessel; Frequency Devices) and sampled on-line by a microcomputer (PDP 11/73, Digital Equipment). For single-channel experiments, currents were recorded on a digital tape recorder (DTR-1200, Biologic, Echirolles, France) then played back off-line and redigitized using commercial hardware (LabMaster DMA, Scientific solutions, Solon, Ohio, USA) and software (pCLAMP software, Axon Instruments, Foster City, Calif., USA). Pipettes were made from borosilicate glass (Kimax, Fisher Scientific). For excised patch experiments, pipettes with resistances of 3–25 MΩ were used, usually coated with Sylgard, whereas for whole-cell recordings, pipettes with resistances of ≈1 MΩ were used. Because we seldom could compensate more than 40% of the series resistance, compensation generally was omitted, thus incurring an error of ≈1 mV/nA. All experiments were performed at room temperature, i.e. 22–25 °C. Cell capacitance was 16–22 pF as previously reported [33], and the input resistance was 5–10 GΩ ($n = 8$).

Solutions

The bath solution for conventional whole-cell, for conventional outside-out and for outside-out-whole-cell recording, as well as the pipette solution for inside-out patches contained (mM) : NaCl 130, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 12.5 and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 10, pH 7.4 with NaOH. In general, various drugs or chemicals including tetraethylammonium·Cl (TEA; Sigma, Product No. T2265) ≤ 2 mM were

added directly to this solution. For experiments with higher concentrations of TEA, an equimolar amount of NaCl was removed. The pipette solution for conventional whole-cell, for conventional outside-out and for outside-out-whole-cell recording contained (mM) : KCl 145, $MgCl_2$ 2, glucose 10, ATP 2, guanosine 5'-triphosphate (GTP) 1 and HEPES 10, pH 7.2 with KOH; $CaCl_2$ 0–4.23 mM and ethylene bis(oxonitrilo) tetraacetic acid (EGTA) 5 mM were added to this solution to obtain the desired concentrations of free Ca^{2+}, as determined using a computer program based on published stability constants, except for a solution with 5 mM EGTA and no added Ca^{2+}, in which free Ca^{2+} was estimated as 0.01 μM. In addition, for some outside-out-whole-cell experiments, 5 mM TEA was added to the pipette solution to reduce background noise. The pipette solution for perforated patch recording contained (mM): KCl 55, K_2SO_4 75, $MgCl_2$ 8, HEPES 10, pH 7.2 as the base solution, with a final concentration of nystatin 165 μg/ml and dimethyl sulfoxide (DMSO) 3.3 μl/ml present in the backfill solution [16]. Experiments with inside-out patches (Figs. 5B, D and 8) were performed with a bath solution containing (mM) : KCl 145, $MgCl_2$ 2, $CaCl_2$ 0–3.66, EGTA 5 ($[Ca^{2+}]$ = 0.01–0.5 μM), glucose 10, and HEPES 10, pH 7.2 with KOH. Charybdotoxin was obtained from the Peptide Institute, Osaka, Japan. Enzymes and chemicals were from Sigma (St. Louis, Mo., USA) or Fisher Scientific (Pittsburgh, Pa., USA)

Data analysis

Current measurements are given with respect to the holding current. End-of-pulse currents for 200-ms pulses were measured as the average current between 175 and 195 ms. The probability of channel opening for a patch ($n \cdot P_O$; Fig. 8D) or for the whole cell ($N \cdot P_O$) was obtained using a 50% threshold-crossing method as implemented in pCLAMP. Because data were filtered at 500 Hz (−3 dB), only events > 360 μs in duration could be resolved and so we ignored transitions < 500 μs. Data were fit to equations using the nonlinear, least-squares method of Marquardt-Levenberg as implemented in Origin (MicroCal Software, Northampton Mass., USA). Data are given as means ± SD.

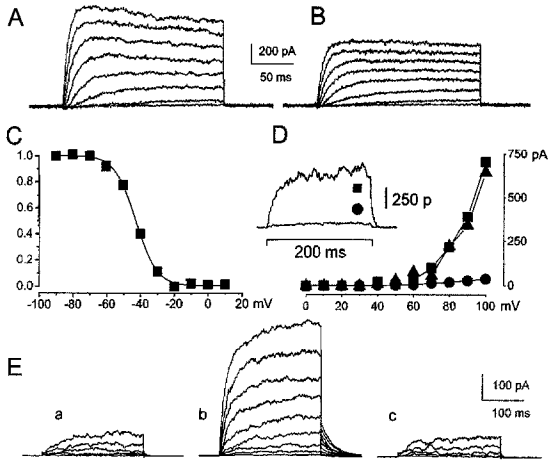

Fig. 1A-E Isolation of large-conductance Ca^{2+}-activated K^+ channel current in smooth muscle cells from the basilar artery of the guinea pig. Outward currents during 200-ms pulses from a holding potential, V_H, of −80 mV to potentials from +10 to +120 mV in steps of 10 mV, before (**A**) and after (**B**) addition of 2 mM tetraethylammonium ion (TEA); conventional whole-cell configuration; $[Ca^{2+}]_i$ = 0.01 μM. **C** Steady-state voltage-dependent inactivation of current recorded in the presence of 2 mM TEA; test pulses to +150 mV from V_H values indicated by *abscissa*; normalized data were fit to the equation, $\{1+\exp[(E-E_{1/2})/k]\}^{-1}$, with $E_{1/2}$ = −42.7 mV and k = 6.1 mV; same cell as **A** and **B**. **D** Outward currents were recorded during 200-ms pulses from a V_H of 0 mV to potentials from +10 to +100 mV in steps of 10 mV; end-of-pulse currents before (■), after addition of 2 mM TEA (●) and after washout (▲) are plotted against test potential; the *inset* shows current records at +100 mV before (■) and after (●) 2 mM TEA; same cell as **A** and **B**. In a different cell, outward currents recorded during 200-ms pulses from a V_H of 0 mV to potentials from +10 to +100 mV in steps of 10 mV after internal perfusion with pipette solution containing $[Ca^{2+}]_i$ = 0.01 μM (**Ea**), $[Ca^{2+}]_i$ = 1.0 μM (**Eb**), and after return to $[Ca^{2+}]_i$ = 0.01 μM (**Ec**)

Results

Isolation of macroscopic BK channel current

Our objective was to study the effect of β-adrenoceptor stimulation on macroscopic current due to BK channels. This required that cells be studied in a whole-cell configuration to clamp $[Ca^{2+}]_i$, with recording conditions adapted to isolate BK channel current from other outward currents. As in other vascular smooth muscle cells [5], the outward current in basilar artery cells from guinea pig was dominated by two components, one attributable to an outward rectifier and the other due to BK channels. Figure 1A shows outward currents recorded from a negative holding potential (V_H = −80 mV) with low $[Ca^{2+}]_i$ in the pipette solution. Under these conditions, a relatively small fraction of the current was blocked by addition of 2 mM TEA to the bath (Fig.1B) and the current that remained required a negative V_H value for availability. Measurements of steady-state inactivation in three cells, made in the presence of 2 mM TEA, indicated that this current was half inactivated at −40 to −45 mV and was completely inactivated at 0 mV (Fig. 1C). A current exhibiting the kinetic features apparent in Fig. 1B, that required a negative V_H for availability, and that was relatively insensitive to TEA was typical of outward rectifier current described in other smooth muscle cells.

We then studied the cells from a V_H of 0 mV to inactivate the outward rectifier and isolate the BK channel current. From a V_H of 0 mV, a noisy outward current was available at positive potentials that was very sensitive to external TEA, with > 90% of the current reversibly blocked by 2 mM TEA (Fig. 1D, inset). In five other cells ($[Ca^{2+}]_i$ = 0.1–1.0 μM; V_H = 0 mV; test potential, 100–120 mV), values of fractional block of 0.77 ± 0.14, 0.97 ± 0.016 and 0.99 ± 0.002 were obtained with 1 mM, 10 mM and 100 mM TEA, respectively, as expected for current due to BK channels [20]. Current available from a V_H of 0 mV also was sensitive to charybdotoxin (100 nM), a more selective blocker of BK channels [23], with values of fractional block of 0.77 ± 0.074 obtained in six cells ($[Ca^{2+}]_i$ = 0.1–1.0 μM; V_H = 0 mV; test potential, 100–140 mV). In addition, the current available from a V_H of 0 mV was very sensitive to $[Ca^{2+}]_i$. Changing $[Ca^{2+}]_i$ from

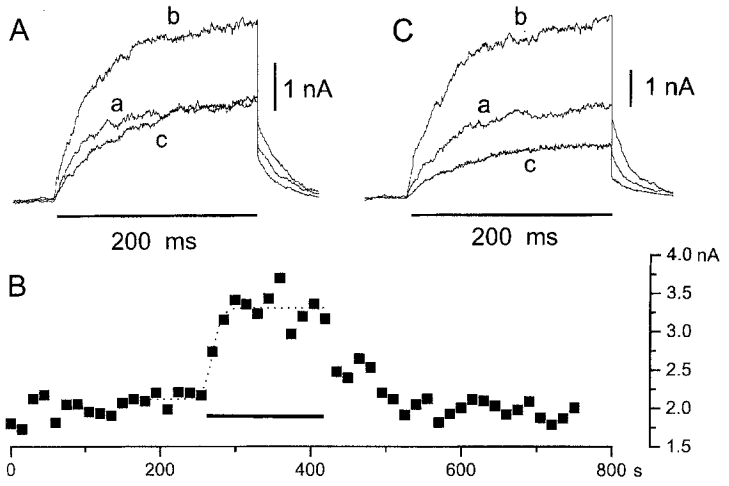

Fig. 2A-C Effect of isoproterenol on large-conductance Ca^{2+}-activated K^+ channel current in smooth muscle cells from the basilar artery of the guinea pig. **A** Current records during 200-ms pulses to +40 mV (V_H = 0 mV) before (*trace a*) and after (*trace b*) addition of 0.4 μM isoproterenol, and after washout of the drug (*trace c*). **B** Time course of the response to isoproterenol, with the magnitude of the current at the end of successive 200-ms depolarizing pulses to +40 mV plotted against time; 0.4 μM isoproterenol was present in the bath during the time indicated by the *horizontal bar*; same cell as in **A**. **C** In a different cell, current records during 200-ms pulses to +50 mV (V_H = 0 mV) obtained under control conditions (*trace a*), after addition of 0.4 μM isoproterenol to the bath (*trace b*), then after addition of 1 mM TEA to the bath (*trace c*)

0.01 μM to 1.0 μM by internal perfusion resulted in a great augmentation of the current available from 0 mV (Fig. 1Eb vs Ea), an effect that was reversed upon returning to the lower $[Ca^{2+}]_i$ (Fig. 1Ec). We took these findings of an outward macroscopic current that was available from a V_H of 0 mV, was sensitive to block by external TEA and charybdotoxin, and was activated by internal Ca^{2+}, as evidence that the current recorded was due to BK channels largely uncontaminated by other channels. Single-channel recordings detailed below gave additional evidence favoring this interpretation.

Effect of isoproterenol

Having characterized the macroscopic outward current available from a V_H of 0 mV, we then examined the effect of isoproterenol on this current. Cells were studied using a pulse program consisting of 200-ms depolarizing pulses to various test potentials repeated every 15 s. In initial experiments on nine cells carried out with a pipette solution containing 0.01 μM Ca^{2+}, application of 0.4 μM isoproterenol to the bath resulted in a small, unreliable increase in the outward current in some cells (Table 1). In contrast, when Ca^{2+} in the pipette was increased to 0.5 μM, application of the same concentration of isoproterenol resulted in a larger, more reliable increase in the outward current. Original records obtained during test pulses to + 40 mV before (Fig. 2A, trace a) and during application of isoproterenol (Fig. 2A, trace b) demonstrate a significant increase in current in this cell. Overall, in six cells studied with 0.5 μM $[Ca^{2+}]_i$ at a test potential of +40 mV, 0.4 μM isoproterenol caused a 59.8 ± 8.7% increase in current.

The time course of the response to isoproterenol for the same cell is shown, illustrating that the response

Table 1 Changes in macroscopic BK current with various chemical agents. All experiments were done using conventional whole-cell recordings except those designated *nys*, which were done with the nystatin-perforated patch method in which $[Ca^{2+}]_i$ is not known; the *number* of cells tested is given in *parentheses*. Effect is expressed as percentage change in current at steady-state after treatment; values of current were measured at the end of 200-ms pulses to test potentials of 20-100 mV from holding potential of 0 mV; values are mean ±SD. (PKA_I Protein kinase A inhibitor)

Chemical agent	Parameter		
	$[Ca^{2+}]_i$ (μM)	Effect (%)	*P*
Isoproterenol (0.4 μM)	0.01 (9)	9.5 ± 9.7	0.02
Isoproterenol (0.4 μM)	0.5 (9), 1.0 (3), *nys* (4)	58.6 ± 17.1	< 0.001
Isoproterenol (0.4 μM) + propranolol (4 μM)	0.5 (4)	−1.7 ± 7.2	0.67
Dibutyryl-cAMP (100 μM)	0.1 (2), 0.5 (3), 1.0 (1)	58.5 ± 24.1	< 0.001
Forskolin (1 μM)	0.1 (1), 1.0 (4), *nys* (1)	59.7 ± 10.3	< 0.001
Isoproterenol (0.4 μM) + PKA_I (10 U/ml)	0.5 (12)	1.3 ± 10.2	0.39

P value for *t*-test with null hypothesis being no effect

required some time to develop (Fig. 2B). The chamber that we used for these experiments allowed changes in solution to be >90% complete in <1s (see Materials and methods), a time much faster than that required to observe the maximum effect of isoproterenol. We examined the time course of effect in eight cells studied with a test pulse repetition rate of one per 5 s. In all cases, two or more pulses were required after the change in solution to obtain the maximum effect. We did not explore further the cause for the relative slowness of the response, and our data do not allow us to distinguish between slow chemical kinetics and a state- or use-dependent effect.

The increase in current due to isoproterenol was completely reversed after washout (Fig. 2A trace a vs trace c, Fig. 2B). Involvement of β-adrenoceptors was confirmed by showing that the increase in current due to 0.4 μM isoproterenol ($[Ca^{2+}]_i$ 0.5–1 μM; test potential +30 to +50 mV) was completely blocked by 4 μM propranolol (Table 1). Also, we tested the sensitivity to block by TEA of the current that was increased by isoproterenol. Records from a cell under control conditions (Fig. 2c, trace a; $[Ca^{2+}]_i = 0.5$ μM; test potential +50 mV), after application of 0.4 μM isoproterenol (Fig. 2C, trace b) and after changing to a solution containing isoproterenol plus 1 mM TEA (Fig. 2c, trace c) demonstrate that the isoproterenol-induced current was highly sensitive to TEA, as expected for current due to BK channels.

We also examined the effect of isoproterenol on cells studied with a nystatin-perforated patch technique. Although this method did not allow control of $[Ca^{2+}]_i$, we used it to prevent loss of any soluble cytoplasmic substances that might be involved in the response to isoproterenol. In four cells studied at test potentials of either +50 or +100 mV, 0.4 μm isoproterenol caused a 53.0 ± 12.3% increase in current, a value that compared favorably with the results obtained with cells studied in the whole-cell mode with membrane rupture (see above). Also, the time course of onset and offset of the drug effect in these cells was similar to that observed in cells studied in the whole-cell mode with membrane rupture. We interpreted this concordance as indicating that the effects measured using a conventional whole-cell mode were not diminished or otherwise altered by loss of soluble, rapidly diffusing substances.

The magnitude of the effect of isoproterenol did not appear to depend on the test potential. Figure 3 shows a family of records at different test potentials before (A) and after (B) application of 0.4 μM isoproterenol to the bath. The relationship between end-of-pulse current and test potential for this cell is shown in Fig. 3C. The two lines in Fig. 3C represent fits to an arbitrary function with identical parameters except that the scaling parameter for line b was increased by 43% with respect to that for line a. Absence of a voltage-dependent effect at potentials > 0 mV was confirmed by analyzing the responses to 0.4 μM isoproterenol in 16 cells studied at

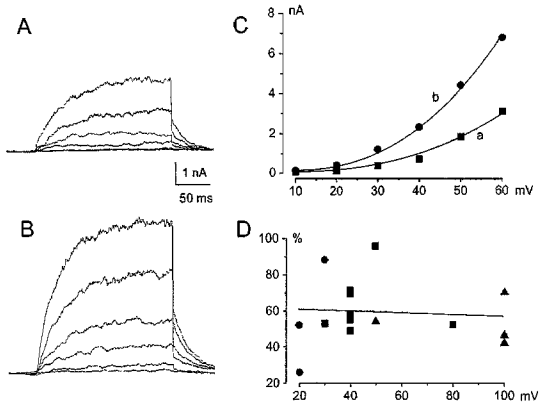

Fig. 3A-D Effect of isoproterenol on large-conductance Ca^{2+}-activated K^+ channel current does not depend on voltage. Outward currents during 200-ms pulses from a V_H of 0 mV to potentials from +10 to +60 mV in steps of 10 mV, before (**A**) and after (**B**) addition of 0.4 μM isoproterenol. **C** End-of-pulse current plotted as a function test potential for records before (■) and after (●) the drug for records shown in **A** and **B**; *lines a* and *b* show fit to the same function, except that that for *b* is scaled for an increase of 43% relative to that for *a*. **D** Relative increase in end-of-pulse current after addition of 0.4 μM isoproterenol plotted against test potential ($V_H = 0$ mV) for 16 cells tested with either 0.5 μM (■) or 1.0 μM Ca^{2+} (●) in the pipette, and for four cells with an unknown concentration of $[Ca^{2+}]_i$ because of the use of nystatin (▲); *regression line* indicates that the effect of isoproterenol was not significantly ($P = 0.81$) related to test potential

different test potentials. This analysis included data given above from six cells tested at +40 mV with a $[Ca^{2+}]_i$ of 0.5 μM, and from ten other cells tested at potentials between +20 and +100 mV, with either 0.5 μM (Fig. 3D, ■) or 1.0 μM Ca^{2+} (Fig. 3D, ●) in the pipette, or an unknown concentration of $[Ca^{2+}]_i$ because of the use of nystatin (Fig. 3D, ▲). Overall, the current increased by 58.6 ± 17.1% (Table 1) in these cells, and no significant ($P = 0.81$) dependence on test potential was apparent (Fig. 3D, line).

Involvement of cAMP and PKA

An initial requirement for implicating the cAMP system in the mechanism of action of isoproterenol would be the mimicking of the effect by agents that activate PKA. We tested the effect of dibutyryl-cAMP (100 μM) on cells studied in the whole-cell configuration with 0.1–1.0 μM Ca^{2+} in the pipette using the test protocol described above. Original records obtained before (Fig. 4A, trace a) and during application of dibutyryl-cAMP (Fig. 4A, trace b) are shown, as is the time course of the response in the same cell (Fig. 4B). As with isoproterenol, the effect took considerably longer to develop than the time required to change the solution. An average increase in current of 58.5 ± 24.1% was observed in six cells (Table 1). The increase in current due to dibutyryl-cAMP was completely reversible after several minutes of washout (Fig. 4A, trace c, 4B).

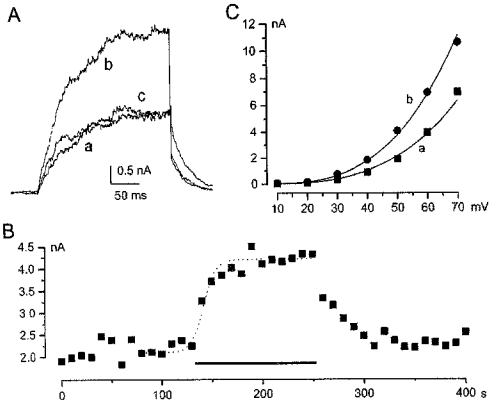

Fig. 4A-C Effect of dibutyryl-cAMP on large-conductance Ca^{2+}-activated K^+ channel current in smooth muscle cells from the basilar artery of the guinea pig. **A** Current records during 200-ms pulses to +50 mV (V_H = 0 mV) before (*trace a*) and after (*trace b*) addition of 100 μM dibutyryl-cAMP, and after washout of the drug (*trace c*). **B** Time course of the response to dibutyryl-cAMP, with the magnitude of the current at the end of successive 200-ms depolarizing pulses to +50 mV plotted against time; 100 μM dibutyryl-cAMP was present in the bath during the time indicated by the *horizontal bar*; same cell as in **A**. **C** End-of-pulse current plotted as a function test potential for records before (■) and after (●) addition of dibutyryl-cAMP; *lines a* and *b* show fit to the same function, except that that for *b* is scaled for an increase of 57 % relative to that for *a*

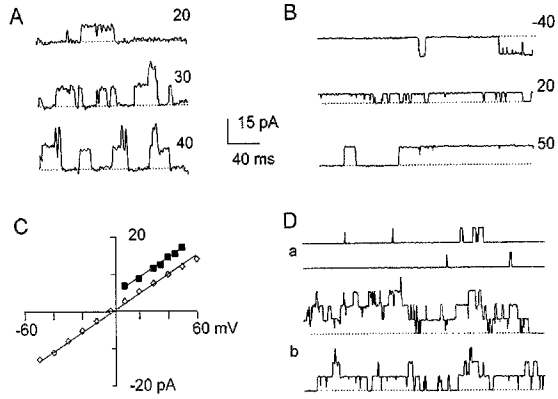

Fig. 5A-D Single-channel recordings of large-conductance Ca^{2+}-activated K^+ channels in smooth muscle cells from the basilar artery of the guinea pig. **A** Current records in outside-out-whole-cell configuration obtained during 200-ms pulses to potentials indicated; V_H = 0 mV; pipette solution with no added Ca^{2+}; filter, 300 Hz (−3 dB). **B** Current records from an inside-out patch obtained at V_H values indicated; records were selected to show openings and do not represent open probability; pipette solution with no added Ca^{2+}; filter, 1 kHz (−3 dB). **C** Current voltage curves for the data shown in **A** and **B**, with *solid lines* indicating a slope conductance of 260 pS. **D** Current records from an inside-out patch obtained at +60 mV with 0.1 μM (**Da**) and 1.0 μM (**Db**) Ca^{2+} in the bath; filter, 1 kHz (−3 dB). *Dotted lines* give zero current levels

Also, the effect of dibutyryl-cAMP was not voltage dependent at potentials > 0 mV, as indicated by the current voltage curves of Fig. 4C. The two lines in Fig. 4C represent fits to an arbitrary function with identical parameters except that the scaling parameter for line b was increased by 57% with respect to that for line a. We also studied the effect of forskolin, a diterpene compound that directly activates adenylate cyclase. In six cells studied, 1 μM forskolin caused a 59.7 ± 10.3% increase in current (Table 1), a value that compared favorably with the results obtained with both isoproterenol and dibutyryl-cAMP. Thus, the effect of 100 μM dibutyryl-cAMP and of 1 μM forskolin appeared to be indistinguishable from that of 0.4 μM isoproterenol in terms of rate of onset of effect, magnitude of effect, reversibility on washout and voltage independence.

Our observations with dibutyryl-cAMP and forskolin suggested that the effect of isoproterenol might be due to activation of PKA. One stringent test of this would be to measure the effect of isoproterenol in the presence of an inhibitor of PKA (PKA_I). Cells were studied in a conventional whole-cell mode with 10 U/ml of PKA_I (Sigma, Product no. P0300) added to the pipette solution ($[Ca^{2+}]_i$ 0.5 μM; test potential +20 to +50 mV). In six out of ten cells exposed to 0.4 μM isoproterenol, the current was either smaller than control or increased by <5%, whereas in four cells, an increase of 7–14% was observed. Overall, exposure to isoproterenol resulted in a +1.3 ± 10.2% increase in current in these cells, an effect that was not statistically significant (Table 1).

Identification of single-channel BK current

Whole-cell recordings were obtained under conditions identical to those described above except that recordings were made at high gain. When the seal resistance was favorable, current fluctuations attributable to single-channel events were observed, a mode of recording designated the "outside-out-whole-cell configuration". From a V_H of 0 mV, small depolarizations elicited single-channel events that were clearly distinguishable from noise (Fig. 5A), demonstrating the underlying unitary currents responsible for the macroscopic currents obtained under the same conditions. Measurements of single-channel amplitude gave a slope conductance of 260 pS (145 mM $[K^+]_i$, 5 mM $[K^+]_o$) over the range of positive potentials tested (Fig. 5C, ■).

Other experiments were carried out to confirm the identity of this channel. Inside-out patches were studied using symmetrical 145 mM $[K^+]$ (Fig. 5B). With no added Ca^{2+} and EGTA (0.5–5 mM), single-channel events were observed at various V_H values that exhibited a slope conductance of 260 pS (Fig. 5C, ◊). In agreement with the findings of Fig. 1E on the macroscopic current, activity of the 260-pS channel was very sensitive to a change in Ca^{2+} concentration in the range of 0.1 to 1.0 μM. In the example shown, $n \cdot P_O$ (number of channels in the patch × open channel probability) increased from 0.00974 with 0.1 μM Ca^{2+} (Fig. 5Da) to 0.715 with 1 μM Ca^{2+} (Fig. 5Db). In three inside-out patches, this channel was found not to be sensitive to addition of 5 mM ATP, regardless of the concentration of Ca^{2+}. We used outside-out patches to assess the sensitivity of this channel to external TEA.

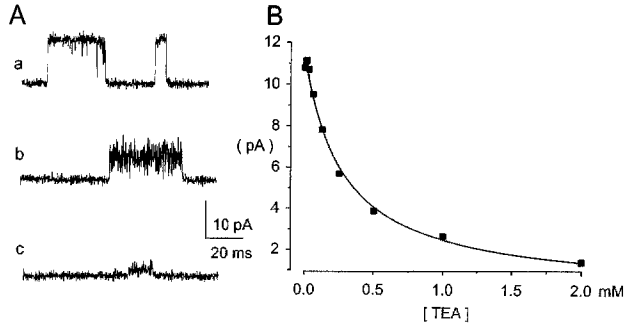

Fig. 6A, B Block of large-conductance Ca^{2+}-activated K^+ channels by external TEA. **A** Original records from an outside-out patch obtained in the presence of 0 mM (**Aa**), 0.25 mM (**Ab**), and 2.0 mM (**Ac**) TEA; V_H = +30 mV; filter, 5.0 kHz (−3 dB). **B** Plot of mean current during channel opening versus concentration of TEA in the bath (■). The data were fit to the equation, $I_{max}/(1 + [TEA]_o/k_{app})$, with values of I_{max} = 11.3 pA and k_{app} = 0.28 mM (*solid line*). Pipette solution with Ca^{2+} = 0.1 μM

Figure 6A shows records before (Fig. 6Aa) and after addition of 250 μM (Fig. 6Ab) and 2.0 mM (Fig. 6Ac) TEA, illustrating the "flickery" block of the BK channel expected for this compound [20]. Measurements of the mean current during channel openings are plotted against concentration of TEA in Fig. 6B, ■. These data were fit to the equation, $I = I_{max}/(1+[TEA]_O/k_{app})$, with values of I_{max}=11.3 pA and k_{app}=280 μM (Fig. 6B, solid line). Similar observations were made in four other patches, with values for k_{app} derived from single-channel studies agreeing well with measurements given above derived from macroscopic currents. Together, these data on slope conductance, sensitivity to internal Ca^{2+} and sensitivity to external TEA confirmed that the current available from a V_H of 0 mV was associated with the typical type I BK channel [22].

Effect of dibutyryl-cAMP with outside-out-whole-cell mode

Having identified the channel under study, we next assessed the effect of dibutyryl-cAMP on its probability of opening. Cells were studied in the outside-out-whole-cell mode. In contrast to the conventional whole-cell method used above, use of the outside-out-whole-cell mode allowed easy identification of the channel and permitted measurements at negative membrane potentials. Figure 7A shows an original record of activity at a V_H of −30 mV with 0.5 μM Ca^{2+} in the pipette. Figure 7B shows a record from the same cell after addition of 100 μM dibutyryl-cAMP to the bath. In this cell, $N \cdot P_O$ (number of channels in the cell × open channel probability) increased 167%, from 0.049 to 0.131. In all, 12 cells were studied in this manner, one at −50 mV, eight at −40 mV, one at −30 mV and two at −20 mV, all with 0.5 μM Ca^{2+} in the pipette. In these cells, addition of 100 μM dibutyryl-cAMP to the bath resulted in a 152 ± 115% increase in $N \cdot P_O$

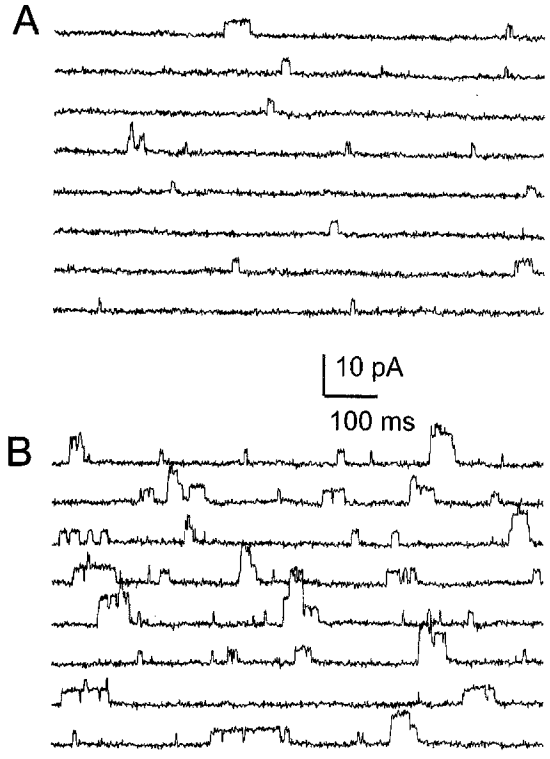

Fig. 7A, B Effect of dibutyryl-cAMP on large-conductance Ca^{2+}-activated K^+ channels recorded using an outside-out-whole-cell configuration. Eight consecutive records representing 7.3 s are shown before (**A**) and after (**B**) addition of dibutyryl-cAMP (100 μM) to the bath; V_H = −30 mV; $[Ca^{2+}]_i$ = 0.5 μM; filter, 1.0 kHz (−3 dB)

Effect of cAMP and PKA on inside-out patches

We examined the effect of direct application of various substrates on the activity of BK channels in inside-out patches. First, we examined the effect of cAMP on inside-out patches studied with 0.5 μM Ca^{2+} and 1 mM Mg^{2+} in the bath. In three patches studied, addition of cAMP (500 μM) had no appreciable effect on $n \cdot P_O$. Conversely, when patches were studied with a bath $[Ca^{2+}]$ of 0.1–0.5 μM, addition of the catalytic subunit of PKA (PKA_{CS}) in the presence of Mg^{2+} (1 mM) and ATP (2 μM) invariably caused a large increase in $n \cdot P_O$. Figure 8A shows a continuous record from an inside-out patch studied at +10 mV before and after application of PKA_{CS} (50 U/ml; added at the arrowhead), with Fig. 8B and C showing portions of the same tracing on an expanded time scale. Values of $n \cdot P_O$, computed for successive 1-min intervals, are plotted against time in Fig. 8D, showing that the response to PKA_{CS} took some time to develop and was partially reversible on washout. This experiment (0.5 μM Ca^{2+} in the bath; V_H = 0 to + 20 mV) was repeated in 14 other patches, with values of $n \cdot P_O$ increasing from 0.04 ± 0.06 to 0.19 ± 0.25, representing an increase of 384 ± 159% ($P < 0.001$) in response to addition of 50 U/ml of PKA_{CS}. As shown in Fig. 8B, removing PKA_{CS} resulted in some diminution of channel activity, but in none of

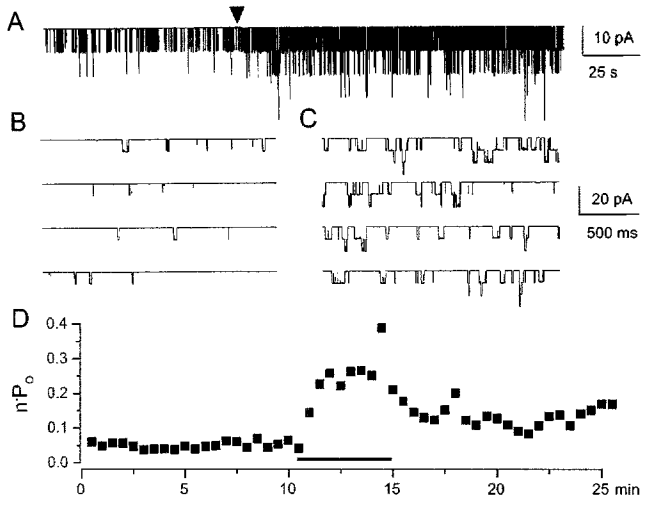

Fig. 8A-D Effect of catalytic subunit of protein kinase A (PKA_{CS}) on large-conductance Ca^{2+}-activated K^+ channels recorded using an inside-out patch configuration. **A** Continuous recording of channel activity at low temporal resolution; 50 U/ml of PKA_{CS} was added to the bath at the *arrowhead.* **B, C** Representative traces at higher temporal resolution of channel openings before and after addition of PKA_{CS}, respectively; filter, 1 kHz (−3 dB). **D** The probability of channel opening ($n \cdot P_O$) during successive 30-s intervals is plotted against time for the same patch; 50 U/ml of PKA_{CS} was present in the bath during the time indicated by the *horizontal bar*

the 14 patches did we observe complete return to baseline activity. To verify that the augmentation in channel activity that we observed with PKA_{CS} was indeed secondary to phosphorylation, we repeated this experiment in the presence of the specific inhibitor of PKA (PKA_I; 5 U/ml) and found an increase in $n \cdot P_O$ that was more than tenfold smaller, only 29 ± 17% ($P = 0.01$; five patches).

As noted above (Table 1), the response of the macroscopic current to isoproterenol was greatly attenuated when cells were studied with a $[Ca^{2+}]_i$ of 0.01 μM. Thus, we examined the effect of PKA_{CS} on the activity of BK channels in inside-out patches studied with the same concentration of Ca^{2+}. Experiments were carried out on six patches using a V_H from 0 mV to +50 mV. Addition of 50 U/ml PKA_{CS} caused an increase in $n \cdot P_O$ from 0.006 ± 0.005 to 0.007 ± 0.005. This small effect, representing an increase of only 11 ± 20% ($P = 0.12$), suggested that much of the Ca^{2+} dependence that we had observed for the effect of isoproterenol on the macroscopic current arose from a terminal step of the reaction scheme.

Discussion

Several features identify the channel that we studied as being a type I, large-conductance Ca^{2+}-activated K^+ channel, including absence of inactivation, sensitivity to internal Ca^{2+}, slope conductance of 260 pS, block by low concentrations of TEA and charybdotoxin, and activation by PKA [22]. In addition, our experiments with inside-out patches indicated that the channel is not directly gated by cyclic nucleotide and that excised patches have no associated or intrinsic kinase activity, as has been reported for BK channels from some other preparations [21, 24].

Most previous work demonstrating a β-adrenergic effect on BK channels has been carried out using cell-attached patches, a method that allows for easy identification of the channel under study [17, 24, 31]. We were unable to use this method because the cAMP system augments availability of Ca^{2+} channels in smooth muscle cells from the basilar artery [38], an effect that would complicate experiments on BK channels that are sensitive to $[Ca^{2+}]_i$. Instead, for experiments on receptor activation and stimulation of endogenous PKA by analogues of cAMP, we used a whole-cell recording method to permit control of $[Ca^{2+}]_i$, and for recordings of macroscopic current, we used a V_H of 0 mV to isolate BK channel current from outward rectifier. With a V_H of 0 mV, we showed that the outward rectifier was completely inactivated, that the current still available was sensitive to internal Ca^{2+} and to low concentrations of external TEA and charybdotoxin, and that small depolarizations elicited single-channel events with a slope conductance of 260 pS, as expected for a BK channel. Moreover, in whole-cell recordings, the macroscopic current that was augmented by isoproterenol exhibited a sensitivity to TEA expected for a BK channel, and single-channel openings that were increased by dibutyryl-cAMP had a slope conductance consistent with a BK channel. In combination, the methods that we used allow us to conclude with confidence that the effects of isoproterenol and of cAMP that we observed were due to direct effects on BK channels, exclusive of effects on other channels, and that the effects were not due indirectly to the expected increase in Ca^{2+} channel availability [38].

The present study is the first to examine in detail the effect of β-adrenoceptor stimulation on BK channel activity in isolated vascular smooth muscle cells. Given the known relationship between β-adrenoceptor stimulation and cAMP production in cerebrovascular smooth muscle [26], coupled with previous reports showing the effect of PKA on BK channels in inside-out patches from smooth muscle [8, 17, 24, 31], the effects of β-adrenoceptor stimulation observed in the present study were not unexpected. Nevertheless, our data showing inhibition of the effect of isoproterenol by PKA_I provide the first direct evidence in cerebrovascular smooth muscle that activation of BK channels by stimulation of β-adrenoceptor is mediated predominantly by cAMP-dependent PKA. This finding suggests that, in basilar artery cells, the PKA system is necessary and sufficient for the effect of β-adrenoceptor stimulation on BK channels. In coronary artery and tracheal smooth muscle cells, a direct G-protein-mediated mechanism that is independent of phosphorylation has been reported for effects of β-adrenoceptor

stimulation on BK channles [18, 32]. Since PKA_I would not be expected to block a direct G-protein effect, our observation that PKA_I effectively blocked the action of isoproterenol suggests that G_s has little direct effect on BK channels in basilar artery cells. A similar conclusion was reached by Fan et al. [11] in their work with myocytes from taenia coli. Also arguing against a direct effect of G_s was the similarity in magnitude and time course of the effect of dibutyryl-cAMP and forskolin compared to isoproterenol, with only the last one expected to involve G_s.

In the experiments with excised patches, addition of PKA_{cs} resulted in a three- to four-fold increase in $n \cdot P_O$, a value similar to that previously reported for this type of experiment on inside-out patches from other preparations [8, 17]. In contrast, the various treatments that we used to activate endogenous PKA, including isoproterenol, dibutyryl-cAMP and forskolin, resulted in a considerably more modest increase in either whole-cell macroscopic current ($\approx$60%) or in $N \cdot P_O$ in outside-out-whole-cell recordings ($\approx$150%). It seems likely that the greater effect observed with excised patches might be due to the loss of phosphatase activity that could occur after patch excision. Intrinsic phosphatase activity previously has been noted in isolated smooth muscle cells, including excised membrane patches [8, 17]. Strong phosphatase activity in intact cells with only partial retention of that activity after patch excision would be consistent with our observation that the effects of isoproterenol and of dibutyryl-cAMP were always completely reversible in whole-cell experiments, but that effects of PKA_{cs} on inside-out patches typically were only partially reversible.

We found that Ca^{2+} plays an important permissive role in the sequence coupling β-adrenoceptor occupation to BK channel activation. The level of $[Ca^{2+}]_i$ was critical for the response of whole-cell macroscopic current to isoproterenol as well as dibutyryl-cAMP, with values of $[Ca^{2+}]_i > 0.1$ μM being required for a robust response, and a value of $[Ca^{2+}]_i = 0.01$ μM giving a small or unreliable response. Although not previously reported for isoproterenol, a similar Ca^{2+} dependence has been observed for stimulation of BK channels in mouse lacrimal cells by vasoactive intestinal polypeptide [21]. Notably, we observed a comparable permissive effect of Ca^{2+} for the direct action of PKA_{cs} on BK channels in inside-out patches, with a 35-fold larger effect of PKA_{cs} observed with 0.1–0.5 μM Ca^{2+} compared to 0.01 μM Ca^{2+}. Earlier reports on the effect of PKA on BK channels in inside-out patches from smooth muscle cells generally have used 0.1 μM Ca^{2+} or more in the bath, and have noted that channel openings were suppressed when perfusing with PKA in Ca^{2+}-free solution [8, 17, 24, 31]. Overall, these findings indicate that β-adrenoceptor-mediated activation of BK channels requires Ca^{2+} at a near-terminal step, possibly at the step involving substrate phosphorylation. Assuming the BK channel to be the substrate [22], it is conceivable that the observed Ca^{2+} dependence reflects a state dependence for phosphorylation, with the proper state of the channel being favored by a higher $[Ca^{2+}]$.

The Ca^{2+} dependence identified here for the effect of β-adrenoceptor-mediated activation of BK channels in basilar artery cells may help to explain our recent finding in these cells of an increase in Ca^{2+} channel availability with stimulation of PKA [38]. In that paper, we hypothesized that the effect of PKA activation on Ca^{2+} channels might play a supportive role in achieving hyperpolarization with β-adrenoceptor stimulation, by helping to ensure sufficient availability of Ca^{2+} for activation of BK channels. Also, given the proposed function of the BK channel in determining the resting membrane potential and myogenic tone of cerebral vascular smooth muscle [3, 4, 7, 39], the observed Ca^{2+} dependence may help to explain the more reliable cerebral vasodilation that has been reported with sympathetic stimulation in hypertension compared to nonhypertension (see [13]), when $[Ca^{2+}]_i$ in vascular smooth muscle is elevated [6].

The results presented here indicate that the BK channel, or a closely associated regulatory protein, in basilar artery cells may be a target phosphoprotein that is activated in the course of β-adrenoceptor-mediated vasorelaxation. As such, our findings resemble those previously made on BK channels in other vascular [31] and nonvascular [2, 10, 11, 17, 18] smooth muscle. As noted in the Introduction, this effect would deactivate voltage-dependent Ca^{2+} channels, thus decreasing steady-state Ca^{2+} influx and so favor vasorelaxation. An important role for the BK channel in mediating smooth muscle relaxation has been identified in trachealis muscle [9, 10, 14] and the data presented here indicate that this channel could serve a comparable role in cerebrovascular smooth muscle. Our observations do not, however, address the crucial question of whether activation of the BK channel is required for vasorelaxation or whether, as recently argued for trachealis muscle [9, 10], activation of this channel plays merely a supportive role.

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Note added in proof Taguchi et al. [36a] recently presented critical evidence indicating that BK channles play an imporatant role in eliciting cAMP-mediated cerebral vasorelaxation.