Regulation of Large Calcium-activated Potassium Channels by Protein Phosphatase 2A

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Vasodilating agents induce relaxation of mesangial cells, in part through cGMP-mediated activation of large calcium-activated potassium channels (BKCa). Normally quiescent in cell-attached patches, the response of BKCa to nitric oxide, atrial natriuretic peptide, and dibutyryl cGMP (Bt2cGMP) is characterized by a biphasic increase and then decrease (“rundown”) in open probability. Using the patch-clamp method in conjunction with phosphatase inhibitors, we investigated whether the run-down phase was the result of dephosphorylation by an endogenous protein phosphatase. In cell-attached patches, cantharidic acid (500 nM), okadaic acid (100 nM), and calyculin A (100 nM), nondiscriminant inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A) at these concentrations, caused a significantly greater and sustained response of BKCa to Bt2cGMP. Within 2 min, the response of BKCa to the combination of cantharidic acid and Bt2cGMP was greater than the response to these agents added separately. Incubation of mesangial cells with okadaic acid for 20 min at a concentration (5 nM) specific for PP2A increased the basal open probability of BKCa and completely inhibited rundown after activation by Bt2cGMP. Incubation with calyculin A (10 nM), a more potent inhibitor of PP1, did not affect BKCa activity. In inside-out patches, Bt2cGMP plus MgATP caused a sustained activation of BKCa that was inhibited by exogenous PP2A but not PP1. It is concluded that either BKCa or a tightly associated regulator of BKCa is a common substrate for endogenous cGMP-activated protein kinase, which activates BKCa and PP2A, which inactivates BKCa in human mesangial cells.

Mesangial cells, which are excitable and have contractile properties similar to smooth muscle, regulate the glomerular filtration rate by modulating the capillary surface area (1–3). Recent patch-clamp studies of human mesangial cells in culture have shown that the mechanism and ion-selective channels involved in maintaining contractile tone are similar or identical to those of vascular smooth muscle (4, 5).

Large calcium-activated potassium channels (BKCa),1 characterized in mesangial cells (5, 6) and vascular smooth muscle (7), are not involved in setting resting potential but respond in a negative feedback manner to agonist-induced increases in contractile tone. Agonists such as angiotensin II elevate intracellular calcium and depolarize the membrane potential, producing an activation of BKCa. The hyperpolarizing membrane potential inhibits further entry of cell calcium by inactivating voltage-gated calcium channels. The gain in this feedback mechanism is increased by smooth muscle relaxants such as nitric oxide and atrial natriuretic peptide that, via cGMP-activated kinase, lower the voltage and calcium thresholds for activating BKCa (8, 9). However, activation of BKCa on cell” by vasorelaxants or Bt2cGMP is followed by an inactivation or rundown phase, in which BKCa returns to base line 20 s after peak activity.

Substrate regulation by phosphorylation is a dynamic balance between the forward kinase phosphorylation and the reverse dephosphorylation by a protein phosphatase. Several studies have now shown that vasorelaxants activate BKCa through guanylyl cyclase and cGMP-dependent protein kinase in both smooth muscle and mesangial cells (9–11); however, the role of protein phosphatase has been addressed only very recently (9, 12). Two such studies on tracheal smooth muscle and neurohypophyseal cells supported the notion that cGMP stimulated BKCa by activating protein phosphatase 2A (12, 13), which activated BKCa by dephosphorylation. In contrast, our laboratory previously showed that the mesangial BKCa was activated by cGMP-activated protein kinase in the presence of okadaic acid, an inhibitor of protein phosphatases 1 and 2A (9, 13), suggesting that these phosphatases inactivated, rather than activated, the mesangial BKCa.

The present studies were performed to elucidate the signaling pathways involved in the rundown phase following activation of BKCa by cGMP-dependent protein kinase. Using the cell-attached configuration and established phosphatase inhibitors, we specifically investigated the specific endogenous protein phosphatase involved in regulating BKCa. Using inside-out patches, we then determined the regulation of BKCa by protein phosphatases 1 and 2A.

EXPERIMENTAL PROCEDURES

Mesangial Cell Cultures—Human mesangial cells were isolated originally by Abboud and co-workers (14) and cultured using standard techniques. Mesangial cells were plated in Waymouth culture medium, pH 7.4, supplemented with 15 mM HEPES buffer, 2.0 mM glutamine, 0.66 mM calcium, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (17% v/v). All experiments were performed using subpassages 6–10. During this generation span, mesangial cells maintained a constant phenotype and typical smooth muscle-like spindle shape (2).

Cells were subcultured onto individual 22 × 22 mm glass coverslips

PP2A, protein phosphatase 2A; ANOVA, analysis of variance.
the effects of cantharidic acid (500 nM), a nonspecific inhibitor

Patch-Clamp Methods—Mesangial cells were prepared for analysis of single BKCa channels using standard patch-clamp techniques previously described (5, 15). Current recordings were made after obtaining gigaohm seals with the patch electrode on the surface of the cell (cell attached) or after withdrawing the patch (excised, inside out). The unitary current (i), defined as zero for the closed state, was determined as the mean of the best fit Gaussian distribution of the amplitude histograms. Channels were considered in an open state when the current was >(n + ½ i) and <(n + ½ i), where n is the maximum number of current levels observed. The probability of a channel existing in an open state (Popen) is defined as the time spent in the open state divided by the total time of the recording. In all cases, Vm represents the holding potential relative to the pipette.

Experimental Design and Solutions—In all cell-attached experiments, the pipette solution contained 140 mM KCl plus 10 mM HEPES buffer, pH 7.4, and the bath solution contained 135 mM NaCl, 5 mM KCl, 1 mM CaCl2, and 10 mM HEPES, pH 7.4. The free Ca2+ concentration of the bath, initially 1.0 mM, was adjusted to lower concentrations by buffering with EGTA as described previously (16). In excised patches, the bathing solution was replaced with 140 mM KCl, 1 mM CaCl2, 10 mM HEPES, pH 7.4. Okadaic acid, cantharidic acid, and calyculin A, established inhibitors of PP1 and PP2A, were used to establish the role of endogenous protein phosphatase in regulation of BKCa. While monitoring BKCa channels in cell-attached patches, these inhibitors were added to the bathing solution in concentrations nonspecific for inhibition of PP1 and PP2A. The holding potential (−Vp) was either 80 mV or 0 mV, which resulted in outward and inward currents, respectively. The effects of these inhibitors on the cGMP-dependent activation of BKCa was determined by adding inhibitor either in the continued presence of 10 μM dibutyryl cGMP (Bt2cGMP) or at least 30 s before the addition of Bt2cGMP. To determine if the endogenous protein phosphatase was either 1 or 2A, cells were incubated in either 5 mM okadaic acid (specific for PP2A) or 10 mM calyculin A for 10–30 min before obtaining a seal. Specific inhibition of BKCa by either exogenous PP2A or PP1 was determined in inside-out patches with 140 mM KCl in the bath and holding potentials of either 40 mV or −40 mV for outward or inward currents, respectively. BKCa were activated either by Bt2cGMP plus MgATP at a holding potential of 40 mV or by Bt2cGMP plus MgATP plus cGMP-activated protein kinase at a holding potential of −40 mV. PP1 (1 unit/ml) or PP2A (0.5 unit/ml) was added in the continued presence of Bt2cGMP plus MgATP with or without cGMP-activated protein kinase. Groups were compared for statistical significance using the paired t test or the ANOVA plus the Student-Newman-Keuls test as appropriate. Cyclic GMP-activated kinase was purchased from Promega. All other chemicals used in this study were purchased from Sigma or Calbiochem.

RESULTS
Effects of Pharmacological Inhibitors of Protein Phosphatases on Rundown of cGMP-activated BKCa—Experiments were performed to determine if the rundown phase after adding Bt2cGMP was the result of either PP1 or PP2A. Fig. 1A shows the effects of cantharidic acid (500 nM), a nonspecific inhibitor of PP1 and PP2A, on the rundown of Bt2cGMP-activated BKCa in cell-attached patches in the absence of a phosphatase inhibitor (upper tracing) and with the simultaneous addition of cantharidic acid (lower tracing). The Popen of BKCa increased from <0.01 to 0.85 after 10 s with the addition of 10 μM Bt2cGMP and then returned to base line in the next 90 s; after 2 min the Popen was <0.01. However, when Bt2cGMP and cantharidic acid were added together, BKCa was activated to a Popen that was sustained at 0.78 after 2 min. Note that the channel amplitude diminished after addition of cantharidic acid. A decrease in the amplitude of BKCa is the result of a decrease in electrochemical potential due to the combination of a decrease in the intracellular potassium concentration and the hyperpolarization of the membrane potential as BKCa is activated in the cell membrane (8, 17).

FIG. 1. Results of cell-attached experiments showing the separate and additive effects of Bt2cGMP (DB-cGMP) and cantharidic acid on the Popen of BKCa. The pipette solutions contained 140 mM KCl, and the bathing solutions were physiological salt solutions (−Vp = 80 mV). Outward currents are positive. Arrows denote the closed state. Tracings showing the effects on BKCa when adding 10 μM Bt2cGMP (DB-cGMP, upper tracing) and 10 μM Bt2cGMP plus 500 nM cantharidic acid (lower tracing) to the bathing solution. The Popen of BKCa was increased by the addition of 10 μM Bt2cGMP from <0.01 to 0.85 after 10 s, after which it returned to base line during the next 90 s and fell to <0.01 after 2 min. The combination of Bt2cGMP plus cantharidic acid activated BKCa to a Popen of 0.78 after 2 min. Note a decrease in channel amplitude due to a decrease in electrochemical driving force for BKCa. Distinct (inward) currents are shown when command potential was changed to 0 mV. B, tracings illustrating the effects on BKCa at 80 and 0 mV on the addition of cantharidic acid followed by cantharidic acid plus Bt2cGMP to the bathing solution. In the control, BKCa were quiescent at 0 mV, and the Popen was <0.001 at 80 mV. Approximately 60 s after the addition of 500 nM cantharidic acid, the Popen increased to 0.084 and 0.005 at 80 and 0 mV, respectively. In the continued presence of cantharidic acid, the Popen was further increased by Bt2cGMP to a sustained value of 0.40 and 0.035 at 80 and 0 mV, respectively. C, bar graph summarizing the effects of cantharidic acid on the run-down phase (120 s) after activation of BKCa by Bt2cGMP (−Vp = 80 mV). Two minutes after the separate additions of 10 μM Bt2cGMP and 500 nM cantharidic acid (Canth) the open probabilities of BKCa were 0.011 ± 0.006 and 0.16 ± 0.05, respectively. Two minutes after the addition of Bt2cGMP plus cantharidic acid the Popen increased to 0.48 ± 0.10 (n = 5). *p < 0.05 compared with the values for Bt2cGMP and cantharidic acid.
Fig. 2. Effects of 100 nM okadaic acid and 100 nM calyculin A on Bt2cGMP-activated BKCa in cell-attached patches. A (0 mV), BKCa was activated within 5 s from <0.001 to 0.10 in response to 10 μM Bt2cGMP (DB-cGMP) and then returned to base line after approximately 5 more seconds. In the continued presence of Bt2cGMP, the addition of okadaic acid reactivated BKCa, to a greater and sustained value of 0.83 after 120 s. Similar results were obtained with 100 nM calyculin A (B, 80 mV). The P+, of BKCa increased from <0.001 to 0.42 within 5 s after the addition of Bt2cGMP and returned to base line within the next 20 s. However, after the addition of Bt2cGMP plus 100 nM calyculin A, the P+ increased and remained at 0.65. The increase in channel amplitude at 0 mV and the reduction in amplitude at 80 mV shows that the membrane potential is hyperpolarizing as BKCa is activated by either okadaic acid or calyculin A. All other conditions are the same as in Fig. 1.

Fig. 3. Results of cell-attached experiments showing typical tracings (A) and a summary (B) of the activation of BKCa by Bt2cGMP after incubating cells for 20–50 min with 5 nM okadaic acid and 10 nM calyculin A. The holding potential (−Vp) was 80 mV. A, in the upper tracing, the P+ of BKCa was 0.063 on obtaining a seal with cells incubated in okadaic acid. The subsequent addition of 10 μM Bt2cGMP (DB-cGMP) increased the P+ to a sustained value of 0.32. In the lower tracing, the basal P+ of BKCa, from cells incubated in calyculin A was <0.005. After adding Bt2cGMP, P+ increased transiently to 0.217 and then returned to 0.024 after 20 s. B, summary of the activation pattern of BKCa after the addition of Bt2cGMP (10 μM) in the control and presence of okadaic acid (5 nM) and calyculin A (10 nM) for 20 to 50 min (−Vp = 80 mV). The blank bars represent the basal P+, and the cross-hatched and solid bars represent the P+ between 5 and 15 s and between 120 and 140 s, respectively, after the addition of Bt2cGMP. In the control cells, the P+ was increased by Bt2cGMP to a value of 0.19 ± 0.04 after 5 s and then ran down to base line after 120 s (n = 6). In the presence of 5 nM okadaic acid, the basal P+ was 0.18 ± 0.07 (n = 4) and increased to 0.29 ± 0.05 and 0.25 ± 0.05 after 5 s and 120 s, respectively. After incubation with calyculin A, the P+ (n = 4) increased from 0.002 ± 0.001 to 0.20 ± 0.05 after 5 s and returned near the base line value (0.008 ± 0.006) after 120 s. The asterisks denote significant (p < 0.05) increases in basal and Bt2cGMP (120 s) when compared with the control and calyculin A groups using the ANOVA plus the Student-Newmann-Keuls test.
Fig. 4. Effects of protein phosphatases on Bt_cGMP-activated BKCa in inside-out patches. The holding potential (\( -V_h \)) was 40 mV. The pipette and bathing solutions contained symmetrical 140 mM KCl. A, the continuous tracing shows the activating response from 0.029 in the control to 0.332 with 10 \( \mu \)M Bt_cGMP (DB-cGMP) plus 0.1 mM MgATP (top). The lower tracing shows the subsequent inactivation to 0.098 by 0.5 unit/ml PP2A. B, summary of the effects of PP2A and PP1 (1 unit/ml) on Bt_cGMP-activated BKCa. PP2A significantly (\( p < 0.025, n = 5 \)) decreased the \( P_o \) of BKCa from 0.57 ± 0.11 to 0.40 ± 0.14. The \( P_o \) of BKCa before and after the addition of PP1 was 0.66 ± 0.11 and 0.70 ± 0.13, respectively (not significant, \( n = 5 \)).

was active (\( P_o = 0.063 \)) after obtaining the seal at 80 mV. The addition of Bt_cGMP increased the \( P_o \) further to a sustained value of 0.32. As shown in Fig. 3B, BKCa was relatively quiescent (\( P_o < 0.005 \)) after the addition of calyculin A and was activated transiently to 0.227 on the addition of Bt_cGMP. This rundown to base line approximately 20 s after peak activation was similar to control experiments (see Fig. 1A). These results are summarized in Fig. 3B. In control cells, the \( P_o \) of BKCa was increased by Bt_cGMP to a value of 0.19 ± 0.04 after 5 s and ran down to base line after 120 s (\( n = 6 \)). After incubation with okadaic acid, the baseline \( P_o \) was 0.18 ± 0.07 and increased to 0.29 ± 0.05 and 0.25 ± 0.05 after 5 and 120 s, respectively (\( n = 4 \)). After incubation with calyculin A (\( n = 4 \)), the \( P_o \) increased from a basal of 0.002 ± 0.001 to 0.20 ± 0.05 after 5 s and returned near the base line value (0.008 ± 0.006) after 120 s. Using the ANOVA plus the Student-Newman-Keuls test, the effects of okadaic acid at basal and Bt_cGMP (120 s) were significantly greater than the respective \( P_o \) values for control and calyculin A. These results indicate that BKCa are maintained quiescent in cell-attached patches by endogenous PP2A. Moreover, specific inhibition of PP2A (by 5 nM okadaic acid) prevents the rundown after activation of BKCa by Bt_cGMP in cell-attached patches.

Effects of Exogenous PP2A and PP1—The inside-out patch configuration was used to determine the effects of exogenous PP2A and PP1 on BKCa. Fig. 4 shows the inactivation of cGMP-activated BKCa by PP2A. As shown in the continuous tracing of Fig. 4A, dibutyryl cyclic GMP plus MgATP activated BKCa from 0.029 to 0.332, and the subsequent addition of PP2A inactivated BKCa to 0.098. However, there was no effect of PP1 on cGMP-activated BKCa (not shown). These data are summarized in the bar graph of Fig. 4B. In each experiment, after activation of BKCa by Bt_cGMP plus MgATP in either the absence or presence of cGMP-activated protein kinase, PP2A (\( n = 5 \)) decreased the \( P_o \) from 0.57 ± 0.11 to 0.40 ± 0.14 (\( p < 0.025 \), paired \( t \) test). However, the \( P_o \) was 0.66 ± 0.11 and 0.70 ± 0.13 (\( n = 3 \); not significant) before and after the addition of PP1.

**DISCUSSION**

This study further defined the signal transduction pathways for regulating BKCa channels in a contractile cell. Three phosphatase inhibitors, in concentrations that inhibit both PP1 and PP2A, caused a larger and more sustained increase in open probability of BKCa in response to Bt_cGMP, the second messenger mediator for relaxation by nitric oxide and atrial natriuretic peptide. Basal open probability of BKCa was increased by okadaic acid in concentrations specific for PP2A but not by calyculin A, a more potent inhibitor of PP1, indicating that endogenous PP2A but not PP1 was maintaining BKCa in a dephosphorylated quiescent state. It was shown that exogenous PP2A, but not PP1, applied to the cytosolic side of BKCa in inside-out patches can specifically inhibit the activation of BKCa by cGMP-dependent protein kinase.

**Biphasic Response of BKCa to cGMP**—In both smooth muscle and mesangial cells, BKCa are activated by cGMP-dependent protein kinase (7, 9). However, after activation by Bt_cGMP in cell-attached patches, the open probability of the mesangial BKCa rapidly runs down to baseline levels. The present study shows that the run-down phase is due to the presence of PP2A, which would dephosphorylate BKCa. Phosphatase-induced channel rundown has been more commonly described for channels in excised patches. Kubokawa et al. (21) found that renal BKCa channels rundown in excised patches due primarily to the presence of PP2A. However, phosphatase-induced rundown is not only found in excised patches; it was also shown that okadaic acid prevents rundown of Ba2+ current (whole cell) in dissociated helix neurons (22).

It is not understood why the effects of cGMP-dependent kinase are transient and ultimately overcome by a phosphatase that presumably dephosphorylates and inactivates BKCa despite the continued presence of Bt_cGMP. However, several mechanisms could be involved in the temporary inhibition and then activation of a protein phosphatase to initiate the rundown phase. A similar type of biphasic activation was demonstrated for Ca2+/calmodulin-dependent protein kinase II, also a substrate for PP2A (23). An increase in intracellular Ca2+ in the rat brain is accompanied by a sequential autophosphorylated increase and then decrease in phosphorylation level of Ca2+/calmodulin-dependent protein kinase II. The decrease in phosphorylation was blocked by 1 mM okadaic acid. It was suggested by these authors that an increase in intracellular calcium autophosphorylated a serine/threonine protein kinase (described by Guo et al. (24)) that would temporarily phosphorlylate and inhibit PP2A. In time, PP2A would autodephosphorylate and inactivate Ca2+/calmodulin-dependent protein kinase II. A similar mechanism may be involved whereby cGMP temporally activates an inhibitor of PP2A. Although a recent study described inhibition of PP1 by cGMP-dependent protein kinase (25), a cGMP-activated inhibitor of PP2A has not been described.

**Regulation of BKCa by Phosphatases**—Although several studies have described regulation of ion-selective channels by cAMP- and cGMP-dependent protein kinases, the reversal of channel phosphorylation by phosphoprotein phosphatases has been investigated only recently (26–31). The present study is one of a few that have now implicated PP2A as a physiological regulator of BKCa channels (12, 13, 32). However, for cGMP-activated protein kinase-activated BKCa, at least two previous studies using three different cell types (12, 13) have shown...
either that PP2A activates BK\textsubscript{Ca} or cGMP-activated protein kinase does not activate BK\textsubscript{Ca} in the presence of inhibitors of PP2A. These results contrast with our study which showed that BK\textsubscript{Ca} was inactivated by PP2A and activated by either cGMP-activated protein kinase or inhibitors of PP2A.

Our disparate results may be explained by another study by Reinhart et al. (32) who have shown that BK\textsubscript{Ca} from the brain expresses two types of channels in planar bilayers with respect to PP2A regulation. Type 1 channels are activated by cAMP-activated protein kinase and inactivated by PP2A. Type 2 BK\textsubscript{Ca} channels are inactivated by cAMP-activated protein kinase and activated by PP2A. Although this was a protein kinase A and not a GMP-activated protein kinase-activated mechanism, it is possible that there are two types of BK\textsubscript{Ca} with respect to regulation of phosphorylation and dephosphorylation by cGMP-activated kinase. However, these type 2 BK\textsubscript{Ca} channels have not been observed in mesangial cells.\textsuperscript{2} When the properties of the PP2A-activated (type 2) channels in tracheal smooth muscle (12) are compared with the mesangial BK\textsubscript{Ca} of this study, we find that the reported single channel conductance (in symmetrical 140 mM KCl) of the tracheal BK\textsubscript{Ca} (257 ± 13 picosiemens) is somewhat larger than the mesangial BK\textsubscript{Ca} (206 ± 18 picosiemens, see Ref. 5). However, voltage-gated activation response in 1 mM Ca\textsuperscript{2+} is similar (see Refs. 5 and 12). It remains to be determined if these are distinct isoforms of BK\textsubscript{Ca}.

The dominant type of BK\textsubscript{Ca} in smooth muscle could be determined by the contractile response to low concentrations of okadaic acid. Since PP1 dephosphorylates myosin light chain kinase, it would be expected that okadaic acid in concentrations expected to induce contractions. If type 1 channels predominated, it would be expected that okadaic acid in concentrations of 1–10 nM would inhibit smooth muscle contraction. That concentrations of okadaic acid would induce smooth muscle contraction if type 2 channels predominate. In support of type 1 channels, at least two studies have shown that low concentrations of okadaic acid relax vascular smooth muscle (34, 35). Although the kinase specific for activation of the mesangial BK\textsubscript{Ca} appears identical to smooth muscle, the effects of okadaic acid on mesangial contraction have not been determined. It would not be surprising if BK\textsubscript{Ca} is regulated by different mechanisms in different cell types. Tseng-Crank et al. (36) found that HSLO, the human gene encoding the calcium-activated potassium channel, contains multiple splice variants in the brain. It was recently found that the mesangial BK\textsubscript{Ca} contains at least two of these variants of HSLO (37). It is therefore feasible that the differential expression of these variants would confer different sites for regulation by kinases and phosphatases.

In summary, we have demonstrated that the mesangial BK\textsubscript{Ca} is not regulated by an all-or-none phosphorylation or dephosphorylation mechanism but rather by a dynamic enzymatic balance between cGMP-activated protein kinase and protein phosphatase 2A, which specifically activate and deactivate BK\textsubscript{Ca}, respectively. It remains to be established, however, if there are multiple molecular variants of BK\textsubscript{Ca} that are differentially modulated by kinase/phosphatase signal transduction mechanisms.

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