Calcium-dependent inhibition of adrenal TREK-1 channels by angiotensin II and ionomycin

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Enyeart JJ, Liu H, Enyeart JA. Calcium-dependent inhibition of adrenal TREK-1 channels by angiotensin II and ionomycin. Am J Physiol Cell Physiol 310: C619–C629, 2011.—Bovine adrenocortical cells express bTREK-1 K⁺ (bovine KCNK2) channels that are inhibited by ANG II through a Gq-coupled receptor by separate Ca²⁺ and ATP hydrolysis-dependent signaling pathways. Whole cell and single patch clamp recording from adrenal zona fasciculata (AZF) cells were used to characterize Ca²⁺-dependent inhibition of bTREK-1. In whole cell recordings with pipette solutions containing 0.5 mM EGTA and no ATP, the Ca²⁺ ionophore ionomycin (1 μM) produced a transient inhibition of bTREK-1 that reversed spontaneously within minutes. At higher concentrations, ionomycin (5–10 μM) produced a sustained inhibition of bTREK-1 that was reversible upon washing, even in the absence of hydrolyzable [ATP]. BAPTA was much more effective than EGTA at suppressing bTREK-1 inhibition by ANG II. When intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was buffered to 20 nM with either 11 mM BAPTA or EGTA, ANG II (10 nM) inhibited bTREK-1 by 12.0 ± 4.5% (n = 11) and 59.3 ± 8.4% (n = 4), respectively. Inclusion of the water-soluble phosphatidylinositol 4,5-bisphosphate (PIP₂) analog DiC₈PI(4,5)P₂ in the pipette failed to increase bTREK-1 expression or reduce its inhibition by ANG II. The open probability (Pₒ) of unitary bTREK-1 channels recorded from inside-out patches was reduced by Ca²⁺ (10–35 μM) in a concentration-dependent manner. These results are consistent with a model in which ANG II inhibits bTREK-1 K⁺ channels by a Ca²⁺-dependent mechanism that does not require the depletion of membrane-associated PIP₂. They further indicate that the Ca²⁺ source is located in close proximity within a “Ca²⁺ nanodomain” of bTREK-1 channels, where [Ca²⁺]ᵢ may reach concentrations of >10 μM. bTREK-1 is the first two-pore K⁺ channel shown to be inhibited by Ca²⁺ through activation of a G protein-coupled receptor.

adrenocortical; potassium channel; ionomycin; phosphatidylinositol 4,5-bisphosphate; nanodomain

BOVINE ADRENOCORTICAL CELLS including cortisol-secreting zona fasciculata (AZF) cells and aldosterone-secreting zona glomerulosa (AZG) cells express bTREK-1 (bovine KCNK2) leak-type K⁺ channels that function pivotally in the physiology of corticosteroid hormone secretion (14, 17, 18, 35). These two-pore, four-transmembrane spanning (2P/4TMS) K⁺ channels set the resting membrane potential of bovine adrenocortical cells and are inhibited by ANG II at concentrations identical to those that trigger membrane depolarization and corticosteroid secretion (14, 35).

The signaling pathways that couple ANG II receptors to bTREK-1 inhibition are complex and only partially understood. In both AZF and AZG cells, ANG II inhibits bTREK-1 through activation of a Gq-coupled AT1 receptor (14, 35, 36). AT1 receptors in these adrenocortical cells have been linked to the activation of multiple signaling pathways (28, 29, 45, 50). However, the Gq-dependent activation of PLCβ, leading to the hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP₂) and the production of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), is the principal signaling mechanism initiating ANG II responses (46).

The activation of PLCδ through multiple Gq-coupled receptors regulates the activity of many neuronal K⁺ channels, including TREK-1, through any of several molecular mechanisms. In particular, M-type K⁺ channels can be inhibited by Ca²⁺ release through IP₃-activated channels of the endoplasmic reticulum (ER) or by depletion of membrane-associated PIP₂ (13, 25, 52, 53). The importance of PLC-induced PIP₂ degradation and IP₃ generated Ca²⁺ release to the inhibition of neuronal TREK-1 channels is controversial. Although one study reported that the inhibition of TREK-1 through PLC-coupled receptors is mediated by the hydrolysis-dependent depletion of PIP₂ (32), others have found that inhibition occurs through different mechanisms (7, 39). Interestingly, none of these studies have reported inhibition of TREK-1 K⁺ channels by Ca²⁺.

In this regard, we have shown that ANG II inhibits native TREK-1 channels in bovine adrenocortical cells by separate Ca²⁺- and ATP hydrolysis-dependent signaling pathways (15, 22, 31). The ATP hydrolysis-dependent inhibition of bTREK-1 by ANG II occurs through a novel mechanism that is independent of PLCδ and its downstream effectors (31). In contrast, the Ca²⁺-dependent pathway for bTREK-1 inhibition by ANG II is independent of ATP hydrolysis, blunted by the PLC antagonist U73122 and blocked by strongly buffering intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) to low levels with 11 mM BAPTA (15).

Separating the effects of increasing [Ca²⁺]ᵢ and PIP₂ depletion on ANG II inhibition of bTREK-1 is complicated by the fact that PLCδ activity is enhanced by Ca²⁺, which could lead to a positive feedback loop fueled by IP₃-stimulated Ca²⁺ release and culminating in bTREK-1 inhibition through the depletion of PIP₂ (27). In the present study, we have investigated the roles of Ca²⁺ and PIP₂ in the regulation of bTREK-1 activity in bovine AZF cells by ionomycin and ANG II.

MATERIALS AND METHODS

Tissue culture media, antibiotics, and fetal bovine sera (FBS) were obtained from Invitrogen (Carlsbad, CA). Coverslips were from Bellco (Vineland, NJ). Collagenase, DNase, phosphate-buffered saline (PBS), bovine plasma fibronectin, tocopherol, selenite, ascorbic acid, BAPTA, EGTA, MgATP, NaUTP, ionomycin, ANG II, and AMP-PNP were obtained from Sigma (St. Louis, MO). Penfluridol was obtained from Janssen Pharmaceutical (Beerse, Belgium). Phosphatidylinositol(4,5) bisphosphate diC₈ [DiC₈PI(4,5)P₂], a water-
soluble derivative of PIP2 was purchased from Echelon Biosciences (Salt Lake City, UT). It was dissolved, aliquoted, and stored at −80°C. Before experiments, a new aliquot was thawed and diluted in the pipette solution, which was used only on that day.

Isolation and culture of AZF cells. Bovine adrenal glands were obtained from steers (age 2–3 yr) at a local slaughterhouse. Isolated AZF cells were obtained and prepared as previously described (16). After isolation, cells were either resuspended in DMEM/F12 (1:1) and plated for immediate use, or resuspended in FBS/5% DMSO, divided into 1-ml aliquots, and stored in liquid nitrogen for future use. For patch clamp experiments, cells were plated in DMEM/F12+ in 35-mm dishes containing 9 × 9-mm2 glass coverslips. To ensure cell attachment, coverslips were treated with bovine plasma fibronectin (10 µg/ml) at 37°C for 30 min and then rinsed with warm, sterile PBS immediately before addition of cells. Cells were maintained at 37°C in a humidified atmosphere of 95% air-5% CO2.

Patch clamp experiments. Patch clamp recordings of K+ channel currents were made in the whole cell and inside-out patch configuration from bovine AZF cells. Although the results reported in this study were obtained by recording currents from AZF cells, preliminary recordings from AZG cells showed no difference in bTREK-1 currents with respect to modulation by ANG II. For whole cell recordings, the standard external solution consisted of (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, and 5 glucose, with pH adjusted to 7.3 using NaOH. The standard pipette solution consisted of (in mM) 120 KCl, 2 MgCl2, 10 HEPES, and 0.2 GTP, with pH titrated to 6.8 using KOH. The buffering capacity of pipette solutions was varied by adding combinations of Ca2+ and BAPTA or EGTA using the Bound and Determined software program (4). Nucleotides, including MgATP, NaUTP, and AMP-PNP, were added to pipette or bath solutions as noted in the text. For inside-out patch recordings, the standard external and pipette solutions used for whole cell recordings were switched. Statistical analysis (Table 1 data) was performed using the unpaired Student’s t-test.

Table 1. Effect of BAPTA, EGTA, and [Ca2+]i on bTREK-1 inhibition by ANG II

<table>
<thead>
<tr>
<th>Pipette Solution</th>
<th>% Block by 10 nM ANG II</th>
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<tr>
<td>11 mM BAPTA, 20 mM Ca2+</td>
<td>12.0 ± 4.5 (n = 11)</td>
</tr>
<tr>
<td>11 mM BAPTA, 100 mM Ca2+</td>
<td>51.7 ± 2.0 (n = 4)</td>
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<tr>
<td>11 mM BAPTA, 200 mM Ca2+</td>
<td>50.5 ± 5.4 (n = 9)</td>
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<tr>
<td>11 mM EGTA, 20 mM Ca2+</td>
<td>59.3 ± 8.4 (n = 4)</td>
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<tr>
<td>11 mM EGTA, 100 mM Ca2+</td>
<td>81.4 ± 1.6 (n = 5)</td>
</tr>
<tr>
<td>30 mM EGTA, 20 mM Ca2+</td>
<td>36.9 ± 9.6 (n = 4)</td>
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Patch clamp amplifier. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve. Pulse generation and data acquisition were done using a personal computer and PCLAMP software with TL-1 interface (Axon Instruments, Burlingame, CA). Currents were digitized at 2–10 kHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using summed scaled hyperpolarizing steps of one-half to one-fourth pulse amplitude. Data were analyzed and histograms generated using PCLAMP (CLAMPFIT 9.2, FETCHAN 6.04, and PSTAT 6.04) and SigmaPlot (version 11.0) software.

RESULTS

Transient and sustained inhibition of bTREK-1 by ionomycin. Bovine AZF cells express both voltage-gated, rapidly inactivating Kv1.4 and noninactivating bTREK-1 K+ channels (18, 35, 37). In whole cell patch clamp recordings, bTREK-1 K+ current typically increases with time to a stable maximum amplitude (35). The absence of time- and voltage-dependent inactivation allows bTREK-1 to be isolated using either of two voltage clamp protocols. When voltage steps of several hundred milliseconds duration are applied from a holding potential of −80 mV, bTREK-1 can be measured near the end of the voltage step when the transient Kv1.4 current has completely inactivated (Fig. 1, A and B, left traces). Alternatively, bTREK-1 current can be selectively activated by an identical voltage step applied immediately after a 10-s prepulse to −20 mV that fully inactivates Kv1.4 (Fig. 1, A and B, right traces).

Previously, we showed that when whole cell recordings of bTREK-1 were made with [Ca2+]i, strongly buffered by 11 mM BAPTA, the Ca2+ ionophore ionomycin at concentrations as high as 10 µM failed to inhibit bTREK-1 (22). However, when whole cell recordings of K+ currents were made with pipette solutions in which [Ca2+]i was weakly buffered to 20 mM with 0.5 mM EGTA, ionomycin inhibited bTREK-1 with distinctive concentration-dependent characteristics. At a concentration of 1 µM, ionomycin produced a rapid inhibition of bTREK-1, after which this current spontaneously recovered to a new steady-state amplitude (Fig. 1, A and B). The maximum transient inhibition occurred within 2 min, reducing bTREK-1 amplitude by an average of 47.7 ± 6.1% (n = 8). After the maximum inhibition of bTREK-1 was achieved, the current returned to an amplitude that was 22.7 ± 7.2% (n = 8) less than the original value (Fig. 1, A–C). Recovery occurred in the continued presence of ionomycin through a process that could be fit with a single exponential time constant of 0.79 ± 0.20 min (n = 8). At concentrations of 5 µM and above, ionomycin produced near total and sustained inhibition of bTREK-1 (Fig. 1, B and C).

The effective inhibition of bTREK-1 by this Ca2+ ionophore suggests a mechanism involving the direct interaction of Ca2+ with bTREK-1 or a channel-associated protein. However, since PLCβ is a Ca2+-dependent enzyme, it is possible that ionomycin could inhibit bTREK-1 by stimulating PLCβ-dependent hydrolysis and depletion of PIP2 (27). In this regard, the transient nature of the inhibition produced by 1 µM ionomycin argues strongly against a role for PIP2 depletion in TREK-1 inhibition. If bTREK-1 inhibition by 1 µM ionomycin were mediated by PIP2 depletion, the observed spontaneous reversal of this inhibition would require the synthesis of this phosphoinositide from phosphatidyl-inositol (PI) by PI4 kinase and...

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PL(4)P5 kinases (21). However, our recordings were made with pipette solutions that contained 2 mM UTP in place of ATP, thus eliminating potential PIP2 synthesis, and bTREK-1 recovery by this mechanism.

The larger, sustained inhibition of bTREK-1 produced by ionomycin at higher concentrations also appeared to be independent of PIP2 hydrolysis. In the experiment illustrated in Fig. 2A, bTREK-1 was recorded with a pipette containing the nonhydrolyzable ATP analog AMP-PNP. Ionomycin (10 μM) produced near-complete inhibition of bTREK-1, and this effect was fully reversed upon washing with saline (Fig. 2A). Full recovery of bTREK-1 current was achieved in each of three similar experiments. Overall, inhibition of bTREK-1 by ionomycin and its reversal appear to be independent of PIP2 hydrolysis and synthesis in these experiments.

At a concentration of 5 μM, ionomycin produced a nearly total sustained inhibition of bTREK-1 (Fig. 1, B and C). In contrast to its effect on bTREK-1 current, ionomycin failed to significantly reduce the amplitude of the voltage-gated Kv1.4 current in these experiments. In the experiment illustrated in Fig. 2B, almost no bTREK-1 current was present upon initiating whole cell recording, allowing Kv1.4 to be viewed and measured in isolation (trace 1). After bTREK-1 grew to a stable amplitude, cells were superfused with ionomycin (1 or 5 μM). A and B: K+ currents were recorded with (right traces) or without (left traces) depolarizing prepulses. bTREK-1 amplitude recorded with (open circles) or without (closed circles) prepulses are plotted against time at right. Numbers on traces correspond to those on plot. C: summary of experiments as in A and B. Bars indicate percent of bTREK-1 remaining after either transient or final block by ionomycin (1 μM), and final block of bTREK-1 by 5 μM ionomycin. Values are means ± SE for the indicated number of determinations in parentheses.
indicate that Ca\(^{2+}\) sources and sensors exist in “nanodomains” separated by 20–50 nm. Processes that are equally sensitive to BAPTA and EGTA are indicative of “Ca\(^{2+}\)-microdomains” where sources and sensors are separated by larger distances of up to several hundred nanometers. The peak [Ca\(^{2+}\)]\(_i\) in microdomains is about 10-fold less that that in nanodomains where [Ca\(^{2+}\)]\(_i\) may reach 50–100 \(\mu\)M (1, 20, 40).

In whole cell recordings from AZF cells, we previously showed that when Ca\(^{2+}\) was weakly buffered by 0.5 mM EGTA, ANG II inhibited bTREK-1 even in the absence of hydrolyzable ATP. In contrast, when [Ca\(^{2+}\)]\(_i\) was strongly buffered with 11 mM BAPTA, ANG II was ineffective (15). In the present study, we compared the inhibition of bTREK-1 by ANG II in whole cell recordings from AZF cells with [Ca\(^{2+}\)]\(_i\) buffered to 20 nM by either 11 mM BAPTA or 11 mM EGTA. BAPTA was far more effective than EGTA at suppressing ANG II-mediated inhibition of bTREK-1. With [Ca\(^{2+}\)]\(_i\) buffered to 20 nM by BAPTA, ANG II inhibited bTREK-1 by only 12.0 ± 4.5% (n = 11). By comparison with [Ca\(^{2+}\)]\(_i\) buffered by EGTA, ANG II inhibited bTREK-1 by 59.3 ± 8.4% (n = 4) (Fig. 3, A and B, and Table 1).

The remarkable effectiveness of BAPTA in suppressing ANG II-mediated bTREK-1 inhibition, combined with the ineffectiveness of EGTA under the same experimental conditions, suggests that the Ca\(^{2+}\) source mediating TREK-1 inhibition is located nearby within a “Ca\(^{2+}\)-nanodomain.” When these experiments were repeated with [Ca\(^{2+}\)]\(_i\) increased from 20 to 100 nM, ANG II (10 nM) was significantly more effective at inhibiting bTREK-1 in the presence of either BAPTA or EGTA. Specifically, with [Ca\(^{2+}\)]\(_i\) buffered to 100 nM by BAPTA and EGTA, ANG II inhibited bTREK-1 by 51.7 ± 2.0% (n = 4) and 81.4 ± 1.6% (n = 5), respectively (Fig. 3, C and D, and Table 1).

In addition, bTREK-1 inhibition by ANG II in the presence of EGTA was accelerated by increasing [Ca\(^{2+}\)]\(_i\) to 100 nM (Fig. 3D). The time-dependent inhibition of bTREK-1 observed with EGTA in the pipette could be fit with a time constant of 2.81 ± 0.12 min (n = 5). These kinetics of inhibition are consistent with a model in which ANG II induces a large increase in [Ca\(^{2+}\)], that, over equal increments of time, inhibits a constant fraction of the remaining active bTREK-1 channels. The accelerated inhibition of
bTREK-1 by ANG II that occurred in the presence of EGTA compared with BAPTA (Fig. 3, C and D) likely reflects the 150-fold difference in their binding rate constants for Ca\(^{2+}\) (40). Increasing the EGTA concentration from 11 to 30 mM with [Ca\(^{2+}\)]\(_i\) buffered to 20 nM blunted bTREK-1 inhibition by ANG II. However, even at this higher concentration, EGTA was far less effective than BAPTA at suppressing TREK-1 inhibition by ANG II (Table 1).

**Effect of PIP\(_2\) on Ca\(^{2+}\)-dependent TREK-1 expression and inhibition by ANG II.** The activation of Gq-coupled AT\(_1\) receptors on AZF cells by ANG II is coupled to the activation of PLC\(_\beta\) and the hydrolysis of membrane-associated PIP\(_2\), producing IP\(_3\) and DAG (28) (Fig. 6). The activation of PLC\(_\beta\) by Gq-coupled receptors is enhanced at elevated [Ca\(^{2+}\)], raising the possibility that depletion of membrane-associated PIP\(_2\) contributes to the greater TREK-1 inhibition by ANG II at higher [Ca\(^{2+}\)] (27). To test this possibility, we measured bTREK-1 inhibition by ANG II with [Ca\(^{2+}\)]\(_i\) buffered to 200 nM with 11 mM BAPTA in the absence and presence of DIC\(_8\)PI(4,5)P\(_2\). The addition of DIC\(_8\)PI(4,5)P\(_2\) (40 \(\mu\)M) to the pipette solution failed to alter TREK-1 inhibition by ANG II (Fig. 4, A–D). Overall, ANG II inhibited TREK-1 by 50.5 ± 5.4% \((n = 9)\) and 51.7 ± 4.7% \((n = 7)\) in the absence and presence of DIC\(_8\)PI(4,5)P\(_2\).
Fig. 4. Effect of the water-soluble PIP2 analog DIC8PI(4,5)P2 on bTREK-1 expression and inhibition by ANG II. bTREK-1 K⁺ currents were recorded from bovine AZF cells at 30-s intervals in response to voltage steps to +20 mV applied from a holding potential of −80 mV with or without a 10-s prepulse to −20 mV. When bTREK-1 reached a stable amplitude, cells were superfused with saline containing ANG II (10 nM). [Ca²⁺]ᵢ in pipette solutions was buffered to 200 nM with 11 mM BAPTA. Pipette solutions were supplemented with 2 mM UTP (A), 2 mM AMP-PNP (C), or 2 mM DIC8PI(4,5)P2 (B). When bTREK-1 reached a stable amplitude, cells were superfused with saline containing ANG II (10 nM). [Ca²⁺]ᵢ in pipette solutions was buffered to 200 nM with 11 mM BAPTA. Pipette solutions were supplemented with 2 mM UTP (A), 2 mM AMP-PNP (C), or 2 mM DIC8PI(4,5)P2 (B). A–C: K⁺ current traces recorded with (right traces) and without (left traces) depolarizing prepulses. bTREK-1 amplitude recorded with (open circles) or without (closed circles) prepulses are plotted against time. Numbers on traces correspond to those on the plot.

D: summary of experiments as in A, B, and C. Bars indicate percentage of bTREK-1 final block by ANG II (10 nM). E: summary of bTREK-1 current densities (pA/pF) as in A and B. Values in D and E are means ± SE for the indicated number of determinations in parentheses.
respectively. DIC₈P(4,5)P₂ also failed to enhance the expression of bTREK-1 in these same experiments.

The mean maximum bTREK-1 current density was almost identical in recordings made in the absence of presence of this compound (Fig. 4E). Also, inhibition of bTREK-1 by ANG II in these experiments was similar when pipette solutions contained either UTP or the nonhydrolyzable ATP analog AMP-PNP (Fig. 4D).

Concentration-dependent inhibition of unitary bTREK-1 currents by Ca²⁺. Results of whole cell patch clamp experiments with ionomycin and ANG II suggested that Ca²⁺ inhibits bTREK-1 by a direct interaction with the channel or a channel-associated protein. The results were also consistent with a model in which Ca²⁺-dependent inhibition of bTREK-1 by ANG II involves Ca²⁺ flux through IP₃-activated channels located within a “Ca²⁺ nanodomain.” Peak [Ca²⁺], near Ca²⁺ channels may reach concentrations up to 100 μM (1, 20).

The potency of Ca²⁺ in inhibiting bTREK-1 channels was determined in single channel recordings from excised inside-out patches of AZF cell membrane patches. In these experiments, membrane patches were excised into an external solution where [Ca²⁺] was buffered to 20 nM and the holding potential was set to −40 mV (inside negative), a potential where Kv.14 channels are inactivated. Voltage steps were applied to a test potential of +20 mV. Under these conditions, bTREK-1 activity typically increased continuously over many minutes of recording.

The experiment illustrated in Fig. 5 shows recordings made from a single membrane patch that contained at least five active channels. Histogram analysis of unitary current amplitudes showed a major peak with a mean of 2.62 ± 0.2 pA and several other peaks with means at approximate multiples of this value (Fig. 5E). Superfusion of the membrane patch with solutions containing 10, 20, and 35 μM Ca²⁺ reversibly inhibited bTREK-1 open probability (Pₒ) by 53.7, 78.6, and 93.6%, respectively (Fig. 5, A–C). In most experiments, we were successful in applying Ca²⁺ to a membrane patch at only one of these three concentrations. In four experiments, 10 μM Ca²⁺ inhibited bTREK-1 Pₒ by 65.7 ± 8.9% (n = 4) (data not shown). At [Ca²⁺] less than 10 μM, the spontaneous increase in bTREK-1 channel activity precluded accurate measurement of the inhibition of channel Pₒ.

Previously, we showed that the diphenylbutyl-piperidine penfluridol potently inhibited bTREK-1 with an IC₅₀ of 0.187 μM (23). Penfluridol (5 μM) inhibited Pₒ by 95.1% as would be expected if the patch contained only bTREK-1 channels (Fig. 5D).

DISCUSSION

In this study, we found that ANG II and ionomycin inhibit bTREK-1 K⁺ channels in adrenocortical cells by a Ca²⁺-dependent mechanism that does not require the depletion of PIP₂. Furthermore, the effectiveness of BAPTA compared with EGTA in suppressing ANG II-mediated TREK-1 inhibition is consistent with a model in which the Ca²⁺ source is located within a “Ca²⁺ nanodomain” of this K⁺ channel. These results, along with the concentration-dependent inhibition of unitary bTREK-1 currents by Ca²⁺ in excised inside-out patches, suggest a model in which the Ca²⁺-dependent inhibition of bTREK-1 channels by ANG II is mediated through PLC by the activation of IP₃-dependent Ca²⁺ channels of the ER (Fig. 6). The findings of this study do not exclude the possibility that, under physiological conditions, ANG II-stimulated depletion of PIP₂ could also contribute to bTREK-1 inhibition.

Ionomycin inhibition of bTREK-1. Ionomycin is a mobile ion carrier that transports Ca²⁺ across cell membranes with a one to one stoichiometry (30). The sustained inhibition of bTREK-1 current produced by ionomycin at high concentrations (≥5 μM) is consistent with a model in which this ionophore induces a persistent increase in [Ca²⁺], that produces near-complete inhibition of bTREK-1 activity.

Since bTREK-1 inhibition occurs in the absence of [ATP], the possible involvement of a Ca²⁺-dependent kinase is excluded. However, the inhibition of bTREK-1 by ionomycin under these conditions does not eliminate the possibility that inhibition could be mediated through Ca²⁺-dependent activation of PLCβ and the subsequent hydrolysis and depletion of PIP₂. However, the spontaneous recovery of bTREK-1 current after inhibition by ionomycin (1 μM) and the total reversal upon washing of sustained bTREK-1 block produced by ionomycin at higher concentrations are proof that inhibition was not mediated by depletion of membrane-associated PIP₂. Specifically, in both cases, recovery of bTREK-1 current occurred in the absence of [ATP]. If bTREK-1 inhibition were mediated by PIP₂ depletion, recovery of the current would require PIP₂ synthesis from PI or PI(4)P, catalyzed by PI4- and PI(4)P 5-kinases (21, 47). Although the mechanism that mediates the spontaneous reversal of TREK-1 block by ANG II is unknown, it likely occurs through the Ca²⁺-dependent activation of the Na⁺/Ca²⁺ exchanger in these cells (5, 38). While PLCβ is a Ca²⁺-dependent enzyme whose activity in response to activation of Gq-coupled receptors is potentiated by [Ca²⁺], we have no evidence indicating that ionomycin alone activates PLC in AZF cells.

Ca²⁺, PIP₂, and TREK-1 inhibition by ANG II. In whole cell recordings, the inhibition of bTREK-1 by ANG II was not altered by including DIC₈P(4,5)P₂ in the pipette solution. The failure of DIC₈P(4,5)P₂ to blunt bTREK-1 inhibition by ANG II indicates that the PLC-dependent effect was not mediated by the depletion of PIP₂. These results are consistent with the hypothesis that the PLC-dependent inhibition of bTREK-1 is mediated by IP₃-dependent release of Ca²⁺ from the ER. Although the molecular mechanism for the putative PLC- and Ca²⁺-dependent inhibition of bTREK-1 by ANG II is not known, it is clear that the effect was not mediated by PLC- or Ca²⁺-activated kinases since these experiments were done in the absence of intracellular ATP. Although our results show that depletion of PIP₂ is not necessary for PLC-dependent inhibition of bTREK-1, they do not exclude the possibility that depletion of this phospholipid alone would be sufficient to suppress channel activity.

The addition of DIC₈P(4,5)P₂ to the pipette solution also failed to significantly increase the expression of bTREK-1 in whole cells recordings. Overall, these findings raise questions about the role of PIP₂ in the regulation of bTREK-1 activation and inhibition. In this regard, it has been reported that PIP₂ and other phospholipids activate TREK-1 by binding to a cluster of positively charged amino acids, located on the carboxyl terminal end of the channel (8, 32). However, PIP₂ has also been reported to inhibit these same channels by interacting with a separate TREK-1 site (6).
The seemingly conflicting results concerning TREK-1 modulation by PIP2 could stem from differences in experimental conditions and the observation that the activity of TREK-1 channels is regulated by many phosphate-containing compounds. bTREK-1 channel activity in AZF cells is increased by ATP, UTP, and other nucleotide triphosphates, as well as by inorganic polyphosphates (16, 51). ATP has also been reported to activate TREK-1 channels of rat ventricular myocytes (49).
These results suggest that selected phosphate-containing compounds may activate TREK-1 by interacting with a common site. In this regard, members of each of the major classes of K⁺ channels including voltage-gated channels, inward rectifiers, and 2P/4TMS leak-type channels have all been reported to be modulated by ATP and PIP₂, in some cases competing for identical or related binding sites on the channels (13, 32, 34, 44, 47).

We measured bTREK-1 in whole cell recordings with nucleotides present at millimolar concentrations in the patch electrode. By comparison, in other studies where phospholipids including PIP₂ have been shown to enhance TREK-1 activity, recordings have been made from excised patches in the absence of ATP or other phosphorylated nucleotides (8, 32). It is not known whether the activity of TREK-1 channels is regulated by PIP₂ under physiological conditions where ATP and other phosphate-containing compounds may be present at millimolar concentrations.

Studies describing the modulation of native and cloned TREK-1 channels by G protein-coupled receptors (GPCRs) and PIP₂ hydrolysis have also produced conflicting results. Only one of three studies that explored the modulation of native and cloned TREK-1 channels by PLC-coupled GPCRs reported that inhibition was due to the hydrolysis-dependent depletion of PIP₂ (32). The other studies found that TREK-1 inhibition was mediated by the activation of protein kinase C or the direct effect of DAG on TREK-1 (7, 39). None reported a direct effect of Ca²⁺ on TREK-1.

**Ang II and TREK-1 inhibition by local Ca²⁺ signaling.** For measuring highly localized Ca²⁺ signals in cells, the relative sensitivity to EGTA compared with BAPTA has proven to be a widely used and reliable tool (1, 20). With [Ca²⁺]ᵢ buffered to 20 nM, BAPTA was far more effective than EGTA in suppressing Ang II-mediated inhibition of bTREK-1, indicating that the Ca²⁺ source is located within a “Ca²⁺ nanodomain” of TREK-1 channels. These results are consistent with a model in which bTREK-1 channels, located near IP₃-activated Ca²⁺-permeable channels of the ER, are inhibited when Ang II stimulates the synthesis of IP₃ from PIP₂ through activation of PLCβ (Fig. 6).

Neuronal M-type K⁺ channels are inhibited through selected Gq-coupled GPCRs by this mechanism (12, 13). In neurons, the effectiveness of this mechanism appears to require that the PLC-coupled receptor, where IP₃ is generated, resides in close proximity to the IP₃-activated channels of the ER (13). In bovine AZF cells, it would appear that the Ca²⁺-dependent inhibition of bTREK-1 by Ang II requires that the AT₁ Ang II receptor PLCβ, the IP₃-activated channel, and the bTREK-1 K⁺ channel coexist together in nanodomains, allowing for efficient bTREK-1 inhibition by Ca²⁺.

In this regard, multiple IP₃ receptor subtypes have been identified in bovine adrenocortical cells (2, 41–43). Interestingly, a microsomal IP₃ binding site that copurified in part with the plasma membrane has been characterized (43). This suggests the existence of an IP₃ receptor in bovine adrenocortical cells that could reside within “Ca²⁺ nanodomains” of bTREK-1 K⁺ channels.

Although relatively high concentrations of [Ca²⁺]ᵢ were required to inhibit bTREK-1 K⁺ channels in excised patches, they were consistent with [Ca²⁺]ᵢ that occur within Ca²⁺ nanodomains near the mouths of Ca²⁺-permeable channels (1, 20). When [Ca²⁺]ᵢ was increased to 100 and 200 nM, BAPTA became far less effective at suppressing bTREK-1 inhibition by Ang II. This result suggests that, under conditions that facilitate PLC activation, the Ca²⁺ buffering capacity provided by 11 mM BAPTA (or EGTA) can be overwhelmed, regardless of the speed of buffering. A positive feedback mechanism for M current inhibition driven by cycles of PLC activation and Ca²⁺ release has been described (27).

While it is clear that the inhibition of bTREK-1 channels by Ca²⁺ is independent of protein kinases, the molecular mechanism is unknown. We have identified two putative calmodulin binding sites on the intracellular loop and carboxyl terminal end of bTREK-1 by analyzing the bTREK-1 protein sequence using The Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/cltdb/cltdb/sequence.html). However, it is not known whether calmodulin binds to either site in a constitutive or Ca²⁺-dependent manner. The Ca²⁺ concentrations required to inhibit bTREK-1 in excised patches are higher that those typically associated with calmodulin-mediated responses (9). It is possible that Ca²⁺ could inhibit bTREK-1 more potently in an intact cell where Ca²⁺-binding proteins such as calmodulin would certainly be present.

In addition to bovine AZG cells, Ang II also regulates the activity of K⁺ channels in AZG cells of other species. Rat AZG cells express 2P/4TMS TASK K⁺ channels that are inhibited by Ang II (10, 11). Ang II has also been reported to activate TASK K⁺ channels in guinea pig ventricular cells (48). The ability of Ang II to inhibit TASK K⁺ currents is likely to be of functional importance, since TASK channels contribute to outward currents that help maintain the resting membrane potential.
to inhibit unidentified voltage-gated K⁺ channels in rat AZG cells (33). mRNA and protein for both TREK-1 and TASK-3 channels have been identified in the human adrenocortical H295R cell line (3). It is not known which of these channels is primarily responsible for setting the resting membrane potential of normal human AZG cells and if either is regulated by ANG II.

Overall, we have established that ANG II inhibits bTREK-1 in bovine adrenocortical cells by a Ca²⁺-dependent mechanism that is independent of PIP₂ depletion. The combined Ca²⁺- and ATP hydrolysis-dependent mechanisms for TREK-1 inhibition in AZF and AZG cells that we have described provide an efficient, failsafe mechanism for membrane depolarization (15, 31). In addition, the inhibition of bTREK-1 by Ca²⁺ suggests a model for ANG II-stimulated corticosteroid secretion that links bTREK-1 inhibition to membrane depolarization and Ca²⁺ entry through voltage-gated channels. In this scheme, the inhibition of bTREK-1 channels by ANG II would produce membrane depolarization and the activation of voltage-gated Ca²⁺ channels, leading to further TREK-1 inhibition in a positive feedback mechanism, culminating in large Ca²⁺-dependent increases in corticosteroid production.

TREK-1 K⁺ channels are widely expressed in CNS neurons as well as endocrine and muscle cells (19, 26, 48). It will be important to find whether TREK-1 channels in these cells are also modulated through Gq-coupled receptors by parallel Ca²⁺- and ATP-dependent mechanisms. Regardless, the finding that TREK-1 channels are inhibited by increasing [Ca²⁺], has broad implications relating to neuronal physiology and pathophysiology.

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DISCLOSURES

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