Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity

Kirill Essin, Birgit Salanova, Ralph Kettritz, Matthias Sausbier, Friedrich C. Luft, Dirk Kraus, Erwin Bohn, Ingo B. Autenrieth, Andreas Peschel, Peter Ruth and Maik Gollasch

Am J Physiol Cell Physiol 293:C45-C54, 2007. First published 28 February 2007;
doi:10.1152/ajpcell.00450.2006

VSOP/Hv1 proton channels sustain calcium entry, neutrophil migration, and superoxide production by limiting cell depolarization and acidification
Antoun El Chemaly, Yoshifumi Okochi, Mari Sasaki, Serge Arnaudeau, Yasushi Okamura and Nicolas Demaurex
J Exp Med, January 18, 2010; 207 (1): 129-139.
[Abstract] [Full Text] [PDF]

VSOP/Hv1 proton channels sustain calcium entry, neutrophil migration, and superoxide production by limiting cell depolarization and acidification
Antoun El Chemaly, Yoshifumi Okochi, Mari Sasaki, Serge Arnaudeau, Yasushi Okamura and Nicolas Demaurex
J Exp Med, January 18, 2010; 207 (1): 129-139.
[Abstract] [Full Text] [PDF]

Am J Physiol Cell Physiol 293: C45–C54, 2007.
First published February 28, 2007; doi:10.1152/ajpcell.00450.2006.

Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity

Kirill Essin,[1,2] Birgit Salanova,[2] Ralph Kettritz,[2] Matthias Sausbier,[4] Friedrich C. Luft,[2] Dirk Kraus,[3] Erwin Bohn,[3] Ingo B. Autenrieth,[3] Andreas Peschel,[3] Peter Ruth,[4] and Maik Gollasch[1,2]

[1]*Department of Nephrology and Medical Intensive Care,* [2]*Franz Volhard Clinic and Max Delbrück Center for Molecular Medicine, HELIOS Klinikum Berlin, Charité-University Medicine Berlin, Humboldt University of Berlin, Berlin; and* [3]*Institute of Medical Microbiology and Hygiene and* [4]*Institute of Pharmacy, Department of Pharmacology and Toxicology, University of Tübingen, Tübingen, Germany*

Submitted 23 August 2006; accepted in final form 22 February 2007

Essin K, Salanova B, Kettritz R, Sausbier M, Luft FC, Kraus D, Bohn E, Autenrieth IB, Peschel A, Ruth P, Gollasch M. Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity. *Am J Physiol Cell Physiol* 293: C45–C54, 2007. First published February 28, 2007; doi:10.1152/ajpcell.00450.2006.— Large-conductance Ca^{2+}-activated K^+ (BK) channels are reported to be essential for NADPH oxidase-dependent microbial killing and innate immunity in leukocytes. Using human peripheral blood and mouse bone marrow neutrophils, pharmacological targeting, and BK channel gene-deficient ($BK^{-/-}$) mice, we stimulated NADPH oxidase activity with 12-*O*-tetradecanoylphorbol-13-acetate (PMA) and performed patch-clamp recordings on isolated neutrophils. Although PMA stimulated NADPH oxidase activity as assessed by O_2^- and H_2O_2 production, our patch-clamp experiments failed to show PMA-activated BK channel currents in neutrophils. In our studies, PMA induced slowly activating currents, which were insensitive to the BK channel inhibitor iberiotoxin. Instead, the currents were blocked by Zn^{2+}, which indicates activation of proton channel currents. BK channels are gated by elevated intracellular Ca^{2+} and membrane depolarization. We did not observe BK channel currents, even during extreme depolarization to +140 mV and after elevation of intracellular Ca^{2+} by *N*-formyl-L-methionyl-L-leucyl-phenylalanine. As a control, we examined BK channel currents in cerebral and tibial artery smooth muscle cells, which showed characteristic BK channel current pharmacology. Iberiotoxin did not block killing of *Staphylococcus aureus* or *Candida albicans*. Moreover, we addressed the role of BK channels in a systemic *S. aureus* and *Yersinia enterocolitica* mouse infection model. After 3 and 5 days of infection, we found no differences in the number of bacteria in spleen and kidney between $BK^{-/-}$ and $BK^{+/+}$ mice. In conclusion, our experiments failed to identify functional BK channels in neutrophils. We therefore conclude that BK channels are not essential for innate immunity.

killing assay; reactive oxygen species; BK-deficient mice; mice infection

NEUTROPHILS ARE THE FIRST-LINE cell defense of the innate immune system. Neutrophils kill microorganisms by ingesting them into phagocytic vacuoles and bombarding them with reactive oxygen species (ROS; e.g., O_2^- and H_2O_2) and enzymatic contents of cellular granules (23). ROS generation is mediated through the activation of NADPH oxidase in the plasma membrane. NADPH oxidase transfers electrons out of the cell into the phagocytic vacuole to reduce O_2 to O_2^-. The resulting negative charge movement (44) should be compensated. Two currents have been suggested to compensate electron flux into the vacuole: an H^+ current through voltage-gated proton channels (16, 25, 34) and a K^+ current (38). K^+ flux activates neutrophil granule proteases, which are necessary to resist staphylococcal and candidal infections (38, 45). Ahluwalia et al. (1) recently proposed that the K^+ flux is caused by opening of big-conductance K^+ (BK) channels in neutrophils.

BK channels, also known as slo and maxi-K^+ channels, are broadly distributed among different cell types (for recent review see Refs. 21 and 32). BK channels are activated by membrane depolarization and by an increase in cytosolic Ca^{2+} and can be suppressed by potent blockers: iberiotoxin (19), charybdotoxin (22), and tetraethylammonium (TEA) (48). The BK channel consists of four pore-forming α-subunits and tissue-specific modulatory β-subunits. BK channel α-subunit-knockout ($BK^{-/-}$) mice suffer from cerebral ataxia and Purkinje cell dysfunction (42), elevated blood pressure (41), progressive hearing loss (40), and erectile dysfunction (49). Immune disorders in these mice have not been reported.

Ahluwalia et al. (1) found that the phorbol ester phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC)-dependent NADPH oxidase activator (8), stimulated a rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and BK channel activity in isolated neutrophils. PMA-activated currents were blocked by iberiotoxin, but not by Zn^{2+} (1), an effective inhibitor of proton current (13). In the study of Ahluwalia et al., iberiotoxin did not affect ROS production but completely inhibited the killing of *Staphylococcus aureus, Serratia marcescens,* and *Candida albicans* by human neutrophils. The authors concluded that the BK channel is essential for innate immunity.

K^+ channels in neutrophils are not as well studied as those in neuronal or muscle cells (26) and even in other leukocytes such as T and B cells (2, 20). The most comprehensive study of K^+ channels in neutrophils was published in 1990 by Krause and Welsh (29). They did not find BK channels in human neutrophils but found two separate K^+ currents: a voltage-dependent current and a Ca^{2+}-activated current. Neither current was sensitive to charybdotoxin. Subsequently, Ca^{2+}-activated intermediate-conductance K^+ channels were identified in cultured HL-60-derived neutrophils (47). ATP-sensitive K^+ channels, which may play a role in neutrophil migration and chemotaxis in the inflammatory response, may

Address for reprint requests and other correspondence: M. Gollasch, Charité Campus Virchow Klinikum, Humboldt Univ., Augustenburger Platz 1, 13353 Berlin, Germany (e-mail: maik.gollasch@charite.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

also be present in neutrophils (10). BK channels had been detected in macrophages but had not been described in neutrophils (15, 18).

Given the proposed importance of BK channels for innate immunity (1), we used BK channel gene-deleted ($BK^{-/-}$) mice to study the role of the BK channel in the immune system. We failed to find that PMA stimulated BK channel activity in mouse and human neutrophils. Iberiotoxin, a specific blocker of the BK channel, did not decrease ROS production and microorganism killing in human neutrophils. Survival of bacteria was not increased in mice after genetic depletion of the BK channel. Thus our results are in agreement with the data obtained by Femling et al. (17), who also could not confirm an essential role of BK channels in neutrophil function.

METHODS

Mice. All the experiments were performed with 2-to 3-mo-old male and female wild-type ($BK^{+/+}$) and $BK^{-/-}$ mice. $BK^{-/-}$ mice were generated as described elsewhere (42). $BK^{+/+}$ and $BK^{-/-}$ mice were of the hybrid SV129/C57BL6 background. Litter- or age-matched animals were randomly assigned to the experimental procedures. All animal experimental protocols were approved by the local animal care committees (Regierungspräsidium, Tübingen and LaGetSi, Berlin, Germany).

Purification of cells. Heparinized venous blood was obtained from healthy volunteers after written informed consent was obtained. Human neutrophils were isolated from blood as previously described (28, 43). Smooth muscle cells were enzymatically isolated from cerebral arteries from mice as previously described (41). Tibial smooth muscle cells were isolated using the same protocol. However, incubation with papain was prolonged to 45 min and isolation with collagenases to 10 min. Mouse neutrophils were isolated from bone marrow by a Ficoll-Histopaque gradient. Mice were killed, the femur and the tibia from both hindlegs were removed and freed of soft tissue attachments, and the extreme distal tip of each extremity was removed. Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS; GIBCO) was forced through the bone with a syringe. The resulting cell suspension was passed over a 70-μm sterile nylon filter (BD Falcon) for removal of cell aggregates and other debris. The filtrate was layered over a double Ficoll-Histopaque gradient (1:1 Histopaque 1119 and 1083, Sigma) and centrifuged at 700 *g* for 30 min. The intermediate layer was collected and washed three times with HBSS. Purity of isolated bone marrow neutrophils was 70% as assessed by staining with Hemacolor (Merck). Neutrophils for further experiments were selected on the basis of their morphology.

Mice infection. Freshly thawed, plasmid-harboring *Yersinia enterocolitica* strain WA-314 serotype *O:8* organisms suspended in 0.1 ml of sterile PBS, pH 7.4, were used for intravenous infection as described previously (5, 7). To determine the actual number of bacteria administered, we plated serial dilutions of the inoculum on Mueller-Hinton agar and counted colony-forming units (CFUs) after 36 h of incubation at 26°C. $BK^{+/+}$ and $BK^{-/-}$ mice were killed by carbon dioxide asphyxiation at 5 days after infection with 5×10^3 bacteria. The spleens were aseptically removed, and a single-cell suspension was prepared using 5 ml of PBS containing 0.1% BSA. Duplicates of 0.1 ml of serial dilutions of these preparations were plated on Mueller-Hinton agar. The limit of detectable CFUs was 25 ($\log_{10} = 1.4$). A total of nine mice were infected per group.

$BK^{+/+}$ and $BK^{-/-}$ mice were also infected intravenously with 10^7 *S. aureus* SA113 and killed 3 days later. The number of bacteria in the spleen was determined by serial dilution and plating on Luria broth plates.

Electrophysiology. Patch-clamp studies were performed on freshly isolated neutrophils and smooth muscle cells. Membrane currents were recorded with an Axopatch 200B amplifier (Axon Instruments). Data were acquired and analyzed with a CED1401 interface and CED Patch and Voltage Clamp Software (version 6.08, Cambridge Electronic Design). Cells were voltage clamped at −30, −40, or 0 mV and pulsed for 300 ms from −100 to +140 or +100 mV in 20-mV increments every 2 s. Currents were measured at the end of the pulse at +140 or +100 mV. In some experiments, currents were recorded from a holding potential of −40 mV during linear voltage ramps at 0.5 V/s from −100 to +100 mV applied every 10 s. In experiments designed to measure proton currents, cells were voltage clamped at −60 mV and pulsed for 8 s in 20-mV increments every 20 s.

If not otherwise indicated, the experiments were performed in the perforated-patch configuration. A stock solution of 100 mg/ml amphotericin B in DMSO was prepared and diluted in the pipette solution to give a final concentration of 200 μg/ml. Stable access was obtained after 10–20 min. Cell capacitance was 3.1 ± 0.2 ($n = 36$, range 1.7–5 pF) for human neutrophils and 2.0 ± 0.1 ($n = 54$, range 1.5–2.5 pF) for mouse neutrophils. We did not routinely correct for series resistance. Agents were applied to the bath with a gravity-driven perfusion system. Several extracellular and pipette solutions were used. In first set of experiments, we used solutions identical to a high-Na^+ extracellular solution containing (in mM) 140 NaCl, 2.5 KCl, 0.5 $MgCl_2$, 1.2 $CaCl_2$, 10 HEPES, and 5 glucose (with pH adjusted to 7.4 with NaOH) and a high-K^+ pipette solution containing (in mM) 140 KCl, 10 NaCl, 2 $MgCl_2$, 0.7 $CaCl_2$, 1 EGTA, and 10 HEPES (with pH adjusted to pH 7.3 with KOH) to record currents. In another set of experiments, we used a high-Na^+ extracellular solution containing (in mM) 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (with pH adjusted to 7.4 with NaOH) and a high-K^+ pipette solution containing (in mM) 110 K^+-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA (with pH adjusted to 7.2 with KOH), which is routinely used in our laboratory to record BK channel currents (37, 41).

We performed some experiments using symmetrical high-K^+ aspartate solutions. The bath solution contained (in mM) 110 K-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 20 glucose (with pH adjusted to 7.2 with KOH), and the pipette solution contained (in mM) 110 K^+-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA (with pH adjusted to 7.2 with KOH). In another set of experiments, the symmetrical high-K^+ aspartate solutions were supplemented with NH_4 (14) to facilitate the measurements of proton currents. The extracellular solution contained (in mM) 80 K^+-aspartate, 25 $(NH_4)_2SO_4$, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 1 EGTA (with pH adjusted to 7.0 with KOH), and the pipette solution contained (in mM) 80 K^+-aspartate, 25 $(NH_4)_2SO_4$, 2 $MgCl_2$, 5 HEPES, and 1 EGTA (with pH adjusted to 7.0 with KOH).

Values are means ± SE. Statistical analysis was performed by one-way analysis of variance and paired *t*-test.

Confocal imaging. For Ca^{2+} measurements, cells were loaded with 5 μM fluo 4-AM (Molecular Probes) and 0.01% pluronic acid (Calbiochem) for 30 min at 5°C in HEPES-PSS (in mM: 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 with NaOH). After they were loaded, the cells were washed with HEPES-PSS three times for removal of extracellular fluo 4-AM and plated at low density on 22-mm glass coverslips, which were mounted on the stage of an inverted Nikon microscope equipped with an UltraVIEW spinning-disk confocal system (Perkin Elmer). Fluo 4-AM was excited at the 488-nm line of an argon laser, and the fluorescence was measured at >510-nm emission. Ca^{2+} signals were monitored in individual cells before and after drug application. Drugs were added to the chamber as concentrated stock solutions to reach the desired final concentration. Two-dimensional fluorescence images were recorded at a rate of 2 frames/s and analyzed with the temporal mode of the UltraVIEW software (Perkin Elmer).

Measurement of O_2^- generation by ferricytochrome c reduction. O_2^- was measured using the assay of SOD-inhibitable reduction of ferricytochrome *c*, as described elsewhere (36). Neutrophils (0.75×10^6) were preincubated with 100 nM iberiotoxin (Sigma) or 10 μM

diphenylene iodonium for 15 min at 37°C and then activated with 0.025 or 1 μg/ml PMA or buffer control. Experiments were done in duplicate. Samples were incubated in 96-well plates at 37°C for up to 60 min, and the absorption of samples with and without 300 U/ml SOD was scanned repetitively at 550 nm using a Microplate Autoreader (Molecular Devices, Munich, Germany). The final ferricytochrome *c* concentration was 250 μM, and the final cell concentration was 3.75×10^6/ml.

Measurement of cellular oxidant stress by dihydrorhodamine oxidation. The generation of reactive oxygen radicals was additionally assessed using dihydrorhodamine-1,2,3 (DHR), as described previously (28). Briefly, prewarmed neutrophils [1×10^7/ml HBSS with Ca^{2+} and Mg^{2+} ($HBSS^{++}$, Biochrom)] were loaded with 1 μM DHR for 10 min at 37°C and then incubated with 100 nM iberiotoxin for 15 min at 37°C. Cells were activated with 0.025 or 1 μg/ml PMA or buffer control at 37°C. After 45 min, the reactions were stopped by addition of ice-cold 1% BSA-PBS. Samples were analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany). Data were collected from 10,000 cells per sample. The shift of green fluorescence in the FL-1 mode was determined, and the mean fluorescence intensity (representing the amount of generated rhodamine 123) is reported.

Killing of C. albicans by human neutrophils. Killing of *C. albicans* was assessed as previously described (12, 35). *C. albicans* were selected from single colonies grown on Sabouraud-agar plates, inoculated into Sabouraud broth, and grown overnight at 30°C. The microorganisms were washed twice in $HBSS^{++}$-HSA and adjusted to a density of 5×10^7 cells/ml. Pooled human serum (Sigma) was added to a final concentration of 10%, and microorganisms were opsonized for 10 min at 37°C. Neutrophils were isolated from peripheral blood as described above and resuspended in $HBSS^{++}$ containing 0.05% human serum albumin (HSA) and 10% pooled human serum. Neutrophils were preincubated for 15 min with 100 nM iberiotoxin at

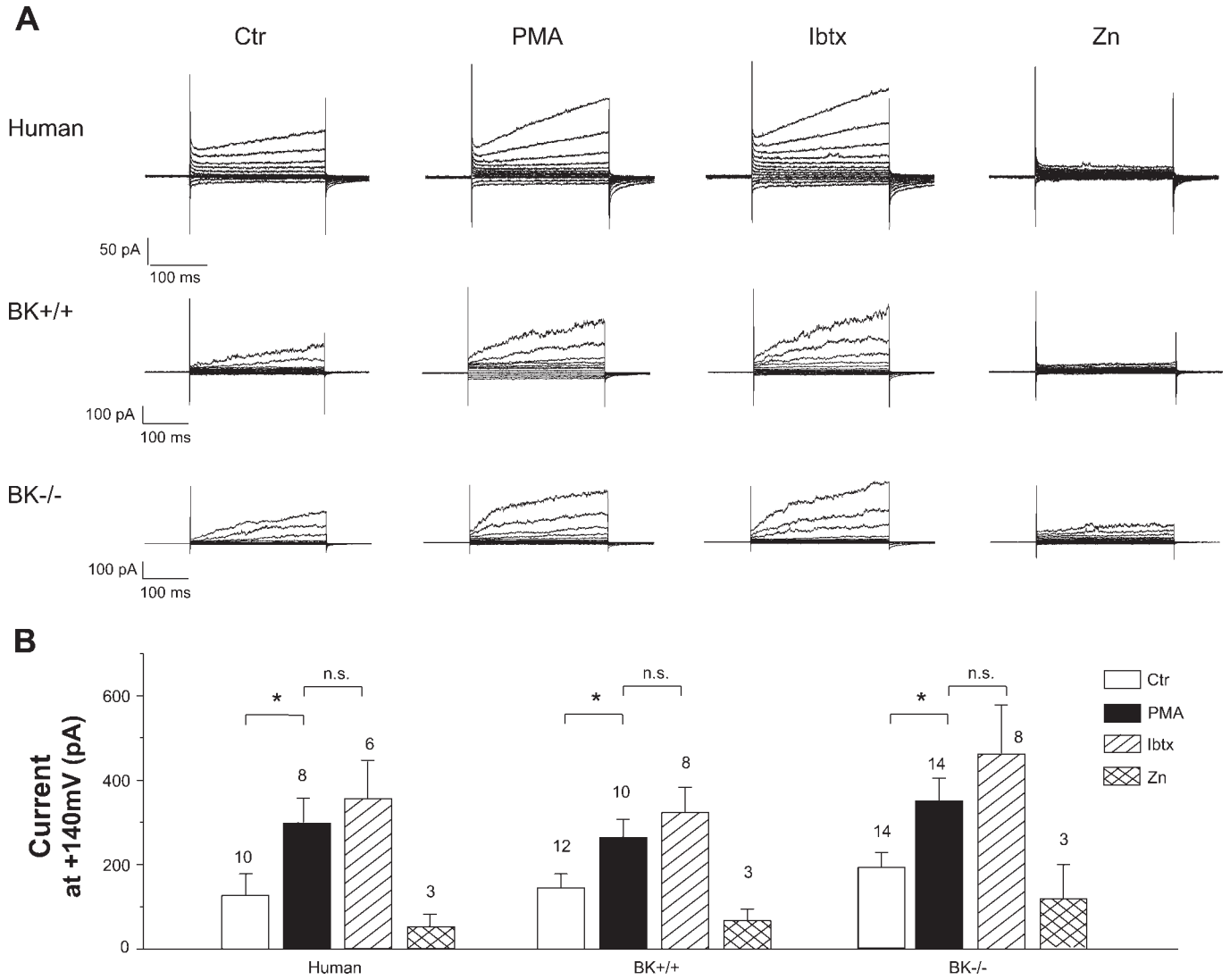

Fig. 1. Big-conductance Ca^{2+}-activated K^+ (BK) channel activity is absent in human and mouse neutrophils. *A*: representative currents in human and mouse $BK^{+/+}$ and $BK^{-/-}$ neutrophils recorded in the absence (Ctr) and presence of 1 μg/ml phorbol 12-myristate 13-acetate (PMA, 5–7 min after application), in the presence of 1 μg/ml PMA + 100 nM iberiotoxin (Ibtx, 10–15 min after application), and in the presence of 1 μg/ml PMA + 3 mM $ZnCl_2$ (Zn, 1–3 min after application). Iberiotoxin did not inhibit PMA-induced currents, indicating that BK channels are not present. Effects of PMA and Zn^{2+} were significant, since only small currents were induced by "sham bath change" within 25 min. Amphotericin-perforated cells were voltage clamped at a holding potential of −30 mV and pulsed for 300 ms from −100 to +140 mV in 20-mV increments every 2 s. High-Na^+ external solution and high-K^+ internal solution were used (1). *B*: mean current amplitudes recorded in human and $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils in response to step depolarization to +140 mV in the absence and presence of 1 μg/ml PMA, in the presence of 1 μg/ml PMA + 100 nM iberiotoxin, and in the presence of 1 μg/ml PMA + 3 mM $ZnCl_2$. Numbers of cells are indicated above bars. *$P < 0.05$. ns, Not significant.

37°C. Opsonized microorganisms were added at a microorganism-to-neutrophil ratio of 2:1. A sample without neutrophils served as a control. The samples were shaken for 90 min at 37°C, and incubation was stopped by addition of 2 ml of ice-cold distilled water to disrupt the neutrophils. Aliquots (25 μl) were spread on Sabouraud agar plates, and colonies were counted after 24 h of incubation at 30°C. The percent killing was calculated as follows: [CFU sample (microorganisms) − CFU sample (neutrophils + microorganisms)]/CFU sample (microorganism) × 100.

Killing of S. aureus by human neutrophils. Neutrophils were isolated from peripheral blood of healthy volunteers as described previously (43) and resuspended in HBSS containing 0.05% HSA. To prepare bacteria, basic medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, and 0.1% K_2HPO_4) was inoculated with 1:100 dilution of an overnight culture and shaken at 37°C until midlogarithmic phase. The bacteria were washed twice in 10 mM potassium phosphate (KP_i) buffer (pH 7.0) containing 0.01% HSA and adjusted to 5×10^7 bacteria/ml. Bacterial and neutrophil suspen-

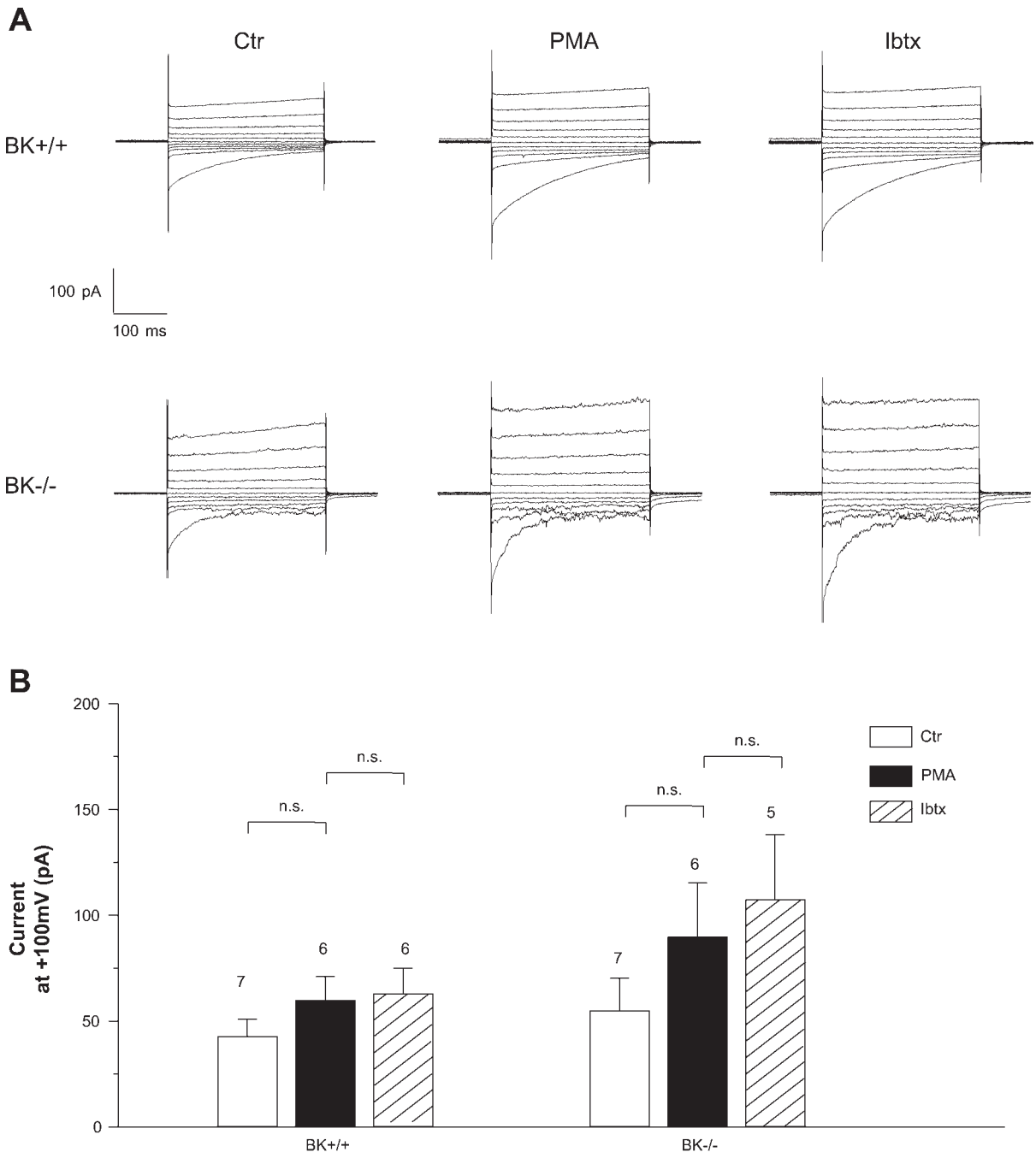

Fig. 2. Currents in symmetrical high-K^+ aspartate solutions were not sensitive to iberiotoxin. *A*: representative currents in $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils recorded in the absence and presence of 1 μg/ml PMA (5–7 min after stimulation) and in the presence of 1 μg/ml PMA + 100 nM iberiotoxin (10–15 min after application). Amphotericin-perforated cells were voltage clamped at a holding potential of 0 mV and pulsed for 300 ms from −100 to +100 mV in 20-mV increments every 2 s. Symmetrical high-K^+ aspartate solutions were used in bath and pipette. *B*: mean current amplitudes recorded in $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils in response to step depolarization to +100 mV in the absence and presence of 1 μg/ml PMA and in the presence of 1 μg/ml PMA + 100 nM iberiotoxin. Numbers of cells are indicated above bars.

sions were mixed to final concentrations of 5×10^6/ml and 2.5×10^6/ml, respectively. Bacteria were opsonized by addition of pooled human serum (Sigma) to a final concentration of 10%. Samples (500 μl) with 100 nM iberiotoxin and without iberiotoxin were shaken at 37°C. Incubation was stopped by dilution of aliquots in ice-cold, distilled water. The neutrophils were disrupted by vigorous vortexing. Appropriate sample volumes were plated on basic medium agar plates, and colonies were counted after 24 h of incubation at 37°C.

RESULTS

Membrane currents in human and mouse neutrophils are not inhibited by iberiotoxin. Iberiotoxin is a potent and specific blocker of the BK channel (19). We did not observe iberiotoxin-sensitive currents in human blood and mouse bone marrow neutrophils (Fig. 1*A*), even after stimulation with PMA. We used the amphotericin B perforated-patch mode of the patch-clamp technique to measure currents in isolated neutrophils. We used the same solutions and protocols routinely used for BK channel current measurements (37, 41) and the solutions and protocols used by Ahluwalia et al. (1) but failed to find iberiotoxin-sensitive currents in neutrophils. Representative currents measured in cells clamped at −30 mV and depolarized for 300 ms from −100 to +140 mV in 20-mV increments in the absence and presence of 1 μg/ml PMA, in the presence of 1 μg/ml PMA + 100 nM iberiotoxin, and in the presence of 1 μg/ml PMA + 3 mM $ZnCl_2$ are shown in Fig. 1*A*. Depolarization-evoked current amplitudes increased on stimulation with PMA and were not inhibited by iberiotoxin (Fig. 1*B*) but were blocked by Zn^{2+}, which abolishes proton currents (17). The BK channel can be also blocked by 1 mM TEA (30, 48). TEA, at 1 mM, had no effect on depolarization-evoked currents in human neutrophils ($n = 6$ cells; data not shown).

Next, using symmetrical high-K^+ aspartate solutions, we attempted to find BK channel currents. Representative currents measured in mouse neutrophils clamped at 0 mV and depolarized for 300 ms from −100 to +100 mV in 20-mV increments in the absence and presence of 1 μg/ml PMA (5–6 min) and in the presence of 1 μg/ml PMA + 100 nM iberiotoxin (10–12 min) are shown in Fig. 2*A*. Depolarization-evoked current amplitudes tended to increase on stimulation with PMA and were not inhibited by iberiotoxin (Fig. 2*B*). External application of 1 mM Ba^{2+} blocked the amplitude of the inward component of the current significantly but did not affect the outward component ($n = 3$ cells each for $BK^{+/+}$ and $BK^{-/-}$ mice; data not shown), indicating the presence of inwardly rectifying K^+ currents previously reported in newt blood neutrophils (27).

The effects of PMA, a well-known PKC activator (39), on BK channel activity are not clear: inhibition (46) and activation have been reported (6). Ahluwalia et al. (1) found that PMA increased $[Ca^{2+}]_i$ in human neutrophils and, therefore, activated BK channels that are Ca^{2+} sensitive. We also found that

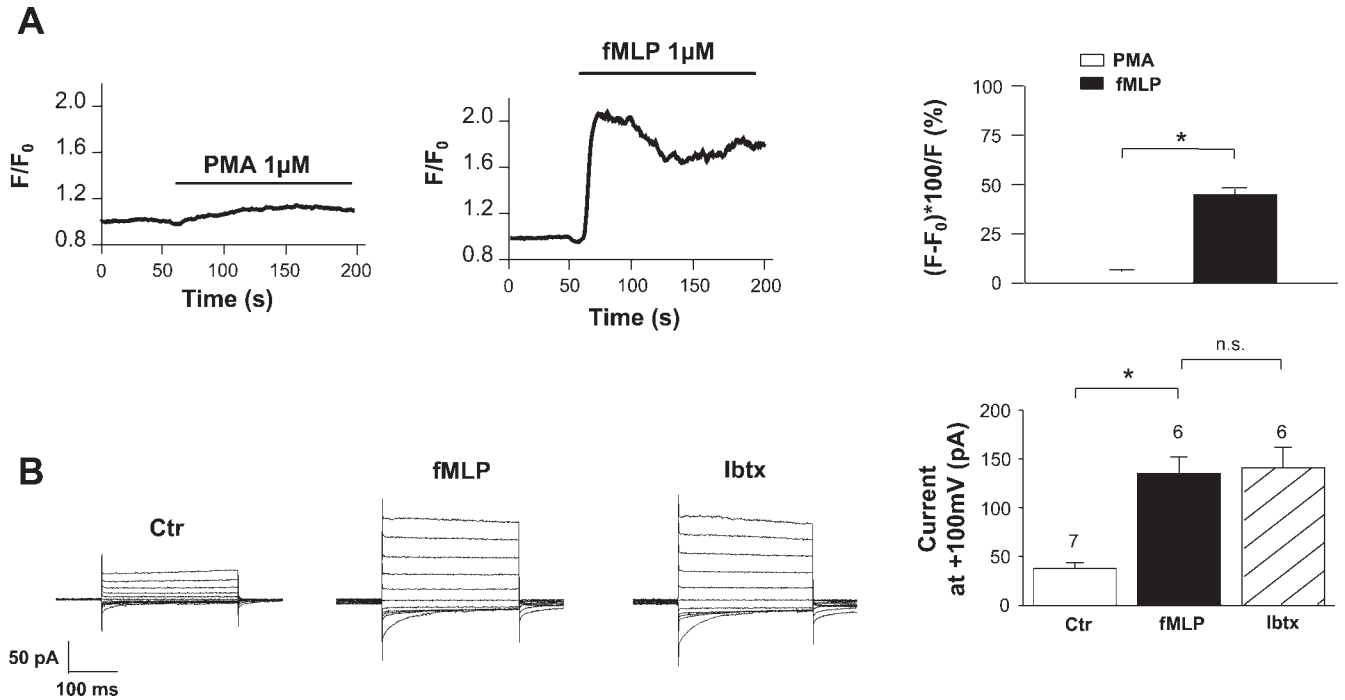

Fig. 3. *N*-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) elevates cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) but fails to stimulate BK channels in human neutrophils. *A*: $[Ca^{2+}]_i$ changes induced by 1 μM PMA and 1 μM fMLP measured using Nipkow spinning-disk confocal microscopy in human neutrophils loaded with the Ca^{2+} indicator dye fluo 4-AM. Time course of mean fluorescence intensity changes was averaged for 5 cells each. Increases in global $[Ca^{2+}]_i$ in human neutrophils induced by 1 μM PMA and 1 μM fMLP are expressed as percentage of fluorescence intensity changes: $(F - F_0) * 100/F_0$, where F is the average intensity over the whole area of the cell in the presence of substance and F_0 is fluorescence before substance application. Values are means ± SE of ≥100 cells for each substance. *B*: representative currents recorded in symmetrical high-K^+ aspartate solutions in a human neutrophil in the absence of fMLP, in the presence of 1 μM fMLP (5–7 min after stimulation), and in the presence of 1 μM fMLP + 100 nM iberiotoxin (10–15 min after application). Amphotericin-perforated cell was voltage clamped at a holding potential of 0 mV and pulsed for 300 ms from −100 to +100 mV in 20-mV increments. Mean current amplitudes were recorded in human neutrophils in response to step depolarization to +100 mV in the absence and presence of 1 μM fMLP and in the presence of 1 μM fMLP + 100 nM iberiotoxin. Numbers of cells in each experimental condition are indicated above bars. *$P < 0.05$.

PMA induced a Ca^{2+} increase in human neutrophils (Fig. 3*A*). Changes in $[Ca^{2+}]_i$ were measured in fluo 4-AM-loaded neutrophils using Nipkow disk confocal microscopy. The time course of changes in fluo-4 fluorescence intensity evoked by 1 μM PMA averaged from five cells in a representative experiment is shown in Fig. 3*A*. The relative fluorescence (F/F_0) increase in the presence of PMA was <20% but was, nonetheless, statistically significant. To achieve a more significant increase in $[Ca^{2+}]_i$, we used *N*-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP). $[Ca^{2+}]_i$ in neutrophils was increased much more effectively by 1 μM fMLP than by PMA at the same concentration. Despite a significant increase in $[Ca^{2+}]_i$, fMLP failed to induce appropriate BK channel activity in human neutrophils (Fig. 3*B*).

As a control, we measured BK currents in smooth muscle cells isolated from mouse tibial and cerebral arteries (Fig. 4). In contrast to the neutrophils, in arterial smooth muscle cells, depolarization-evoked currents were effectively blocked by 100 nM iberiotoxin. BK channel activity in $BK^{+/+}$ cerebral smooth muscle cells was abolished by iberiotoxin (Fig. 4*A*). In contrast, BK channel currents were absent in $BK^{-/-}$ cells. BK channel currents in $BK^{+/+}$ tibial smooth muscle cells were effectively blocked by iberiotoxin (Fig. 4*B*). In tibial and cerebral smooth muscle cells, iberiotoxin also blocked spontaneous transient outward currents,

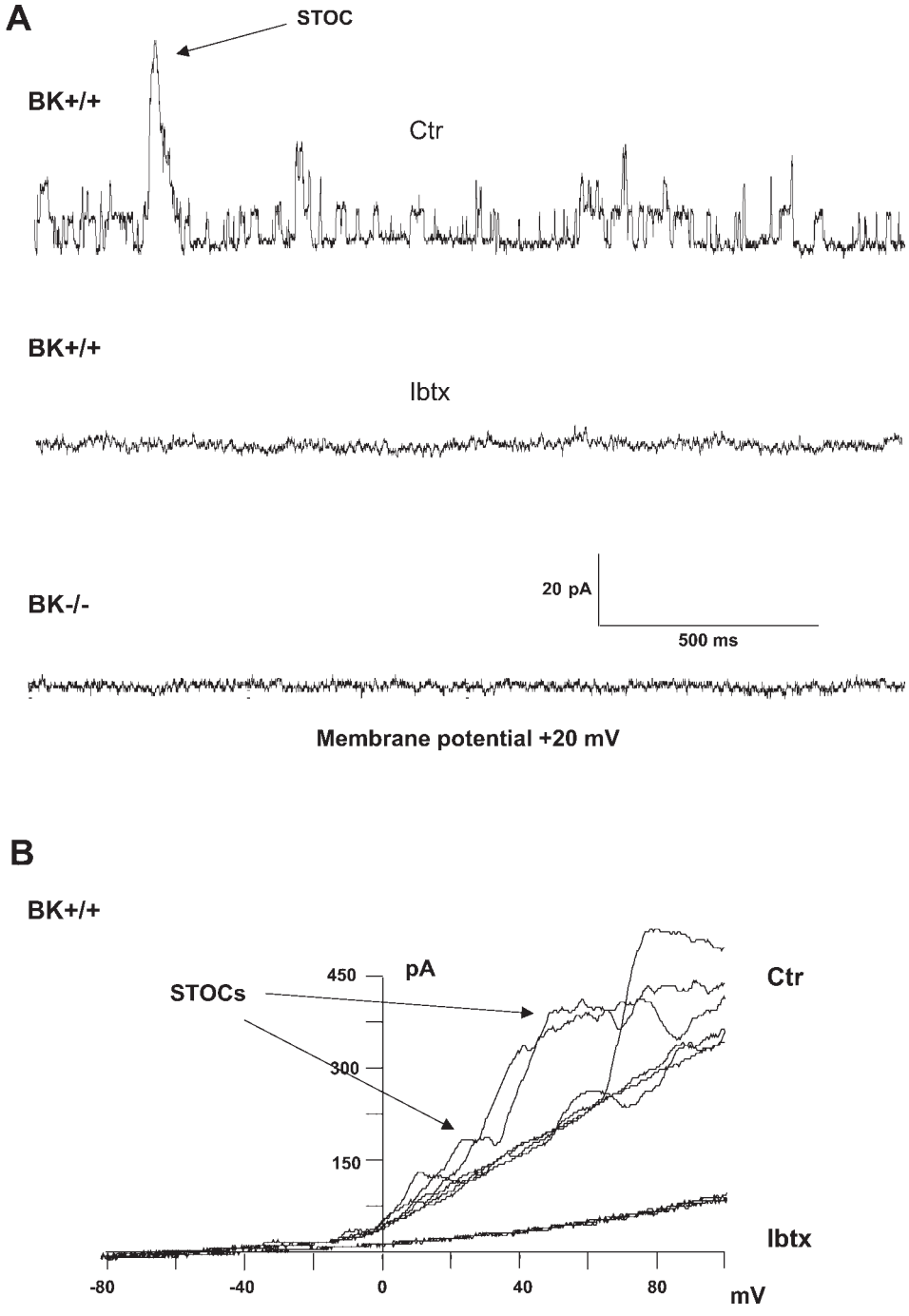

Fig. 4. BK channel activity in arterial smooth muscle cells. *A*: BK channel activity in a smooth muscle cell isolated from a cerebral artery of a $BK^{+/+}$ mouse (*top trace*) was blocked by 100 nM iberiotoxin (10 min after application, *middle trace*). BK channel activity was absent in a smooth muscle cell isolated from a cerebral artery of a $BK^{-/-}$ mouse (*bottom trace*). Amphotericin-perforated cells were voltage clamped at a holding potential of +20 mV. High-Na^+ external and high-K^+ internal pipette solutions were used (41). Data were filtered at 1 kHz and sampled at 2 kHz. Arrow indicates coordinated openings of several BK channels, which are known as spontaneous transient outward current (STOC) (37). Similar results were obtained in 3 other $BK^{+/+}$ and 3 other $BK^{-/-}$ cerebral smooth muscle cells (see Ref. 41 for our previous recordings). *B*: 100 nM iberiotoxin blocked STOCs and whole cell K^+ current in a smooth muscle cell isolated from the tibial artery of a $BK^{+/+}$ mouse. Amphotericin-perforated cell was voltage clamped at −40 mV, and linear voltage ramps at 0.5 V/s from −100 to +100 mV were applied every 10 s. High-Na^+ external and high-K^+ internal pipette solutions were used (41). The 6 superimposed records are shown in the absence and presence of 100 nM iberiotoxin (10 min after application). Similar results were obtained in 3 other cells.

which represent coordinated openings of a cluster of BK channels caused by Ca^{2+} sparks (37, 41).

As another control, proton-like currents were recorded in human and mouse neutrophils. Symmetrical high-K^+ aspartate solutions were supplemented with NH_4 (14). Representative current traces are shown in Fig. 5. Amphotericin-perforated cells were voltage clamped at a holding potential of −60 mV and pulsed for 8 s from −60 to +60 mV in 20-mV increments every 20 s. PMA-stimulated currents were not sensitive to iberiotoxin. However, the currents were almost completely blocked by Zn^{2+}, which is consistent with previously reported results (17).

Iberiotoxin does not inhibit killing of human neutrophils. Ahluwalia et al. (1) reported that iberiotoxin is able to inhibit neutrophil killing. We tested the effect of iberiotoxin on the ability of human neutrophils to kill *S. aureus* and *C. albicans* (Fig. 6, *A* and *B*). Survival of *S. aureus* at the end of 15, 30, and 60 min of incubation with neutrophils at 37°C compared with the initial number of bacteria is shown in Fig. 6*A*. The ratio of bacteria to neutrophils was taken as 2:1. Almost all bacteria were killed at 60 min. Bacterial survival was not significantly changed by 100 nM iberiotoxin at 15, 30, or 60 min. The ability of neutrophils to kill *C. albicans* at the end of 90 min of incubation with neutrophils at 37°C is shown in Fig. 6*B*. Opsonized microorganisms were added at a microorganism-to-neutrophil ratio of 2:1. The ~40% of *C. albicans* killed in control was not decreased by 100 nM iberiotoxin. Therefore, our data indicate that iberiotoxin does not inhibit killing of *S. aureus* and *C. albicans* by neutrophils, in contrast to the data presented by Ahluwalia et al. (1) but in agreement with other reports (11, 17).

Iberiotoxin does not inhibit ROS production in human neutrophils. Effective killing of *S. aureus* and *C. albicans* requires NADPH oxidase activity and generation of ROS (4, 24). We tested the effect of iberiotoxin on generation of ROS in human neutrophils with two independent assays. O_2^- was measured using the assay of SOD-inhibitable reduction of ferricytochrome *c* (Fig. 6*E*). At 25 ng/ml–1 μg/ml, PMA, a known NADPH oxidase activator (8), stimulated O_2^- production. Iberiotoxin (100 nM) did not block O_2^- production in neutrophils in the absence or presence of PMA. Similar results were obtained with the DHR oxidation assay. The generation of reactive oxidants was stimulated by 25 ng/ml and 1 μg/ml PMA and was not inhibited by 100 nM iberiotoxin (Fig. 6*F*). In contrast to iberiotoxin, diphenylene iodonium, a classical inhibitor of NADPH-dependent ROS production (9), strongly reduced the oxidant generation in PMA-stimulated cells (n = 6; data not shown). Therefore, our data show that ROS production was not inhibited by iberiotoxin. The data are in agreement with the absence of an inhibitory effect of iberiotoxin on killing activity of human neutrophils and confirm data obtained in previous studies (1, 17).

BK channel knockout does not reduce resistance to S. aureus and Yersinia infection in mice. If BK channels are essential for innate immunity, a reasonable expectation would be that $BK^{-/-}$ mice are less resistant to infections than $BK^{+/+}$ mice. We performed experiments with *S. aureus*- and *Yersinia*-infected mice to explore this possibility (Fig. 6*C*). $BK^{+/+}$ and

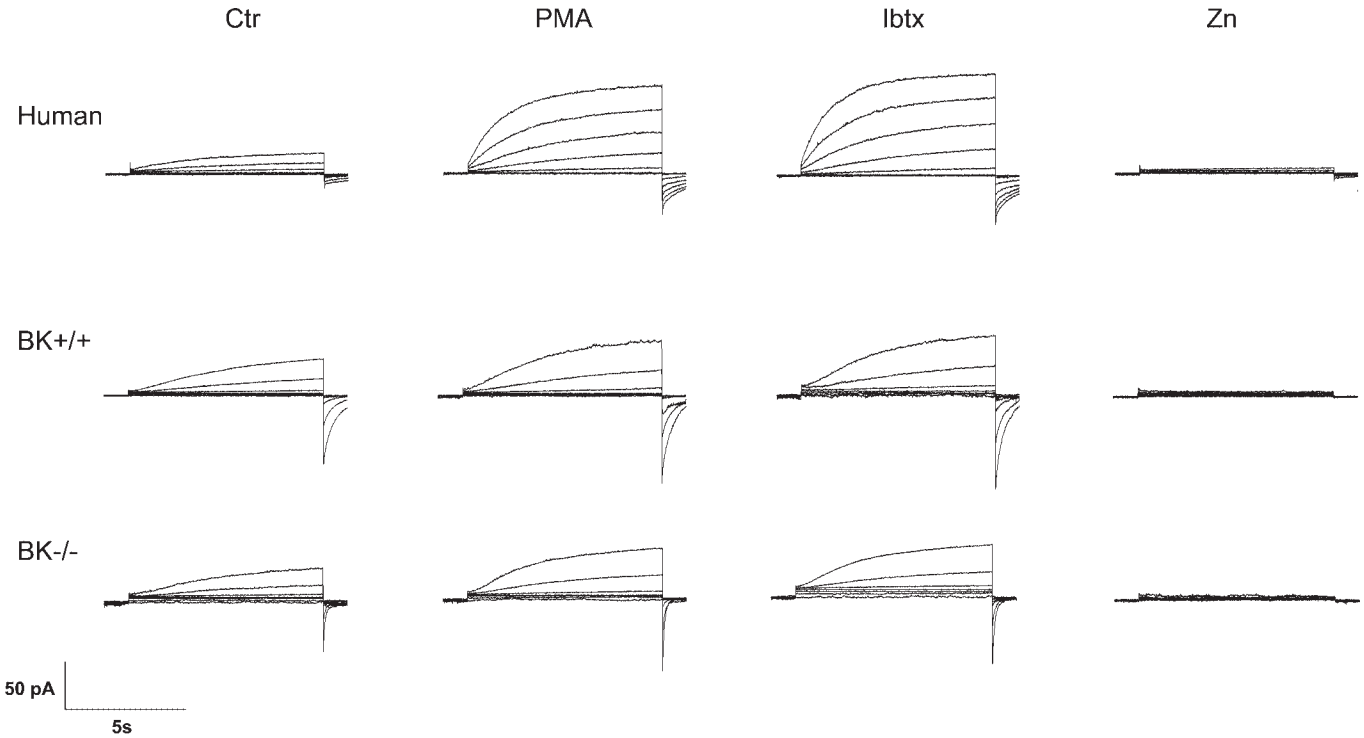

Fig. 5. Proton-like currents in human and mouse neutrophils were not sensitive to iberiotoxin but were blocked by Zn^{2+}. Currents in human and $BK^{+/+}$ and $BK^{-/-}$ mouse neutrophils were recorded in the absence and presence of 1 μg/ml PMA (5–7 min after stimulation), in the presence of 1 μg/ml PMA + 100 nM iberiotoxin (10–15 min after application), and in the presence of 1 μg/ml PMA + 3 mM $ZnCl_2$ (1–3 min after application). Amphotericin-perforated cells were voltage clamped at a holding potential of −60 mV and pulsed for 8 s from −60 to +60 mV in 20-mV increments every 20 s. Recordings were performed in symmetrical high-K^+ aspartate solutions supplemented with NH_4 (14). PMA-stimulated currents were not sensitive to iberiotoxin. However, they were blocked by Zn^{2+}. Similar results were obtained in 4 other human and 3 other $BK^{+/+}$ and 3 other $BK^{-/-}$ mouse neutrophils.

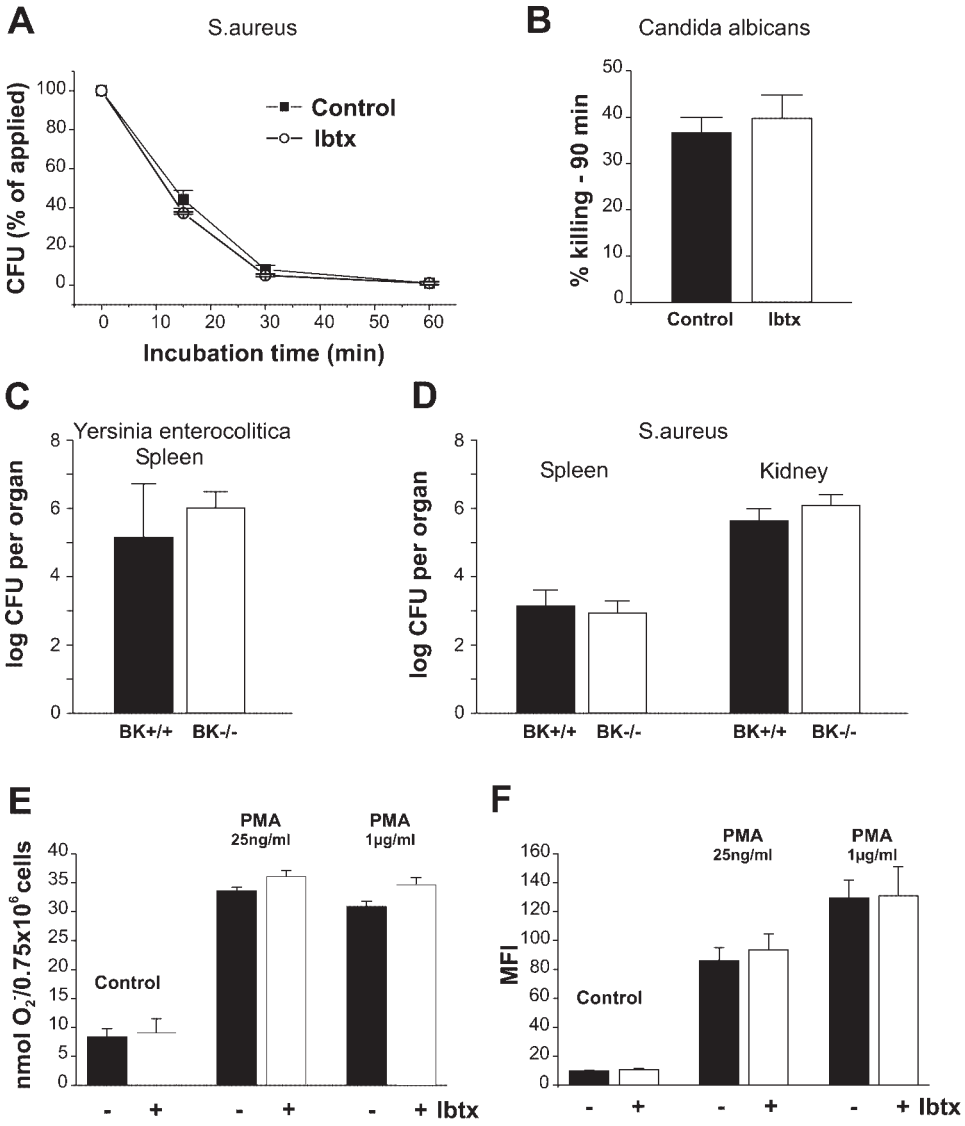

Fig. 6. BK channel inhibition does not influence killing activity of neutrophils and reactive oxygen species production. *A*: effect of BK channel inhibition on killing of opsonized *Staphylococcus aureus* by human neutrophils. Number of viable *S. aureus* colony-forming units (CFUs) after incubation with human neutrophils is expressed as percentage of initial count. Samples were incubated in the absence and presence of 100 nM iberiotoxin. Values are means ± SE of 2 independent experiments run in duplicate. BK channel inhibitor iberiotoxin had no effect on killing of *S. aureus* by human neutrophils. *B*: effect of BK channel inhibition on the killing of *C. albicans* by human neutrophils. BK channel inhibitor iberiotoxin had no effect on killing of *C. albicans* by human neutrophils. *C*: BK channel knockout does not reduce resistance to *Yersinia* infection in mice. Number of bacteria were counted in spleens isolated 5 days after $BK^{+/+}$ and $BK^{-/-}$ mice were infected with *Yersinia*. Values are means ± SE for 9 mice. BK channel is not essential for *Yersinia* killing. *D*: BK channel knockout does not reduce resistance to *S. aureus* infection in mice. Number of bacteria were counted in spleens and kidneys isolated 3 days after $BK^{+/+}$ and $BK^{-/-}$ mice were infected with *S. aureus*. Values are means ± SE from 10 mice. BK channel is not essential for *S. aureus* killing. *E*: effect of BK channel inhibition on O_2^- generation. Data are from samples incubated for 45 min. Values are means ± SE ($n = 7$). BK channel inhibitor iberiotoxin had no effect on respiratory burst activity of PMA-treated neutrophils. *F*: effect of BK channel inhibition on oxidant generation of PMA-treated neutrophils. Oxidants were assessed using dihydrorhodamine (DHR) oxidation assay. Values are means ± SE ($n = 6$). Independent assay to estimate respiratory burst activity indicates that BK channel inhibitor iberiotoxin had no effect on respiratory burst of PMA-stimulated neutrophils. MFI, mean fluorescence units (i.e., amount of rhodamine-1,2,3 generated).

$BK^{-/-}$ mice were intravenously infected with *S. aureus* or *Y. enterocolitica*. Three days after infection with *S. aureus* and 5 days after infection with *Yersinia*, the mice were killed, and the number of viable bacteria recovered from spleen and kidney was determined. The number of viable *Yersinia* and *S. aureus* was not increased by the absence of BK channels in mice (Fig. 6, *C* and *D*). Thus the data do not support the idea that BK channels are essential for innate immunity and, thus, for protection against bacterial infections.

DISCUSSION

We did not find that PMA stimulated BK channel activity in human or mouse neutrophils. For a positive control, we recorded iberiotoxin-sensitive BK channel currents in arterial smooth muscle cells, where their existence is well established (for recent review see Ref. 31). Electrophysiological measurements were done using the protocol and solutions routinely used in our laboratory (37, 41) and the solutions and protocol used by Ahluwalia et al. (1). They found that PMA significantly increases intracellular Ca^{2+} in neutrophils and activates Ca^{2+}-dependent K^+ currents blocked by iberiotoxin. In contrast to iberiotoxin, PMA is not a classical pharmacological tool to study BK channels. The action of PMA is variable, ranging from channel activation (6) to inhibition (46), depending on the cell type. The reported effects were not associated with a rise in $[Ca^{2+}]_i$ but, rather, with PKC-dependent protein phosphorylation. For example, in pulmonary arterial smooth muscle cells, PMA stimulated PKC and, thereby, activated BK channels via cGMP-dependent protein kinase (6), which directly phosphorylates the pore-forming channel α-subunits (3). Also, the increase in $[Ca^{2+}]_i$ in neutrophils is not the commonly observed effect of PMA (33). We detected the PMA-induced increase in neutrophil $[Ca^{2+}]_i$; however, the response we observed was much more modest than that reported by Ahluwalia et al. On the other hand, a similar concentration of fMLP increased $[Ca^{2+}]_i$ significantly but failed to stimulate BK channel activity.

Although BK channel activity was not detected in neutrophils in our study and in the report by Femling et al. (17), these cells successfully kill *S. aureus* and *C. albicans* (17) (Fig. 6, *A* and *B*). Iberiotoxin, a specific blocker of the BK channel, did not decrease the ability of neutrophils to eliminate microor-

ganisms. The killing assays were performed in two independent laboratories (Berlin and Tübingen) using different experimental methods and conditions. Although Femling et al. (17) also obtained their results in two separate laboratories, four independent sources report no essential role for BK channels in the neutrophil killing function. Our experiments with $BK^{-/-}$ mice also do not support the idea that BK channels are essential for innate immunity. $BK^{-/-}$ mice were not less resistant to *S. aureus* and *Yersinia* infection than their $BK^{+/+}$ littermates. The notion that neutrophils function via BK channel activity should be revised.

ACKNOWLEDGMENTS

We thank Dr. S. Herroeder and A. Nuernberg for demonstrating the method of neutrophil extraction from mouse bone marrow, Yoland Anistan for help with cell isolation, and Juliane Krejci for expert technical assistance.

GRANTS

This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Ahluwalia J, Tinker A, Clapp LH, Duchen MR, Abramov AY, Pope S, Nobles M, Segal AW.** The large-conductance Ca^{2+}-activated K^+ channel is essential for innate immunity. *Nature* 427: 853–858, 2004.
2. **Aiyar J.** Potassium channels in leukocytes and toxins that block them: structure, function and therapeutic implications. *Perspect Drug Discovery Design* 15–16: 257–280, 1999.
3. **Alioua A, Tanaka Y, Wallner M, Hofmann F, Ruth P, Meera P, Toro L.** The large conductance, voltage-dependent, and calcium-sensitive K^+ channel, Hslo, is a target of cGMP-dependent protein kinase phosphorylation in vivo. *J Biol Chem* 273: 32950–32956, 1998.
4. **Aratani Y, Kura F, Watanabe H, Akagawa H, Takano Y, Suzuki K, Dinauer MC, Maeda N, Koyama H.** Relative contributions of myeloperoxidase and NADPH-oxidase to the early host defense against pulmonary infections with *Candida albicans* and *Aspergillus fumigatus*. *Med Mycol* 40: 557–563, 2002.
5. **Autenrieth IB, Beer M, Bohn E, Kaufmann SH, Heesemann J.** Immune responses to *Yersinia enterocolitica* in susceptible BALB/c and resistant C57BL/6 mice: an essential role for γ-interferon. *Infect Immun* 62: 2590–2599, 1994.
6. **Barman SA, Zhu S, White RE.** PKC activates BK_{Ca} channels in rat pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol* 286: L1275–L1281, 2004.
7. **Bohn E, Autenrieth IB.** IL-12 is essential for resistance against *Yersinia enterocolitica* by triggering IFN-γ production in NK cells and $CD4^+$ T cells. *J Immunol* 156: 1458–1468, 1996.
8. **Cox JA, Jeng AY, Sharkey NA, Blumberg PM, Tauber AI.** Activation of the human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C. *J Clin Invest* 76: 1932–1938, 1985.
9. **Cross AR, Jones OT.** The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. *Biochem J* 237: 111–116, 1986.
10. **Da Silva-Santos JE, Santos-Silva MC, Cunha Fde Q, Assreuy J.** The role of ATP-sensitive potassium channels in neutrophil migration and plasma exudation. *J Pharmacol Exp Ther* 300: 946–951, 2002.
11. **Decleva E, Defendi R, Menegazzi R, Busetto S, Patriarca P, Dri P.** Essential role of myeloperoxidase in the microbicidal activity of neutrophils (Abstract). *40th Annu Sci Meeting Eur Soc Clin Invest Prague* 2006, p. 36.
12. **Decleva E, Menegazzi R, Busetto S, Patriarca P, Dri P.** Common methodology is inadequate for studies on the microbicidal activity of neutrophils. *J Leukoc Biol* 79: 87–94, 2006.
13. **Decoursey TE.** Voltage-gated proton channels and other proton transfer pathways. *Physiol Rev* 83: 475–579, 2003.
14. **DeCoursey TE, Cherny VV, Zhou W, Thomas LL.** Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils. *Proc Natl Acad Sci USA* 97: 6885–6889, 2000.
15. **DeCoursey TE, Kim SY, Silver MR, Quandt FN.** Ion channel expression in PMA-differentiated human THP-1 macrophages. *J Membr Biol* 152: 141–157, 1996.
16. **DeCoursey TE, Morgan D, Cherny VV.** The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. *Nature* 422: 531–534, 2003.
17. **Femling JK, Cherny VV, Morgan D, Rada B, Davis AP, Czirjak G, Enyedi P, England SK, Moreland JG, Ligeti E, Nauseef WM, DeCoursey TE.** The antibacterial activity of human neutrophils and eosinophils requires proton channels but not BK channels. *J Gen Physiol* 127: 659–672, 2006.
18. **Gallin EK.** Calcium- and voltage-activated potassium channels in human macrophages. *Biophys J* 46: 821–825, 1984.
19. **Galvez A, Gimenez-Gallego G, Reuben JP, Roy-Contancin L, Feigenbaum P, Kaczorowski GJ, Garcia ML.** Purification and characterization of a unique, potent, peptidyl probe for the high-conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*. *J Biol Chem* 265: 11083–11090, 1990.
20. **George Chandy K, Wulff H, Beeton C, Pennington M, Gutman GA, Cahalan MD.** K^+ channels as targets for specific immunomodulation. *Trends Pharmacol Sci* 25: 280–289, 2004.
21. **Ghatta S, Nimmagadda D, Xu X, O'Rourke ST.** Large-conductance, calcium-activated potassium channels: structural and functional implications. *Pharmacol Ther* 110: 103–116, 2006.
22. **Gimenez-Gallego G, Navia MA, Reuben JP, Katz GM, Kaczorowski GJ, Garcia ML.** Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc Natl Acad Sci USA* 85: 3329–3333, 1988.
23. **Hampton MB, Kettle AJ, Winterbourn CC.** Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 92: 3007–3017, 1998.
24. **Hampton MB, Kettle AJ, Winterbourn CC.** Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infect Immun* 64: 3512–3517, 1996.
25. **Henderson LM, Chappell JB, Jones OT.** The superoxide-generating NADPH oxidase of human neutrophils is electrogenic and associated with an H^+ channel. *Biochem J* 246: 325–329, 1987.
26. **Hille B.** *Ion Channels of Excitable Membranes*. Sunderland, MA: Sinauer, 2001.
27. **Kawa K.** Electrophysiological properties of three types of granulocytes in circulating blood of the newt. *J Physiol* 415: 211–231, 1989.
28. **Kettritz R, Schreiber A, Luft FC, Haller H.** Role of mitogen-activated protein kinases in activation of human neutrophils by antineutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 12: 37–46, 2001.
29. **Krause KH, Welsh MJ.** Voltage-dependent and Ca^{2+}-activated ion channels in human neutrophils. *J Clin Invest* 85: 491–498, 1990.
30. **Langton PD, Nelson MT, Huang Y, Standen NB.** Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. *Am J Physiol Heart Circ Physiol* 260: H927–H934, 1991.
31. **Ledoux J, Werner ME, Brayden JE, Nelson MT.** Calcium-activated potassium channels and the regulation of vascular tone. *Physiology Bethesda* 21: 69–78, 2006.
32. **Lu R, Alioua A, Kumar Y, Eghbali M, Stefani E, Toro L.** MaxiK channel partners: physiological impact. *J Physiol* 570: 65–72, 2006.
33. **Mahomed AG, Anderson R.** Activation of human neutrophils with chemotactic peptide, opsonized zymosan and the calcium ionophore A23187, but not with a phorbol ester, is accompanied by efflux and store-operated influx of calcium. *Inflammation* 24: 559–569, 2000.
34. **Nanda A, Grinstein S.** Protein kinase C activates an H^+ (equivalent) conductance in the plasma membrane of human neutrophils. *Proc Natl Acad Sci USA* 88: 10816–10820, 1991.
35. **Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, van Kessel KP, van Strijp JA.** *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J Exp Med* 193: 1067–1076, 2001.
36. **Pick E, Mizel D.** Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* 46: 211–226, 1981.
37. **Pluger S, Faulhaber J, Furstenau M, Lohn M, Waldschutz R, Gollasch M, Haller H, Luft FC, Ehmke H, Pongs O.** Mice with disrupted

BK channel β_1-subunit gene feature abnormal Ca^{2+} spark/STOC coupling and elevated blood pressure. *Circ Res* 87: E53–E60, 2000.

38. **Reeves EP, Lu H, Jacobs HL, Messina CG, Bolsover S, Gabella G, Potma EO, Warley A, Roes J, Segal AW.** Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. *Nature* 416: 291–297, 2002.
39. **Ron D, Kazanietz MG.** New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J* 13: 1658–1676, 1999.
40. **Ruttiger L, Sausbier M, Zimmermann U, Winter H, Braig C, Engel J, Knirsch M, Arntz C, Langer P, Hirt B, Muller M, Kopschall I, Pfister M, Munkner S, Rohbock K, Pfaff I, Rusch A, Ruth P, Knipper M.** Deletion of the Ca^{2+}-activated potassium (BK) α-subunit but not the BK β_1-subunit leads to progressive hearing loss. *Proc Natl Acad Sci USA* 101: 12922–12927, 2004.
41. **Sausbier M, Arntz C, Bucurenciu I, Zhao H, Zhou XB, Sausbier U, Feil S, Kamm S, Essin K, Sailer CA, Abdullah U, Krippeit-Drews P, Feil R, Hofmann F, Knaus HG, Kenyon C, Shipston MJ, Storm JF, Neuhuber W, Korth M, Schubert R, Gollasch M, Ruth P.** Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 112: 60–68, 2005.
42. **Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus HG, Wolfer DP, Pedroarena CM, Storm JF, Ruth P.** Cerebellar ataxia and Purkinje cell dysfunction caused by Ca^{2+}-activated K^+ channel deficiency. *Proc Natl Acad Sci USA* 101: 9474–9478, 2004.
43. **Schmitz FJ, Veldkamp KE, Van Kessel KP, Verhoef J, Van Strijp JA.** Delta-toxin from *Staphylococcus aureus* as a costimulator of human neutrophil oxidative burst. *J Infect Dis* 176: 1531–1537, 1997.
44. **Schrenzel J, Serrander L, Banfi B, Nusse O, Fouyouzi R, Lew DP, Demaurex N, Krause KH.** Electron currents generated by the human phagocyte NADPH oxidase. *Nature* 392: 734–737, 1998.
45. **Segal AW.** How neutrophils kill microbes. *Annu Rev Immunol* 23: 197–223, 2005.
46. **Tian L, Philp JA, Shipston MJ.** Glucocorticoid block of protein kinase C signalling in mouse pituitary corticotroph AtT20 D16:16 cells. *J Physiol* 516: 757–768, 1999.
47. **Varnai P, Demaurex N, Jaconi M, Schlegel W, Lew DP, Krause KH.** Highly co-operative Ca^{2+} activation of intermediate-conductance K^+ channels in granulocytes from a human cell line. *J Physiol* 472: 373–390, 1993.
48. **Villarroel A, Alvarez O, Oberhauser A, Latorre R.** Probing a Ca^{2+}-activated K^+ channel with quaternary ammonium ions. *Pflügers Arch* 413: 118–126, 1988.
49. **Werner ME, Zvara P, Meredith AL, Aldrich RW, Nelson MT.** Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. *J Physiol* 567: 545–556, 2005.