Modulation of Mouse Paneth Cell α-Defensin Secretion by mIKCa1, a Ca\textsuperscript{2+}-activated, Intermediate Conductance Potassium Channel*

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Paneth cells in small intestinal crypts secrete microbicidal α-defensins in response to bacteria and bacterial antigens (Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E., and Ouellette, A. J. (2000) Nat. Immunol. 1, 113–138). We now report that the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel mIKCa1 modulates mouse Paneth cell secretion. mIKCa1 cDNA clones identified in a mouse small intestinal crypt library by hybridization to human IKCa1 cDNA probes were isolated, and DNA sequence analysis showed that they were identical to mIKCa1 cDNAs isolated from erythroid cells and liver. The genomic organization was found to be conserved between mouse and human IKCa1 as shown by comparisons of the respective cDNA and genomic sequences. Reverse transcriptase-PCR experiments using nested primers amplified mIKCa1 from the lower half of bisected crypts and from single Paneth cells, but not from the upper half of bisected crypts, villus epithelium, or undifferentiated crypt epithelial cells, suggesting a lineage-specific role for mIKCa1 in mouse small bowel epithelium. The cloned mIKCa1 channel was calcium-activated and blocked by ten structurally diverse peptide and nonpeptide inhibitors with potencies spanning 9 orders of magnitude and indistinguishable from that of the human homologue. Consistent with channel blockade, charybdoxin, clotrimazole, and the highly selective IKCa1 inhibitors, TRAM-34 and TRAM-39, inhibited (~50%) Paneth cell secretion stimulated by bacteria or bacterial lipopolysaccharide, measured both as bactericidal activity and secreted cryptdin protein, but the inactive analog, TRAM-7, did not block secretion. These results demonstrate that mIKCa1 is modulator of Paneth cell α-defensin secretion and disclose an involvement in mucosal defense of the intestinal epithelium against ingested bacterial pathogens.

Gene-encoded antimicrobial peptides are evolutionarily conserved molecules that all known species elaborate as components of innate immunity (1). Generally containing fewer than 40 amino acids, these biochemically diverse peptides have secondary structures that range from linear α-helical molecules to β-sheet peptides constrained by up to four disulfide connectivities, including the covalently closed circular α-defensins (2). In mammals, the α-defensins are cationic, 3–4-kDa peptides with a characteristic trisulfide array, and they occur in phagocytic leukocyte granules from which they mediate nonoxidative killing of ingested microbial cells following phagolysosomal fusion (3–5). Varied epithelia also express α- and β-defensins, secreting them onto mucosal surfaces by apparent constitutive pathways or as secretory granule components of exocytotic cells (6–13). In crypts of the small intestinal epithelium, Paneth cells accumulate high levels of α-defensins that are termed cryptdins in mice, and which they secrete in response to bacterial or pharmacologic stimuli. Paneth cells participate in innate mucosal immunity by discharging α-defensins at millimolar concentrations. Bacteria or bacterial antigens stimulate mouse Paneth cells to release apical secretory granules that contain several bactericidal peptides and proteins of which the cryptdins account for ~70% of the secreted microbicidal peptide activity (10). The secretory responses occur within minutes of exposure to soluble bacterial antigens or to carbamyl choline and are dose-dependent, suggesting a receptor-mediated process (10). In mouse small intestinal crypts stimulated with carbamyl choline, the cytosolic calcium dynamics change only in Paneth cells in a biphasic pattern consistent with mobilization of intracellular Ca\textsuperscript{2+} stores followed by influx of extracellular Ca\textsuperscript{2+} (14, 15). These observations led to the hypothesis that Paneth cell secretion in response to bacterial stimuli may be modulated by a cation-selective channel that could regulate the influx of extracellular Ca\textsuperscript{2+}.

Intermediate conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels are the product of the IKCa1 genes (also known as IK1, hKCa4, hSK4, KCNN4) and are important in regulating the membrane potential of colonic epithelial cells, and lymphocytes, and in the volume regulation of red blood cells (16–20). IKCa\textsubscript{n} channels have an intermediate single channel conductance of 11 picosiemens in sodium and 40 picosiemens in potassium Ringer, are voltage-independent, and open in response to changes in intracellular Ca\textsuperscript{2+}. The protein has six transmembrane segments, internal N and C termini, and its C terminus is complexed to calmodulin, the channel calcium sensor (21). The azole antmycotic, clotrimazole (CLT), blocks this channel

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1 The abbreviations used are: CLT, clotrimazole; ChTX, charybdoxin; KCa\textsubscript{n}, calcium-activated K\textsuperscript{+} channel; IKCa\textsubscript{n}, intermediate conductance K\textsuperscript{+}; LPS, lipopolysaccharide; NCR, noncoding region; RT, reverse transcriptase; TRAM, triaryl methane; TRAM-3, (2-chlorophenyl)diphenylmethane; TRAM-7, 1-tritylmethylene; TRAM-34, 1-(2-chlorophenyl)diphenylmethyl-1H-pyrazole; TRAM-39, 2-(2-chlorophenyl)-2,2-diphenylacetonitrile; PIPES, 1,4-piperazinediethanesulfonic acid; cfu, colony-stimulating unit(s).
with nanomolar affinity, and triaryl methane analogs of CT are highly selective and potent inhibitors of the human IKCa1 channel (22). Scorpion toxin charybdoxin (ChTX) also blocks hIKCa1, and a selective analog, ChTX-Glu2, has been generated by structure-guided design (23). These highly selective reagents provide probes for analyzing the role of IKCa1 in Ca2+-mediated signaling events.

Because hIKCa1 regulates specific immune responses mediated by T lymphocytes (20, 24), we tested whether a KC channel could participate in innate immune responses to bacteria by modulating calcium signaling during Paneth cell secretion. In this report, mouse Paneth cells in small intestinal epithelium are shown to express mIKCa1, and pharmacologic inhibition of IKCa1 with highly selective triaryl methane (22) diminished cryptdin secretion in response to bacteria and lipopolysaccharide (LPS).

EXPERIMENTAL PROCEDURES
Preparation of a Mouse Small Intestinal Crypt cDNA Library—Crypts from the small intestines of adult Swiss Webster mice were prepared using EDTA dissociation (10, 25–27). Fractions consisting of >90% crypts were obtained by passage through small intestinal segments in nominally Ca2+-, Mg2+-phosphate-buffered saline with 30 mM EDTA. Total crypt cellular RNA was isolated (28, 29), from which poly(A)-containing mRNA was purified by oligo(dT)-cellulose chromatography. A custom cDNA library was constructed by Stratagene Cloning Systems, Inc. (La Jolla, CA) using crypt mRNA as template for reverse transcription of single-stranded cDNA with oligo(dT)16-20 to prime the reverse transcriptase reaction from the mRNA 5′ termini. Double-stranded cDNAs with adapted termini were cloned in the EcoRI and XhoI sites of the phagemid vector Uni-ZAP XR (Stratagene Cloning Systems). Cloning was performed without polymerase chain reaction (PCR) amplification of sequences or size exclusion of low molecular weight mRNAs. The library contained ~9.8 × 106 primary clones and was amplified to a working titer of 9.9 × 1010 plaque-forming units/ml.

Cloning of Mouse Crypt mIKCa1 cDNA—Bacteriophages containing mIKCa1 were identified in the crypt library by screening ~5 × 106 plaque-forming units in duplicate by hybridization at 42 °C in 1× NaCl, 50% formamide, 10% dextran sulfate, and 1× SSC with 0.5 × 106 primary clones and 1 × 108 secondary clones (10). For each hybridization, 10 ml of isotonic PIPES buffer, consisting of 10 m M PIPES with 0.8% sodium gluconate (pH 7.4) and 2 mg actinomycin D, was used to activate mIKCa1. To eliminate native COS cell chloride conductances, 10 ml of RNAguard RNase inhibitor, and 40 ml of 1× RT-PCR reaction mix lacking AmpliTaq DNA Polymerase were preheated at 94 °C for 2 min, then complete reaction mixes were cycled as before. Samples of the PCR reactions were analyzed by separation in 2% agarose gels to visualize the 293-bp product, and the fragments were purified with QIAEX II gel extraction kit (Qiagen, Valencia, CA) or transferred to nylon membranes to identify mIKCa1 by hybridization with an hIKCa1 probe (29, 30). Amplification of mIKCa1 was performed a minimum of four times on uninfected, freshly prepared crypts. The 293-bp amplification product was cloned into the TOPO II TA cloning vector (Invitrogen), and its identity as the appropriate region of mIKCa1 sequence was confirmed by DNA sequence (data not shown).

Inhibition of Paneth Cell Secretion by mIKCa1-Specific Blockers—To test for a role for mIKCa1 in Paneth cell secretion, the inhibitory effects of K+ channel blockers were tested in an ex vivo crypt assay system as described previously (10, 27). One-thousand crypts were incubated in 1 ml of isotonic PIPES buffer, consisting of 10 mM PIPES with 0.8% sodium gluconate (pH 7.4), and the fragments were quantitated by autoradiography. The mIKCa1 coding region was amplified from COS-7 cells with FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions, excised to plasmid forms and subjected to digestion within the mIKCa1 coding region were identical to mIKCa1. Plaqueforming units in duplicate by hybridization at 42 °C in 1× NaCl, 50% formamide, 10% dextran sulfate, and 1× SSC with 0.5 × 106 primary clones and 1 × 108 secondary clones (10). For each hybridization, 10 ml of isotonic PIPES buffer, consisting of 10 mM PIPES with 0.8% sodium gluconate (pH 7.4) and 2 mg actinomycin D, was used to activate mIKCa1. To eliminate native COS cell chloride conductances, 10 ml of RNAguard RNase inhibitor, and 40 ml of 1× RT-PCR reaction mix lacking AmpliTaq DNA Polymerase were preheated at 94 °C for 2 min, then complete reaction mixes were cycled as before. Samples of the PCR reactions were analyzed by separation in 2% agarose gels to visualize the 293-bp product, and the fragments were purified with QIAEX II gel extraction kit (Qiagen, Valencia, CA) or transferred to nylon membranes to identify mIKCa1 by hybridization with an hIKCa1 probe (29, 30). Amplification of mIKCa1 was performed a minimum of four times on uninfected, freshly prepared crypts. The 293-bp amplification product was cloned into the TOPO II TA cloning vector (Invitrogen), and its identity as the appropriate region of mIKCa1 sequence was confirmed by DNA sequence (data not shown).

Electrophysiology of mIKCa1—The mIKCa1 cDNA cloning was performed in mouse Paneth cell secretion (10). All experiments were repeated a minimum of three times on freshly prepared crypts.

Western Blot Analysis—Proteins extracted from collected secretions were analyzed for cryptdins by Western blotting following separation by 4%–12% acrylamide–10% acrylamide–urea PAGE gel containing 5 M urea as described previously (10, 27). One-thousand crypts were incubated in 1 ml of isotonic PIPES buffer, consisting of 10 mM PIPES with 0.8% sodium gluconate (pH 7.4) and 2 mg actinomycin D, and the fragments were quantitated by autoradiography. The mIKCa1 coding region was amplified from COS-7 cells with FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions, excised to plasmid forms and subjected to digestion within the mIKCa1 coding region were identical to mIKCa1. Plaque-forming units in duplicate by hybridization at 42 °C in 1× NaCl, 50% formamide, 10% dextran sulfate, and 1× SSC with 0.5 × 106 primary clones and 1 × 108 secondary clones (10). For each hybridization, 10 ml of isotonic PIPES buffer, consisting of 10 mM PIPES with 0.8% sodium gluconate (pH 7.4) and 2 mg actinomycin D, was used to activate mIKCa1. To eliminate native COS cell chloride conductances, 10 ml of RNAguard RNase inhibitor, and 40 ml of 1× RT-PCR reaction mix lacking AmpliTaq DNA Polymerase were preheated at 94 °C for 2 min, then complete reaction mixes were cycled as before. Samples of the PCR reactions were analyzed by separation in 2% agarose gels to visualize the 293-bp product, and the fragments were purified with QIAEX II gel extraction kit (Qiagen, Valencia, CA) or transferred to nylon membranes to identify mIKCa1 by hybridization with an hIKCa1 probe (29, 30). Amplification of mIKCa1 was performed a minimum of four times on uninfected, freshly prepared crypts. The 293-bp amplification product was cloned into the TOPO II TA cloning vector (Invitrogen), and its identity as the appropriate region of mIKCa1 sequence was confirmed by DNA sequence (data not shown).

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mIKCa1 in Paneth Cell Secretion

RESULTS

Cloning of mIKCa1 cDNA from Adult Mouse Small Intestinal Crypts—A cDNA library prepared from isolated adult mouse small intestinal crypts was screened in duplicate with a probe from the 5'-NCR of hIKCa1 (GenBankTM accession number AF033021). Forty-six positive clones with identical or overlapping restriction patterns were identified in the mouse crypt library. The complete 1278-bp coding sequence was obtained in sequences (GenBankTM accession numbers AF022797 and AC073810) showing that the intron-exon organization of the mIKCa1 gene is identical to that in the hIKCa1 homologue (Fig. 1). Comparison of the hIKCa1 cDNA and genomic sequences (GenBankTM accession numbers AF024487, AF072884, and NM_008433) and with full-length mIKCa1 cDNA from mouse embryonic liver (GenBankTM accession number AK010943). mIKCa1 cDNA alignments with existing mouse genomic DNA sequences (GenBankTM accession numbers AC073693 and AC073810) showed that the intron-exon organization of the mIKCa1 gene is identical to that in the hIKCa1 homologue (Fig. 2). Comparison of the hIKCa1 cDNA and genomic sequences (GenBankTM accession numbers AF022797 and AF305731–AF305735) with mIKCa1 sequences revealed >90% nucleotide sequence identity from nucleotide 97 of the human transcript through the coding region and 3'-NCR. The hIKCa1 coding region is 6 nucleotides longer than mIKCa1, coding for two additional residues in the S3-S4 loop. The deduced mIKCa1 protein differs from hIKCa1 at 53 additional positions, two additional residues in the S3-S4 loop. The deduced mIKCa1 protein differs from hIKCa1 at 53 additional positions, two additional residues in the S3-S4 loop. The deduced mIKCa1 protein differs from hIKCa1 at 53 additional positions, two additional residues in the S3-S4 loop.

mIKCa1 mRNA Is Expressed Selectively in Paneth Cells in the Mouse Small Intestine—The intestinal epithelial cell lineages that express the mIKCa1 gene in mouse small intestinal epithelium were determined using a nested RT-PCR assay. Consistent with previous reports (18, 20), RT-PCR analysis of whole organ RNA showed that mIKCa1 mRNA was present in adult mouse bone marrow, spleen, liver, kidney, testis, heart, stomach, small intestine, cecum, and colon (data not shown). Also, mIKCa1 mRNA was amplified equivalently from whole organ RNA throughout the gastrointestinal tract, and its detection in neonatal mouse small bowel RNA (not shown) showed that mIKCa1 gene expression precedes crypt ontogeny and the differentiation of epithelial cell lineages. Because amplification of whole organ RNA provides no information as to sites of gene expression, we attempted to determine the cell type(s) expressing mIKCa1 mRNA in small bowel by in situ hybridization. However, mIKCa1 mRNA levels were insufficient to detect hybridization, and therefore small intestinal mIKCa1 expression was investigated by the RT-PCR analysis of isolated epithelial structures and cells.

In mice, Paneth cells were the primary small intestinal epithelial cell lineage found to express mIKCa1. To identify the small intestinal cell types expressing mIKCa1, nested RT-PCR assays were performed on isolated intact villus epithelium (Fig. 2A, “arrow a”), intact crypts (Fig. 2A, “arrow b”), biassed crypts (Fig. 2A, “arrows c and d”), single Paneth cells (Fig. 2A, “arrows g and h”), or single undifferentiated crypt cells (Fig. 2A, “arrows i and j”). Individual structures or cells were transferred to separate microfuge tubes, mIKCa1 cDNA was amplified using sequence-specific nested amplimers, and the products were analyzed by gel electrophoresis (see “Experimental Procedures”). mIKCa1 mRNA was detected in intact crypts (Fig. 2B, lane b) but not in villus epithelium (Fig. 2B, lane a), and analyses of biassed crypts showed that mIKCa1 mRNA was present only in the lower portion of the crypt (Fig. 2B, lane d) but not in the upper half (Fig. 2B, lane e). Since the lower half of the crypt contains Paneth cells as well as undifferentiated crypt epithelial cells that are agranular, RT-PCR assays were conducted on both these cell populations isolated from single cell suspensions of isolated crypts (see “Experimental Procedures”). Although Fig. 2 shows Paneth cells in crypts stained selectively with Amido Black (see “Experimental Procedures”), all RT-PCR experiments reported here were performed on unstained crypts and are representative of four separate determinations. Single cell RT-PCR detected mIKCa1 mRNA only in Paneth cells (Fig. 2B, lanes g and h, respectively, containing one or five individual Paneth cells), but not in agranular crypt epithelial cells (Fig. 2B, lanes i and j, one or five cells, respectively). Glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified using RNA from all sources in Fig. 2 that were negative for mIKCa1 products (data not shown). Hybridization of a mIKCa1 cDNA probe to a Southern blot of the gel shown in the upper panel of Fig. 2B was consistent with identification of the amplification products as mIKCa1 sequences (Fig. 2B, lower panel). The authenticity of the amplified products was verified by DNA sequence analysis (data not shown).

mIKCa1 and hIKCa1 Are Pharmacologically Indistinguishable—Because mouse and human IKCa1 differ at 55 amino acid residue positions (13% difference), we compared the properties of the mouse channel heterollogously expressed in COS-7 cells with that of human IKCa1. In the representative inside-out patch shown in Fig. 3A, mIKCa1 currents were induced by 1 μM free Ca2+, but not by 50 nM Ca2+, confirming the Ca2+-dependence of this channel. In experiments done in the whole cell mode, the reversal potential of the mIKCa1 current shifted from ~80 mV in sodium Ringer solution to 0 mV in potassium Ringer, consistent with the channel being potassium-selective (Fig. 3B). We also conducted a detailed pharmacological analysis of mIKCa1 using a panel of ten inhibitors that are known to block hIKCa1 in a potency range spanning 9 log units (20, 21). Representative traces for four compounds (TRAM-34, ChTX-Glu32, CLT, and TRAM-7) are shown in Fig. 3, C–F, and dose-response curves for all ten compounds are shown in detail in Fig. 3G. The Kd values (mean ± S.D.) obtained for the mouse channel are identical to those reported for the human homologue, hIKCa1 (20, 21). Earlier published data demonstrated that the single channel conductances of mIKCa1 and hIKCa1 are identical (17, 18). Taken together, these findings show that the biophysical and pharmacological properties of the mIKCa1 channel are indistinguishable from those of hIKCa1, allowing the previously characterized and selective inhibitors of hIKCa1, TRAM-34, TRAM-39, and ChTX-Glu32 to be applied to functional studies of mIKCa1 in Paneth cells.

mIKCa1 Blockers Suppress Paneth Cell Secretory Responses to Bacteria and LPS—The selective expression of mIKCa1 in Paneth cells of mouse small intestinal epithelium suggested that mIKCa1 may regulate Ca2+-mediated events in Paneth cell secretion (15). To test this concept, the effects of mIKCa1

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mm KCl, 2 mm CaCl2, 1 mm MgCl2, 5 mm HEPES (pH 7.4), 290–310 mosm) was used as an external solution. Currents were elicited by 200-ms voltage ramps from −120 to 40 mV every 10 s, and the reduction of slope conductance at ~80 mV by channel antagonists were taken as a measure of channel blocking. The recording in Fig. 3A was done on excised inside-out patches with the external solution described above as pipette solution. K+ aspartate solutions containing 1 μM and 50 μM free [Ca2+] were applied to the cytoplasmic side of the patch. CLT, econazole, and tetrathyraminon were from Sigma; nifedipine was from RBI (Natick, MA); ChTX, ChTX-Glu32, and SrH (Stichodactyla helianthus toxin) were from Bachem Biosciences (King of Prussia, PA). The synthesis and specificities of ChTX-Glu32 (23) and the tritylamethanes TRAM-3, TRAM-7, TRAM-34, and TRAM-39 have been described previously (22).
FIG. 1. Comparison of genomic organization and intron-exon junctions of mouse and human IKCa1. The putative transmembrane segments (S1–S6) in the coding region, the 5'- and 3'-NCR, and the intron-exon junctions (arrowheads) are depicted in the schematic at the top of the figure. Donor and acceptor splice site sequences at each of the conserved exon-intron boundaries between mIKCa1 and hIKCa1 are shown below. Consensus GT-AG (5'-3') splice site sequences are observed at each junction.
channel blockers were tested as inhibitors of Paneth cell α-defensin secretion following exposure to S. typhimurium or E. coli or to 100 ng/ml LPS for 30 min (10). Because cryptdins localize exclusively to Paneth cell secretory granules in the small intestine and account for 70% of the bactericidal activity in Paneth cells (10), bactericidal peptide activity assays provide a sensitive and accurate index of Paneth cell secretion. Accordingly, the effects of IKCa1 blockers on secretory activity were evaluated by measuring antimicrobial peptide activity in Paneth cell secretions for innate immunity in small intestinal crypts exposed to bacteria and bacterial antigens, Paneth cells are inferred to participate in innate immunity in the crypt microenvironment and perhaps above the crypt-villus junction as well (10, 27). The highly restricted expression of mIKCa1 in Paneth cells of the small bowel, and the inhibition of bacterial stimulated Paneth cell secretion by highly selective mIKCa1 blockers, identify mIKCa1 as a functional marker for Paneth cells. Other intestinal cells may express mIKCa1 at levels below the detection limits of our assays.

In the presence of 1 μM ChTX-Glu32 or CLT, the quantity of bactericidal activity secreted by Paneth cells in crypts exposed to S. typhimurium was inhibited by ~50% compared with crypts not exposed to blockers (Fig. 4A). Further studies with CLT showed that the inhibition was dose-dependent (Fig. 4B), and similar results were obtained with crypts exposed to 1000 cfu/crypt E. coli (data not shown). Although these findings were consistent with the involvement of mIKCa1 in Paneth cell secretory responses to bacteria, we perceived the need to test additional inhibitory agents with greater specificities. For example, ChTX, an agent used in preliminary experiments (Fig. 4C and data not shown) also blocks the BKCa, Kv1.2, and Kv1.3 channels (31), and CLT inhibits cytochrome P450-dependent enzymes (32–34) in the concentration range that blocks mIKCa1 (Fig. 3). Therefore, to test for mIKCa1 involvement in Paneth cell secretion of bactericidal peptide activity more specifically, the inhibitory effects of more selective mIKCa1 blockers were evaluated. As shown in Fig. 4, A and C, ChTX-Glu32, selective for IKCa1 over Kv1.2 and Kv1.3 (23) (Fig. 3), inhibited bacterial and LPS-stimulated secretion of bactericidal peptide activity to the same extent as ChTX (Fig. 4C). The highly selective IKCa1 blockers TRAM-34 and TRAM-39 also inhibited LPS-triggered Paneth cell release of bactericidal peptide activity at 200 nM, nearly equivalent to the level of inhibition obtained using 1 μM CLT. On the other hand, the inactive analog TRAM-7 had no measurable inhibitory effect on secretion (Fig. 4C). None of the inhibitors was inherently bactericidal, and none stimulated Paneth cells to release granules (data not shown). These findings implicate the mIKCa1 channel as a specific modulator of Paneth cell α-defensin release in response to bacteria or LPS.

To evaluate the effects of mIKCa1 blockers on cryptdin secretion, Paneth cell secretions collected from crypts exposed to bacteria (Fig. 4, A and B) or LPS (Fig. 4C) were dialyzed, separated by acid-urea PAGE, and probed in Western blots with anti-cryptdin-1 antibody (Fig. 5). As reported previously (10), S. typhimurium evoked secretion of activated cryptdins (Fig. 5, A and B, lanes 1), and no measurable cryptdin was released when crypts were incubated for 30 min in isotonic buffer without stimuli (Fig. 5A, lane 4; Fig. 5B, lane 5). Consistent with the inhibitory effects of ChTX-Glu32 and CLT on release of bactericidal peptide activity (Fig. 4, A and B), 1 μM ChTX-Glu32 or 1 μM CLT reduced cryptdin-specific immunoreactivity in Paneth cell secretions elicited by bacteria (Fig. 5A), and this effect was dose-dependent (Fig. 5B). CLT (1 μM) and the specific IKCa1 inhibitors TRAM-34 and TRAM-39 (200 nM) also diminished LPS-induced cryptdin release from Paneth cells (Fig. 5C). These findings and those in Fig. 4 provide evidence that mIKCa1 has a role in Paneth cell secretion in response to infectious challenge, disclosing potential implications for innate immunity in small intestinal crypts exposed to bacteria.

DISCUSSION

From evidence of their secretion of cryptdins in response to bacteria and bacterial antigens, Paneth cells are inferred to participate in innate immunity in the crypt microenvironment and perhaps above the crypt-villus junction as well (10, 27). The highly restricted expression of mIKCa1 in Paneth cells of the small bowel, and the inhibition of bacterial stimulated Paneth cell secretion by highly selective mIKCa1 blockers, identify mIKCa1 as a functional marker for Paneth cells. Other intestinal cells may express mIKCa1 at levels below the detection limits of our assays.

Carbamyl choline-induced Paneth cell secretion is associated with a biphasic increase in cytosolic [Ca2+]i, where the first rise in [Ca2+]i derives from intracellular stores and the second is dependent on uptake of exogenous Ca2+ (15). The involvement of the Ca2+-activated mIKCa1 channel in Paneth cell secretory responses to bacteria and LPS suggests a role for cytosolic [Ca2+]i in this process as well. By analogy to events in human lymphocytes during the specific immune response, mIKCa1 channels in the Paneth cell membrane would open as cytosolic [Ca2+]i approaches 300 nM, providing the counterbalancing cation efflux necessary to sustain Ca2+ entry from the external environment.
**Fig. 3.** Electrophysiological properties of mIKCa1 expressed in COS-7 cells. 

**A**, representative IK conductance in a single excised inside-out patch in the presence of 1 μM and 50 nM free [Ca^{2+}], demonstrating the calcium dependence of the current. This experiment was repeated on five additional patches. 

**B**, representative IK current recorded in the whole-cell mode in a COS-7 cell transfected with mIKCa1. In this experiment, the bath solution was changed from 160 mM sodium aspartate to 160 mM potassium, and the shift in reversal potential from -80 mV to 0 mV demonstrated the potassium selectivity of the current. This experiment was repeated on 10 additional cells. 

**C**–**F**, inhibitory effects of 25 mM mIKCa1 in Paneth cell secretion. 

**G**, normalized current plotted against compound concentration.
milieu (20, 24, 35). Blockade of mIKCa1 would depolarize the membrane and attenuate the calcium signaling response required to generate a complete Paneth cell secretory response. The mIKCa1 channel may also influence secretion of antimicrobial peptides from other mucosa in the airway, gingival crevice, oropharynx, or urogenital epithelium. The involvement of mIKCa1 in Paneth cell secretion as shown here does not exclude potential roles for other subtypes of calcium-activated K⁺/H11001 channels in antimicrobial peptide secretion by any of these cells.

CLT is being evaluated in human clinical trials for the treatment of secretory diarrheas due to its ability to block IKCa1 in the colonic epithelium (36, 37). Our finding that selective blockers of the IKCa1 channel diminish secretion of Paneth cell antimicrobial peptides in response to bacterial exposure suggests that IKCa1 blockade may have deleterious effects on innate immune mechanisms in the small intestine. To our knowledge, Paneth cell dysfunction has not been identified with human disease. However, in mice genetically deficient for the procryptdin-activating metalloproteinase matrilysin (TRAM-34, 100 nM TRAM-34, TRAM-39, or 1 μM CLT, ChTX, or ChTX-Glu32 as in B) the bactericidal activity of 10-μl samples was measured and expressed as described in B, relative to secretions released by stimulation with LPS in the absence of IKCa1 inhibitors.

FIG. 4. Effects of mIKCa1 inhibition on Paneth cell secretion of bactericidal peptide activity. A, secretions were collected from crypts (1000) resuspended in isotonic sodium gluconate buffer containing 1 μM CLT or 1 μM ChTX-Glu32 coincubated with S. typhimurium at 37 °C for 30 min. Stimulated secretions or control supernatants were collected, and the bactericidal activity of 10-μl samples were tested against ~500 cfu of defensin-sensitive S. typhimurium (10). Surviving bacteria were quantitated as colony-forming units following overnight growth. Data points represent individual triplicate determinations in two separate experiments. For each series of data points, plus characters denote presence of S. typhimurium with crypts as bacterial secretory stimuli, and minus signs denote crypts incubated in the absence of bacteria as negative controls for secretion. Similarly, CLT or ChTX-Glu32 blocking agents were present as noted, and minus signs denote crypts incubated in the absence of either blocker. B, secretions were collected from crypts stimulated with S. typhimurium in the presence of increasing concentrations of CLT, and the bactericidal activity of 10-μl samples of secretions was assayed as described in A and expressed as mean percent inhibition of bacterial cell killing ± S.D. (n = 3), relative to secretions stimulated by S. typhimurium in the absence of blocking agent. C, secretions were collected from crypts stimulated with 100 ng/ml LPS in the presence or absence of 200 nM TRAM-34, TRAM-39, or TRAM-7 or 1 μM CLT, ChTX, or ChTX-Glu32 as in B. The bactericidal activity of 10-μl samples was measured and expressed as described in B, relative to secretions released by stimulation with LPS in the absence of IKCa1 inhibitors.

nm TRAM-34, 100 nm ChTX-Glu32, 100 nm CLT, and 10 μM TRAM-7 on representative whole-cell IK currents. G, pharmacology of mIKCa1. Kd values were determined by testing every compound three times at four concentrations and fitting the Hill equation (nH) to the reduction of slope conductance at ~80 mV. Mean ± S.D. are shown. ■, ChTX (Kd = 4 ± 1 nm, nH = 1.05); △, TRAM-34 (Kd = 21 ± 3 nm, nH = 1.18); ○, ShK (Kd = 26 ± 3 nm, nH = 1.08); ○, ChTX-Glu32 (Kd = 37 ± 4 nm, nH = 0.97); ●, TRAM-39 (Kd = 75 ± 10 nm, nH = 1.14); ○, CLT (Kd = 80 ± 9 nm, nH = 1.12); ●, TRAM-3 (Kd = 490 ± 30 nm, nH = 1.05); ○, nifedipine (Kd = 4.2 ± 0.4 μM, nH = 1.08); ●, econazole (Kd = 11 ± 0.9 μM, nH = 1.1); ○, TRAM-7 (Kd > 25 μM); □, tetraethylammonium (Kd = 28 ± 3 nm, nH = 1.63).
sin, the lack of activated intestinal α-defensins correlates with a diminished ability of the mice to clear orally administered enteric infections, and matrilysin-null mice are 10-fold more susceptible to systemic disease caused by S. typhimurium infection (27). Defects in mIKCa1 expression or function could adversely affect enteric host defense by attenuating secretory responses to infection and perhaps increasing host susceptibility to bacterial colonization.

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REFERENCES


