

The molecular candidate encoding for this channel has remained elusive, although a number of suggestions have been investigated. The latest contender to emerge is TMEM16A (Anoctamin). Evidence so far confirms that over-expression of TMEM16A generates Cl^- currents with the same time-dependent kinetics and Ca^{2+} sensitivity as recorded through Cl_{Ca} channels in smooth muscle cells.

This study investigated expression profiles of TMEM16A in a number of vascular tissues across a number of species. The aim of this study was to confirm the role of TMEM16A as a potential candidate encoding for Cl_{Ca} currents (I_{ClCa}) in native vascular myocytes.

Smooth muscle cells were enzymatically isolated from the mouse portal vein (PV), aorta and carotid artery (CA), rat pulmonary artery (PA), and the rabbit PA. Robust I_{ClCa} , exhibiting distinctive voltage-dependent kinetics, were recorded from all of the tissue preparations mentioned.

RT-PCR was performed, using species-specific primers designed against the available sequences for TMEM16A. Mouse aorta, PA, CA and PV, rat aorta, PA and PV, and rabbit PA tissues were studied. All samples showed clear expression of TMEM16A.

Immunocytochemistry revealed specific expression of TMEM16A in rat PA, and mouse PV, CA and aorta isolated myocytes. This was apparent throughout the cytoplasm, with punctuate "hotspots". Western blot analysis showed bands of the expected molecular weight in mouse aorta, PV and CA.

In summary, we have demonstrated that TMEM16A is a viable candidate for the encoding of Cl_{Ca} channels in vascular smooth muscle.

1657-Pos

TMEM16A is a Calcium-Activated Chloride Channel in Pulmonary Artery Smooth Muscle Cells

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Calcium-activated chloride channels (CaCCs) play pivotal roles in many physiological processes. In vascular smooth muscle, activation of these ion channels by agonist-induced Ca^{2+} release provokes membrane depolarisation, increased Ca^{2+} entry through L-type Ca^{2+} channels and ultimately vasoconstriction. The molecular identity of vascular CaCCs is not fully understood. Here we present evidence that TMEM16A (or Anoctamin1), a member of the transmembrane 16 (TMEM16) protein family forms CaCCs in pulmonary artery smooth muscle cells (PASMCs). Patch-clamp analysis in acutely isolated PASMCs revealed strongly outward rectifying Ca^{2+} -activated Cl^- currents which activated slowly at positive potentials and showed large deactivating tail currents upon repolarisation, very similar to heterologous TMEM16A currents (Caputo *et al.* (2008) *Science* 322, 590-594; Yang *et al.* (2008) *Nature* 455, 1210-1215; Schroeder *et al.* (2008) *Cell* 134, 1019-1029). High levels of TMEM16A mRNA were identified in rat pulmonary arteries and various other vascular smooth muscle cell types. Downregulation of TMEM16A gene expression in primary cultured PASMCs, with small interfering RNA, was accompanied by almost total loss of whole-cell CaCC currents. Our data suggest that TMEM16A forms calcium-activated chloride channels in rat pulmonary artery smooth muscle.

1658-Pos

Regulation and Gating of mAno1 by Voltage and Calcium

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Ca-activated chloride channels play important roles in epithelial secretion, regulation of vascular tone, control of membrane excitability, olfactory transduction, and photoreception. Recently, Ano1 (TMEM16a) has been identified as a Ca^{2+} -activated chloride channel. Activation of Ano1 exhibits both Ca^{2+} - and voltage-dependence. However, the structures and mechanisms responsible for Ca^{2+} - and voltage-dependent activation remain unknown. mAno1 exhibits a strong outward rectification at $<0.5 \mu\text{M}$ Ca^{2+} concentration with little inward current. As Ca^{2+} concentration is increased, inward current increases. We hypothesized that Ano1 has two Ca^{2+} binding sites, a high affinity site that controls the outward current and a low affinity site that controls inward current. We further hypothesized that a region in the first intracellular loop characterized by five contiguous glutamates (444EEEE448) was the potential high-affinity Ca^{2+} sensor. To test this, HEK-293 cells were transfected with wild type mAno1 and mutants in the region surrounding the five glutamates and studied by whole cell and inside-out excised patch recording. In the absence of intracellular Ca^{2+} , wild type mAno1 could be activated by very strong depolarizations ($> +100 \text{ mV}$). In the presence of Ca^{2+} , the G-V relations were well fit with the Boltzmann equation. $V_{1/2}$ was 73 mV at 10 μM intracellular free Ca^{2+} . Increasing

Ca^{2+} shifted the G-V relation to the left. We mutated the putative Ca^{2+} -binding site by deleting the last glutamate of the cluster plus the 3 trailing amino acids (del448EAVK451), substituting the first four glutamates with alanines (444EEEE/AAAA447), and substituting of each negatively charged amino acid with alanine. These mutations altered both voltage-dependent and Ca^{2+} -dependent gating of the channel in complex ways that are consistent with this region being an integral component of Ca^{2+} dependent gating of the channel.

1659-Pos

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1660-Pos

The Preference of the CLC-0 Pore for Charged Methanethiosulfonate Reagents

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Previous experiments from our laboratory showed that the negatively-charged methanethiosulfonate (MTS) reagent, 2-sulfonatoethyl MTS (MTSES) modified a cysteine residue at the Y512 position of CLC-0 faster than the positively-charged 2-(trimethylammonium)ethyl MTS (MTSET). This observation suggested a hypothesis that the pore of CLC-0 may be built with a positive intrinsic pore potential. The hypothesis, however, is challenged by our most recent finding that the preference for the negatively charged MTS reagent is significantly reduced when the cysteine is placed at a deeper pore position, E166. In this study, we examine the discrepancy in the preference for charged MTS reagents between the Y512C and E166C mutants. We find that the inhibition of E166C by intracellularly-applied MTS reagents is contaminated by the modification of an endogenous cysteine because MTS modification rates of the E166A and E166C mutants are similar to each other. We identify that C229, which is located at the dimer interface of the channel, is the endogenous cysteine responsible for this contamination. After C229 is mutated, CLC-0 resumes a preference for selecting the negatively-charged over the positively-charged MTS reagents in modifying E166C, re-confirming the idea of a positive intrinsic potential in the pore. Our study also suggests a communication between the pore region near E166 and the dimer interface near C229 because the inhibition of the channel due to the modification of C229 is dependent upon the amino acid placed at position 166.

1661-Pos

Regulation of the Chloride/Proton Exchanger CLC-5 By Internal pH

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CIC-5 transports anions and protons across intracellular membranes and is necessary for endosomal/lysosomal acidification. Similar to other CIC transporters, CIC-5 can switch between two different transport modes and operate either as anion-proton exchanger or as anion channel, depending on the type of external anion. So far, only some regulatory mechanisms that affect the functional mode of these transporters have been described. We here use combined whole-cell patch clamp and fluorescent intracellular pH recordings in transfected tsA201 cells to characterize CIC-5 mediated transport under various ionic conditions. Asymmetric lowering of intracellular pH results in small increases of both total and proton current amplitudes that might be well explained by changes in the electrochemical gradient imposed by such a maneuver. With internal Cl^- -based solution (pH 7.4) exchange of external Cl^- with SCN^- results in ~5-fold increase in total CIC-5 currents at positive voltages. At higher internal proton concentration (pH 6) the same change of external anion composition results in a 70-fold increase in total currents. The voltage dependence of channel opening is not significantly altered, excluding shifts of the activation curve as a mechanistic explanation. For mutant CIC-5 lacking the "proton glutamate", E268H CIC-5, the simultaneous effect of internal protons and external SCN^- is significantly less pronounced. These findings imply that acidic internal pHs change the protonation state of the proton glutamate and promote the action of external uncoupling anions. In addition, they imply that CIC-5 might even mediate uncoupled anion transport at physiological anion compositions. CIC exchangers may utilize part of the proton electrochemical driving force between the intra/extracellular sites of intracellular compartments to establish anion concentration gradients. The described effects of intracellular pH might be an important part in this pathway and contribute to regulation of endosomal/lysosomal physiological function.