Explaining Calcium-Dependent Gating of Anoctamin-1 Chloride Channels Requires a Revised Topology

Kuai Yu, Charity Duran, Zhiqiang Qu, Yuan-Yuan Cui, H. Criss Hartzell

Rationale: Ca^{2+}-activated Cl channels play pivotal roles in the cardiovascular system. They regulate vascular smooth muscle tone and participate in cardiac action potential repolarization in some species. Ca^{2+}-activated Cl channels were recently discovered to be encoded by members of the anoctamin (Ano, also called Tmem16) superfamily, but the mechanisms of Ano1 gating by Ca^{2+} remain enigmatic.

Objective: The objective was to identify regions of Ano1 involved in channel gating by Ca^{2+}.

Methods and Results: The Ca^{2+} sensitivity of Ano1 was estimated from rates of current activation, and deactivation in excised patches rapidly switched between zero and high Ca^{2+} on the cytoplasmic side. Mutation of glutamates E702 and E705 dramatically altered Ca^{2+} sensitivity. E702 and E705 are predicted to be in an extracellular loop, but antigenic epitopes introduced into this loop are not accessible to extracellular antibodies, suggesting this loop is intracellular. Cytoplasmically applied membrane-impermeant sulfhydryl reagents alter the Ca^{2+} sensitivity of Ano1 E702C and E705C as expected if E702 and E705 are intracellular. Substituted cysteine accessibility mutagenesis of the putative re-entrant loop suggests that E702 and E705 are located adjacent to the Cl conduction pathway.

Conclusions: We propose an alternative model of Ano1 topology based on mutagenesis, epitope accessibility, and cysteine-scanning accessibility. These data contradict the popular re-entrant loop model by showing that the putative fourth extracellular loop (ECL 4) is intracellular and may contain a Ca^{2+} binding site. These studies provide new perspectives on regulation of Ano1 by Ca^{2+}.

Key Words: calcium ▪ ion channel ▪ chloride channel ▪ patch clamp ▪ transmembrane topology

Calcium-activated chloride channels (CaCCs) play vital roles in the cardiovascular system.\(^1^,\(^4\) In vascular smooth muscle, vasoconstrictor-stimulated mobilization of Ca^{2+} from intracellular stores opens CaCCs that serve in a positive feedback loop to sustain contraction by depolarizing the membrane and activating Ca^{2+} influx through voltage-gated Ca^{2+} channels. In portal vein smooth muscle, Ca^{2+} sparks generated from ryanodine-sensitive stores can activate CaCCs to generate spontaneous transient inward currents, which depolarize and activate voltage-gated Ca^{2+} channels. In cardiac myocytes of some species, including rabbit, pig, dog, and sheep, but probably not human, CaCC currents have been shown to play a role in cardiac action potential repolarization and to participate in arrhythmogenesis.\(^5^,\(^6\)\) Recently, it has been proposed that the health benefits (including reduced risk of cardiovascular disease) of red wine and green tea may be explained by the direct effects of gallotannins on CaCCs.\(^7\)

Although the cardiovascular significance of CaCCs has long been apparent, understanding how these channels operate has been slow to develop because the molecular identity of CaCCs was not discovered until 2008, when three laboratories identified Tmem16A, now known as anoctamin-1 (Ano1), as an essential subunit of CaCCs.\(^8^–^10\) Ano1 is a member of a 10-gene superfamily, of which two members, Ano1 and Ano2, have been clearly shown to encode CaCCs that participate in fluid and salt transport by epithelia, slow wave activity in the gut, regulation of smooth muscle contraction, and control of cellular excitability.\(^11^–^13\) Ano1 has been shown to encode CaCCs in vascular smooth muscles.\(^14^–^16\)

Understanding how Ano1 channels operate requires knowledge of how Ca^{2+} causes the channel to open. Even before CaCCs were identified at the molecular level, it was noticed that the biophysical properties of CaCC currents depend on Ca^{2+} concentration. Submicromolar Ca^{2+} activates a current that strongly outwardly rectifies and is time-dependent, whereas higher concentrations activate an instantaneous current with no rectification. This difference is
physiologically relevant because it determines, for example, whether epithelial CaCCs function in a secretory or absorptive capacity or whether neuronal CaCCs carry outward or inward current.17,18 However, it is enigmatic how voltage-dependent and Ca$^{2+}$-dependent gating comes about in molecularly, largely because the location of the Ca$^{2+}$ binding sites and the anion-selective pore remain unknown.

Studies on the structure-function of Ano1 to date have been guided by a topology model of eight transmembrane helices 5 and 6. This model is based on hydropathy analysis19 and experiments performed on Ano7.20 However, the validity of this model for Ano1 has not been experimentally established. This is especially critical because Ano7 has not yet been shown to be a Cl$^{-}$ channel and because the amino acid sequences of Ano1 and Ano7 are only 32% identical.11,21 The putative re-entrant loop has been a prime suspect for the channel pore in Ano1 because the R621E mutation was reported to drastically alter the anion-to-cation selectivity of the channel.8 Experiments described raised questions in our mind about the location of the pore and stimulated us to re-examine the topology of Ano1 and potential Ca$^{2+}$ binding sites. Our revised model moves the previously designated fourth extracellular loop to an intracellular location and identifies a potential Ca$^{2+}$ binding site in this domain. This model has the attractive feature that it places a Ca$^{2+}$ binding site immediately adjacent to the pore and provides insights into Ano1 channel gating.

### Methods

Methods have previously been described18 and detailed Methods are provided in the Online Supplement. The mAno1 a,c splice variant (Accession: Q8BHY3) was used. Mutations were made using polymerase chain reaction-based mutagenesis. The mAno1 was transfected into HEK293 cells using Fugene-6; (Roche Molecular Biochemicals). Transfected HEK293 cells were patch-clamped using a transmembrane solution (570, 614, 672, 700, or 824; Figure 1A), expressed the constructs in HEK cells, and then evaluated the accessibility of the putative channel pore by confocal microscopy. We first performed patch-clamp recording to verify that the introduction of the epitope did not destroy channel function. All of the constructs exhibited currents characteristic of Ano1 (Figure 1A, right table) and mAno1-EGFP fluorescence at the membrane (Figure 1A, left panels), indicating that the HA insertions did not significantly alter the tertiary structure of the channel. In nonpermeabilized cells, Ha epitopes were accessible to extracellularly applied antibodies in permeabilized and nonpermeabilized cells by confocal microscopy. We first performed patch-clamp recording to verify that the introduction of the epitope did not destroy channel function. All of the constructs exhibited currents characteristic of Ano1 (Figure 1A, right table) and mAno1-EGFP fluorescence at the membrane (Figure 1A, left panels), indicating that the HA insertions did not significantly alter the tertiary structure of the channel. In nonpermeabilized cells, Ha epitopes were accessible to extracellular anti-HA antibody only at positions 614 and 824 (Figure 1A, middle panels); all the other positions were inaccessible. The accessibility of the epitopes introduced at 672 and 700 is not consistent with the topology shown in Figure 1B (left panel) and suggests that the putative extracellular loop 4 (amino acids 650–706) is oriented intracellularly.

### Non-standard Abbreviations and Acronyms

Ano1: anoctamin-1, Tmem16a
CaCC: calcium-activated chloride channel
HA: human influenza hemagglutinin
MTSEA: [2-aminomethyl]-methanethiosulfonate hydrobromide
MTSES: sodium [2-sulfonatoethyl]-methanethiosulfonate
MTSET: [2-Trimethylammonioethyl]-methanethiosulfonate bromide

### Results

**Topology of mAno1**

We used 14 different web servers using different strategies to predict mAno1 transmembrane domains (Online Table I). All algorithms consistently identified seven segments corresponding to transmembrane domains 1 to 4 and 6 to 8 in Figure 1B (left panel). Two algorithms failed to identify transmembrane domain 5 and five algorithms identified the proposed re-entrant loop as a transmembrane domain. In light of this ambiguity, we wanted to clarify Ano1 topology. We introduced HA epitopes into mAno1-EGFP at various positions (570, 614, 672, 700, or 824; Figure 1A), expressed the constructs in HEK cells, and then evaluated the accessibility of the putative epitopes to extracellularly applied antibodies in permeabilized and nonpermeabilized cells by confocal microscopy. We first performed patch-clamp recording to verify that the introduction of the epitope did not destroy channel function. All of the constructs exhibited currents characteristic of Ano1 (Figure 1A, right table) and mAno1-EGFP fluorescence at the membrane (Figure 1A, left panels), indicating that the HA insertions did not significantly alter the tertiary structure of the channel. In nonpermeabilized cells, Ha epitopes were accessible to extracellular anti-HA antibody only at positions 614 and 824 (Figure 1A, middle panels); all the other positions were inaccessible. The accessibility of the epitopes introduced at 672 and 700 is not consistent with the topology shown in Figure 1B (left panel) and suggests that the putative extracellular loop 4 (amino acids 650–706) is oriented intracellularly.

**E702 and E705 Contribute to Ca$^{2+}$ Gating**

This revised Ano1 topology suggests new possibilities for the mechanism of channel gating by Ca$^{2+}$. Amino acids 650 to 706, which were previously thought to form an extracellular loop, contain a sequence that is highly conserved among all members of the Ano superfamily: [E/D]-[Y/F]-[M/L/Q]-E-[M/T/L/Q]. In Ano1 and Ano2, this sequence is invariably 702EYMEM. To test whether this region is involved in channel function, E702 and E705 in mAno1 were replaced...
with glutamines. This double mutation exhibited an apparent reduction in Ca\(^{2+}\) sensitivity (Figure 2). The E702Q/E705Q mutant was activated only a small amount by 20 \(\mu\)mol/L Ca\(^{2+}\), a concentration that maximally activated wild-type (WT) Ano1, but E702Q/E705Q was significantly activated by 100-fold higher Ca\(^{2+}\) concentrations (2 mmol/L Ca\(^{2+}\)). This concentration of Ca\(^{2+}\) had no effect on Cl\(^{-}\) currents in untransfected HEK cells (Online Figure I).

We have previously shown that Ano1 can be activated by high voltage in the absence of Ca\(^{2+}\). High-voltage-activated currents for E702Q/E705Q and WT were similar in amplitude (WT: 46.1±10.4 pA/pF, \(n=10\); E702Q/E705Q: 45.0±7.6 pA/pF, \(n=5\) at +200 mV; \(P=0.66\)), supporting the suggestion that this mutation mainly affects Ca\(^{2+}\)-dependent gating while having little effect on voltage-dependent gating. Furthermore, the current activated by 500 \(\mu\)mol/L Ca\(^{2+}\) was very strongly outwardly rectifying, which is characteristic of WT Ano1 currents that are activated by submaximal \([\text{Ca}^{2+}]\). Voltage-dependent activation and deactivation of the current were accelerated as expected if the apparent affinity of the channel for Ca\(^{2+}\) were decreased.\(^{18}\)

We then examined the effects of mutation of E702 and E705 individually. The conservative E702D substitution produced currents that were similar to WT but with slightly more outward rectification. In contrast, the charge-reversal E702K dramatically decreased Ca\(^{2+}\)-activated current (Figure 2E). Both the conservative E705D and the charge-reversal E705K mutations exhibited markedly reduced Ca\(^{2+}\)-dependent activation (Figure 2E). These results suggest that both E702 and E705 are important in Ca\(^{2+}\) sensing or gating.

To quantitate the effects of E702 and E705 substitutions on Ca\(^{2+}\)-dependent gating, we performed experiments in which inside-out excised patches were rapidly switched between zero and high Ca\(^{2+}\) within several milliseconds\(^{18}\) (Figure 3). Current decay was well-fit by a monoexponential equation. The time constant of deactivation (\(\tau_{\text{off}}\)) was \(V_m\)-dependent and was greatly accelerated by the E702Q and E705Q mutations (Figure 3E). At +120 mV, \(\tau_{\text{off}}\) was 408.4±67.3 ms for WT, 40.4±4.1 ms for E702Q, and 96.3±6.6 ms for E705Q.

In principle, the process of current deactivation on switching to zero Ca\(^{2+}\) involves two steps: Ca\(^{2+}\) dissociation from its binding site, followed by channel closure. However, the process is likely dominated by Ca\(^{2+}\) dissociation because \(\tau_{\text{off}}\) is strongly dependent on the ligand (Ba\(^{2+}\) or Ca\(^{2+}\)) used to activate the channel\(^{18}\) (see Discussion).
The time constant of activation ($\tau_{on}$) of Ano1 current on switching to $\text{Ca}^{2+}$-containing solutions is dependent on $[\text{Ca}^{2+}]$, as expected if $\text{Ca}^{2+}$ binding is a rate-limiting step in channel activation. At $+120 \text{ mV}$ with $82 \mu\text{mol/L} [\text{Ca}^{2+}]$, $\tau_{on}$ for WT current was $5.1 \pm 1.1 \text{ ms}$, but was approximately 25-times slower for E705Q at $131.1 \pm 20.2 \text{ ms}$ (Figure 3D). At very high $[\text{Ca}^{2+}]$ of $2 \text{ mmol/L}$, $\tau_{on}$ for E705Q was still slower than wild-type ($35.9 \pm 7.8 \text{ ms}$). The E702Q mutation had a smaller effect on $\tau_{on}$ than the E705Q mutation (Figure 3D). The $\tau_{on}$ of E702Q was indistinguishable from WT at $82 \mu\text{mol/L} [\text{Ca}^{2+}]$, but under these conditions $\tau_{on}$ is close to the switching time of the perfusion system and therefore not quantitatively reliable. However, lower $\text{Ca}^{2+}$ concentrations could not be tested for E702Q because the current amplitude with low $[\text{Ca}^{2+}]$ was too small for accurate measurement.

The very slow $\tau_{on}$ observed with E705Q raised questions in our minds about possible artifacts of the fast perfusion system. To test the validity of this approach, we measured the activation of currents in response to photolysis of caged $\text{Ca}^{2+}$ (NP-EGTA) under whole-cell patch clamp. For the same intensity UV flash, WT currents activated much more quickly than E705Q currents (Online Figure II). At the same flash intensity...
Thiol Reagents Alter Ca\(^{2+}\) Sensitivity of E702C and E705C

Alteration of the Ca\(^{2+}\) sensitivity of mAno1 by changing the charge at E702 and E705 by mutagenesis is consistent with the hypothesis that these amino acids contribute to a Ca\(^{2+}\) binding site. To test this hypothesis using a different approach, we asked whether modification of thiols introduced at these positions by charged MTS reagents would also alter Ca\(^{2+}\) sensitivity. The E702 or E705 were replaced with cysteine and the effects of charged thiol reagents, MTSET\(^+\) and MTSES\(^-\), on channel gating by Ca\(^{2+}\) were measured in fast perfusion experiments. The MTS reagents were applied to the cytoplasmic face of inside-out excised patches. The mAno1 has at least five cysteines that are predicted to be cytoplasmic, but neither MTSET\(^+\) nor MTSES\(^-\) had any significant effect on the amplitude or kinetics of activation or deactivation of wild-type mAno1 when applied to the cytoplasmic face of the patch (Figure 4C–E). The E702C (unmodified by MTS reagent) was much less sensitive to Ca\(^{2+}\) than wild-type mAno1. At +120 mV, the EC\(_{50}\) for E702C was 114 \(\mu\)mol/L compared to 0.94 \(\mu\)mol/L for WT and 21 \(\mu\)mol/L for E702Q. The larger EC\(_{50}\) for E702C compared to E702Q might be explained if the side chain oxygen of glutamine participates in Ca\(^{2+}\) coordination. MTSET\(^+\) increased the EC\(_{50}\) of E702C approximately 30-fold (from 114 \(\mu\)mol/L to approximately 3 mmol/L at 120 mV) by slowing \(\tau_{on}\) and accelerating \(\tau_{off}\) (Figure 4F–H). The decrease in Ca\(^{2+}\) affinity was accompanied by a decrease in current amplitude (56.4\%±10.8\%), as expected. MTSES\(^-\) had the opposite effect on EC\(_{50}\) (Figure 4F–H) and the uncharged MTSEH had no effect (Online Figure IIIA). At 0 mV, the EC\(_{50}\) was estimated to be 550 \(\mu\)mol/L for unmodified E702C, approximately 3890 \(\mu\)mol/L (extrapolated for MTSET\(^+\)-modified currents, and 144 \(\mu\)mol/L for MTSES\(^-\)-modified currents. The results with E705C exhibited a similar trend but were decidedly less dramatic, possibly because of reduced accessibility of MTS reagents to this residue, which is surrounded by very hydrophobic amino acids.

If E702 and E705 are located on the cytoplasmic side of the membrane, we would predict that MTS reagents applied from the extracellular side would have no effect on E702C or E705C currents. We tested this prediction using whole-cell recording because outside-out patches could be obtained with only very low success. We also tested several other cysteine-substituted amino acids nearby. In whole-cell recording, extracellular application of MTSET\(^+\) and MTSES\(^-\) had no significant effect on E702C or E705C currents (MTSET\(^+\) increased E702C currents by 4.7\%±6.6\% and decreased E705C by −3.1\%±0.9\%). This compares to a decrease of 56.4\%±10.8\% for E702C in excised patches exposed to cytosolic MTSET\(^+\). MTSET\(^+\) had no significant effect on any of the currents generated by these cysteine-substituted mutants (Online Figure IIIB).

Online Figure IV shows the time courses of the current changes caused by cytoplasmic MTS reagents on E702C and WT in inside-out excised patches. Currents were activated by...
a low concentration of Ca\(^{2+}\) to maximize the change in current amplitude that was produced by a change in Ca\(^{2+}\) affinity. MTSET\(^+\) (1 mmol/L) decreased the current with \(\tau\) of 52 ms and a rate constant of approximately \(2 \times 10^4\) M\(^{-1}\)s\(^{-1}\). This rate is close to the rate of modification of mercaptoethanol by MTSET\(^+\) in solution (approximately \(10^2\) M\(^{-1}\)s\(^{-1}\)), suggesting that these residues are freely accessible to the aqueous environment. The effect of MTSES\(^-\) is slower than MTSET\(^+\), but because MTSES\(^-\) reacts with mercaptoethanol in solution approximately five-times slower than MTSET\(^-\), this result is also consistent with high aqueous accessibility.

### Cysteine Accessibility to Probe Residues Near the Pore

The region between F620 and N650 has been proposed to be important in forming the Ano1 pore because mutagenesis of positively charged amino acids (notably R621) in this region were reported to drastically alter ion selectivity.\(^8\) The R621E mutant was reported to exhibit a \(P_{Na}/P_K\) ratio of 0.87 compared to 0.03 for wild-type. We have tried without success to confirm these observations using several different methods. In Figure 5, \(P_{Na}/P_{Cl}\) and \(P_{Cs}/P_{Cl}\) were determined by the dilution potential method.\(^23\) The ionic selectivity was found to be identical for WT \((P_{Na}/P_{Cl}=0.13; P_{Cs}/P_{Cl}=0.08)\) and R621E \((P_{Na}/P_{Cl}=0.14; P_{Cs}/P_{Cl}=0.1)\). The lack of effect of the R621E mutation on ion selectivity raised questions in our mind regarding whether the pore of Ano1 was located in this region.

We used substituted cysteine accessibility mutagenesis\(^23\) to glean more information about the topology of the putative re-entrant loop. The mAno1 has 16 native cysteines that could possibly compromise the interpretation of the effects of thiol reagents on introduced cysteines. To determine which endogenous cysteines might be essential for Ano1 function, we first synthesized a cysteine-less mAno1 in which all cysteines were replaced with serines. The cysteine-less mutant trafficked to the plasma membrane, as judged by the presence of a bright ring of fluorescence of the mAno1-EGFP tag at the cell surface, but it did not generate currents. To determine which cysteines were important for function, a series of mutants were generated with individual cysteines or groups of cysteines replaced with serine (Online Table II). Six cysteines (C370, C379, C383, C386, C395, or C386) were essential for Ano1 current. The essential cysteines are predicted to be extracellular. Five are located in the first extracellular loop and one is located in the last extracellular loop. An Ano1 construct having the six essential cysteines intact and the remaining 10 cysteines substituted with serine (mAno1\(_{6C}\): C23S/C49S/C166S/C259S/C357S/C559S/C625S/C630S/C635S/C933S) exhibited identical biophysical properties to the wild-type channel but had a reduced current amplitude. Replacing any one of the remaining six cysteines (C370, C379, C383, C386, C395, or C386) with serine in Ano1\(_{6C}\) completely eliminated the current (Figure 6A). Because these six cysteines were essential for mAno1 function, the mAno1\(_{6C}\) construct was used as the template for cysteine scanning mutagenesis. Extracellular MTSET\(^+,\) MTSES\(^-\), or MTSEA\(^+\) had little effect on Ano1\(_{6C}\) (Figure 6C).

![Figure 5. Effect of R621E mutation on anion:cation permeability of mAno1. Whole-cell recordings of (A) WT mAno1 in symmetrical 150 mmol/L NaCl with 180 nmol/L Ca\(^{2+}\) and (B) R621E mAno1 in symmetrical 150 mmol/L NaCl with 1.1 mmol/L Ca\(^{2+}\)). R621E mAno1 with different extracellular [NaCl]. E, Change in reversal potential (\(\Delta E_{rev}\)) for different extracellular [NaCl] or [CsCl] determined from experiments like those in (C) and (D). Lines are best fits to the Goldman-Hodgkin-Katz equation.

Each amino acid from F620 to Q646 in mAno1\(_{6C}\) was replaced with cysteine and extracellular MTS reagents were tested in whole-cell recording (an example is shown in Figure 6B). Cysteine substitution at positions 620 to 629, 633 to 634, and 637 to 638 significantly reduced current amplitude (Figure 6C). MTSET\(^+\) significantly stimulated G628C, G629C, and S630C\(^*\) currents, whereas MTSES\(^-\) decreased S625C\(^*\), G628C, and M632C currents (Figure 6D). *denotes that this position is a native cysteine in wild-type mAno1 but is mutated from S to C in mAno1\(_{6C}\). These results support the idea that this region is important in channel function.

To locate amino acids that might be deeper in the pore, we tested the effect of MTSEA\(^+\), which is more membrane-
permeant than MTSET+. MTSEA+ significantly increased I636C and Q637C currents, suggesting that these amino acids are located deeper in the membrane. In addition, MTSET+ increased K645C current extremely slowly over approximately 10 minutes (this is not reflected in summary data because the increase was so slow). Although our analysis cannot formally distinguish between a re-entrant loop and a membrane-spanning segment, these data are consistent with amino acids 625 to 630 contributing to an outer vestibule that is easily accessible to the extracellular fluid and amino acids beyond 635 being located deep in the pore. To determine whether these amino acids are located in the permeation pathway, we measured the relative iodide permeability ($P_i/P_d$) and conductance ($G_i/G_d$) of the cysteine-substituted mAno16C with the expectation that amino acids in the permeation pathway might affect ionic selectivity. However, none of the mutations significantly altered the ionic selectivity (Figure 7). We also measured the relative Na+ permeability and conductance of selected mutants and did not detect any significant differences. These data suggest that this region of the protein may contribute to the permeation pathway but is not critical for forming the selectivity filter.

**Discussion**

**Ca2+ Sensor of Ano1**

One contender for the Ca2+ binding site is a stretch of five glutamic acids located in the first intracellular loop because of its resemblance to the “Ca bowl” of the large-conductance K channel. Although a naturally occurring splice variant ($\Delta_{448}$EAVK451) that deletes the fifth glutamic acid increases the EC50 for Ca2+ approximately 50-fold, neutralization of the first four glutamic acids (444EEEE/AAAA447) also alter the voltage-dependent gating of the channel in the absence of Ca2+, we have suggested that the pentaglutamate region couples Ca2+ and voltage to channel opening but is unlikely to be a major Ca2+ binding site itself. Other studies have implicated sites in the N-terminus as possible Ca2+ or CaM binding sites, but there remains no consensus about the mechanisms of Ca2+ regulation.

The data presented here strongly implicate another region in Ca2+ regulation. Surprisingly, this region was predicted to be an extracellular loop from hydropathy analysis and, by analogy to Ano7, another member of the anoctamin superfamily. However, there is no convincing evidence that Ano7...
is a Cl$^-$ channel. In our experience, Ano7 does not traffic to the plasma membrane or generate currents in transfected cells and is largely intracellular in adult human prostate, where it is normally expressed. In a study by Schreiber et al., it was suggested that Ano7 might function as a CaCC. However, in iodide flux assays, the ATP-stimulated flux in Ano7-transfected cells was similar to untransfected cells and less than 10% as large as in Ano1-transfected cells. The ionomycin-stimulated iodide flux in Ano7-transfected cells was actually less than in untransfected cells. Furthermore, the short form of Ano7 (Ano7S), which is a 179-amino-acid-protein with no predicted transmembrane domains, produced approximately the same iodide flux as the long form of Ano7. This supports the idea that Ano7 is not a plasma membrane CaCC and raises the possibility that the topology of Ano7 is different than that of Ano1.

Revised Topology Is Required to Explain Our Data

Our data show that HA tags introduced at positions 672 and 700 in Ano1 are not accessible to extracellular antibody. Furthermore, when E702 and E705 are replaced with cysteine, exposure of the cytosolic face of the membrane to membrane-impermeant MTS reagents causes a rapid change in current amplitude or Ca$^2+$ sensitivity. This provides an independent piece of data that these residues are intracellular and not extracellular.

If this region is intracellular, then a transmembrane segment must exist somewhere between positions 614, where the introduced HA epitope is extracellular, and 672, where the HA epitope is intracellular. Although computer algorithms for predicting transmembrane domains do not consistently identify a transmembrane domain between amino acids 614 and 672, between positions 631 and 648, 12 of the 18 amino acids are hydrophobic. Based on secondary structure predictions, all but one of the hydrophobic amino acids are located on one face of this segment. On this basis, we believe that residues 631 to 648 may form a transmembrane segment. The problem with this model is that the HA epitope introduced at position 824 is clearly extracellular, which means that there must be an even number of transmembrane segments between 614 and 824. Attempts to probe the topology of this region were unsuccessful because introduction of HA epitopes at positions 631 to 648 may form a transmembrane segment. The side chains of lysine and arginine interact with the hydrophobic aspects of the long transmembrane segments. Tryptophan has a strong preference for lipid carbonyls and the hydrophobic aspects of the long transmembrane segments. This is a common feature of membrane interfaces. This is a common feature of mAno1 are consistently identified by transmembrane prediction algorithms. These segments all exhibit a characteristic feature: tryptophan and basic residues are located at the predicted membrane interfaces. This is a common feature of transmembrane segments. Tryptophan has a strong preference for lipid carbonyls and the hydrophobic aspects of the long side chains of lysine and arginine interact with the hydrophobic core of the membrane. However, in contrast to the six transmembrane segments that exhibit these characteristic features, the two putative transmembrane segments located between them lack these features. This suggests that the central part of the protein may have a more complex topology than previously predicted. A similar uncertainty existed regarding the transmembrane topology of the CIC transporters before they were crystallized. The uncertainty stemmed from the fact that the CIC transmembrane α-helices are variable in length and tilted at different angles relative to the plane of the membrane. Understanding the organization of mAno1 further will likely have to await direct structural studies.

Effects of Thiol Reagents Support an Intracellular Location of E702 and E705

One observation that we believe strongly supports the hypothesis that E702 and E705 are intracellular is the finding that altering the charge on E702C and E705C with membrane-impermeant charged MTS reagents changes the Ca$^2+$ affinity of the channel. MTSET$^+$ renders the channel less sensitive to Ca$^2+$ activation, whereas MTSES$^-$ shifts the Ca$^2+$ sensitivity of the channel closer to that of wild-type. The size and charge of MTSES$^-$-modified cysteine makes it an excellent surrogate for glutamic acid because MTSES$^-$-modified cysteine is almost exactly the same volume as glutamic acid (142 Å$^3$ for MTSES$^-$-modified cysteine compared to 138 Å$^3$ for glutamic acid) and the negative charge is relatively localized with an estimated charge of −0.79 on each oxygen (a preferred Ca$^2+$ ligand) on the sulfonatoethyl group. In contrast, MTSET$^+$ is larger (approximately 173 Å$^3$) and its charge density is relatively diffuse. The +1 charge on MTSET$^+$ is distributed over approximately 17 atoms. This explains why addition of positive charge by cysteine modification by MTSET$^+$ does not itself activate the channel, because its charge density is low compared to Ca$^2+$, which has a +2 charge in a volume of approximately 20 Å$^3$.

Separation of Effects on Ca$^{2+}$ Binding and Channel Gating

Channel gating by Ca$^{2+}$ involves at least two steps: Ca$^{2+}$ binding and channel opening/closing. Because these steps are allosterically coupled, it is difficult to unambiguously separate the effect of a mutation on Ca$^{2+}$ binding from channel gating. However, we believe that the effects of the E702 and E705 mutations on the activation and deactivation of Ano1 in response to Ca$^{2+}$ jumps are most simply interpreted in terms of changes in Ca$^{2+}$ binding. We have shown that the rates of Ano1 activation and deactivation depend on the ligand used. For example, channel deactivation is more rapid and activation is slower using Ba$^{2+}$ as ligand compared to Ca$^{2+}$. The simplest explanation of these data is that ligand binding and unbinding are the predominant rate-limiting steps and that the probability of the channel being open in the absence of bound ligand is negligible. It is difficult to imagine a mechanism that would change the open time that is dependent on the species of ligand (Ca, Ba) but is independent of ligand dissociation. Although one could argue that Ba$^{2+}$ and Ca$^{2+}$ place the channel into different conformations that relax with different kinetics on ligand dissociation, there is no precedent supporting such a hypothesis.

Physiological Significance of Ca$^{2+}$ Gating of Ano1

CaCCs participate in the transient outward current (I$\text{to}$) that plays a role in repolarization of the action potential in some
species. The best-studied component of Ito is a K⁺ current mediated by Kᵡ₄.₅/K₁.₄ channels; however, in some species, CaCC currents comprise a significant fraction of Ito.²¹

The contribution of CaCC current, because of its dependence on Ca²⁺, would be expected to increase under conditions of Ca²⁺ overload and could lead to arrhythmogenesis. CaCCs have been implicated in T-wave alternans, a prequel to malignant ventricular arrhythmias that occur during left ventricular hypertrophy,²² and delayed afterdepolarizations that may lead to arrhythmias.²³,²⁴ Drugs targeting Ano1 could potentially provide new therapies for hypertension or cardiac arrhythmias.

Elucidating the molecular mechanisms of Ano1 gating is essential for developing new therapies targeting these channels. High-throughput screens have identified small molecule inhibitors and activators of Ano1.²⁵–²⁸ However, evaluating these drugs requires a molecular understanding of how the Ano1 channel is gated. For example, drugs could activate or inhibit Ano1 channels by altering the affinity of the channel for Ca²⁺ or by affecting how the channel gates open in response to Ca²⁺ or to voltage. Drugs working by these different mechanisms are likely to have different pharmaceutical effects. For this reason, it is of paramount importance to understand how this channel operates. Another important question is that of possible side effects of drugs that might occur because Ano1 is ubiquitously expressed in epithelia and smooth muscle of various types. However, Ano1 has multiple splice variants,²⁹,³⁰ some of which affecting the regions that we have identified as important in Ca²⁺ and voltage-dependent gating. This not only raises the possibility that variant-specific drugs might be developed but also emphasizes the importance of structure-function studies to establish the functions of various domains that are drug targets.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- The ion channel anoctamin-1 (Ano1) regulates vascular smooth muscle tone and participates in cardiac action potential repolarization in some species (comprising the current known as I_{to2}).
- Ano1 conducts chloride ions and is activated by increases in intracellular calcium.

What New Information Does This Article Contribute?

- The study describes the structural determinants of calcium activation of Ano1.
- The protein domain involved in Ano1 calcium regulation was formerly thought to be extracellular; hence, a new model for the orientation of the channel in the membrane is proposed.
- The study provides insights that may be helpful in designing and evaluating new drugs that target Ano1.

Although chloride channels are known to contribute to cardiovascular regulation, relatively little is known at the molecular level about how they are governed. Hence, we studied how the newly discovered chloride ion channel Ano1 is turned on by intracellular calcium. We have identified two glutamic acid residues that drastically alter the sensitivity of the channel to intracellular calcium when they are mutated. Surprisingly, these amino acids were previously thought to be located in an extracellular loop. Using a variety of techniques, we suggest that this loop is located on the cytoplasmic side of the membrane and is involved in calcium sensing by the channel. These data contradict the current re-entrant loop model by showing that the putative fourth extracellular loop (ECL 4) is intracellular and may contain a Ca\(^{2+}\) binding site. Elucidating the molecular mechanisms of Ano1 regulation by calcium is essential for developing new therapies that target these channels. Drugs that target Ano1 could be useful in treating hypertension or arrhythmias.
Explaining Calcium-Dependent Gating of Anoctamin-1 Chloride Channels Requires a Revised Topology
Kuai Yu, Charity Duran, Zhiqiang Qu, Yuan-Yuan Cui and H. Criss Hartzell

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**Supplemental Material**

**Detailed Methods.**

**Electrophysiology.** The α,c splice variant 1 of mAno1 2 (Accession: Q8BHY3) was used in all experiments and numbering refers to this sequence. mAno1 tagged with enhanced green fluorescent protein (EGFP) at the C-terminus was provided by Dr. Uhtaek Oh, Seoul National University. Mutations were made using PCR-based mutagenesis (Quickchanger; Stratagene, La Jolla, CA). WT or mutant mAno1 was transfected into HEK293 cells (1 μg total DNA per 35-mm plate), using a blend of lipids (Fugene-6; Roche Molecular Biochemicals, Indianapolis, IN). Single cells identified by EGFP fluorescence were used for whole-cell patch clamp experiments within 72 hours. Transfected HEK293 cells were recorded using conventional whole-cell and excised inside-out patch-clamp technique (EPC-7, HEKA). Fire-polished borosilicate glass patch pipettes were 3-5 MΩ. Experiments were conducted at room temperature (20-24°C). Since the liquid junction potentials were small (<2 mV), no correction was made. The zero Ca\(^{2+}\) intracellular solution contained (mmol/L): 146 CsCl, 2 MgCl\(_2\), 5 EGTA, 10 HEPES, 10 sucrose, pH 7.3, adjusted with NMDG. High Ca\(^{2+}\) pipette solution contained 5 mmol/L Ca\(^{2+}\)-EGTA, instead of EGTA (free Ca\(^{2+}\) \(\sim\) 20 μM). The 126 μM and 2 mmol/L Ca\(^{2+}\) were made by adding 0.2 mmol/L and 2 mmol/L CaCl\(_2\) to high-Ca\(^{2+}\) solution. The standard extracellular solution contained (mmol/L): 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 15 glucose, 10 HEPES, pH 7.4 with NaOH. This combination of intracellular and extracellular solutions set \(E_{\text{rev}}\) for Cl\(^{-}\) currents to zero. Relative anion conductance was determined by measuring the slope of the current–voltage (I-V) curve determined from voltage ramps with different extracellular anions. Relative anion permeability was determined by measuring the shift in zero-current \(E_{\text{rev}}\) after changing the bath solution from 151 mmol/L Cl\(^{-}\) to 140 mmol/L substitute anion (X\(^{-}\)) plus 11 mmol/L Cl\(^{-}\). The permeability (P) ratio was calculated by using the Goldman-Hodgkin-Katz equation: 

\[
P_X/P_{\text{Cl}} = \frac{[\text{Cl}]_o /[\text{X}]_o \exp(\Delta E_{\text{rev}} F / RT)}{[\text{Cl}]_i /[\text{X}]_i} = \frac{[\text{Cl}]_o /[\text{X}]_o \exp(\Delta E_{\text{rev}} F / RT)}{[\text{Cl}]_i /[\text{X}]_i}
\]

where \(\Delta E_{\text{rev}}\) is the difference between \(E_{\text{rev}}\) with the test anion X\(^{-}\) and that observed with symmetrical Cl\(^{-}\). F (Faraday’s constant), R (ideal gas content), and T (temperature in °Kelvin) are thermodynamic parameters. Permeability of Na\(^{+}\) or Cs\(^{+}\) relative to Cl\(^{-}\) was determined by measuring changes in zero-current \(E_{\text{rev}}\) when the concentration of extracellular NaCl or CsCl was changed (“dilution potential” method) 3. Traces were analyzed with Clampfit 9 (Molecular Devices). Data are presented as mean ± SEM. Statistical difference between means was evaluated by two-tailed t-test. Statistical significance was assumed at \(p<0.05\).

**Rapid Perfusion.** The fast application of Ca\(^{2+}\) to excised inside-out patches was performed using a double-barreled theta tubing (1.5 mm o.d.; Sutter Instruments) with a tip diameter of \(\sim\)50 μm attached to a piezobimorph on a micromanipulator 4, 5. One barrel was filled with standard zero-[Ca\(^{2+}\)] solution, and the other barrel was filled with intracellular solution containing the indicated free Ca\(^{2+}\). Excised patches were switched between streams by applying \(\sim\)100 V to the piezobimorph. The time course of solution exchange across the laminar flow interface was estimated by liquid junction potential measurements to be 0.5 ms (10–90% rise time) for a 10-fold difference in ionic strength. The onset of current upon switching to high [Ca] frequently exhibited a sigmoid time course. Some of this may reflect kinetics of access of Ca\(^{2+}\) to its binding site. For simplicity, the onset was fit to an exponential and the sigmoid onset was ignored.

**Photolysis of caged Ca\(^{2+}\).** Cells were placed on the stage of a Zeiss Axiovert inverted microscope. The microscope condenser assembly was replaced with a 100-W xenon model JML flash lamp (Rapp Optoelektronik GmbH, Hamburg, Germany) focused onto the cell (4-mm diameter spot) with an 18-mm focal length lens 6. Short-wavelength UV light was reduced with a UG11 filter. We typically used 45 mJ, 90 mJ, and 135 mJ flashes. The duration of the flash was
< 2 ms. Cells were loaded with caged Ca\(^{2+}\), o-nitrophenyl EGTA (NP-EGTA, Invitrogen) \(^7\), from the patch pipet. The pipet solution contained (mM) 2 NP-EGTA, 1.5 mM CaCl\(_2\), 25 mM HEPES-NMDG pH 7.5, 1 mM MgCl\(_2\), and 136 mM CsCl. Free Ca\(^{2+}\) concentration was calculated to be 80 nM. Before photolysis, Ano1 current was typically less than 100 pA in amplitude.

**Sulfhydryl modification.** Stock solutions of [2-Trimethylammonioethyl]-methanethiosulfonate bromide (MTSET), sodium [2-sulfonatoethyl]-methanethiosulfonate (MTSES) and [2-aminoethyl]-methanethiosulfonate hydrobromide (MTSEA) (Toronto Research Chemicals, Toronto, Canada) were prepared in water, stored on ice, and used within 90 min. The reagents were diluted to the indicated working concentration immediately before use. Effects of MTSES and MTSET were not reversed by washout of the reagent and presumably reflect modification of cysteine residues. MTS reagents were applied on the intracellular side of excised inside-out patches, and were washed out before recording.

**Cysteine-less mAno1.** All 16 native cysteines in mAno1 were replaced with serines using the following strategy. DNA molecules were synthesized by Integrated DNA Technologies, Coralville, Iowa, corresponding to bases 1000-1279 (encoding amino acids ~333-426 including 6 cysteines) and 1582-1980 (encoding amino acids ~527-660 including 4 cysteines) with a serine codon (AGT or AGC) replacing each cysteine codon. Fragments corresponding to bases 1-438 (2 cysteines), 399-1035 (2 cysteines), 1242-1617 (0 cysteines), and 1945-2883 (2 cysteines) were amplified from the mAno1 cDNA by PCR and the cysteine codons replaced with serine codons by PCR-based mutagenesis. The fragments were assembled by overlapping PCR \(^8\) using primers that added Nhe1 and EcoRI restriction endonuclease sites at the N- and C- termini for ligation into the pEGFP-N1 vector. A variety of constructs were made in this way with different combinations of substituted cysteines in order to determine which cysteines were essential for mAno1 function (Supplementary Table 1). All constructs were verified by complete full-length sequencing.

**HA-tagged mAno1.** Tandem human influenza haemagglutinin (HA) epitopes (YPYDVPDYA) were introduced into various locations in mAno1 where EcoRI restriction endonuclease sites had been added by PCR-based mutagenesis. The EcoRI-augmented mAno1 constructs were digested with EcoR1 and ligated with two primers that encoded the HA tag: Forward primer A: ccggaattctacccatacgatgttccagattacgcttacccatacgatgttccagattacgctgaattccgg
Reverse primer B: ccggaattcagcgtaatctggaacatcgtatgggtaagcgtaatctggaacatcgtatgggtagaattccgg
All constructs were confirmed by sequencing. Only constructs that generated currents were used for immunofluorescent staining and topology determination.

**Immunofluorescent staining.** mAno1 with inserted HA epitopes at various positions were used to determine the topology by assessing the accessibility of the HA epitope to extracellularly applied antibody. Cells were fixed for 15 min at room temperature in 1% paraformaldehyde in 0.1mol/L phosphate buffer pH 7. Non-permeabilized cells were washed in 3 times in blocking buffer (PBS containing 1% cold water fish gelatin (Sigma)) for 15 min each. Permeabilized cells were incubated in blocking buffer containing 0.15% - 0.3% Triton-X100. Cells were incubated with anti-HA antibody diluted 1:750 in blocking buffer for 2 h at room temperature, washed 3 times in blocking buffer, and then incubated in goat-anti-rabbit IgG conjugated with Dylight-549 (Jackson Immunochemicals) diluted 1:1000 in blocking buffer. Images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope.
Online Figure I. 2 mM Ca\(^{2+}\) does not activate Cl\(^{-}\) current in untransfected HEK cells. Untransfected HEK cells were whole-cell patch clamped with 2 mM Ca\(^{2+}\) in the pipet solution.
Online Figure II. Kinetics of Ano1 current activation in response to photolysis of caged Ca$^{2+}$ in whole cell recording. Cells were patch clamped with pipet solutions containing NP-EGTA loaded with Ca$^{2+}$. Ca$^{2+}$ was released by photolysis using a 90 mJ flash from a flashlamp at 100 ms on the x-axis. The activation of the current was fit to an exponential equation.
Online Figure III. Effects of MTS reagents on Ano1 and various cysteine-substituted mutants. 

A-C. Effects of uncharged MTSEH on $\tau_{on}$, $\tau_{off}$, and EC$^{50}$ for Ca$^{2+}$ for the E702C mutant of Ano1. Experiments were performed as in Fig. 4 except that cells expressing E702C were exposed to 1 mM MTSEH. 

D. Effects of MTSET$^+$ on currents generated by Ano1 and various cysteine-substituted mutants. HEK cells were transfected with Ano1 mutants and the effect of 1 mM MTSET$^+$ applied in the bath were measured. The effect of extracellular MTSET$^+$on wild type Ano1 is shown in Fig. 6.
Online Figure IV. Timecourse of the effect of MTSES⁻ (A, C) and MTSET⁺ (B,D) applied to the cytoplasmic side of an inside-out patch on current amplitudes of E702C (A,B) and WT Ano1 (C,D) currents. The patch was excised in zero Ca²⁺ and then switched to high Ca²⁺ (20 µM in A,C,D; 200 µM in B) and then rapidly switched to 1 mM MTSET⁺ or MTSES⁻ in high-Ca²⁺ solution. With WT Ano1, the current spontaneously runs down with time.
**Online Table I.**

**Transmembrane Prediction Analysis of mAno1**

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Φ - hydrophobicity, inside - inside positive, HMM - hidden Markov Model, NN - neural network, consensus - consensus of multiple predictions, ΔG - free energy of partition, SVM - support vector machine
Online Table II
Current amplitudes of cysteine-substituted mAno1 constructs.

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Filled boxes indicate cysteines that were replaced with serines. Current amplitude was measured with 20 µM Ca^{2+} by whole-cell recording. Currents indicated by "-" were smaller than 200 pA and considered negligible. Essential cysteines are bold.
Supplementary References


