Fluorescence resonance energy transfer analysis of subunit stoichiometry of the epithelial Na\textsuperscript{+} channel

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Activity of the epithelial Na\textsuperscript{+} channel (ENaC) is rate limiting for Na\textsuperscript{+} (re)absorption across electrically tight epithelia. ENaC is a heteromeric channel comprised of three subunits, \(\alpha\), \(\beta\) and \(\gamma\), with each subunit contributing to the functional channel pore. The subunit stoichiometry of ENaC remains uncertain with electrophysiology and biochemical experiments supporting both a tetramer with a \(2\alpha:1\beta:1\gamma\) stoichiometry and a higher ordered channel with a \(3\alpha:3\beta:3\gamma\) stoichiometry. Here we used an independent biophysical approach based upon fluorescence resonance energy transfer (FRET) between differentially fluorophore-tagged ENaC subunits to determine the subunit composition of mouse ENaC functionally reconstituted in CHO and COS-7 cells. We find that when all three subunits are co-expressed, ENaC contains at least 2 of each type of subunit. Findings showing that ENaC subunits interact with like subunits in immunoprecipitation studies are consistent with these FRET results. Upon native polyacrylamide gel electrophoresis, moreover, oligomerized ENaC runs predominantly as a single species with a molecular mass > 600 kDa. Since single ENaC subunits have a molecular mass \(\sim 90\) kDa, these results also agree with the FRET results. The current results as a whole, thus, are most consistent with a higher ordered channel possibly with a \(3\alpha:3\beta:3\gamma\) stoichiometry.
Ion channels are integral membrane proteins that form selective pores in the plasma membrane through which ions move down their electrochemical gradients. The epithelial Na\(^+\) channel (ENaC) is a member of the ENaC/Deg gene superfamily (1-4). This family contains several ion channels (e.g. ASIC, BNaC, DEG, DRASIC, ENaC and FaNaCH) of similar structure that serve diverse physiological functions from modulating mechanosensory transduction and neuronal signaling to control of electrolyte movement across epithelial barriers.

Functional ENaC, similar to most other ion channels, is a heteromeric protein complex containing several distinct channel subunits. Four ENaC subunits have been identified: \(\alpha\), \(\beta\), \(\gamma\) and \(\delta\) (1;5;6). The first three subunits are widely expressed and together form the channel resident to the luminal plasma membrane of epithelial cells capable of electrogenic Na\(^+\) (re)absorption. Consistent with this idea are findings showing that co-expression of only two of these three subunits produces little to no current in heterologous expression systems (1;5;7;8). The \(\delta\)ENaC subunit localizes to brain and reproductive tissues where it is believed to substitute for \(\alpha\)ENaC in the functional channel (6). Importantly, the stoichiometric relationship between ENaC subunits is uncertain.

Each ENaC subunit has a cytosolic NH\(_2\)- and COOH-terminus with two transmembrane domains separated by a single large extracellular loop with all three ENaC subunits, \(\alpha\), \(\beta\) and \(\gamma\), contributing the functional pore (1;5;9;10). Several laboratories have tested ENaC subunit stoichiometry with a biophysical approach ultimately using MacKinnon’s formulation to establish titration and inhibition curves (9;11-13). This approach relies on patch clamp electrophysiology to discriminate between channels containing wild-type subunits from those containing mutant subunits with differential sensitivities to channel blockers and cysteine modifying methanethiosulfonates. Results from these types of experiments support both a \(2\alpha:1\beta:1\gamma\) and a \(3\alpha:3\beta:3\gamma\) stoichiometry. Results from biochemical experiments using sucrose
gradient sedimentation and quantitative analysis of cell surface expression of ENaC subunits, moreover, provide support for both the 2:1:1 and 3:3:3 stoichiometry (9;13;14). Results testing functionality of ENaC concatamers supporting a 2:1:1 stoichiometry, similarly, contrast with freeze-fracture electron microscopy results supporting a higher ordered stoichiometry (9;15).

We use here a biophysical approach distinct from that used previously by others to distinguish between the 2:1:1 and 3:3:3 subunit stoichiometry proposed for ENaC. Our approach was based on fluorescence resonance energy transfer between ENaC subunits differentially tagged with distinct fluorophores. The current results demonstrate that when all three subunits are co-expressed, ENaC can contain more than one copy of each type of subunit and thus, our findings are more consistent with a higher ordered channel.
EXPERIMENTAL PROCEDURES

Plasmids – Full-length mouse α, β, γENaC subunit cDNAs were ligated in-frame behind either eCFP or eYFP into pECFP-C1 or pEYFP-C1 (Clontech; Palo Alto, CA) at the XhoI and EcoRI restriction endonuclease sites. Initially, channel subunit cDNAs were amplified with standard PCR from the respective pCMV-Myc-ENaC plasmids described previously by our laboratory (16). For α, β and γENaC, the upstream and downstream primers were 5’-

CGAACTCGAGTTATGCTGGACCACACCAGAGC and 5’-

GCTAGAATTTCAGAGTGCCATGGCCGGAGC;
CGAACTCGAGTTATGCCAGTGAAGAAGTACC and 5’-

GCTAGAATTTCAGATGGCCTCCACCTCACTG;
CGAACTCGAGTTATGGCGCCTGGAGAGAAG and 5’-

GCTAGAATTTCAGACTCATGGTGCAACTG, respectively. These primer sets engineered upstream XhoI and downstream EcoRI sites (underlined) into each of the respective subunit cDNAs. The newly generated pECFP-ENaC and pEYFP-ENaC plasmids encode fusion proteins containing the full-length mENaC subunit following an NH2-terminal eCFP or eYFP with expression driven by the CMV promoter. All plasmids were sequenced to ensure proper reading frame, orientation and sequence.

Fluorescence resonance energy transfer (FRET) – For FRET microscopy, COS-7 and CHO cells were transfected with vectors encoding tagged ENaC subunits and/or control proteins. Cells were transfected with Polyfect reagent (Qiagen; Valencia, CA) per the manufacturer’s instruction as described previously (16-18). Fluorescence signals were collected with a 510 LSM confocal microscope (Zeiss; Thornwood, NY) using laser excitation at 458 nm (eCFP) and 514 nm (eYFP) and emission windows of 468.5-505.5 nm (eCFP; HQ 487/37 bandpass; Chroma...
Technology, Brattleboro, VT) and 525-575 nm (eYFP; HQ 550/50 bandpass; Chroma Technology). Preceding the emission filters in the light path was a NFT 515 nm dichroic mirror that split the eCFP and eYFP signals to distinct photomultiplier tubes. This system has been described previously (19). All fluorescence data were collected with a high-resolution (1.4 NA), 60x oil-immersion DIC lens. The software controlled 510 LSM confocal microscope enabled timed regional photobleaching with photobleaching performed selectively at 514 nm with a mean reduction of 525-575 nm (eYFP) emissions to less than 15% in the photobleached region. Images were acquired before and after photobleaching.

Percent FRET efficiency between eCFP (donor) and eYFP (acceptor) was quantified at steady-state in fixed cells with acceptor photobleaching methods (also called donor dequenching; (19) using the following formula:

\[
\text{% FRET efficiency} = \left[ (A_1 - A_0) \times \frac{B_0}{B_1} \right] \times 100\%
\]

where \(A_0\) and \(A_1\) are eCFP emissions in the photobleached region before and after photobleaching, respectively; and \(B_0\) and \(B_1\) are eCFP emissions in a non-bleached region before and after photobleaching, respectively. With this approach, % FRET efficiency is the percent increase in donor emissions upon dequenching following selective acceptor photobleaching with spurious changes in donor emissions normalized to an internal control.

Electrophysiology – Macroscopic ENaC currents were recorded as previously described (16-18). In brief, CHO cells were transiently transfected using the Polyfect reagent per the manufacturer’s instructions with eCFP- and eYFP-tagged \(\alpha, \beta, \gamma\)-ENaC cDNA (0.5 \(\mu\)g ea. subunit/35 mm dish). Cells were maintained in culture in a humidified incubator at 37 \(^\circ\)C in DMEM plus 10% fetal
bovine serum supplemented with standard antibiotics and 10 µM amiloride. Electrophysiological measurements were made 24-72 hrs. after transfection. Transfected cells were identified by eYFP fluorescence. Internal pipette and extracellular bath solutions were (in mM): 120 CsCl, 5 NaCl, 5 EGTA, 2 MgCl₂, 2 ATP, 0.1 GTP, 10 HEPES (pH 7.4); and 160 NaCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES (pH 7.4), respectively. Voltage clamp experiments were performed with an Axopatch 200B (Axon Instr.) patch clamp amplifier interfaced with a PC running pClamp 9.0 (Axon Instr.). For whole-cells experiments, macroscopic currents were elicited by voltage ramping from a holding potential of 40 mV to –100 mV over a 500 msec period with ramps delivered every 5 sec.

Native gel electrophoresis, immunoprecipitation and Western blot analysis – All NuPAGE electrophoresis products were from Invitrogen (Carlsbad, CA). Native molecular weight standards were from Amersham Biosciences (Piscataway, NJ). Cells transfected (using Polyfect) with myc-tagged α, β and γENaC were extracted in gentle lysis buffer (see (16;20); 1% NP-40). Samples were prepared in NuPAGE Sample Buffer without heating and reducing reagents with 100 µg/well loaded onto a 2-12% NuPAGE gel. In some instances, samples were treated with a final concentration of 50 mM dithiothreitol (DTT) and 1% lithium dodecyl sulfate (LDS) and heated (100 °C, 5 min.) to denature and reduce sample proteins in order to disrupt ENaC subunit complexes. Protein was separated by PAGE in NuPAGE running buffer at 30 volts for 24 hours at 4°C and then subsequently electroblotted to nitrocellulose. Blots were probed with anti-Myc antibody as described previously (16;17).

Co-immunoprecipitation experiments were performed to determine possible interactions between like ENaC subunits. Immunoprecipitation experiments followed published protocols (16;18;20). In brief, cells were co-transfected with Myc- and HA-tagged, as well as fluorophore-
tagged ENaC subunits in such a manner that like subunits contained either a Myc or HA tag and the other two subunits contained an eYFP tag (i.e. Myc-αENaC + HA-αENaC + eYFP-βENaC + eYFP-γENaC). Fluorescence of eYFP was monitored to determine expression levels. Cells were extracted with gentle lysis buffer and 400 µg (in 400 µl) of total cell lysate exposed overnight to anti-HA-agarose (Roche). Anti-HA interacting proteins and their associated proteins were precipitated, separated with SDS-PAGE and electroblotted onto nitrocellulose. Blots were probed with anti-Myc antibody (Clontech) to determine if like subunits interacted.

Statistics – All data are mean ± SEM. P ≤ 0.05 was significant with population data analyzed with the appropriate t-test.
RESULTS

We used, in the current study, a biophysical approach based on FRET between differentially fluorophore-tagged channel subunits to determine ENaC subunit stoichiometry. This method has been used successfully previously by Zheng and colleagues to determine stoichiometry of rod cyclic-nucleotide-gated (CNG) channels (21) and by Erickson and colleagues to establish that calmodulin is pre-tethered to voltage-gated Ca\(^{2+}\) channels (22). Our general experimental design and rationale closely followed that of Zheng and colleagues (21). Figure 1 shows a schematic representation of fluorescence resonance energy transfer from eCFP to eYFP (A), as well as predicted results from FRET experiments testing the proposed 2:1:1 (B) and the 3:3:3 (C) stoichiometry of ENaC. ENaC subunits fused to eYFP and eCFP are shown as yellow and blue circles (D), respectively. For either a 2\(\alpha\):1\(\beta\):1\(\gamma\) or a 3\(\alpha\):3\(\beta\):3\(\gamma\) stoichiometry, co-expression of any eYFP-tagged subunit with a distinct eCFP-tagged second subunit in the presence of the untagged third subunit would result in formation of a channel complex containing both fluorophores leading to nonradiative fluorescence resonance energy transfer from the fluorophore emitting at a shorter wavelength (eCFP; donor) to that emitting at a longer wavelength (eYFP, acceptor). Similarly, eCFP-\(\alpha\)ENaC would be expected to FRET with eYFP-\(\alpha\)ENaC in channel complexes (containing untagged \(\beta\)- and \(\gamma\)ENaC) of either stoichiometry. Discrimination between these proposed subunit arrangements then arises only from energy transfer between co-expression of two \(\beta\)ENaC or two \(\gamma\)ENaC subunits bearing distinct fluorophores. For a 2:1:1 stoichiometry, co-expression of eCFP-\(\beta\) with eYFP-\(\beta\) in the presence of untagged \(\alpha\) and \(\gamma\)ENaC would result in formation of channel complexes containing only one \(\beta\)ENaC subunit and thus, only one fluorophore resulting in no appreciable FRET. A similar prediction is made for co-expression of eCFP-\(\gamma\) with eYFP-\(\gamma\) in the presence of untagged \(\alpha\) and \(\beta\)ENaC considering a 2:1:1 stoichiometry. In contrast, co-expression of eCFP-\(\beta\) with eYFP-\(\beta\) in
the presence of untagged α and γENaC, as well as eCFP-γ with eYFP-γ in the presence of untagged α and βENaC are predicted at the higher ordered 3:3:3 stoichiometry to produce channels containing both fluorophores resulting in substantial FRET.

Figure 2 shows a montage of confocal fluorescence micrographs of COS-7 cells co-expressing eCFP and eYFP (2A, negative control), a concatamer containing these two proteins linked by a six glycine spacer (CGY), which served as a positive control (2B), and eCFP-αENaC plus eYFP-αENaC with untagged β and γENaC (2C). The top panels in each group show emissions from eYFP and eCFP, as well as merged images prior to photobleaching eYFP in a controlled region (outlined by the white rectangle). The lower panels show eYFP, eCFP and merged signals in the same cells following photobleaching. Photobleaching eYFP did not affect eCFP emission in cells expression non-colocalized eCFP and eYFP (2A), but increased eCFP emission in bleached regions in cells expression colocalized eCFP and eYFP in the form of CGY (2B). Similar to the positive control and as predicted, substantial energy transfer was observed between eCFP-αENaC and eYFP-αENaC in the presence of untagged β and γENaC upon photobleaching (2C).

Summarized in figure 3 are FRET results from both COS-7 and CHO cells expressing combinations of control, tagged ENaC and untagged ENaC subunit cDNAs. The positive control of CGY had significant energy transfer with a % FRET efficiency of 20 ± 0.9 and 28.7 ± 3.8 for COS-7 and CHO cells, respectively. Because the linker in CGY is a noncleavable track of six glycines, these efficiencies represent the maximums possible with the cell types and imaging system used in the current study (19). No FRET was observed, as expected, in the negative control of co-expression of eCFP with eYFP. Also as expected, FRET was observed when any tagged subunit was co-expressed with a differently tagged distinct second subunit and an untagged distinct third subunit; and when eCFP-αENaC and eYFP-αENaC were co-expressed
with untagged β and γENaC. All of these combinations resulted in significantly greater FRET compared to a negative control where the respective eCFP-ENaC subunit was replaced with eCFP-KCNQ4 (represented by X in the figure). KCNQ4 is a potassium channel subunit not expected to interact with ENaC subunits. These combinations, in addition, resulted in significantly greater FRET compared to the eCFP plus eYFP negative control in COS-7 cells (not tested in CHO cells.) As noted above, co-expression of eCFP-βENaC with eYFP-βENaC plus untagged α and γENaC; and eCFP-γENaC with eYFP-γENaC plus untagged α and βENaC would discriminate between the proposed 2:1:1 and 3:3:3 stoichiometry. In both cell types, we observed significantly greater FRET when eCFP-βENaC was co-expressed with eYFP-βENaC plus untagged α and γENaC compared to co-expressing eCFP-KCNQ4 in place of eCFP-βENaC. Similarly, we observed significantly greater FRET when eCFP-γENaC was co-expressed with eYFP-γENaC plus untagged α and βENaC compared to co-expressing eCFP-KCNQ4 in place of eCFP-γENaC. To exclude the possibility that our results were unduly influenced by formation of homomeric channel complexes containing both fluorophores, we co-expressed eCFP-γENaC with eYFP-γENaC in the absence of α and βENaC. This combination resulted in no appreciable FRET indicating a low formation frequency for homomeric channels. These results are consistent with previous findings showing that expression of only a single type of ENaC subunit or any two of the three subunits in contrast to all three subunits together results in few functional channels and that when all three subunits are co-expressed, formation of heteromeric channels containing all 3 subunits is preferred over homomeric channels and channels containing only two subunits (5;9); also see below). Thus, both the current results and those reported previously support the idea that when all three subunits are co-expressed, channels containing all three subunits are preferentially assembled at a fixed stoichiometry. We conclude from our FRET
results that ENaC reconstituted in COS-7 and CHO cells contains at least two of each type of channel subunit supporting a higher ordered stoichiometry over a heterotetrameric arrangement.

In complement to our FRET studies, we performed co-immunoprecipitation experiments testing possible subunit interactions. The top blot in figure 4 shows a typical western blot containing whole cell lysate from cells co-expressing Myc-αENaC + HA-αENaC + eYFP-βENaC + eYFP-γENaC; Myc-βENaC + HA-βENaC + eYFP-αENaC + eYFP-γENaC; and Myc-γENaC + HA-γENaC + eYFP-αENaC + eYFP-βENaC. This blot was probed with anti-Myc antibody. The lower blot in figure 4 contains the anti-HA-immunoprecipitant from the respective lysates. This blot was probed with anti-Myc antibody. Anti-HA and anti-Myc antibodies did not inappropriately recognize Myc- and HA-tagged ENaC subunits, respectively (not shown). Consistent with the FRET results, these biochemical studies demonstrated that α, β and γENaC all have the capacity to interact with like subunits. Such findings are consistent with a stoichiometry where the channel contains at least 2 of each type of subunit.

The electrophysiology experiments reported in figure 5 and summarized in table 1 demonstrate that the tagged ENaC subunits used for FRET analysis form functional channels in CHO cells. Co-expression of eCFP-αENaC with eYFP-βENaC and eYFP-γENaC (5A); and eYFP-αENaC with eCFP-βENaC and eCFP-γENaC (5B) resulted in robust amiloride-sensitive inward Na⁺ currents similar to those described previously by us for ENaC reconstituted in CHO cells (16-18). To exclude the possibility that currents were formed by homomeric channels or channels containing only two subunits, tagged subunits were expressed in combinations allowing formation of only homomeric channel complexes and channels containing at most only two types of subunits. As summarized in table 1, very modest amiloride-sensitive currents were observed when eCFP-αENaC was co-expressed with eYFP-αENaC, and no currents were observed for any other homomeric channel complex or any other heteromeric channel complex containing
only two types of ENaC subunits. These results support the idea that functional ENaC contains all 3 channel subunits and that the tagged channel subunits used in the current study form functional channels. In addition, comparison of patch clamp and FRET results where eCFP-γENaC and eYFP-γENaC are co-expressed in the presence and absence of α and βENaC demonstrate that formation of the heteromeric channel containing all 3 subunits is preferred over homomorphic channels.

Similar to the results obtained form other approaches used to investigate ENaC stoichiometry, interpretation of the current FRET results is simplified if a single major species of channel is present in the cell. Figure 6 shows a Western blot probed with anti-Myc antibody of a native gel containing whole cell lysate from CHO cells transfected with myc-tagged α, β and γENaC. The first lane contains sample protein that was denatured and reduced by addition of LDS and DTT. The last three lanes contain sample preparations from 3 distinct transfections with the proteins in these samples not being denatured or reduced. This blot demonstrates that a single ENaC species with a molecular mass > 600 kDa is observed in native conditions when all three subunits are co-expressed. This complex is reduced by DTT and LDS to monomers, which have molecular masses near 90 kDa. This mass for monomers is similar to what we have previously reported for ENaC subunits expressed in CHO cells (16).
DISCUSSION

Several laboratories now have investigated the subunit stoichiometry of ENaC. These studies have lead to the proposal of two possible subunit arrangements. Firsov and colleagues (9), Dijkink and colleagues (14), and Kosari and colleagues (12) propose that ENaC is a tetramer with a subunit stoichiometry of $2\alpha:1\beta:1\gamma$. This is an attractive arrangement for it parallels the stoichiometry of other cation channels, such as $K_v$ and voltage-gated $Na^+$ and $Ca^{2+}$ channels that have a 4-fold internal symmetry (11;23;24). Moreover, for $K^+$ channels, such as $K_{ir}$, comprised of subunits having only two transmembrane domains, a feature shared with all ENaC subunits, the established stoichiometry is a tetramer (25). In contrast to this arrangement, Snyder and colleagues (13) and Eskanderi and colleagues (15) propose that for a cation channel, ENaC has a unique stoichiometry resulting in a higher ordered structure with a possible $3\alpha:3\beta:3\gamma$ arrangement. Interestingly, many of the same methods and experimental approaches were used to provide support for both the $2:1:1$ and $3:3:3$ stoichiometry. Thus, it has become critical to test ENaC subunit stoichiometry with several independent assays. Here we tested between the two previously proposed models of ENaC subunit stoichiometry using a biophysical approach, distinct from that used previously, dependent on fluorescence resonance energy transfer between ENaC subunits differentially tagged with fluorophores. We find that ENaC contains at least two of each type of subunit. Biochemical results demonstrating that oligomerized ENaC has a molecular mass greater than 600 kDa with subunits alone having masses ~ 90 kDa, and that each ENaC subunit is capable of interacting with like subunits also support the idea that the fully complexed channel contains more than four subunits. Our FRET and biochemical findings are most consistent with the sucrose gradient and freeze-fracture results of others suggesting a subunit stoichiometry of $3\alpha:3\beta:3\gamma$ (13;15).
Similar to the earlier studies testing ENaC subunit stoichiometry, our experimental approach has several advantages and limitations. The current experiments were performed in fixed cells under steady-state conditions at equilibrium. Thus, our results were not influenced by possible dynamic rearrangement of channel subunits during the experiment. This becomes particularly important considering the recent findings of others suggesting that in the plasma membrane, ENaC stoichiometry and possibly oligomerization is not fixed (26).

Another advantage of using FRET is that this method yields binary results with respect to determining protein interaction and thus, it simplifies analysis of whether two ENaC subunits interact. Moreover, FRET directly quantifies protein interactions. These two advantages are in contrast to previous studies of ENaC subunit stoichiometry where changes in wild type and mutant channel activity in response to blockers and modifying reagents were assessed with electrophysiology and results subsequently fitted by idealized equations to establish subunit relationships (9;12;13). These earlier electrophysiology studies, moreover, required the assumption that mutant effects are dominant. Such an assumption was not necessary with the current approach. In contrast to these earlier studies, however, the current investigation cannot definitively determine absolute subunit stoichiometry but rather determines relative subunit levels with our results suggesting that ENaC contains at least 2 of each type of subunit.

The current study, similar to the earlier electrophysiological probing of ENaC subunit stoichiometry, also assumes that single monomers do not readily form homomeric channels. Two lines of evidence from the current study and many findings by others support this contention. Firstly, our patch results (table 1) demonstrate that expression of single subunits and co-expression of any two of the three subunits do not readily form functional channels; whereas, co-expression of all three subunits results in abundant functional channels. These observations are consistent with similar findings by others (5;7;27;28). Secondly, FRET between
differentially tagged but similar monomers (e.g. CFP-γENaC & YFP-γENaC) was not observed. Thus, formation of homomeric channels is rare and does not interfere to a significant extent with our results.

Another limitation of the approach used in the current study was that it established subunit stoichiometry for the entire cellular pool of ENaC. This is distinct from the earlier electrophysiology studies that investigate stoichiometry for ENaC in the plasma membrane (9;12;13). However, our biochemical results showing that ENaC reconstituted in CHO and COS-7 cells is defined by a single major species that has a molecular mass greater than 600 kDa provides support for the idea that when all three ENaC subunits are co-expressed they quickly and readily oligomerize into the final channel complex. These data argue that the stoichiometry of the total cellular pool of channels is representative of those in the membrane. In addition, our electrophysiology results indicated that tagged ENaC subunits used for FRET reconstitute functional channels demonstrating that they can oligomerize into channels that are functional at the plasma membrane. Finally, our results similar to that of others (5;9;27;28), support the idea that when all three channel subunits are available, ENaC prefers to oligomerize into a heteromeric complex containing each type of subunit.

An alternative interpretation of our results that cannot be excluded with the current data set is that tetrameric ENaC cluster and our results reflect inter- rather then intra-channel subunit FRET. We believe this not to be the case for we see no consistent channel clustering when assaying ENaC reconstituted in CHO cells at the single channel level (16;17). In addition, this notion is not well supported in the literature (8;29;30), though, Jovov and colleagues (31) have suggested that ENaC expressed in planar lipid bilayers and Xenopus laevis oocytes may cluster in an actin-dependent manner to form a channel with distinct biophysical properties. One additional possibility that cannot be fully excluded with the current results but that is not
consistent with all of major findings reported in the current study is that ENaC is a tetramer and that we are observing FRET between like subunits within tetramers containing only two types of subunits. While the FRET results cannot exclude this possibility, results from electrophysiology and native gels are fully consistent with such an arrangement. Thus, the current results add to the understanding of possible subunit arrangements in ENaC and are most consistent with a channel complex containing at least 2 of each type of subunit.
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FIGURE LEGENDS

Figure 1. Determination of ENaC subunit stoichiometry using FRET. A. This schematic shows fluorescence resonance energy transfer from the donor CFP fluorophore (blue) to the acceptor YFP fluorophore (yellow). Also shown is FRET between like subunits that have been differentially tagged with CFP and YFP predicted by a ENaC subunit stoichiometry of $2\alpha:1\beta:1\gamma$ (B) versus $3\alpha:3\beta:3\gamma$ (C). Co-expression of YFP-$\beta$ plus CFP-$\beta$ and YFP-$\gamma$ plus CYP-$\gamma$ in the presence of untagged other subunits would discriminate between these stoichiometries (D). Arrows denote FRET and X denotes a lack of energy transfer.

Figure 2. Acceptor photobleaching. A. Fluorescence micrographs of negative control COS-7 cells co-expressing eCFP and eYFP. The top and bottom rows show cells before and after photobleaching eYFP in the region of interest, which is outlined by a white rectangle. The first and second columns show the eCFP and eYFP signal, respectively, with merged images shown in the last row. B. Fluorescence micrographs of positive control COS-7 cells co-expressing the CGY concatemer containing eCFP tethered to eYFP by a 6 glycine spacer. Conditions identical to A. C. Fluorescence micrographs of COS-7 cells co-expressing eCFP-$\alpha$ENaC and eYFP-$\alpha$ENaC plus untagged $\beta$ and $\gamma$ENaC. Conditions identical to A.

Figure 3. ENaC contains at least 2 copies of each type of subunit. Summary graph of % FRET efficiency between YFP-tagged ENaC subunits and either a similar or distinct CFP-tagged subunit or a CFP-control (CFP-KCNQ4) in the presence of the other untagged-subunit(s) in COS-7 and CHO cells. *P<0.05 vs. CGY and/or co-expression with control plasmid denoted by X (CFP-KCNQ4).
**Figure 4.** ENaC subunits can interact with like subunits.  

A. This typical Western blot contains whole cell lysate from cells co-expressing Myc-αENaC + HA-αENaC + eYFP-βENaC + eYFP-γENaC; Myc-βENaC + HA-βENaC + eYFP-αENaC + eYFP-γENaC; and Myc-γENaC + HA-γENaC + eYFP-αENaC + eYFP-βENaC.  This blot was probed with anti-Myc antibody.  

B. This typical Western blot contains the anti-HA immunoprecipitant from the respective lysates in A.  This blot was probed with anti-Myc antibody.

**Figure 5.** Fluorophore-tagged ENaC subunits form functional channels.  

Currents in the absence (control) and presence of amiloride in CHO cells transfected with the indicated fluorophore-tagged ENaC constructs.  Currents were elicited by a voltage ramp (40 mV down to –100 mV) applied to voltage-clamped cells with Cs⁺ and Na⁺ being the predominant cations in intracellular and extracellular fluids with symmetrical Cl⁻. Little background current was seen in the absence of transfection and none of this current was amiloride sensitive (see ref. 16-18).

**Figure 6.** ENaC has a molecular mass > 600 kDa.  

Western blot probed with anti-Myc antibody of a native gel containing whole cell lysate from CHO cells co-expressing myc-tagged α, β and γENaC. The first lane contains denatured and reduced lysate. The next lane is blank. The 3rd lane contains the same lysate as lane 1, but in the native state. The last two lanes contain native lysates from two other distinct transfections.
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Table 1. Heterologous expression of tagged ENaC in CHO cells.

| cDNA                        | observations | current density (pA/pF) |
|-----------------------------|--------------|-------------------------|
| eCFP-α, eYFP-β, eYFP-γ      | 3/3          | 202 ± 89                |
| eCFP-α, eYFP-α             | 2/4          | 9 ± 4                   |
| eCFP-β, eYFP-β             | 0/7          | -                       |
| eCFP-γ, eYFP-γ             | 0/6          | -                       |
| eCFP-α, eYFP-β             | 0/4          | -                       |
| eCFP-γ, eYFP-β             | 0/5          | -                       |
| eCFP-α, eYFP-γ             | 0/6          | -                       |

*Number of cells with amiloride-sensitive Na⁺ currents vs. total number tested.

**Amiloride-sensitive current at –80 mV.
A. CFP and YFP images with energy transfer and FRET indicators.

B. Diagram of energy transfer in 2α:1β:1γ:

\[ \alpha \rightarrow \alpha \]
\[ \beta \rightarrow \beta \]
\[ \gamma \rightarrow \gamma \]

C. Diagram of energy transfer in 3α:3β:3γ:

\[ \alpha \rightarrow \alpha \]
\[ \beta \rightarrow \beta \]
\[ \gamma \rightarrow \gamma \]

D. cDNA schemes with combinations of α, β, γ.

- α, α, β, γ
- α, β, β, γ
- α, β, γ, γ

Staruschenko, Fig. 1
Staruschenko, Fig. 2

A. | eYFP | eCFP | merged
---|---|---|---
bleach

B. | CGY
bleach

C. | eCFP-αENaC | eYFP-αENaC | β, γENaC
bleach
COS cells

CHO cells

Staruschenko, Fig. 3
| IB: Myc | IP: HA | IB: Myc |
|--------|--------|--------|
| Myc-α | HA-α   | 105    |
| Myc-β | HA-β   | 75     |
| Myc-γ | HA-γ   |        |
| YFP-β | YFP-α  |        |
| YFP-γ | YFP-γ  |        |
| YFP-β |        |        |

A.

B.
A. eCFP-αENaC + eYFP-βENaC + eYFP-γENaC

B. eYFP-αENaC + eCFP-βENaC + eCFP-γENaC

Staruschenko, Fig. 5
Staruschenko, Fig. 6
Fluorescence resonance energy transfer analysis of subunit stoichiometry of the epithelial Na\(^+\) channel

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