JGP Journal of General Physiology

COMMENTARY

PANX1 hexamers work but cells prefer heptamers

Andrew L. Harris[1]

Gupta et al. (https://doi.org/10.1085/jgp.202413676) reconcile a disconnect between structural and functional data regarding stoichiometry of PANX1 channels and provide new insights about channel activation.

Pannexin 1 (PANX1) forms a wide-pore channel that is permeable to ATP, amino acids, and a variety of other cellular molecules of similar size (Syrjanen et al., 2021). It is perhaps most widely appreciated as a mediator of ATP release in multiple physiological and pathological processes (Chiu et al., 2018; Koval et al., 2021; Syrjanen et al., 2021; Wu et al., 2023). PANX1 is known to be activated by caspase cleavage of its cytosolic C-terminal domain and by activation of G protein–coupled receptors (GPCRs), among other proposed mechanisms (Chiu et al., 2018; Koval et al., 2021; Wu et al., 2023). The molecular mechanism(s) by which these activating treatments produce channel opening is a topic of active interest and investigation. There is agreement from multiple high-resolution cryo-EM studies that the native form of the channel is a heptamer of PANX1 subunits (Syrjanen et al., 2021; Wu et al., 2023).

The structural data stand in contrast to the conclusion regarding subunit stoichiometry that was part of a detailed study of the role of the C-terminal domains in PANX1 channel activation (Chiu et al., 2017). In that study, concatemeric PANX1 channels were used to show that, following cleavage of the intersubunit linkers, unitary conductance and molecular permeability increased as a function of truncated C-terminal domains (the segment normally cleaved by caspase). In that work, the unitary conductance of native PANX1 channels (formed by expression of individual PANX1 subunits) corresponded to that of (cleaved) hexameric concatemers. It was inferred on this basis that the native channels were hexameric. This finding was not surprising since earlier work had indicated that PANX1 channels were hexameric (Boassa et al., 2007; Ambrosi et al., 2010; Wang et al., 2014), there were no high-resolution structures yet, and at the time there was a tacit expectation that homomultimeric channels would have an even number of subunits; other wide-pore channels, such as connexin hemichannels, were known to be hexameric.

The disconnect between this conclusion and the structural data has remained unresolved until now. In a recent issue of *JGP*, the same group that concluded that the native PANX1 channels were hexameric addresses this conundrum head-on, comprehensively, and conclusively (Gupta et al., 2025). The new data show that indeed native PANX1 channels are heptameric, provide clues as to what contributed to the previous conclusion, and in the process provide unexpected information about the activation mechanisms of PANX1 channels that would not have otherwise emerged.

In the previous work, hexameric concatemers were generated in which the subunits were linked (C-terminus to N-terminus) by a segment containing a TEVp cleavage site. Following expression in HEK293T cells, TEVp was applied via patch pipette to whole cells or via bath solution to excised inside-out patches, to cleave the linkers between the subunits.

When concatemers of PANX1 monomers were exposed to TEVp to cleave the linkers, no channel activity was seen, as expected, consistent with findings from expression of single monomers not exposed to caspase. To define a progressive mechanism by which truncation of the C-terminal domain by caspase activates the channel, concatemers were generated composed of increasing numbers of monomers in which the segment of the C-terminal domain normally cleaved by caspase was deleted. These C-terminal–truncated concatemers produced channel activity only after linker cleavage, consistent with results obtained when single monomers were expressed and the channels exposed to caspase or expressed with the C-terminal domain deleted. These results demonstrated that the engineered truncation of the C-terminal domain in the (cleaved) concatemers mimicked caspase cleavage-mediated activation. The absence of channel activity prior to linker cleavage suggested that the linkage between the N- and C-termini prevented channel activity, even though the caspase-sensitive segment of the C-terminal domain was already deleted.

Gupta et al. (2025) employed the same overall expression strategy but targeted explicitly the stoichiometry issue. Four concatemeric constructs were generated: hexamers and heptamers, composed of subunits either with or without the C-terminal segment. These constructs, and single PANX1 monomers, were expressed in HEK293T cells from which the PANX1 gene was deleted by CRISPR/Cas9 to guarantee that no endogenous PANX1 monomers would be present.

[1]Department of Pharmacology, Physiology, and Neuroscience, Rutgers New Jersey Medical School, Newark, NJ, USA.

Correspondence to Andrew L. Harris: aharris@njms.rutgers.edu.

When each of the four concatemeric constructs was expressed, no whole-cell currents developed. Following linker cleavage, no currents developed from the hexameric or the heptameric full-length constructs. However, linker cleavage produced robust whole-cell currents in cells expressing the hexameric or the heptameric constructs with the C-terminal deletion. The whole-cell currents were comparable, and both cleaved concatemeric forms were shown to be permeable to ATP and to the dye TO-PRO-3 (the permeabilities were greater for the heptameric channels). These results demonstrated that both the hexameric and the heptameric forms of PANX1 could produce functional channels activated by loss of the regulatory C-terminal segment and that the C- and N-termini needed to be unlinked to permit this activation.

To address the issue of the stoichiometry of native PANX1 channels, single-channel recordings were made using excised inside-out patches from cells expressing the C-terminal–deleted hexamers and heptamers following bath-applied TEVp and also from cells expressing single PANX1 monomers in which a TEVp cleavage site was engineered to produce the same C-terminal truncation. This allowed the same treatment (bath-applied TEVp) to activate the concatemeric channels as well as the channels formed by expression of PANX1 monomers in the excised patches (Fig. 1 A).

Each of the resulting three channel forms - C-tail–truncated hexameric and heptameric concatemers and channels from singly expressed monomers - was characterized regarding unitary conductance, open channel rectification, open/closed dwell times, and open probability. For each of these parameters, the data from the channels composed of singly expressed PANX1 monomers closely corresponded to those from the cleaved heptamers and not with those from the cleaved hexamers. Of note, the unitary conductance of the heptameric channels and of the native channels was significantly greater than that of the hexameric channels and corresponded closely to that seen in the previous work (Fig. 1 B).

The results are clear: These single-channel data show that the native form of PANX1 channels is the heptamer, consistent with the cryo-EM data. Furthermore, and perhaps surprisingly, the non-native (hexameric) form of the channels is quite functional, able to be activated by C-terminal deletion and permeable to ATP and TO-PRO-3.

So, why in the earlier work did the single-channel conductance of the hexameric concatemers match that of the native channels? The principal experimental difference was that endogenous PANX1 expression was eliminated in the current study unlike in the earlier work (in which there had been no evidence of endogenous PANX1 activity or expression). This suggests that the misleading finding in the earlier work was due in some fashion to endogenous PANX1 expression. But how? A possibility is that there was rapid incorporation of endogenous (albeit cryptic) monomers into the cleaved hexameric concatemers, rendering them heptameric. Such a process would infer that the heptameric form is energetically far more favored, if achievable, than a hexameric form. In any case, such a process would be unusual. However, activation of the channels in the excised inside-out patches took several minutes following bath application of TEVp, perhaps allowing time for heptamer formation from hexamers.

In support of such a rapid change in stoichiometry, the authors note that an equilibration between tetrameric and pentameric forms of TRPV3 on the order of 2–3 min was recently reported from high-speed atomic force microscopy (Lansky et al., 2023). In that study, evidence was presented to indicate individual TRPV3 monomers leaving and entering multimeric structures in a dynamic equilibrium between the pentameric and tetrameric forms. Such monomer exchange raises challenging and intriguing thermodynamic issues. However, in the case of PANX1 there is no evidence for this kind of equilibrium since singly expressed monomers produced channels with a single unitary conductance; since hexamers and heptamers both form functional channels, but with clearly distinct unitary conductances, any functional native hexamers following expression of single monomers would be evident electrophysiologically, and that was not the case. Nevertheless, a specific mechanism by which PANX1 monomers are recruited by hexamers to form heptamers, or more agnostically regarding mechanism, how suppression of cryptic endogenous expression of PANX1 enabled formation of heptameric channels from (cleaved) concatemeric hexamers, remains to be explored. Such studies could involve attempting to resolve the kinetics of heptamer formation in the plasma membrane of the parental cells (cells without suppression of endogenous PANX1) expressing hexameric concatemers, though this would be difficult at the single-channel level. Alternatively, perhaps high-speed AFM, as in Lansky et al. (2023), could provide some insight. It seems unlikely that a monomer would be able to associate with or incorporate into an uncleaved hexameric concatemer, but this might be investigated by cross-linking studies.

These studies raise a caution regarding interpretation of experiments using cleaved concatemeric channel constructs. Even though the mechanism involved is unclear, the possibility exists that endogenous monomers, even though undetected, may confound certainty about the structural form presumed to be established by concatemeric constructs after linker cleavage.

However, the story does not end here. Recall that PANX1 channels can also be activated by GPCR stimulation in the absence of caspase or C-terminal truncation. The full-length (C-terminal domain intact) hexameric and heptameric PANX1 constructs described above were also assessed for their ability to be activated by GPCR stimulation. They were co-expressed with the α1D adrenoceptor (α1DAR), and the cells treated with phenylephrine (PE). It was expected that treatment with PE would have no effect on the uncleaved concatemers and would stimulate channel activity only following linker cleavage by TEVp. Indeed, PE treatment after linker cleavage did stimulate robust PANX1 channel activity of both the hexameric and heptameric forms.

The surprise was that PE application also produced robust PANX1 activity *before* linker cleavage: activation by α1DAR does not require cleavage of the N–C-terminal link, whereas activation by C-terminal domain deletion does. This paper and the earlier one showed that channels “pre-activated” by C-terminal domain deletion of the component monomers, but with their N- and C-termini linked, were not active, and that only cleavage of the linker permitted the channels to open. The new data imply that the gating reactions initiated by these two physiological

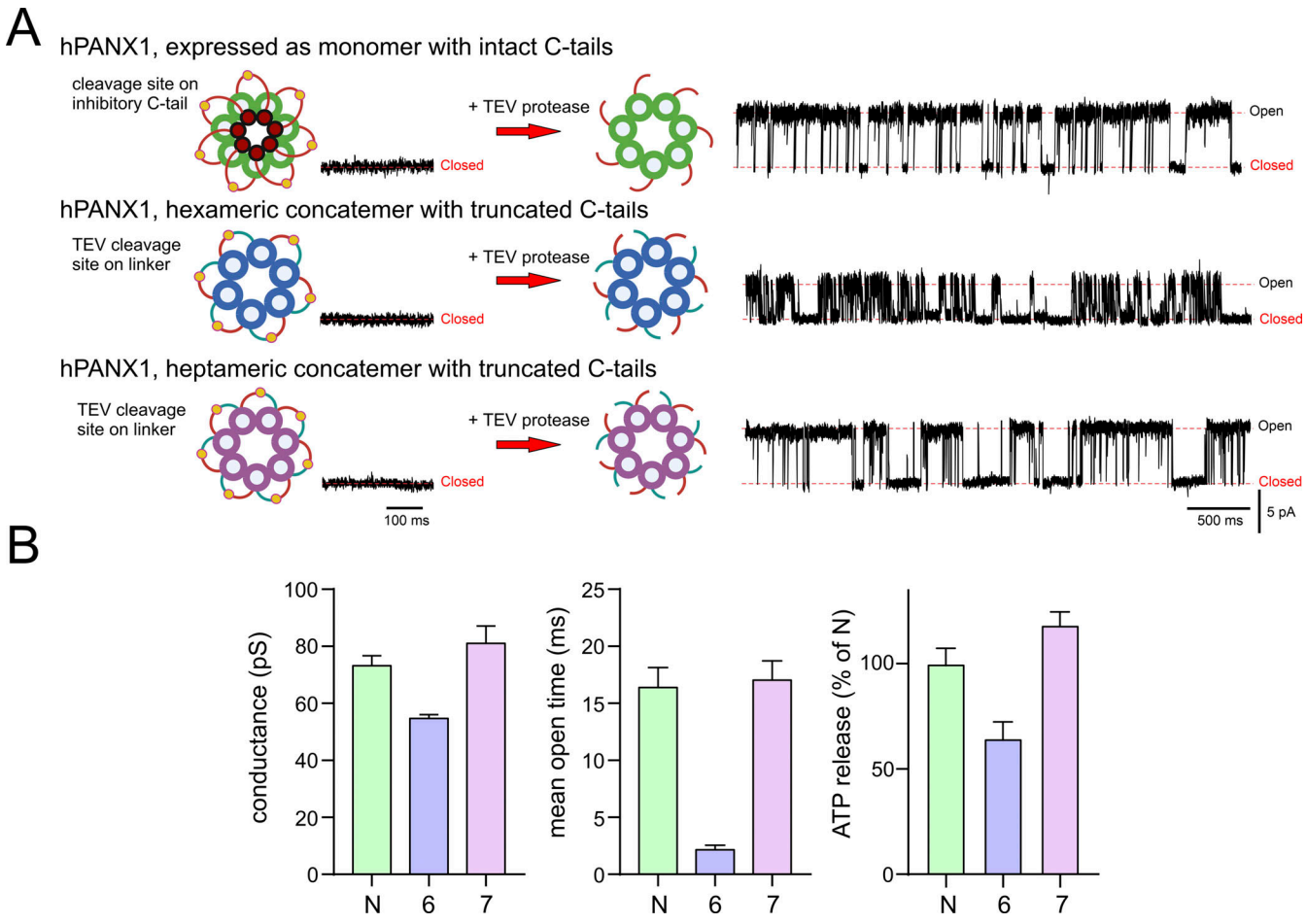

Figure 1. **Functional data show that even though hPANX1 channels can work as hexamers, the native form is heptameric. (A)** Diagram depicting the experimental strategy used by Gupta et al. (2025) to generate the desired forms of hPANX1 channels and the corresponding single-channel activity (obtained from excised inside-out patches at +80 mV). Top: Native hPANX1 channel formed by hPANX1 expressed as monomers. The full-length C-terminal (CT) domain (red) is depicted as ending in a pore-lining ball to indicate a nonconductive state. Caspase truncation of the CT domain activates hPANX1 channels. In Gupta et al., this activating truncation was achieved by TEV protease cleavage at an engineered TEV cleavage site (orange-filled circle). Middle: Hexameric concatemer in which the same portion of the CT was deleted, and followed by a TEV cleavage site and short linker (green) to the N-terminus of the adjacent subunit. TEV cleavage of the linker was required to activate the channels. Lower: Heptameric concatemer in which the same portion of the CT was deleted, followed by a TEV cleavage site and short linker to the N-terminus of the adjacent subunit. TEV cleavage of the linker was required to activate the channels. (Channel traces shown were adapted from Gupta et al. [2025] Fig. S3 B [native], Fig. 3 B [hexamer], and Fig. 4 B [heptamer]). **(B)** Comparison of properties of native hPANX1 channels (N; hPANX1 expressed as monomers) activated by CT truncation, with those of concatemeric hexamers (6) and heptamers (7) with CT truncations, active after intersubunit linker cleavage. From left to right: Graphs show unitary conductance and mean open times obtained from single-channel recordings in excised patches (+80 mV) and normalized ATP release. Bars show means and SE. (Conductance data shown are as stated in the paper; mean open times and normalized ATP release data are taken from Gupta et al. (2025) Fig. 5, D and E; and Fig. 6 A, respectively, and replotted as means and SE).

activators of PANX1 channels (C-terminal truncation and GPCR activation) operate by differing mechanisms - the former apparently requiring unlinked N- and/or C-termini and the latter without this requirement. It is worth noting that this novel insight about distinct physiological gating requirements emerged from an add-on experiment in support of an investigation of stoichiometry, not a study targeted at gating mechanisms.

What does this imply? Linker cleavage obviously eliminates nonphysiological configurational constraint on both the N- and C-termini, but what is the relevant effect of that on channel gating? For example, is it that the N-terminal domain directly controls ion permeation by flipping into the pore as proposed recently in a model of caspase-mediated PANX1 gating (Henze et al., 2024), or is the effect more allosteric in nature, involving as yet unknown more global conformational changes? If the former, how can the N-terminal domain play a direct role in activation of linked concatemers by α1DAR? It is known that specific posttranslational modifications (PTMs) in the cytosolic loop and the C-terminal domain of PANX1 can activate the channel (Chiu et al., 2021; Koval et al., 2021; Wu et al., 2023), implying a more distributed conformational change in gating. Whether such PTMs can activate channels formed of linked concatemers is, of course, unknown at this time but would be interesting to explore.

As noted above, the present work strongly suggests that PANX1 channels have a single native heptameric stoichiometry but also that a non-native, hexameric stoichiometry retains several key aspects of channels with the native stoichiometry: activation by C-terminal deletion, activation by α1DAR, and

permeability to ATP and TO-PRO-3. There are differences, of course: compared with the heptameric channels, the hexameric channels have smaller unitary conductance, are more rectifying, have shorter open dwell times, and are less permeable to ATP, but the fundamentals are present in the non-native (non-physiological) form of the channel. In other words, it seems that the non-native hexameric PANX1 channels are about as different functionally from the native heptamers as are the varieties of CALHM1 channels that natively form with differing stoichiometries (Ren et al., 2022; Polfer and Furukawa, 2024).

In addition to TRPV3 and CALHM, other homomeric channels reported to exist with more than a single stoichiometry include 5-HT3a receptors (tetrameric and pentameric; Introini et al., 2024) and Orai1 channels (pentameric and hexameric; Lu et al., 2024). From a biological point of view, what matters are the form(s) in which such channels are natively expressed, and, if more than one form is natively expressed, how do their functional properties differ, and are they natively expressed in the same cells, or are the stoichiometries cell type specific? If the latter, how is that achieved, and if the former, what determines their relative expression levels (e.g., are they in a dynamic equilibrium with each other)? From a biophysical point of view, functional channel forms of differing stoichiometry offer the opportunity to investigate precisely how the different stoichiometries generate, modify, or lose specific functional properties (e.g., activation mechanisms, voltage dependence, ion selectivity). Prior to the discovery of functional channels with different stoichiometries, such questions could not even be asked.

Gupta et al. (2025) provide an excellent example of how to approach and resolve an apparent contradiction in a field, even if the controversy derives from one's own data. The experimental plan was comprehensive and unbiased, designed to get at the truth of matter and to dive deeply enough to reveal not only the correct answer, but also to provide a basis for understanding previous work. The detailed analysis and multiple levels of positive and negative controls provided certainty, as well as new information about both the native and non-native stoichiometries, and along the way yielded unexpected information about how a conformational constraint differentially affected activation by two physiological activators.

Acknowledgments

Crina M. Nimigean served as editor.

Author contributions: A.L. Harris: conceptualization and writing original draft, review, and editing.

Disclosures: The author declares no competing interests exist.

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