Response to the Letter: "About a new method to measure fractional Ca^{2+} currents through ligand-gated ion channels"

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In the February 2009 issue of the *Journal of General Physiology*, we published a paper in which we presented (among several other results) a novel, single-channel–based approach to estimating the fractional Ca^{2+} currents through neurotransmitter-gated ion channels (Elenes et al., 2009). We applied the method to a variety of mutant and wild-type muscle nicotinic acetylcholine receptors (AChRs) and to the NMDA receptor composed of NR1 and NR2A subunits, all heterologously expressed in HEK-293 cells. Our goal was to estimate the impact of a panel of naturally occurring mutations on the Ca^{2+}-permeation properties of the AChR.

Our methodology is based on the observation that (in the presence of saturating concentrations of, say, Na^+ in the pipette solution of cell-attached patches) increasing concentrations of Ca^{2+} in the pipette reduce the inward single-channel conductance, eventually reaching a nonzero value that coincides with the single-channel conductance measured in the presence of Ca^{2+} alone (at saturating concentrations) in the pipette. Furthermore, this phenomenon turned out to be quantitatively consistent with Na^+ and Ca^{2+} competing for a single site in the channel's pore, and hence, only three parameters are needed to characterize it: (1) the single-channel conductance in the absence of Ca^{2+}; (2) the single-channel conductance in the limit of infinite Ca^{2+} concentration; and (3) an apparent Ca^{2+}-dissociation equilibrium constant that is a measure of how much Ca^{2+} is needed to displace the other ion (in this example, Na^+) from the pore. If the values of these three parameters are known, the fraction of the total current that is carried by Ca^{2+} at any desired extracellular Ca^{2+} concentration can be estimated using very simple expressions (Eqs. 2–5 in Elenes et al., 2009).

For most constructs, our estimates of the fractional Ca^{2+} currents using this approach (Table II in Elenes et al., 2009) are in remarkable agreement with the values obtained by some groups using other methods, most notably, the fluorometry/patch clamp approach (Zhou and Neher, 1993). For example, our estimate of 12.8% for the fractional Ca^{2+} current through the NR1-NR2A NMDAR compares very well with the estimate of 11% from Burnashev et al. (1995), 13.5% from Jatzke et al. (2002), and 14% from Egan and Khakh (2004), all applying the fluorometry/patch clamp approach to the same channel expressed in the same cell line, and using nearly the same ion and voltage conditions as we have. Another example of close agreement between these two approaches is the human adult-type muscle AChR; our value is 9.3%, whereas that from Fucile et al. (2006) was 7.2% and that from Di Castro et al. (2007) was 7.8%. Yet another example is the AChR containing the congenital myasthenic syndrome mutation T264P in the ε subunit; our number is 15.0%, whereas that from Di Castro et al. (2007) was 11.8%. Interestingly, the value of 15.4% estimated by Di Castro et al. (2007) for the AChR containing the εV259F congenital myasthenic syndrome mutation is also very close to the average value estimated by us for wild-type and mutant adult-type AChRs (~13%; we have not estimated the fractional Ca^{2+} current through this particular mutant using our method). This finding seems most sensible, considering that a V-to-F mutation at the 7′ position of the M2 pore-lining segment, a position that is not expected to be exposed to the channel's lumen in the open conformation (Cymes et al., 2005), is unlikely to have a major effect on the Ca^{2+} permeation properties of the AChR.

For a few constructs, however (specifically, the adult and fetal wild-type AChRs from mouse muscle), our estimates of the fractional Ca^{2+} currents are higher than those reported previously using the fluorometry/patch-clamp method, an intriguing discrepancy in light of the reasonable agreement between the values yielded by both approaches for all other constructs. In a letter to the editor (see p. 259 of this issue), Fucile and Grassi claim to have identified the reasons for these divergent results, a development we were eager to learn about. Quite disappointingly, however, none of the issues raised in this letter is valid, as we elaborate below. We also note that the quoted work of Villarroel and Sakmann (1996) was performed on rat not mouse muscle receptors, and that, in that paper, fractional Ca^{2+} currents were

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Abbreviation used in this paper: AChR, acetylcholine receptor.

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calculated from reversal potential estimates assuming the validity of the Goldman-Hodgkin-Katz equation, not using fluorometry.

Fucile and Grassi suggest that our approach is wrong because we only take into account the slopes (and disregard the displacements along the voltage axis) of I-V curves recorded with increasing concentrations of Ca^{2+} in the pipette solution of cell-attached patches, and because we consider that the limit of the inward conductance as the concentration of Ca^{2+} is increased (in the presence of a constant concentration of another cation in the pipette) represents currents carried by Ca^{2+} and not by the other cation in the mixture.

The reason why we only consider the slopes of the recorded I-V curves in Eqs. 2–5 (Elenes et al., 2009) was explained in the paper, and it has to do with the particular voltage dependence of the fractional Ca^{2+} currents. In the presence of a monovalent cation such as Na^+ or K^+, at a concentration of ~150 mM in the pipette of cell-attached patches, the fractional Ca^{2+} current (at any extracellular Ca^{2+} concentration higher than the cytosol's) is unity at zero voltage and decreases as the voltage is hyperpolarized; eventually, this fractional current reaches a nonzero plateau. It is this (voltage-independent) limit that we calculated and reported for each construct in Table II of Elenes et al. (2009), as we explicitly indicated. Moreover, we suggested that these values are expected to be very close to those corresponding to the resting membrane potential. Because the recorded I-V relationships (at hyperpolarized potentials) were adequately fit by straight lines, the fractional Ca^{2+} current in this voltage-independent limit (and at any given extracellular Ca^{2+} concentration) is given by the ratio between the channel's conductance to Ca^{2+} and that to the entire mixture of cations. It is the conductances (and hence, the slopes) that matter in this limit; not the lateral displacements of the straight lines.

With respect to the competition between ions, the reason why we consider that Ca^{2+} displaces other cations in the mixture, eventually becoming the sole charge carrier as its concentration increases, is that the limiting value of this conductance is nearly identical to that measured in the presence of saturating (100–200 mM) Ca^{2+} alone. Note that, with 100–200 mM Ca^{2+} in the pipette solution of cell-attached patches, the inward conductance is 28 pS (Fig. 1 A), whereas in the additional presence of 150 mM Na^+, the conductance only increases to 33 pS (Fig. 1 B).

In their letter, Fucile and Grassi argue that this reasoning is fundamentally wrong, and that the finding that these conductance values coincide is perhaps a mere coincidence (compare their Fig. 1, A and B). Instead, they suggest that, even in the presence of mixtures of 150 mM Na^+ and 100 mM Ca^{2+} on the extracellular side, the currents at hyperpolarized potentials are mostly carried by Na^+ with only a small contribution by Ca^{2+}. Most disconcerting in their analysis is the assumption of ohmic behavior across the entire voltage range, even under extremely asymmetric conditions ($[Ca^{2+}]_o/[Ca^{2+}]_i$ of ~1,000 and [monovalent cations]$_o$/[monovalent cations]$_i$ of $\sim 10^{-3}$; see their Fig. 1), and the calculation of reversal potentials from our I-V data in Fig. 15 of Elenes et al. (2009), which correspond exclusively to inward currents.

To resolve the question as to whether monovalent cations contribute substantial inward current at high $[Ca^{2+}]_o$ and hyperpolarized potentials, we compared the effect of increasing the concentration of extracellular

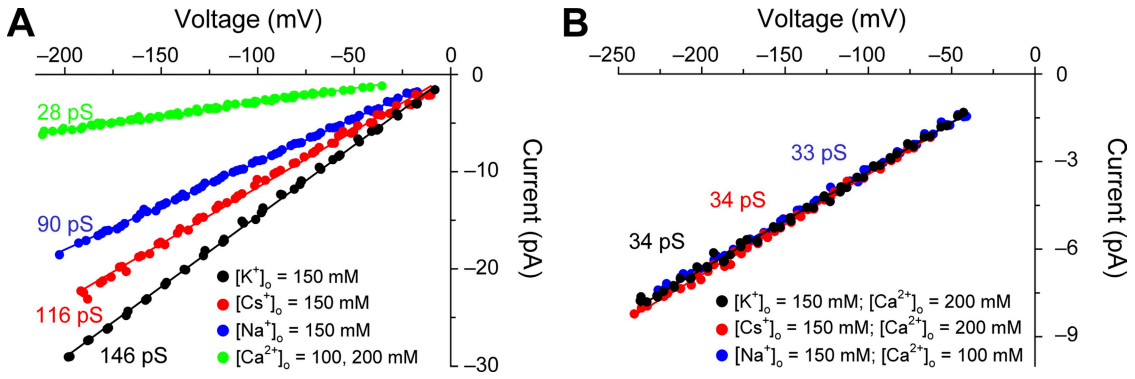

Figure 1. Single-channel I-V curves recorded in the cell-attached configuration from the mouse muscle AChR containing the εT264P mutation, one of the constructs studied in Elenes et al. (2009). Note the different current axis scales of the two panels. (A) I-V relationships in the presence of saturating concentrations of the indicated cations (as their chloride salts) in the pipette solution. In the case of Ca^{2+}, the slopes estimated with 100 or 200 mM Ca^{2+} were indistinguishable, and hence, the corresponding data points were pooled together. (B) I-V relationships in the presence of the indicated binary mixtures of cations in the pipette solution. In the case of Na^+, 100 mM Ca^{2+} was enough to bring the single-channel conductance to a value close to that estimated in the presence of Ca^{2+} alone; however, in the cases of K^+ and Cs^+, 200 mM Ca^{2+} was needed. The pipette solutions also contained 10 mM HEPES, and the final pH was 7.4. To facilitate the visual comparison of slopes, all I-V curves were shifted along the voltage axis so that their linear projections go through the origin.

Ca^{2+} in mixtures containing a constant, saturating concentration (~150 mM) of Na^+, K^+, or Cs^+ (Fig. 1). If Fucile and Grassi's reasoning were correct, mixtures of 100–200 mM Ca^{2+} and any of these monovalent cations are expected to have different inward conductance values because the currents at hyperpolarized potentials would be carried mostly by the monovalent cations, and these exit the channel at very different rates. Indeed, the inward single-channel conductance is 146 pS in the presence of 150 mM K^+ on the extracellular side, 116 pS in the presence of 150 mM Cs^+, and 90 pS in the presence of 150 mM Na^+ (all in the nominal absence of extracellular divalent cations; Fig. 1 A). Conversely, if it were true that Ca^{2+} at sufficiently high concentrations becomes the only charge carrier of inward currents at hyperpolarized potentials (regardless of whether the other ion on the extracellular side is Na^+, K^+, or Cs^+), then all three mixtures should have a similar single-channel conductance, which in turn should asymptotically approximate that in the presence of saturating Ca^{2+} alone (28 pS; Fig. 1 A). From the I-V plots in Fig. 1 B, the inward conductances are 34 pS with 150 mM K^+ and 200 mM Ca^{2+} on the extracellular side, 34 pS with 150 mM Cs^+ and 200 mM Ca^{2+}, and 33 pS with 150 mM Na^+ and 100 mM Ca^{2+}, which are certainly all very close to the conductance estimated in the presence of 100–200 mM Ca^{2+} alone. Evidently, the elementary notion that different cations compete with each other for a site in the channel's pore and that, eventually, one of them may dominate the currents is correct.

In their letter, Fucile and Grassi argue that the fact that the reversal potential of I-V curves (recorded in the presence of, say, extracellular 100 mM Ca^{2+} and intracellular 150 mM of monovalent cations) shifts in the depolarizing direction upon the addition of Na^+ to the extracellular side indicates that monovalent cations do flow through the channel, even when a 100-mM Ca^{2+} solution is present. This is, of course, a well-known experimental observation that does not contradict our position at all. Instead, this simply indicates that, in the absence of extracellular Na^+ and around zero voltage, intracellular monovalent cations flow outwardly faster than Ca^{2+} does inwardly; hence, the negative sign of the reversal potential under these ion conditions and its rightward shift upon the addition of extracellular Na^+. But it is important to understand that the balance of ion-specific currents can change steeply as a function of voltage. Indeed, at hyperpolarized potentials, the inward flow of saturating Ca^{2+} through the muscle AChR dominates the inward currents, even with 150 mM Na^+ (or K^+ or Cs^+) on the extracellular side, as we unequivocally show in Fig. 1.

In a nutshell, we also would like to know the reasons behind the few noted discrepancies. What is clear, however, is that the reasons argued by Fucile and Grassi in their letter are not the culprit.

Edward N. Pugh Jr. served as editor.

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