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COMMENTARY

Manganese and intracellular Ca^{2+} handling

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The presence of significant amounts of the transition metal, manganese, is essential for living cells where it is bound to some intracellular enzymes. The free (i.e., unbound) Mn^{2+} concentration in both extracellular and intracellular space is tightly regulated and thought to be considerably lower than the free calcium ion (Ca^{2+}) concentration. Mn^{2+} can pass through plasmalemma Ca^{2+} ion channels, but under normal circumstances due to channel selectivity and relative concentrations, this event is rare. But when extracellular Mn^{2+} is increased to mM levels, significant Mn^{2+} influx occurs through Ca^{2+} channels in the plasma membrane and intracellular manganese levels increase above normal physiological levels. Mn^{2+} ions also have the property of binding to and quenching the fluorescence of fluorophores. This property can be used to detect Mn^{2+} influx and is the basis of the use of raised extracellular Mn^{2+} in experiments designed to detect pathways for Ca^{2+} influx. This commentary features the manganese quench technique as used in a recently published article and discusses in detail the potential consequences for the intracellular Ca^{2+} handling when intracellular Mn^{2+} is increased, as it now competes to a greater extent than normal with Ca^{2+} for intracellular buffers.

Adding manganese ions to the extracellular medium is used as an experimental tool to study the phenomenon of store-operated Ca^{2+} entry (SOCE) (Hallam and Rink 1985). Like many divalent cations, including Sr^{2+} and Ba^{2+}, manganese (Mn^{2+}) is chemically similar enough to Ca^{2+} that it passes through sarcolemmal Ca^{2+} channels. Another less common feature is that Mn^{2+} binding to fluorescent molecules reduces fluorescence, such that when fully bound, the fluorophore has negligible fluorescence, i.e., fluorescence quenching. In the case of manganese, the quench is a consequence of the collision of quencher (Mn^{2+}) with the excited form of the fluorophore that results in the dissipation of the high-energy excited state of the fluorophore to heat (instead of light!). This property of manganese is related to the ability of transition metals to form multiple oxidation states. Therefore, when Mn^{2+} is added to the extracellular fluid, the magnitude of the quench provides a measure of the cytoplasmic manganese concentration ($[Mn^{2+}]$) and, by proxy, the entry of Ca^{2+} via SOCE.

In a recent article, Jacque-Fernandez et al. (2025) investigated the complex time course of the manganese ion quench of the fluorescence signal from an intracellular Ca^{2+} indicator (Fura-2) in isolated fast skeletal muscle fibers from the zebrafish. By exciting the dye at a Ca^{2+}-sensitive wavelength and a Ca^{2+}-insensitive (isosbestic) wavelength, the authors were able to monitor intracellular Ca^{2+} and Mn^{2+} signals, a technique first used to study SOCE in skeletal muscle by Collet and Ma (2004). The zebrafish twitch muscle preparation was chosen because the Ca^{2+} cycling that underlies the twitch is purely an intracellular event involving SR Ca^{2+} release and uptake. The authors noted that when mM levels of Mn^{2+} were added to the extracellular space, the Fura-2 fluorescence was gradually quenched (over minutes) by a slow increase in intracellular Mn^{2+} as it crossed the cell membrane via a constant leak. Most importantly, they showed a transient decrease in the Fura2 fluorescence during a twitch in association with the intracellular Ca^{2+} transient (Fig. 1 A). They hypothesized that the secondary transient quench signal is due to Mn^{2+} displacement from slow intracellular Ca^{2+} buffers and not due to Ca^{2+} influx via SOCE. Through a series of elegant experiments, the authors provide evidence that SR Ca^{2+} release is the cause of the secondary transient quench, therefore this purely intracellular event will complicate the interpretation of quench signals in studies of SOCE.

This research highlights the fascinating properties of manganese, normally present in cells at trace quantities and mainly bound to cellular proteins. Manganese binding plays a key role in the structure and function of many proteins, a well-known example is Mn superoxide dismutase, an enzyme that protects the cells against oxygen radical damage. The total manganese concentration in tissues, including skeletal muscle, has been estimated at between 20 and 50 µM (Tuschl et al., 2013; Kondo et al., 1991), the concentration of free manganese (Mn^{2+}) within the cytosol is less well studied and is assumed to be low (<10 nM). Interestingly, even low levels of biological transition metal cations such as Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} were a cause for concern for the designers of calcium ion indicators, as generally these ions bind to the Ca^{2+} chelation site with high affinity, and

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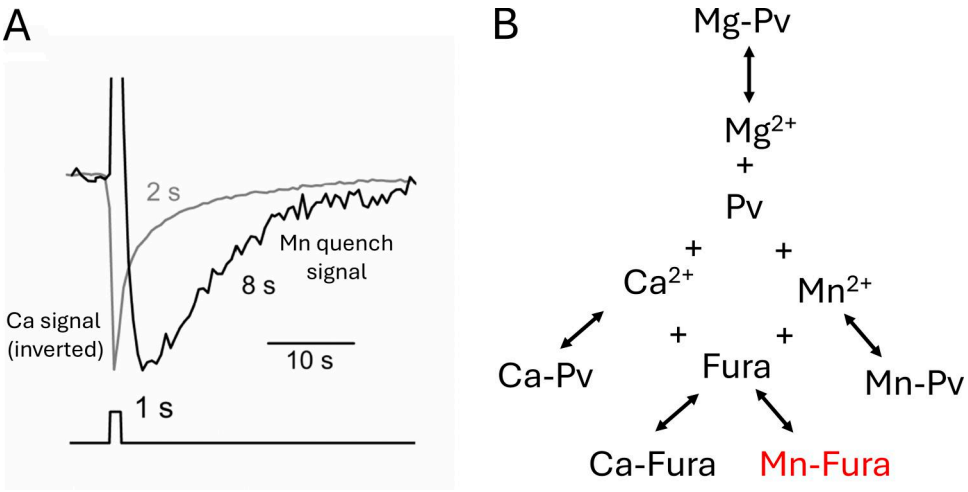

Figure 1. **The equilibria associated with the Mn^{2+} quench. (A)** Recordings of Fura-2 fluorescence changes induced by a depolarizing pulse to 0 mV for 1 s. The grey trace shows $[Ca^{2+}]_i$ with a decrease of fluorescence corresponding to an increase of $[Ca^{2+}]_i$. The dark trace is the Mn^{2+} quench signal taken from Fig. 5 A of Jacque-Fernandez et al. (2025). **(B)** The network of important equilibria involving Mn^{2+}, Ca^{2+}, Mg^{2+}, Fura-2, and parvalbumin (Pv). Note that Fura has a relatively low affinity for Mg^{2+}; therefore, this reaction can be ignored. The species Mn-Fura (in red) represents the form of Fura that does not fluoresce and is therefore the basis of the Mn^{2+} quench transient shown in panel A.

the summed competitive effect would substantially lower the Ca^{2+} sensitivity and also quench the fluorescence signal (Grynkiewicz et al., 1985). The cross interference of these endogenous divalent cations with Ca^{2+} binding to indicators makes measurement of physiological free ion levels challenging. A recent paper using a novel sensor suggests a free Mn^{2+} concentration of ~1 μM in HEK293 cells (Kahali et al., 2024). This is more than 100-fold greater than previous estimates and is at a value that would result in the substantial quenching (by >90%) of a Fura indicator within the cells. This level of endogenous quenching seems unlikely, as Fura-based indicators have been successfully used in many cell types; clearly this is a conundrum that deserves further investigation. Like calcium, the extracellular and intracellular concentrations of manganese are maintained within narrow limits. Too low a value would interfere with key manganese-dependent enzymes. Conversely, increasing above normal by a factor of two- to threefold results in toxic actions primary through sustained mitochondrial dysfunction (Tuschl et al., 2013).

The intracellular manganese levels used in the manganese quench protocol are almost certainly disruptive to the cell's metabolism in the long term, but it is assumed that these effects are minimal immediately after application of extracellular Mn^{2+}. Fig. 1 A is taken from the featured article (Jacque-Fernandez et al, 2025), showing the Ca^{2+} transient. The time course of the quench transient is slower than that of the Ca^{2+} transient. The reason for this, suggested by the authors, is that the exchange of Ca^{2+} with Mn^{2+} bound to intracellular proteins is a slow process. This makes sense since muscle cells contain the Ca^{2+} buffer protein, parvalbumin (Pv). This protein has a role in slowly binding/buffering Ca^{2+} ions released by the SR. The origin of the slow interaction is that the Mn^{2+}/Ca^{2+}-binding sites bind Mg^{2+} ions at rest. The slow kinetics of Ca^{2+} binding arises from the need for Mg^{2+} to dissociate before Ca^{2+} (or Mn^{2+}) can bind (see Fig. 1 B) (See Eisner et al. [2023] for review).

This work suggests an interesting set of questions. First, does the time course of the transient manganese quench give us insight into the time course of calcium binding to Pv? A second related question would be whether the competition between intracellular Mn^{2+} and Ca^{2+} affects Ca^{2+} buffering and the time course of the Ca^{2+} transient and associated Ca^{2+}-triggered events.

The complex interactions between Ca^{2+}, Mg^{2+}, and the intracellular buffers of these ions have been the focus of many computational models designed to help understand the underlying physiology (Baylor and Hollingworth, 1988). To incorporate Mn^{2+} binding into these models requires knowledge of the Mn^{2+} affinity for intracellular buffers. For indicators such as Fura-2 that are based on the binding site of the chelator EGTA, the affinity for Mn^{2+} is ~10 times that of Ca^{2+} (Martell and Smith 1974; Grynkiewicz et al., 1985). The affinity of Mn^{2+} for Pv, specifically the EF hand domains, has not been quantified, but assuming the same relative effects to small molecule buffers with a similar binding site design, the affinity of Mn^{2+} would be expected to be ~10 times that of Ca^{2+}. The Mn^{2+} dissociation rate constants were estimated by assuming that the Mn^{2+} association rate constant, like for Ca^{2+}, is diffusion limited (Mirti, 1979). Interestingly, structural studies of Pv suggest that Mn^{2+} binding modifies the tertiary structure of the protein in a different way compared with Ca^{2+}(Declercq et al., 1991; Nara et al., 1994).

We have modelled the expected results of adding Mn^{2+} in the simulation of Fig. 2. It should be noted that the simulation is not designed as an exact replica of the data by Jacque-Fernandez et al., simply to illustrate the sorts of behavior expected. The solid black traces show a simulation of the exchange of Ca^{2+} and Mg^{2+} on Pv in the absence of Mn^{2+}. Under typical cellular resting conditions ($[Ca^{2+}]$ = 100 nM and $[Mg^{2+}]$ = 1 mM), most of the Pv has either Ca^{2+} or Mg^{2+} bound (in roughly equal amounts) with only about 5% unbound. The addition of a bolus of Ca^{2+} immediately increases $[Ca^{2+}]_i$. Some of this Ca^{2+} will bind to the free

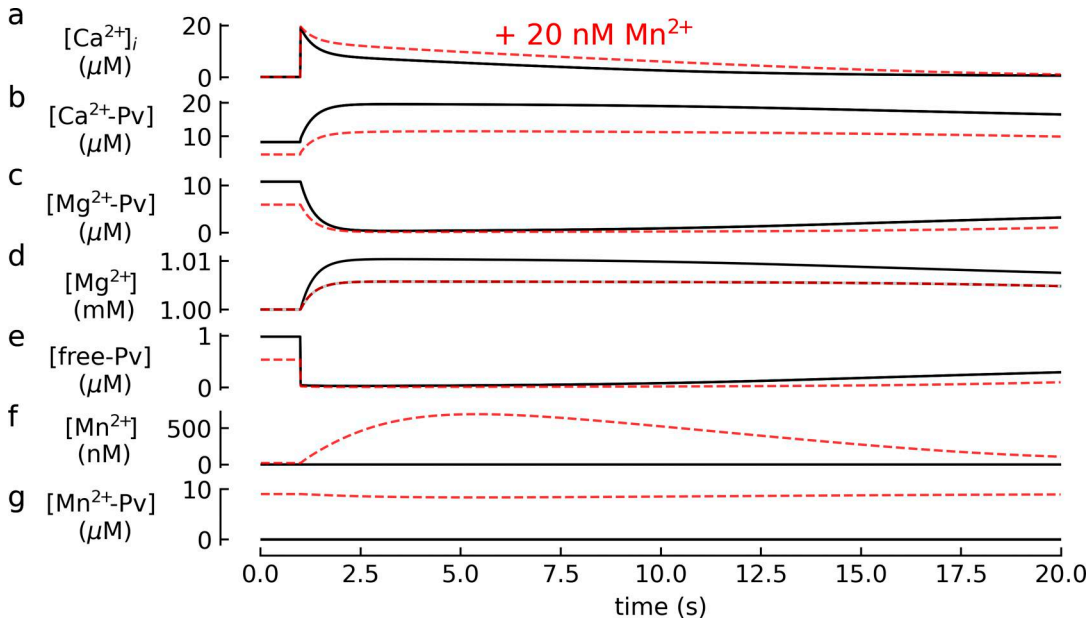

Figure 2. **Simulation of the interactions of Mn^{2+} with a slow buffer (Pv).** Traces show (from top to bottom): a, free $[Ca^{2+}]$; b, Ca^{2+} bound to Pv; c, Mg^{2+} bound to Pv; d, free $[Mg^{2+}]$; e, free Pv; f, free $[Mn^{2+}]$; g, Mn^{2+} bound to Pv. A bolus of Ca^{2+} was added at 1 s. The solid black trace was obtained in the absence, and the red dashed in the presence of 20 nM free Mn^{2+}. (See Eisner et al., 2023 for model). The equilibrium constants and therefore the associated rate constants for Mn^{2+} binding to the intracellular buffers, including Fura2, were estimated from the reported values relative to Ca^{2+} Martell and Smith (1974), i.e., EGTA $\log K_{Ca}$ 11.0, $\log K_{Mn}$ = 12.3, $\log K_{Mg}$ = 5.28; EDTA: $\log K_{Ca}$ = 10.7, $\log K_{Mn}$ = 12.0, $\log K_{Mg}$ = 8.8.

Pv, decreasing its concentration (e). The law of mass action then dictates that Mg-Pv will dissociate to replenish the pool of free Pv at the expense of a decrease of [Mg-Pv] (c), releasing Mg^{2+} into the cytoplasm (d). The time course of these events is limited by the slow dissociation of Mg^{2+} from Pv, resulting in a slow decrease of [Mg-Pv] and increase of $[Mg^{2+}]$. The physiological importance of this mechanism is that it allows Pv to accelerate relaxation without decreasing the maximum rise of $[Ca^{2+}]_i$ (Cannell, 1986).

The dashed red traces simulate the behavior after having added Mn^{2+} at a free concentration of 20 nM. In the absence of direct measurements, we assume that the K_d of Mn^{2+} binding to Pv is 0.1 of that for Ca^{2+} because Mn^{2+} dissociates from Pv at one tenth the speed as does Ca^{2+}. This results (g) in about 50% of the Pv being in the Mn^{2+} bound form, with corresponding decreases in the Ca^{2+}-, Mg^{2+}-, and free forms of Pv. Now the transient decrease of free Pv concentration produced by Ca^{2+} addition will release Mn^{2+} to maintain the equilibrium of the binding reaction, resulting in an increase of $[Mn^{2+}]$ (f). As $[Ca^{2+}]$ decreases, free Pv increases, so $[Mn^{2+}]$ decays. Because much of the Pv is in the Mn^{2+} form, and there is less free Pv available, the rise of $[Ca^{2+}]_i$ decays (red dashed line) more slowly than in the absence of Mn^{2+}. In other words, the addition of Mn^{2+} has decreased the effective Pv concentration and therefore its ability to lower $[Ca^{2+}]_i$.

Consistent with the data by Jacque-Fernandez et al. (Fig. 1 A), the model predicts that the change of $[Mn^{2+}]$ lags that of $[Ca^{2+}]$. It is also interesting to note that the model predicts that the addition of Ca^{2+} has very different effects on the concentration of the two competing cations, Mg^{2+} and Mn^{2+}, with the latter having much faster kinetics. Two factors are relevant here: (1) The fast kinetics of Mn^{2+} interaction with free Pv means that the concentration of Mn-Pv can track that of free PV, whereas this is not the case for the slower Mg^{2+}. (2) More importantly, if free Pv decreases to a fraction *r* of its initial value then the ratios [Mg-PV]/$[Mg^{2+}]$ and [Mn-PV]/$[Mn^{2+}]$ must also change by the same fraction in the steady state. In the simulation, when Mn^{2+} has been added, the concentration of Mn-Pv and Mg-Pv are very similar yet free Mg^{2+} is 50,000 times greater than free Mn^{2+}. A given change of *r* will therefore result in a much larger fractional change of Mn^{2+} than Mg^{2+}.

Different results are predicted if one considers the effects of Mn^{2+} on a fast buffer. Although the addition of Ca^{2+} to the cytoplasm will again produce a transient rise of Mn^{2+} concentration, the decrease of Ca^{2+} buffer power will now accelerate the kinetics of the decay of $[Ca^{2+}]_i$ (not shown).

These simulations therefore suggest that the addition of Mn^{2+} will decrease the availability of Ca^{2+} buffers and thence their buffer power. They also reproduce the observation by Jacque-Fernandez et al. that the exchange of Mn^{2+} bound to intracellular buffers (i.e., Pv) for Ca^{2+} causes a transient rise of cytoplasmic Mn^{2+} and therefore a transient Mn^{2+} quench, which occurs over a longer timescale than does the initiating Ca^{2+} transient. The exact interrelationship depends critically on the relative affinities of manganese, magnesium, and calcium for the intracellular buffers. Finally, increasing Mn^{2+} within the cell will decrease the total Ca^{2+} buffer power of the cell as manganese binds to Pv. This should be another feature to consider when interpreting SOCE signals using Mn^{2+} quench procedures.

While Mn^{2+} quench signals may simply be a biophysical curiosity, it is worth remembering that Mn^{2+} and other heavy metals are required for life and that these exchanges must occur

to a much lesser extent under conditions of normal cellular manganese levels. The extent to which this exchange and that of other trace divalent cations occur and the extent to which they influence endogenous Ca^{2+} buffering requires further research.

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