



A new set of equations for the simplified calibration of fluorescence Ca^{2+} transients in skeletal muscle fibers

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Abstract

The classical approach for calibrating non-ratiometric fluorescent Ca^{2+} dyes entails the measurement of the fluorescence maximum (F_{max}) and minimum (F_{min}), as well as the dissociation constant (Kd) of the Ca^{2+} —Dye reaction (model 1). An alternative equation does not need the F_{min} but requires the rate constants k_{on} and k_{off} (model 2). However, both approaches are experimentally time consuming and the rate constants for several dyes are unknown. Here, we propose a set of equations (model 3) that simplify the calibration of fluorescent Ca^{2+} transients obtained with non-ratiometric dyes. This equation allows the calibration of signals without using the F_{min} : $[\text{Ca}^{2+}] = Kd(F - F_{rest}/F_{max} - F) + [\text{Ca}^{2+}]_{IR}(F_{max} - F_{rest}/F_{max} - F)$, where $[\text{Ca}^{2+}]_{IR}$ is the resting $[\text{Ca}^{2+}]$. If the classical calibration approach is followed, the F_{min} can be estimated from: $F_{min} = F_{rest} - ([\text{Ca}^{2+}]_{IR}(F_{max} - F_{rest})/Kd)$. We tested the models' performance using signals obtained from enzymatically dissociated *flexor digitorum brevis* fibers of C57BL/6 mice loaded with Fluo-4, AM. Model 3 performed the same as model 2, and both gave peak $[\text{Ca}^{2+}]$ values $15 \pm 0.3\%$ ($n=3$) lower than model 1, when we used our experimental F_{min} (1.24 ± 0.11 A.U., $n=4$). However, when we used the mathematically estimated F_{min} (6.78 ± 0.2 A.U) for model 1, the peak $[\text{Ca}^{2+}]$ were similar for all three models. This suggests that the dye leakage makes a correct determination of the F_{min} unlikely and induces errors in the estimation of $[\text{Ca}^{2+}]$. In conclusion, we propose simpler and time-saving equations that help to reliably calibrate cytosolic Ca^{2+} transients obtained with non-ratiometric fluorescent dyes. The use of the estimated F_{min} avoids the uncertainties associated with its experimental measurement.

Keywords Ca^{2+} · Fluorescence · Dyes · Skeletal muscle · Calibration

Introduction

The Ca^{2+} ion is a universal intracellular messenger that is involved in many different cellular processes (Berridge et al. 2003). In skeletal muscle cells, the Ca^{2+} ion links membrane depolarization with fiber contraction through a process known as excitation–contraction coupling (ECC) (Calderón et al. 2014a). For decades, ECC has served as a testing ground for experiments that assess the interaction between Ca^{2+} and intracellular fluorescent dyes.

Measuring intracellular Ca^{2+} has a long history (Jöbsis and O'Connor 1966; Ridgway and Ashley 1967). However, it was Tsien and coworkers' work who generalized the use of intracellular dyes for determining the dynamic concentration of intracellular Ca^{2+} , $[\text{Ca}^{2+}]$ (Grynkiewicz et al. 1985; Minta et al. 1989; Tsien et al. 1982). Polycarboxylate non-ratiometric Ca^{2+} dyes are a class of non-genetically encoded sensors extensively used nowadays in skeletal muscle (Bakker et al. 2017; Calderón et al. 2014b; Caputo et al. 1994; Cully et al. 2016; Jaque-Fernandez et al. 2020; Katerinopoulos and Foukaraki 2002; Komatsu et al. 2018; Olivera and Pizarro 2018; Sébastien et al. 2018; Wang et al. 2018). By either injecting the salt form of the dye with a micropipette or by allowing the passive diffusion of a modified molecule of the dye (Kao et al. 2010; Minta et al. 1989; Tsien 1981) it is possible to measure $[\text{Ca}^{2+}]$ within the cells (Calderón et al. 2014a; Grynkiewicz et al. 1985; Kao et al. 2010; Katerinopoulos and Foukaraki 2002; Tsien et al. 1982).

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In order to convert the fluorescent Ca^{2+} signals into the ionic concentration, a calibration procedure must be undertaken (Kao et al. 2010). The classical approach (model 1) for calibrating single wavelength dyes requires the knowledge of the maximum fluorescence (F_{max}), the minimum fluorescence (F_{min}) and the dissociation constant (Kd) of the Ca^{2+} —Dye reaction (Gryniewicz et al. 1985). However, the determination of the F_{min} is time-consuming and fraught with technical difficulties due to compartmentalization and leakage of the dye. Also, the use of equipment with low sensitivity and poor quality filters may produce low signal to noise ratio (parasite light), and the binding of the dye to intracellular components can modify the expected intrinsic fluorescence of the dye even in absence of Ca^{2+} . Moreover, the presence of traces of Ca^{2+} in many reagents (including water) make it difficult to guarantee that a solution is devoid of Ca^{2+} . Additionally, Kd values are highly variable, depending whether they are obtained in vitro or in situ, and are also affected by temperature, pH, ionic strength and the presence of proteins (Baylor and Hollingworth 2007; Harkins et al. 1993; Konishi et al. 1988; Thomas et al. 2000; Woodruff et al. 2002; Zhao et al. 1996). The effect of these factors on the Kd has not yet been determined for all available dyes.

An alternative equation (model 2) for calibrating single wavelength dyes that did not need the F_{min} and avoided the uncertainty of the Kd was then proposed (Caputo et al. 1994). It requires, however, to know the values of k_{on} , k_{off} and the resting Ca^{2+} concentration $[\text{Ca}^{2+}]_{JR}$ as well as to calculate the derivative of the experimentally obtained signal, dF/dt . Nevertheless, the experimental determination of k_{on} and k_{off} has only been calculated for a few dyes (Baylor and Hollingworth 2007; Berlin and Konishi 1993; Caputo et al. 1994; Lattanzio and Banschat 1991; Zhao et al. 1996), since some have kinetics faster than the mixing time required to measure them. Also, the numerical calculation of dF/dt propagates rounding and estimation errors thus adding noise to the final results (Shukla and Rusling 1985).

Consequently, both approaches for calibrating single wavelength dyes have clear limitations. This may explain why most of the papers using non-ratiometric Ca^{2+} measurements do not calibrate their signals. In this work we propose and experimentally validate a new equation (model 3) to calibrate Ca^{2+} signals without the need to know the F_{min} , k_{on} , k_{off} or dF/dt . We also present an equation to calculate the F_{min} parameter needed for determining Ca^{2+} from fluorescence signals according to the current published models (Caputo et al. 1994; Gryniewicz et al. 1985). The comparison of the performance of the three models on the calibration of Ca^{2+} fluorescence signal in skeletal muscle is also carried out.

Methods

Mathematical approach

To obtain Ca^{2+} calibration equations, we started with the most basic chemical reaction between two reactant agents, that is, the Ca^{2+} ion and the dye. The binding of these two chemical agents produces a third agent, Ca^{2+} -D. Here, D is the Ca^{2+} dye, and its concentration depends on the concentrations of the reactants.

The *mass action law* is a phenomenological model used to describe chemical kinetics wherein the *speed* or *reaction rate* is considered to be proportional to the concentration of the reactants involved. Ordinary differential equations are well suited to model chemical reactions when the spatial distribution of the chemical species can be disregarded as is the case inside a well stirred container. Consider the 1:1 chemical reaction scheme, which applies to most reactions of Ca^{2+} and a dye:



The values k_{on} and k_{off} are known as rate coefficients and express the proportionality of the reaction speeds v_{on} and v_{off} on the concentrations of the reactants. The rate coefficient k_{on} has units of $\text{M}^{-1} \text{s}^{-1}$, while k_{off} has units of s^{-1} .

When the system reaches equilibrium, Ca^{2+} , D, and Ca^{2+} -D remain constant, their time derivatives are zero, and the speeds v_{on} and v_{off} are constant and equal. We can define the equilibrium constant Kd in terms of k_{on} and k_{off} as,

$$K_d = \frac{k_{off}}{k_{on}} \quad (2)$$

Starting from (1), we come to a first order reaction to measure the $[\text{Ca}^{2+}]$ in terms of the remaining equilibrium concentrations,

$$[\text{Ca}^{2+}] = K_d \frac{[\text{Ca} - D]}{[D]} \quad (3)$$

In the framework of mass action law kinetics, we can obtain a set of three differential equations that describe the time evolution of the concentrations of each species,

$$\frac{d}{dt} [\text{Ca}^{2+}] = -k_{on} [\text{Ca}^{2+}] [D] + k_{off} [\text{Ca} - D] = \frac{d}{dt} [D] = -\frac{d}{dt} [\text{Ca} - D] \quad (4)$$

By continuing with this analysis, it is possible to come to a well-known calibration equation (Gryniewicz et al. 1985; Kao et al. 2010; Minta et al. 1989) to express Ca^{2+} measured with a single wavelength intracellular dye:

$$[Ca^{2+}] = K_d \left(\frac{F - F_{min}}{F_{max} - F} \right) \quad (5)$$

This equation is considered to be model 1. As stated (Gryniewicz et al. 1985), it assumes that the dye: (i) behaves in the cell as it does in calibration media, (ii) forms a simple 1:1 complex with Ca^{2+} , and (iii) is sufficiently diluted so that the intensity of the fluorescence is linearly proportional to the concentrations of the fluorescent species.

An alternative equation proposed by Caputo and colleagues (Caputo et al. 1994) exploits the reported experimental value of the $[Ca^{2+}]_{IR}$ for skeletal muscle cells (70 nM in frog in the original work by Caputo et al. 1994, and 100 nM in mammals) and the time derivative of the fluorescence signal. If $\Delta F = F - F_{rest}$, where F_{rest} is the resting fluorescence of the cell, the proposed equation is:

$$[Ca^{2+}] = \frac{k_{off} \Delta F + [Ca^{2+}]_{IR} (F_{max} - F_{rest}) k_{on} + \frac{dF}{dt}}{(F_{max} - F_{rest} - \Delta F) k_{on}} \quad (6)$$

This equation is considered to be model 2. Equations (5) and (6) were used as the starting points for developing the new equations.

Experimental approach

All manipulations and procedures carried out with mice during the development of this work were approved by the Committee for Ethics in experiments with animals of the University of Antioquia (minutes of June 2016).

Stocks of Cyclopiazonic acid (CPA, Tocris, USA), *N*-benzyl-*p*-toluene sulphonamide (BTS, Tocris, USA) and Fluo-4, AM (F14201, Thermo Fisher, USA) were prepared in Dimethyl sulfoxide (DMSO, Merck, Germany). Frozen aliquots of these reagents were thawed and dissolved in the experimental solution to achieve final working concentrations just before starting the experiments. The maximum final concentration of DMSO in the solutions was 1.3%. Saponin (Sigma-Aldrich, USA) stock was prepared in distilled water. Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) and *N*-(2-Hydroxyethyl) piperazine-*N*O-2-ethanesulfonic acid (HEPES) were from Amresco (USA). Other salts and glucose were from Amresco (USA) and Merck (Germany).

The method for enzymatic dissociation of skeletal muscle is a modification of previously published ones (Caldern et al. 2014b). Briefly, 66 ± 4.8 day-old male mice (C57BL6/J), with a mean weight of 21.7 ± 0.4 g (n = 4), were euthanized by rapid cervical dislocation. Flexor digitorum brevis (FDB) muscles were dissected out and incubated in a modified Ringer solution (in mM: 2.7 KCl, 1.2 KH₂PO₄, 0.5 MgCl₂, 138 NaCl, 0.1 Na₂HPO₄, 1 CaCl₂, pH 7.4) containing 2.5 mg ml⁻¹ collagenase (Worthington CLS2; 230 u/mg

dw) for 82–84 min at 36.8 °C. After collagenase treatment, the muscles were washed twice with Tyrode (in mM: 5.4 KCl, 1 MgCl₂, 140 NaCl, 0.33 NaH₂PO₄, 2 CaCl₂, 10 glucose, 10 HEPES, pH 7.3) at room temperature, and gently separated from tendons and remaining tissue with a set of fire-polished Pasteur pipettes. Fibers rendered via this procedure remained excitable and contracted briskly for up to 24 h. Dissociated fibers were transferred to the experimental chamber (RC-27NE, Warner instruments, USA) and were incubated for 12 min at room temperature in Tyrode solution containing 6 μM of the Ca²⁺ dye Fluo-4, AM. After loaded, the cells were washed and rested during 25 min to allow for the acetoxymethyl ester form of the dye be fully de-esterified.

For Ca²⁺ recordings, the chamber was mounted onto the stage of an inverted Axio Observer A1 (Carl Zeiss, Germany) microscope equipped for epifluorescence and the fibers were illuminated with a LED device. The wavelengths (in nm) of the filter combination (excitation/dichroic/emission) for Ca²⁺ transients were 450–490/510/515. Light signals were collected with a photomultiplier (R928P tube in a D104 photometer, Horiba, Japan) connected to a 1550A Digidata (Molecular Devices, USA). Intracellular Ca²⁺ transients were elicited by applying suprathreshold rectangular current pulses (1–1.4 ms) through two platinum plate electrodes placed along either side of the experimental chamber. Data were acquired and analyzed using pCLAMP 10 software (Molecular Devices, USA).

In all experiments, the fiber viability was verified by eliciting a few Ca²⁺ transients in Tyrode solution. The F_{rest} was considered as the mean fluorescence in a window of 5 s in a stable part of the recording, before exchanging to the F_{min} and F_{max} solutions. For the F_{min} experiments, a modified Tyrode solution with 0.002% saponin, 15 μM CPA, 50 μM BTS, 10 mM EGTA, without Ca²⁺ and Mg²⁺, was used. The F_{min} values were measured at different points of the signal, as explained in detail in the ‘Results’ section. For the F_{max} assays, the above modified Tyrode solution was supplemented with 100.01 mM Ca²⁺. The F_{max} was taken as the mean of a 500 ms segment, at the peak of a time-expanded recording. Saponin works as a rapid membrane permeabilizing agent. BTS allowed us to acquire recordings free of movement artifacts. CPA was used to inhibit the uptake of Ca²⁺ by the sarcoplasmic reticulum upon membrane permeabilization, in order to keep the Ca²⁺ in the cytosol and rapidly saturate the dye. All experiments were done at 20 °C.

For the calibration of the Ca²⁺ transients, the following Fluo-4 values were used: K_d in vitro: 0.345 μM; K_d in situ: 1 μM; k_{off} : 60 s⁻¹; k_{on} : 60 μM⁻¹ s⁻¹; $[Ca^{2+}]_{IR}$: 0.1 μM (Thomas et al. 2000). The F_{rest} , F_{min} and F_{max} were experimentally obtained in the present work.

Statistical analyses and graphs were performed using OriginPro 2019 software (OriginLab Corporation, USA).

Results are given as mean \pm standard error of the mean (SEM).

Results

A new calibration equation for non-ratiometric Ca^{2+} dyes

Because of the abovementioned difficulties associated with obtaining the F_{min} and the k_{off} of some dyes, we first developed a new calibration equation that was not dependent on these two parameters.

Rather, it exploits the $[\text{Ca}^{2+}]_{IR}$ and the F_{rest} since the latter already carries the information regarding the F_{min} . From Eq. (5) we obtain,

$$[\text{Ca}^{2+}]_{IR} = K_d \left(\frac{F_{rest} - F_{min}}{F_{max} - F_{rest}} \right) \quad (7)$$

Rearranging,

$$\frac{1}{K_d} [\text{Ca}^{2+}]_{IR} (F_{max} - F_{rest}) = (F_{rest} - F_{min}) \quad (8)$$

It is possible to rewrite as,

$$F - F_{min} = (F - F_{rest}) + (F_{rest} - F_{min}) \quad (9)$$

$$= F - F_{rest} + \frac{1}{K_d} [\text{Ca}^{2+}]_{IR} (F_{max} - F_{rest}) \quad (10)$$

With the result being:

$$[\text{Ca}^{2+}] = K_d \left(\frac{F - F_{rest}}{F_{max} - F} \right) + [\text{Ca}^{2+}]_{IR} \left(\frac{F_{max} - F_{rest}}{F_{max} - F} \right) \quad (11)$$

Which is a new calibration equation for non-ratiometric Ca^{2+} dyes and is herein referred to as model 3. It is clear that when $F = F_{rest}$ the equation produces the result $[\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{IR}$, returning the expected value for $[\text{Ca}^{2+}]_{IR}$.

Validation of the model in skeletal muscle fibers

We tested the performance of the Eqs. (5), (6) and (11), by calibrating Ca^{2+} transients obtained with Fluo-4 in skeletal muscle fibers. For that, we experimentally determined the F_{min} and the F_{max} in mouse FDB fibers loaded with the same dye. The results are shown in Fig. 1. Panels a and b illustrate typical F_{max} and F_{min} recordings, respectively. The F_{max} value was 41.728 ± 3.170 A.U. ($n=5$). Since no clear guidelines for measuring the real F_{min} are described, four points were considered when estimating four potential F_{min} , as shown in the panel c. Point one assumes the lowest, stable value as the F_{min} . Points two and three assume a change in slope as the

true F_{min} , since the first point may be highly affected by dye leakage. Point four explores the possibility that the very first change in slope is the actual F_{min} . The values obtained were 0.267 ± 0.03 , 0.629 ± 0.059 , 1.240 ± 0.11 , and 7.648 ± 0.59 A.U. for points 1 to 4, respectively ($n=4$ in all cases).

The effect of the uncertainties in the determination of the F_{min} on the calibration results, using Eq. (5), is shown in panel d (vertical arrows). A change of up to 17.8% in the peak $[\text{Ca}^{2+}]$ was estimated from points 1 to 4.

The comparison of the performance of the three calibration models is shown in Fig. 2. When the experimental F_{min} value of the point 1 is used for the model 1, the peak $[\text{Ca}^{2+}]$ was overestimated by 17% (Model 1e, black trace), compared to models 2 and 3, which are independent of the F_{min} and performed the same (red and blue traces). Since the difference between model 1 versus models 2 and 3 can be mostly explained by the effect of the F_{min} on the peak $[\text{Ca}^{2+}]$, as previously shown in the Fig. 1d, we devised an approach to mathematically estimate the F_{min} without these experimental uncertainties.

Estimating F_{min} from F_{rest}

The F_{min} value can be estimated by rearranging Eq. (8):

$$F_{min} = F_{rest} - \frac{1}{K_d} [\text{Ca}^{2+}]_{IR} (F_{max} - F_{rest}) \quad (12)$$

This means that the F_{min} could be regarded as a correction to the F_{rest} .

The F_{min} predicted by Eq. (12), when using the K_d in situ for Fluo-4 and the F_{rest} of our experiments, was 6.78 ± 0.13 A.U. ($n=4$). It calls our attention the fact that the predicted value did not coincide with any of the previously identified 4 points (Fig. 1c) and was therefore designated as the 5th point. The peak $[\text{Ca}^{2+}]$ obtained with this F_{min} is pointed out by the horizontal arrow in Fig. 1d, and the calibrated Ca^{2+} transient is graphed in Fig. 2. This figure demonstrates that if the calibration of model 1 is performed with the mathematically estimated F_{min} (Model 1m, green trace), the peak $[\text{Ca}^{2+}]$ compares very well to that obtained with models 2 and 3 (in red and blue, respectively).

These results demonstrate that there is a high degree of uncertainty about how to reliably identify the best point to measure the F_{min} . This further suggests that the typical recordings obtained with F_{min} experiments are strongly affected by leakage of the dye.

Finally, we tested if the assumptions for the K_d and $[\text{Ca}^{2+}]_{IR}$ values ($1 \mu\text{M}/0.1 \mu\text{M} = 10$) were correct, by using a set of fibers in which we determined the F_{max} and the F_{rest} , but from which the F_{min} was not measured. In this case, with a mean F_{rest}/F_{max} of 0.235 ± 0.007 ($n=5$), we came to a $K_d/[\text{Ca}^{2+}]_{IR}$ value of 10.78 ± 0.39 , confirming the validity of the Eq. (12).

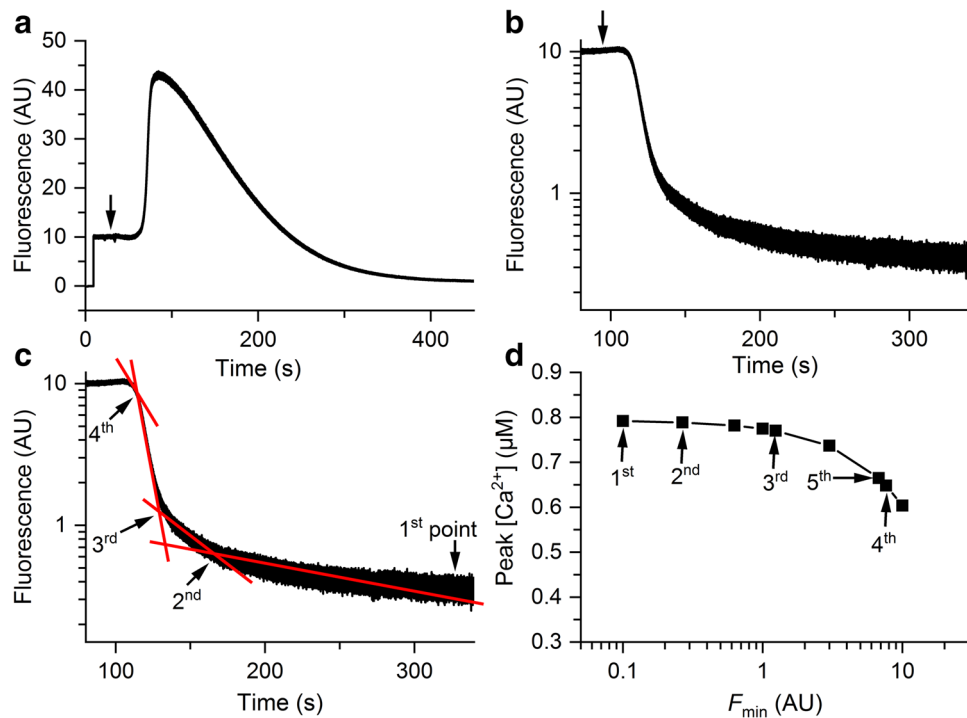


Fig. 1 Calibration of Ca^{2+} transients obtained in skeletal muscle fibers loaded with Fluo-4 and using the Eq. (5). Representative F_{max} and F_{min} recordings are shown in **a** and **b**, respectively. The arrows indicate the start of the solution exchange. Two approaches to estimate the F_{min} value were followed (**c**): identifying a stable part at the end of the signal (1st point), or identifying clear changes in the slope, as labelled by the red lines (2nd to 4th points). If the classical cali-

bration is performed using the F_{min} values obtained in points 1 to 4, the peak $[\text{Ca}^{2+}]$ pointed out by the vertical arrows are obtained (**d**). Points without arrows indicate peak $[\text{Ca}^{2+}]$ calculated when other F_{min} points were arbitrarily selected and not indicated in the panel **c**. The $[\text{Ca}^{2+}]$ pointed out by the horizontal arrow was obtained by using the F_{min} estimated with the Eq. (12). (Color figure online)

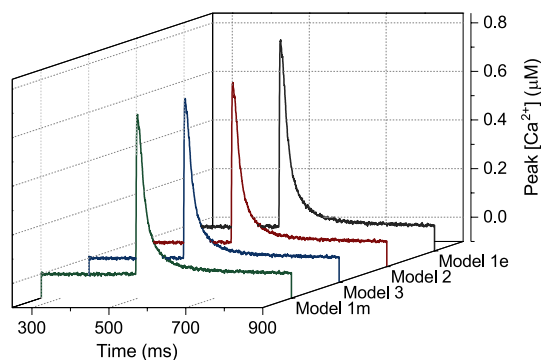


Fig. 2 Comparison of the performance of the models 1 to 3 on the calibration of a Ca^{2+} transient of a muscle fiber obtained with Fluo-4. The black trace is a Ca^{2+} transient calibrated with the Eq. (5) and the F_{min} of the 1st point, which was experimentally (**e**) measured. The red and blue traces are the same Ca^{2+} transient calibrated with the Eqs. (6) and (11), respectively. The green signal is the same record, calibrated with the Eqs. (5) and (12); its peak corresponds to the value pointed out by the horizontal arrow in the Fig. 1d. In this case, m stands for mathematically estimated. Note that red, blue and green are kinetically identical. (Color figure online)

Discussion

We addressed the issue of the calibration equations for non-ratiometric Ca^{2+} dyes. The main results of our work are the following: (i) we validated a new, simpler equation for the calibration of signals obtained with non-ratiometric Ca^{2+} dyes, (ii) we devised a new equation that corrects for the expected dye leakage when estimating the F_{min} , (iii) under some conditions, the three calibration models perform similarly in mammalian skeletal muscle fibers.

The importance of a reliable Ca^{2+} calibration

Knowledge of precise cytoplasmic $[\text{Ca}^{2+}]$ have implications for our understanding of physiological and pathophysiological phenomena, as well as in the development and evaluation of new drugs, for conditions that involve alterations in, for instance, cardiac, skeletal muscle, neuronal or endocrine intracellular Ca^{2+} kinetics. Given that mathematical models of the ECC allow for the prediction of Ca^{2+} dynamics and

the role of the binding sites involved (Bakker et al. 2017; Baylor and Hollingworth 2007), a complete and proficient understanding of the shape, duration and amplitude of the Ca^{2+} transient is crucial.

The present work tackles several difficulties inherent to current non-ratiometric Ca^{2+} dye calibration procedures. Our new calibration model presents advantages over those discussed in previous sections since it avoids the measurement of the F_{min} , sparing time, and the numerical differentiation of the fluorescence recordings, avoiding the addition of noise to the final reported $[\text{Ca}^{2+}]$. The new equation requires the researcher to only know the Kd , $[\text{Ca}^{2+}]_{IR}$, F_{max} , F_{rest} and F . The values of these parameters obtained either experimentally, or sourced from the literature are, in general, reliable. In this regard, the Kd of most of Ca^{2+} dyes (Paredes et al. 2008; Takahashi et al. 1999), and the $[\text{Ca}^{2+}]_{IR}$ are readily available across the literature (Konishi 2005; Williams et al. 1990). Both the F_{rest} and F come from the experimental signal to be calibrated and the F_{max} can be determined for each cellular model and experimental set up at the end of each experiment.

The performance of different Ca^{2+} calibration models

Equations (6) and (11) performed equally because they do not depend on the F_{min} , and the differences between them and the classical Eq. (5) can be explained by the F_{min} values used. The fact that the two calibration models that do not use the F_{min} yield similar results that are lower than the classical model, suggest that the F_{min} is usually underestimated, which in turn overestimates the peak $[\text{Ca}^{2+}]$. Our results show that model 3 performs the same as models 1 and 2, making it reliable and ready for use in routine calibration procedures in skeletal muscle fibers.

Although we did not present any results to prove this, the fact that the parameters in the equations have been calculated for many cell types (Thomas et al. 2000; Woodruff et al. 2002) suggests that besides skeletal muscle fibers, it is plausible that Eqs. (6) and (11) will perform well across a range of cells. Moreover, we expect both equations to work well for the calibration of genetically-encoded Ca^{2+} dyes (Tang et al. 2011) that, based on their kinetic characteristics, were originally calibrated with the classical model 1.

The uncertainties of the experimental measurement of the F_{min}

A useful relationship was derived to estimate the F_{min} in terms of Kd , $[\text{Ca}^{2+}]_{IR}$, F_{max} and F_{rest} values. The discrepancy observed between the estimated and the measured F_{min} rises concerns around the lack of a standardized approach to determine the F_{min} in experimental recordings. A typical

F_{min} recording shows several slopes which are difficult to interpret. Thus, we assessed the most common and intuitive approaches to measure the F_{min} , either at a clear change in the slope, or after a clear stabilization of the signal. We then mathematically predicted the F_{min} using the in situ Kd , in order to take into account the importance of the intracellular components on the function of the Ca^{2+} dyes. Using this approach, we uncovered a large difference between the experimentally measured F_{min} and the mathematically estimated F_{min} . This difference likely arises from leakage of the dye upon permeabilization in the F_{min} experiments and it could be quantified as the difference in the F_{min} experimentally measured in the point 1, and the F_{min} estimated with the Eq. (12). This means that the last two thirds of the decay of the signal in our F_{min} recordings may be related to leakage of the dye.

Although some groups have shown their elegant calibration procedures (Caputo et al. 1994; Cully et al. 2016; Olivera and Pizarro 2018), the majority of papers using non-ratiometric dyes to measure Ca^{2+} in skeletal muscle do not calibrate their signals, nor do they show their F_{min} traces or identify where the F_{min} values were measured. It is possible that several of those calibrations have overestimated up to 17% the peak $[\text{Ca}^{2+}]$, particularly if the values of the F_{min} had been obtained with approaches that only consider points 1 and 2. Then, using the estimated F_{min} corrects for the uncertainties in the measurement of the F_{min} and their effects on the peak Ca^{2+} concentration, further making the results obtained with the three calibration models (Eqs. 5, 6 and 11) comparable.

Conclusion

We presented new equations to calibrate fluorescent Ca^{2+} signals obtained in skeletal muscle fibers with non-ratiometric dyes in a precise, time-saving, reliable way. Provided that the effect of dye leakage on the F_{min} can be corrected, all three models evaluated perform similarly and may be considered interchangeable. Moving forward, our new model may help and encourage researchers to present their Ca^{2+} results calibrated.

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Author contributions All authors listed met the conditions required for full authorship. JCC conceived and designed the study. JCC and MAG obtained funding and ethics approval. DMR and MAG developed the equations and AFM performed the experiments in muscle fibers. AFM and JCC jointly analyzed the results. JCC wrote the first draft of the manuscript and all the authors reviewed and approved the final version.

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Availability of data and materials Data may be shared upon reasonable request to the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval All manipulations and procedures carried out with mice during the development of this work were approved by the Committee for Ethics in experiments with animals of the University of Antioquia (minutes of June 2016).

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