

Analysing force–pCa curves

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Received: 22 December 2009 / Accepted: 8 April 2010 / Published online: 20 May 2010
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Abstract We investigated three forms of the Hill equation used to fit force–calcium data from skinned muscle experiments; Two hyperbolic forms that relate force to calcium concentration directly, and a sigmoid form that relates force to the $-\log_{10}$ of the calcium concentration (pCa). The equations were fit to force–calcium data from 39 cardiac myocytes (up to five myocytes from each of nine mice) and the Hill coefficient and the calcium required for half maximal activation, expressed as a concentration (EC_{50}) and as a pCa value (pCa_{50}) were obtained. The pCa_{50} values were normally distributed and the EC_{50} values were found to approximate a log-normal distribution. Monte Carlo simulations confirmed that these distributions were intrinsic to the Hill equation. Statistical tests such as the *t*-test are robust to moderate levels of departure from normality as seen here, and either EC_{50} or pCa_{50} may be used to test for significant differences so long as it is kept in mind that ΔEC_{50} is an additive measure of change and that ΔpCa_{50} is a ratiometric measure of change. The Hill coefficient was found to be sufficiently log-normally distributed that log-transformed values should be used to test for statistically significant differences.

Keywords Muscle · Contraction · Calcium · Statistics

Introduction

A sigmoid or hyperbolic relation between calcium and muscle contraction or ATPase activity was well known in

the late 1960s (Weber and Murray 1973; Hellam and Podolsky 1969). Several labs noted that the relation was well described by an equation used by Hill (1910) to describe the cooperative binding of oxygen by haemoglobin (Donaldson and Kerrick 1975; Best et al. 1977; Fabiato and Fabiato 1975). The Hill equation was originally:

$$y = 100 \frac{Kx^n}{1 + Kx^n} \quad (1)$$

where x was the partial pressure of O_2 , y was the percent saturation of haemoglobin, and K and n were constants. As Hill pointed out at the time “My object was rather to see if an equation of *this type* can satisfy all the observations, than to base any direct physical meaning on n and K ” (Hill 1910). Hence n and K can be seen as arbitrary fitting constants that can be used to describe any given effect on a dissociation curve relating x and y . When applied to muscle, x is the Ca^{2+} concentration and y is the force. The constant n is often denoted by nh or h and is termed the Hill coefficient, and K is often replaced in modified equations by EC_{50} or by pCa_{50} . The use of the Hill equation has allowed muscle physiologists to summarise the results of their experiments in terms of two constants, the Hill coefficient and the calcium required for half maximal force development, the EC_{50} .

At first the EC_{50} was found by finding the midpoint of a smoothly drawn sigmoid curve, however other means have been used historically including linearised forms of the Hill equation (Kerrick et al. 1985), equations including association constants (Brandt et al. 1980) and Hill plots (Best et al. 1977), but the advent of the digital computer allowed Donaldson and Kerrick to be among the first to fit the data directly to the Hill equation (Kerrick and Donaldson 1972).

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In addition to Eq. 1, two forms of the Hill equation have fallen into common use by muscle physiologists. A form closely related to the original Hill formulation:

$$\frac{P}{P_0} = \frac{Ca^h}{EC_{50}^h + Ca^h} \quad (2)$$

where P is the tension, P_0 is the maximum force, Ca is the calcium concentration and h is the Hill coefficient. This form has been frequently used to fit force–[Ca] data and to obtain a value for the EC_{50} as a measure of calcium sensitivity (e.g. Konhilas et al. 2002).

Another form, perhaps more common, is derived in part from the use of logarithmically spaced calcium values in the design of the typical force–calcium experiment:

$$\frac{P}{P_0} = \frac{1}{1 + 10^h \cdot (pCa_{50} - pCa)} \quad (3)$$

where P , P_0 and h are as before and pCa is the $-\log_{10}[Ca]$ and pCa_{50} is the pCa when the force is equal to $P_0/2$. Both forms are mathematically equivalent and while the terms EC_{50} and pCa_{50} are related by a simple log transform, the tendency is to specify the form of the equation which directly estimates the parameter of interest, pCa_{50} or EC_{50} . Good accounts of the various mathematical forms of the Hill equation can be found in the literature and the interested reader can consult these for further information (Bindslev 2008; Colquhoun 2006; Goutelle et al. 2008).

The midpoint of the force–calcium curve, whether expressed as EC_{50} or pCa_{50} , has a simple interpretation as an index of the “calcium sensitivity” of the muscle cell. When calcium sensitivity is decreased more calcium is required to get force to the midpoint and when calcium sensitivity is increased, less calcium is required to get the force to the midpoint. The Hill curve is often fit to force–calcium data to find the best estimate of the calcium required for 50% of maximum force under a specified set of conditions. Changes in calcium sensitivity have been reported as a function of muscle length, sarcomeric protein phosphorylation, temperature, and pH. It has become a common question as to whether a treatment has altered the calcium sensitivity of a muscle fibre, however it should be kept in mind that calcium sensitivity does not correspond to an equilibrium constant nor a binding constant nor to any single physical property of the muscle. As pointed out by Shiner and Solaro, the force–calcium curve is a stationary state fuelled by ATP breakdown and consequently many factors other than binding affinities, such as crossbridge number or turnover, can affect the midpoint of the force– pCa curve (Shiner and Solaro 1984).

In a recent review in this journal, Fuchs and Martyn (2005) described the fitting of force–calcium data from skinned muscle experiments and pointedly noted that little was known about the statistical distribution of the two

candidates (EC_{50} and pCa_{50}) for a potentially standard measure of calcium sensitivity. They highlighted an experiment by Konhilas et al. (2003) that showed a significant change in calcium sensitivity when measured as a change in the EC_{50} but showed no significant change when it was measured as the change in pCa_{50} .

We address some of the issues raised by Fuchs and Martyn (2005) by examining the distribution of EC_{50} and pCa_{50} in force–calcium experiments using skinned human cardiac myocytes and show that while pCa_{50} is normally distributed and EC_{50} is log-normally distributed, the departures from normality are not so significant as to invalidate statistical tests performed on EC_{50} values. Monte Carlo simulations show that the skewness of the EC_{50} distribution is a property of the Hill equation regardless of its particular form. We also show that the Hill coefficient is log-normally distributed and that log transformation is recommended before statistical tests are used to test for treatment differences. We also reconsider the results of Konhilas et al. (2003) and show that there is only an apparent disparity in EC_{50} and pCa_{50} results and that they are in fact congruent.

Materials and methods

Measurements of force as a function of bathing calcium concentration were conducted on multiple myocytes prepared from nine different mice for a total of 39 individual experiments. This gives the distribution of pCa_{50} and EC_{50} values in a typical experimental series.

Tissues

C57Bl mice were killed by cervical dislocation, the hearts were rapidly removed to ice cold Krebs–Henseleit buffer and the heart dissected into small pieces that were then frozen and stored in liquid nitrogen. Frozen pieces were, on the day of the experiment, dissected on a liquid N_2 cooled metal block and 10 mg was removed to an Eppendorf tube containing ice cold rigor solution and 0.3% Triton X-100. The sample was homogenised at 1,000 rpm using a Tissue Tearor tissue homogeniser and the resultant slurry was then centrifuged at $800 \times g$ for 5 min and the supernatant discarded. The pellet was resuspended in fresh rigor buffer and spun again at $800 \times g$ and the supernatant discarded. Myocytes fragments were resuspended in rigor solution and stored at 4°C until required.

Force– pCa experiments

All experiments were carried out at a temperature of 15°C on a temperature controlled plate that had multiple small

wells with coverslip bottoms and a mounting area. The small wells were used to hold a ‘bubble’ of solution and changes in bathing calcium concentration were achieved by moving the suspended myocyte rapidly from well to well. The system was built around a Nikon T2000 microscope with phase contrast ability at magnifications up to $400\times$. Sarcomere length was measured for each myocyte using an image based system (Aurora Scientific 901A HSVL).

An aliquot of the myocyte suspension was placed on a glass slide to permit myocyte selection and mounting. Myocyte fragments with rectangular shapes were selected from the visual field and attached, using a silicone based aquarium cement, to a force transducer at one end and a motor arm at the other end. Force was measured using a modified force transducer (Aurora scientific 406A) that had a resolution of $1\ \mu\text{N}$ and a resonant frequency of $1\ \text{kHz}$. The motor arm used to adjust length (Aurora Scientific 315C) had a maximum range of $3\ \text{mm}$ and a step response time of $1\ \text{ms}$.

At the beginning of each experiment, sarcomere length was fixed at $2.2\ \mu\text{m}$ by adjusting the length of the myocyte using the motor. Sarcomere length was monitored throughout the experiment and if the sarcomere length varied by more than $0.1\ \mu\text{m}$ from the set length of $2.2\ \mu\text{m}$, the preparation was discarded.

The myocytes were activated by moving the myocyte from a bath containing relaxing solution to one containing pre-activating solution for a few seconds, and then moving it to a bath containing calcium. Once the contraction was stable the myocyte was moved back to a well containing relaxing solution. The myocytes were exposed to a solution with a pCa of 4.3 initially to record a maximum force, then from 5 to 7 pCa values from the series 6.4, 6.0, 5.8, 5.7, 5.6, 5.4, and 4.3, presented in random order. Another maximal contraction (pCa 4.3) concluded the series. If the first and last pCa 4.3 contractions differed by more than 20% the preparation and data were discarded, if there was a lesser difference, or no difference, then a Hill curve was fit using only the random order pCa contractions (i.e. the first and last pCa 4.3 contractions were discarded to avoid introducing an order-dependent bias into the fitted curve (Fisher 1990)).

Solutions

The composition of activating, relaxing and pre-activating solutions were calculated using a custom computer program based on the work of Fabiato (Fabiato and Fabiato 1979). They had an ionic strength of about $180\ \text{mM}$ and compositions are described in Table 1.

Table 1 Composition of solutions used in force–calcium experiments

Species	Concentrations (mM)		
	Relaxing	Pre-activating	Activating
Ionic strength (mM)	180	180	180
pH	7.0	7.0	7.0
pCa	9	9	4.3
Free Mg^{2+} (mM)	1	1	1
Mg-ATP (mM)	5	5	5
EGTA (mM)	20	0.5	20
HDTA (mM)	0	19.5	0
Creatine phosphate	10.0	10.0	10.0
K-propionate	40.07	40.73	0.52 ^a
DTT	1	1	1

Equimolar amounts of CaCl_2 and EGTA were used to make Ca–EGTA. Solutions contained $10\ \text{mM}$ creatine phosphate, $40\ \text{mM}$ *N,N*-bis(2-hydroxy-ethyl)-2-aminoethanesulfonic acid (BES), $0.1\ \text{mM}$ PMSF, $10\ \text{mM}$ leupeptin and $1\ \text{mM}$ pepstatin. The Mg-ATP and free Mg^{2+} concentrations were calculated to be 5 and $1\ \text{mM}$ respectively. Ionic strength was adjusted to $180\ \text{mM}$ by the addition of potassium propionate. Potassium hydroxide was used to adjust the pH to 7.0 at 20°C . EGTA Ethylene glycol-bis(β -amino-ethyl ether)-*N,N,N,N*-tetraacetic acid, HDTA hexamethylenediamine tetraacetic acid

^a A greater amount of KOH was required to bring the activating solution to pH 7 than was required for the relaxing solution. With the Cl in the Ca–EGTA solution, this was equivalent to adding about $40\ \text{mM}$ KCl to the activating solution

Curve fitting

Force–calcium data were fit to Eq. 3 using the NLS library in the R statistical language (Ihaka and Gentleman 1996). Force values were expressed as fractions of maximum force and calcium was expressed as pCa during the fitting process and pCa₅₀ values were obtained and tabulated for each experiment.

Calcium concentrations were calculated from pCa values using $[\text{Ca}] \mu\text{M} = 10^{(6-\text{pCa})}$. Force–[Ca] data were fit to Eq. 2 and to the original Hill equation (Eq. 1) using the NLS library for R (Ihaka and Gentleman 1996). For the fit to the Hill equation, the EC₅₀ was calculated as $1/(K^n)$ where K is the fitting constant and n is the Hill coefficient.

Monte Carlo simulations

To examine the propagation of experimental error into the resulting distribution of EC₅₀ and pCa₅₀ in data from force–calcium experiments, we used a Monte Carlo simulation to look at the distribution of fitted EC₅₀ and pCa₅₀ estimates when the underlying values of pCa₅₀ and EC₅₀ were known and when the force estimates were subject to a small amount of normally distributed error, as might be seen in a typical experiment. We used an approach

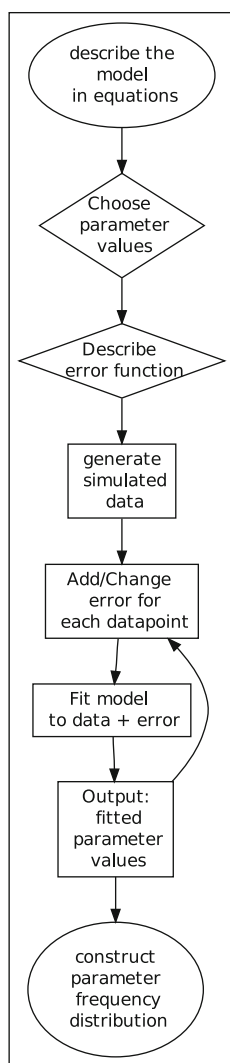
described by Christopoulos to fit agonist dose-response data (Fig. 1 and c.f. Christopoulos (1998)).

To generate a theoretical curve we used the following form of the Hill equation:

$$P = \frac{P_0}{1 + 10^h \cdot (pCa - pCa_{50})}$$

The parameter values selected were based on the values obtained from the experimental data with $pCa_{50} = 5.7$, $P_0 = 25 \text{ mN/mm}^2$ and $h = 2.1$. The model was evaluated at the following pCa values: 6, 5.75, 5.5, 5.25, 4.75, 4.5, and 4.25, to obtain values for the force. The Monte Carlo simulation added random errors to the estimates of force and fit both model equations to obtain estimates for the pCa_{50} , EC_{50} and the Hill coefficient. The random errors were drawn from a normal distribution with mean of 0.00 and a standard deviation of 7% of P_0). This was repeated 1,000 times to obtain distributions for the fitted parameters.

Fig. 1 This figure describes the steps used to construct the Monte Carlo simulation used to model the distribution of EC_{50} and pCa_{50} in fits of a Hill equation to the data. The figure was adapted from Christopoulos (1998)



Note that the intent of this simulation was to examine how typical experimental errors affected the distribution of fitted values EC_{50} and pCa_{50} values and not to reproduce the variance of the original data set.

Statistical calculations

All statistics, including fitting of probability density functions, and Monte Carlo simulations were performed using the R statistical language version 2.8 (Ihaka and Gentleman 1996). Differences were held to be statistically significant at $P < 0.05$ (Fisher 1990).

Results

Thirty-nine experiments gave data that met the criteria of being within the set sarcomere length range of $2.2 \pm 0.1 \mu\text{m}$ and less than 20% rundown in maximum force.

In each experiment, the data were well fit by both forms of the equation. The fits using Eq. 3 resulting in pCa_{50} values are shown in Fig. 2. Similarly good fits were obtained for the hyperbolic form of the equation (Eq. 2) and for fits to the original Hill formulation (Eq. 1) (data not shown).

The distributions of the fitted EC_{50} values and the fitted pCa_{50} values are shown in Fig. 3. The histograms are density histograms where the y-axis displays the relative frequency density. The product of the dimensions of a individual bar is the relative frequency and the total area under the histogram is 1. This presentation allows direct comparison with other forms of the probability density function. The overlaid function (red line) in each plot is a probability density function fitted to the relative frequency data.

The EC_{50} distribution from the fit to the hyperbolic and the original forms of the Hill equation are skewed to the left (skewness = 0.72 for the hyperbolic form and 0.54 for the original Hill equation) while the pCa_{50} distribution is relatively symmetrical (skewness = 0.04). A quantile-quantile plot (QQ plot) permits comparison between two distributions by plotting quantiles of each of the two distributions on each axis (Cleveland 1994). Similarly shaped distributions lie along a straight line. In Fig. 3 the distributions of EC_{50} and pCa_{50} are compared to a normal distribution. As can be seen in Fig. 3 panels e and f, large values of EC_{50} tend to be more numerous than would be expected from a normal distribution. Figure 3, panel d, shows that the pCa_{50} distribution also has some moderate deviations from normality, with a few more values at the extremes than might be expected from a normal distribution.

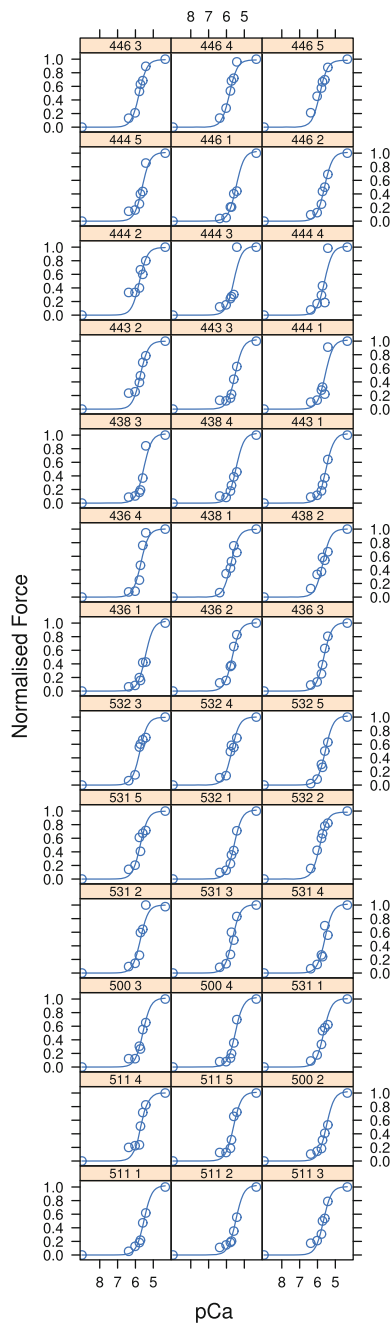


Fig. 2 The results of each experiment with the fitted force–pCa curve used to calculate the distribution of pCa₅₀. The curves were fitted to normalised force data. The number above each data set is the experiment number, a space and the myocyte number

The Shapiro–Wilk test can be used to detect strong departures from normality (Shapiro and Wilk 1965); the test did not show a significant departure from normality for pCa₅₀ ($W = 0.98$; $P = 0.76$) or for EC₅₀ values derived from Eq. 1 ($W = 0.96$; $P = 0.20$), but showed a borderline significant departure for EC₅₀ distributions from Eq. 2 ($W = 0.94$; $P = 0.06$). A log-normal distribution is one in which the logarithms of a variate are normally distributed

(Brown and Rothery 1993). Taking the log of the EC₅₀ values produced, as expected, a distribution very close to the pCa₅₀ distribution, reducing the skewness and showing a near normal distribution. This is consistent with EC₅₀ having a log-normal distribution.

The Hill coefficient distribution was also examined to see if there were significant departures from normality. The Hill coefficient distribution was the same regardless of the form of the Hill equation. The Hill coefficient distribution and the QQ plot are shown in Fig. 4. As can be seen the Hill coefficient is positively skewed (skewness = 1.26) and the Shapiro–Wilk (1965) test shows a strong departure from normality ($W = 0.89$; $P = 0.001$). This is not an artefact of the fitting equation since all forms of the Hill equation give the same distribution. A log transform of the Hill coefficient is shown on the right in Fig. 4 and is much less skewed (skewness = 0.32) and the Shapiro–Wilk test does not show a significant departure from normality ($W = 0.98$; $P = 0.70$), although the QQ plot shows that the agreement is not perfect. The log-transformed Hill coefficient is more suitable for use with parametric tests such as Student’s *t*-test and ANOVA than the untransformed value.

Monte Carlo simulations of force–pCa data and subsequent fits to that data were used to examine whether the distribution of the pCa₅₀, EC₅₀ and Hill coefficients were inherent to the form of the Hill equation, the fitting process or the data. All three equations were fit to 1,000 simulated datasets and the EC₅₀, pCa₅₀ and Hill parameters tabulated.

Surprisingly, the distribution of pCa₅₀ values from the simulated data were slightly negatively skewed (skewness = -0.21) and the EC₅₀ distributions were identical for both the hyperbolic and original Hill equation and were positively skewed (skewness = 0.61), although to a lesser extent than seen in the experimental data (Fig. 5). While both distributions showed significant differences from normality using the Shapiro–Wilk test ($P = 0.014$ and <0.001 for pCa₅₀ and EC₅₀ respectively), this is likely due to the large number of ‘data’ present revealing small but significant differences from normal that are, however, unlikely to affect conclusions drawn from tests that assume normality such as Student’s *t*-test. This suggests that the fitting data to the Hill equation produces EC₅₀ and pCa₅₀ distributions that have small to moderate departures from normality, pCa₅₀ being the less skewed of the two measure. It also shows that a simple model of normal variation in the measured force values for a fixed underlying description of the force as a function of calcium does not effectively reproduce the distribution seen in the experimental data from skinned cardiac myocytes.

The effect of redesigning the underlying experiment to use evenly spaced calcium concentrations to produce the force–pCa curve is shown in Fig. 6. The simulation used

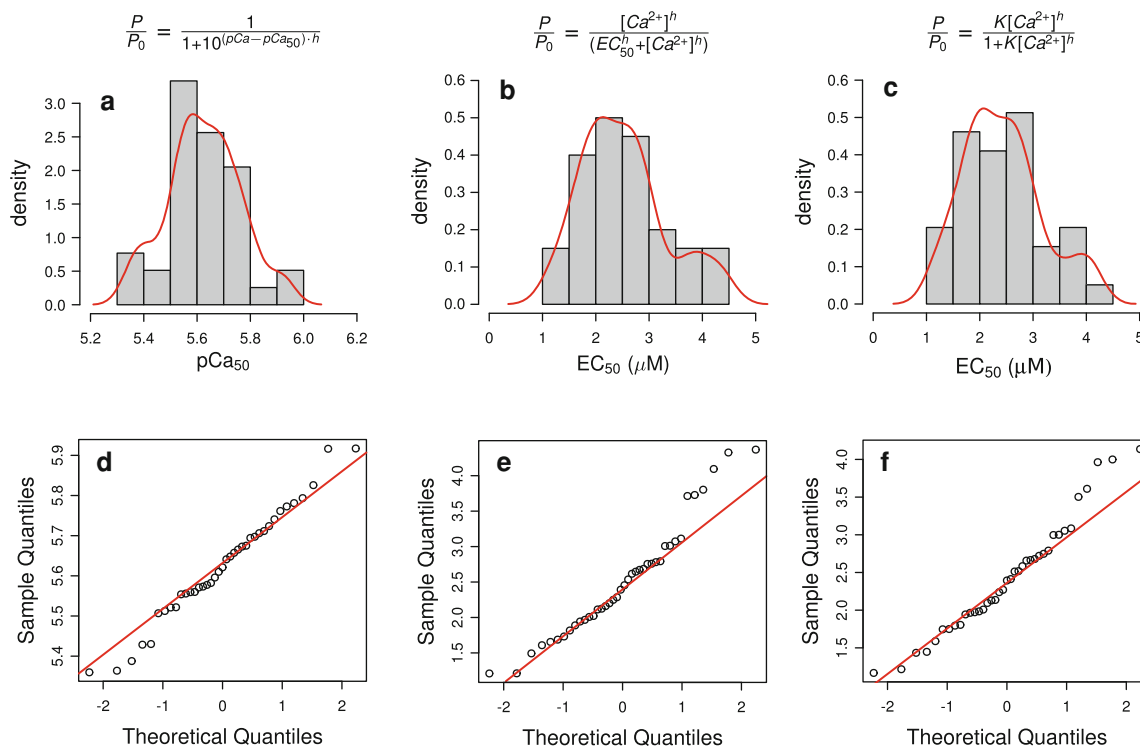
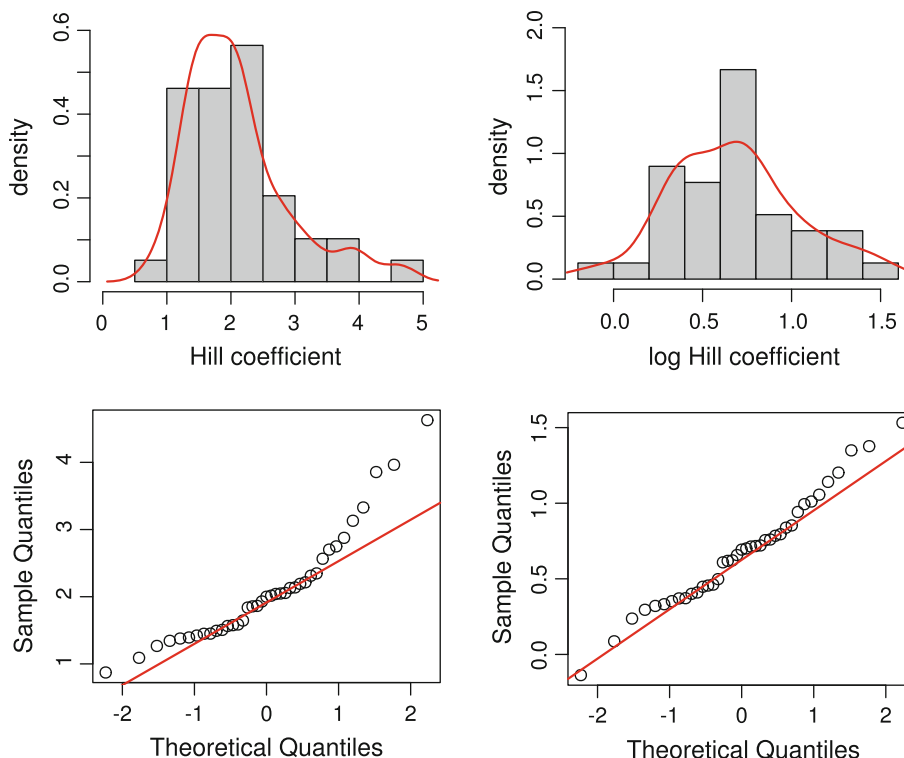


Fig. 3 Distribution of pCa_{50} and EC_{50} (in μM) from curves fitted to force–calcium data from 39 experiments. The equations used to fit the data are given in the *top row*. The *histograms* are density histograms where the *y-axis* displays the relative frequency density. The product of the dimensions of a individual bar is the relative frequency and the total area under the histogram is 1. **a** Density histogram and overlaid density function for pCa_{50} data. **b** Density histogram and overlaid

density function for EC_{50} from the same data as **(a)** fitted to Eq. 2. **c** Density histogram and overlaid density function for fit for EC_{50} values calculated from K and the Hill coefficient from fits of the original Hill equation to the experimental data. **d–f** QQ plots comparing distribution of pCa_{50} and EC_{50} data (*y-axis*) from **a–c** to a normal distribution (*x-axis*) in each case the *line* is drawn through the 1st and 3rd quartiles

Fig. 4 The distribution of the Hill coefficient from the fit of the data to the modified Hill equation. *Top left* A relative density histogram of the Hill coefficient values from 39 fitted curves. *Bottom left* A QQ plot of the Hill coefficient values against a normal distribution. The distribution is positively skewed. *Top right* A relative density histogram of the log-transformed Hill coefficients from fits to the experimental data and a probability density function overlaid on top of it. *Bottom right* A QQ plot of the Hill coefficient values against a normal distribution. The distribution of the log-transformed values is more symmetrical than the distribution of raw values and is likely to be sufficiently close to normal for *t*-tests and ANOVAs



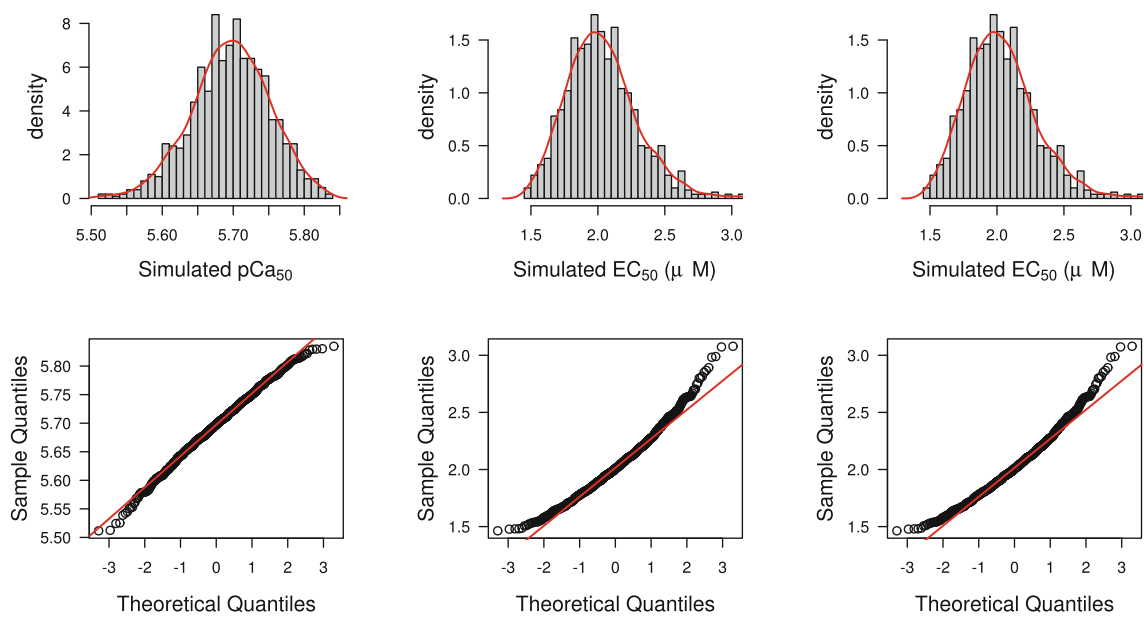


Fig. 5 The distribution of pCa_{50} (left) and EC_{50} (center and right) from fits to 1,000 simulated data sets. Represented as relative frequency histograms (upper row) and as QQ plots (lower row). The

distribution of pCa_{50} is slightly negatively skewed while the distribution of EC_{50} is slightly positively skewed

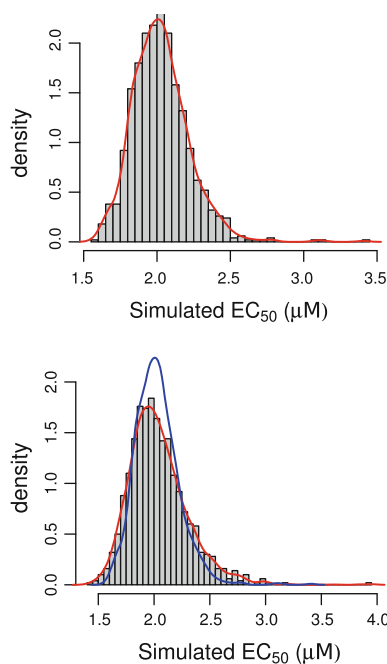


Fig. 6 Histograms of EC_{50} values when the calcium concentrations used to create the force–calcium curve are evenly spaced (top) or logarithmically spaced (bottom). Red lines on each panel indicate overlaid probability density functions. The blue line in the bottom panel indicates the probability density distribution represented in the top panel. The simulation used the same parameters, range of concentrations and number of data points except that the datapoints were evenly spaced in concentration for the top panel and logarithmically spaced in the bottom panel. The EC_{50} distribution from evenly spaced data points is marginally less skewed than the distribution from logarithmically spaced data points (Color figure online)

the same simple variance model as used to create Fig. 6 but with a standard deviation of 5% and used two different sequences of calcium concentrations; one was logarithmically spaced, the other was evenly spaced. Each sequence had 10 points and each covered the same concentration range (0.03–10 μM). Each data set was independently fit to the hyperbolic form of the Hill equation. The EC_{50} distribution for evenly spaced calcium concentrations had skewness of 1.08 while the logarithmically spaced calcium concentrations had a skewness of 1.12. This suggests that, all other factors being equal, the spacing of the data points has only a small affect on the distribution of the EC_{50} parameter.

The simulation studies also gave estimates of the Hill coefficient for each fit. The histogram shows a positively skewed distribution as seen in the experimental data (Fig. 7).

Discussion

The experimental data showed that the distribution of EC_{50} was approximately log-normal and that of pCa_{50} was approximately normal. The Shapiro–Wilk test did show a borderline significant ($0.1 > P > 0.05$) departure from normality for the EC_{50} distribution, however tests for normality should be viewed with some caution as they are relatively insensitive (Dyer 1974). More informative is the QQ plot showing a long right hand tail on the EC_{50} distribution and a more symmetric distribution for the pCa_{50} (Fig. 3).

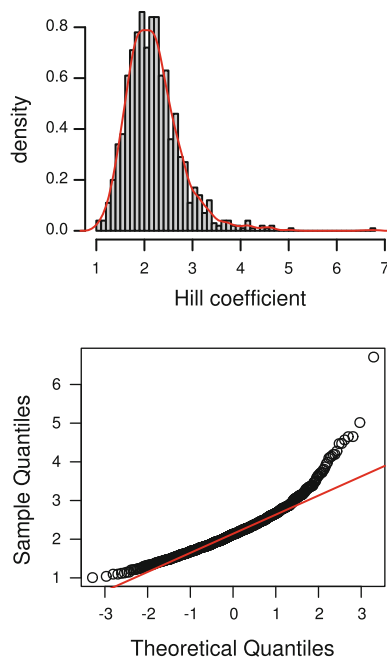


Fig. 7 Relative frequency histogram (*top*) and QQ plot (*bottom*) of Hill coefficient values from fits to 1,000 simulated datasets. The QQ-plot is against quantiles of the normal distribution

The Monte Carlo simulations with simple force variations for a fixed underlying model showed that some of the skew in the distribution of EC_{50} was an inherent property of the EC_{50} term and that pCa_{50} was more normally distributed. Using the original form of the Hill equation to calculate the EC_{50} values did reduce the amount of skew, and this was due to small differences in the fitted values of the parameters probably due to the slightly different weighting of data points under the different equations. The simulation also showed that the distribution of EC_{50} values was log-normal although to a lesser extent than that shown by the experimental data. This discrepancy was likely due to an overly simplistic model of the causes of variance in the data and we specifically ignored the role played by variance in the underlying pCa_{50}/EC_{50} values from animal to animal. A study of the role of different sources of variance in the distribution of fitted parameters in the Hill equation would be interesting and useful but is beyond the scope of this study.

The skew in the EC_{50} distribution was not greatly affected by the use of evenly spaced calcium concentrations instead of logarithmically spaced calcium concentrations. This suggests that the asymmetry is inherent to the use of a term such as EC_{50} in the Hill equation.

This suggests that the propagation of experimental errors through the Hill equation and into estimates of K , EC_{50} or pCa_{50} deserves a more thorough and systematic examination that includes the effect of the experimental design. Preliminary examination of these effects (data not

shown) suggests that the amount of skewness in the distribution of EC_{50} is greatly affected by both the variance function used to generate the simulated error, a uniform distribution produces more positive skew than a normal distribution, an error standard deviation proportional to the data point magnitude also produces more skew than an error with a standard deviation that is constant. Also important is the spacing of the independent variable, and the magnitude of the Hill coefficient, a lower value of the Hill coefficient produces more positive skewness than a higher value.

Both the experimental and the simulated data concur in that the distribution of EC_{50} is best approximated by a log-normal distribution and that the pCa_{50} is approximately normally distributed.

While the finding that EC_{50} has a log-normal distribution has not previously been well described in muscle, the finding that the parameter equivalent to K in the Hill equation has a log-normal distribution has been described previously in other fields. Fleming et al examined nor-adrenaline dose response curves in five different muscle preparation types, performing between 17 (guinea pig atrium) and 77 (rat vas deferens) experiments and found that the dose required to elicit 50% of the maximal response (ED_{50}) was positively skewed and that the $\log(ED_{50})$ was normally distributed (Fleming et al. 1972). This finding was extended and confirmed by DeLean et al using measures of radioligand binding and Monte Carlo based modelling of experimental data that showed that the geometric mean of half maximal doses was a more reliable estimator of affinity constants than the arithmetic mean (De Lean et al. 1982). Other simulations of the Hill equation in fields such as toxicology and pharmacology have also shown that the distribution of ED_{50} is log-normally distributed (Christopoulos 1998). Consequently, the use of logarithms of the K or ED_{50} values is routinely recommended in guides to fitting dose-response curves (e.g. Motulsky and Christopoulos 2005).

While several studies have found skewed distributions for EC_{50} or its equivalent, the amount of skew and the effect on tests of parameter significance, varies from study to study. This is because the skewness of the EC_{50} distribution is not a fixed property of the Hill equation but depends on the specific values of the Hill coefficient, the spacing of the independent variable and the type of variation seen in the experimental data. Therefore, each field using the Hill equation needs to examine the distribution of EC_{50} , or its equivalent, for combinations of typical values, to assess the amount of skew in the distribution.

Hill coefficient

The Hill coefficient is often used as an index of cooperativity based on the fact that when the Hill coefficient is 1,

the Hill equation reduces to the Langmuir isotherm which describes the adsorption of molecules to a surface as a function of the concentration of molecules above the surface. A Hill coefficient greater than one implies a positive cooperativity, that is the affinity of the remaining binding sites for ligand is increased by the binding of one ligand. A negative cooperativity implies the reverse, that binding of a ligand reduces the affinity of the remaining binding sites (Colquhoun 2006; Weiss 1997). While attempts have been made to assign a specific physical meaning to the Hill coefficient (Brandt et al. 1980), it should be kept in mind that the Hill equation is descriptive and not a mechanistic equation. This was the intent of Hill and has been recognised by pharmacologists (Christopoulos 1998; Colquhoun 2006) and some muscle physiologists (Shiner and Solaro 1984). While it may be interpreted as the minimum number of binding sites required by the functional contractile unit to explain an observed level of cooperativity, the Hill coefficient is best seen as an *index* of cooperativity rather than as a measure of a physical quantity such as numbers of ligands or binding sites (Shiner and Solaro 1984).

The Hill coefficient from the experimental data was not normally distributed when fit by either equation, although a log transform of the Hill coefficient was approximately normal. This was also found in the results of the Monte Carlo simulation. While little has been reported about experimental distribution of fitted Hill coefficients, non-normal distributions have been previously reported for Hill coefficients in *simulations* of drug binding studies (Christopoulos 1998). The marked deviation of the Hill coefficient from normality and the large reduction in skew of the log-transformed Hill coefficient suggests that log-transforms of the Hill coefficient should be used in statistical tests of simple differences in the value of the Hill coefficient. Similar recommendations have been made for the testing of Hill coefficient differences in drug–receptor assays (Christopoulos 1998; Motulsky and Christopoulos 2005).

EC₅₀ or pCa₅₀?

A common question in studies of skinned muscle is whether there is a difference in calcium sensitivity due to an underlying treatment. This is usually tested by use of Student's *t*-test for two conditions, treatment and control, and an ANOVA for more than two conditions. One of the assumptions of Student's *t*-test, as an example of a parametric test of significance, is that the data to be tested has a normal or near-normal distribution. This requirement would seem to favour the use of pCa₅₀ over EC₅₀ since the pCa₅₀ distribution is more nearly normal while the EC₅₀ is log-normal. But the EC₅₀ distribution shows only a moderate departure from normality and the *t*-test is very robust

to moderate departures from normality (Fisher 1990). If the distributions are similarly skewed, as might occur in comparisons of two EC₅₀ values, and the *n* in both groups of the two-sample *t*-test are approximately equal then the effect on the *t*-test is likely very small (Heeren and D'Agostino 1987; Box and Watson 1962). Therefore studies that have used EC₅₀ in *t*-tests and ANOVAs are unlikely to be invalid because of the moderate departure from normality of the EC₅₀ distribution, nor is there sufficient reason to discourage the use of tests of EC₅₀ values in future studies. If there is concern that a lack of normality may jeopardise conclusions then tests that do not rely on distributional assumptions, such as randomisation tests, can be used for either EC₅₀ or pCa₅₀ (Ludbrook 2000).

ΔEC₅₀ and ΔpCa₅₀

When looking at changes or differences in calcium sensitivity, there are additional considerations apart from statistical significance, and these can be best illustrated by example. Fuchs and Martyn (2005), in addition to calling for data regarding the distribution of pCa₅₀ and EC₅₀, also discussed the results reported by Konhilas et al. (2003) regarding changes in length dependent activation as a result of the exchange of ssTnI for cardiac TnI.

Konhilas et al. (2003) found that when slow skeletal TnI was exchanged for cardiac TnI in isolated mouse myocytes, the change in calcium sensitivity due to a change in sarcomere length from 1.95 to 2.25 differed when measured as ΔEC₅₀. The ΔEC₅₀ went from 0.41 with cardiac TnI to 0.26 with slow skeletal TnI, but when the calcium sensitivity was measured as ΔpCa₅₀ it did not seem to alter very much (0.12–0.13). Fuchs and Martyn (2005) asked, was the calcium sensitivity different or not? However, the data provided by Konhilas are not in fact incongruent. If two force–[Ca²⁺] curves are displaced relative to one another along the *x*-axis then ΔEC₅₀ will show how much calcium is required to be added to the lesser to match the greater. The ΔpCa₅₀ is, on the other hand, a multiplicative factor, i.e. it is how much you have to multiply one EC₅₀ to get to another EC₅₀. On the log scale of pCa this becomes addition. In Konhilas et al., the EC₅₀ at 1.95 μm was 1.72 μM, at 2.25 μm it was 1.31 μM giving the shift of 0.41 μM and a ratio of 10^{0.12} = 1.31 (from the delta pCa value). With slow skeletal troponin the starting EC₅₀ is different, as might be expected with a different troponin, with the EC₅₀ at 1.95 μm being 1.04 μM and at 2.25 μm being 0.78 μM. The difference in the amount of calcium required for 50% activation (ΔEC₅₀) is different at 0.26 μM, but the ratio of concentration change is nearly the same at 10^{0.13} = 1.34 as with cardiac troponin. The questions of whether the calcium sensitivity is different and which measure is correct, are perhaps poorly posed ones in this instance. The data are

consistent with two troponins having different underlying affinities for calcium, hence the dissimilar ΔEC_{50} values, and a length dependent effect that proportionately reduces calcium sensitivity regardless of the troponin present hence the similar ΔpCa_{50} values. This is only a single finding in a broader set of experiments and is discussed here only as an exercise in the understanding of the Hill equation and not as a reinterpretation of the complete set of experiments of Konhilas et al. (2003).

The calcium sensitivity is a descriptive index that can help to summarise an experiment and is not a measure of a physical property of the underlying system. EC_{50} and pCa_{50} can each be used to describe calcium sensitivity, and changes in calcium sensitivity, so long as we keep in mind the mathematical basis of each measure and the limitations of curve fitting as opposed to model building in describing the results of an experiment.

Conclusion

Based on the empirical and theoretical distributions presented here, either EC_{50} or pCa_{50} may be used to compare treatment effects on calcium sensitivity using Student's *t*-test or ANOVA provided the numbers in each group are approximately equal. The pCa_{50} is marginally preferred given that it is more like a normal distribution and it should be the form used if numbers in treatment groups are unequal. If EC_{50} is used for comparisons with unequal numbers in each group then randomisation tests would be more appropriate than the standard parametric tests.

Beyond significance testing it should be kept in mind that point estimates of EC_{50} and pCa_{50} provide the same information related by a simple log transformation. When interpreting changes in pCa_{50} or EC_{50} it should be kept in mind that, when expressed as deltas, one represents a multiplicative factor and the other an additive factor, and that both should be considered in the interpretation of any given experiment.

The distribution of the Hill coefficient had marked departures from normality in both the experimental and simulation studies and on this basis we recommend that significance testing be performed on log-transformed Hill coefficients rather than the raw Hill coefficient.

As previously noted, the Hill equation does not describe any physically realizable reaction scheme and is useful only to describe and summarise simple dose-response experiments. It may well be that the Hill equation has outlived its usefulness as a descriptive equation and the use of calcium sensitivity as a summary measure is obstructing further inquiry into the underlying mechanisms of action. For example the phosphorylation of sarcomeric proteins is often described as having altered calcium sensitivity, however

with multiple contributing processes such as calcium affinity, transduction of the calcium binding into molecular rearrangement of the troponin molecule, alteration of the equilibrium position of troponin and altered energetic requirements for strong binding of myosin to actin, there are many ways in which calcium sensitivity could be altered and many possible ways in which the changes in calcium sensitivity can interact beyond sums or ratios of EC_{50} or pCa_{50} . Using more realistic models of calcium/troponin/tropomyosin/actomyosin interaction may aid the interpretation of such changes and further our understanding of muscle contraction.

Acknowledgements We would like to thank Prof. Pieter de Tombe for his helpful discussions. This work was supported in part by NIH grants HL62426 and HL077195.

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