Protein and Acidosis Alter Calcium-Binding and Fluorescence Spectra of the Calcium Indicator Indo-1

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ABSTRACT The fluorescent indicator indo-1 is widely used to monitor intracellular calcium concentration. However, quantitation is limited by uncertain effects of the intracellular environment on indicator properties. The goal of this study was to determine the effects of protein and acidosis on the fluorescence spectra and calcium dissociation constant (K_d) of indo-1. With 350 nm excitation light, the ratio of indo-1 fluorescence in the absence versus the presence of saturating Ca^{2+} at wavelength λ (S_λ) and K_d increased with [protein]. At pH 7.3, K_d, S_{400}, and S_{470}, which were 210 nM, 0.033, and 1.433 in the absence of protein, increased to 808 nM, 0.161, and 2.641, respectively, by adding proteins from frog muscle and to 638 nM, 0.304, and 3.039, respectively, by adding proteins from rat heart. Effects of protein on indo-1 fluorescence were reduced at higher [indo-1]. Acidosis (pH 6.3) had separate effects, which were additive to those of protein: in the absence of protein, acidosis increased K_d to 640 nM; frog muscle proteins further increased K_d to 1700 nM. Acidosis also changed S_λ slightly. In summary, interaction with protein or protons alters indo-1 calcium-binding and fluorescence. These findings are consistent with several previous studies and suggest that indo-1 calibration constants need to be derived in the presence of appropriate types of protein, ratio of [indo-1]/[protein], and pH.

INTRODUCTION

Indo-1 is widely used to monitor intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$); however, quantitation of $[Ca^{2+}]_i$ is hampered by uncertain effects of intracellular milieu on the response of indo-1 to calcium. For example, although it is becoming increasingly recognized that indo-1 fluorescence spectra and the calcium dissociation constant (K_d) may be altered by interactions with intracellular proteins, or by changes of intracellular pH, uncertainty still exists on the magnitude of these changes.

Several studies suggest that indo-1 fluorescence spectra are altered by interaction with protein. Indo-1 fluorescence spectra were blue-shifted in permeabilized myocytes compared with protein-free solution (Hove-Madsen and Bers, 1992); these effects were partly attributed to light absorption by the sample and also to indo-1 interaction with protein (Hove-Madsen and Bers, 1992). The fluorescence spectrum of calcium free indo-1 was blue-shifted by albumin or protein mixtures; however, the spectrum of the calcium-bound species was little affected (Brandes et al., 1993). Finally, the ratio of indo-1 fluorescence at two wavelengths in the absence of calcium (R_{min}) was increased by a factor of seven in-vivo compared with in-vitro; however, the ratio with saturating calcium (R_{max}) was unaltered (Westerblad and Allen, 1993). Similar to the report of Brandes et al. (1993), this finding may also be caused by interaction of indo-1 with cell protein causing a blue-shift in the fluorescence of calcium-free- but not calcium-bound indo-1.

Several studies have suggested that indo-1 K_d for calcium is not altered in the cell compared with protein-free solution (Spurgeon et al. 1990; Ikenouchi et al., 1991; Westerblad and Allen, 1993), whereas others have suggested that K_d increases in the cell by up to a factor of five (Hove-Madsen and Bers, 1992). A similar uncertainty exists concerning the effects of cell protein on the K_d of the related calcium indicator fura-2: intracellular milieu was suggested to have little effect on fura-2 K_d for calcium (Backx and Ter Keurs, 1993), whereas others found fura-2 K_d increased by a factor of three to four in the presence of protein solutions (Konishi et al., 1988). Recently, the K_d of two other tetracarboxylate indicators (fura-red and fluo-3) were found to increase in the presence of proteins (Kurebayashi et al., 1993; Harkins et al., 1993).

Significant decreases in intracellular pH may occur in tissue during a variety of conditions such as skeletal muscle fatigue or during myocardial ischemia. However, the effects of decreased pH on indo-1 properties has not been studied in detail: the effect of changes of pH on indo-1 fluorescence is little known (Lee et al., 1988); furthermore, no studies have examined the combined effects of protein and acidosis on fluorescence and K_d. In-vitro, and in the absence of protein, a decline in pH from pH 7.0 to 6.0 caused indo-1 K_d to increase from 190 to 530 nM (Lattanzio, 1990). Over a narrower range of proton concentration, the K_d of indo-1 increased slightly from 180 to 240 nM with a decline in pH from 7.9 to 6.8 (Westerblad and Allen, 1993).

Because of the uncertain effects of proteins and pH on indo-1, and the importance of indo-1 as a tool for monitoring $[Ca^{2+}]_i$, the goal of this study was to determine the effects of protons and proteins (from both frog skeletal muscle or rat

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heart) on indo-1 K_d for calcium and indo-1 fluorescence spectra. We used conditions relevant to our in-vivo studies (Brandes et al., 1993; Baker et al., 1993, 1994); however, generalizations for other studies may be made concerning the effects of proteins and protons on indo-1.

The present studies found that proteins and protons had separate and additive effects on indo-1 K_d for calcium and fluorescence spectra. Calcium calibration constants used to derive $[Ca^{2+}]_i$ from indo-1 fluorescence, therefore, were significantly altered by [protein] and changes of pH.

MATERIALS AND METHODS

Animal methods

Frog

Bullfrogs (*Rana catesbeiana*) (length ≈ 6 inches), $n = 5$, were killed by double pithing. Thigh musculature was removed to prepare muscle homogenates ($n = 3$). To record calcium signals during contraction, the ventral head of the semitendinosus muscle was removed and loaded with indo-1 by arterial perfusion with indo-1-AM (acetoxymethyl ester) (Molecular Probes, Eugene, OR) as previously described (Baker et al., 1993) ($n = 2$).

Rat

Male Sprague-Dawley rats (400–500 g), $n = 20$, were anticoagulated with heparin (1 ml i.p.) and anesthetized with ketamine (1 ml i.p.). Hearts were excised and arrested in cold isosmotic saline containing 30 mM KCl. Isolated hearts were briefly perfused retrograde with a modified Krebs-Heinseleit (Brandes et al., 1993) perfusate to remove ventricular blood, and the left ventricle was isolated and homogenized.

Preparation of protein extracts from tissue homogenates

Frog muscle

In three separate preparations, ≈50 g of thigh musculature from each frog was homogenized over ice using a Polytron blender (Brinkman Instruments, Ontario, Canada) in 65 ml of buffer containing 80 mM KCl, 10 mM PIPES, and 2 mM NaN_3 to inhibit bacterial growth: in two preparations the pH of the buffer was adjusted to 7.3, and in the other to pH 6.3. pH adjustment required ≈20 mM KOH; thus, total $[K^+]$ in the buffers was ≈100 mM. The protease inhibitor phenylmethylsulfonyl fluoride (0.2 mM) was added to the homogenate. The homogenate was centrifuged at 100,000 × *g* for 1 h at 4°C, which yielded ≈75 ml of supernatant ([protein] ≈ 15 mg/ml), which was lyophilized. The dried protein powder was resuspended in 10 ml of the PIPES buffer (see above), sealed in dialysis tubing (which retained protein with a molecular weight >12,000; Sigma Chemical Co, St. Louis, MO) and exhaustively dialyzed at 4°C against 400 ml of the PIPES buffer for 2.5 days with a solution change every 12 h. In two preparations, protein extracts were dialyzed against buffer at pH 7.3, and in one preparation proteins were dialyzed against buffer at pH 6.3. [protein] was measured spectrophotometrically using a Bradford assay. The pH of the protein extract was readjusted to pH 7.3 or 6.3. Finally, to remove any precipitate that formed during dialysis, the protein extract was centrifuged at 3000 × *g* for 10 min at 4°C. Yield was ≈10 ml with 80 mg/ml [protein]. The protein extract was kept on ice and used within 3 days (freezing caused some denaturation).

Rat heart

≈25 g of rat left ventricle from a total of 20 rat hearts was homogenized over ice in 125 ml of PIPES buffer (pH 7.3) (see above). Thereafter, preparation of proteins was similar to that described above. After lyophilization, powder was resuspended in 6.5 ml of buffer; after dialysis (see above), final [protein] was adjusted to 80 mg/ml by addition of buffer.

Measurement of indo-1 fluorescence and K_d

Instrumentation

Fluorescence was measured using an SLM 48000S spectrofluorometer (SLM instruments, Urbana, IL). Light from a 450 W xenon arclamp was reflected from a dichroic reflector (Oriel, Stratford, CT) that reflected ultraviolet light (340–450 nm) passed through an infra-red absorption filter (absorbing >750 nm) (Schott KG-1, Duryea, PA), filtered at 350 or 360 nm with interference filters (10-nm bandwidth) (Corion, Holliston, MA) and passed through a quartz fiber optical cable (Dolan-Jenner Industries, Woburn, MA), and focused onto a cuvette containing 2 ml of sample plus indo-1 free acid (12 μM, Molecular Probes). Fluorescent light, collected perpendicular to the illumination, was collected above 380 nm using a high pass filter (Schott, KV 380; to absorb scattered excitation light), and the intensity was measured with an emission monochromator at wavelengths between 360 and 640 nm with the bandwidth set to 1 nm.

Measurement protocol

Free $[Ca^{2+}]$ was controlled by varying the ratio of EGTA to CaEGTA, from which the resultant free $[Ca^{2+}]$ could be calculated after accounting for the effect of pH on the calcium dissociation constant of EGTA. Using the method of Tsien and Pozzan (1989), the K_d of EGTA for calcium was calculated to be 95.4 nM at pH 7.3 and 9.35 μM at pH 6.3 and 20°C. The adequacy of calcium-buffering was verified by use of different concentrations of total EGTA, which gave comparable results.

Protein extract containing indo-1 free acid (12 μM) was divided into two samples; EGTA (10 mM) was added to one (zero calcium), and CaEGTA (10 mM) was added to the other (high calcium). The free $[Ca^{2+}]$ of the high calcium sample was sufficient to saturate indo-1 as verified by an unchanged Ca^{2+}-bound indo-1 emission spectrum after addition of excess $CaCl_2$. EGTA and CaEGTA were from 100 mM stock solutions made up in 100 mM KCl (Molecular Probes) and adjusted to pH 7.3 or 6.3. After recording the emission spectrum of the zero calcium sample, free $[Ca^{2+}]$ was progressively increased by successive removal of aliquots of the zero calcium sample and replacement with equal volumes of the high calcium sample, with spectra recorded at each $[Ca^{2+}]$. All indo-1 fluorescence spectra were corrected for the influence of protein and/or instrumental background fluorescence by subtraction, at each [protein], of a spectrum recorded in the absence of indo-1.

Data analysis

Indo-1 fluorescence emission spectra obtained in protein-free solution in the absence of Ca^{2+} and with saturating Ca^{2+} are shown in Fig. 1 *A*. The ratio of these spectra gives the calibration parameter *S* as a function of wavelength (Grynkiewicz et al., 1985). At emission wavelengths below the isosbestic wavelength (λ_{iso}, at $S = 1$), indo-1 fluorescence intensity increases with increased $[Ca^{2+}]$ and vice versa.

With fluorescence measured in-vivo at wavelengths λ1 and λ2, the *S* values at these wavelengths, $S_{\lambda 1}$ and $S_{\lambda 2}$, would be required to calculate $[Ca^{2+}]_i$ (Brandes et al., 1993) (see below). Different investigators use different λ1 and λ2; in this study, the effect of [protein] on *S* values was determined at wavelengths that were used to measure indo-1 in-vivo fluorescence in this laboratory. However, the effects of [protein] on fluorescence can be generalized qualitatively to all wavelengths.

K_d was determined by fitting K_d to the relationship between the fluorescence ratio (*R*; measured using emission at 400 and 470 nm) and $[Ca^{2+}]$ according to Eq. 1 (Grynkiewicz et al., 1985):

$$[Ca^{2+}]^N = K_d \cdot S_{470} \cdot (R - R_{min})/(R_{max} - R), \quad (1)$$

where R_{min} and R_{max} are the ratios of fluorescence (at 400 and 470 nm)

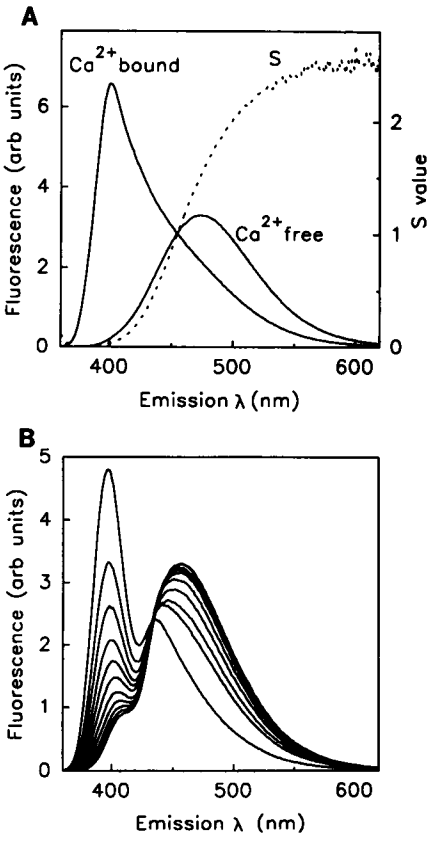

FIGURE 1 (*A*) Emission spectra of Ca^{2+}-free and Ca^{2+}-bound indo-1 in the absence of protein (350-nm excitation). The ratio of Ca^{2+}-free/Ca^{2+}-bound spectra yields the spectrum of *S* values (Grynkiewicz et al., 1985). (*B*) Indo-1 emission spectra obtained in the presence of frog muscle proteins (12 mg/ml) at different [Ca^{2+}] (different experiment than *A*). Inflections in the spectra occurred around 420 nm because of light absorption by the sample.

obtained at zero and saturating [Ca^{2+}], respectively, and *N* is the number of Ca^{2+} binding to indo-1.

K_d and *S* values considerably increased with [protein], whereas λ_{iso} decreased. These parameters were fit to a simple binding relationship described by

$$Y = Y_0 + \Delta Y_{sat} \cdot [\text{protein}]/([\text{protein}] + P_{50}), \quad (2)$$

where *Y* is the parameter value (of K_d, *S* or λ_{iso}) at a particular [protein], Y_0 is the value measured in the absence of protein, ΔY_{sat} is the maximum change in *Y* extrapolated to saturating [protein], and P_{50} is the [protein] causing a 50% change in *Y*.

In-vivo measurements

Methods used to monitor $[Ca^{2+}]_i$ in frog skeletal muscle have been described in detail (Baker et al., 1993).

Calibration of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined from the fluorescence ratio *R* measured in-vivo at wavelengths λ_1 and λ_2 using Eq. 1 and calculating R_{max} and R_{min} according to

$$R_{max} = S_R/bH \quad (3a)$$

$$R_{min} = R_{max} \cdot S_{\lambda 1}/S_{\lambda 2}, \quad (3b)$$

where $S_R = (1 - S_{\lambda 2})/(1 - S_{\lambda 1})$. The values of $S_{\lambda 1}$ and $S_{\lambda 2}$ were taken from the in-vitro calibrations, whereas *bH*, defined as the slope of the relationship between fluorescence intensities at wavelengths λ2 vs. λ1 (Brandes et al., 1993), was obtained from the in-vivo measurements (see Results).

Calculations and statistics

Pooled data are expressed as means ± SE. Tests of significance used a significance level of $p < 0.05$. Curve-fitting was performed using the nonlinear least-squares (Marquardt-Levenberg) method (Sigmaplot 4.1, Jandel Scientific, Corte Madera, CA).

RESULTS

Effect of proteins on indo-1 emission spectra

Fig. 1 *A* shows indo-1 emission spectra obtained with zero and saturating [Ca^{2+}] in the absence of protein. From a different experiment, Fig. 1 *B* shows superimposed indo-1 spectra obtained in the presence of proteins from frog muscle (12 mg/ml [protein]) at differing [Ca^{2+}], ranging from zero- to saturating. The presence of the protein extract caused several changes in indo-1 emission spectra compared with spectra obtained in the absence of protein (Fig. 1 *A*). Protein caused a reduction of fluorescence intensity centered in the region of 410–423 nm. As previously suggested (Fralix et al., 1990), an intensity reduction in this region is most likely caused by absorption of light by hemoglobin or myoglobin. Light absorption by proteins from frog muscle (which lacks myoglobin) likely arose from hemoglobin from blood that had been in the muscle vasculature. The presence of an inner filter effect was confirmed in the absorption spectrum of the protein extract, which showed increased absorption centered at 412 nm (data not shown). Using protein extracts from rat heart, there was also substantial filtering of light from both hemoglobin and myoglobin.

Light absorption by the protein extract caused changes in indo-1 emission spectra relative to emission spectra in the absence of protein. There was a decrease in the intensity of the spectrum of Ca^{2+}-bound indo-1 relative to the spectrum of Ca^{2+}-free indo-1. Furthermore, there was a slight blue-shift in the intensity of the spectrum of Ca^{2+}-bound indo-1, where the emission maximum moved from 401 nm in the absence of protein (Fig. 1 *A*) to 397 nm in the presence of protein (Fig. 1 *B*). Similar changes were previously observed using permeabilized myocytes (Hove-Madsen and Bers, 1992).

The presence of the protein extract also caused a large blue-shift of the spectrum of Ca^{2+}-free indo-1, where the emission maximum moved from 474 nm in the absence of protein (Fig. 1 *A*) to 458 nm in the presence of protein (Fig. 1 *B*). These changes were similar to those found with purified proteins (Brandes et al., 1993).

The presence of an inner filter does not easily allow the effects of protein on the emission spectra to be discerned separately from the effects of absorption. However, apparent changes in the spectra caused by light absorption by the protein extract do not represent real changes in calcium-sensitive fluorescence and, thus, will not affect either λ_{iso} or *S* values (Brandes et al., 1993).

In the presence of protein, λ_{iso} decreased and S values increased (see below), which indicates that protein caused real changes in Ca^{2+}-sensitive indo-1 fluorescence, as previously reported for purified protein (Brandes et al., 1993).

Effect of protein and acidosis on indo-1 fluorescence

Fig. 2 *A* shows examples of spectra of S values recorded in the presence of frog muscle proteins (pH 7.3) at several different [protein]. The S spectra were elevated by increased [protein]; in consequence, λ_{iso} (defined at $S = 1$) decreased. Fig. 2 *B* shows the relationship between λ_{iso} and [protein] for frog muscle proteins (at pH 7.3 and 6.3) and rat heart proteins (pH 7.3). λ_{iso} decreased with increased [protein] from frog muscle or from rat heart. Decreases of λ_{iso} were well described by Eq. 2. The maximum decrease of λ_{iso} was similar under all three conditions (range -18.2 to -24.3 nm); however, P_{50} calculated to each fit (equivalent to the [protein] causing a 50% change) was larger ($p < 0.05$) for rat heart proteins (8.5 ± 1.9 mg/ml) than for frog muscle proteins at pH 7.3 (2.95 ± 0.66 mg/ml), and was lowest ($p < 0.05$) with acidosis (0.89 ± 0.24 mg/ml).

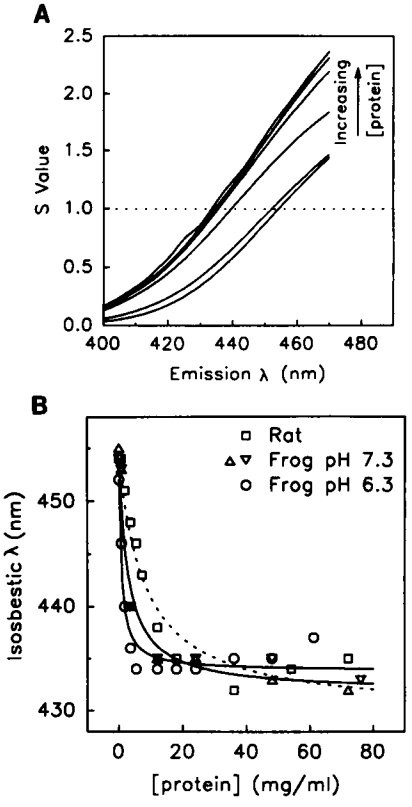

FIGURE 2 (*A*) S values versus emission wavelength for samples containing no protein (lowest curve) and with increasing [protein] up to a maximum of 72 mg/ml (350-nm excitation). Protein caused an increase in S values at all wavelengths and a decrease in the isosbestic wavelength (at $S = 1$). (*B*) Relationship between isosbestic wavelength and [protein] for proteins from frog muscle at pH 7.3 (△, ▽), pH 6.3 (○), and from rat heart (pH 7.3) (□). [Ca^{2+}] was controlled by 10 mM total [EGTA] in all experiments except one series where 5 mM total [EGTA] (▽) was used (see text).

The relationships between S measured at 400 and 470 nm (S_{400} and S_{470}), using frog muscle protein are shown in Fig. 3, *A* and *B*. These wavelengths were used to monitor $[Ca^{2+}]_i$ in-vivo from frog muscle (Baker et al., 1993, 1994). Both S_{400} and S_{470} increased with [protein] (pH 7.3, *triangles*; pH 6.3, *circles*); appearing to saturate at high [protein] as described by Eq. 2.

Acidosis resulted in an increase of S_{400} both in the absence and presence of protein (Fig. 3 *A*). Although the absolute increase of S_{400} upon addition of protein was not changed with acidosis ($p > 0.05$), S_{400} reached higher values with acidosis ($p < 0.01$). Furthermore, P_{50} was lower at pH 6.3 than at pH 7.3 (0.64 ± 0.16 mg/ml vs. 2.01 ± 0.37 mg/ml, $p < 0.01$). In contrast, acidosis did not appreciably affect the relationship between S_{470} and [protein] (Fig. 3 *B*).

The results of fitting λ_{iso}, S values, and K_d (see below) versus [protein] to Eq. 2 for proteins from frog muscle and rat heart are summarized in Table 1. Proteins from rat heart had different effects on indo-1 fluorescence spectra compared with those from frog muscle: both S and P_{50} were considerably higher ($p < 0.01$).

The changes of indo-1 fluorescence spectral properties with [protein] are consistent with indo-1 binding to protein. This suggests that the ratio of [indo-1] to [protein] may determine changes of fluorescence. To examine this possibility, the effects of frog muscle protein on indo-1 fluorescence

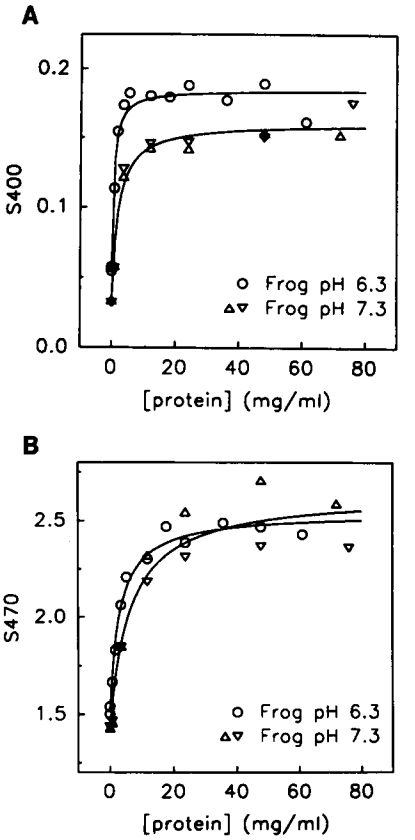

FIGURE 3 Relationships between S values and [protein] for frog muscle proteins (350-nm excitation). S values were determined at emission wavelengths of (*A*) 400 nm and (*B*) 470 nm. Symbols as for Fig. 2.

TABLE 1 Effect of protein on indo-1 K_d and fluorescence spectra

	ΔY_{sat}	P_{50} (mg/ml)	Y_0
Frog muscle pH 7.3			
K_d	598 nM (26)$^{**}_{*}$	3.52 (0.73)$^{ns}_{ns}$	210 nM
S_{400}	0.128 (0.004)$^{ns}_{**}$	2.01 (0.37)$^{**}_{**}$	0.033
S_{470}	1.208 (0.083)$^{ns}_{ns}$	6.49 (1.88)$^{ns}_{*}$	1.433
λ_{iso}	−22.2 nm (0.93)$^{*}_{ns}$	2.95 (0.66)$^{*}_{*}$	454 nm
	mean P_{50} (n = 4) = 3.74 (0.97)$^{ns}_{**}$		
Frog muscle pH 6.3			
K_d	1079 nM (60)$_{**}$	2.29 (0.65)$_{*}$	641 nM
S_{400}	0.129 (0.004)$_{**}$	0.64 (0.16)$_{**}$	0.056
S_{470}	1.021 (0.029)$_{**}$	3.28 (0.44)$_{**}$	1.520
λ_{iso}	−18.2 nm (0.71)$_{*}$	0.89 (0.24)$_{**}$	452 nm
	mean P_{50} (n = 4) = 1.78 (0.62)$_{**}$		
Rat heart pH 7.3			
K_d	404 nM (72)	19.9 (8.9)	234 nM
S_{400}	0.273 (0.019)	14.9 (2.3)	0.031
S_{470}	1.580 (0.172)	22.1 (5.9)	1.459
λ_{iso}	−24.3 nm (1.78)	8.5 (1.9)	454 nm
	mean P_{50} (n = 4) = 16.35 (3.02)		

Values of K_d, S values, and λ_{iso} measured (using 12 μM indo-1) as a function of [protein] (from frog or rat) were fit to Eq. 2. The table shows the calculated fit values ± SE.

Statistics: parameters were compared among the three preparations from their confidence intervals, ** ($p < 0.01$), * ($p < 0.05$), ns (not significant); superscript (comparison with frog at pH 6.3), subscript (comparison with rat). Mean P_{50} of all four parameters were compared among protein solutions using a t-test.

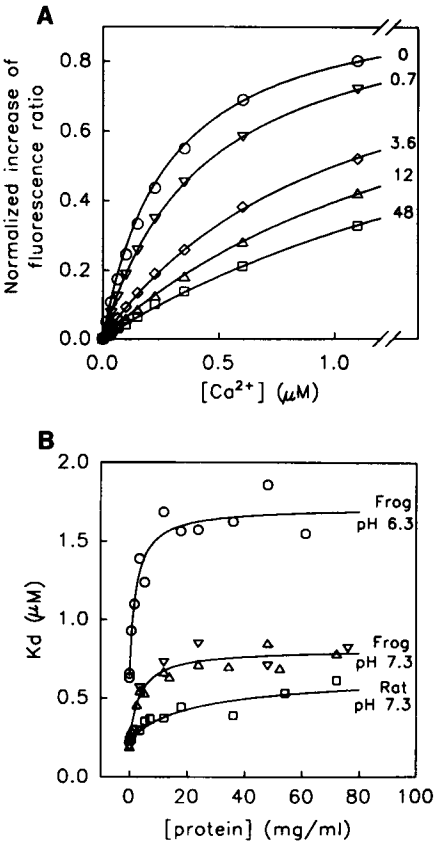

FIGURE 4 (*A*) Relationship between the normalized increase in fluorescence ratio and [Ca^{2+}] for indo-1 in the presence of different frog muscle [protein] (shown in mg/ml next to each curve) (pH 7.3, different symbol for each [protein]). Measures of the ratio of fluorescence (R) at 400 and 470 nm at different [Ca^{2+}] were fit to Eq. 1 (using $N = 1$) to determine K_d. Data and fits were normalized to allow comparison between experiments (R was normalized to $(R - R_{min})/(R_{max} - R_{min})$, where R_{min} and R_{max} were the ratios measured in zero calcium and saturating calcium solutions, respectively). Increased indo-1 K_d for Ca^{2+} with increased [protein] is evidenced by the shallower curves at higher [protein]. (*B*) Relationship between K_d and [protein] for proteins from frog muscle and rat heart. Symbols as for Fig. 2.

were compared at low and high [indo-1]. At [protein] = 3.6 mg/ml and [indo-1] = 12 μM, values for λ_{iso}, S_{400}, and S_{470} (439 nm, 0.132, and 1.914, respectively) were close to those expected from Eq. 2 and the data given in Table 1 (i.e. 441 nm, 0.115, and 1.864, respectively). However, at [protein] = 3.6 mg/ml and [indo-1] = 48 μM, values for λ_{iso}, S_{400}, and S_{470} (455 nm, 0.067, and 1.308, respectively) were closer to those found in protein-free solution (i.e., 454, 0.033, and 1.433, respectively). Thus, the reduced effects of protein on indo-1 K_d and fluorescence spectra observed at high [indo-1] may result from a lower fraction of indo-1 bound to protein.

Effect of protein and acidosis on indo-1 calcium binding

The dissociation constant (K_d) of indo-1 for calcium was determined from measurements of the ratio of fluorescence (R) (measured at 400 and 470 nm) versus [Ca^{2+}]. Data were fit to Eq. 1 to determine K_d (see Materials and Methods). Fig. 4 *A* shows examples of the relationship between the normalized increase of R and [Ca^{2+}] at several different frog muscle [protein] (pH 7.3). Data and fitted values of R were normalized as $(R - R_{min})/(R_{max} - R_{min})$, where R_{min} and R_{max} were the ratios measured in zero calcium and saturating calcium solutions, respectively. This normalization was to allow data and fits from experiments at different [protein] to be compared on the same graph. Fig. 4 *A* shows that at each [protein] the data were well described by Eq. 1. The figure also shows that the apparent K_d (i.e., true $K_d \cdot S_{470}$), equal to the [Ca^{2+}] where the normalized increase of $R = 0.5$, was increased with [protein]. The apparent K_d and S_{470} increased from 259 nM and 1.34 in the absence of protein to 2239 nM and 2.7, respectively, at the highest protein shown (48 mg/ml). These changes represented a change in true K_d from 193 to 828 nM. The stoichiometry of indo-1 binding to Ca^{2+} (as assessed by N, in Eq. 1) did not vary significantly from 1:1 binding ($p > 0.05$).

The effects of [protein] and acidosis on K_d are shown for all experiments in Fig. 4 *B* and are summarized in Table 1. Proteins from both frog muscle and rat heart significantly increased indo-1 K_d for calcium. At pH 7.3, the K_d at saturating [protein] was greater ($p < 0.05$) for frog muscle proteins (808 ± 26 nM) than for rat heart proteins (638 ± 72 nM) (values from Table 1). At pH 6.3, and in the absence of protein, K_d was 641 nM; addition of frog muscle proteins increased K_d to a maximum of 1720 ± 60 nM. Thus, K_d was increased by acidosis in the absence of protein; and K_d was increased by frog muscle protein in the absence of acidosis. In addition, the maximum K_d obtained using protein increased further in the presence of acidosis. These findings

suggests that protein and protons caused increased K_d through separate mechanisms.

Fig. 4 *B* also shows that similar results were obtained using frog muscle proteins when $[Ca^{2+}]$ was controlled with either 10 mM total [EGTA] (*triangles*) or 5 mM total [EGTA] (*inverted triangles*). Using 10 mM total [EGTA], the K_d of indo-1 calculated for saturating [protein] (791 ± 26 nM) was not different ($p > 0.05$) from that found using 5 mM total [EGTA] (837 ± 50 nM). This suggests that $[Ca^{2+}]$ was adequately buffered during the measurements.

Determination of in-vivo calibration values

Intracellular [protein] is reported to be ≈100 mg/ml (Konishi et al., 1988; Hove-Madsen and Bers, 1992). However, appropriate calibration constants cannot be simply derived from in-vitro measurements extrapolated to this [protein] because, as suggested above, the extent of indo-1 interaction with intracellular proteins may depend on the relative [protein] versus [indo-1]. In the present studies, determination of the appropriate [protein] (at a fixed [indo-1]) to derive calibration constants relied on matching measurable indo-1 spectral parameters in-vitro and in-vivo.

Frog muscle

λ_{iso} was determined in-vivo, and calibration constants were derived in-vitro at a [protein] that resulted in a similar λ_{iso} (see Brandes et al., 1993).

Fig. 5 *A* shows superimposed in-vivo recordings of indo-1 fluorescence transients elicited by twitch stimulation (each record is the average of 6 twitches). Transients were recorded at different emission wavelengths ranging between 400 and 440 nm. As expected from the indo-1 emission spectra, the amplitude of fluorescence change was largest at low wavelengths, becoming smaller and then negative at higher wavelengths. Fig. 5 *B* shows the peak rates of change of fluorescence (dF/dt) during transients versus detection wavelength for two different experiments. This measure was previously determined to emphasize the initial rapid Ca^{2+}-dependent component of the transient and to minimize any motion artifacts that would occur more slowly and later in the transient (Brandes et al., 1993). Data were fit to a quadratic polynomial, and λ_{iso} found at the wavelength where the fitted polynomial was zero. λ_{iso} in these experiments were 429.2 and 432 nm (mean 430.6 nm).

The in-vitro relationship between λ_{iso} and [protein] (Fig. 2 *B*, Table 1) indicates that at saturating [protein], λ_{iso} would be 431.8 nm (i.e., 454–22.22 nm, Table 1). The similar λ_{iso} found in-vivo and in-vitro at saturating [protein] suggests that indo-1 loaded into frog muscle would be in an environment corresponding to saturating [protein]. For frog muscle proteins, all calibration constants reached close to their maximum values at relatively low [protein] (mean P_{50} was 3.74 ± 0.97 mg/ml, $n = 4$). Therefore, at about 100 mg/ml [protein], the effect of [protein] on the calibration constants was almost saturated; consequently, Eq. 2 was used

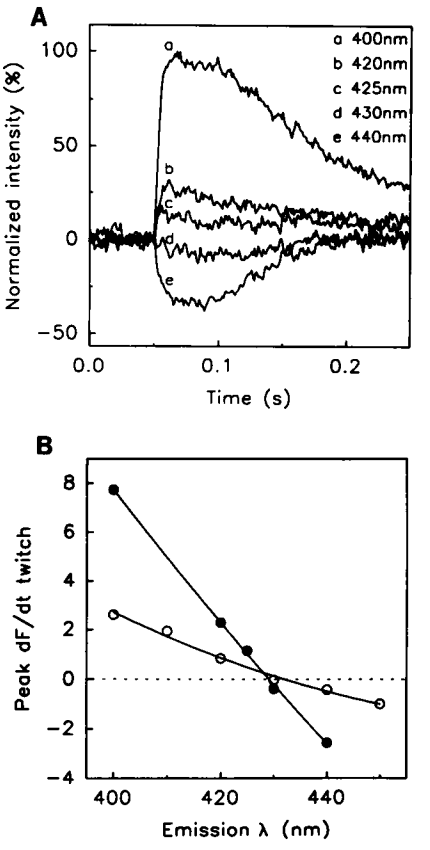

FIGURE 5 (*A*) Fluorescence transients recorded during twitch stimulation of frog muscle at different emission wavelengths (indicated next to each curve). Intensities were normalized to the maximum intensity at 400 nm (350-nm excitation). (*B*) Relationship between the peak rate of change of fluorescence intensities (dF/dt; arbitrary units) and detection wavelength. Data shown for two experiments. Isosbestic wavelength was determined at $dF/dt = 0$.

to estimate calibration constants (K_d, S_{400}, and S_{470}) assuming [protein] = 100 mg/ml and using the fitted values of ΔY_{sat}, P_{50}, and Y_0 (Table 1).

Rat heart

In a previous study (Brandes et al., 1993), λ_{iso} of indo-1 in a perfused heart was estimated to be ≈427 nm. Fig. 2 *B* and Table 1 suggest that in-vitro this λ_{iso} was closely matched at saturating [protein] (where λ_{iso} = 429.7 nM). Thus, in-vivo in the rat heart, as in frog muscle, the [protein] (relative to [indo-1]) was also high enough to saturate the effect of protein on indo-1 λ_{iso}.

The effective [protein] used to calculate calibration parameters for the rat heart was estimated by matching the variable S_R (see Eq. 3), determined in-vitro, to that previously found in-vivo (Brandes et al., 1993). S_R (defined in Eq. 3a) was previously calculated in the heart from measurements of R_{max} (according to Eq. 3a) and was found to be −1.34 (using 350-nm excitation and emission at 385 and 456 nm) (Brandes et al., 1993). For measurements at the same excitation and emission wavelengths in the present experiments, a value for

S_R of −1.34 could be achieved at a [protein] of 158 mg/ml. At this [protein] (and with 12 μM [indo-1]), the effect of protein on calibration parameters was also close to saturated.

Table 2 lists calibration parameters derived from proteins of frog muscle and rat heart that can be used to calculate $[Ca^{2+}]_i$ in-vivo under differing experimental conditions of pH, excitation, and emission wavelengths. Calibration values S_{385} and S_{456} for indo-1 determined for rat heart proteins were both considerably higher when using excitation at 360 nm compared with 350 nm.

DISCUSSION

The major new findings of this study were: 1) protein and protons have marked additive effects on indo-1 calcium-binding and fluorescence; 2) the effects of protein on indo-1 depended on both [indo-1] and [protein]; and 3) effects were different using proteins from different tissues. These findings suggest that calculation of $[Ca^{2+}]_i$ from indo-1 fluorescence requires use of calibration constants derived in the presence of appropriate types of protein, ratio of [indo-1] to [protein] and pH. This suggestion is consistent with many previous studies that recognize the importance of calibration conditions.

Interaction of indo-1 with protein

The relationships between [protein] and the indo-1 calcium calibration constants are consistent with a simple binding interaction between indo-1 and protein. These relationships suggest that the degree of interaction between indo-1 and protein should depend not simply on the [protein] but on the relative concentrations of indo-1 and protein. Consistent with this, the effects of protein on indo-1 fluorescence properties were reduced at a higher [indo-1]. These studies, therefore, offer a warning that [protein] versus [indo-1] may be an important factor to consider for calibration. Conceivably, some of the discrepancies between previous reports concerning the effects of proteins on K_d (or other calibration values) may arise because of differing indo-1 loading between studies. Possibly, the relationships between [protein] and indo-1 calcium calibration parameters described in this study may be equated to other studies after accounting for any differences in [indo-1] used.

TABLE 2 Indo-1 calcium calibration parameters used to calculate $[Ca^{2+}]_i$ in-vivo

	[protein] (mg/ml)	K_d (nM)	$S_{\lambda 1}$	$S_{\lambda 2}$
Frog (350-nm excitation) pH = 7.3	100	788	0.158	2.567
Frog (350-nm excitation) pH = 6.3	100	1696	0.184	2.509
Rat (350-nm excitation) pH = 7.3	158	594	0.098	2.209
Rat (360-nm excitation) pH = 7.3	158	594	0.174	4.083

Calibration constants derived using Eq. 2 and fitted values in Table 1. [protein] was extrapolated based on indo-1 spectral characteristics (see text). Values for frog muscle at 350-nm excitation (S values measured at 400- and 470-nm emission); values for rat heart at both 350- and 360-nm excitation (S values measured at 385- and 456-nm emission).

Values for P_{50} (determined in fitting Eq. 2) for each calcium calibration parameter were similar within each type of protein (Table 1) but were larger ($p < 0.01$) for protein from rat heart (16.35 ± 3.02 mg/ml, $n = 4$) compared with frog muscle (3.74 ± 0.94 mg/ml, $n = 4$, pH 7.3). The similarity of P_{50} within each protein type suggests that interaction of indo-1 with protein has multiple effects on indo-1, simultaneously affecting indo-1 calcium binding and fluorescence spectra. However, with frog muscle proteins the P_{50} for S_{400} was slightly but significantly lower than that for S_{470} at both pH 7.3 ($p < 0.05$) and pH 6.3 ($p < 0.01$). This finding may indicate that interaction of indo-1 with protein involves more than a single binding reaction. The larger P_{50} for proteins from rat heart may be explained by 1) a lower affinity for indo-1 to rat heart protein compared with frog muscle proteins, or 2) that indo-1 may bind to the same kind of protein, present in both protein extracts, but which may exist at a lower concentration in the rat heart extract. The results showed that for saturating [protein], the calculated maximum S_{470} was similar for proteins from rat or frog. However, the corresponding K_d was lower and S_{400} was higher for proteins from rat than from frog. These results suggest, therefore, that rat and frog have different protein types that have different effects on indo-1 calcium-binding and fluorescence.

Effect of protein on indo-1 fluorescence spectra

Previously, protein was found to have only a small effect on the emission spectrum of Ca^{2+}-bound indo-1 (Brandes et al., 1993); however, protein caused a large blue-shift and increase of the intensity of the emission spectrum of Ca^{2+}-free indo-1 (Brandes et al., 1993). Similar changes could not be observed in the present studies using tissue extracts or in previous studies using permeabilized myocytes (Hove-Madsen and Bers, 1992) because of the complicating effects of light absorption by the sample. However, it is possible that in the present study, similar influences of protein on the emission spectra of indo-1 may have given rise to the effects of protein extracts on S values and λ_{iso}. For example, the changes in S_{400}, S_{470}, and λ_{iso} induced with frog muscle proteins at 100 mg/ml [protein] (pH 7.3) could be accounted for assuming that 1) proteins did not affect the emission spectra of Ca^{2+}-bound indo-1 (Brandes et al., 1993) or the shape of the emission spectrum of Ca^{2+}-free indo-1 and 2. proteins caused a 12-nm blue-shift and 1.96 times increase of intensity of the Ca^{2+}-free indo-1 emission spectrum. Constancy of the shape and position of the Ca^{2+}-bound emission spectrum would imply that R_{max} would not be affected by protein; however, a blue-shift and intensity increase of the Ca^{2+}-free spectrum would cause an increase of R_{min} with [protein]. From the relation between R_{min} and R_{max} (Eq. 3b), the changes in S values found in this study would suggest that R_{min} would increase by a factor of 2.7 upon addition of 100 mg/ml frog muscle protein (pH 7.3). Consistent with this, the presence of calf serum or mouse muscle protein caused an increase of R_{min}, whereas R_{max} was unchanged (Westerblad and Allen, 1993).

Effect of acidosis on indo-1 fluorescence spectra

Acidosis had a relatively small effect on fluorescence spectra compared with its effect on K_d. S_{400} was increased by acidosis in the absence of protein and was increased by protein in the absence of acidosis. The maximum S_{400} obtained using protein at pH 7.3 was increased further in the presence of acidosis. These findings suggest that protein and protons caused increased S_{400} through separate mechanisms. In contrast, acidosis had little effect on the maximum S_{470} or λ_{iso} (at saturating [protein]). However, the P_{50} values for fits of S_{400}, and λ_{iso} versus [protein], were reduced with acidosis compared with pH 7.3, suggesting that acidosis increased the binding of indo-1 to protein in addition to the direct effect on indo-1.

Effects of protein on Ca binding by indo-1

Interactions between intracellular proteins and indo-1 increases indo-1 K_d for calcium. Proteins from frog and rat muscle both caused increased K_d; however, frog muscle proteins affected K_d more: the K_d at saturating protein was higher, and K_d was affected by frog proteins at lower concentrations compared with rat muscle. Previous studies found that the effect of proteins on the fluorescence spectra of indo-1 were dependent on the type of protein (Brandes et al., 1993). The present studies are consistent with this and further suggest that effects on K_d also vary with the type of protein.

Previously, the 1:1 stoichiometry of indo-1:Ca found in protein-free solution was found to change appreciably in the presence of protein (Hove-Madsen and Bers, 1992). In contrast, in the present study a 1:1 stoichiometry was maintained even at high [protein].

Effect of acidosis on Ca binding by indo-1

Consistent with previous studies (Lattanzio, 1990; Lattanzio and Bartschat, 1991), in the present study, in the absence of protein, acidosis increased the indo-1 K_d for calcium. The elevated K_d of indo-1 with acidosis was further increased by protein. The relative increase of K_d with and without saturating [protein] was similar at pH 7.3 and 6.3. These findings, consistent with the separate and additive effects of proteins and acidosis on S_{400}, suggest that proteins and acidosis also caused increased K_d through separate and additive mechanisms. However the P_{50} value for fits of K_d versus [protein] was reduced with acidosis compared with pH 7.3, suggesting, as mentioned above, that acidosis increased the binding of indo-1 to protein in addition to the direct effect on indo-1 Ca^{2+} binding.

Calculation of $[Ca^{2+}]_i$

The calibration parameters presented in Table 2 have been used to calculate $[Ca^{2+}]_i$ using data previously obtained in this laboratory from isolated perfused rat heart (Brandes et al., 1993) and intact frog skeletal muscle (Baker et al., 1993).

In rat heart loaded using indo-1 AM, diastolic and systolic $[Ca^{2+}]_i$ were previously estimated as 187 and 464 nM, respectively (Brandes et al., 1993). These estimates were similar to those of other studies using AM loading (Backx and ter Keurs, 1993); however, they did not account for the increased K_d of indo-1 for Ca^{2+} in the presence of proteins. Accounting for the increased K_d (by using the calibration constants presented in Table 2), these previous estimates would be increased to 391 and 1015 nM, respectively.

From our previous studies of frog muscle (Baker et al., 1993), calibration using values in Table 2 suggests a $[Ca^{2+}]_i$ in resting muscle of 353 ± 25 nM (mean ± SE, $n = 7$). This is similar to recent estimates using fura red (Kurebayashi et al., 1993) and fluo-3 (Harkins et al., 1993), where both indicators had increased K_d for Ca^{2+} in the presence of protein.

Problems and limitations

Several problems limit the findings of this study. The effects of proteins and acidosis on indo-1 calcium calibration parameters was defined; however, other constituents of the milieu may also affect indo-1 calcium calibration parameters.

Determination of K_d relied on controlling $[Ca^{2+}]$ by varying the proportion of EGTA to CaEGTA. Although this method is well established and has been commonly used, it is possible that the calculated $[Ca^{2+}]$ does not accurately reflect that actually obtained in solution, especially in the presence of high [protein]. However, the similarity of results with different concentrations of calcium buffer suggests that a consistent $[Ca^{2+}]$ is attained for a given ratio of EGTA/CaEGTA.

The effects of protein on indo-1 calcium calibration constants are dependent on the protein type. The present studies have examined only the effects of soluble proteins on indo-1. Insoluble proteins also interact with indo-1 (Hove-Madsen and Bers, 1992) and may have different effects on indo-1 compared with the soluble proteins.

The interaction of indo-1 with protein from tissue homogenates may differ from interactions in-vivo. For example, the accessibility of sites on proteins that indo-1 interacts with may differ in-vivo and in-vitro.

Use of indo-1-AM loading limits calibration due to the presence of noncytosolic indo-1. For example, mitochondria, which may load indo-1, represent a large volume fraction of heart muscle (see Backx and ter Keurs, 1993). In contrast, in the mainly glycolytic frog muscle, the mitochondrial fraction is very low. Use of indo-1-AM loading in this study would not influence measurement of λ_{isos} because only indo-1 in the cytosol would be expected to show rapid $[Ca^{2+}]_i$ transients.

CONCLUSIONS

Indo-1 K_d for calcium and fluorescence spectra are markedly altered by interaction with intracellular protein and by changes of pH. Thus, consistent with previous studies that recognized the importance of calibration conditions, the

present studies suggest that estimation of $[Ca^{2+}]_i$ from indo-1 fluorescence requires calcium calibration parameters derived in the presence of appropriate 1) types of protein, 2)ratio of [indo-1]/[protein], and 3) pH.

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