single-muscle fibers from mouse were used, the fluorescence of indo-1 was measured in the standard ratiometric fashion, and ratios were converted to $[Ca^{2+}]_i$ according to Grynkiewicz et al. (1985). The ratio at a very low $[Ca^{2+}]_i$ (R_{min}) was obtained by microinjection of EGTA. $CaCl_2$ was injected to establish the ratio at high $[Ca^{2+}]_i$ (R_{max}). Finally, to establish K_D an intermediate $[Ca^{2+}]_i$ was obtained by injection of a solution containing equal amounts of EGTA and Ca^{2+}-EGTA.

The most important advantage of our approach is that all the relevant properties of the indicator are established in the intracellular environment. In addition, the method is relatively easy to use in large cells, such as muscle, and it is not limited to Ca^{2+} indicators; for example, we have used the same approach to calibrate furaptra for Mg^{2+} (Westerblad and Allen, 1992). The disadvantage is that the calibration will damage the cell. Furthermore, ratios at all three $[Ca^{2+}]_i$ cannot be obtained in one cell; this means that the calibration depends on mean values, which may be a problem if the variation between cells is large.

Employing our calibration method we obtained a $[Ca^{2+}]_i$ at rest in mouse muscle fibers of about 30 nM, which is similar to other estimates in mouse muscle where "intracellular" calibration techniques have been used (e.g., Head, 1993). Our method can also be used in the null method fashion. With injection of EGTA we observed a clear-cut reduction of the fluorescent ratio. The solution with equal amounts of EGTA and Ca^{2+}-EGTA had a $[Ca^{2+}]$ of about 100 nM (pH set to 7.3, which is the intracellular pH), and injection of this solution resulted in a marked increase of the ratio. Thus, the resting $[Ca^{2+}]_i$ must lie somewhere between 0 and 100 nM.

It is possible that frog fibers have a higher resting $[Ca^{2+}]_i$ than mouse fibers. However, most careful estimates in frog fibers give values below 100 nM (e.g., Blatter and Blinks, 1991). We have recently applied our method to fibers from *Xenopus* frogs, and we then get a resting $[Ca^{2+}]_i$ of about 40 nM (unpublished observations). The solution with intermediate $[Ca^{2+}]$ was set in these experiments to 200 nM, and it gave a clear-cut increase of the ratio, which indicates that the resting $[Ca^{2+}]_i$ was markedly lower than 200 nM. Thus, the values of resting $[Ca^{2+}]_i$ reported by Baylor and co-workers are higher than those obtained by other methods. A possibility that needs to be excluded is that the discrepancy arises because in their experiments all properties of the indicator have not been established in the intracellular environment.

REFERENCES

Blatter, L. A., and J. R. Blinks. 1991. Simultaneous measurement of Ca^{2+} in muscle with Ca electrodes and aequorin. Diffusible cytoplasmic constituent reduces Ca^{2+}-independent luminescence of aequorin. *J. Gen. Physiol.* 98:1141–1160.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Harkins, A. B., N. Kurebayashi, and S. M. Baylor. 1993. Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3. *Biophys. J.* 65:865–881.

Head, S. I. 1993. Membrane potential, resting calcium and calcium transients in isolated muscle fibres from normal and dystrophic mice. *J. Physiol.* 469:11–19.

Kurebayashi, N., A. B. Harkins, and S. M. Baylor. 1993. Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers. *Biophys. J.* 64:1934–1960.

Morgan, K. G. 1993. $Ca^{2+}{}_i$ versus $[Ca^{2+}]_i$. *Biophys. J.* 65:561–562.

Ross, W. N. 1993. Calcium on the level. *Biophys. J.* 64:1655–1656.

Westerblad, H., and D. G. Allen. 1992. Myoplasmic free Mg^{2+} concentration during repetitive stimulation of single fibres from mouse skeletal muscle. *J. Physiol.* 453:413–434.

Westerblad, H., and D. G. Allen, 1993. The influence of intracellular pH on contraction, relaxation and $[Ca^{2+}]_i$ in intact single fibres from mouse muscle. *J. Physiol.* 466:611–628.

Response to Westerblad and Allen

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The published work of Westerblad and Allen (1993) employed a different Ca^{2+} indicator (indo-1) on a different preparation (mammalian muscle fibers) than used by us (fura-red and fluo-3 on frog fibers (Kurebayashi et al., 1993; Harkins et al., 1993)). They reported that R_{max}/R_{min}, the ratio of two important fluorescence calibration constants of indo-1, was 6-fold smaller in myoplasm than in a simple salt solution and that addition of protein (5% calf serum) to the calibrating salt solution caused a 7-fold decrease in R_{max}/R_{min} (Westerblad and Allen, 1993). These large changes likely result from the binding of indicator to protein (Westerblad and Allen, 1993; Konishi et al., 1988) and raise the question whether indo-1's dissociation constant for Ca^{2+} (K_D), a key parameter in the calibration of $[Ca^{2+}]$, might also be altered by protein. We and others have found that the K_D values of tetracarboxylate Ca^{2+} indicators are substantially increased by addition of protein, 50–100 mg/ml, to the calibration solutions (Konishi et al., 1988; Uto et al., 1991; Hove-Madsen and Bers, 1992; Kurebayashi et al., 1993; Harkins et al., 1993). Moreover, these elevated K_D values appear to be similar to the K_D values of the indicators when in the myoplasm of frog fibers (Kurebayashi et al., 1993; Harkins

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et al., 1993; Hollingworth et al., 1992). The factors we estimated for the increases in K_D were 3–4-fold for fura-2 (Konishi et al., 1988; Hollingworth et al., 1992), 3–4-fold for fura-red (Kurebayashi et al., 1993) and 2–5-fold for fluo-3 (Harkins et al., 1993). We are therefore surprised that Westerblad and Allen (1993) found that indo-1's K_D in myoplasm, 182 nM, differs little from that measured in a simple salt solution, 210 nM. Although Westerblad and Allen did not report the effect of 5% calf serum on indo-1's K_D, Hove-Madsen and Bers (1992) found that the K_D for this indicator was increased 4–5-fold in calibrations carried out in the presence of cellular protein (100 mg/ml) or with permeabilized cardiac myocytes. It therefore seems possible that indo-1's K_D, and hence resting $[Ca^{2+}]$, is substantially higher in the myoplasm of mammalian muscle than concluded by Westerblad and Allen.

The in situ calibration technique of Westerblad and Allen (1992, 1993) relied on the injection of fibers with calibration solutions of high ionic strength (up to 1.5 M) and osmolarity. These solutions produced marked fiber swelling and damage to the cell. The swelling of the fiber (attributable to water movements induced by the hypertonicity of the injected solutions) must have diluted the concentrations of the normal myoplasmic constituents. We suggest that this dilution, and possibly the elevation in ionic strength (Westerblad and Allen, 1992), may have altered the binding reaction between indicator and intracellular protein, which, as mentioned above, strongly affects the calibration of tetracarboxylate Ca^{2+} indicators. More generally, introduction into the fiber of a solution that does not contain protein would be expected to alter the in situ calibration toward that of an in vitro calibration without protein. Thus, we question whether, with this calibration method, "all the relevant properties of the indicator are established in the intracellular environment."

Regarding our experiments (Harkins et al., 1993), Westerblad and Allen suggest that F_{max}/F_{min} of fluo-3 (a quantity somewhat analogous to R_{max}/R_{min} of indo-1) might be ~6-fold smaller in myoplasm than in a simple salt solution and consequently that our estimate of resting $[Ca^{2+}]$ may be erroneously high. We agree that, if F_{max}/F_{min} in the fiber were so affected, our estimation of resting $[Ca^{2+}]$ should be reduced, but only by ~30% (cf. Eqs. 1 and 4 of Harkins et al. (1993)). Against this suggestion, however, is our finding that a calibration solution that contained a large concentration of intracellular protein (122 mg/ml aldolase) reduced F_{max}/F_{min} by only 1.5-fold (from 174 to 114; see Fig. 3 *B* of Harkins et al. (1993)). Although we acknowledge that uncertainty exists concerning the actual value of fluo-3's F_{max}/F_{min} in frog myoplasm, the value of 100 that we assumed for our final calibration seems reasonable.

In summary, the determination of resting $[Ca^{2+}]$ presents a difficult measurement problem and we doubt that current knowledge justifies placement of a high degree of confidence in any single indicator or calibration method. Probably the best estimates will derive from a comparison of results with several indicators and calibration techniques. We also emphasize that a growing body of evidence indicates that protein, at intracellular concentrations, has major effects on the properties of tetracarboxylate indicators, including K_D. Hopefully, future experiments will increase our understanding of indicator-protein interactions and related calibration difficulties that complicate the reliability of $[Ca^{2+}]$ estimation.

REFERENCES

Harkins, A. B., N. Kurebayashi, and S. M. Baylor. 1993. Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3. *Biophys. J.* 65:865–881.

Hollingworth, S., A. B. Harkins, N. Kurebayashi, M. Konishi, and S. M. Baylor. 1992. Excitation-contraction coupling in intact frog skeletal muscle fibers injected with mmolar concentrations of fura-2. *Biophys. J.* 63:224-234.

Hove-Madsen, L., and D. M. Bers. 1992. Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. *Biophys. J.* 63:89–97.

Konishi, M., A. Olson, S. Hollingworth, and S. M. Baylor. 1988. Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys. J.* 54:1089-1104.

Kurebayashi, N., A. B. Harkins, and S. M. Baylor. 1993. Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers. *Biophys. J.* 64:1934–1960.

Uto, A., H. Arai, and Y. Ogawa. 1991. Reassessment of fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell Calcium.* 12:29–37.

Westerblad, H., and D. G. Allen. 1992. Myoplasmic free Mg^{2+} concentration during repetitive stimulation of single fibres from mouse skeletal muscle. *J. Physiol.* 453:413–434.

Westerblad, H., and D. G. Allen. 1993. The influence of intracellular pH on contraction, relaxation and $[Ca^{2+}]_i$ in intact single fibres from mouse muscle. *J. Physiol.* 466:611–628.