tionship originally derived by Malkin and Kok (1966) still holds in the framework of the exciton-radical pair equilibrium model.

Finally, I would like to point out that the interpretation of fluorescence induction curves with blocked electron transfer between Q_A and Q_B has been subject to critique for different reasons for a long time (Doschek and Kok, 1972; Hemelrijk and van Gorkom, 1992; France et al., 1992). A critical review of the widely used commercial PAM-fluorimeter has been published recently (Büchel and Wilhelm, 1993). It is also worth mentioning that even experimental data on the shape of fluorescence induction curves obtained with the pump-probe technique are contradictory (Hemelrijk and van Gorkom, 1992; France et al., 1992), and thus appear not to be a "better" alternative to conventional methods. In my view a complete interpretation of experimental fluorescence induction curves is still an open subject that will progress by comparison with model predictions. Experimental observations like those offered by Falkowski et al. do neither prove nor disprove the self-consistency of any conclusions drawn from a purely theoretical treatment of the problem.

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Methods for Calibration of Fluorescent Calcium Indicators in Skeletal Muscle Fibers

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In a recent paper in this journal Baylor and co-workers concluded that the free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in frog skeletal muscle fibers at rest is at least 100 nM and may be as large as 300 nM (Harkins et al., 1993; see also Kurebayashi et al., 1993). This $[Ca^{2+}]_i$ is higher than that frequently reported for muscle fibers, and hence, it raises the question about the accuracy of various methods used to measure $[Ca^{2+}]_i$ (Morgan, 1993; Ross, 1993).

In the paper by Kurebayashi et al. (1993), $[Ca^{2+}]_i$ was measured with the fluorescent indicator fura red, whereas Harkins et al. (1993) used another fluorescent Ca^{2+} indicator, fluo-3. The methods of calibration of these two indicators were similar in that the fraction of indicator in the Ca^{2+}-bound form at rest (f_r) was established by a combination of in situ and in vitro measurements. The value of f_r was then combined with estimates of the apparent dissociation constant (K_D) to get $[Ca^{2+}]_i$ at rest; estimates of K_D were obtained either in vitro or from rate constants obtained from $[Ca^{2+}]_i$ transients due to stimulation. The estimates of f_r and K_D depended on the calibration technique and showed a large spread both with fura red and fluo-3. However, the authors conclude that the $[Ca^{2+}]_i$ at rest is at least 100 nM and can be as large as 300 nM (Harkins et al., 1993).

The methods described by Baylor and co-workers are attractive in that a complete calibration can be performed without causing damage to the cell. The disadvantage is that the techniques are technically rather cumbersome, and to some degree, they depend on in vitro measurements in uncertain solutions. For instance, Harkins et al. (1993) show that when F_{max}/F_{min} of fluo-3 (the fluorescence intensity of the Ca^{2+}-bound and Ca^{2+}-free form, respectively) is large, a term in the calibration equation (their Eq. 4) is of little importance. In simple ionic solutions in vitro they found F_{max}/F_{min} to be 200. To mimic the intracellular environment, they added protein to their calibration solutions, and this markedly reduced the ratio; hence, they assumed an intracellular F_{max}/F_{min} of 100, which required a small (5%) reduction to their estimate of $[Ca^{2+}]_i$. In a recent study (Westerblad and Allen, 1993; see below), we measured R_{max}/R_{min} (the equivalent expression for a ratiometric dye) for another fluorescent Ca^{2+} indicator, indo-1, both in simple ionic solutions in vitro and in situ, and the resulting ratios were 68 and 11, respectively. If a similar intracellular change occurs with fluo-3, then the error in estimated resting $[Ca^{2+}]_i$ by Harkins et al. (1993) becomes rather large, and their value of resting $[Ca^{2+}]_i$ should be reduced by a considerable amount.

We have recently used another method for intracellular calibration of indo-1 (Westerblad and Allen, 1993). Intact,

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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.

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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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