*B ochimica et Biophysica Acta*, 462 (1977) 314–322


BBA 47382

# COOPERATIVE INTERACTIONS BETWEEN CALCIUM-BINDING SITES ON GLYCERINATED MUSCLE FIBERS

## THE INFLUENCE OF CROSS-BRIDGE ATTACHMENT

FRANKLIN FUCHS

*Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 15261 (U.S.A)*

(Received February 7th, 1977)

SUMMARY

A double isotope technique and EGTA buffers were used to measure the binding of $Ca^{2+}$ to rabbit psoas muscle fibers extracted with detergent and glycerol. These experiments were designed to test the effect of rigor complex formation, determined by the degree of filament overlap, on the properties of the $Ca^{2+}$-binding sites in the intact filament lattice. In the presence of 5 mM $MgCl_2$ (no ATP), reduction of filament overlap was associated with a reduced binding of $Ca^{2+}$ over the entire range of free $Ca^{2+}$ concentrations ($5 \cdot 10^{-8}$–$2 \cdot 10^{-5}$ M). With maximum filament overlap (sarcomere length 2.1–2.2 $\mu$m) the maximum bound $Ca^{2+}$ was equivalent to 4 mol $Ca^{2+}$/mol troponin and there was significant positive interaction between binding sites, as shown by Scatchard and Hill plots. With no filament overlap (sarcomere length 3.8–4.4 $\mu$m) the maximum bound $Ca^{2+}$ was equivalent to 3 $\mu$mol $Ca^{2+}$/mol troponin and graphical analysis indicated a single class of non-interacting sites. The data provide evidence that when cross-bridge attachments between actin and myosin filaments are formed not only does an additional $Ca^{2+}$ binding site appear, but cooperative properties are imposed upon the binding sites.

## INTRODUCTION

Force development in striated muscle is activated by the binding of $Ca^{2+}$ to troponin [1, 2], a protein localized on the actin filament in close association with tropomyosin [3]. Purified troponin has been found to bind up to 4 mol $Ca^{2+}$/mol troponin [4] and it has been suggested that all four binding sites must be occupied for contraction to occur [5]. Bremel and Weber [6] first showed that when troponin was incorporated into a reconstituted contractile system the binding of $Ca^{2+}$ was enhanced when myosin cross-bridges formed attachments to the actin (rigor complexes). This finding raises the possibility that the $Ca^{2+}$ receptor sites in more highly organized contractile systems have properties which may be quite different from those of the purified troponin and which might have considerable physiological importance in the intracellular regulation of force development [7, 8].

Recent work in this laboratory has focused on the behavior of $Ca^{2+}$-binding sites located in what is presumably an intact myofilament lattice, namely the glycerinated rabbit psoas fiber. Fuchs and Bayuk [9] presented evidence for positive cooperativity in the binding of $Ca^{2+}$ to glycerinated fibers in rigor at rest length. More recently it has been found that the total number of $Ca^{2+}$-binding sites in the glycerinated fiber is a function of the number of cross-bridge attachments between the myosin and actin filaments [10]. Fibers in rigor with maximum filament overlap bound a maximal amount of $Ca^{2+}$ equivalent to 4 mol $Ca^{2+}$/mol troponin. If the filaments were detached, either by pre-stretch of the fibers to a length at which filament overlap was eliminated, or by immersion in MgATP, the number of $Ca^{2+}$-binding sites was reduced by an amount equivalent to 1 mol $Ca^{2+}$/mol troponin.

With regard to cooperativity it is noteworthy that, in contrast to the results of Fuchs and Bayuk [9], no evidence for positive interaction between binding sites was found by Potter and Gergely [4] in their careful study of $Ca^{2+}$-binding to purified troponin. If, as suggested above, the properties of troponin are modified by incorporation into more organized systems, then the results which have been cited are not necessarily contradictory. In this report evidence is presented that cross-bridge attachments, determined by the amount of filament overlap, influence not only the total number of binding sites, but also the degree of cooperativity between sites.

## METHODS AND MATERIALS

Rabbits were killed by intravenous injection of sodium pentobarbital. Thin strips of psoas muscle were tied to sticks either at rest length or after stretch or shortening. The glycerol extraction procedure was essentially that of Taylor [11], with minor modifications. The bundles were first extracted for at least 6 h at 0 °C with a solution containing 80 mM KCl, 20 mM imidazole (pH 7.0), 2.5 mM dithiothreitol, 5 mM ethylene glycol($\beta$-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), and 1 % Triton X-100. The bundles were then extracted for 24 h at 0 °C with a solution containing 80 mM KCl, 20 mM imidazole (pH 7.0), 2.5 mM dithiothreitol, and 50 % glycerol. After the second excretion, the bundles were split into thinner strands, transferred to fresh glycerol solution (same composition) and stored at −20 °C. Fiber bundles were extracted for at least 5 days before being used for binding measurements.

Several individual fibers were separated at random from each of the bundles for measurement of sarcomere length. This measurement was made with a phase contrast microscope equipped with an ocular micrometer. Between 8 and 12 measurements were made for each bundle, each measurement being an average value calculated from the length of a row of 10 sarcomeres.

Binding measurements were made with thin bundles, 2.0–2.5 cm in length, containing no more than 5–6 fibers. These were separated under a dissecting microscope and transferred to beakers containing 2 ml of buffer solution of the following composition: 100 mM KCl, 20 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 5 mM[$^3$H]-glucose, 0.1 mM $^{45}CaCl_2$, and varying concentrations of EGTA to adjust the free $Ca^{2+}$ concentration. The latter was calculated according to Ogawa et al. [12] on the basis of an apparent CaEGTA binding constant (at pH 7.0) of $10^{6.14}$ [13]. The total $Ca^{2+}$ concentration included contaminant $Ca^{2+}$, as determined by atomic absorption

spectroscopy.

The fiber bundle was incubated in the buffered $Ca^{2+}$ solution for 20 min at room temperature. An aliquot of the buffer solution was removed and analyzed for both $^{45}Ca$ and $^{3}H$ with a Beckman LS-100 liquid scintillation counter [9]. At the end of the incubation period, the fiber bundle was transferred to 0.5 ml of fresh buffer containing excess non-radioactive carrier (10 mM glucose, 5 mM $CaCl_2$) to elute $^{45}Ca$ and $^{3}H$. Following isotope extraction, the fiber bundle was analyzed for protein as described by Fuchs and Bayuk [9] and the eluate was analyzed for $^{45}Ca$ and $^{3}H$. The [$^{3}H$]glucose served as a marker for the solvent space. Bound $Ca^{2+}$ was calculated from the $^{45}Ca/^{3}H$ ratio of the fibers relative to that of the buffer solution.

## RESULTS

Binding measurements were made with three groups of fibers having sarcomere length ranges of 2.1–2.2 $\mu$m (short), 2.8–2.9 $\mu$m (rest length) and 3.8–4.4 $\mu$m (stretched). The data are listed in Table I and the relation between bound $Ca^{2+}$ and free $Ca^{2+}$ is plotted in Fig. 1. At the lowest $Ca^{2+}$ concentrations, the three curves overlap but as the concentration of $Ca^{2+}$ rises above $1 \cdot 10^{-7}$ M the curve for the stretched fibers clearly diverges from those of the short and rest length fibers. The differences between the short and rest length fibers reach the level of statistical significance only as the free $Ca^{2+}$ concentration approaches saturation.

According to the measurements of Page and Huxley [14] the myosin filament of rabbit psoas is 1.6 $\mu$m in length and the total actin filament length is 2.24 $\mu$m. Thus, the fibers in the short group would have maximum filament overlap (and maximum rigor complex formation) whereas most of the fibers in the stretched group would have no filament overlap. As shown previously [10], fiber bundles extracted sequentially with Triton X-100 and glycerol contain, on the average, 87 % myofibrillar

TABLE I

BOUND $Ca^{2+}$ AS A FUNCTION OF FREE $Ca^{2+}$ CONCENTRATION AND SARCOMERE LENGTH RANGE

Values are given as mean±S.E.M. in $\mu$mol/g. Number of measurements is indicated in parentheses. See text for experimental conditions.

| $[Ca^{2+}]$(M) | Sarcomere length range ($\mu$m) | | |
|---|---|---|---|
| | 2.1–2.2 | 2.8–2.9 | 3.8–4.4 |
| $5 \cdot 10^{-8}$ | 0.07±0.01 (10) | 0.07±0.02 (11) | 0.08±0.02 (10) |
| $1 \cdot 10^{-7}$ | 0.18±0.04 (10) | 0.19±0.04 (11) | 0.17±0.04 (10) |
| $1.5 \cdot 10^{-7}$ | 0.28±0.04 (10) | 0.28±0.03 (11) | 0.20±0.04 (10) |
| $2.0 \cdot 10^{-7}$ | 0.40±0.02 (10) | 0.36±0.03 (11) | 0.26±0.03 (9) |
| $2.5 \cdot 10^{-7}$ | 0.48±0.04 (10) | 0.46±0.03 (11) | 0.33±0.05 (9) |
| $3 \cdot 10^{-7}$ | 0.57±0.04 (10) | 0.56±0.03 (11) | 0.45±0.06 (10) |
| $5 \cdot 10^{-7}$ | 0.86±0.03 (10) | 0.75±0.07 (11) | 0.57±0.07 (10) |
| $1 \cdot 10^{-6}$ | 1.13±0.02 (10) | 0.98±0.08 (11) | 0.85±0.09 (10) |
| $2 \cdot 10^{-6}$ | 1.35±0.08 (10) | 1.24±0.07 (11) | 1.01±0.11 (10) |
| $5 \cdot 10^{-6}$ | 1.64±0.05 (10) | 1.46±0.09 (11) | 1.22±0.09 (10) |
| $1 \cdot 10^{-5}$ | 1.70±0.05 (10) | 1.49±0.06 (11) | 1.31±0.09 (9) |
| $2 \cdot 10^{-5}$ | 1.88±0.06 (10) | 1.68±0.07 (11) | 1.36±0.10 (10) |

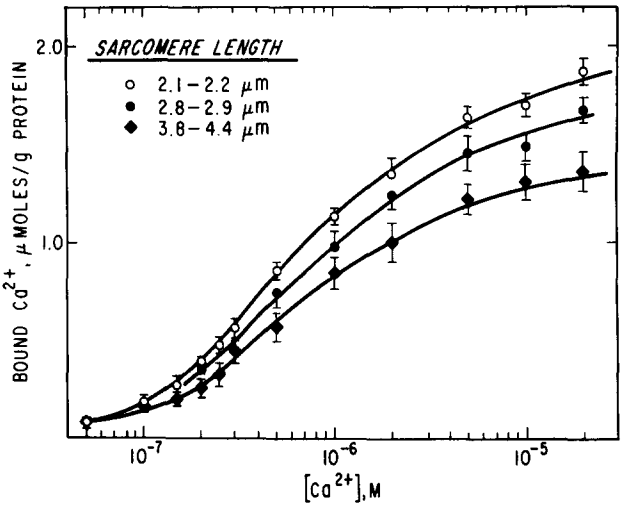


Fig. 1 The binding of $Ca^{2+}$ to glycerinated rabbit psoas fibers of different sarcomere lengths, as indicated. Data are listed in Table I. Vertical bars indicate standard errors.

protein. Assuming a troponin content of 0.6 μmol/g myofibril protein, the amount of $Ca^{2+}$ bound by the short fibers at the highest free $Ca^{2+}$ concentration, 1.88 μmol $Ca^{2+}$/g protein (S.E.±0.06, $n = 10$), is equivalent to 3.6 mol $Ca^{2+}$/mol troponin. The maximum $Ca^{2+}$ bound by the stretched fibers, 1.36 μmol $Ca^{2+}$/g protein (S.E.± 0.10, $n = 10$) is equivalant to 2.6 mol $Ca^{2+}$/mol troponin. This result is in agreement with the earlier conclusion from this laboratory [10] that troponin, in the intact filament lattice, has four $Ca^{2+}$-binding sites per molecule when the cross-bridges are attached to actin and three $Ca^{2+}$-binding sites when the filaments are completely detached from each other. It is of interest to compare the binding curves in Fig. 1 to those of glycerinated insect flight muscle published by Marston and Tregear [15]. These workers showed that reduction in rigor complex formation by the addition of MgATP reduced the amount of bound $Ca^{2+}$ at all levels of free $Ca^{2+}$. Maximal $Ca^{2+}$-binding in the presence of MgATP was about 30 % less than in the rigor state.

In interpreting these data, the possibility must be considered that the differences in maximum $Ca^{2+}$ binding as a function of sarcomere length could arise from a loss of troponin during glycerol extraction, this loss being greater in stretched fibers. This is a somewhat unlikely explanation in view of the recent results from this laboratory [10] showing that detachment of the filaments either by stretch or by immersion of shortened fibers in MgATP produces the same reduction in the number of $Ca^{2+}$-binding sites. However, for a more direct test of this possibility, an additional set of experiments was carried out. Stretched fiber bundles (mean sarcomere length 3.5–3.7 μm) were teased into thin bundles, as described. Calcium-binding measurements were made on one group of bundles by the usual procedure. The other group was allowed to shorten in buffer containing 5 mM MgATP and $2 \cdot 10^{-5}$ M $Ca^{2+}$. The MgATP was washed out and the fibers were then analyzed with respect to sarcomere length and maximum $Ca^{2+}$ binding. If the reported variation in maximal $Ca^{2+}$ binding with sarcomere length was simply due to differential loss of troponin, then active shortening

TABLE II

THE EFFECT OF ATP-INDUCED SHORTENING ON THE NUMBER OF CALCIUM BINDING SITES OF GLYCERINATED MUSCLE FIBERS

Values are given as mean ± S.E.M. Number of measurements is indicated in parentheses. $p$ value based on Student $t$ test for difference between two means.

| | Sarcomere length ($\mu$m) | Bound $Ca^{2+}$ ($\mu$mol/g) |
|---|---|---|
| Before shortening | 3.68 | 1.41 ± 0.06 (5) |
| After shortening | 2.05 | 1.88 ± 0.12 (5) |
| | | $p < 0.01$ |

should have no effect on the number of $Ca^{2+}$-binding sites. The results in Table II show a significant enhancement in bound $Ca^{2+}$ with ATP-induced shortening. The measured value at sarcomere length 2.05 $\mu$m corresponds to that seen with fiber pre-shortened in the living state. Hence, the variation in number of $Ca^{2+}$-binding sites is considered to be a consequence of differences in the number of cross-bridge attachments rather than differences in the content of $Ca^{2+}$-binding protein.

Scatchard plots of the data are shown in Fig. 2. The data points for the stretched fibers are best fitted by a straight line, thus indicating a single class of non-interactive sites. The intercept on the abcissa is 1.4 $\mu$mol $Ca^{2+}$/g and the apparent affinity constant is $1.2 \cdot 10^6$ $M^{-1}$. The plots for the two groups of fibers with filament overlap have a more complex shape. At lower degrees of saturation there is a convexity with respect to the abcissa which is characteristic of a binding system with positive cooperativity.

Hill plots of the same data are shown in Fig. 3 and the estimated Hill coefficients are listed in Table III. The plot of the data for the stretched fibers is a straight line with a slope of 1.0. The other two Hill plots have a bend at approximately half-saturation, in

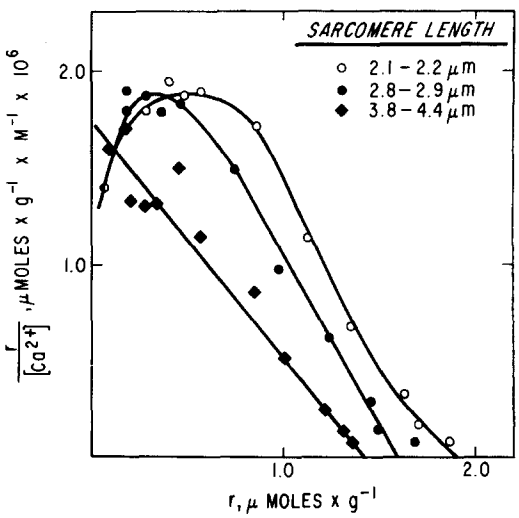


Fig. 2 Scatchard plots of the data in Figure 1. Symbol: $r$, bound $Ca^{2+}$.

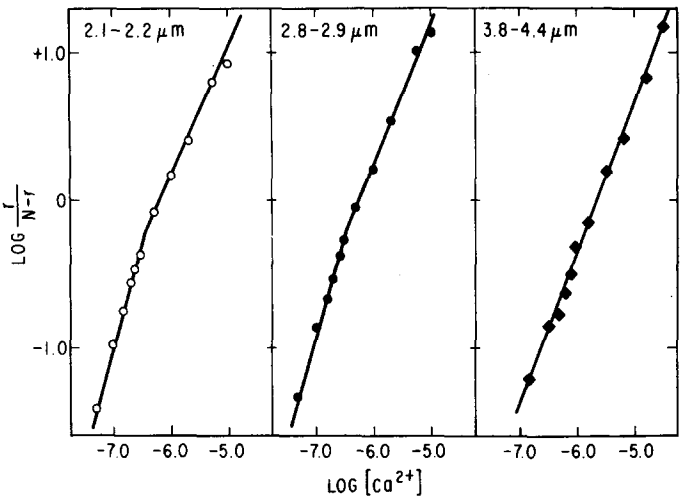


Fig. 3 Hill plots of the data in Figure 1. Symbols: $r$, bound $Ca^{2+}$; $N$, bound $Ca^{2+}$ at saturation, as determined from Scatchard plot intercepts in Fig. 2.

agreement with earlier results of Fuchs and Bayuk [9]. At greater than half saturation, the slopes of the plot for the short and rest length fibers are 0.9 and 1.0, respectively. At less than half-saturation the slopes are 1.6 and 1.4, respectively. These results suggest that when the cross-bridges are attached to actin half of the binding sites interact positively.

TABLE III

ESTIMATED HILL COEFFICIENTS AS A FUNCTION OF SARCOMERE LENGTH AND DEGREE OF SATURATION

| Sarcomere length ($\mu$m) | < 50 % saturation | > 50 % saturation |
|---|---|---|
| 2.1–2.2 | 1.6 | 0.9 |
| 2.8–2.9 | 1.4 | 1.0 |
| 3.8–4.4 | 1.0 | 1.0 |

## DISCUSSION

The ability of rigor complex formation to enhance the binding of $Ca^{2+}$ to troponin was first described by Bremel and Weber [6] and has since been confirmed in several different systems [15–17]. In an extension of this work to the glycerinated rabbit psoas fiber, it has been found that the number of $Ca^{2+}$-binding sites is reduced by 25 % when the cross-bridges are disconnected either by elimination of filament overlap or treatment with MgATP under relaxing conditions [10]. The data in this report suggest that cross-bridge attachments also influence the degree of cooperativity between binding sites.

The role of cooperative interactions in the control of muscle contraction has been the subject of much recent discussion [7, 18]. The most compelling evidence for

cooperativity is the steepness of the curve relating free $Ca^{2+}$ concentration to force development in various "skinned" muscle fiber preparations [19–22]. The ATPase activity vs. [$Ca^{2+}$] curve also has a steeper slope than would be expected for a simple non-cooperative system [4, 7], although it is of interest that in those instances [23–25] in which force, ATPase activity and free $Ca^{2+}$ were correlated in the same preparation, the force vs. [$Ca^{2+}$] slope was greater than the ATPase activity vs. [$Ca^{2+}$] slope. The force vs. [$Ca^{2+}$] relationship is markedly affected by, among other things, the MgATP [13] and free $Mg^{2+}$ [26] concentrations and since the true intracellular concentrations of these species are still uncertain the force vs. [$Ca^{2+}$] relationship under physiological conditions cannot be rigorously specified. In the case of glycerinated psoas fibers, the force vs. [$Ca^{2+}$] data [27, 28] are much less extensive and they show more variation. The slopes of the $Ca^{2+}$-binding curves obtained in this laboratory also show considerable variability from one series of measurements to the next. Whether this variability is due to biological variation or inadequacy of $Ca^{2+}$ buffering is not known.

On the other hand, certain findings have been quite consistent. For fibers lacking appreciable filament overlap, the Scatchard plot is linear throughout and the Hill coefficient is unity (see also ref. 10). Thus in the absence of cross-bridge attachments, binding appears to be non-cooperative, with just a single class of sites present. Potter and Gergely [4] showed that purified troponin, in the presence of 2 mM $MgCl_2$, also has a single class of non-interactive sites. Their estimated binding constant, $5.4 \cdot 10^6\ M^{-1}$, compares favorably with the value of $1 \cdot 10^6$–$2 \cdot 10^6\ M^{-1}$, obtained for stretched glycerinated psoas fibers. The main discrepancy between the two sets of data is with respect to the number of binding sites. Purified troponin binds 4 mol $Ca^{2+}$/mol troponin while the calculated bound $Ca^{2+}$ of the stretched fibers is equivalent to 3 mol $Ca^{2+}$/mol troponin. This calculation rests on the assumption that under the stated experimental conditions there is no significant binding to proteins other than troponin. If this assumption should prove to be incorrect, the discrepancy becomes even greater.

For fibers with a high degree of filament overlap (sarcomere length $< 3\ \mu m$) the Scatchard plot is convex with respect to the abcissa at less than half-saturation and the Hill coefficient is 1.5–2.0 (see also ref. 9). With increasing saturation, the Hill coefficient becomes unity (see Table I) and the maximum bound $Ca^{2+}$ is equivalent to 4 mol $Ca^{2+}$/mol troponin [10]. Thus in the intact filament lattice with cross-bridges attached the binding sites display a degree of cooperativity which is not evident with either purified troponin or troponin bound to actin filaments free of rigor complexes. If it is assumed that the force-generating complexes formed in the presence of ATP have the same effect on $Ca^{2+}$-binding, an assumption supported by recent experiments of Solaro et al. [16], then it is possible to postulate the existence of a positive feedback system in which $Ca^{2+}$-binding promotes cross-bridge attachment which, in turn, favors the binding of additional $Ca^{2+}$ [7, 8]. Several lines of evidence point to the existence of a mechanism whereby force feeds back positively on the molecular control system. To cite one example, it is well known that in both skeletal [29] and cardiac [30] muscle, the magnitude of the force developed determines the time course of mechanical activity. Since the number of cross-bridge attachments is a function of the force developed [31] the data reported here might provide a mechanistic basis for coupling between force and the amount of $Ca^{2+}$ bound to the filaments. Such a

coupling was postulated by Kaufmann et al. [30] to account for the shortening deactivation of cardiac muscle.

In this context, it is of interest to re-examine Endo's [32] data showing that the force vs. $[Ca^{2+}]$ slope of skinned frog fibers is much steeper at sarcomere length 2.0–2.3 $\mu$m than at sarcomere length 3.0–3.3 $\mu$m. These results would also suggest that the number of cross-bridge attachments plays some role in determining the degree of cooperativity of the $Ca^{2+}$ response.

These considerations naturally lead to the possibility that the $Ca^{2+}$-binding curve of a system actively developing force is quantitatively different in shape from that of extracted systems (myofibrils, actomyosin) which do not generate force. If so, the differences alluded to above between the binding curves and the force vs. $[Ca^{2+}]$ curves may be more apparent than real. There is a clear need for carefully executed experiments relating free $Ca^{2+}$ to bound $Ca^{2+}$ and force development in the same muscle fiber.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (AM 10551) and the Muscular Dystrophy Association. Ms. Beverly Black provided outstanding technical assistance.

REFERENCES

1 Fuchs, F. and Briggs, F. N. (1968) J. Gen Physiol. 51, 655–676
2 Ebashi, S., Kodama, A. and Ebashi, F., (1968) J. Biochem 64, 465–477
3 Ebashi, S., Endo, M. and Ohtuski, I. (1969) Quart. Rev. Biophys. 2, 351–384
4 Potter, J. and Gergely, J. (1975) J. Biol. Chem. 250, 4528–4533
5 Murray, J. M., Weber, A. and Bremel, R. D. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, E., Clementi, F., Drabikowski, W., and Margreth, A., eds.) pp. 489–496, North-Holland, Amsterdam
6 Bremel, R. D. and Weber, A. (1972) Nature New Biol. 238, 97–101
7 Weber, A. and Murray, J. M. (1973) Physiol. Rev. 53, 612–673
8 Fuchs, F. (1974) Ann. Rev. Physiol. 36, 461–502
9 Fuchs, F. and Bayuk, M. (1976) Biochim. Biophys. Acta 440, 448–455
10 Fuchs, F. (1977) Biochim. Biophys Acta 491, 523–531
11 Taylor, D. L. (1976) J. Cell Biol. 68, 497–511
12 Ogawa, Y., Harigaya, S., Ebashi, S. and Lee, K. S. (1971) in Methods in Pharmacology (Schwartz, A., ed.) Vol. I, pp. 327–346, Appleton-Century-Crofts, New York
13 Godt, R. (1974) J. Gen Physiol. 63, 722–739
14 Page, S. G. and Huxley, H. E. (1963) J. Cell Biol. 19, 369–390
15 Marston, S. and Tregear, R. T. (1974) Biochim. Biophys. Acta 347, 311–318
16 Solaro, R. J., Bruni, F. D. and Gleason, E. W. (1976) Biochim. Biophys. Acta 449, 304–309
17 Chaplain, R. A. and Gergs, U. (1974) Biochem. Biophys. Res. Commun. 61, 297–305
18 Gergely, J. (1976) in Cell Motility (Goldman, R., Pollard, T. and Rosenbaum, J., ed.) Book A, pp. 137–149, Cold Spring Harbor, New York
19 Hellam, D. C. and Podolsky, R. J. (1969) J. Physiol. 200, 807–819
20 Julian, F. J. (1971) J. Physiol. 218, 107–145
21 Fabiato, A. and Fabiato, F. (1975) J. Physiol. 249, 497–517
22 Kerrick, W. G. L., Secrist, D., Coby, R. and Lucas, S. (1976) Nature 260, 440–441
23 Schadler, M. (1967) Pflugers Archiv. 296, 70–90
24 Solaro, R. J., Wise, R. M., Shiner, J. S. and Briggs, F. N. (1974) Circ. Res. 34, 525–530
25 Levy, R. M., Umazume, V. and Kushmerick, M. J. (1976) Biochim. Biophys. Acta 430, 352–365

26 Donaldson, S. K. B. and Kerrick, W. G. L. (1975) J. Gen. Physiol. 66, 427–444
27 Filo, R. S., Bohr, D. F. and Ruegg, J. C. (1965) Science 147, 1581–1583
28 Chaplain, R. A. and Gergs, U. (1974) Biochem. Biophys. Res. Commum. 61, 517–524
29 Jewell, B. R. and Wilkie, D. R. (1960) J. Physiol. 152, 30–47
30 Kaufmann, R. L., Bayer, R. M. and Harnasch, C. (1972) Pflugers Archiv. 332, 96–116
31 Huxley, A. F. (1957) Prog. Biophys. Biophys. Chem. 7, 255–318
32 Endo, M. (1972) Nature New Biol. 237, 211–213