hind the cytochrome c marker, suggesting a molecular weight of about 12,000. The behavior on gel filtration of media from lymphocytes incubated in the presence of lectins was not determined. Crude LM or activity partially purified by gel filtration did not increase the proliferation of synovial cells over controls as judged by cell counting or by uptake of [^{3}H]thymidine (not shown), nor did it stimulate lactate production (Fig. 3), suggesting an effect different from the so-called connective tissue–activating peptide which characteristically stimulates glycolysis in its target cells (*12*). The synthesis of lysozyme, a characteristic product of macrophages (*13*), was also not stimulated by the factor, providing additional evidence that the responsive synovial cells are distinct from typical macrophages (*3*). Furthermore, we could not detect collagenase activity in cultures of human peripheral blood monocytes exposed to lymphocyte media. The levels of collagenase in guinea pig macrophages stimulated by lymphocyte products as reported by Wahl *et al.* (*7*) are calculated to be less than 0.1 percent of those of the stimulated human synovial cells in the present study.

The biological significance and specificity of the production and effects of this lymphocyte factor remain to be determined. Lymphocytes are abundant in the subsynovial region in rheumatoid synovitis, and the production of this stimulating factor might serve as a cell-to-cell mediator acting between lymphocytes and susceptible target cells in the proliferative synovial lining layer. If the factor can stimulate collagenase in vivo, it may have an important role in regulating connective tissue turnover, and may be responsible for some of the deleterious effects on joint structures in rheumatoid arthritis and connective tissue resorption in a variety of other inflammatory disorders.

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References and Notes

1. E. D. Harris, Jr., and S. M. Krane, *N. Engl. J. Med.* **291**, 557, 605, and 652 (1974).
2. S. M. Krane, *Ann. N.Y. Acad. Sci.* **256**, 289 (1975).
3. J.-M. Dayer, S. M. Krane, R. G. G. Russell, D. R. Robinson, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 945 (1976).
4. H. Ishikawa and M. Ziff, *Arthritis Rheum.* **19**, 1 (1976); J. A. van Boxel and S. A. Paget, *N. Engl. J. Med.* **293**, 517 (1975).
5. D. T. Y. Yu and J. B. Peter, *Semin. Arthritis Rheum.* **4**, 25 (1974).
6. P. Stastny, M. Rosenthal, M. Andreis, D. Cooke, M. Ziff, *Ann. N.Y. Acad. Sci.* **256**, 117 (1975).
7. L. M. Wahl, S. M. Wahl, S. E. Mergenhagen, G. R. Martin, *Science* **187**, 261 (1975).
8. A. Böyum, *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97), 77 (1968).
9. J. M. Evanson, J. J. Jeffrey, S. M. Krane, *J. Clin. Invest.* **47**, 2639 (1968).
10. G. J. Todaro and C. A. Meyer, *J. Natl. Cancer Inst.* **52**, 167 (1974).
11. H. Arvilommi and L. Räsänen, *Nature (London)* **257**, 144 (1975).
12. C. W. Castor, *Ann. N.Y. Acad. Sci.* **256**, 304 (1975).
13. S. Gordon and Z. A. Cohn, *Int. Rev. Cytol.* **36**, 171 (1974).
14. O. H. Lowry, J. V. Passonneau, F. X. Hasselberger, D. W. Schulz, *J. Biol. Chem.* **239**, 18 (1964).
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Localization of Cyclic GMP and Cyclic AMP in Cardiac and Skeletal Muscle: Immunocytochemical Demonstration

Abstract. *When rat cardiac and skeletal muscle are explored by immunocytochemical procedures designed to show sites of localization of adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP), distinct staining patterns for the two nucleotides are seen. Antibody to cyclic AMP is found in the area of the sarcoplasmic reticulum, while antibody to cyclic GMP is found with a periodic distribution corresponding to that of the A band. This suggests a role for cyclic GMP in the regulation of myosin.*

The regulation of calcium concentration by the membranes of the sarcoplasmic reticulum is important in coupling excitation and contraction in muscle. Evidence suggests that adenosine 3',5'-monophosphate (cyclic AMP) is involved in the sequestration of calcium by the sarcoplasmic reticulum (*1*). Cyclic AMP stimulates the activity of protein kinase and increases calcium uptake by enriched preparations of fragmented sarcoplasmic reticulum isolated from cardiac and fast skeletal muscle (*1*). Calcium uptake by the sarcoplasmic reticulum is associated with relaxation of both cardiac and skeletal muscle (*2*).

Changes in the intracellular concentrations of cyclic AMP and cyclic GMP have been observed in each contraction cycle in heart (*3*). In addition, cyclic GMP levels increase in cardiac muscle after stimulation with acetylcholine (*4*). However, the role of cyclic GMP in the control of contraction is not understood. In a number of tissues in vitro the increased concentration of cyclic GMP observed after the addition of acetylcholine and other agents depends upon the presence of extracellular calcium (*5*). This suggests that calcium fluxes participate in the control of intracellular cyclic GMP concentrations. These and other experiments have led to the postulate that cyclic GMP might participate in the feedback regulation of calcium transport across membranes (*6*).

To investigate the roles of both cyclic nucleotides in cardiac and skeletal muscle, we have used an immunocytochemical technique that reveals sites of localization of cyclic AMP and cyclic GMP in specific cell types and in a variety of tissues (*7*). We have found that in a number of rat tissues cyclic GMP is predominantly located in the plasma membrane area and in the nucleus, whereas cyclic AMP is often found in cytoplasmic elements and in the plasma and nuclear membrane areas. Since cyclic nucleotide immunocytochemistry utilizes unfixed tissues, and free cyclic nucleotide might be lost during the staining procedure, sites of localization of these cyclic nucleotides appear to indicate sites of binding or receptor proteins.

Cardiac and skeletal muscle were obtained from rats killed by cervical dislocation. Skeletal muscle was taken from the lower part of either the fore or hind limbs and the skin quickly dissected away. Tissues were immersed in OCT compound (optimal cutting temperature; Miles, Elkhart, Indiana) and frozen in an aluminum container in a mixture of acetone and solid CO_2. Tissues were frozen within 1 minute after removal, and were later cut into sections, 3 to 4 μm thick, with a cryostat. The sites of localization of both nucleotides were determined by an indirect immunofluorescent technique (*7*) for which we used rabbit immunoglobulin (Ig) specific to either cyclic AMP or to cyclic GMP, prepared by the method of Steiner *et al.* (*8*). Air-dried slides were treated sequentially as described previously (*7*). To test the specificity of the procedure, we showed that similarly prepared Ig fractions from unimmunized rabbits failed to produce significant staining. In addition, passage of the specific

antiserum over an affinity column of Sepharose coupled to the antigen eliminated the specific nucleotide staining pattern (*9*).

Localization of fluorescence with respect to the A and I bands of muscle was determined by photographing fluorescent areas under identical geometric optical conditions between crossed polarizers in visible light and in fluorescent light, and matching the fluorescent and polarization optical images of the very same area.

Fluorescent antibody against cyclic AMP appeared in the area of the sarcoplasmic reticulum and the sarcolemma in both skeletal and cardiac muscle (Fig. 1). This pattern of localization supports biochemical data identifying a cyclic AMP–dependent protein kinase in microsomal fractions of both cardiac and slow skeletal muscle (*1*). Strong fluorescence attributed to cyclic AMP was also located at the intercalated disks of cardiac muscle (Fig. 1B). In addition, in longitudinal sections of cardiac muscle, but not of skeletal muscle, faint periodic cross bands stained with the antibody to cyclic AMP. When identical tissue sections photographed sequentially by fluorescence and polarized light were compared, fluorescence was found to superimpose on both the I and A bands. This pattern suggests that cyclic AMP could be associated with elements of the sarcoplasmic reticulum, which ensheaths both bands, though it is possible that some cyclic AMP is associated with structural proteins present in both bands of the myofibrils. Analysis of the cyclic AMP content and binding capabilities of the principle proteins of the contractile elements should help to answer this question. The association of cyclic AMP with proteins of the tropinin-tropomyosin complex which are associated with the thin filaments would be of particular interest because biochemical data suggest a possible role of cyclic AMP in the phosphorylation of troponin I in cardiac muscle (*10*).

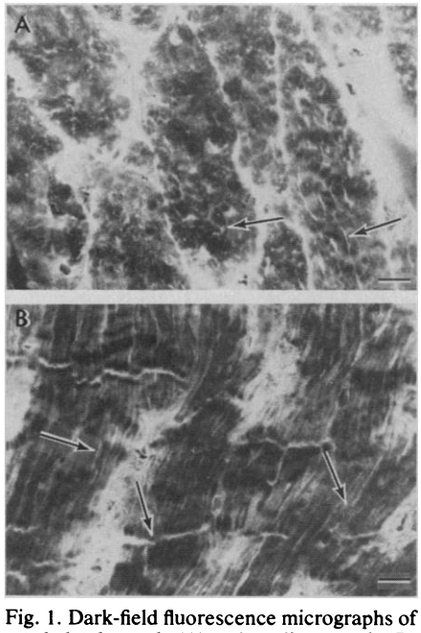

Fig. 1. Dark-field fluorescence micrographs of rat skeletal muscle (A) and cardiac muscle (B) stained with fluorescent antibody to cyclic AMP. In (A) the arrows are directed to fluorescence of sarcoplasmic reticulum from fibers cut in cross section (left) and longitudinal section (right). In (B) the arrow on the left points to fluorescence on the sarcoplasmic reticulum; the middle arrow depicts fluorescence on the intercalated disk; the arrow on the right identifies the faintly fluorescent periodic banding. Scale bar, 10 μm.

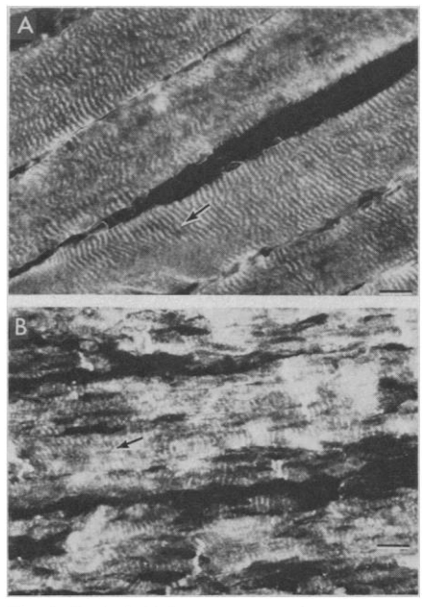

Fig. 2. Dark-field fluorescence micrographs of longitudinal sections of rat skeletal muscle (A) and cardiac muscle (B) stained with antibody to cyclic GMP. In (A) the arrow points to a cross band stained with cyclic GMP antibody. In (B) the arrow points to GMP on a cross band. Note that the intercalated disks fluoresce in cardiac muscle. Scale bar, 10 μm.

The pattern of localization, with fluorescent antibody specific for cyclic GMP, was distinctly different from that specific for cyclic AMP (Fig. 2). Evidence for cyclic GMP appeared predominantly in both tissues in bands with a distinct periodicity ranging from 1.2 to 2.5 μm. Fluorescence was the most striking in skeletal muscle and was observed in contracted and in relaxed regions. Fluorescence appears to be more intense in contracted portions, but we have not attempted to quantitate the differences. In addition, in cardiac muscle the intercalated disks fluoresced brightly (Fig. 2B). Neither the sarcoplasmic reticulum nor the sarcolemma fluoresced in either cardiac or skeletal muscle when fluorescent antibody against cyclic GMP was applied.

Matched polarization and fluorescence photographs of the same area showed that the fluorescence of antibody to cyclic GMP corresponded to the anisotropic bands (Fig. 3B). This coincidence indicates that bound cyclic GMP is present in the A bands, which contain myosin. The absence of fluorescence in the I band suggests that cyclic GMP is not bound to receptors in actin, troponin, or tropomyosin in amounts sufficient to be detected by this immunocytochemical method.

The differential localization of the two nucleotides in both cardiac and skeletal muscle suggests diverse roles for cyclic AMP and cyclic GMP in the regulation of muscle contraction. Our studies support biochemical data indicating a role for cyclic AMP in regulating calcium uptake by the sarcoplasmic reticulum (*1*). The binding of fluorescent antibody to cyclic GMP in the A band region suggests that cyclic GMP may have a role in regulating a function of myosin. Recently, Pires *et al.* (*11*) described a calcium-sensitive protein kinase which phosphorylates the myosin light chain component of molecular weight 18,500 from white and red skeletal muscle and from cardiac muscle. This protein kinase does not appear to be regulated by cyclic AMP, either in the absence or presence of calcium ion (*11*). Perhaps cyclic GMP participates in the control of phosphorylation by this light chain kinase or in the dephosphorylation

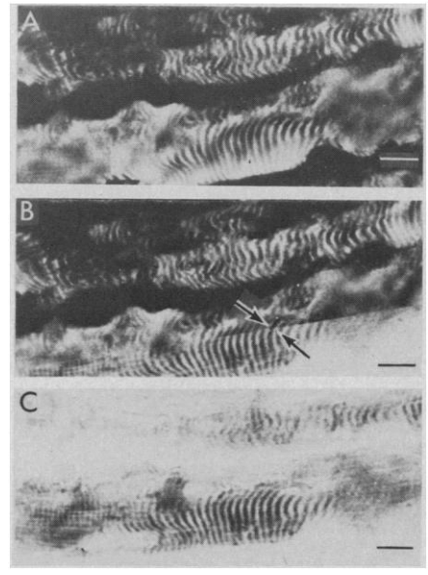

Fig. 3. Evidence for the localization of antibody to cyclic GMP in the A band of rat skeletal muscle. The same section was photographed (A) by fluorescence and (C) by polarization microscopy. In (B) the photographs from (A) and (C) are aligned and matched along the line indicated by arrows. The fluorescent and anisotropic bands superimpose, thus specifying the A band as the site of binding of fluorescent antibody to cyclic GMP. Scale bar, 10 μm.

of the light chain by a phosphatase. Cyclic GMP might also in some manner modulate the binding of cations, perhaps calcium, in the components of myosin, since evidence in other tissues suggests a close interrelation between calcium uptake and changes in cyclic GMP concentrations (*5, 6*). Cyclic GMP might also be involved in the regulation of the structural integrity of myosin in some other manner.

These findings might have wider applicability in the study of cellular motion. Actin and myosin, in addition to their role in muscle contraction, are also found in association with plasma membrane and other intracellular organelles which may regulate intracellular rearrangement (*12*). Furthermore, cyclic nucleotides have been postulated to play a role in the modulation of cell surface and cytoskeletal movement (*13*). Thus, cyclic nucleotide immunocytochemistry should be helpful in establishing in further detail the role of the cyclic nucleotides in these contractile processes.

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References and Notes

1. M. A. Kirchberger, M. Tada, D. I. Repke, A. M. Katz, *J. Mol. Cell. Cardiol.* **4**, 673 (1972); M. A. Kirchberger, M. Tada, A. M. Katz, *J. Biol. Chem.* **249**, 6166 (1974); H. Wray, R. R. Gray, R. A. Olsson, *ibid.* **248**, 1496 (1973).
2. S. Ebashi, M. Endo, I. Ohtsuki, *Q. Rev. Biophys.* **2**, 251 (1969).
3. G. Brooker, *Science* **182**, 933 (1973); A. Wollenberger, E. Babskii, E. Krause, S. Genz, D. Blohm, E. Bogdanova, *Biochem. Biophys. Res. Commun.* **55**, 446 (1973).
4. W. J. George, J. B. Polson, A. G. O'Toole, N. D. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 398 (1970).
5. G. Schultz, J. G. Hardmann, K. Shultz, C. E. Baird, E. W. Sutherland, *ibid.* **70**, 3889 (1973).
6. M. J. Berridge, *Adv. Cyclic Nucleotide Res.* **6**, 1 (1975).
7. H. J. Wedner, B. J. Hoffer, E. Battenberg, A. L. Steiner, C. W. Parker, F. E. Bloom, *J. Histochem. Cytochem.* **20**, 293 (1972); S. H. Ong, T. H. Whitley, N. W. Stowe, A. L. Steiner, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2022 (1975); J. W. Kebabian, F. E. Bloom, A. L. Steiner, P. Greengard, *Science* **190**, 157 (1975); A. L. Steiner, S. H. Ong, H. J. Wedner, *Adv. Cyclic Nucleotide Res.* **7**, 115 (1976).
8. A. L. Steiner, D. M. Kipnis, R. Utiger, C. W. Parker, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 367 (1969); A. L. Steiner, C. W. Parker, D. M. Kipnis, *J. Biol. Chem.* **247**, 1106 (1972).
9. E. F. Fallon, R. Agrawal, E. Furth, A. L. Steiner, R. Cowden, *Science* **184**, 1089 (1974).
10. R. Rubio, C. Bailey, C. Villar-Palasi, *J. Cyclic Nucleotide Res.* **1**, 143 (1975).
11. P. E. Pires, S. V. Perry, M. A. W. Thomas, *FEBS Lett.* **41**, 292 (1974).
12. T. D. Pollard and R. R. Weihing, *Crit. Rev. Biochem.* **2**, 1 (1973); Y. Yang and J. G. Perdue, *J. Biol. Chem.* **247**, 4503 (1972); R. S. Adelstein, M. A. Conti, G. S. Johnson, I. Pastan, T. D. Pollard, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3693 (1972); R. Pollack, M. Osborn, K. Weber, *ibid.* **72**, 994 (1975); G. M. Edelman, *Science* **192**, 218 (1976).
13. I. Pastan and G. S. Johnson, *Adv. Cancer Res.* (1974), p. 303.
14. We thank H. Stanley Bennett for his invaluable advice and criticism of this work. Supported by PHS grant AM17438.

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Perceived Lightness Depends on Perceived Spatial Arrangement

Abstract. *The perceived shade of gray depends primarily on the luminance relationship between surfaces perceived to lie in the same plane and not between surfaces that are merely adjacent in the retinal image. This result implies that depth perception must precede lightness perception and that lateral inhibition cannot explain lightness constancy.*

A change in the perceived spatial position of a surface can change its perceived color from black to white or from white to black. This finding challenges the widespread view that denies any substantial role of depth perception in the perception of surface lightness (the shade of gray between white and black).

Since 1948, when Hans Wallach published his classic experiments in lightness constancy (*1*), a consensus in this field has held that perceived lightness is a function of luminance ratios between adjacent parts of the retinal image, regardless of where those parts are perceived to lie in three-dimensional space. Moreover, because of Wallach's emphasis on retinal adjacency, many researchers (*2*) have concluded that lateral inhibitory connections among retinal cells provide the neural mechanism underlying the ratio principle.

A number of investigators (*3–7*) have sought to show that retinal ratios do not tell the whole story. Essentially the approach has been to change the apparent spatial position of a target surface so that it either appears to lie in the same plane as that of its surrounding surface or in a different plane in order to determine whether the apparent spatial separation between the surfaces reduces their interaction and thus produces a different perceived color in the target even though the two-dimensional retinal pattern remains unchanged. Two studies (*3, 4*) reported changes as great as one and a quarter steps on the Munsell scale (*8*), or 17 percent of the difference between black and white. Most (*5–7*) have reported little or no change.

With a few exceptions (*9*), it is now generally agreed (*10*) that perceived lightness is essentially determined by the relative intensities of adjacent parts of the retinal array. The experiments that I report here grew out of a seeming inconsistency between the retinal ratio theory and everyday experience. Rarely are black, white, and gray surfaces grossly misperceived. Yet the retinal ratio theory would predict consistently accurate lightness perception only when the difference in luminance at the retina is produced by a difference in the reflectance of the external surfaces. When the difference occurs because external surfaces that receive unequal amounts of illumination are imaged on adjacent parts of the retina, sizable lightness illusions should be expected. This difficulty is mitigated by the fact that the boundary between different levels of illumination is frequently gradual. However, illumination boundaries are by no means always gradual. For example, the retinal image can con-

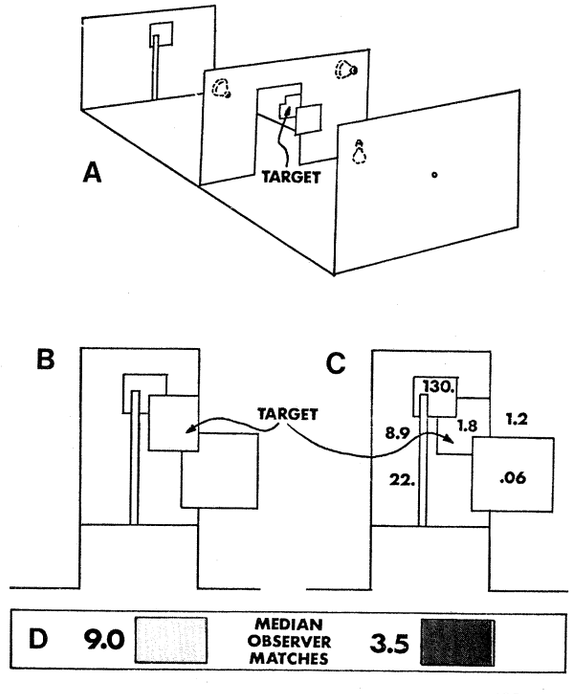

Fig. 1. (A) Perspective view of the apparatus showing hidden light bulbs. The displays (as seen through the pinhole) in which the target appeared to be located either (B) in the near plane or (C) in the far plane. (D) The average match from a Munsell chart for the two displays. Luminances (C) are in foot-lamberts.