Rapid Publication

Calcium–Calmodulin-Dependent Myosin Phosphorylation by Pancreatic Islets

MICHAEL J. MACDONALD AND ANJANEYULU KOWLURU

SUMMARY

Pancreatic islets contain enzyme activity which catalyzes the phosphorylation by MgATP of cardiac, skeletal, or smooth muscle myosin light chains. The enzyme is activated by calcium (Ka = 10 μM) and calmodulin (Ka = 2 nM) and inhibited by trifluoperazine (Ki = 10 μM), a known inhibitor of calmodulin and of insulin secretion. The enzyme binds to a calmodulin affinity column when Ca^{2+} is present and is eluted when Ca^{2+} is omitted. These are the properties of myosin light chain kinase. Since phosphorylation of smooth muscle myosin is necessary for its activation by actin, the kinase may have a key role in coupling stimuli that increase intracellular calcium to the contractile processes involved in insulin secretion.
DIABETES *31*:566–570, June 1982.

Insulin secretion from the pancreatic beta-cell, induced by a number of secretagogues, is accompanied by an increase in intracellular calcium, and secretion may well depend on contractile proteins similar to those of muscle (microfilaments) and cilia (microtubules). Available evidence is consistent with a saltatory movement of insulin granules from the Golgi complex to the plasma membrane of the beta-cell oriented by a lattice of microtubules and powered by actomyosin contraction (reviewed in refs. 1 and 2). Microfilaments may also participate in opening of the plasma membrane during the extrusion of insulin granules. The requirement for extracellular calcium, depolarization of the beta-cell plasma membrane, the requirement for ATP (reviewed in ref. 3), and potentiation of insulin release by oubain[4] are each reminiscent of muscle contraction.

Although calcium is generally believed to be important in coupling stimuli to the insulin secretory process, little specific information exists on how calcium acts in the beta-cell. A prime target for calcium might be myosin light chain kinase (MLCK). This enzyme is inhibited by micromolar concentrations of trifluoperazine,[5] an inhibitor of calmodulin-requiring enzymes[6] and, indeed, similar low concentrations of trifluoperazine potently inhibit insulin secretion in vitro.[7,8] MLCK is a recently discovered calcium-dependent enzyme found in smooth, skeletal, and cardiac muscle as well as in nonmuscle tissues such as brain and platelets.[6] Since calmodulin is a regulatory subunit of the enzyme,[9] this accounts for its susceptibility to inhibition by trifluoperazine. The enzyme is an ATP-myosin light chain phosphotransferase. Current evidence suggests that the MgATPase activity of smooth muscle myosin in the phosphorylated state can be activated by actin, whereas the ATPase activity of the unphosphorylated myosin is not affected by actin and remains at a low level.[10] Since MLCK is active only in the presence of Ca^{2+}, a Ca^{2+}-dependent activation of contraction is achieved. The possibility of pancreatic islets containing MLCK has been mentioned recently in the literature,[1,2,11] but attempts to detect the enzyme have not been reported, possibly because standard assay procedures for its activity are more suitable for large amounts of pure enzyme and its substrate (myosin light chains)[12] than for the impure enzyme in small amounts of tissue, such as in pancreatic islets. We have used procedures that enabled us to demonstrate MLCK activity in pancreatic islets.

MATERIALS AND METHODS

Materials. Cardiac (calmodulin-free) and skeletal muscle myosin light chains (not calmodulin-free) were generous gifts of Dr. S. James Stull and Donald Blumenthal[12] and bull testis calmodulin was a generous gift of Dr. Frank Siegel. Smooth muscle myosin light chains were purified from fresh turkey gizzards.[13]

Tissue preparation. Pancreatic islets were isolated from well-fed Sprague-Dawley rats, washed twice in 200 mM mannitol, 70 mM sucrose, 10 mM triethanolamine chloride, pH 7.5, and 1 mM ethylene-bis (oxyethylene-nitrilo) tetra-acetic acid (EGTA) and then washed twice more and ho-

From the Institute for Enzyme Research and Department of Pediatrics, University of Wisconsin, Madison, Wisconsin.
Address reprint requests to Michael J. MacDonald, Institute for Enzyme Research, 1710 University Avenue, Madison, Wisconsin 53706.
Received for publication 16 February 1982.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/31/6/566/506178/31-6-566.pdf by guest on 02 December 2023

mogenized in the same solution without EGTA.[14] Cytosol was the 105,000 × g × 1 h supernatant fraction.

MLCK assay. MLCK activity was assayed at 37°C in a volume of 40 μl containing 50 mM 3-(N-morpholino)-propanesulfonic acid, pH 7.0, 10 mM magnesium acetate, 15 mM 2-mercaptoethanol, 55 mM mannitol, 18 mM sucrose 25 μM [γ-^{32}P]ATP (2500 cpm/pmol), and unless stated otherwise, 150 nM calmodulin, 50 μM cardiac or skeletal muscle myosin phosphorylatable light chains, and 100 μM free $CaCl_2$. The reaction was started by adding ATP and stopped after 10 min by adding electrophoresis sample buffer to give a final sodium dodecyl sulfate (SDS) concentration of 1%. The samples were immediately heated at 100°C for 2 min and electrophoresis in 0.1% SDS 10% polyacrylamide slab gels was done by the method of Laemmli.[15] ^{32}P incorporation into protein was demonstrated by exposing the gel to X-Omat AR film (E. Kodak Co.) and Cronex Lightning Plus intensifying screens (E. I. DuPont De Nemours Co.)[16] at −70°C for 12–24 h and quantitated by densitometry. The peaks under the densitometric tracings were cut out of the paper on which they were drawn, weighed to estimate their area, and MLCK activity was expressed as a percent of maximum labeling. After the autofluorography the gels were stained for protein with Comassie blue to identify molecular-weight marker proteins. Proteins with the following relative subunit molecular weights were used: rabbit muscle phosphorylase a (92,500), pyruvate kinase (57,000), and aldolase (40,000); cow's milk lactoperoxidase (82,000), bovine serum albumin (68,000), pig heart fumarase (38,000), bovine pancreas chymotrypsinogen (25,000) and RNAse (13,700), and horse heart myoglobin c (17,200) and cytochrome c (12,500).

Since a crude homogenate was used in the experiment depicted in Figure 1, NaF (30 mM) and oligomycin (3 μg/ml) were added to the assay mixture to inhibit ATPases. Skeletal muscle myosin light chains (5 μM phosphorylatable light chains) were used in experiments depicted in the two upper panels and cardiac muscle myosin light chains (15 μM of phosphorylatable light chains) were used in the experiments depicted in the lowest panel of Figure 2. Free calcium concentrations of 5 μM or lower were maintained by varying the ratio of $CaCl_2$ to 10 mM EGTA.[17] Higher calcium concentrations were calculated from the measured free calcium in the assay mixture and the added calcium. Free calcium in the assay mixture was measured with antipyrylazo III[18] and in the experiment depicted in the uppermost panel of Figure 2 the free Ca^{2+} was 10 μM before the addition of Ca^{2+}. Smooth muscle myosin light chains (8 μg) were used, when added, in the experiment depicted in Figure 4.

DEAE chromatography. To study calmodulin activation of MLCK, cytosol was chromatographed on diethylaminoethyl-(DEAE)-Sephacel in the presence of EGTA to remove endogenous calmodulin.[19] Cytosol from 1500 islets was dissolved in 1.5 ml of 10 mM sodium phosphate, pH 7.5, 1 mM dithiothreitol, 1 mM EGTA (column buffer), and applied to a 3.7 cm × 0.1 cm² DEAE Sephacel column. The column was washed with 0.5 ml of column buffer and then 0.5 ml of column buffer containing 0.15 M KCl. MLCK was eluted with 0.3 M KCl.

Calmodulin-Sepharose chromatography. Calmodulin was coupled to cyanogen bromide-activated Sepharose-4B.[20] Cytosol from 1500 islets was dissolved in 1.0 ml of 75 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM dithiothreitol and 20 mM Tris chloride, pH 7.5 (column buffer), and applied to a 3.0 × 0.1 cm² calmodulin Sepharose column.[12] The column was washed with 1.0 ml of column buffer and MLCK was eluted with column buffer containing 1 mM EGTA in place of $CaCl_2$. Five fractions of 0.2 ml were collected and 20 μl of these fractions, the "wash" fraction and the original sample, was used in the MLCK assay.

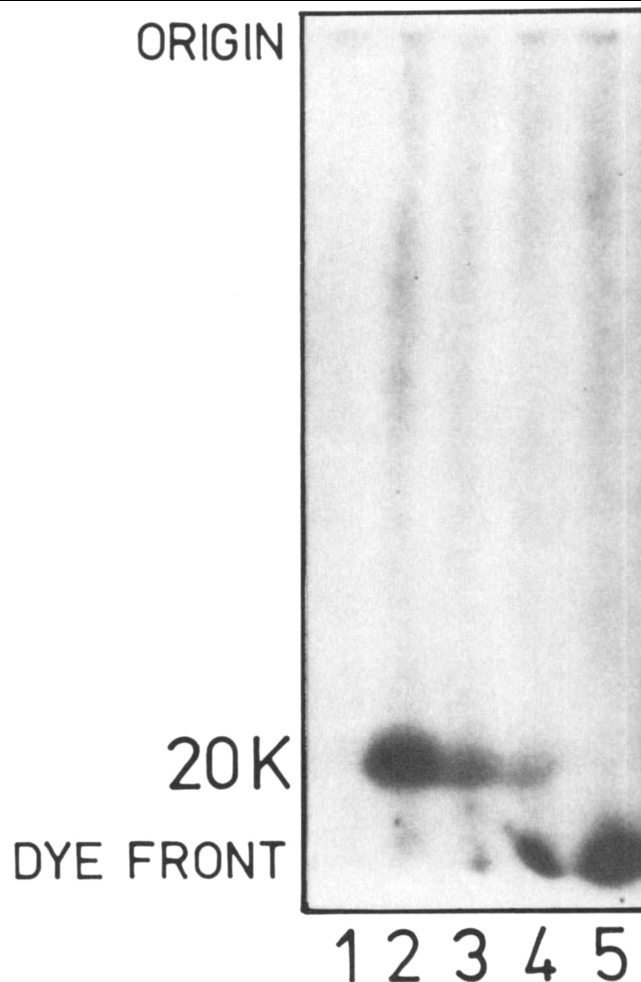


FIGURE 1. Autofluorograph of an SDS polyacrylamide gel showing islet MLCK activity. Phosphorylation by [γ-^{32}P]MgATP of cardiac myosin light chains (20K) catalyzed by an islet homogenate. Lane 1, homogenate omitted; lane 2, complete assay mixture; lane 3, 50 μM trifluoperazine added; lane 4, 6 mM EGTA added; and lane 5, light chains omitted.

RESULTS

Figure 1 is a photograph of a film exposed to an SDS polyacrylamide gel. It shows that an homogenate of pancreatic islets catalyzes labeling by [γ-^{32}P]ATP of cardiac muscle light chains (mol wt 20,000). Labeling occurred only when both homogenate and light chains were present and it was strongly inhibited by trifluoperazine or by chelating calcium with EGTA. Only the added light chains, in contrast to islet proteins, were labeled. This was accomplished by using a low amount of homogenate equivalent to that from about eight islets. In other experiments, greater than 95% of the myosin light chain kinase activity was found in the islet cytosol; therefore cytosol was used for subsequent studies.

The effects of various calcium, trifluoperazine, and cal-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/31/6/566/506178/31-6-566.pdf by guest on 02 December 2023

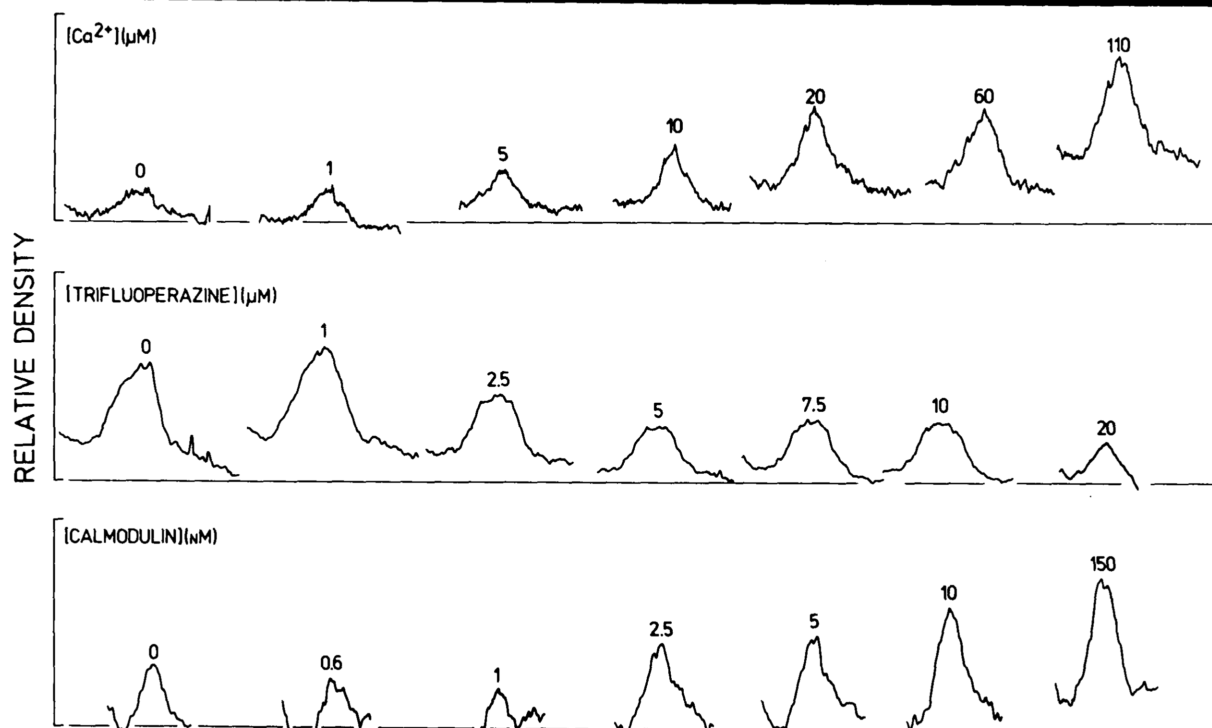


FIGURE 2. Effect of calcium, trifluoperazine, and calmodulin on pancreatic islet MLCK activity. The effects of various concentrations of the three agents on densitometric tracings of the myosin light chain band on autofluoragraphs are shown.

modulin concentrations on the amount of ^{32}P labeling of the myosin light chain band were estimated by densitometry (Figure 2). Figure 3 shows the influence of the three effectors on the areas under the peaks from Figure 2 combined with data from additional experiments. The data show that enzyme activity is correlated positively with the calcium and calmodulin concentrations and negatively with the trifluoperazine concentration. One-half maximal enzyme activity was attained at about 10 μM Ca^{2+} in the presence of maximal stimulating calmodulin (150 nM). The average concentration of trifluoperazine that gave 50% inhibition was about 10 μM. The enzyme was partially purified from islet cytosol by chromatography in the presence of EGTA on DEAE Sephacel to remove calmodulin in order to study activation by exogenous calmodulin. The calmodulin concentration that gave one-half maximal enzyme activity was about 2 nM (Figure 3) and 10 nM calmodulin gave 90% of maximal activity. The low enzyme activity present in the absence of added calmodulin might be due to a trace of calmodulin present in the enzyme preparation.

The islet MLCK activity binds to a calmodulin affinity column (Figure 4) and this strongly indicates that the enzyme is

FIGURE 3. Effect of calcium, trifluoperazine, and calmodulin on pancreatic islet MLCK activity. MLCK activity is expressed as a percent of maximal labeling of the myosin light chain band.

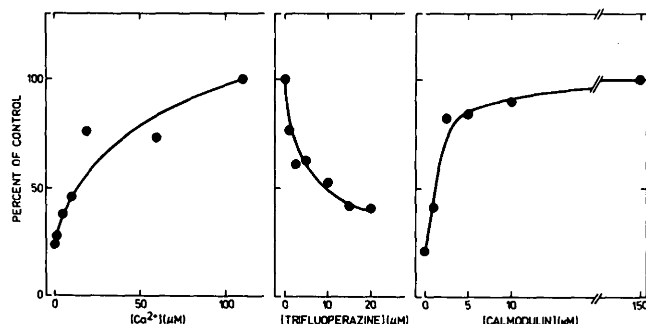
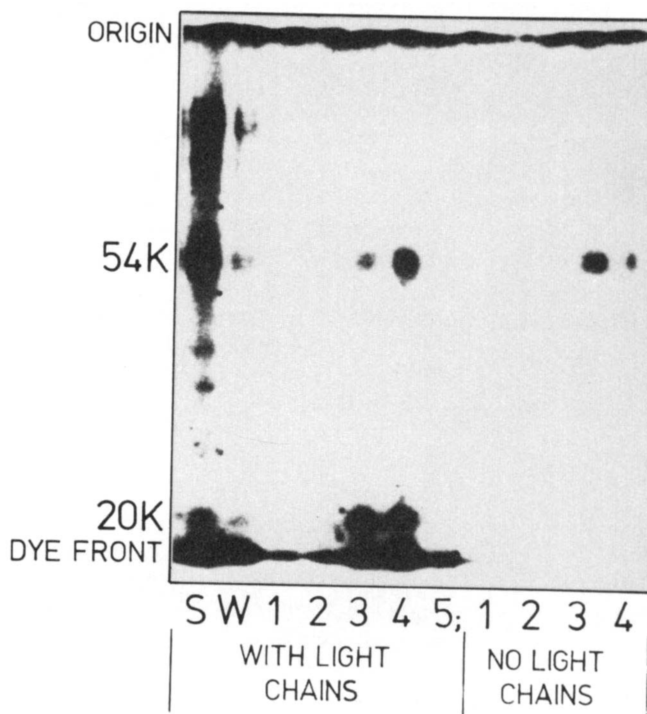


FIGURE 4. Binding of pancreatic islet MLCK to calmodulin-Sepharose. MLCK activity was measured in the sample before (S) and after (W) it was applied to the calmodulin-Sepharose column (Ca^{2+} present) and in the eluate when Ca^{2+} was omitted from the column buffer (Fractions 1–5) in the presence and absence of smooth muscle light chains.



Downloaded from http://diabetesjournals.org/diabetes/article-pdf/31/6/566/506178/31-6-566.pdf by guest on 02 December 2023

a calmodulin-requiring enzyme. When Ca^{2+} was present in the column buffer during the application of the islet cytosol sample and during washing of the column, the column eluate contained negligible MLCK activity, but when Ca^{2+} was omitted from, and EGTA added to, the column buffer (fractions 1–5) MLCK activity was eluted from the column. (MLCK activity was concentrated in fractions 3 and 4.) Smooth muscle myosin light chains were used as the substrate for MLCK in this experiment. When the light chains were omitted from the assay mixture, none of the column fractions catalyzed labeling of a 20,000-mol-wt band.

A phosphoprotein with a subunit mol wt of 54,000, and apparently a protein kinase besides MLCK, was eluted from the calmodulin column. These proteins are the subjects of a separate investigation.

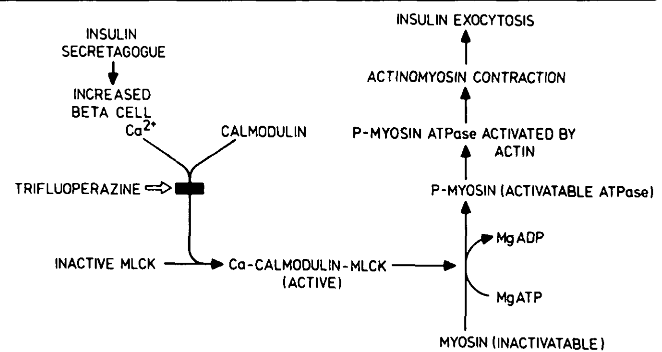


FIGURE 5. Theoretical scheme for MLCK participation in insulin exocytosis and site of trifluoperazine inhibition.

DISCUSSION

Cells normally maintain free calcium in the cytoplasm at a concentration of about 10^{-7} M. An increase of intracellular-free calcium to above 10 μM is believed to activate many calcium-dependent processes, such as muscle contraction or insulin secretion. The present work shows that the concentration of Ca^{2+}, which gives one-half maximal activation of pancreatic islet MLCK, is approximately 10 μM (Figures 2 and 3). The concentrations of Ca^{2+} and calmodulin that activate the islet MLCK are very similar to those that activate the purified enzyme from skeletal muscle[12] and the concentrations of trifluoperazine that inhibit the islet MLCK (Figures 2 and 3) are similar to those that inhibit insulin secretion by isolated pancreatic islets (10–20 μM).[7,8,11]

Maximal inhibition of MLCK of 60% by 20 μM trifluoperazine was observed (Figure 3). It is interesting that higher concentrations of trifluoperazine were either no more inhibitory or less inhibitory (data not shown). This apparent dual effect of trifluoperazine matches the effects of calmodulin inhibitors on insulin release and on activities of other secretory tissues. We have noticed that 100 μM trifluoperazine is sometimes less or no more inhibitory of insulin release from isolated pancreatic islets than is 10 μM trifluoperazine. Others have noticed that 50 μM trifluoperazine[7] or 200 μM W-7 (also a calmodulin inhibitor at 25 μM)[21] increased basal insulin release from pancreatic islets. In experiments on human neutrophils, Smolen et al.[22] found that 30 μM trifluoperazine inhibited lysosomal enzyme release essentially 100%, whereas 100 μM trifluoperazine inhibited enzyme release only 0–17%. Similarly, Egrie (working in F. Siegel's laboratory)[23] found that 100 μM desipramine inhibited calmodulin-dependent activation of adrenal medullary cyclic nucleotide phosphodiesterase 83%, but 1–10 mM desipramine was less inhibitory. It is possible that at high concentrations the calmodulin "inhibitors" may act like calmodulin itself and be stimulatory.

The effect of insulin secretagogues on calcium handling by the beta-cell is believed to be of fundamental importance in stimulus-secretion coupling.[3] Besides the fact that the beta-cell contains microfilamentous proteins, such as actin and myosin, and that calcium is often involved in contractile processes, there seems to be little direct biochemical evidence for the interaction of calcium with microfilaments in the beta-cell. The work reported here strongly points toward MLCK being a potential target for calcium in the beta-cell. When the binding sites of calmodulin are saturated with calcium, myosin light chain kinase should catalyze the phosphorylation of myosin which, in turn, should permit myosin to interact with actin so that contraction can occur. Trifluoperazine, by preventing the activation of the myosin kinase by calmodulin, should inhibit insulin release (Figure 5).

ACKNOWLEDGMENTS

M.J.M. thanks the Juvenile Diabetes Foundation for a Research Career Development Award. This work was supported by NIH grant AM28348 and a grant from the American Diabetes Association, Wisconsin Affiliate, Inc.

REFERENCES

1 Ostlund, R. E.: Contractile proteins and pancreatic beta-cell secretion. Diabetes *26*:245–52, 1977.

2 Means, A. R., and Dedman, J. R.: Calmodulin in endocrine cells and its multiple roles in hormone action. Mol. Cell. Endocrinol. *19*:215–27, 1980.

3 Malaisse, W. J., Sener, A., Herchuelz, A., and Hutton, J. D.: Insulin release: the fuel hypothesis. Metabolism *28*:373–86, 1979.

4 Hales, C. N., and Milner, R. D. G.: The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol. *194*:725–43, 1968.

5 Sparrow, M. P., Mrwa, U., Hofmann, F., and Rüegg, J. D.: Calmodulin is essential for smooth muscle contraction. FEBS Lett. *125*:141–45, 1981.

6 Klee, C. B., Crouch, T. H., and Richman, P. G.: Calmodulin. Ann. Rev. Biochem. *49*:489–515, 1980.

7 Sugden, M. D., C' .istie, M. R., and Ashcroft, S. J. H.: Presence and possible role of calcium-dependent regulator (calmodulin) in rat islets of Langerhans. FEBS Lett. *105*:95–100, 1979.

8 Valverde, I., Sener, A., Lebrun, P., Herchuelz, A., and Malaisse, W. J.: The stimulus-secretion coupling of glucose-induced insulin release. XLVII. The possible role of calmodulin. Endocrinology *108*:1305–12, 1981.

9 Mrwa, U., Troschka, M., Gross, C., and Katzinski, L.: Calcium-sensitivity of pig-carotid-actomyosin ATPase in relation to phosphorylation of the regulatory light chain. Eur. J. Biochem. *103*:415–19, 1980.

10 Persechini, A., and Hartshorne, D. J.: Phosphorylation of smooth muscle myosin: evidence for cooperativity between the myosin heads. Science *213*:1383–85, 1981.

11 Gagliardino, J. J., Harrison, D. E., Christie, M. R., Gagliardino, E. E., and Ashcroft, S. J. H.: Evidence for the participation of calmodulin in stimulus-secretion coupling in the pancreatic B-cell. Biochem. J. *192*:919–27, 1980.

12 Blumenthal, D. K., and Stull, J. T.: Activation of skeletal muscle myosin light chain kinase by calcium (2+) and calmodulin. Biochemistry *19*: 5608–14, 1980.

13 Adlestein, R. S., and Klee, C. B.: Purification and characterization of smooth muscle myosin light chain kinase. J. Biol. Chem. *256*:7501–7509, 1981.

14 MacDonald, M. J.: High content of mitochondrial glycerol-3-phosphate dehydrogenase in pancreatic islets and its inhibition by diazoxide. J. Biol. Chem. *256*:8287–90, 1981.

15 Laemmli, U. K.: Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature *227*:680–85, 1970.

16 Swanstrom, R., and Shank, P. R.: X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I. Anal. Biochem. *86*:184–92, 1978.

17 Portzehl, H., Caldwell, P. C., and Rüegg, J. C.: The dependence of

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/31/6/566/506178/31-6-566.pdf by guest on 02 December 2023

contraction and relaxation of muscle fibres from the crab Maia Squinado on the internal concentration of free calcium ions. Biochim. Biophys. Acta *79*:581–91, 1964.

[18] Scarpa, A., Brinley, F. J., and Dubyak, G.: Antipyrylazo III, a "middle range" Ca^{2+} metallochromic indicator. Biochemistry *17*:1378–85, 1978.

[19] Mrwa, U., and Hartshorne, D. J.: Phosphorylation of smooth muscle myosin and myosin light chains. Fed. Proc. *39*:1564–68, 1980.

[20] Klee, C. B., and Krinks, M. H.: Purification of cyclic 3′,5′-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to Sepharose. Biochemistry *17*:120–26, 1978.

[21] Niki, A., Niki, H., and Koide, T.: Effects of a new calmodulin inhibitor (W-7) on glucose-induced insulin release and biosynthesis. Diabetologia *21*:309, 1981. Abstract.

[22] Smolen, J. E., Korchak, H. M., and Weissman, G.: The roles of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human neutrophils. Biochim. Biophys. Acta *677*:512–20, 1981.

[23] Egrie, J. C.: Regulation of cyclic nucleotide phosphodiesterase activity in brain and adrenal medulla. Ph.D. thesis, University of Wisconsin, 1975.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/31/6/566/506178/31-6-566.pdf by guest on 02 December 2023