brane. In the presence of the proper cation, the internal protons may flow out through the carrier-mediated antiport mechanism. Na^+/H^+ antiport system was described both in intact *E. coli* cells and in membrane preparations (West & Mitchell, 1974; Schuldiner & Fishkes, 1978). Indeed, addition of 20 mM NaCl to the washed cells 2 min before lactose induces an increased exit of H^+ during the lactose pulse at pH 7.9 (Figure 6B). At pH 6.4, a slow exit rate was observed regardless of whether the cells were washed or whether Na^+ was added externally (Figure 6A). These results imply that the Na^+/H^+ antiport mechanism in *E. coli* cells is more effective at the basic pH values. A similar pH dependence was previously proposed to explain a different phenomenon: acidification of the cytoplasm at basic external pH while the primary energy transducing event is still proton extrusion from the cell.

The results described in this communication pinpoint several differences in the behavior of $\Delta\tilde{\mu}_{H^+}$ and lactose transport in intact cells as compared to isolated membrane vesicles. Thus, in intact cells, $\Delta\psi$ values increase when the external pH is raised and significantly compensate for the decrease in ΔpH, yielding a $\Delta\tilde{\mu}_{H^+}$ value relatively constant through the pH range tested. From a comparison of the lactose steady-state gradients and $\Delta\tilde{\mu}_{H^+}$, we conclude that in intact cells one proton is translocated with each molecule of lactose, both at pH 6 and pH 8. This is supported by direct measurements of H^+ and lactose fluxes.

It is still too early to conclude whether the differences observed are only apparent and due to the differing complexities of the two systems or if they are due to the functioning of some regulatory mechanism present only in one of them.

References

Adams, M. H., Ed. (1959) *Bacteriophages*, pp 446, Interscience, New York.
Chance, B., & Montal, M. (1971) *Curr. Top. Membr. Transp. 2*, 99.
Colowick, S., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774.
Davis, G. D., & Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17.
Flagg, J. L., & Wilson, H. (1977) *J. Membr. Biol. 31*, 233.
Greville, G. D. (1969) *Curr. Top. Bioenerg. 3*, 1.
Harold, F. M. (1972) *Bacteriol. Rev. 36*, 172.
Harold, F. M. (1977) *Curr. Top. Bioenerg. 6*, 84.
Heinz, E., Geck, P., & Pietrzyk, C. (1975) *Ann. N.Y. Acad. Sci. 264*, 428.
Hoffman, J. F., & Laris, P. C. (1974) *J. Physiol. (London) 239*, 519.
Mitchell, P. (1968) in *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd., Bodmin, Cornwall, U.K.
Padan, E., Zilberstein, D., & Rottenberg, H. (1976) *Eur. J. Biochem. 63*, 533.
Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16*, 4271.
Ramos, S., Schuldiner, S., & Kaback H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1892.
Ramos, S., Schuldiner, S., & Kaback, H. R. (1978) *Methods Enzymol.* (in press).
Rottenberg, H. (1975) *J. Bioenerg. 7*, 61.
Rottenberg, H. (1976) *FEBS Lett. 66*, 159.
Rottenberg, H. (1978) *Methods Enzymol.* (in press).
Schuldiner, S., & Fishkes, H. (1978) *Biochemistry 17*, 706.
Schuldiner, S., Rottenberg, H., & Avron, M. (1972) *Eur. J. Biochem. 25*, 64.
Skulachev, V. P. (1971) *Curr. Top. Bioenerg. 4*, 127.
Tsuchiya, T., & Rosen, B. P. (1975) *J. Biol. Chem. 250*, 7687.
West, C. I., & Mitchell, P. (1973) *Biochem. J. 132*, 587.
West, C. F., & Mitchell, P. (1974) *Biochem. J. 144*, 87.

Isolation and Characterization of Baby Hamster Kidney (BHK-21) Cell Modulator Protein†

Marie-Jeanne Yerna,* David J. Hartshorne,‡ and Robert D. Goldman

ABSTRACT: A Ca^{2+}-dependent modulator protein has been isolated from BHK-21 cells. The purification requires heat treatment, ion-exchange chromatography, and gel filtration. The protein appears homogeneous on sodium dodecyl sulfate–polyacrylamide and isoelectric focusing gels. The protein comigrates with purified smooth muscle and brain modulators. BHK-21 modulator is characterized by a high content of aspartic and glutamic acids and by a high phenylalanine/tyrosine ratio. It lacks both cysteine and tryptophan. The protein is effective in activating brain-modulator-deficient phosphodiesterase. It can also be used in assay systems to generate Ca^{2+}-sensitive actin activation of both BHK-21 and smooth muscle myosins. Therefore, it is proposed that the BHK-21 modulator protein is a component of the Ca^{2+}-dependent mechanism involved in the regulation of actin–myosin interactions in BHK-21 cells.

Modulator protein has been described as a cofactor for phosphodiesterase activity in rat brain by Cheung (1970) and Kakiuchi et al. (1970). Following these initial observations, similar proteins have been isolated from a wide variety of tissues (see Wang, 1977, for review). Further studies have indicated that the modulator protein binds Ca^{2+} and is involved in the Ca^{2+}-mediated regulation of several aspects of cyclic nucleotide metabolism (Cheung et al., 1975; Brostrom et al., 1975; Lin & Cheung, 1975; Teo & Wang, 1973; Stevens et al., 1976; Watterson et al., 1976a,b).

The central role of Ca^{2+} in the regulation of muscle contraction has also been extensively documented (Weber &

† From the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213. *Received July 5, 1978; revised manuscript received November 20, 1978.* This work represents partial fulfillment for the requirements of Marie-Jeanne Yerna's Ph.D. degree. This work has been supported by National Cancer Institute funds granted to Robert D. Goldman (No. 1-R01-CA-17210-03).

‡ Present address: Department of Nutrition and Food Science, Agricultural Sciences Building, University of Arizona, Tucson, AZ 85721.

0006-2960/79/0418-0673$01.00/0

Murray, 1973; Hartshorne & Gorecka, 1979). In skeletal muscle, troponin C (TnC) has been shown to be a Ca^{2+}-binding protein which in association with tropomyosin and the other troponin subunits mediates the Ca^{2+}-dependent interaction of actin and myosin (Weber & Murray, 1973). In the regulation of smooth muscle activity, it has been demonstrated that a specific Ca^{2+}-dependent kinase is involved in phosphorylation of the 20000-dalton light chains of myosin. This phosphorylation allows cyclic myosin–actin interactions to take place (Gorecka et al., 1976). This kinase has been characterized and found to consist of two components (Dabrowska et al., 1977), one of which was shown to be indistinguishable from the Ca^{2+}-binding modulator protein found in brain and other tissues (Dabrowska et al., 1978).

Myosin has been isolated from cultured BHK-21 cells (Yerna et al., 1978). This myosin exhibits very low Mg^{2+}-dependent actin-activated ATPase activity. On the basis of hybrid experiments with purified BHK-21 myosin and the smooth muscle kinase, it has been proposed that a similar endogenous myosin regulatory system exists in both the BHK-21 and smooth muscle cells (Yerna et al., 1978).

In light of these observations and our interest in further characterizing the actomyosin regulatory system of nonmuscle cells, we have isolated the modulator protein from BHK-21 cells. This protein has been characterized with respect to some of its physicochemical and functional properties. In addition, it has been compared directly with smooth muscle and brain modulator, as well as with skeletal muscle troponin C.

Materials and Methods

Cell Culture. Baby hamster kidney (BHK-21/C13) cells were grown in a CO_2 incubator at 37 °C in Dulbecco's modified Eagle's medium containing 10% calf serum, 10% tryptose phosphate broth, 50 units/mL of penicillin, and 50 μg/mL of streptomycin. Petri dishes containing confluent cultures were transferred to glass roller bottles following removal with 0.05% trypsin–ethylenediaminetetraacetate (EDTA) solution (Grand Island Biological Co.). Cells from confluent cultures were harvested from roller bottles in 0.54 mM EDTA in phosphate-buffered saline (PBS;[1] 0.17 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and washed three times in PBS.

Preparation of BHK-21 Modulator Protein. After the final wash in PBS, the cell pellet was suspended in 5 volumes of a solution containing 20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 0.2 mM dithiothreitol (DTT), sonicated for 15 s (frequency 5 using a Sonifier, Bronson Ultrasonics Corp., Model W185), and centrifuged at 30000*g* for 30 min. Solid ammonium sulfate was added to the supernatant to obtain 50% saturation and the sample was centrifuged at 10000*g* for 15 min. The pellet was discarded, additional ammonium sulfate was added to the supernatant to 100% saturation, and the pH was adjusted to pH 4.0 with 1 N HCl. This solution was left overnight at 4 °C and then centrifuged at 10000*g* for 15 min. The pellet was resuspended in 20 mM Tris-Cl, pH 7.5, 0.2 mM DTT and dialyzed overnight against three changes of the same buffer. Following the addition of $CaCl_2$ to a final concentration of 10 mM, the resulting solution was incubated for 6 min in a boiling water bath and then cooled in an ice bath. Coagulated proteins were removed by centrifugation at 10000*g* for 15 min. The supernatant was exhaustively dialyzed against 20 mM Tris-Cl, pH 7.5, 0.2 mM DTT, 1 mM EGTA (buffer A) containing 0.1 M NaCl and then applied to a DEAE-cellulose (DE52, Whatman) column (1.5 × 10 cm) which had been preequilibrated with the same buffer. The column was washed with 100 mL of buffer A containing 0.1 M NaCl. Adsorbed proteins were eluted at ~30 mL/h with a linear NaCl gradient consisting of 250 mL each of 0.1 M and 0.7 M NaCl in buffer A as the starting and limiting buffers, respectively. Column fractions were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and the appropriate fractions were pooled, dialyzed, and concentrated by lyophilization prior to the last purification step. The lyophilized protein was dissolved in 20 mM Tris-Cl, pH 7.5, 0.2 mM DTT and applied to a column (95 × 2.6 cm) of Sephadex G-100 (Pharmacia) in the same buffer. The column was eluted at a flow rate of 20 mL/h and column fractions were monitored as described above. Fractions under the peak region of the modulator protein were collected, dialyzed against distilled water, and then lyophilized.

Preparation of Other Proteins. Chicken gizzard myosin was prepared according to the technique of Hartshorne et al. (1977); rabbit skeletal muscle actin and tropomyosin were prepared by the method of Driska & Hartshorne (1975); chicken gizzard kinase and modulator were prepared as described by Dabrowska et al. (1977); activator-deficient phosphodiesterase and bovine brain modulator protein were prepared by the method of Watterson et al. (1976a); and BHK-21 myosin was prepared as described by Yerna et al. (1978). Troponin C was kindly provided by Dr. Sarah Hitchcock. The amino acid compositions of these gizzard and brain modulators were determined and were identical with those published in the literature cited above.

Measurement of Adenosine Triphosphatase (ATPase) Activity. ATPase assays were carried out at 25 °C in a final volume of 2 mL containing 25 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, and 2.5 mM ATP (Hartshorne & Mueller, 1969). The amount of inorganic phosphate released during the reaction was determined by the method of Fiske & Subbarow (1925). For assays done in the absence of Ca^{2+}, 1 mM EGTA was added.

Assay of Phosphodiesterase Activator Activity. The activity of cyclic nucleotide phosphodiesterase was measured using the basic procedure outlined by Lin et al. (1974a). The method involved coupling of the phosphodiesterase reaction to a 5′-nucleotidase reaction which was monitored by measuring the liberated phosphate by the method of Fiske & Subbarow (1925). The enzyme activity was measured at 30 °C in a reaction mixture containing 40 mM Tris–acetate, pH 8.0, 10 mM $Mg(CH_3COO)_2$, 0.1 mM $CaCl_2$, 2 mM cAMP, 0.5 unit of snake venom 5′-nucleotidase (Sigma) and appropriate amounts of enzyme and modulator proteins.

Protein Determination. Protein concentrations were determined either by the microbiuret method (Itzhaki & Gill, 1974) using bovine serum albumin as a standard or by the micro-Kjeldahl method assuming a 16% nitrogen content.

Gel Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed by a modification of the procedure of Fairbanks et al. (1971). The gels contained 10% acrylamide and 0.08% *N*,*N*′-methylenebis(acrylamide). Electrophoresis was also carried out in 7.5% polyacrylamide gels containing 7 M urea as described by Perrie & Perry (1970).

Gel Electrofocusing. Isoelectric gels were prepared with 7.3% (w/v) acrylamide, 0.2% (w/v) *N*,*N*′-methylenebis(acrylamide), 6 M urea, 2% (w/v) Bio-Rad ampholines of the appropriate pH range, 0.03% (w/v) ammonium persulfate, and

[1] Abbreviations used: PBS, phosphate-buffered saline; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

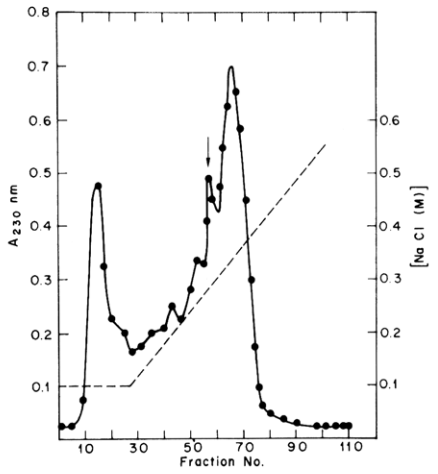

FIGURE 1: DEAE-cellulose chromatography of BHK modulator. The protein obtained from ammonium sulfate fractionation was chromatographed on a column of DEAE-cellulose. Proteins were eluted with a linear NaCl gradient (100–700 mM). Fractions of approximately 3 mL each were collected and analyzed for the presence of modulator. The peak indicated by the arrow corresponds to fractions enriched for protein comigrating with smooth muscle modulator.

0.06% (v/v) *N,N,N′,N′*-tetramethylethylenediamine. The cathode electrode solution was 1% (v/v) ethylenediamine and the anode electrode solution was 1.4% orthophosphoric acid. Protein samples containing 6 M urea, 1% β-mercaptoethanol were loaded onto the gels and the electrofocusing was performed at constant voltage (200 V) for about 20 h. Gels were stained and destained according to the procedure of Righetti & Drysdale (1974).

The pH gradient was determined as follows: the isoelectric focusing gel was cut into 5-mm sections which were placed in vials containing 2 mL of degassed H_2O. These vials were capped and, after 1–2 h, the pH was measured.

Amino Acid Analysis. Analyses were performed using a Durrum D-500 analyzer following hydrolysis for 22 h in 6 N HCl at 110 °C in vacuo. Trimethyllysine was identified as a distinct peak before lysine by comigration with a trimethyllysine standard and quantitated using a color constant of 0.89 × the color constant of lysine as determined from the trimethyllysine standard. Half-cystine was determined as cysteic acid following performic acid oxidation according to the procedure of Hirs et al. (1956).

Results

Purification of BHK-21 Modulator. The purification procedure described above is based on some properties common to modulator proteins isolated from several sources, i.e., heat stability, low molecular weight, and the acidic nature of the protein (Cheung, 1970; 1971; Kakiuchi et al., 1970). These three properties allow the purification of BHK-21 modulator following heat treatment of a low ionic strength extract and conventional chromatographic procedures.

Figure 1 shows the elution profile obtained when the supernatant resulting from the boiling step is chromatographed on DEAE-cellulose. Absorbance is monitored at 230 nm. $NaDodSO_4$–polyacrylamide gel electrophoresis indicated that a protein similar to modulator protein was eluted at approximately 0.28 M NaCl (arrow). The last elution peak did not contain protein as determined by discontinuous electrophoresis and by a A_{260} nm/A_{278} nm ratio higher than 5 indicating the presence of nucleic acid. The appropriate column fractions were pooled, dialyzed, and freeze-dried. Following the procedure outlined in Materials and Methods, we then subjected the protein to chromatography on Sephadex G-100.

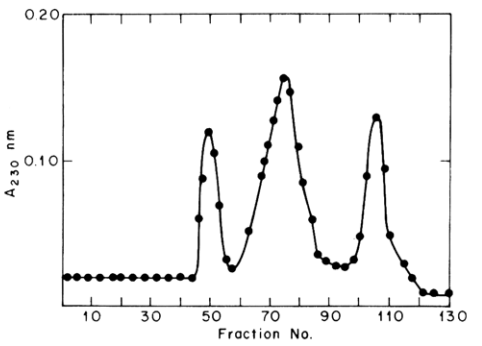

FIGURE 2: Gel filtration. The fractions recovered from the DEAE-cellulose column were pooled and concentrated prior to chromatography on a column of Sephadex G-100. Three-milliliter fractions were collected and assayed for modulator by their ability to activate brain phosphodiesterase. The second peak corresponds to the purified modulator.

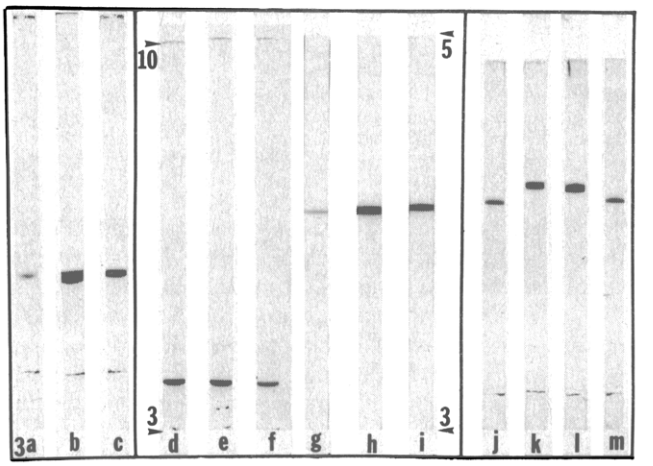

FIGURE 3: Polyacrylamide gel comparisons of BHK-21 and other modulator proteins. (a–c) Ten percent polyacrylamide–1% $NaDodSO_4$: (a) 3 μg of smooth muscle modulator; (b) mixture of 3 μg of smooth muscle modulator and 5 μg of BHK-21 modulator; (c) 5 μg of BHK-21 modulator; (d–i) gel isoelectric focusing in 6 M urea; (d–f) pH 3–10; (g–i) pH 3–5; (d) 40 μg of BHK-21 modulator; (e) mixture of 25 μg of BHK-21 modulator and 25 μg of brain modulator; (f) 25 μg of brain modulator; (g) 10 μg of brain modulator; (h) mixture of 10 μg of brain modulator and 25 μg of BHK-21 modulator; (i) 25 μg of BHK-21 modulator. (j–m) Urea (7 M)–10% polyacrylamide gels: (j) and (m) 3 μg of rabbit skeletal muscle troponin c; (k) 5 μg of BHK-21 modulator; (l) mixture of BHK-21 modulator and smooth muscle modulator, 3 μg each.

Protein was monitored at 230 nm. As shown in Figure 2, BHK-21 modulator eluted as a single, symmetrical peak. Fractions containing modulator were pooled and concentrated as described above. In order to estimate the purity of the final protein preparation, both $NaDodSO_4$–polyacrylamide gels and isoelectric focusing gels were used. As shown in Figure 3, a single protein band was observed in both cases. The overall purification required 4 to 5 days and yielded 2 to 3 mg of pure protein per 30 g of packed cells. BHK-21 modulator comigrates on $NaDodSO_4$–polyacrylamide gels with purified smooth muscle modulator, which has an approximate molecular weight of 17 000 (Dabrowska et al., 1978), and with brain modulator (not shown).

Physical Characterization. BHK-21 modulator is an acidic protein as determined by isoelectric focusing. It comigrates with brain modulator (Figure 3). A direct estimate of the p*I* by determining the pH of gel slices resulted in a value of 4.1, which is in close agreement with the published p*I* value for brain modulator of 4.3 (Lin et al., 1974b).

The amino acid composition of BHK-21 modulator is presented in Table I. The values reported are the average

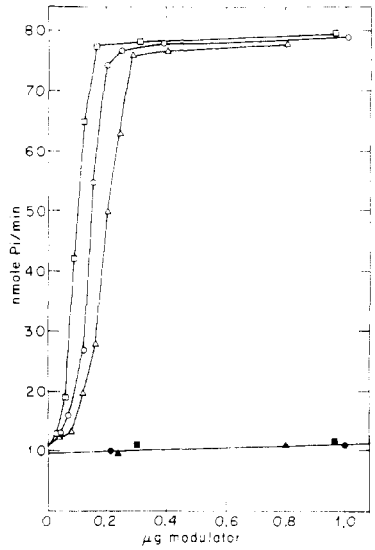

FIGURE 4: Activation of activator-depleted cyclic nucleotide phosphodiesterase by brain modulator (□); smooth muscle modulator (○); BHK-21 modulator (△). Assay method given in text. Phosphodiesterase level was constant, and the amount of modulator was varied. Closed symbols indicate inclusion of 1 mM EGTA.

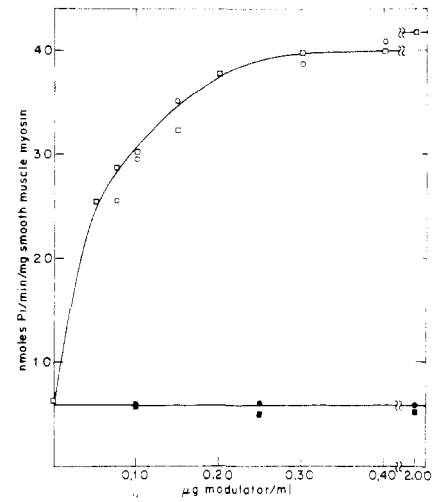

FIGURE 5: Effects of gizzard modulator (□) and BHK-21 modulator (○) at constant level of gizzard 105K protein on the actin activation of gizzard myosin at low ionic strength. Assay conditions: 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM ATP, 20 mM Tris-Cl, pH 7.5, 25 °C, gizzard myosin 0.45 mg/mL, skeletal muscle actin 0.25 mg/mL, skeletal muscle tropomyosin 0.075 mg/mL, gizzard 105K 5 μg/mL. Closed symbols indicate the inclusion of 1 mM EGTA.

of three different 22-h hydrolyses analyses performed on three different preparations (nine determinations). The amino acid composition obtained for BHK-21 modulator is very similar to those of modulator proteins isolated from different sources. Glutamic acid and aspartic acid compose about 40% of the molecule. BHK-21 modulator also contains 1 mol of an unusual amino acid, trimethyllysine, which has been reported previously for brain modulator protein (Amphlett et al., 1976). BHK modulator lacks cysteine. It also lacks tryptophan as indicated by the absence of tryptophan fluorescence emission and by its UV spectrum.

In order to distinguish between modulator and skeletal muscle troponin C which are known to share common physical properties, electrophoresis in polyacrylamide gels containing 7 M urea was carried out. Under these conditions, the two proteins are known to exhibit different mobilities (Dabrowska et al., 1978; Amphlett et al., 1976). BHK-21 modulator coelectrophoreses with smooth muscle modulator. However, the modulator proteins migrated towards the anode with a mobility slightly less than that of troponin C (Figure 3).

Biological Characterization. In earlier studies it was recognized that the modulator protein is involved in the Ca^{2+}-dependent regulation of cyclic nucleotide metabolism. Therefore, BHK-21 modulator was tested for its ability to activate activator-depleted cyclic nucleotide phosphodiesterase isolated from bovine brain. These data are shown in Figure 4 and represent the average obtained for three experiments. The activator titration curve obtained for BHK-21 modulator was similar to that obtained for modulator protein of bovine brain and smooth muscle gizzard. It is not known if the differences in activation obtained at subsaturating levels are significant. Figure 4 also confirms the previous findings (Wang et al., 1975) that the enzyme is not activated by the protein activator in the absence of Ca^{2+}.

Recently, another biological function has been assigned to modulator, namely, as a cofactor of the smooth muscle myosin light chain kinase. Figure 5 shows the effects of increasing amounts of BHK-21 modulator and gizzard modulator on the actin-moderated ATPase activity of smooth muscle myosin. At a constant level of gizzard 105K protein (Dabrowska et al., 1977) and in the presence of Ca^{2+}, both modulators were

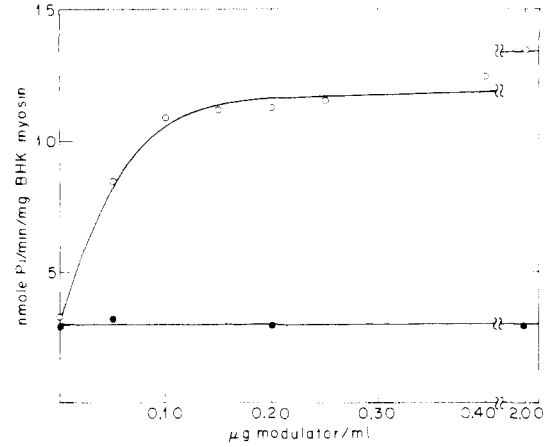

FIGURE 6: Actin activation of BHK-21 myosin at low ionic strength at constant level of gizzard 105K protein and increasing concentration of BHK-21 modulator protein. Assay conditions as in Figure 5. BHK myosin concentration: 0.35 mg/mL. Closed sumbols indicate the inclusion of 1 mM EGTA.

equally effective in activating the ATPase activity. In the absence of Ca^{2+}, neither modulator had any effect on ATPase activity. Figure 6 describes a similar experiment with BHK-21 myosin. Although the final level of ATPase activity was much lower, the overall activation profile observed was very similar to that obtained using smooth muscle myosin. In this case, it is also clear that Ca^{2+} was necessary to generate activation of ATPase activity. Activation of ATPase activity results from the modulator-mediated Ca^{2+} activation of the 105K protein, which transfers phosphate from ATP to the myosin 20 000-dalton light chain (Gorecka et al., 1976; Yerna et al., 1978, 1979).

Discussion

On the basis of the above physicochemical and biological properties, the protein isolated from BHK-21 cells appears similar to modulator proteins isolated from several tissues (Watterson et al., 1976a; Stevens et al., 1976; Dedman et al., 1977a; Dabrowska et al., 1978). Its presence has also been reported in crude preparations obtained from cultured chicken embryo fibroblasts (Watterson et al., 1976b).

Table I: Amino Acid Compositions[a] of BHK-21 and Other Modulator Proteins

	modulators from						
amino acid	BHK-21[b]	bovine pancreas[c]	bovine brain[d]	bovine heart[e]	gizzard smooth muscle[f]	adrenal medulla[g]	range
Lys	7 (1.1)	8	8	9	7	8	7-9
His	1 (0.2)	1	1	1	1	1	1
trimethyllysine	1 (0.1)	1	1		1		1
Arg	6 (0.3)	6	7	6	6	5	5-7
Asp	24 (1.2)	25	24	25	23	22	22-25
Thr[h]	9 (0.6)	13	12	12	12	11	11-13
Ser[i]	5 (1.2)	5	5	3	5	5	3-5
Glu	39 (2.9)	33	29	30	28	25	25-33
Pro	2 (0.3)	3	2	2	2	2	2-3
Gly	12 (0.8)	13	12	12	12	12	12-13
Ala	11 (0.7)	13	12	12	11	11	11-13
$^1/_2$-cystine	0	0	0	0		1	0-1
Val	8 (0.4)	8	8	9	7	9	7-9
Met	6 (0.5)	9	10	9	9	8	8-10
Ile	5 (0.7)	8	8	8	8	7	7-8
Leu	7 (1.2)	10	10	10	9	8	8-10
Tyr	2 (0.4)	2	2	2	2	2	2
Phe	6 (0.9)	9	8	9	8	7	7-9
Trp[j]	0	0	0	0		0	0

[a] Given as moles of amino acid/mole of protein. [b] The numbers in parentheses correspond to the standard deviations. [c] From Vandermeers et al. (1977). [d] From Watterson et al. (1976a). [e] From Stevens et al. (1976). [f] From Dabrowska et al. (1978). [g] From Kuo & Coffee (1976). [h] Corrected assuming a 6% loss during 22 h of hydrolysis. [i] Corrected assuming a 10% loss during 22 h of hydrolysis. [j] Determined by emission fluorescence.

In this paper a simple and convenient method is described for the purification of modulator protein from cultured BHK-21 cells which involves four steps: ammonium sulfate fractionation, heat treatment, ion-exchange chromatography, and gel filtration. The purified protein has the same molecular weight as smooth muscle and brain modulators and a similar isoelectric point (Figure 3). It also shares several other important physicochemical properties with modulators isolated from various tissues. These properties include amino acid composition, the presence of trimethyllysine, the absence of tryptophan (Dabrowska et al., 1978; Dedman et al., 1977b; Stevens et al., 1976; Watterson et al., 1976b), and a typical UV spectrum which reflects the amino acid composition (Dabrowska et al., 1978; Liu & Cheung, 1976; Stevens et al., 1976; Watterson et al., 1976a).

Several biological functions have been reported for modulator proteins including the stimulation of phosphodiesterase activity (Cheung, 1970; Kakiuchi et al., 1970), the activation of adenylate cyclase activity (Cheung et al., 1975; Brostrom et al., 1975), the regulation of the (Ca^{2+}-Mg^{2+})ATPase activity of red blood cell membranes (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), stimulation of phosphorylation of brain membrane protein (Schulman & Greengard, 1978), and as a cofactor of the smooth muscle myosin light chain kinase (Dabrowska et al., 1978). The results of this study indicate that BHK-21 modulator can stimulate phosphodiesterase activity and myosin ATPase activity to the same extent as modulator proteins isolated from brain and smooth muscle. These data also illustrate that the protein isolated from BHK-21 cells differs from troponin C; it has been shown that troponin C does not activate chicken gizzard myosin kinase (Dabrowska et al., 1978) and that it fails to activate phosphodiesterase at the concentrations tested (Stevens et al., 1976).

On the basis of previous studies (Yerna et al., 1978) which indicated that a Ca^{2+}-sensitive regulatory system, similar to the kinase system reported in smooth muscle, might be present in BHK-21 cells, we have been able to isolate from these cells a crude kinase preparation with properties similar to those of the smooth muscle myosin light chain kinase (Yerna et al., 1979). Therefore, the isolation and the characterization of BHK-21 modulator represent an important step in characterizing a Ca^{2+}-sensitive system which could regulate the interaction of actin and myosin in cultured cells.

Acknowledgments

We wish to thank Dr. W. E. Brown for help with the amino acid analyses and Drs. C. Coffee and A. Grimaldi for help with the phosphodiesterase assays.

References

Amphlett, G. W., Vanaman, T. C., & Perry, S. V. (1976) *FEBS Lett. 72*, 163.

Brostrom, C. O., Huang, Y. C., Breckenridge, B. McL., & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 64.

Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun. 33*, 533.

Cheung, W. Y. (1971) *J. Biol. Chem. 246*, 2859.

Cheung, W. Y., Bradham, L. S., Lynch, R. J., Lin, Y. M., & Tallant, E. A. (1975) *Biochem. Biophys. Res. Commun. 66*, 1055.

Dabrowska, R., Aromatorio, D., Sherry, J. M. F., & Hartshorne, D. J. (1977) *Biochem. Biophys. Res. Commun. 78*, 1263.

Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., & Hartshorne, D. J. (1978) *Biochemistry 17*, 253.

Dedman, J. R., Potter, J. D., & Means, A. R. (1977a) *J. Biol. Chem. 252*, 2437.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977b) *J. Biol. Chem. 252*, 8415.

Driska, S., & Hartshorne, D. J. (1975) *Arch. Biochem. Biophys. 167*, 203.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375.

Gopinath, R. M., & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun. 77*, 1203.

Gorecka, A., Aksoy, M. O., & Hartshorne, D. J. (1976) *Biochem. Biophys. Res. Commun. 71*, 325.

Hartshorne, D. J., & Mueller, H. (1969) *Biochim. Biophys. Acta 175*, 301.
Hartshorne, D. J., & Gorecka, A. (1979) *Handbook of Physiology* (in press).
Hartshorne, D. J., Gorecka, A., & Aksoy, M. O. (1977) in *Excitation-Contraction Coupling in Smooth Muscle* (Casteels, R., Godfraind, T., & Ruegg, J. C., Eds.) p 377, Elsevier, Amsterdam.
Hirs, C. H. W., Moore, S., & Stern, W. H. (1956) *J. Biol. Chem. 219*, 623.
Itzhaki, R. F., & Gill, D. M. (1964) *Anal. Biochem. 9*, 401.
Jarrett, H. W., & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun. 77*, 1210.
Kakiuchi, S., Yamazaki, R., & Nakajima, H. (1970) *Proc. Jpn. Acad. 46*, 587.
Kuo, I. C. Y., & Coffee, C. J. (1976) *J. Biol. Chem. 251*, 1603.
Lin, Y. J., & Cheung, W. Y. (1975) *Int. J. Biochem. 6*, 271.
Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974a) *Methods Enzymol. 38*, 262.
Lin, Y. M., Liu, Y. P., & Cheung, W. Y. (1974b) *J. Biol. Chem. 249*, 4943.
Liu, Y. P., & Cheung, W. Y. (1976) *J. Biol. Chem. 251*, 4193.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31.
Righetti, P. G., & Drysdale, J. W. (1974) *J. Chromatogr. 98*, 271.
Schulman, H., & Greengard, P. (1978) *Nature (London) 271*, 478.
Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S., & Wang, J. H. (1976) *J. Biol. Chem. 251*, 4495.
Teo, T. S., & Wang, J. H. (1973) *J. Biol. Chem. 248*, 5950.
Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., Kutzner, R., Delforge, A., & Christophe, J. (1977) *Eur. J. Biochem. 81*, 379.
Wang, J. H. (1977) in *Cyclic Nucleotides: Mechanisms of Action* (Cramer, H., & Schultz, J., Eds.) Vol. 5, p 179, Raven Press, New York.
Wang, J. H., Teo, T. S., Ho, H. C., & Stevens, F. C. (1975) in *Advances in Cyclic Nucleotide Research* (Drummond, G. I., Greengard, P., & Robison, G. A., Eds.) p 179, Raven Press, New York.
Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976a) *J. Biol. Chem. 251*, 4501.
Watterson, D. M., Van Eldik, L. J., Smith, R. E., & Vanaman, T. C. (1976b) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2711.
Weber, A., & Murray, J. M. (1973) *Physiol. Rev. 53*, 612.
Yerna, M.-J., Aksoy, M., Hartshorne, D. J., & Goldman, R. D. (1978) *J. Cell Sci. 31*, 411.
Yerna, M.-J., Dabrowska, R., Hartshorne, D. J., & Goldman, R. D. (1979) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Amino Acid Sequence and Disulfide Bond Assignment of Myotoxin *a* Isolated from the Venom of Prairie Rattlesnake (*Crotalus viridis viridis*)†

Jay W. Fox, Marshall Elzinga, and Anthony T. Tu*

ABSTRACT: The primary structure of myotoxin *a*, a myotoxin protein from the venom of the North American rattlesnake *Crotalus viridis viridis*, was determined and the position of the disulfide bonds assigned. The toxin was isolated, carboxymethylated, and cleaved by cyanogen bromide, and the resultant peptides were isolated. The cyanogen bromide peptides were subjected to amino acid sequence analysis. In order to assign the positions of the three disulfide bonds, the native toxin was cleaved sequentially with cyanogen bromide and trypsin. A two-peptide unit connected by one disulfide bond was isolated and characterized, and a three-peptide unit connected by two disulfide bonds was isolated. One peptide in the three-peptide unit was identified as Cys-Cys-Lys. In order to establish the linkages between the peptides and Cys-Cys-Lys, one cycle of Edman degradation was carried out such that the Cys-Cys bond was cleaved. Upon isolation and analysis of the cleavage products, the disulfide bonds connecting the three peptides were determined. The positions of the disulfide bridges of myotoxin *a* were determined to be totally different from those of neurotoxins isolated from snake venoms. The sequence of myotoxin *a* was compared with the sequences of other snake venom toxins using the computer program RELATE to determine whether myotoxin *a* is similar to any other types of toxins. From the computer analysis, myotoxin *a* did not show any close relationship to other toxins except crotamine from the South American rattlesnake *Crotalus durissus terrificus*.

Muscle degeneration is commonly observed upon rattlesnake envenomation. Rattlesnake venoms exhibit high levels of proteolytic activity and therefore it has been suggested that the myonecrotic effects of the venom were the results of the action of venom proteases (Porges, 1953). However, a heat-stable component of *Timersurus flavoviridis* venom and a basic polypeptide from black widow spider venom (Lee et al., 1974) (neither of which has proteolytic activity) have been shown to elicit myonecrotic effects. Thus, venom-induced myonecrosis appears to be the result of more complex action than that attributable solely to venom proteases. It is likely that myonecrosis results from both independent and synergistic effects of venom components.

† From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523 (J.W.F. and A.T.T.), and the Department of Biology, Brookhaven National Laboratory, Upton, New York 11973 (M.E.). *Received July 21, 1978.* This work is based on the dissertation submitted by J. W. Fox in partial fulfillment of the degree of Ph.D. at Colorado State University. This work was supported by grants from the National Institutes of Health, 2 R01 GM15991 (A.T.T.) and HL-21471 (M.E.). The research carried out at Brookhaven National Laboratory was under contract with the U.S. Energy Research and Development Administration.

0006-2960/79/0418-0678$01.00/0