A Calcium-Sensitizing System from Human Platelets and its Activity on Muscle and Platelet Actomyosin

Muscle contraction is known to be regulated by trace amounts of Ca^{2+}-ions. In vertebrate muscles the tropomyosin-troponin complex is involved[1], whereas a myosin-dependent regulatory system has been described in molluscs[2]. Thrombosthenin, the actomyosin-like, contractile protein of blood platelets may be considered as a prototype for contractile proteins of non-muscular cells. It therefore appears of particular importance to know more about its mode of activation. According to a recent report[3], the activity of the Mg^{2+}-stimulated ATPase of thrombosthenin is indeed regulated by Ca^{2+}-ions. In the present work, an attempt of characterizing the proteins responsible for this effect has been made; these regulatory proteins have been separated from the contractile proteins proper, and their activity has been assessed by comparison and hybridization with a system derived from striated muscle.

Methods. For the preparation of thrombosthenin from human blood platelets, the procedure described by BETTEX-GALLAND et al.[4] was essentially followed, with the exception that all steps leading to the first crude extract were carried out in the presence of 0.5 m*M* dithiothreitol (DTT). Desensitized actomyosin and the regulatory protein system (containing tropomyosin and troponin components) from rabbit skeletal muscle were prepared as described earlier[5,6]. The conditions for the estimation of ATPase-activities are given in the Tables; in all cases the incubation time was 20 min at 22 °C. Superprecipitation was assessed by a turbidimetric method, whereby thrombosthenin was stirred slowly in the presence of 5 m*M* ATP (see text of Figure 1 for details).

Results. The Mg^{2+}-stimulated ATPase of thrombosthenin preparations, obtained by reprecipitation of crude extract in the absence of DTT, was invariably inhibited in the order of magnitude from 20 to 50% by the addition of ethanol dioxy-*bis*-(ethylamine) tetra-acetic acid (EGTA) to a final concentration of 2 m*M*. Superprecipitation was accordingly affected by the Ca^{2+}-chelator (Figure 1).

If, however, crude thrombosthenin was reprecipitated in the presence of 0.5 m*M* DTT, and the precipitate thus obtained was stirred gently at low ionic strength for 10 min, EGTA no longer inhibited the Mg^{2+}-stimulated ATPase of such preparations, which are subsequently termed 'desensitized thrombosthenin'. Independently of the presence or absence of DTT, half or more of the total protein remained in solution after precipitation of thrombosthenin at low ionic strength at pH 6.2 (cf.[4]). The ATPase activities of these supernatants corresponded to those of thrombosthenin, both with Mg^{2+} or Ca^{2+} (2.5 m*M* final concentrations); the presence of EGTA was without effect on the Mg^{2+}-stimulated ATPase of either supernatant.

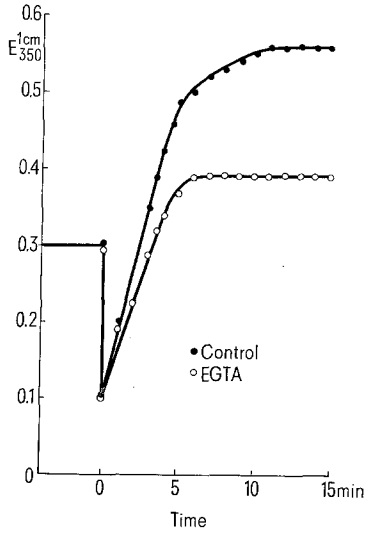

Fig. 1. Time course of superprecipitation of thrombosthenin (0.8 mg/ml) in 50 m*M* KCl, 10 m*M* imidazole buffer (pH 7) and 5 m*M* $MgCl_2$ in the presence and absence of 2 m*M* EGTA. 5 m*M* ATP was added at zero time.

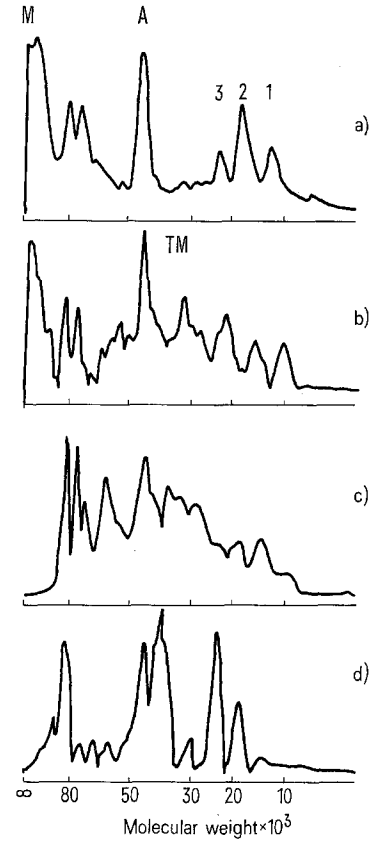

Fig. 2. Densitometric tracings of electropherograms of 80–100 μg protein on 10% polyacrylamide gels in the presence of 0.1% sodium dodecylsulphate[11]. The abscissa gives the molecular weights as inferred from the known molecular weights of muscle actin[15], the 3 light chains of myosin[12] and platelet tropomyosin[3]. a) Actomyosin from striated rabbit muscle. M, myosin heavy chains; A, actin; 1, 2, 3, myosin light chains. b) Thrombosthenin from human blood platelets. TM, tropomyosin. c) Fraction containing regulatory proteins from thrombosthenin. d) Fraction containing regulatory proteins from rabbit actomyosin.

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Table I. Effect of Ca^{2+}-sensitizing protein fraction (CFS) from platelets on desensitized thrombosthenin (DTS) and actomyosin (DAM)

DTS (mg/ml)	DAM (mg/ml)	CSF (mg/ml)	Ratio of CSF to DTS or to DAM	Specific Mg-ATPase activity	Mg-ATPase activity (%)
0.4		0		0.0124	100
0.4		0.55	1.38	0.0085	68
0.4		1.10	2.75	0.0086	69
0.4		2.20	5.50	0.0085	68
	0.15	0		0.485	100
	0.15	0.2	1.33	0.488	101
	0.15	0.4	2.67	0.380	79
	0.15	0.8	5.33	0.322	67

Experimental conditions: 25 m*M* KCl, 25 m*M* *tris*-HCl (pH 7.6), 2.5 m*M* $MgCl_2$, 2.5 m*M* ATP and 2 m*M* EGTA. The ATPase activity expressed as μmoles P_i liberated per mg of DTS or DAM per min (cf.[7]) was determined on CSF separately and subtracted in each case.

Table II. Effect of regulatory protein system (RPS) from skeletal muscle on desensitized actomyosin (DAM) and thrombosthenin (DTS)

DAM (mg/ml)	DTS (mg/ml)	RPS (mg/ml)	Ratio of RPS to DAM or to DTS	Specific Mg-ATPase activity	Mg-ATPase activity (%)
0.23		0		0.564	100
0.23		0.06	0.26	0.385	68
0.23		0.12	0.52	0.330	59
0.23		0.24	1.04	0.234	41
0.23		0.48	2.09	0.250	44
	0.58	0		0.0138	100
	0.58	0.15	0.26	0.0124	90
	0.58	0.30	0.52	0.0102	74
	0.58	0.60	1.03	0.0080	58
	0.58	1.20	2.07	0.0083	60

The incubation medium in which the proteins tabulated above were contained, had the following composition: 5 m*M* KCl, 10 m*M* imidazole buffer (pH 7), 2,5 m*M* $MgCl_2$, 1 m*M* ATP, and 2 m*M* EGTA. Specific ATPase-activity is given in μmoles P_i liberated per mg of DAM or DTS per min (cf.[7]).

Desensitized thrombosthenin regained its Ca^{2+}-sensitivity only after the addition of the supernatant obtained in the presence of 0.5 m*M* DTT (Table I). This finding corresponds to similar observations made on actomyosin preparations from rabbit skeletal muscle, where regulatory proteins are obtained by extraction of crude preparation at low ionic strength[5].

Therefore, it was attempted to isolate these proteins from a thrombosthenin supernatant, using the techniques developed for the separation of the tropomyosin-troponin complex from muscle proteins[6,8,9]. Unfortunately no defined activities were obtained. However, by gel-filtration of a DTT-supernatant of thrombosthenin on Sephadex G-200 in 80 m*M* KCl, 20 m*M* *Tris*-HCl, pH 8.0, and 0.5 m*M* DTT, followed by chromatography on DEAE-Sephadex A-50 in 20 m*M* *Tris*-HCl and 0.5 m*M* DTT, and elution with a KCl-gradient (from zero to 0.4 *M*), a Ca^{2+}-sensitizing fraction was obtained at KCl concentrations ranging from 0.2–0.3 *M*. However, it was still contaminated with a Mg^{2+}-stimulated ATPase, most likely the one described by ROSSI[10], since its activity was strongly inhibited by small amounts of Ca^{2+}-ions.

As shown in Table I, this protein fraction not only restored Ca^{2+}-sensitivity to desensitized thrombosthenin, but also to desensitized actomyosin from rabbit skeletal muscle (Table I). On the other hand, the regulatory protein containing fraction from rabbit muscle was able to confer Ca^{2+}-sensitivity to desensitized thrombosthenin, provided the ATPase assays were carried out at low ionic strength ($\mu = 0.02$) and in the presence of an excess of Mg^{2+} over ATP (Table II).

Disc-electrophoresis patterns of the actomyosins from rabbit muscle and from human blood platelets, as well as of the regulatory protein containing fractions used in these hybridization experiments, are shown in Figure 2.

Discussion. The results presented here clearly demonstrate that the activity of thrombosthenin is controlled by Ca^{2+} in a way similar to actomyosin. Evidence for the presence of regulatory proteins has been found. Tropomyosin has been isolated and characterized before by COHEN and COHEN[3]. The question then arises whether platelets also contain a troponin-like material. Whereas the isolation and characterization of the troponin complex from these cells has as yet not been achieved, the hybridization experiments with material obtained from rabbit muscle clearly indicate that platelet proteins can substitute for the regulatory proteins of striated muscle and vice versa.

The similarities in the electrophoretic pattern of desensitized thrombosthenin with that of rabbit actomyosin, with its easily identifiable bands of the myosin heavy chains, actin and the 3 myosin light chains (cf.[12]), are quite striking. It is obvious that thrombosthenin most likely contains the same myosin chains, and furthermore that these are also comparable with respect to molecular weight. Different from desensitized actomyosin, desensitized thrombosthenin shows the presence of an additional band which is inserted between the positions of actin (thrombosthenin A) and the largest myosin light chain. The apparent molecular weight of this fraction, 30,000 Daltons, makes it likely that this is tropomyosin (cf.[3]), which seems to separate more easily from the muscle than from the platelet contractile proteins.

Neither the Ca^{2+}-sensitizing fraction from platelets, nor the fraction containing the regulatory proteins of muscular origin, contain heavy myosin chains, but in both actin is still present. The two fastest moving bands in the muscle preparation correspond to the components of 18,000 and 23,000 Daltons of the troponin complex[13,14]. Unfortunately it is not yet possible to attribute the bands of the platelet Ca^{2+}-sensitizing system to either troponin or to myosin light chains. The fact, however, that this system can be removed from intact thrombosthenin and confers Ca^{2+}-sensitivity to desensitized muscle-actomyo-

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sin seems to indicate that it resembles much more the tropomyosin-troponin type of regulatory proteins found in skeletal muscle than the myosin-linked type present in the muscles of molluscs.

Zusammenfassung. Die Mg-ATPase des kontraktilen Systems von Blutplättchen, Thrombosthenin, wird durch Spuren von Calciumionen reguliert. Eine Regulationseiweisse enthaltende Fraktion kann von Thrombosthenin abgetrennt werden. Zugabe dieser Eiweissfraktion zu sogenanntem «desensibilisierten» Thrombosthenin aber auch zu «desensibilisiertem» Actomyosin aus Skelettmuskel vermag die Mg-ATPase beider Systeme wieder von Spuren von Calciumionen abhängig zu machen. Die Regulationseiweisse, d.h. der Tropomyosin-Troponin-Komplex aus Skelettmuskeln, vermögen ebenfalls «desensibilisiertes» Thrombosthenin wieder Calcium-abhängig zu machen.

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Immunoassay for Lysine^8-Vasopressin (LVP): Comparison of Biological and Immunological Activity of Lysine-Vasopressin and Some of its Synthetic Analogues

In radioimmunossay of vasopressin already reported (Klein[1]; Permutt[2]; Miller[3]; Edwards et al.[4]; Robertson[5]; Johnston[6]) no details about the biological activity of radioiodinated molecule and the role of the various amino-acid residues in the immunological reactivity are given. In this report, the antidiuretic activity of ^{125}I-LVP is measured and the cross reactivity of vasopressin analogues with a LVP antiserum studied with a view to identification of the antigenic sites of antidiuretic hormone and to comparing the structural requirements for its immunological and biological activity.

Materials and methods. Synthetic LVP (Sandoz) is conjugated to rabbit serum albumin by the method of Goodfriend et al.[7]. 0.5 ml of conjugate containing 100 U LVP is emulsified with an equal volume of Freund's adjuvant. About 0.4 ml is injected directly into rabbit spleen and the other part into the toe pads. Subsequent weekly i.m. and i.p. injections are administered, as well as toe pad injections (on the whole 100 U). 10 days after the 6th immunization, the serum is screened. Injections of similar materials are carried out at 3 week intervals.

Synthetic LVP (5 to 8 μg) is labelled according to Hunter and Greenwood[8] using 2 mC ^{125}I Na specific radioactivity higher than 14 mC/μg (Radiochemical Center, Amersham). ^{125}I-LVP is separated from free ^{125}I by passage through a 1 × 10 cm column (I) of DEAE cellulose developed with 0.02 *M* ammonium acetate buffer pH = 5.4. For a further purification, ^{125}I-LVP from column I (1 ml) is applied to a G15 Sephadex column

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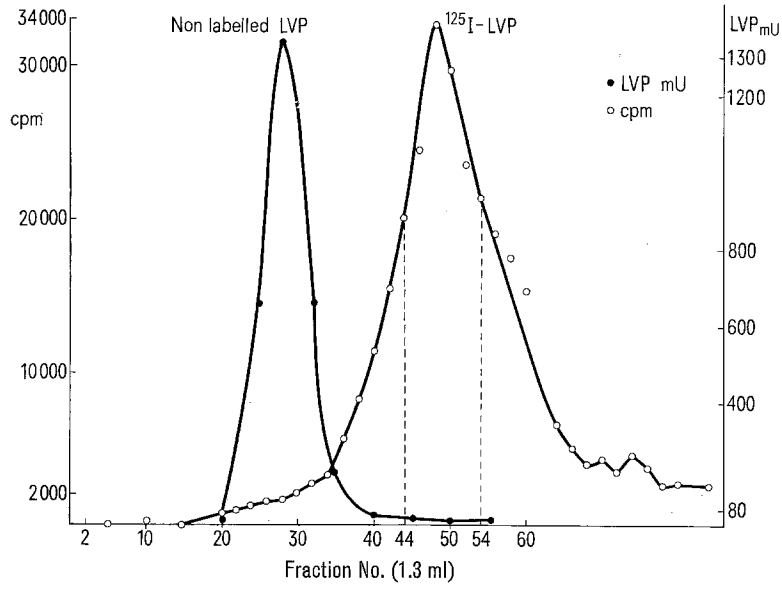

Fig. 1. Comparison between gel filtration of labellen ^{125}I-LVP (○—○) and antidiuretic activity of non labelled LVP (●—●) on a G15 Sephadex columd (30 × 0.9 cm).