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SUPERPRECIPITATION OF VASCULAR ACTOMYOSIN—THE EFFECT OF VASOACTIVE COMPOUNDS AND ANTI-INFLAMMATORY AGENTS ON THE PROCESS

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Abstract—Adenosinetriphosphate(ATP)-induced superprecipitation of actomyosin extracted from cow carotid has been studied with the turbidimetric method. Dependence of the reaction on the concentration of ATP and Ca^{2+} ion was established. Superprecipitation was inhibited by adenosinediphosphate (ADP) added in equal concentration to that of ATP, while adenosinemonophosphate (AMP) had no inhibitory effect. Superprecipitation was inhibited by vasodilators (papaverine, sodium nitrite), as well as by certain anti-inflammatory compounds (flufenamic, mefenamic acid, acetylsalicylic acid). The rate and intensity of superprecipitation was greatly enhanced by adrenaline and noradrenaline in low concentrations. Similar increasing effects were noted upon the use of higher concentrations of histamine, serotonin and bradykinin. The superprecipitation increasing effect of adrenaline and histamine were inhibited by papaverine, nitrite and flufenamic acid in very low concentrations.

NUMEROUS papers have appeared concerning the physical transformation of the contractile proteins of skeletal muscle upon the addition of adenosinetriphosphate (ATP). Superprecipitation can be followed by recording the change in optical density of the suspension of actomyosin and this simple *in vitro* model has yielded a considerable amount of information about the mechanism of the contraction of the protein complex at the molecular level.

Unlike skeletal muscle relatively few workers have examined the superprecipitation phenomena of the contractile proteins of the vascular wall,[1,2] a turbidimetric study of this process has only recently been reported.[2] The physical and enzymatic properties of actomyosin of various kinds of muscle are known to differ considerably and the results therefore obtained with skeletal muscle actomyosin are not directly transferable to the smooth muscle system. Still less data is available concerning the effect of drugs on actomyosin–ATP interaction.

In a study of the superprecipitation of skeletal muscle actomyosin we have found that the reaction was effectively inhibited by certain non-steroidal anti-inflammatory compounds.[3] In the present experiments we have extended these investigations to the study of the superprecipitation of actomyosin extracted from cow carotid tissue and explored the use of a turbidimetric assay system to follow the event. This study of the effects of various vasoactive compounds has led us to the conclusion that certain agents are capable of exerting a significant effect on the actomyosin–ATP interaction of vascular smooth muscle preparations.

MATERIALS AND METHODS

Crude actomyosin was extracted from cow carotids according to the procedure of Mallin.[1] Shortly after the animal had been killed the excised pieces of the carotids were placed into ice-cold saline and transferred to the laboratory. The adventitia was cleaned of surrounding connective tissue, the tissue finely minced under continuous cooling and then left to stand for 24 hr in 3 vol. of weakly alkaline 0·6 M KCl,[4] with occasional stirring. The mixture was centrifuged and the supernatant was diluted with 14 vol. of distilled water. The protein precipitated by this dilution, during 12 hr standing at 4°, was separated by centrifugation and then redissolved in 0·6 M KCl. The protein was again precipitated by dilution, redissolved and stored at 4°, it was used within 7 days of the time of extraction.

The present experiments have been performed with proteins prepared on six different occasions. The first two experiments were preliminary investigations and the remaining four experiments were conducted under identical conditions.

Measurement of superprecipitation

In the presence of added Ca^{2+} ion, superprecipitation was performed by the method of Ebashi[5] and by that of Levy and Fleisher.[6] The standard composition of incubation medium was as follows: 0·25 mg/ml protien; 30 mM KCl; 60 mM tris–HCl; 2·5 mM $MgCl_2$ and 5 mM $CaCl_2$; pH 7·4, total volume 2·0 ml. This mixture was incubated with the compound to be tested for 2 min in the optical cell, whereafter the reaction was started by the addition of 0·05 ml ATP. Changes in optical density were recorded at 545 nm usually during the first 15 min, following the addition of ATP. During measurement, the contents of the cell were stirred (1000 rev/min) by the aid of a small magnetic rod at the bottom of the cell. Reactions were performed at room temperature (25°). In the investigations of the effects of the various compounds every third tube served as control, whereby changes of control values were ruled out.

Superprecipitation of actomyosin in the presence of added Ca^{2+} was estimated in one experiment also from the protein concentration in the precipitate after centrifugation at 2000 rev/min for 1 min, according to Weber *et al.*[7]

RESULTS

Superprecipitation of skeletal muscle actomyosin takes place when only exogenous Mg^{2+} ion is added to the system and no Ca^{2+} ion. Yet it has been demonstrated that a minute amount of Ca^{2+} ion is nevertheless essential for the reaction.[5] ATP added with constant stirring to the medium containing no exogenous Ca^{2+} ion does not produce increased optical density. However, if stirring is stopped aggregation and rapid sedimentation of protein gel takes place in the tube containing ATP, while hardly any sedimentation occurs in actomyosin gel-suspension containing no ATP. The phenomenon can be followed turbidimetrically if the photocell "sees" sedimentation of the gel-column through an adequate aperture between the optical cell and the photocell. Figure 1 gives a sketch diagram of the set-up.

As shown in the experiment illustrated in Fig. 2, a certain time after the addition of ATP (a few minutes of which are necessary to allow the top of the protein column to reach the upper level of the optic aperture) even sedimentation set in, manifested by the gradual decrease in optical density. The process depends on the minute amount of Ca^{2+} ion being present in the medium, which is shown by the fact that sedimentation

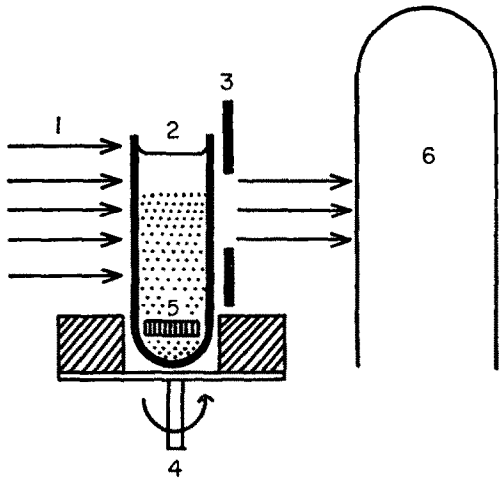

FIG. 1. Experimental set-up for the study of the measurement of rapid sedimentation of actomyosin suspension induced by ATP. 1—monochromatic light; 2—optical cell; 3—aperture; 4—magnetic stirring device; 5—plastic-covered magnetic rod; 6—detector.

of gel can be fully inhibited not only with EDTA, but also by EGTA which actually binds only Ca^{2+} ion at neutral pH. As revealed by Fig. 3 actomyosin gel sedimentation was strongly enhanced by low concentration of ATP (0·1 mM). At higher concentrations (0·5 mM) the effect decreased and in concentrations higher than 1 mM ATP did not bring about any change in the sedimentation of actomyosin gel. Table 1 shows that the effect of ATP was inhibited by papaverine, sodium nitrite, flufenamate, acetylsalicylate, while phenylbutazone and indomethacin were practically ineffective.

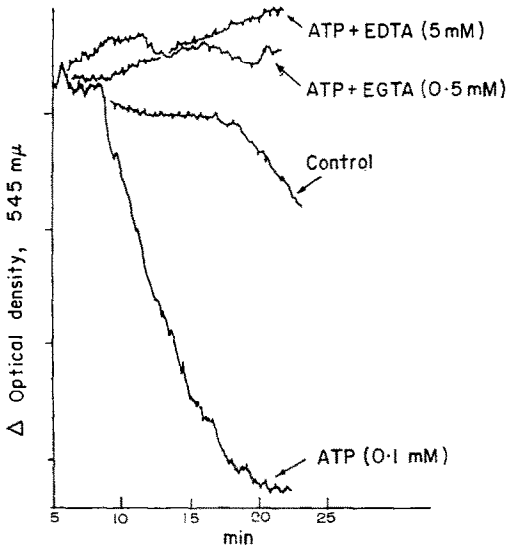

FIG. 2. Turbidimetric measurement of sedimentation of actomyosin suspension induced by ATP. On the ordinate graduations indicate 0·1 OD changes. Reaction conditions the same as in Table 1.

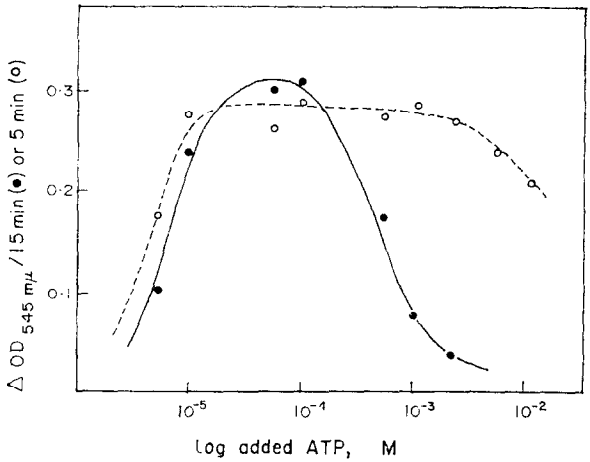

FIG. 3. Dependence of increased sedimentation or superprecipitation on concentration of ATP (●) Reaction conditions the same as in Table 1. Incubation medium contained no added Ca^{2+} ion. (○) Reaction conditions the same as in Table 2, incubation medium contained both Ca^{2+} and Mg^{2+} ions alike.

In further series of experiments the incubation-medium contained Ca^{2+} ion. In the presence of added Ca^{2+} ion, the optical density, registered during continuous stirring, was increased by the addition of ATP.

It may be supposed that P_i liberated as a result of ATPase activity of the protein form insoluble calcium phosphate with the Ca^{2+} present in the medium and that may result in an increase in optical density. To exclude this possibility we have performed two experiments. As it is evident from Fig. 4, superprecipitation of actomyosin, measured from the protein concentration in the precipitate after centrifugation runs parallel with the increase of optical density, measured turbidimetrically.

TABLE 1. EFFECT OF VARIOUS COMPOUNDS ON ATP-INDUCED ENHANCED SEDIMENTATION OF VASCULAR SMOOTH MUSCLE ACTOMYOSIN

Compounds	Concentration (μg/ml)	Decrease of $OD_{545\,nm}$/15 min Mean ± S.E.M.
Control (no added ATP)		0·05 ± 0·01
Control (ATP)		0·32 ± 0·05
Papaverine	50	0·12 ± 0·01*
$NaNO_2$	25	0·18 ± 0·03*
Flufenamic acid	50	0·13 ± 0·02*
Acetylsalicylic acid	200	0·18 ± 0·02*
Phenylbutazone	100	0·28 ± 0·03
Indomethacin	100	0·29 ± 0·04

Reaction conditions (mM): KCl 30, Tris–HCl (pH 7·4) 60, $MgCl_2$ 2·5, ATP 0·1 and protein 0·25 mg/ml. Reaction mixture was stirred 30 sec after the addition of ATP, then stirring was stopped and the decrease of optical density ($OD_{545\,nm}$) was registered 15 min following the addition of ATP.

* Deviation from the controls (ATP) is significant ($P < 0·01$).

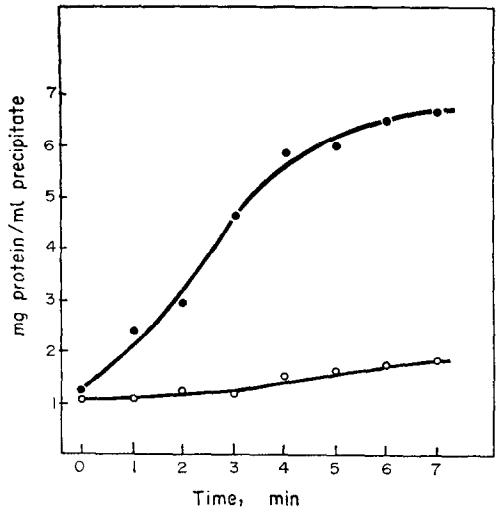

FIG. 4. Superprecipitation of vascular actomyosin measured from the protein concentrations in the precipitate after centrifugation. Reaction conditions the same as in Table 2, except protein concentration, which was 1 mg/ml and the total volume was 5 ml.

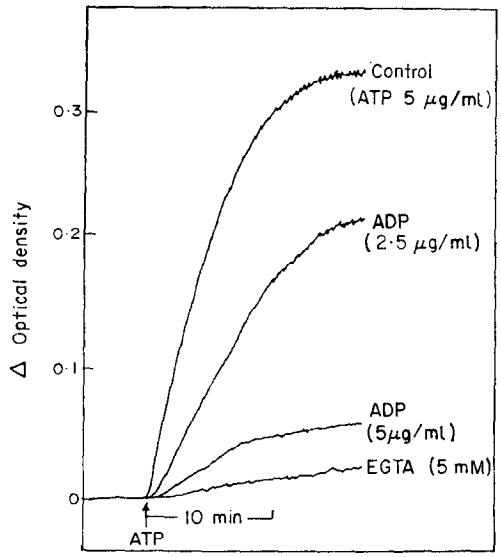

FIG. 5. Effect of ADP and chelation of Ca^{2+} on ATP-induced superprecipitation. Reaction conditions the same as in Table 2. ADP or EGTA was added to the medium 2 min before ATP.

Mallin[1] measured ATPase activity to be 4·2 μmoles P_i/mg protein/hr. Transferring this value to our conditions (0·5 mg protein, 15 min observation time) this means 0·52 μmoles P_i. Addition of the five times of this P_i amount to the system containing Ca^{2+} ion but not protein, resulted in 0·025 changes in the optical density. On the basis of these two experiments it may be stated that the measured increase in optical density of actomyosin suspension containing Ca^{2+} ion is the result of conformational changes of protein molecules induced by ATP.

As demonstrated in Fig. 3, ATP exerted full effect at 10 μM, with higher concentration of ATP its effect decreased from 5 mM. The results of one experiment presented in Fig. 5 shows that by chelating Ca^{2+} ion EGTA completely inhibited superprecipitation. The Fig. 5 furthermore shows that ATP-induced superprecipitation was practically fully inhibited by a similar amount of adenosinediphosphate (ADP). Even in high concentration (50 μg/ml) adenosinemonophosphate (AMP) exerted only a minimal inhibitory effect. As revealed in Fig. 6 and Table 2, the rate and intensity of

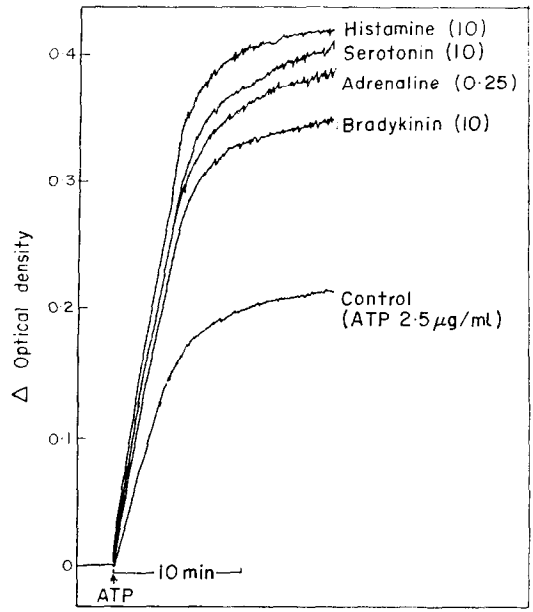

FIG. 6. Increasing effect of various vasoactive compounds on ATP-induced superprecipitation. Reaction conditions as in Table 2.

ATP-induced superprecipitation are strongly enhanced by catecholamines in quite low concentrations. Curiously enough, in concentrations higher than 2·5 μg/ml their effects failed to assert themselves. Acetylcholine in concentrations of 0·1 and 10 μg/ml did not influence superprecipitation. In much higher concentrations than catecholamines, histamine, serotonin and bradykinin strongly enhanced actomyosin superprecipitation elicited by low concentration of ATP.

The respective effects of adrenaline, histamine and serotonin were in fact fully inhibited by specific antagonists (phenoxybenzamine, cyproheptadine, methysergide) added 1 min before the former compounds.

TABLE 2. EFFECT OF VASOACTIVE COMPOUNDS ON ATP-INDUCED SUPERPRECIPITATION OF VASCULAR SMOOTH MUSCLE ACTOMYOSIN

Compounds	Concentration (μg/ml)	$OD_{545\ nm}$ increase/5 min Mean $\pm$ S.E.M.
Control (ATP)		0·18 $\pm$ 0·03
Adrenaline	0·25	0·38 $\pm$ 0·05*
Noradrenaline	0·25	0·43 $\pm$ 0·08
Adrenaline	1·0	0·32 $\pm$ 0·03*
Noradrenaline	1·0	0·28 $\pm$ 0·02*
Adrenaline	10·0	0·24 $\pm$ 0·03
Noradrenaline	10·0	0·21 $\pm$ 0·03
Histamine	1·0	0·26 $\pm$ 0.03
	10·0	0·39 $\pm$ 0·05*
Serotonin	10·0	0·37 $\pm$ 0·04*
Bradykinin	10·0	0·39 $\pm$ 0·03*

Reaction conditions (mM): KCl 30, Tris–HCl (pH 7·4) 60, $MgCl_2$ 2·5, $CaCl_2$ 5, ATP 0·008, protein 0·25 mg/ml. Increase of $OD_{545\ nm}$ after the addition of ATP to the reaction mixture was recorded under continuous stirring 5 min following the addition of ATP.

* Deviation from the controls is significant ($P < 0·01$).

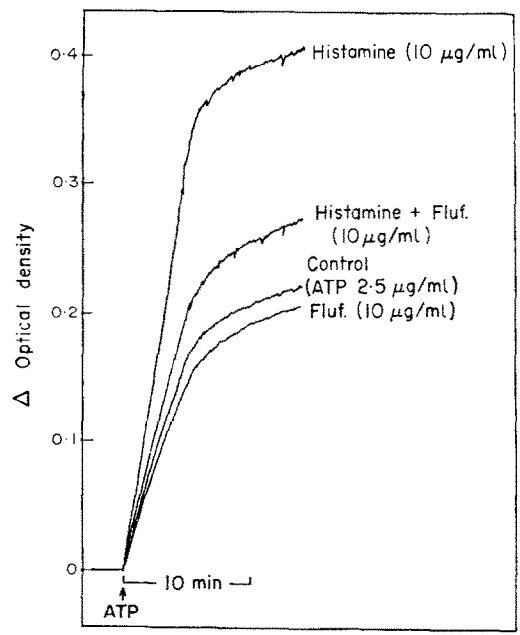

FIG. 7. Effect of flufenamic acid on ATP-induced and histamine enhanced superprecipitation of vascular actomyosin. Reaction conditions the same as in Table 2.

TABLE 3. EFFECT OF VARIOUS COMPOUNDS ON SUPERPRECIPITATION OF VASCULAR SMOOTH MUSCLE ACTOMYOSIN

Compounds		Concentration – range (μg/ml) in which the compound produces 20–50% inhibition (–) or increase (+) of the reaction compared to control
Papaverine	(–)	25–75
Sodium nitrite	(–)	5–25
Flufenamic acid	(–)	20–50
Mefenamic acid	(–)	20–50
Acetylsalicylic acid	(–)	200–400
Phenylbutazone	(–)	100–150
Indomethacin	(–)	>100
Adrenaline	(+)	0·1–1
Noradrenaline	(+)	0·1–1
Histamine	(+)	5–25
Serotonin	(+)	5–25
Bradykinin	(+)	5–10

Reaction conditions the same as in Table 2. At each compound the given concentration-range was calculated on the basis of minimum six measurements.

Table 3 shows that ATP-induced superprecipitation was inhibited by the vasodilators papaverine and sodium nitrite. An inhibitory effect was also produced by the anti-inflammatory drugs flufenamic acid, mefenamic acid, acetylsalicylic acid. Phenylbutazone had only a minimal effect, while indomethacin failed to exhibit any inhibitory action. The effect of the most potent compound flufenamic acid is presented in Fig. 7.

The data of Table 4 is evidence that the superprecipitation increasing effect of adrenaline and histamine were strongly inhibited by very low concentrations of papaverine, nitrite and flufenamic acid.

TABLE 4. EFFECT OF SOME SELECTED COMPOUNDS ON SUPERPRECIPITATION ENHANCED BY ADRENALINE OR HISTAMINE

Compounds	Concentration (μg/ml)	Inhibition (%)* of superprecipitation increased by adrenaline or histamine	
		Adrenaline (0·25 μg/ml)	Histamine (10 μg/ml)
Papaverine	5	20 (3)†	35 (3)
	25	85 (5)	90 (4)
Sodiumnitrite	1	55 (3)	35 (3)
	10	90 (3)	85 (3)
Flufenamic acid	5	58 (6)	70 (5)

Reaction conditions the same as in Table 2.
* Increasing effect of the stimulant is taken for 100 per cent.
† Number of measurement in parenthesis.

DISCUSSION

The mechanism of muscle contraction comprises the following processes: (a) depolarization of the membrane or withdrawal of Ca^{2+} ions from the stabilizing sites; (b) as a result of this depolarization the membrane structure undergoes a conformational change which leads to an increased permeability to Ca^{2+} and the Ca^{2+} ion is released from the inner surface of the membrane ("sequestered" Ca^{2+} bound to sarcoplasma reticulum); (c) Ca^{2+} ion having released into myoplasma is closely bound to troponine, whereby the inhibitory action of the tropomyosin–troponine complex on actomyosin–ATP interaction is abolished; so that muscle contraction can take place.

At present it is the generally accepted view that in all three types of muscle (skeletal, cardiac and smooth muscle) contraction is controlled by depolarization of the membrane or by the direct effect of drugs on the membrane so as to cause the release of bound calcium [see above (a) and (b)].[8] According to this concept the explanation of the mechanism of the effect of active compounds at the molecular level is by increasing or reducing the free calcium level of the myoplasma. From the theoretical aspect a third mechanism is possible, i.e. that the active compounds may interfere with the binding of Ca^{2+} ion to troponin, or interfere with the actomyosin–ATP interaction.

Contrary to skeletal muscle, in vascular smooth muscle only a small proportion of Ca^{2+} ions, eliciting contraction are derived from the poorly developed sarcoplasmic reticulum.[9] The bulk of Ca^{2+} ions which induce contraction of smooth muscle have their origin in the extracellular medium. Few investigations have been made to explain the effect of compounds on vascular smooth muscle at molecular level. The effect of catecholamines can be explained by an increase of the intracellular Ca^{2+} ion concentration. Presumably the effect is indirect, asserting itself through cyclic 3′,5′-AMP. Cyclic AMP formation may alter the permeability of the membrane, thereby controlling the release and uptake of Ca^{2+} ion.[10] However, according to a recent finding, the membrane calcium permeability does not increase during noradrenaline stimulation of arterial smooth muscle.[11]

Many authors ascribe the positive inotropic effect of catecholamines to the accumulation of cyclic AMP.[12,13] On the other hand, the results of Sulakhe and Dhalla[14] do not suggest an action of cyclic AMP on calcium transport heart sarcotubular membranes.

The inhibitory effect of papaverine on vasoconstriction can be explained by suggesting that the compound occupies in the membrane the sites to which Ca^{2+} ion is normally bound, modifying thereby the electric and mechanical activity controlled by calcium.[15] Another hypothesis is that papaverine increases the rate of uptake of calcium by sarcoplasmic vesicles.[16]

An interesting experiment has been performed by Northover[17] who found that contractions of depolarized vascular segments, elicited by various agonists, were inhibited by certain non-steroidal anti-inflammatory compounds (flufenamic acid, indomethacin) which, however did not influence the rate of ^{45}Ca influx.[18] As an explanation the author put forward the hypothesis that the compounds in question may act directly on the contractile proteins. In a recent study,[19] however, Northover has abandoned this assumption because indomethacin,—in a way similar to local anaesthetics—has been found to inhibit Ca^{2+} influx.

Superprecipitation of vascular smooth muscle actomyosin has only been studied so

far at an exceedingly high protein concentration. The present sensitive method of investigation has allowed us to follow the reaction at protein concentration as low as those previously used in investigations of the superprecipitation of skeletal muscle actomyosin. Because extraction procedure is similar (at high ionic strength) and the identical protein concentration used in the assay, it is justified to compare the superprecipitation of skeletal muscle actomyosin to the reaction of vascular smooth muscle protein. The two processes are similar in so far as (a) both require Mg^{2+} ions, while in high concentration magnesium is inhibitory; (b) small amount of calcium is necessary for the reaction; (c) superprecipitation-inducing effect of ATP decreases over a certain concentration; in high concentration ATP inhibits the reaction; (d) in case of Ca^{2+} ion excess ATP does not act as inhibitor even in high concentration.

The two processes differ inasmuch as (a) the rate and intensity of superprecipitation is less in the case of vascular actomyosin than in the case of striated muscle protein; (b) the minute amount of Ca^{2+} ion is not enough to get an increase in the optical density of protein gel upon the addition of ATP; it only suffices to make one phase of the reaction possible, namely the aggregation and rapid sedimentation of the protein. Increase of optical density can be measured only at high concentration of added calcium.

In our experiments ATP-induced superprecipitation was inhibited by ADP. Our observation contradict the results of Honig[28] who was unable to inhibit vascular smooth muscle adenosinetriphosphatase (ATPase) with ADP, while AMP and inorganic phosphate did inhibit the enzymatic activity. Contrary to Honig's finding, Herlihy and Murphy[21] could not find any direct effect of AMP or phosphate on the superprecipitation and enzymatic activity of the contractile protein of hog carotid arteries. The present findings that ADP can inhibit the superprecipitation of vascular contractile protein may provide a basis for an explanation of the mechanism of functional (ischaemic) vasodilation.

The rate and intensity of superprecipitation can be strongly enhanced by the addition of adrenaline, noradrenaline as well as by serotonin, histamine and bradykinin to the incubation medium. A remarkable but still unknown feature of the effect of catecholamines is that only low concentrations produce an enhancing effect. Comparison of our results and *in vivo* effects is rendered exceedingly difficult by the fact that of the above listed compounds only catecholamines exert a uniform effect on vascular smooth muscle (vasoconstriction), while histamine, serotonin and bradykinin (the mediators of inflammatory reaction) in isolated system produce vasoconstriction, *in vivo* vasoconstriction or vasodilation but *in situ* (applied topically on the microcirculation) invariably vasodilation.

Effects contrary to those of the above mentioned compounds were exerted by vasodilators, such as papaverine and nitrite which significantly inhibited the superprecipitation of vascular smooth muscle protein. Of the tested non-steroidal anti-inflammatory agents a similar inhibitory effect was shown by flufenamic and mefenamic acid and acetylsalicylic acid in fairly high concentrations. It is, however, much more remarkable that very low concentrations of the selected compounds (papaverine, nitrite and flufenamic acid) strongly inhibited the respective effects of adrenaline and histamine, perhaps serotonin and bradykinin-effect can also be inhibited but this was not examined in the present study.

In the vascular system vascular smooth muscle cells are not the only sites where

actomyosin–ATP interaction may play a role. The cytoplasma of endothelial cells is known to contain myofilaments which are supposed to play a part in morphological changes of the cells and so also in the formation of intercellular gaps with a consequent increase in capillary permeability.[9, 22] According to Spector[23] mediators of inflammation which appear to exert their effect by increasing capillary permeability do so by contraction of endothelial cells. It is possible that the enhancing effect of such inflammatory-mediators on the superprecipitation of vascular actomyosin is related to their capillary permeability increasing activity, while some of the non-steroidal anti-inflammatory compounds inhibit the increase via an essentially similar mechanism.

Our experiments indicate that in the interpretation of the effects of various vasoactive compounds it would be unwise to consider membrane-effects only, since the compounds may also influence the function of the contractile proteins.

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REFERENCES

1. M. L. Mallin, *J. Cell. Physiol.* **65,** 355 (1965).
2. K. F. Guthe and D. F. Bohr, *Am. J. Physiol.* **219,** 1366 (1970).
3. P. Görög and I. B. Kovács, *Biochem. Pharmac.* **19,** 2289 (1970).
4. H. H. Weber and K. Meyer, *Biochem. Z.* **266,** 137 (1933).
5. S. Ebashi, *J. Biochem.* **50,** 236 (1961).
6. H. M. Levy and M. Fleisher, *Biochim. biophys. Acta* **100,** 471 (1965).
7. A. Weber, R. Herz and I. Reiss, *J. gen. Physiol.* **36,** 679 (1963).
8. C. P. Bianchi, *Fedn Proc.* **28,** 1624 (1969).
9. J. A. G. Rhodin, *Physiol. Rev.* **42,** Suppl. No. 5 48 (1962).
10. W. G. Nayler, *Am. Heart J.* **73,** 379 (1967).
11. C. van Breemen and P. Lesser, *Microvasc. Res.* **3,** 113 (1971).
12. E. W. Sutherland and G. A. Robinson, *Pharmac. Rev.* **18,** 145 (1966).
13. J. R. Williamson, *Pharmac. Rev.* **18,** 205 (1966).
14. P. V. Sulakhe and N. S. Dhalla, *Molec. Pharmac.* **6,** 659 (1970).
15. N. Tashiro and T. Tomita, *Br. J. Pharmac.* **39,** 608 (1970).
16. F. Carpendedo, G. C. Toson, M. Furlanut and M. Ferrari, *J. Pharm. Pharmac.* **23,** 502 (1971).
17. B. J. Northover, *Br. J. Pharmac. Chemother.* **31,** 483 (1967).
18. B. J. Northover, *Br. J. Pharmac. Chemother.* **31,** 417 (1968).
19. B. J. Northover, *Br. J. Pharmac.* **41,** 540 (1971).
20. C. R. Honig, *Microvasc. Res.* **1,** 133 (1968).
21. J. T. Herlihy and R. A. Murphy, *Circulation Res.* **28,** 434 (1971).
22. G. Majno and G. E. Palade, *J. biophys. biochem. Cytol.* **11,** 571 (1961).
23. W. G. Spector, *Pharmac. Rev.* **10,** 475 (1958).