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EFFECT OF ANTI-INFLAMMATORY COMPOUNDS ON ACTOMYOSIN–ADENOSINETRIPHOSPHATE INTERACTION

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Abstract—The influence of several non-steroid anti-inflammatory agents (NAIA) on the superprecipitation and adenosinetriphosphatase (ATP-ase) activity of natural actomyosin has been studied. Superprecipitation and the ATP-induced reduction of light-scattering of actomyosin solution have been significantly inhibited by some of the investigated compounds. Inhibitory action showed the following order: flufenamic acid > mefenamic acid > indomethacin > ibuprofen > ibufenac > acetylsalicylic acid > phenylbutazone > salicylic acid. Mg and Ca ion activated ATP-ase activity of actomyosin was inhibited with similar relative potencies. The compounds proved to be much stronger inhibitors of Ca than of Mg ion activated ATP-ase. Positive correlation has been found between the inhibitory effect of NAIA on vascular smooth muscle constriction and antagonism of actomyosin-ATP–interaction.

THE INTERACTION of actomyosin and adenosinetriphosphate (ATP) implies on the one hand the hydrolysis of nucleoside triphosphate, on the other hand the physical transformation of actomyosin gel. The latter process, during which the transparent gel becomes dense, crystalline and shows rapid sedimentation, is termed superprecipitation.[1] Superprecipitation together with the investigation of the adenosinetriphosphatase (ATP-ase) activity of the contractile protein provide an *in vitro* model of muscular contraction.

In the past few years a contractile protein, similar to the actomyosin of the striated muscle has been found to be present on the outer surface of the membrane of several kinds of cells. The first "ecto-ATP-ase" enzyme was discovered by Engelhard on the membrane of nucleated erythrocytes.[2] Similar enzyme activity was observed by Hoffman on non-nucleated erythrocytes,[3] by Bettex-Galland on the surface of platelets[4] and by Diamant on the membrane of peritoneal mast cells in the rat.[5] The contractile protein situated on the outer surface of erythrocytes and platelets is assumed to play an important role in the control of the size and shape of cells. Several authors have explained cell aggregation by a change in the activity of this contractile protein.[6–9] Non-steroid anti-inflammatory agents (NAIA) have been proved to be capable of inhibiting the aggregation of platelets[10,11] and erythrocytes[12] induced by various aggregating agents. In the case of platelet[13] and erythrocyte aggregation[14] the inhibitory compounds are supposed to act by the alteration of the contractile and enzyme activity of the protein on the outer surface of the cell-membrane.

The vascular effects of certain NAIA also suggest that the function of the contractile

protein is influenced by these compounds. Contractions produced by various vasoactive substances in isolated vessels have been strongly inhibited by NAIA.[15, 16]

In the light of these findings it seemed worthwhile to investigate the influence of NAIA on actomyosin–ATP interaction. The results obtained indicate that even in low concentrations certain NAIA strongly inhibit the physical transformation and enzymic activity of actomyosin.

MATERIALS AND METHODS

Actomyosin gel (Myosin B or "natural actomyosin") was prepared from rabbit muscle essentially according to the procedure of Levy and Fleisher.[17] The ground muscle was extracted with alkaline 0·6 M KCl for 24 hr, the actomyosin was precipitated twice and used within 8 days after the time of extraction. In the experiments performed on trypsin-treated "natural actomyosin", the suspension of actomyosin (2 mg/ml) was incubated with trypsin (10 μg/ml) for 20 min at 25°. After stopping the reaction by sufficient amount of trypsin-inhibitor the actomyosin was centrifuged and resuspended for use. Salicylic acid was used as sodium salt, flufenamic acid, mefenamic acid, acetylsalicylic acid, indomethacin, phenylbutazone, ibufenac (4-isobutylphenylacetic acid) and ibuprofen (4-isopropylphenylacetic acid) as Tris salts. The investigated compounds were incubated for 5 min with actomyosin gel prior to the addition of ATP. All readings were performed at room temperature (25°).

Measurement of superprecipitation

The standard medium contained actomyosin gel 0·2 mg/ml, 30 mM KCl, 60 mM Tris-maleate buffer (pH 7·4), 5 mM $MgCl_2$, the total volume amounting to 2·0 ml. Superprecipitation was measured with the turbidimetric method of Levy and Fleisher.[17] During the whole time of measurement the content of the cell was stirred (1000 rpm) with a small magnetic rod at the bottom of the cell. Reaction was started by the addition of 0·05 ml ATP (8 μM) and the increase of turbidity was determined 1 min later at 545 mμ.

Measurement of the "clearing" effect of ATP

The standard medium contained 0·5 mg/ml actomyosin, 0·6 M KCl, 10 mM $MgCl_2$, 25 mM Tris-maleate buffer (pH 7·4), the total volume amounting to 4·0 ml. The light-scatter of the actomyosin solution observable at a 90° angle was measured with the fluorimetric equipment of the spectrophotometer. As control, the intensity-decrease of scattered light of actomyosin solution after the addition of 0·2 mM ATP was taken for 100 per cent and the effect of the investigated compounds was calculated on this basis as percentage values.

Measurement of ATP hydrolysis

The standard medium consisted of 0·2 mg/ml actomyosin, 30 mM KCl, 25 mM Tris-maleate buffer (pH 7·4), 2·5 mM ATP and 5 mM $MgCl_2$ or 10 mM $CaCl_2$. Five min after the addition of ATP, reaction was stopped with ice cold trichloracetic acid applied in 5% final concentration. Activity was estimated by measuring the production of inorganic phosphate (P_i) by the method of Lowry and Lopez.[18]

RESULTS

The respective effects of the investigated compounds on superprecipitation and on ATP-induced "clearing" are illustrated in Table 1. Superprecipitation has been

significantly inhibited by the compounds in the following order: flufenamic acid > mefenamic acid > indomethacin > ibuprofen > ibufenac > acetylsalicylic acid > phenylbutazone > salicylic acid. The respective values of pentachlorophenol, *p*-chloromercurybenzoate (PCMB) and Mersalyl have also been tabulated for comparison. It must be remarked here that the antranilates (flufenamic and mefenamic acid), arylacetic acids (ibufenac and ibuprofen) and acetylsalicylic acid produced almost com-

TABLE 1. EFFECT OF ANTI-INFLAMMATORY COMPOUNDS ON SUPERPRECIPITATION AND ATP-INDUCED CLEARING OF ACTOMYOSIN

Compounds	Concentration (μg/ml)	Superprecipitation		Clearing	
		ΔE_{545} mμ*	IC_{30}†	Decrease of light-scattering (%)	IC_{30}†
Control (only ATP)	—	0·54†	—	100	—
Acetylsalicylic acid	100	0·48		88	
	200	0·36	210	73	225
	400	0·18		44	
Salicylic acid	200	0·51		82	
	400	0·47	730	80	800
	800	0·36		70	
Flufenamic acid	10	0·45		92	
	20	0·32	16	77	24
	40	0·15		41	
Mefenamic acid	20	0·50		84	
	40	0·31	33	68	37
	80	0·07		32	
Indomethacin	20	0·42		83	
	40	0·38	39	76	63
	80	0·30		65	
Phenylbutazone	100	0·46		78	
	200	0·38	200	73	255
	400	0·32		62	
Ibufenac	100	0·52		76	
	200	0·36	190	65	155
	400	0·12		41	
Ibuprofen	50	0·48		80	
	100	0·35	88	70	105
	200	0·08		51	
Pentachlorophenol			52		6
PCMB			12		32
Mersalyl			25		35

* = Before the addition of ATP $OD_{545} = 0·162 \pm 0·008$.
† Concentration producing 30 per cent inhibition (μg/ml).
‡ = Every value is the mean of five parallel readings.

plete concentration-related inhibition, while in the case of indomethacin and phenylbutazone inhibitory effect did not exceed 50 per cent notwithstanding the use of considerably higher concentrations. This finding explains why comparison of the compounds has been based on the concentrations producing 30 per cent inhibition.

Investigating the superprecipitation of trypsin-treated "natural actomyosin", the nature of which is similar to that of reconstituted actomyosin,[19] flufenamic acid inhibited the superprecipitation in a similar order of magnitude as in the case of "natural actomyosin".

As demonstrated in Table 1, the compounds exerted a similar influence on super-

precipitation and on the "clearing" induced by ATP. In the latter test the IC_{30} values were slightly higher than in conjunction with superprecipitation, which may be ascribed partly to the inevitably higher concentration of actomyosin applied.

The effects of NAIA on Mg^{2+} and Ca^{2+} activated ATP-ase of actomyosin are illustrated in Table 2. The influence of lower concentrations of the compounds on "basic" ATP-ase activity of actomyosin estimated without the addition of any activator has been studied separately. Since the chemicals had not been subjected to any special

TABLE 2. EFFECT OF ANTI-INFLAMMATORY COMPOUNDS ON THE Ca AND Mg ION ACTIVATED ATP-ASE ACTIVITY OF ACTOMYOSIN

Compounds	Activator	μmoles P_i/mg N/5 min							
		at various conc. of the compounds (μg/ml)							
		—	10	20	40	80	100	200	400
Control	—	2·4							
	Mg	9·8							
	Ca	14·3							
Flufenamic acid	—		0·42						
	Mg		7·3	6·2	5·2				
	Ca		9·6	5·4	2·1				
Indomethacin	—			1·65					
	Mg			9·1	8·5	6·4			
	Ca			10·1	7·3	5·1			
Ibuprofen	—				1·2				
	Mg				8·3		7·6	7·2	
	Ca				11·5		8·2		
Acetylsalicylic acid	—						0·62		
	Mg						8·6	7·5	6·3
	Ca						10·3	8·4	3·8
Phenylbutazone	—						1·45		
	Mg						9·7	8·4	7·5
	Ca						12·6	9·3	5·2

Every value is the mean of three parallel readings.

purification, the presence of minimal amounts of Ca and/or Mg ions has to be reckoned with here too. The results reveal that the Mg and Ca ion activated ATP-ase activity of actomyosin has been inhibited by certain NAIA. The relative efficacy of the compounds corresponds to the order of effectiveness observed in connection with superprecipitation. The inhibitory effect on Ca ion activated ATP-ase was significantly stronger than on Mg ion activated enzyme. Low concentrations of the effective compounds inhibit the activity of ATP-ase measured without the addition of any activator.

DISCUSSION

The results of the present experiments support the hypothesis that low concentrations of NAIA exert a significant influence on the enzymatic and physical properties of actomyosin. Of the compounds investigated, flufenamic and mefenamic acid, ibufenac ibuprofen and perhaps acetylsalicylic acid possess this effect at concentrations which were usually attained in the blood of patients after oral medication of the drugs in question. In our experiments effects on the physical transformation of actomyosin gel and on the ATP-ase activity have been found to run a parallel course. The effects of the NAIA investigated by ourselves have been proved to differ from the effects of

such compounds as pentachlorophenol or PCMB, low concentrations of which have been found to enhance the ATP-ase activity of actomyosin and even to play the role of activator, whereas in higher concentrations they strongly inhibit both ATP-ase activity and superprecipitation.[20,21] Contrary to this biphasic effect in the case of NAIA only inhibitory action has been demonstrable by the applied methods.

The sensitivity of superprecipitation to inhibition, measured at low ATP concentration and ionic strength correspond to the sensitivity to inhibition of "clearing" observed at high ATP concentration and ionic strength. This is also a proof that the precipitation of protein gel or the dissociation of actomyosin molecule rest on the same mechanism.

According to our present knowledge Ca ion plays a decisive role in muscular contraction.[22] The free calcium ions cause contraction of the myofibrils by eliminating the control by which the troponin–tropomyosin complex suppresses the magnesium activated interaction of actomyosin and ATP. The "relaxant" effect of effective NAIA derive from the direct inhibitory effect on magnesium-activated interaction of actomyosin and ATP, as the inhibitory effect of the most active compound (flufenamic acid) can be estimated on trypsin-treated "natural actomyosin" which protein is insensitive to calcium ion.

Our experiment shows a favourable relationship to the results of Northover[15,16] who estimated the inhibitory effect of certain NAIA on the various contractions of isolated arteries and veins. Particularly in reducing constriction of the smooth muscle and in inhibition of actomyosin–ATP interaction the compounds investigated in both studies (flufenamic and mefenamic acid, indomethacin, ibufenac, phenylbutazone and salicylic acid) have shown a very similar order of efficacy. In the experiments of Northover, effective NAIA, unlike local anaesthetics did not exert any influence on the calcium permeability of smooth muscle cells, therefore the author assumed that the compounds in question acted directly on the contractile protein of the smooth muscle. Our present findings confirm this assumption. The observation, however, that the effective NAIA have a marked inhibitory effect on Ca^{2+} dependent ATP-ase activity raise the possibility that these drugs—beside the direct effect on the contraction of actomyosin—may alter the ability of sarcoplasmic reticulum to accumulate and release Ca ion.

The correlation between the inhibitory effects of these compounds on cellular aggregation and on actomyosin–ATP interaction is less clear-cut. Aggregation of erythrocytes[12] and of platelets[10, 11] has been strongly inhibited and with equal potency by phenylbutazone and flufenamic acid. On the other hand, contrary to flufenamic acid, phenylbutazone exerted a minimal inhibitory effect on actomyosin–ATP interaction. It is remarkable that in inhibiting erythrocytes and platelets aggregation and in antagonizing actomyosin–ATP interaction acetylsalicylic acid has proved to be far more effective than salicylic acid. These findings are consistent with the observations that acetylsalicylic acid is much more efficient than salicylic acid against bradykinin-induced bronchoconstriction in the guinea-pig[23] or as an inhibitor of erythema-reaction[24]—and clearly show that the mechanism of action of the two drugs is quite different.

Our studies indicate that the effect of NAIA on actomyosin may play a role in the inhibitory effect of these compounds on cellular aggregation and on experimental inflammation. The justification of this assumption will have to be proved by studies which elucidate the effects exerted on actomyosin isolated from smooth muscle and from cellular membranes.

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