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TRIFLUOPERAZINE CAN DISTINGUISH BETWEEN MYOSIN LIGHT CHAIN KINASE-LINKED AND TROPONIN C-LINKED CONTROL OF ACTOMYOSIN INTERACTION BY Ca^{++}

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SUMMARY

Trifluoperazine specifically inhibits both the increased ATPase and the degree of phosphorylation of myosin light chains induced by 5×10^{-5}M Ca^{++} in chicken gizzard muscle actomyosin preparations. Half maximal inhibition occurs at a concentration of trifluoperazine of 3×10^{-6}M. Pig skeletal muscle actomyosin ATPase was not inhibited by concentrations of trifluoperazine below 10^{-4}M. It is suggested that trifluoperazine can be used to distinguish between the two different mechanisms of Ca^{++} regulation of actomyosin interaction, operational in smooth and skeletal muscle, and can thus be used to investigate the class of Ca^{++} control of actomyosin operating in various aspects of non-muscle cell motility.

INTRODUCTION

There are two distinct models by which calcium ions are believed to exert a regulatory influence in the interaction between actin filaments and myosin in vertebrate muscle. In striated muscle there is compelling evidence that Ca^{++} mediates exposure of myosin binding sites on the thin filaments by inducing a shift in the position of tropomyosin on the actin helix (1). This shift in the position of tropomyosin results from the binding of Ca^{++} to troponin C, the Ca^{++} receptor in the troponin complex (2,3), and thus acts to relieve an inhibition.

Calcium regulation of smooth muscle contraction on the other hand does not require the participation of the thin filaments in the control process per se (4) but instead appears to be mediated by the direct activation of myosin ATPase by control of the level of phosphorylation of one of the two pairs of myosin light chains by interaction between a specific light chain protein phosphokinase and a phospho-protein phosphatase (5,6). The positive aspect of the control process being activation of the light chain kinase by a calmodulin-Ca^{++} complex (7). Thus in the two cases cited the concentration

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dependence of activation by calcium of the actomyosin ATPase reflects the binding chacteristics of troponin C and calmodulin respectively for Ca^{++}.

The mechanism of regulation of actomyosin ATPase in non-muscle cells is far less clear, due in part to the diffuculty of preparing Ca^{++}-sensitive actomyosin from tissues or cells in which these proteins constitute only a small proportion of the total cell protein. There is evidence for the presence of myosin light chain kinase activity in several tissues (see 8) but only the kinase isolated from blood platelets and brain shows any Ca^{++}-dependence (9) and since both striated (10) and smooth muscle (11) contain Ca^{++}-dependent myosin light kinases its presence does not a priori imply a particular class of control.

Certain antipsychotic drugs have been shown to cause vascular relaxation and to prevent superprecipitation of smooth muscle actomyosin (12) by inhibition of the phosphorylation of myosin light chain (13). In this report I present evidence that the phenothiazine antipsychotic trifluoperazine (TFP) can distinguish between actimyosin ATPase regulated via myosin light chain kinase, and that regulated via troponin C, by specific inhibition of Ca^{++}-induced phosphorylation of light chains. This drug could thus prove a useful probe into the mechanisms of Ca^{++}-regulation of actomyosin ATPase operating in non-muscle cell motility.

MATERIALS AND METHODS

Materials

(γ-^{32}P)ATP as the triethylammonium salt was purchased from the Radiochemical Centre, Amersham, Bucks., U.K., in 50% (v/v) ethanol. The ethanol was removed at 23°C under vacuum and the (γ-^{32}P)ATP stored as a 10mM solution in 50mM-Na-Pipes* pH 6.8 at -30°C at an initial specific radioactivity of 25 mCi/mmole. Butyl-PBD* was obtained from Birchover Instruments, Letchworth, Herts.,U.K., and incorporated into a scintillation 'cocktail' containing 6g of butyl-PBD, 80g of naphthalene, 600 ml of toluene and 400 ml of 2-methoxyethanol. Trifluoperazine hydrochloride was a generous gift from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K.. Fresh chicken gizzards were generously provided by Country Produce Ltd., Queensferry, Clwyd, U.K., and were stored

*Abbreviations

Pipes, 1.4 piperazinediethanesulphonate; Butyl-PBD, 5 - (4 - Biphenylyl)-2 - (4-t-butylphenyl) - 1 - oxa - 3, 4 - diazole; SDS, sodium dodecyl sulphate; EGTA, - Ethyleneglycol-bis-(β-aminoethyl ether)-N,N'tetracetic acid; TFP, trifluoperazine.

at -30°C. Chemicals for electrophoresis were obtained from BDH Chemicals, Poole, Dorset, U.K., and all other chemicals from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Preparation of chicken gizzard actomyosin - Crude actomyosin was prepared from frozen chicken gizzard muscle essentially as described by Driska and Hartshorne (14). Triton X-100 at 1%(v/v) was included in the 3rd and 4th of a total of six washes. After the final low ionic strength precipitation the washed pellet was resuspended in 10mM-Na-Pipes pH 6.8 and stored on ice in this buffer; under these conditions Ca^{++}-sensitivity remained stable for several days. Yields varied between 6-10mg actomyosin per g of frozen gizzard.

Preparation of pig skeletal actomyosin - Fresh pig muscle was minced into 50% (v/v) glycerol containing 3mM-Tris-HCl pH 7.5 and stored at -30°C. The glycerol solution was changed by centrifuging myofibres at 9, $500g_{av}$ for 10' and resuspending them in 10 volumes of fresh glycerol solution. Several changes were made over a period of a week until the myofibres were almost white, they could be stored for many months without loss of calcium sensitivity.

To extract crude actomyosin the myofibres were washed in 10mM Tris-HCl pH 7.5 at 0°C then extracted with 0.6M KCl, 10mM Na_2CO_3, 40mM $NaHCO_3$ at 0°C (15). The viscous myofibre suspension was then centrifuged at $50,000g_{av}$ for 30 min at 2°C to remove myofibres. The clear layer of supernatant was filtered through glass wool and stored as a 10mg/ml solution on ice.

Measurement of ATPase activity and phosphorylation - All ATPase measurements were made in 50mM-Na-Pipes pH 6.8, 60mM-KCl, 1mM-$MgCl_2$, at 37°C, the reaction was started by addition of (γ-^{32}P)ATP to 1mM. All incubation media contained 5mM-EGTA and various $CaCl_2$ concentrations, calculated to give the quoted free calcium ion concentrations by a multiple ligand/metal ion programme written by Dr.P.J.England, Biochemistry Department, Bristol University, BS8 1TD, U.K.

Samples for ATPase measurements were taken at zero time and 10 minutes. The concentration of protein used was adjusted to give a maximum of 50% hydrolysis of (γ-^{32}P)ATP over the incubation time. Aliquots (10µl) were removed from the incubation mixture and quenched into 1 ml 5%(v/v) perchloric acid containing 10mg/ml Norit-GSX charcoal (16). The charcoal was removed by centrifugation and 0.5ml aliquots of the supernatant assayed for radioactivity in 10ml H_2O by Cerenkov radiation.

Phosphorylation was determined in 20µl-40µl aliquots of incubation mixture added onto 2 x 1cm rectangles of Whatman 3MM paper. The paper rectangles were washed x 3 in 5%(v/v) trichloracetic acid by stirring in a wire cage, generally over a 2-4h period. The paper rectangles then received a final short wash in ethanol and were assayed for radioactivity in the butyl-PBD scintillation 'cocktail' described in Materials and Methods. The specific radioactivity in each assay was calculated from an internal standard. TFP was dissolved just prior to each experiment into distilled water and added at 1% of the final assay volume to give the quoted concentrations. All actomyosin preparations were assayed at 10^{-8}M-Ca^{++} and 5×10^{-5}M-Ca^{++} prior to inhibition experiments, preparations showing less than a 4 fold stimulation were discarded.

RESULTS AND DISCUSSION

By using the isolation procedures described with frozen gizzard muscle, the actomyosin generally exhibited a 4-10 fold stimulation of both ATPase activity and degree of phosphorylation in the presence of 5×10^{-5}M-Ca^{++} compared to that at 10^{-8}M-Ca^{++}, the maximal degree of phosphorylation noted at 5×10^{-5}M-Ca^{++} was 2.7 nmoles mg^{-1}, which in these preparations approximates to 2 moles of Pi per mole of myosin.

The Ca^{++}-sensitivity curves for both gizzard and skeletal actomyosin ATPase and gizzard phosphorylation are almost superimposable (Fig.1) with half maximal stimulation occurring at 10^{-6}M. The actual figures for this half maximal point vary in the literature between 10^{-6} and 10^{-5}M-Ca^{++} (17,18). These differences of course may reflect the different association constants used and whether correction was made for ATP and Mg^{++} concentration in calculating Ca^{++}-concentration. On the data presented here Ca^{++}, Mg^{++}, EGTA and ATP concentrations were all taken into account; Pipes does not bind divalent cations significantly (19).

In the presence of increasing concentrations of TFP the Ca^{++}-sensitive portion of the ATPase of gizzard actomyosin was progressively inhibited showing kinetically some tendency towards negative cooperativity. The half maximal inhibition occurred at 3×10^{-6}M TFP. The non Ca^{++}-dependent ATPase

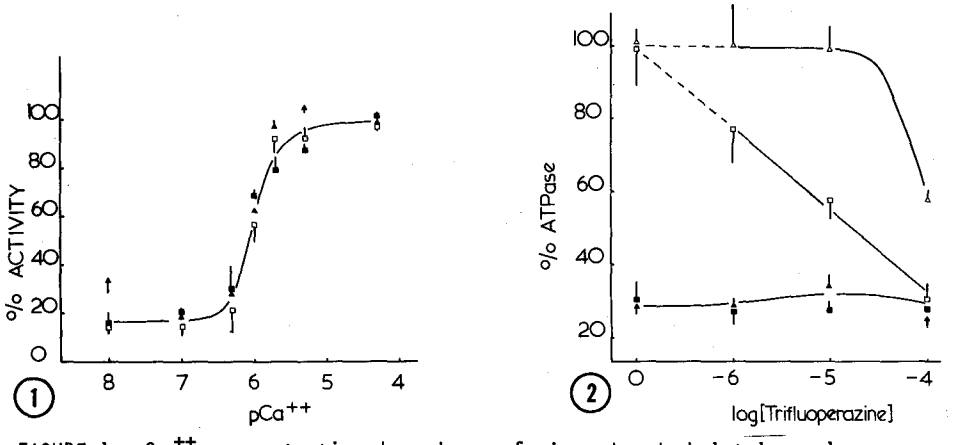

FIGURE 1 - Ca^{++} concentration dependence of gizzard and skeletal muscle actomyosin ATPase and gizzard phosphorylation.
ATPase activity was measured in actomyosin preparations from gizzard and skeletal muscle as described in Materials and Methods. Ca^{++} concentrations were maintained by using 5mM-EGTA to buffer free Ca^{++}. Phosphorylation was assayed as tricholoracetic acid - insoluble material under the same conditions. Activity is expressed as a % of that at 5×10^{-5}M Ca^{++}.
■ - gizzard ATPase; □ - gizzard phosphorylation; ▲ - skeletal ATPase.
Bars indicate S.E.M.

FIGURE 2 - The effect of trifluoperazine on gizzard and skeletal muscle actomyosin ATPase.
ATPase activity was measured as described in Materials and Methods at 10^{-8}M-Ca^{++} (solid symbols) and 5×10^{-5}M-Ca^{++} (open symbols) in the presence of various concentrations of TFP. ■,□ - chicken gizzard actomyosin, ▲, △ - pig skeletal actomyosin. Activity is expressed as a % of the ATPase activity at 5×10^{-5}M-Ca^{++} in the absence of TFP.
Bars indicate S.E.M.

activity was not inhibited at concentrations of TFP up to 10^{-4}M (Fig.2). The Ca^{++}-activated ATPase of pig skeletal actomyosin on the other hand was only inhibited at 10^{-4}M TFP and the slope of inhibition appeared much steeper, perhaps reflecting a different mechanism of inhibition.

The mechanism of action of TFP has been extensively described in the publications of Weiss and coworkers (see review 20). They quote two high affinity binding sites for TFP on calmodulin, Kd=10^{-6}M, showing a degree of negative cooperativity which appears to be reflected in the kinetics of inhibition of the gizzard actomyosin ATPase reported here. They also found that TFP only binds to the Ca^{++}-calmodulin complex and not to free calmodulin, thus presumably preventing the interaction of the activating complex with its target enzyme. Since calmodulins isolated from various tissues are similar (but not identical) in biological properties and structure to Troponin C (21,22), it was considered necessary to demonstrate a lack of inhibition of skeletal muscle Ca^{++}-activation to avoid the possibility that TN-C itself retains the binding sites for TFP, which appear to be a ubiquitous feature of calmodulin (2,3). Although the mode of action of TN-C would not of course present a free Ca^{++}-TN-C complex to TFP.

That TFP operates through the phosphorylation of myosin light chains can be seen in Table 1 where at 10^{-4}M, a concentration which completely inhibits the Ca^{++}-dependent ATPase, phosphorylation is totally prevented.

TABLE 1

Effect of Trifluoperazine on Gizzard Actomyosin Phosphorylation

Incubation conditions	Free Ca^{++} concentration	
	10^{-8}M	5×10^{-5}M
	Gizzard actomyosin phosphorylation nmoles mg^{-1}	
control	0.38±0.02(4)	3.39±0.14(4)
TFP(10^{-4}M)	0.21±0.07(4)	0.31±0.02(4)

Phosphorylation was assayed in gizzard actomyosin preparations as described in Materials and Methods at 10^{-8}M-Ca^{++} and 5×10^{-5}M-Ca^{++} and in the absence or presence of 10^{-4}M-TFP. Figures are means ± S.E.M. for the no.of data points indicated in parentheses from two actomyosin preparations.

These data show that TFP can distinguish between the two classes of control of actomyosin interaction thus far descibed in vitro by inhibition of light chain phosphorylation and may serve as a means to identification of the control mechanisms operating in non muscle cells, and possibly also the role of the myosin light chain kinase in skeletal muscle in which the P light chain appears to be fully phosphorylated in resting muscle (24). Thus far there have been reports of light chain phosphorylation in platelet actomyosin which was not Ca^{++}-dependent (25) and isolation of myosin light chain kinases from various tissues which are (9) or not (see 8) themselves Ca^{++}-dependent. Given that both smooth and skeletal muscle systems contain the enzymes required for controlling the phosphorylation of light chains, but appear to use two entirely different systems operationally to control Ca^{++}-activation of contraction, a probe or method is required which can distinguish between the operational control mechanism rather than the potential mechanisms available to the system. Experiments are underway to probe aspects of non muscle cell motility, which mainly by virtue of the cells containing actin and myosin,or of a process being sensitive to one of the cytochalasins (see review 26) have been thought to involve actomyosin interaction. It must be noted however that a negative result from the use of TFP in such situations is not specific i.e. it could mean either that actomyosin is not the driving force for the motile event or that the control system operating is of the TN-C linked type, or of another class.

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