Parallel Response of Myofibrillar Contraction and Relaxation to Four Different Nucleoside Triphosphates

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ABSTRACT   The Mg chelates of ITP, GTP, and UTP in addition to that of ATP were shown to be capable of causing complete relaxation of myofibrils as indicated by the complete inhibition of syneresis and the reduction of the NTPase activity to that of isolated myosin. For ITP and GTP the required concentrations were about 100 times higher than those for ATP, whereas UTP was maximally effective also in low concentrations (0.2 mM). For all NTP's the concentrations for relaxation were related to those necessary for contraction so that NTP concentrations which gave 80–90 % maximal NTPase activity in the presence of Ca caused complete relaxation in the absence of Ca. Thus, these experiments do not support the view that relaxation is caused by the binding of NTP to a special inhibitory site but are quite compatible with the idea that relaxation depends on the extent to which the hydrolytic site is saturated with NTP. The Ca concentration required for contraction depends on the nature of the NTP base and its concentration; it is lower for ITP than for ATP and decreases with decreasing concentrations of ITP.

INTRODUCTION

Relaxation of muscle depends on ATP in a twofold manner. ATP is required for the removal of Ca by reticulum (Hasselbach and Makinose, 1961; Ebashi and Lipmann, 1962) and ATP must interact directly with the myofibrils (Weber and Herz, 1962; Endo, 1964; Levy and Ryan, 1965).

ATP is not specific as an energy source for the transport of Ca from the medium into the reticulum but can be replaced by inosine triphosphate (ITP) (Martonosi and Feretos, 1964) and other nucleoside triphosphates (NTP) (Hasselbach, 1964; Carsten and Mommaerts, 1964; Makinose and The, 1966). Makinose and The (1966) showed that all NTP's are capable of lowering the Ca^{2+} in the medium to concentrations below 10^{-8} M; i.e., concentrations sufficiently low for relaxation to occur. Nevertheless, it was reported that glycerol-extracted muscle fibers could not be made to relax by sarco-

plasmic reticulum (then known as the Marsh-Bendall factor) in the presence of NTP's other than ATP, acetylATP, and cytosine triphosphate (CTP) (Hasselbach, 1956; Bendall, 1958). Since the data on the reticulum rule it out as the site of this NTP specificity, one is forced to assume that NTP's with bases like guanine, inosine, or uridine are incapable of the direct interaction with myofibrils responsible for relaxation. In support of this view Bendall (1958) and Watanabe and Sleator (1957) found that ITP could also not relax tension if Ca was removed by MgEDTA instead of by the reticulum.

This ATP specificity of myofibrillar relaxation would indicate that the myofibrillar site for NTP interaction which causes relaxation is quite distinct from that for contraction, because all NTP's can cause contraction.

However, the finding that ITP cannot cause relaxation is quite surprising in view of the observation that the rate at which ITP is hydrolyzed by myofibrils is inhibited on removal of Ca (Bendall, 1958). Unchanged mechanical activity combined with a decrease in the rate of hydrolysis would indicate changes in the coupling between chemical energy and mechanical work. It would suggest that with ATP as a substrate the mechanism operates at a lower efficiency than with ITP after removal of Ca. However, it would be surprising if on removal of Ca the interaction between actin and myosin is suppressed in the presence of ATP on the one hand and made more efficient in the presence of ITP on the other.

Therefore, it seemed of interest to reinvestigate the relaxing action of NTP's other than ATP and we measured the effect of ITP, guanosine triphosphate (GTP), and uridine triphosphate (UTP) on the syneresis and the enzymic activity of myofibrils.

EXPERIMENTAL

Myofibrils were prepared as previously described (Weber et al., 1963).

Syneresis was estimated from the protein concentration in the precipitate after centrifugation at 3000 g for 1 min (Weber et al., 1963). In the experiments without a phosphate donor (all experiments except those of Fig. 1) an estimate of the NTP concentration at the end of the incubation period, but before completion of sedimentation, was obtained by measuring the liberation of inorganic phosphate into the supernatant (according to Taussky and Schorr, 1953). The NTP concentration given for each experimental point is the arithmetic mean of the initial and the final concentrations.

Hydrolytic Activity   Myofibrils were incubated with the NTP for 1 min under vigorous stirring. The ionic conditions are described in each figure legend. In the experiments of Fig. 1 the ATP concentration was kept constant by creatine phosphate (CP) and creatinephosphokinase (CPK). The incubation was terminated by the addition of pCMB, $ZnSO_4$, and $Ba(OH)_2$ in the presence of CP (Somogyi, 1945) and with 4% trichloroacetic acid (TCA) in the other experiments. In the presence of CP the liberation of creatine was measured (Eggleton et al., 1943), otherwise that of inor-

ganic phosphate (Taussky and Schorr, 1953). For the creatine determination Ca was added, after the incubation was over, in an amount equal to that of the free EGTA present in order to avoid the formation of a precipitate during the development of the color reaction.

In the experiments without a phosphate donor the concentration of NTP at the end of the incubation period was calculated and the rate of hydrolysis related to the arithmetic mean of the initial and final concentrations. In the experiments illustrated by Fig. 5 the myofibril concentrations were varied, on the basis of trial and error, so that for each Ca concentration the ITP or ATP concentration during hydrolysis was reduced by the same amount.

Calculation of the Ca Concentration The Ca^{2+} concentrations in the experiments illustrated by Fig. 4 were calculated (as in previous experiments (Weber and Winicur [1961]) from the ratio CaEGTA/EGTA and the dissociation constant for

$$\frac{Ca \times (EGTA^{4-} + EGTA^{3-} + EGTA^{2-})}{CaEGTA^{2-}} = K = 2 \times 10^{-7} \text{ for pH 7.0}$$

(the binding constants for Ca and H^+ and EGTA were taken from Chaberek and Martell, 1959).

Reagents CP and CPK were purchased from Boehringer Mannheim Corp., and the NTP from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Relaxation, i.e. inhibition of syneresis and ATPase activity on removal of Ca, was not observed at concentrations of MgATP below 2 μM (Fig. 1). Inhibition became significant for ATPase activity at a concentration of about 3 μM (Fig. 1 A) and for syneresis at about 10 μM MgATP (Fig. 1 B) and was complete for both at about 20–50 μM. The *extent* of syneresis appears to be inhibited over the same concentration range as the *rate* of syneresis as measured by Levy and Ryan (1967). From these data it may appear that the range of MgATP concentration over which syneresis was inhibited was unusually narrow, unless one takes into consideration the finding that the extent of syneresis in the presence of EGTA (2 mM) was significantly higher than in its absence (a similar observation was made for the rate of syneresis as measured by changes in the optical density at 545 nm by Levy and Ryan, 1967). Possibly the first sign of inhibition is the failure of syneresis to increase with increasing ATP concentration rather than a depression below the extent of syneresis in the absence of EGTA. Thus one may consider that syneresis was significantly inhibited by 6 μM ATP because syneresis had not increased with increasing ATP but had remained the same as at 3 μM ATP.

The nature of the activation of syneresis in the presence of EGTA is not understood and it is not known whether the activation is related to the low concentrations of Ca or to the presence of EGTA. It should be pointed out that there was no corresponding acceleration of ATPase activity (Fig. 1 A).

Figs. 2 and 3 show that inhibition of myofibrillar ATPase and syneresis in the absence of Ca was not specific for MgATP but could be produced by the Mg chelates of other NTP's if they were present in sufficiently high concentrations. In order to insure that all NTP's were present as the Mg chelates, Mg was added in a 5 mM excess over the concentration of the NTP's.

The inhibition of syneresis was the same for all compounds studied and was complete. The pellet formed by uncontracted myofibrils in the absence of ATP contains protein in a concentration of about 20–25 mg/ml; therefore,
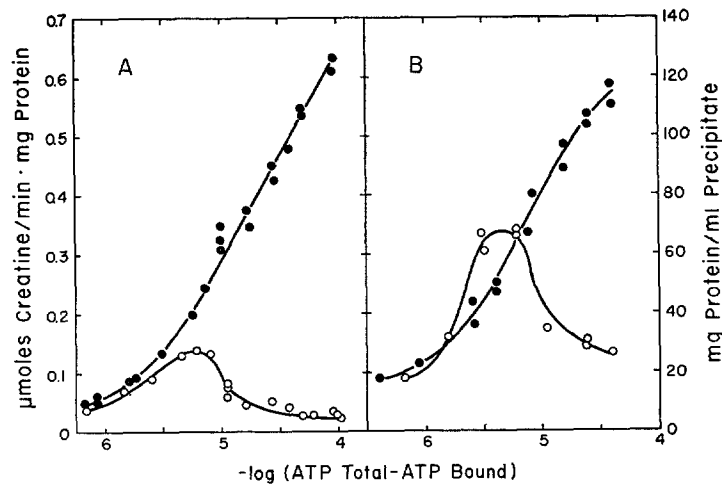

FIGURE 1. Syneresis and ATPase activity as a function of the ATP concentration of the medium in the absence (open circles) and presence (filled circles) of Ca. A, ATPase activity, 2.6 mg/ml myofibrillar protein, 5 ml; $I = 0.08$; 16 mM imidazole, pH 7.0; 8 mM CP, 2 mg/ml CPK; 1 mM Mg^{2+}; open circles, 4 mM EGTA; filled circles, 40 μM Ca; 1 min incubation at 24°C. B, syneresis, 4.2 mg/ml myofibrillar protein, 5 ml; $I = 0.11$; 20 mM imidazole, pH 7.0; 10 mM CP, 0.6 mg/ml CPK; 1 mM Mg^{2+}; open circles, 2 mM EGTA; filled circles, 40 μM Ca.

this protein concentration is equated with zero syneresis. The inhibition of the hydrolytic activity, however, may appear on first glance to have been incomplete with ITP and GTP since the lowest rates were much higher than those with ATP or UTP. Since relaxation is generally considered to be characterized by a cessation of actin-myosin interaction, the rate of NTP hydrolysis during complete relaxation should be reduced to the rate by myosin alone. Table I shows that myosin hydrolyzed ITP and GTP at much higher rates than ATP and UTP in confirmation of previous results by Hasselbach (1957). When the myofibrillar rate of hydrolysis is related to the myosin content of myofibrils (0.5 mg/mg protein, Hanson and Huxley, 1957) by doubling the myofibrillar rate, a comparison of the data in Table I and Figs. 2 A and 3 A shows that the rate of hydrolysis of ITP and GTP by myofibrils had been
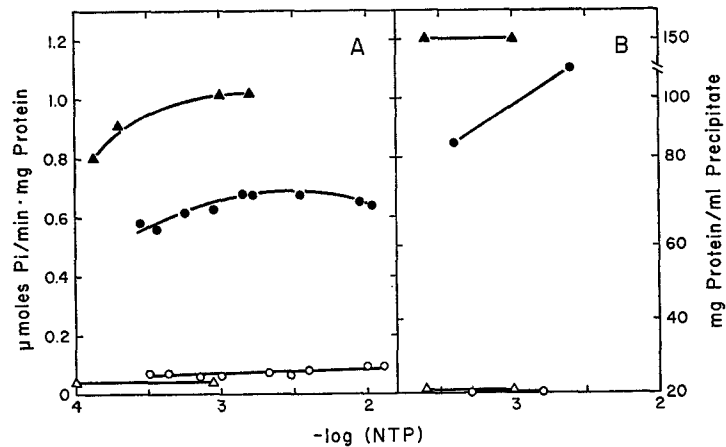
FIGURE 2. Syneresis and NTPase activity in the absence (1 mM EGTA) open circles and open triangles, and in the presence of Ca (0.1 mM) filled circles and filled triangles. Open and filled triangles, ATP; filled and open circles, UTP. A, NTPase activity, 0.8 mg/ml myofibrillar protein, 5 ml; I = 0.12; 20 mM imidazole, pH 7.0; 5 mM Mg^{2+}. B, syneresis, 1.3 mg/ml myofibrillar protein, 10 ml; ionic conditions as for A.

reduced to that by myosin alone whereas ATP and UTP were hydrolyzed somewhat more rapidly by relaxed myofibrils than by isolated myosin.

The concentrations of ITP and GTP necessary to obtain maximal relaxation were similar and with 2–4 mM were nearly 100 times higher than the concentration of ATP.
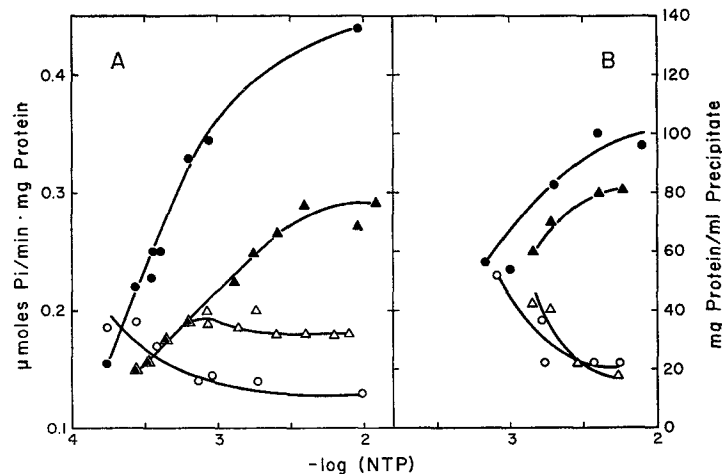

FIGURE 3. Syneresis and NTPase activity in the absence (open circles and open triangles) and presence (filled circles and filled triangles) of Ca. Ionic conditions as for Fig. 2. A, NTPase activity, 0.8 mg/ml myofibrillar protein, 5 ml. B, syneresis, 1.8 mg/ml myofibrillar protein; 10 ml. Filled and open circles, MgITP; filled and open triangles, MgGTP.

The following control experiments were performed to check whether relaxation at high concentrations of ITP was caused by ITP and not by ATP formed by transphosphorylation from ITP to ADP. ADP may have been present as a contaminant of ITP. An estimate of the possible ADP contamination was obtained in the following manner. ITP was hydrolyzed to IDP by myofibrils and after the removal of the myofibrils the rate of phosphoryla-

TABLE I
NTPASE ACTIVITY OF MYOSIN*

4.8 mM MgNTP	Rate of hydrolysis
	$\mu moles/min/mg$ protein
ATP	0.03
UTP	0.08
ITP	0.24
GTP	0.33

Conditions for NTPase test similar to that with myofibrils: $I = 0.12$; 20 mM imidazole, pH 7.0; 1 mM EGTA; 5 mM Mg^{2+}.

* Myosin characterized by the rate of K^+-activated ATP hydrolysis (0.6 M KCl, pH 8.0; 5.0 mM EDTA) 4.0 $\mu moles/min/mg$.
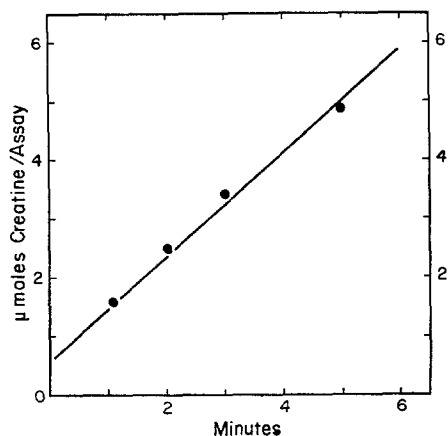

FIGURE 4. Liberation of creatine by creatine phosphokinase (CPK) in the presence of IDP. 120 $\mu moles$ of ITP were incubated with 20 mg myofibrillar protein for 20 min. The neutralized perchloric acid filtrate was incubated with 0.3 mg CPK and 20 $\mu moles$ of creatine phosphate. 10 mM imidazole, pH 7.0; 5.7 mM IDP, 8 mM Mg.

tion of IDP by creatinephosphokinase was measured. IDP was phosphorylated at a constant rate of 4 $\mu moles/min/mg$ kinase (Fig. 4) which is about 7 % of the rate at which ADP was phosphorylated under the same conditions (60 $\mu moles/min/mg$). However, a small amount of creatine, about 1 $\mu mole/$ 120 $\mu moles$ ITP, was liberated rapidly in the beginning, probably by phosphate transfer to contaminating ADP. If the washed myofibrils had contained sufficient enzyme to maintain the contaminant as ATP, the relaxation in the presence of 5 mM MgITP could have been caused by the 50 μM ATP. However, the data of Table II indicate that a significant transphosphorylation

from ITP to ADP was not catalyzed by the washed myofibril preparations. Table II shows that the addition of 0.2 mM ADP to 1 mM ITP did not result in relaxation although the phosphorylation of only 10–20 % of this ADP would have produced enough ATP to cause complete relaxation.

The concentrations of NTP necessary for relaxation were related to the concentrations necessary for contraction. ITP and GTP, which required a much higher concentration for maximal syneresis and hydrolytic activity than UTP and ATP, also needed a higher concentration for inhibition. Although the maximal extent of syneresis and the maximal rate of hydrolysis were

TABLE II

ITP	ADP	Syneresis
m M	*m M*	*mg protein/ml precipitate*
0.6	—	57
0.6	0.1	50
0.6	0.2	65
1.0	—	57
1.0	0.1	63
1.0	0.2	63
6.0	—	20
6.0	0.1	21
6.0	0.2	22

At maximal syneresis in the presence of Ca 130 mg protein per ml precipitate was found. Conditions of assays: $I = 0.12$; 10 mM imidazole, pH 7.0; 20 mg myofibrillar protein; 10 ml assay; 6 mM Mg; 1 mM EGTA; 1 min centrifugation.

quite different for the four NTP's and were significantly lower for GTP than for ATP, with ITP and UTP taking intermediate positions, all four NTP's caused inhibition of syneresis and NTPase activity to the same extent.

Fig. 5 illustrates the unexpected finding that the concentration of Ca^{2+} that removes inhibition and reactivates hydrolysis (and syneresis, not shown here) varies with the nature of the base of the NTP and its concentration. It was four times lower for 6 mM MgITP than for 6 mM MgATP and decreased further on lowering the MgITP concentration, so that the Ca concentration for half-saturation decreased from 4 μM for 6 mM MgATP to 0.35 μM for 0.4 mM MgITP.

DISCUSSION

It was shown that in the presence of four different NTP's myofibrils responded to the removal of Ca by complete inhibition of syneresis and the reduction of the rate of NTP hydrolysis to that of isolated myosin. For ATP it has been

shown that this inhibition on removal of Ca is equivalent to relaxation, and it appears therefore plausible to conclude from these experiments that myofibrillar relaxation is not specific for ATP but can be caused by many NTP's.

It is not unlikely that the previous failures to produce relaxation of tension can be explained as the result of artefact. One can think of several reasons why the concentration of ITP in the interior of the fibers was too low under the conditions used by Bendall (1958) and Watanabe and Sleator (1957), if one keeps in mind that ITP is a hundred times less effective for relaxation
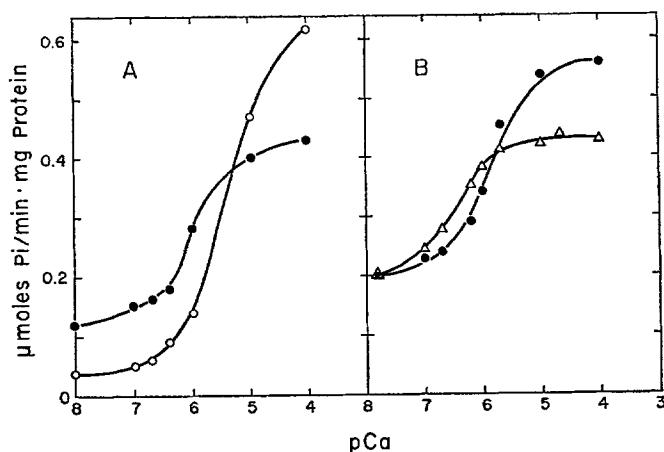

FIGURE 5. Hydrolysis of ATP and ITP as a function of pCa. A, comparison of MgATP with MgITP, B of two different concentrations of Mg-ITP. As described in the experimental section the concentration of myofibrils was varied from 1.9 mg/ml at low Ca^{2+} to 0.2 mg/ml at high Ca^{2+}. The NTP concentrations at the beginning and end of the incubation period were for MgATP (open circles) 6.0 and 5.6 mM, for high MgITP (filled circles) 6.0 and 5.8 mM, and for low MgITP (open triangles) 0.55 and 0.3 mM. Ionic strength = 0.1 for A and 0.12 for B; 20 mM imidazole, pH 7.0; total EGTA 1 mM; 5 mM Mg^{2+}.

than ATP. First, the ratio between the rate of penetration by diffusion and the rate of disappearance by hydrolysis is much lower for ITP than ATP because relaxed myofibrils hydrolyze ITP at a much higher rate than ATP. Second, myokinase reconstitutes ADP more readily than IDP. Third, lower concentrations of Ca cause contraction in the presence of ITP, especially when it is present in low concentrations, than in the presence of ATP. This is significant because the conditions for the removal of Ca in these experiments (by the displacement of Mg from 1.0 mM MgEDTA) were not optimal. In Hasselbach's experiments with UTP (1956), the reticulum may not have been capable of removing Ca, because UTP is the least effective NTP to drive Ca transport (Makinose and The, 1966). The drop in NTP concentration in these fiber bundles from the medium to the interior must have been very large because 2.5 mM ATP was required for relaxation although actin-

myosin interaction is completely inhibited in myofibrils by 50 μM ATP (Fig. 1; Levy and Ryan, 1965). It would be desirable to repeat the experiments on tension relaxation on a well-controlled test system such as for instance the perfused skinned fiber.

The experiments with the different NTP's were originally undertaken to obtain evidence in support of the concept that relaxation requires the binding of NTP to an inhibitory site that is distinct from the site on myosin responsible for actin-activated hydrolysis and the energy transfer resulting in contraction (Weber et al., 1964; Levy and Ryan, 1965). If for instance it had been found that GTP, which has the lowest affinity for the hydrolytic site, caused inhibition in concentrations lower than any other NTP, this concept of two different sites would have received strong support. Instead the same sequence for affinity was found for relaxation as for contraction: ATP > UTP > ITP = GTP. This sequence differs from that for the nucleotide-binding site of G actin[1] which apparently has a higher affinity for ITP than for UTP (Strohmann and Samorodin, 1962; Martonosi, 1962). The finding that at low concentrations of NTP, syneresis and hydrolysis are not inhibited could also be explained by a cooperative effect of NTP saturation of the enzymatic site of myosin. For instance, one could speculate that in the absence of Ca, actin-myosin interaction could take place as long as NTP was bound to only one head of the myosin molecule whereas with NTP bound to both heads (Slayter and Lowey, 1967) interaction was inhibited in the absence of Ca but not in its presence. The data presented here, which show that relaxation was complete at concentrations of NTP which caused 80–90 % of maximal hydrolytic activity in the presence of Ca, are compatible with such a possibility.

It has been established by the work of Ebashi and his group (Ebashi and Endo, 1968) and by Fuchs and Briggs (1968) that myosin does not bind Ca^{2+} in the range of Ca^{2+} concentrations which cause contraction but that troponin does. This troponin-bound ion regulates contraction as was proved unequivocally by the work of Ebashi et al. (1967). Therefore the finding, that the concentration of Ca required for contraction depends on the base of the NTP and its concentration, suggests that troponin binds NTP. However, it appears from a recent experiment that ATP in concentrations just sufficient for maximal relaxation is not bound to actin, tropomyosin, or troponin. Furthermore, Ebashi et al., (1968) found that Ca binding to troponin is not modified by NTP in any concentration. Therefore one may speculate that the degree of saturation of myosin with NTP determines to what extent troponin must be saturated with Ca for contraction to be possible.

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[1] I would like to draw attention to the fact that relative affinities of the various NTP's for the site responsible for syneresis, ATPase activity, and relaxation are quite different from the relative affinities of the nucleotides for actin. This suggests that the nucleotide-binding site of actin is not directly involved in contraction or relaxation.

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