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Propiverine hydrochloride, an anti-pollakiuric agent, inhibits the activity of actomyosin ATPase from the urinary bladder

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Abstract

The present study was performed to investigate the effects of propiverine hydrochloride (1-methyl-4-piperidyl diphenylpropoxyacetate hydrochloride, P-4), a novel anti-pollakiuric agent, on the contractile proteins of smooth muscle. P-4 (30–300 μM) inhibited the activity of native actomyosin adenosine triphosphatase (ATPase) that had been freshly purified from canine urinary bladder, and calmodulin at 10 μM overcame this inhibition. P-4 also inhibited myosin light chain kinase from smooth muscle in a dose-dependent manner. However, at 300 μM, P-4 was unable to inhibit by 50% the activity of trypsin-treated myosin light chain kinase, which was independent of Ca^{2+}/calmodulin. 1 mol of calmodulin bound 4 to 5 mol of [^{14}C]P-4 in a Ca^{2+}-dependent manner with a K_d of 77.4 μM. These results indicate that calmodulin is one of the intracellular target molecules for P-4 and that inhibition of the action of calmodulin by P-4 might cause the inhibition of actomyosin ATPase activity, with subsequent relaxation of the smooth muscle of the urinary bladder. © 1997 Elsevier Science B.V.

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1. Introduction

Propiverine hydrochloride (1-methyl-4-piperidyl diphenylpropoxyacetate hydrochloride, P-4), a derivative of benzyl acid, is a drug that was synthesized by Klosa and Delmar (1962). P-4 reduces the frequency of micturition and it is useful for the treatment of patients with pollakiuria. It has been reported that the compound has an anticholinergic effect and a direct inhibitory effect on contraction of the smooth muscle of the urinary bladder (Nagai et al., 1983; Haruno, 1992a,b). Recently, Tokuno et al. (1993) demonstrated that P-4 blocked voltage-dependent Ca^{2+} channels by recording inward Ca^{2+} currents in single muscle cells from the urinary bladder. Their results also suggested that P-4 might have some intracellular inhibitory actions, in addition to its anticholinergic and Ca^{2+} channel-blocking actions, because the recovery of the current after washout occurred more rapidly than recovery after mechanical inhibition. However, the intracellular target molecule of P-4 for inhibition of contraction of the urinary bladder remains to be identified.

The phosphorylation of myosin is an important feature of smooth muscle contraction (Hartshorne, 1987). An increase in the intracellular concentration of Ca^{2+} ions leads to the formation of a Ca^{2+}/calmodulin complex and this complex activates myosin light chain kinase. The phosphorylation of two 20-kDa myosin light chains by myosin light chain kinase results in activation of the contractile apparatus, namely, an increase in the rate of hydrolysis of ATP by the actin-activated myosin adenosine triphosphatase (ATPase) and the cycling of cross-bridges. Therefore, it seems important to examine whether P-4 affects the contractile apparatus of the smooth muscle of the urinary bladder in our attempt to characterize the mechanism of the inhibition of urinary bladder contraction by P-4.

In this study, we investigated the effect of P-4 on the activity of native actomyosin ATPase that had been freshly purified from canine urinary bladder.

2. Materials and methods

2.1. Chemicals

Propiverine hydrochloride (P-4) was obtained from Apogepha Artzneimittel (Dresden, Germany) and [^{14}C]P-4

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was obtained from the Taiho Pharmaceutical (Tokushima, Japan). The samples were dissolved in distilled water for assays of enzymatic activity and binding. All other chemicals were of reagent grade or higher.

2.2. Preparation of proteins

Native actomyosin was freshly prepared from canine urinary bladder. The muscle tissue was minced, suspended in 5 volumes of buffer A (20 mM imidazole (pH 6.9), 80 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM ATP, 1 mM NaN_3, 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF), 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor) and homogenized for 30 s in a Polytron homogenizer (Kinematica, Switzerland). The homogenate was stirred for 4 h and then centrifuged at $16\,000 \times g$ for 30 min. The supernatant was dialyzed against buffer B (20 mM imidazole (pH 6.9), 25 mM KCl, 25 mM $MgCl_2$, 0.2 mM EGTA, 1 mM dithiothreitol and 0.5 μM *p*-APMSF). After dialysis, the crude actomyosin was collected by centrifugation ($10\,000 \times g$, 20 min) and purified by sequential washing and centrifugation in buffer C (20 mM imidazole (pH 6.9), 60 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 1 mM dithiothreitol and 1 μM *p*-APMSF), first in the presence and then in the absence of 0.5% Triton X-100. The final pellet was resuspended in 40 mM imidazole (pH 6.9), 60 mM KCl and 5 mM dithiothreitol.

Thiophosphorylated actomyosin was prepared, as described by Szymanski et al. (1992), by incubation of actomyosin (2 mg/ml) in a solution of 25 mM imidazole (pH 6.9), 40 mM KCl, 5 mM $MgCl_2$ and 0.2 mM $CaCl_2$ that contained 1 mM ATPγS for 40 min at 25°C. The reaction was terminated by centrifugation at $12\,000 \times g$ for 5 min and resuspension of the pellet in a solution containing 40 mM imidazole (pH 6.9), 60 mM KCl and 5 mM dithiothreitol. After two further washes, the thiophosphorylated actomyosin was resuspended in the same buffer.

Other proteins were prepared from various tissues by the procedures described in the respective references: calmodulin from bovine brain (Yazawa et al., 1980), myosin light chain kinase from chicken gizzard (Ikebe et al., 1987) and the 20-kDa light chain of myosin from chicken gizzard (Hathaway and Haeberle, 1983).

2.3. Assay of the activity of actomyosin ATPase

The activity of native actomyosin ATPase was measured as described by Ikebe and Hartshorne (1985) with some modifications. All assays were carried out at 25°C in a solution of 25 mM imidazole (pH 6.9), 40 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ or 1 mM EGTA, 1 mM [γ-^{32}P]ATP, 1–2 mg/ml actomyosin and various concentrations of P-4. Reactions were stopped by the addition of an aliquot (0.5 ml) of the assay mixture to a tube that contained 1 ml of a 2% (w/w) suspension of activated charcoal and 0.5 ml of a solution of 1 M perchloric acid and 0.35 M NaH_2PO_4. The samples were mixed, kept on ice and centrifuged and radioactivity in aliquots of supernatant was determined by Cerenkov counting.

2.4. Assay of the activity of myosin light chain kinase

The activity of myosin light chain kinase was assayed as described previously (Sasaki et al., 1992). The reaction mixture contained 30 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 60 nM calmodulin, 0.2 mg/ml 20-kDa myosin light chain, 30 mM [γ-^{32}P]ATP, 4 nM of the kinase and various concentrations of P-4.

Myosin light chain kinase was treated with trypsin as described previously (Tanaka et al., 1980). For the reactions with trypsin-treated myosin light chain kinase, we used a buffer containing 1 mM EGTA instead of $CaCl_2$ and calmodulin.

2.5. Equilibrium dialysis

The binding of [^{14}C]P-4 to calmodulin was examined by the equilibrium dialysis method. A dialysis bag containing 25 mM Tris–HCl (pH 7.4), 0.1 M NaCl, 0.2 mM $CaCl_2$ or 1 mM EGTA, and 10 μM calmodulin was immersed in a protein-free buffer with various concentrations of P-4 plus 10 μM [^{14}C]P-4. After dialysis for 20 h, the radioactivity of aliquots of the solution in the bag and the external buffer was determined by scintillation counting.

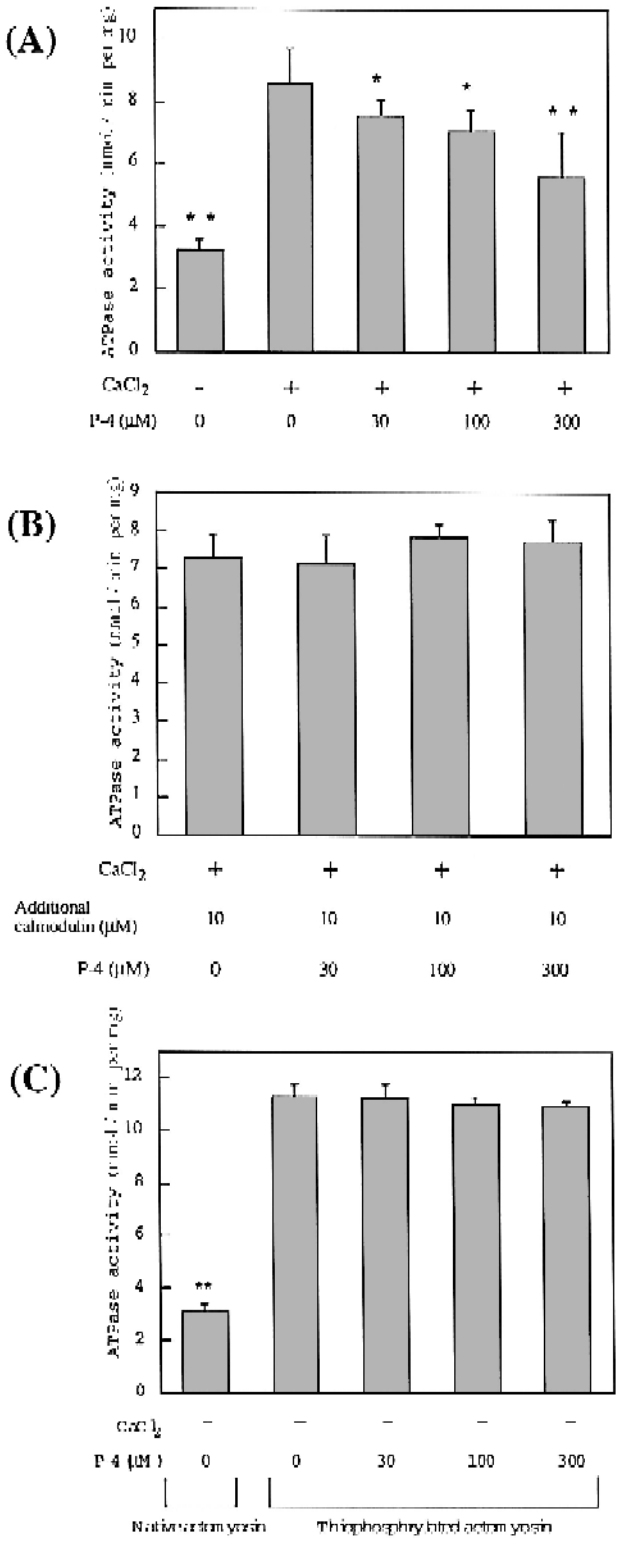
3. Results

3.1. Effects of P-4 on the activity of actomyosin ATPase from the canine urinary bladder

Native actomyosin, freshly prepared from the canine urinary bladder, was used to investigate the effects of P-4 on the activity of actomyosin ATPase. The specific activity of native actomyosin ATPase from the canine urinary bladder was 8.6 ± 1.1 nmol/mg · min in the presence of 0.2 mM $CaCl_2$, as determined in a neutral buffer that contained 40 mM KCl and 5 mM $MgCl_2$ (Fig. 1A). By contrast, the activity was 3.3 ± 0.3 nmol/mg · min in the presence of 1 mM EGTA. The activity of actomyosin ATPase in the presence of Ca^{2+} ions was 2.6 ± 0.7 times that in the presence of 1 mM EGTA.

The inhibitory effect of P-4 on the activity of native actomyosin ATPase is shown in Fig. 1A. At concentrations from 30 to 300 μM, P-4 inhibited the Ca^{2+}-dependent activity of actomyosin ATPase in a dose-dependent manner.

Fig. 1B shows the effects of the addition of exogenous purified calmodulin (final concentration, 10 μM) on the inhibition of ATPase activity by P-4. P-4, even at 300 μM,



did not inhibit the ATPase activity in the presence of 10 μM calmodulin.

To determine whether P-4 affects the activity of the actomyosin ATPase directly, we measured the actomyosin ATPase activity of thiophosphorylated myosin and the

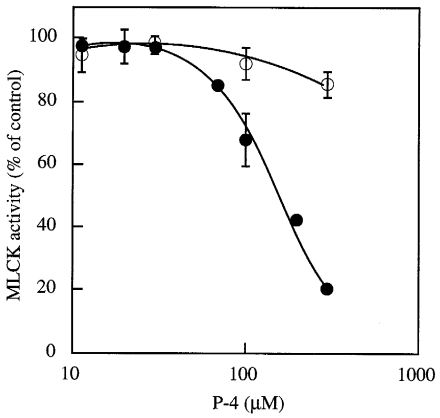


Fig. 2. Effects of P-4 on the activity of myosin light chain kinase (MLCK) from chicken gizzard. The activities of Ca^{2+}/calmodulin-dependent (●) and trypsin-treated (○) myosin light chain kinases were measured with a reaction mixture that consisted of 30 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 0.2 mM $CaCl_2$ or 1 mM EGTA, 60 nM calmodulin, 0.2 mg/ml 20 kDa myosin light chain, 30 mM [γ-^{32}P]ATP and various concentrations of P-4. Results are expressed as a percentages of the activity of the vehicle control. Data represent means ± S.D. (vertical bars) of results from 4 experiments.

effects of P-4 on this activity. As shown in Fig. 1C, the activity of thiophosphorylated actomyosin was independent of Ca^{2+}/calmodulin and it was not inhibited by P-4.

Moreover, we measured the level of myosin phosphorylation in the native actomyosin preparation. The Ca^{2+}-dependent phosphorylation of 20-kDa myosin light chain in native actomyosin was inhibited by P-4 in a dose-dependent manner.

3.2. Effects of P-4 on the activity of myosin light chain kinase

To examine the effect of P-4 on the activity of the myosin light chain kinase, it would have been appropriate

Fig. 1. (A) Effects of P-4 on the activity of actomyosin ATPase freshly prepared from canine urinary bladder. The activity of native actomyosin ATPase was measured as described in Section 2. All assays were carried out at 25°C in a solution containing 25 mM imidazole (pH 6.9), 40 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ (+) or 1 mM EGTA (−), 1 mM [γ-^{32}P]ATP, 1–2 mg/ml actomyosin and various concentrations of P-4. Data are expressed as means ± S.D. (vertical bars) of results from 4–8 experiments. * $p < 0.05$; ** $p < 0.01$ compared to the value of the vehicle control in the presence of 0.2 mM $CaCl_2$. (B) Effects of calmodulin on the P-4-induced inhibition of actomyosin ATPase activity. The assay was carried out at 25°C in a solution containing 25 mM imidazole (pH 6.9), 40 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM [γ-^{32}P]ATP, 1–2 mg/ml actomyosin, various concentrations of P-4 and 10 μM calmodulin. Data are expressed as the means ± S.D. (vertical bars) of results from 4 experiments. (C) Effect of P-4 on the activity of thiophosphorylated actomyosin ATPase. The assay was carried out at 25°C in a solution of 25 mM imidazole (pH 6.9), 40 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM [γ-^{32}P]ATP, 2 mg/ml thiophosphorylated actomyosin and various concentrations of P-4. Data are expressed as the means ± S.D. (vertical bars) of results from 4 experiments. ** $p < 0.01$.

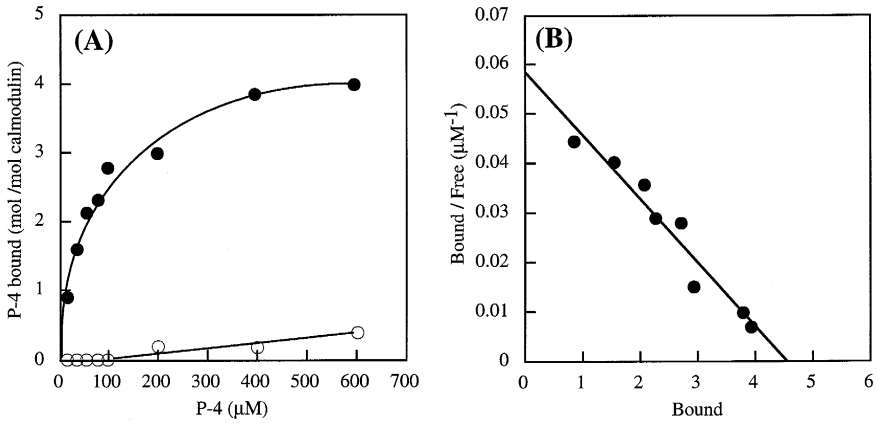


Fig. 3. Binding of [^{14}C]P-4 to calmodulin. (A) The binding of [^{14}C]P-4 to calmodulin was determined by equilibrium dialysis. The number of moles of P-4 bound per mole of calmodulin is plotted as a function of the concentration of free P-4. Reactions were performed in the presence of 0.2 mM $CaCl_2$ (●) or 1 mM EGTA (○). (B) Scatchard plot of the data obtained in the presence of 0.2 mM $CaCl_2$.

to use the kinase from the canine urinary bladder. However, our preparation of native actomyosin was too small and too unstable to allow us to purify myosin light chain kinase, so we used myosin light chain kinase from chicken gizzard instead. As shown in Fig. 2, P-4 inhibited the activity of the myosin light chain kinase from chicken gizzard in a dose-dependent manner. By contrast, trypsin-treated myosin light chain kinase, which was constitutively active (in other word, it was independent of Ca^{2+}/calmodulin), was not inhibited by P-4 at concentrations up to 300 μM.

3.3. Binding of P-4 to calmodulin

To confirm the direct interaction of P-4 with calmodulin, we examined the binding of P-4 to calmodulin. The results of equilibrium dialysis with [^{14}C]P-4 are shown in Fig. 3. In the presence of 0.2 mM $CaCl_2$, [^{14}C]P-4 bound to calmodulin. However, [^{14}C]P-4 did not bind to calmodulin in the presence of 1 mM EGTA. The data are shown as a Scatchard plot in Fig. 3B. It appears that the binding of P-4 to calmodulin is Ca^{2+}-dependent and that there are four to five binding sites for P-4 per calmodulin molecule and that the affinity constant is 77.4 μM.

4. Discussion

In the present study, we found that P-4, a drug that is used to treat pollakiuria, inhibited the activity of native actomyosin ATPase that had been freshly prepared from canine urinary bladder. Several studies have suggested that P-4 has a direct inhibitory effect on the smooth muscle contraction of the urinary bladder (Nagai et al., 1983; Haruno, 1992a,b; Tokuno et al., 1993). However, there has been no information to date about the intracellular actions of the drug. Thus, the present study is the first to link P-4 to an effect at the level of the contractile apparatus.

This study also showed that the addition of exogenous purified calmodulin overcame the inhibition of ATPase activity by P-4 and that P-4 inhibited the activity of Ca^{2+}/calmodulin-dependent myosin light chain kinase. However, P-4 had no effect on the activity of thiophosphorylated actomyosin ATPase and on a constitutively active (Ca^{2+}/calmodulin-independent) myosin light chain kinase. These results suggest that the inhibitory effect on native actomyosin ATPase of P-4 is due to an interaction with calmodulin. Moreover, we demonstrated the Ca^{2+}-dependent binding of P-4 to calmodulin by equilibrium dialysis, using [^{14}C]P-4.

The contractile activity of smooth muscles, such as those of the urinary bladder, is controlled by the intracellular concentration of Ca^{2+} ions. The major target for binding of Ca^{2+} ions is calmodulin; the binding is associated with the contractile apparatus and the development of tension via myosin light chain kinase. The apoenzyme of myosin light chain kinase is inactive and a ternary complex of Ca^{2+} ions, calmodulin and myosin light chain kinase phosphorylates the 20-kDa myosin light chain. This phosphorylation of myosin increases the activity of the actin-activated ATPase and 'activation' of myosin is equated with the initiation of the contractile response. Therefore, the binding of P-4 to calmodulin might cause inhibition of the activity of actomyosin ATPase, with subsequent relaxation of the smooth muscles of the urinary bladder.

P-4 has multiple actions, being both an anticholinergic agent and a Ca^{2+}-blocking agent, and it has been suggested that the target molecules for the actions of P-4 are muscarinic receptors and voltage-dependent Ca^{2+} channels in the smooth muscle of the urinary bladder (Tokuno et al., 1993). In addition, this study showed that calmodulin is an intracellular target molecule for P-4. However, we did not check the direct interaction of P-4 with other members of the contractile apparatus, such as myosin, actin and tropomyosin. Like calmodulin, the 20-kDa myosin light

chain belongs to the E-F hand protein family, therefore, P-4 may be able to bind to the 20-kDa myosin light chain.

Haruno (1992a,b) reported that P-4 (1–100 μM) inhibited the KCl-induced contraction of a preparation of isolated urinary bladder muscle from the guinea pig and the author also showed that P-4 (1–10 μM) affected the spontaneous contraction of isolated strips of guinea pig urinary bladder and the rhythmic contractions of the urinary bladder in anesthetized dogs. Moreover, Tokuno et al. (1993) reported that P-4 (3–10 μM) inhibited carbachol-induced contractions in the presence of verapamil and Ca^{2+}-induced contractions in a medium that contained excess K^+ ions and atropine.

In summary, the present study showed that calmodulin is one of the intracellular target molecules for P-4 and that the inhibition of the action of calmodulin by P-4 might cause inhibition of the activity of actomyosin ATPase resulting in relaxation of the smooth muscle of the urinary bladder. Therefore, inhibition of actomyosin ATPase by P-4 might contribute to the action of P-4 in improving the condition of patients with pollakiuria.

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