Interaction of smooth muscle relaxant drugs with calmodulin and cyclic nucleotide phosphodiesterase

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Summary. Some smooth muscle relaxant drugs with an unknown mechanism of action have been tested for their interaction with calmodulin and with calmodulin-induced cyclic nucleotide phosphodiesterase (PDE) activity. The affinity of these drugs for calmodulin does not parallel their inhibitory effect on the calmodulin activation of PDE. The lack of parallelism could be due to a binding of the drugs to different sites on calmodulin; furthermore a binding of papaverine, octylonium bromide and felodipine to PDE molecule might also be considered to explain their inhibitory effect on PDE basal activity. The myolytic effect of octylonium bromide and pinaverium bromide may be due to their interaction with calmodulin-dependent systems.
Key words. Calmodulin; phosphodiesterase, cyclic dinucleotide; smooth muscle relaxants; relaxant drugs, smooth muscle.

Calmodulin regulates many cellular Ca^{2+}-dependent processes including smooth muscle contraction[2]. Recently, a variety of hydrophobic compounds has been reported to bind to calmodulin and to inhibit calmodulin-mediated events[3,4]; these ligands have been classed as calmodulin-antagonists. Moreover, some Ca antagonists have also been shown to interact with calmodulin[5].

Some drugs devoid of anticholinergic action exert a direct smooth muscle relaxant activity. Their mechanisms of action are poorly understood though for some of them an effect on PDE activity has been suggested[6], because of the role played by cAMP in smooth muscle contraction.

We have tested the interaction of different smooth muscle relaxing agents with calmodulin, taking advantage of the fluorescence characteristics of dansyl calmodulin and of felodipine, which undergoes a fluorescence increase when it binds to native calmodulin[7].

We have also investigated the effect of these drugs on the Ca^{2+}-induced calmodulin-dependent activation of PDE.

Materials and methods. Felodipine was kindly given by Dr B. Ljung of Hassle, Molndal; octylonium bromide by Menarini, Florence; pinaverium bromide by Farmades, Rome; verapamil by Knoll, Milan; trimebutine maleate by Sigma-Tau, Rome; tiropramide by Rorer, Monza. All other reagents were of analytical grade. Calmodulin-deficient PDE from bovine heart was purchased from Sigma, St. Louis, Mo. Calmodulin from bovine brain was prepared by affinity chromatography on Affi-gel phenothiazine (Bio-Rad) by a modification of the method of Jamieson and Vanaman[8]. Calmodulin (1 mg/ml) was dansylated with dansyl chloride (1.5 mol/mol calmodulin) in 10 mM $NaHCO_3$ pH 10.0 at 4 °C for 12 h[9]. The reaction mixture was dialyzed overnight against 10 mM morpholinopropane sulfonic acid (MOPS), 90 mM KCl, 2 mM EGTA, pH 7.0 to remove excess free label. The bound dye concentration was determined according to Chen[10]. Under these conditions 0.4–0.5 mol of the dansyl label was incorporated per mol of calmodulin. Dansyl calmodulin was indistinguishable from native calmodulin in its ability to activate purified PDE.

Fluorescence measurements were made in 3 ml of 10 mM MOPS, 90 mM KCl, 2 mM EGTA and 3 mM $CaCl_2$ (pCa = 3), pH 7.0 using a Perkin Elmer MPFIV spectrofluorometer, at an excitation wavelength of 340 nm[11].

Felodipine was excited at 365 nm and its fluorescence was monitored at 445 nm.

PDE activity was measured by the procedure of Butcher and Sutherland[12], modified by Wang and Desai[13], which involves the coupling of phosphodiesterase with a 5'-nucleotidase reaction and measuring the inorganic phosphate produced by a modification of the method of Martin and Doty[14].

Results. The fluorescence spectrum of dansyl calmodulin in the presence of 1 mM Ca^{2+} exhibits a maximum at 490–500 nm. The addition of increasing amounts of drugs produced an increase in fluorescence at 500 nm (F/F_o) between 60% and 80% (fig. 1). The half-maximal binding values (M/2) were 2.4, 3.6, 6.0, 8.5, 31.0, and 36.0 μM for fendiline, octylonium bromide, felodipine, pinaverium bromide, verapamil, and emepronium bromide, respectively.

Nifedipine, tiropramide, trimebutine maleate, papaverine and N-butylscopolammonium bromide (up to 100 μM) had no effect in increasing the fluorescence intensity of dansyl calmodulin.

In the absence of added calcium none of these ligands produced a change in dansyl calmodulin fluorescence. Titrations of 1 μM felodipine, 0.2 μM native calmodulin were performed with fendiline, octylonium bromide and pinaverium bromide in the same buffer as in figure 1. M/2 values (0.9, 1.5, and 4.5 μM, respectively) are in good agreement with those obtained from the titration of 0.2 μM dansyl calmodulin (data not shown). Papaverine, N-butylscopolammonium bromide, trimebutine maleate and tiropramide gave only a slight increasing effect, not higher than 8%. Drug titration of calmodulin-felodipine in the absence of Ca^{2+} or of felodipine alone produced no change in fluorescence. The table shows the effect of some drugs (100 μM) on PDE activity. For comparison, trifluoperazine, a well known inhibitor of calmodulin-stimulated PDE[4], is also included. Tiropramide, emepronium bromide and verapamil were ineffective or had little inhibitory effect (< 15%). Octylonium bromide and felodipine also inhibited basal PDE activity and their effect was investigated at different drug concentrations. At low concentration (< 25 μM) the inhibition by octylonium bromide is more marked on the calmodulin-activated enzyme than on basal activity (fig. 2). Felodipine behaved similarly (data not shown).

Discussion. Johnson et al.[5] observed a good correlation between the binding to calmodulin and the inhibition of myosin light chain kinase activity by five different Ca antagonists. Their order of potency was: fendiline > prenylamine > D600 > verapamil > diltiazem.

The affinity of the drugs examined here for calmodulin does not parallel their inhibitory effect on the calmodulin activation of PDE. Fendiline, which shows the highest affinity for calmodulin in our experimental conditions, is a weak inhibitor of the

Effect of some drugs on cyclic nucleotide phosphodiesterase

Added drug (100 μM)	Enzyme activity without calmodulin	with calmodulin
None	100	232
Trifluoperazine	100	100
Fendiline	100	199
Pinaverium bromide	100	159
Papaverine	92	150
Octylonium bromide	12	12
Felodipine	0	0

The activity obtained in the absence of calmodulin and drugs was taken as 100. PDE was incubated with 1.2 mM cAMP in 40 mM Tris-HCl, 40 mM imidazole, 3 mM Mg acetate, 0.1 mM KCl, 0.1 mM $CaCl_2$, pH 7.5 at 37 °C for 30 min. Calmodulin was 0.3 μM.

calmodulin-stimulated enzyme. Felodipine is the most effective in inhibiting PDE but it has an M/2 value intermediate between octylonium and pinaverium bromide. Papaverine markedly inhibits the calmodulin activation of PDE but does not enhance the fluorescence of dansyl calmodulin and of calmodulin-bound felodipine.

Much evidence indicates that the binding of calcium to calmodulin results in structural changes and in the exposure of a large hydrophobic region with different sites for a variety of hydrophobic molecules and proteins[15,16].

Some drugs which are able to bind to calmodulin (like phenothiazines, butyrophenones and possibly local anesthetics) can compete with calmodulin-activated proteins, such as PDE, affecting the calmodulin-mediated enzyme reactions[4,17]. The compounds we have tested are structurally different and may bind to different sites on the same hydrophobic region. Some of them are amphiphilic amines with a large hydrophobic moiety and they could be expected to bind through hydrophobic bonds and ionic attractions with negatively charged residues on calmodulin as suggested by Prozialeck and Weiss[18]. The binding of the drugs to different sites could explain the lack of parallelism between calmodulin affinity and enzyme inhibition.

Furthermore a possible binding of the drugs to the PDE molecule must be considered. Vincenzi suggested for some anti-calmodulin drugs a direct interaction with calmodulin effector enzymes[19]. The binding of papaverine to the calmodulin site on the PDE molecule may explain its inhibitory effect. The inhibition of PDE basal activity by octylonium bromide and felodipine could be also due to this mechanism.

Calmodulin plays a central role in smooth muscle contraction since it activates, directly or through protein kinases, adenylate cyclase, PDE, actomyosin Mg^{2+}-ATPase and Ca^{2+} pumps. By these reactions calmodulin contributes to the control of cAMP level, actomyosin cross-bridge cycling rates and calcium movements in the muscle cell.

Some of the postneurotropic mechanisms reported for papaverine-like compounds may be mediated by calmodulin. This is suggested by the muscle relaxant side-effect of some calmodulin antagonists, such as chlorpromazine, trifluoperazine, prenylamine[3].

The myolytic activity of octylonium bromide[20] and pinaverium bromide may be due to their interaction with calmodulin-regulated systems since these compounds bind to calmodulin with high affinity and inhibit at least one calmodulin-dependent reaction.

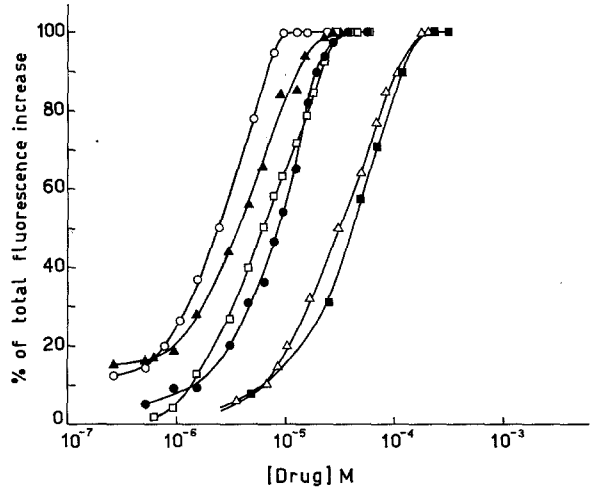

Figure 1. Titration curves of dansyl calmodulin with different smooth muscle relaxing agents. The percentage of total fluorescence increase is reported as a function of fendiline (○), octylonium bromide (▲), felodipine (□), pinaverium bromide (●) verapamil (△), and emepronium bromide (■). Aliquots of 5–10 µl of drug aqueous solutions (0.1–1 mM) were added to 3 ml of 2 µM dansyl calmodulin in 10 mM MOPS, 90 mM KCl, 2 mM EGTA and 2 mM $CaCl_2$, pH 7.0. Felodipine and fendiline were dissolved in ethanol. Felodipine excitation was at 290 nm to prevent drug fluorescence; excitation wavelenght was 340 nm for all the other compounds.

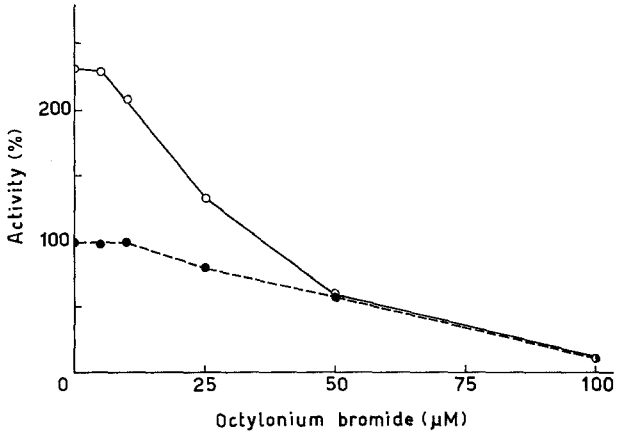

Figure 2. Effect of octylonium bromide on basal and calmodulin-stimulated phosphodiesterase activity. Enzyme activity was determined in the absence (●) and in the presence (○) of calmodulin (0.1 µM). Results are expressed as percent of basal activity.

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