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Interactions with calmodulin: potential mechanism for some inhibitory actions of tetracyclines and calcium channel blockers

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A role for calcium–CaM* in the hydroosmotic action of vasopressin (VP) has been postulated based on results obtained with phenothiazines [1–3], drugs that inhibit the action of the calcium–CaM complex [4]. Other drugs that inhibit the action of VP include calcium channel blockers [5] and various tetracyclines [6].

The inhibition by tetracyclines and calcium blockers may involve interaction with cAMP-related enzymes [6–8] and correlates with their binding to specific proteins [9].

The observations and their similarities to the effects of trifluoperazine on the action of VP [1–3] led us to consider the possibility that these different drugs may all interfere with calcium–CaM-dependent steps.

Methods

Determination of CaM activity. CaM activity was determined by the activation of CaM-deficient phosphodiesterase as outlined by Cheung [10] and previously described in detail from this laboratory [1]. The standard assay contained 4 μM [^{3}H]cyclic AMP (200,000 cpm) 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.05 to 0.5 mM $CaCl_2$ (as indicated), 40 mM Tris, pH 8.0, and 2.5 μg of CaM-deficient phosphodiesterase. Various amounts of purified CaM (sp. act. 40,000 units/mg) were added as indicated in Results. Assays using heat-inactivated phosphodiesterase served as blanks and were less than 15% of the lowest activity. The various tetracyclines and most calcium antagonists were made up fresh daily in assay buffer and kept in the dark. Nifedipine was prepared daily as a concentrated solution in ethanol–PEG 4000 (1:1) and diluted in buffer prior to the experiment. Incubations were carried out in duplicate at 30° for 30 min while protected from light.

The reaction was terminated by the addition of 5 μl of a stopping solution (containing 0.2 M EDTA and adenosine, 5'-AMP and cAMP, each at 12.5 M), and the tubes were transferred on ice. The amount of [^{3}H]cyclic AMP hydrolyzed during the reaction was determined by thin-layer chromatography using the method of Rangel-Aldao *et al.* [11] as previously described [1]. Each result was calculated as the mean of the duplicate determinations which varied by less than 5%.

Results

Effects of tetracyclines on in vitro *CaM activity*. Purified CaM caused a dose-dependent activation of CaM-deficient phosphodiesterase activity that was calcium dependent (Fig. 1). Addition of calcium from 0.02 to 0.5 mM resulted in comparable and full stimulation. Addition of equal concentrations (0.05 mg/ml or 125 μM) of various tetracyclines caused the CaM dose–response curve to shift to the right to different degrees (Fig. 1). The tetracyclines showed little effect on basal phosphodiesterase activity. By far the strongest inhibitor of CaM activation was 5,6-DMC. At 0.05 mg/ml (125 μM) of 5,6-DMC, the inhibition could not be totally overcome by CaM up to 10 μg/assay (i.e. 100-fold excess over that needed for maximal activation). At a lower concentration of 5,6-DMC (0.01 mg/ml or 25 μM), its inhibitory effect could be overridden by higher concentrations of CaM (Fig. 1). The inhibitory action of these tetracyclines on CaM activation of phosphodiesterase cannot be explained by chelation of calcium, as increasing the calcium concentration did not attenuate the effect of DMC or 5,6-DMC. [Under control conditions, 92 pmoles cAMP were hydrolyzed and values with DMC or 5,6-DMC (125 μM) were inhibited to 24.5 and 18.9 at 0.05 mM Ca^{2+}, 25.0 and 20.2 at 0.5 mM Ca^{2+}, and 24.9 and 20.1 at 1 mM Ca^{2+} respectively.] Figure 2 shows the effect of increasing concentrations of tetracyclines on phosphodiesterase activity in the absence and presence of CaM (0.1 μg or 25 μM). Half-maximal inhibition for CaM-stimulated activity occurred at about 0.008 mg/ml (approximately 20 μM) for 5,6-DMC and at about 0.04 mg/ml (approximately 100 μM) for DMC and 100% inhibition at 0.025 (60 μM) and 0.05 mg/ml (125 μM) respectively. These concentrations are almost one order of magnitude lower than those required for the inhibition of VP-induced water flow in the toad bladder [6, 9]. Thus, effective inhibitory intracellular concentration could be attained with the concentrations used in the transport experiments. Also the inhibitory potency for CaM activation by the various tetracyclines was (by decreasing inhibition): 5,6-DMC > DMC ≫ DOC > TC > OTC. This sequence is identical to that described for the effects of tetracyclines on VP-induced water flow in the toad bladder [6, 9].

Effect of "calcium-blockers" on in vitro *CaM activity*. At 100 μM, diltiazem caused a small (20%) but reproducible inhibition of the CaM-activated phosphodiesterase whereas verapamil and its analogue D600 resulted in a 40–50% inhibition (Fig. 3). The inhibitory effect of 100 μM D600 could be overcome by increasing the CaM concentration 10-fold (two experiments). Nifedipine (15–30 μM) progressively inhibited phosphodiesterase activity in both the absence and presence of CaM. The inhibitory effect of nifedipine (15 μM) was unaffected by increasing CaM up to 1.0 μg/assay. This pattern may indicate an unspecific effect of nifedipine on the phosphodiesterase enzyme itself.

Discussion

The inhibition of the action of VP by tetracyclines may involve binding of the drugs to cellular proteins that have a role in VP-mediated water transport [6, 9]. Based on the

* Abbreviations: VP, vasopressin; CaM, calmodulin; TFP, trifluoperazine; 5,6-DMC, 5,6-anhydrodemeclocycline; DMC, demeclocycline; DOC, doxycycline; OTC, oxytetracycline; and TC, tetracycline.

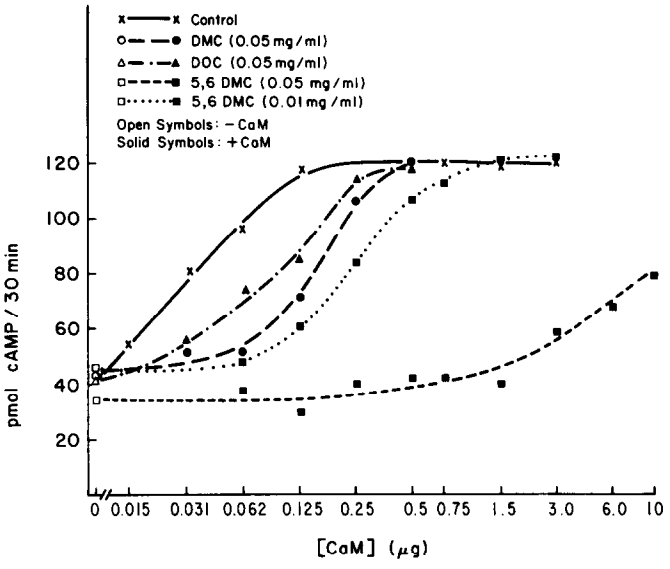

Fig. 1. Effects of several tetracyclines on calmodulin activation of calmodulin-deficient phosphodiesterase activity for cAMP. Each point represents the means of three different experimental determinations, carried out in duplicate. Interexperimental variation was less than 10%. Abbreviations: DMC, demeclocycline; DOC, doxycycline; and 5,6-DMC, 5,6-anhydrodemeclocycline. Concentration of 0.05 mg/ml correspond to 125 μM.

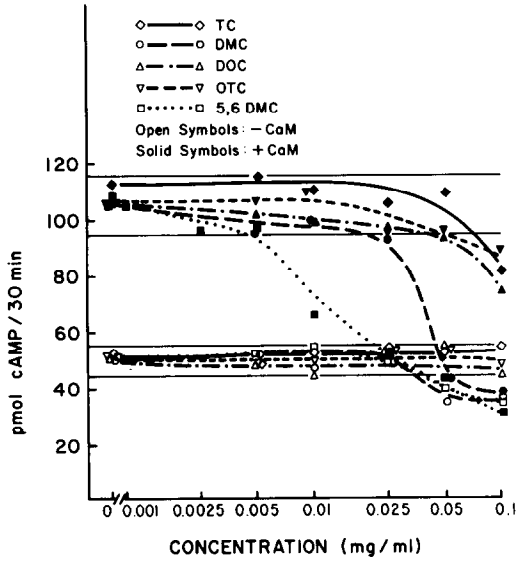

Fig. 2. Effect of increasing concentrations of tetracyclines on the activity of phosphodiesterase in the absence and presence of CaM (0.1 μg/assay). The two lower horizontal lines indicate the range of activity in the absence of CaM and the upper ones those in the presence of CaM (0.1 μg/assay) under control conditions. Each point represents the mean of three different experimental determinations. Concentrations of tetracyclines from 0.001 to 0.1 mg/ml correspond to 2.5 to 250 μM. Abbreviations: TC, tetracycline; and OTC oxytetracycline.

present results we would suggest that CaM may be such a protein. This hypothesis is based on the following considerations.

The tetracyclines *in vitro* show dose-dependent inhibition of CaM activation with the same order as that for protein binding and for inhibition of the hydroosmotic action of VP [6, 9]. Several considerations about the structure–activity relationship for drugs inhibiting the action of CaM [12] seem to apply to the tetracyclines. For example, an increase in the hydrophobicity of the rings, by introduction of halogens, increases the anti-CaM potency of phenothiazines [12]. Both DMC and 5,6-DMC contain a chloride in the ring structure, whereas the less potent congeners do not. The increased effectiveness of 5,6-DMC over DMC is associated with a further increase in the hydrophobicity due to loss of water and the resulting formation of conjugated double bonds in the ring structure. The binding of tetracyclines to protein of the toad bladder is enhanced markedly in the presence of calcium [9]. In general, binding of CaM inhibitory drugs to CaM is increased by calcium [4].

Similar considerations can be applied to the *in vitro* effects of several "calcium-channel blockers". In the present study, calcium channel blockers inhibited CaM activation of phosphodiesterase as also reported for nimodipin and verapamil [13]. The order of inhibition was similar to that for the hydroosmotic response in the toad bladder [1].

In vitro some tetracyclines and calcium blockers antagonise CaM-activation of phosphodiesterase. Interference with CaM-dependent enzymes, including adenylate cyclase, may be related to the inhibition of the hydroosmotic action of VP by these drugs. The different drugs may differentially distribute to cellular compartments and thus inhibit CaM-dependent enzymes differentially.

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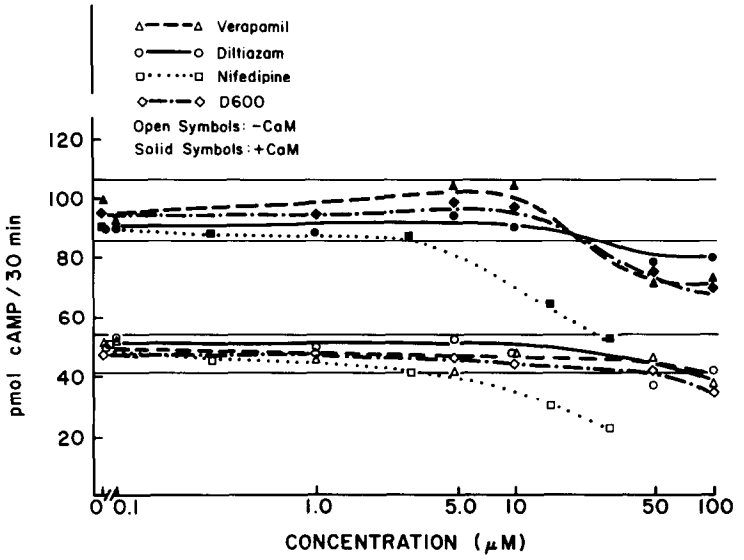

Fig. 3. Effect of increasing concentrations of calcium blockers on the activity of phosphodiesterase in the absence and presence of CaM. Each point represents the mean of three different experimental determinations.

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Effect of superior cervical ganglionectomy on melatonin stimulation by specific MAO-A inhibition

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Monoamine oxidase (MAO) (EC 1.4.3.4) inhibitors have been shown to increase melatonin content of the rat pineal gland [1]. Direct or indirect stimulation of β-adrenergic receptors as well as increased availability of the substrate (serotonin) for melatonin synthesis have been proposed as explanations for this effect of MAO inhibitors [2, 3]. We have observed recently that inhibition of MAO-A, but not MAO-B, increases rat pineal melatonin [4]. The rat pineal is innervated solely by sympathetic nerves that originate in the superior cervical ganglia [5]. MAO-A is found within these sympathetic nerve endings [6, 7], while MAO-B is found within pinealocytes [7]. Considering that superior cervical ganglionectomy almost completely eliminates MAO-A from the pineal gland [6, 7], it is of interest to explore whether ganglionectomy will alter the clorgyline-induced increase of rat pineal melatonin.

Methods

Male Sprague–Dawley rats (200–250 g) were kept two per cage under constant temperature (22°) and a 12 hr light–12 hr dark schedule with free access to food and water for at least 1 week before the experiment.

Superior cervical ganglionectomy (bilateral) was performed according to procedures described elsewhere. Two weeks after the operation (time necessary for completion of the denervating process—the development of ptosis was observed in all animals), saline or clorgyline (2.5 mg/kg, i.p.; a dose which almost completely inhibits MAO-A activity in rat brain basal ganglia [4]) was injected at 10:00 a.m. into sham-operated and ganglionectomized animals. Pineals were removed 2 hr later.

Each pineal was used for the determination of melatonin, serotonin (5-HT), *N*-acetylserotonin (NAS), tryptophan