The involvement of a calmodulin-dependent phosphodiesterase in the negative control of carbamylcholine on cyclic AMP levels in dog thyroid slices

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1. INTRODUCTION

In dog thyroid slices TSH expresses most of its physiological effects through an increase of the intracellular concentration of cAMP [1]. In the same tissue, carbamylcholine enhances cGMP accumulation and in TSH stimulated slices decreases cAMP level. These effects are reproduced by agents which increase free Ca^{2+} intracellular levels [2] and are abolished in calcium depleted media and cells [3]. The inhibition of cAMP accumulation by carbamylcholine reflects an increased disposal rate, it is relieved by 1-methyl-3-isobutylxanthine (MIX) [4,5] but not by 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724). Three cyclic nucleotide phosphodiesterases have been previously identified in horse thyroid [6]. Here, we present data suggesting that the effect of carbamylcholine is secondary to a direct activation by Ca^{2+} of a calmodulin-dependent cyclic nucleotide phosphodiesterase of dog thyroid.

Abbreviations: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; MIX, 1-methyl-3-isobutylxanthine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone

2. MATERIALS AND METHODS

cAMP and phosphodiesterase assays have been reported in [4,6]. The 3 soluble phosphodiesterases were separated from a 40000 × *g* dog thyroid crude supernatant [6]. Homogenization and purification were performed in buffers containing the following protease inhibitors: 75 mg/ml phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 μM leupeptin, 25 mg/l trypsin inhibitor. The crude thyroid supernatant was applied to a DEAE-cellulose column where the cAMP-specific and the cGMP-activated phosphodiesterases were prepared as in [6]. The calmodulin-dependent phosphodiesterase was obtained as in [7]. After the DEAE-cellulose step, the calmodulin-dependent phosphodiesterase was applied to a calmodulin-sepharose column (0.9 × 10 cm) in the presence of 0.5 mM $CaCl_2$.

The column was first washed with 40 mM Tris–HCl pH 7.5 buffer containing 0.5 mM $CaCl_2$ and NaCl at two salt concentrations: 50 mM and 200 mM. The phosphodiesterase was further eluted with 2 mM EGTA in the presence of 200 mM NaCl. Calmodulin was purified from bovine brain as in [8]. Phenylmethylsulfonyl fluoride, benzamidine, trypsin inhibitor, 1-methyl-3-isobutylxanthine were from Sigma (St Louis, MO). Leupeptin was supplied by Peptide Institute (Osaka).

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DEAE-cellulose (DE-52, DE-23) was from Whatman. Ro 20-1724 was provided by Hoffman-La Roche.

3. RESULTS

The effects of Ro 20-1724 and MIX, two phosphodiesterase inhibitors, have been studied on dog thyroid slices and on phosphodiesterases isolated from the same tissue. Ro 20-1724 and MIX were added to the incubation medium of dog thyroid slices stimulated by TSH (1 mU/ml) in the presence of either 10 μM carbamylcholine or 5 μM ionophore A 23187. Table 1 shows that the decrease of cAMP concentration observed with carbamylcholine or ionophore A 23187 was suppressed in the presence of 0.1 mM MIX but not with 0.1 mM Ro 20-1724.

Three different soluble phosphodiesterases hydrolyzing cGMP and/or cAMP were separated after DEAE-cellulose chromatography (fig.1). The first enzymatic peak hydrolyzed cAMP and cGMP and was activated by a saturated concentration of calmodulin in the presence of 2 mM Ca^{2+}; the second form also hydrolyzed both cyclic nucleotides, cAMP hydrolysis being stimulated by 3 μM cGMP; the third peak specifically hydrolyzed cAMP. MIX and Ro 20-1724 were tested on these 3 soluble phosphodiesterases:

(i) cAMP-specific phosphodiesterase was more sensitive to inhibition by Ro 20-1724 than to inhibition by MIX; this was observed in a range of inhibitor concentration from 0.1 μM–100 μM (fig.2);

(ii) The calmodulin-dependent phosphodiesterase was inhibited by MIX in a dose-dependent manner. This inhibition was much more potent than the inhibitory effect of Ro 20-1724 (fig.3A,B). Inhibition by the xanthine was more effective when the enzyme was studied under saturated calmodulin stimulated-assay conditions: at 10 μM MIX, basal phosphodiesterase activity was inhibited by 60%, in contrast activity of the enzyme stimulated by

Table 1

Effect of MIX and Ro 20-1724 on the carbamylcholine and ionophore A 23187 inhibition of cAMP accumulation in dog thyroid slices stimulated by TSH

	Ro 20-1724 (0.1 mM)	MIX (0.1 mM)
TSH (1 munit/ml)	2163 ± 292	1575 ± 42
TSH (1 munit/ml) + carbamylcholine (10 μM)	673 ± 115	1339 ± 88
TSH (1 munit/ml) + A 23187 (5 μM)	396 ± 91	1462 ± 118

Dog thyroid slices were first incubated for 60 min in Krebs–Ringer-bicarbonate buffer with for the last 20 min 5 μM ionophore A 23187 (when tested). This was followed by a 30 min incubation in the presence of 10 μM carbamylcholine, 5 μM ionophore, 1 munit/ml TSH and 0.1 mM Ro 20-1724 or MIX (when included). Results are expressed as pmol cAMP/100 mg wet tissue (means of triplicates ± SE). In the absence of TSH, cAMP accumulation reached 94 ± 23 pmol/100 mg wet tissue

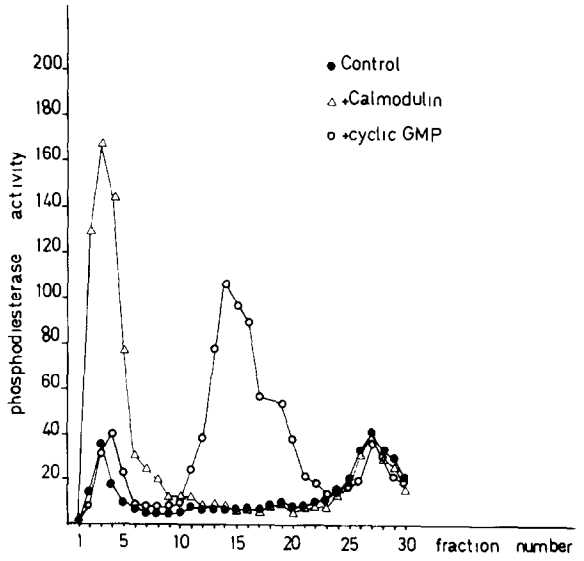


Fig.1. Profile of dog thyroid cyclic nucleotide phosphodiesterase activities resolved by DEAE-cellulose chromatography. Experimental details are given in section 2. cAMP phosphodiesterase activity was assayed at 3 μM substrate level in the presence of a saturated concentration of calmodulin and 2 mM $CaCl_2$ (△—△), or in its absence (●—●). Hydrolysis of 3 μM cAMP was also determined with added cGMP, 3 μM (○—○). Fractions of 10 ml each were collected. Activity is expressed as pmol cAMP hydrolyzed . 20 μl^{-1} . 30 min^{-1}.

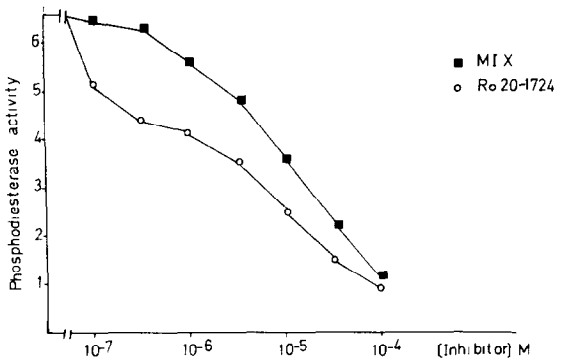


Fig.2. Effect of MIX and Ro 20-1724 on the activity of dog thyroid cAMP-specific phosphodiesterase. cAMP hydrolysis was measured at 1 μM substrate level. Results are expressed as pmol cAMP hydrolyzed.min^{-1}.mg protein^{-1}.

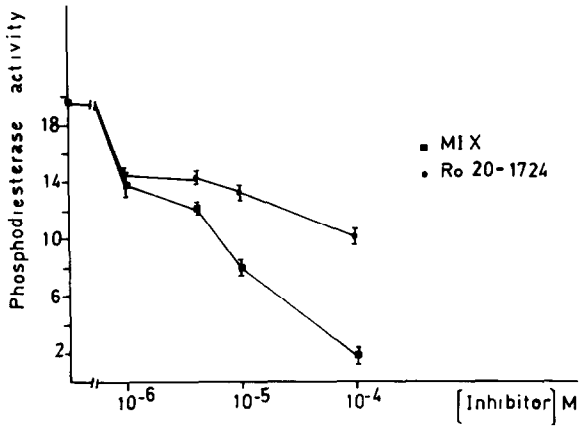
calmodulin was inhibited by 77% (fig.3A and 3B, respectively);

(iii) At 50 μM Ro 20-1724 had no effect on the cGMP-activated phosphodiesterase, whereas MIX at the same concentration inhibited at about 60% cAMP hydrolysis at 3 μM substrate level in the presence of 3 μM cGMP (added as an activator, not shown). In the absence of cGMP, 50 μM MIX stimulated cAMP hydrolysis; this effect was described with the rat liver cGMP-stimulated phosphodiesterase [9].

4. DISCUSSION

When dog thyroid slices are stimulated by TSH, carbamylcholine lowers the accumulation of cAMP [4]. Several types of experimental data presented earlier have suggested that the inhibition by carbamylcholine is mediated by an increase of the free intracellular Ca^{2+} concentration [2]. As shown in table 1, the effect of carbamylcholine is reproduced by the ionophore A 23187 in the presence of Ca^{2+} and both controls are relieved by MIX, suggesting a common biochemical mechanism of inhibition of cAMP accumulation. Further studies established that the decrease of cAMP level observed in the presence of carbamylcholine bears on cAMP degradation (i.e., most probably on the phosphodiesterases) rather than on cAMP synthesis [4]. Here, the particular effect of MIX which abolishes the carbamylcholine and Ca^{2+} effects, has been used in our system as a criterion to evaluate the role of the various phosphodiesterases (table 1). This contrasts with the negative control of norepinephrine on cAMP accumulation in the same tissue which bears on cAMP synthesis (i.e., adenylate cyclase) and is not affected by MIX [10].

We previously reported that micromolar concentrations of cGMP stimulate cAMP phosphodiesterase activity in crude thyroid homogenate



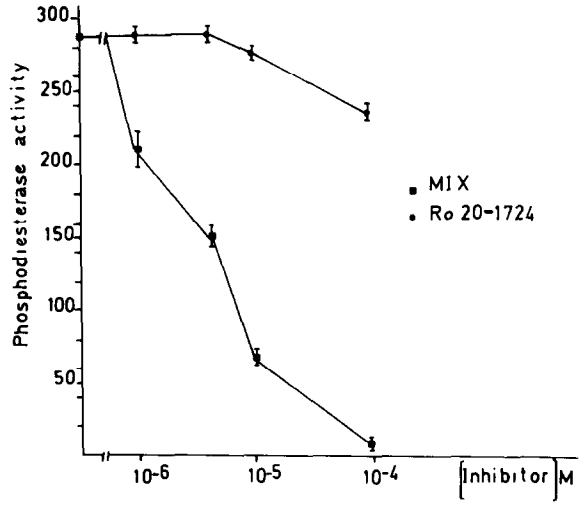


Fig.3. Effect of MIX and Ro 20-1724 on the activity of purified dog thyroid calmodulin-dependent phosphodiesterase in the absence (A), or in the presence of saturated calmodulin and 2 mM $CaCl_2$ (B). cAMP hydrolysis was assayed at 1 μM substrate level. Stimulation factors by calmodulin were 15, 13, 8 and 4-fold at 0, 5, 10 and 100 μM MIX, respectively. Results are expressed as pmol cAMP hydrolyzed.min^{-1}.mg protein^{-1}.

and that this activation is no longer observed in the presence of 0.1 mM MIX [6]. Such data are confirmed with the purified cGMP-stimulated rat liver phosphodiesterase [9]. Since cGMP accumulation is increased in the presence of carbamylcholine, such data argue in favor of a direct effect of cGMP as an activator of cAMP degradation [3]. However, several indirect arguments bear against this hypothesis:

(i) the cGMP-activated phosphodiesterase is clearly a Ca^{2+}-independent enzyme [6], and therefore can not be controlled directly by changes in intracellular Ca^{2+} concentration;

(ii) Mn^{2+} increases cGMP without decreasing cAMP accumulation [2];

(iii) sodium nitroprusside enhances cGMP accumulation in thyroid slices whether they are depleted of Ca^{2+} or not; it does not decrease TSH induced cAMP accumulation. Moreover, at the same concentration, sodium nitroprusside does not relieve the carbamylcholine inhibition of cAMP accumulation (C. Decoster et al., unpublished).

We show here the presence of a calmodulin-dependent phosphodiesterase in dog thyroid (fig.1). We further show that MIX, a competitive inhibitor of the phosphodiesterases [11], is a more potent inhibitor of that enzyme than Ro 20-1724. Saturated by calmodulin, this phosphodiesterase is more sensitive to MIX inhibition than in its absence, so that the factor of stimulation by calmodulin of the enzyme activity is progressively reduced as the concentration of the xanthine increases. Since MIX, but not Ro 20-1724, is known to suppress the negative control of carbamylcholine on cAMP accumulation, the data suggest a direct calmodulin activation of cAMP phosphodiesterase activity. The effect of the xanthine on the calmodulin-dependent phosphodiesterase appears to be specific since Ro 20-1724, as in the erythrocytes [12], shows more selectivity as an inhibitor of the thyroid cAMP-specific phosphodiesterase.

In conclusion, the data obtained from the comparison of MIX and Ro 20-1724 on the phosphodiesterase present in dog thyroid suggest that, when used in vitro (i.e., with thyroid slices incubated in the presence of TSH and carbamylcholine) MIX reduces the activation by calmodulin of a specific phosphodiesterase which appears as the major catabolic enzyme for cAMP. Whether, and how this mechanism is general and totally independent of a cGMP control of the other phosphodiesterase is not known. A systematic investigation using multiple derivatives of MIX is now being carried out to study this problem.

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REFERENCES

[1] Dumont, J.E. (1971) Vitam. Horm. 29, 287–412.
[2] Decoster, C., Mockel, J., Van Sande, J., Unger, J. and Dumont, J.E. (1980) Eur. J. Biochem. 104, 199–208.
[3] Van Sande, J., Decoster, C. and Dumont, J.E. (1979) Mol. Cell. Endocrinol. 14, 45–57.
[4] Van Sande, J., Erneux, C. and Dumont, J.E. (1977) J. Cycl. Nucl. Res. 3, 335–345.
[5] Erneux, C., Van Sande, J., Dumont, J.E. and Boeynaems, J.M. (1977) Eur. J. Biochem. 72, 137–147.
[6] Erneux, C., Couchie, D. and Dumont, J.E. (1980) Eur. J. Biochem. 104, 297–304.
[7] Klee, C.B. and Krinks, M.H. (1978) Biochemistry 17, 120–126.
[8] Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313–1320.
[9] Erneux, C., Miot, F., Boeynaems, J.M. and Dumont, J.E. (1982) FEBS Lett. 142, 251–254.
[10] Cochaux, P., Van Sande, J. and Dumont, J.E. (1982) Biochim. Biophys. Acta 101, 39–46.
[11] Wells, J.N., Wu, Y.J., Baird, C.E. and Hardman, J.G. (1975) Mol. Pharmacol. 11, 775–783.
[12] Sheppard, H. and Wiggan, G. (1971) Mol. Pharmacol. 7, 111–115.