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METHYLXANTHINE EFFECTS ON CYCLIC ADENOSINE 3':5' MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY IN PREPARATIONS OF NEONATAL RAT CEREBELLUM: MODIFICATION BY TRIFLUOPERAZINE.

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Summary: Paradoxically, caffeine was found to stimulate the activity of 3':5'-cyclic AMP phosphodiesterase at substrate concentrations of 14μM, in cerebellar tissue from 10-day-old rats. Pretreatment with trifluoperazine, an inhibitor of calmodulin-dependent enzyme activation, converted the stimulatory effect of caffeine to the expected inhibitory action. Trifluoperazine pretreatment also increased the inhibitory action of theophylline on the cerebellar phosphodiesterase, but had no effect on the inhibitory action of 3-isobutyl-1-methylxanthine. It is suggested that caffeine and to a lesser extent theophylline in addition to their intrinsic phosphodiesterase inhibitory activity can also cause calmodulin dependent effects on cerebellar phosphodiesterase due to calcium mobilisation.

The purine methylxanthines, caffeine, theophylline and 3-isobutyl-1-methylxanthine (IBMX) exhibit 3':5'-cyclic AMP phosphodiesterase (EC.3.1.4.17) (PDE) inhibitory activity in a variety of tissues (1). However it has also been suggested that they may directly alter intracellular Ca^{2+} distribution (2,3) and it is often unclear whether observed biological effects are due to sustained cyclic-AMP levels, or to cyclic-AMP independent effects on intracellular Ca^{2+} distribution. Direct measurements of intracellular calcium fluxes are necessarily performed under unphysiologic conditions, so some doubt must exist about the validity of such measurements. In the adult brain many of the actions of calcium are mediated by a heat stable Ca^{2+} regulator

Cyclic AMP; cyclic adenosine 3':5' monophosphate.
PDE; cyclic AMP phosphodiesterase (EC.3.1.4.17).
IBMX; 3-isobutyl-1-methylxanthine.
MX; methylxanthine.

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protein (calmodulin) and recent studies suggest that the concentration of available calcium within the cell regulates the activity of calmodulin (4).

The early studies of Cheung and his co-workers have shown the importance of calmodulin as a regulator of brain PDE (5). Infant rat cerebellum contains PDE which increases markedly after birth due to an increase of endogenous activator (6). In this tissue the activity of calmodulin dependent enzymes could be expected to mirror alterations in the free calcium ion concentration in the cell cytosol. This report shows that the methylxanthines caffeine and theophylline have effects on neonatal rat cerebellum PDE activity apparently due to activation of calmodulin and demonstrates the relative activity of the methylxanthines with respect to calcium dependent processes and intrinsic PDE inhibitory activity.

METHODS

Infant Wistar rats were allowed to suckle until time of sacrifice. After decapitation, brains were removed rapidly, the cerebellum dissected and sectioned in 200µm slices at 4°C. The cerebellar slices were incubated in McCoy medium containing glucose and saturated with $95\%O_2 + 5\%CO_2$ at 37°C. Trifluoperazine (TFP) (Trifluoperazine HCl., Merke, Sharpe and Dohme) was added to a final concentration of 100µM for 30 minutes preincubation. Control incubations were performed without the addition of phenothiazine. The methylxanthines studied were added in a second incubation in the concentrations indicated under Results. At the termination of the incubation step, cerebellar slices were rinsed and homogenised in 0.05M Tris pH 7.4 and a cytosolic fraction obtained by centrifugation at 30,000g for 30 minutes.

The activity and kinetic properties of the PDE present in this crude cytosolic preparation were determined at 37°C by a coupled enzyme procedure modified from the method of Loten and Sneyd (7). Cytosol was added to a reaction mixture of 105µl with the following final concentrations Tris 40mM; $MgCl_2$ 5mM; [^{3}H]cyclic AMP 64 nM; cyclic AMP 0.3-300µM and 10µg 5'nucleotidase (*Crotalus atrox* venom, Sigma Chemical Co.). Hydrolysis was terminated by immersion of the reaction tubes in a water bath at 98-100°C for 5 minutes. Separation of hydrolysed adenosine was performed after addition of 500µl of AG1-X2 ion exchange resin (Biorad Laboratories) containing 250mg resin in water. After filtration the liquid phase was collected and radioactivity determined following the addition of 10ml Instagel (Packard Instrument Corp.). The action of caffeine on the cytosolic fraction was determined by the direct addition of methylxanthine in a final concentration of 10mM to the cytosol of a preparation which had been incubated without methylxanthine or TFP.

Phosphodiesterase activity was expressed as pmol cyclic AMP hydrolysed per 10 minutes. Double reciprocal plots of cyclic AMP concentration versus PDE velocity were constructed to estimate the apparent V_{max} and K_m of the PDE isolated in this procedure.

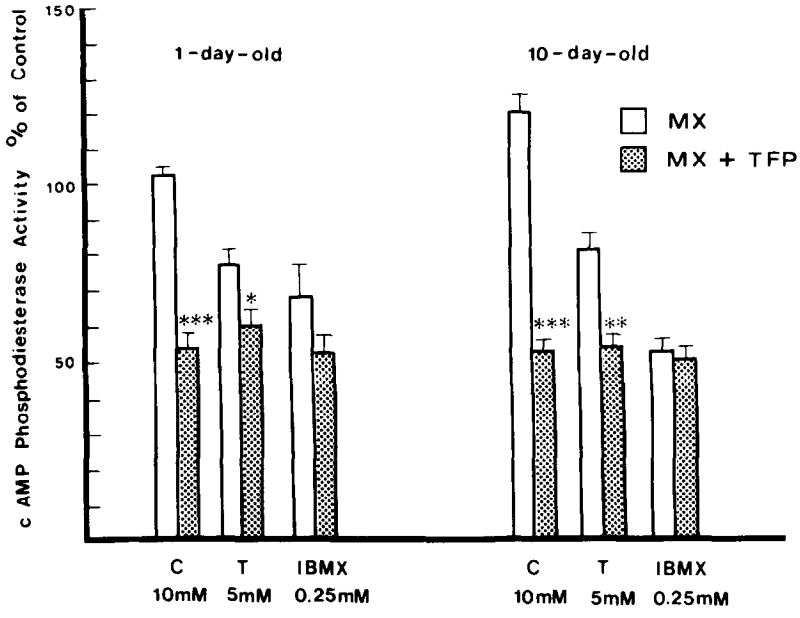

Figure 1. Phosphodiesterase activity in cerebellum from 1-day-old and 10-day-old rats.

PDE activity was estimated at a final cyclic AMP concentration of 14μM following incubation of cerebellar slices with methylxanthine alone or TFP followed by methylxanthine addition. The values are expressed as a percentage of control estimated from an aliquot of each preparation without methylxanthine. Values are mean ± SEM for eight observations. Significant difference from TFP-free incubation is indicated:
* $p < .05$; ** $p < .005$; *** $p < .001$. (Student Distribution).

RESULTS

Figure 1. shows that the effect of 10mM caffeine and 5mM theophylline on PDE activity in slices of cerebella from 1 and 10-day-old rats is considerably modified by preincubation of slices with TFP. Without preincubation with TFP, caffeine had no significant effect on PDE activity in cerebella from 1-day-old rats, and stimulated the activity in cerebella from 10-day-old rats. However after preincubation in 100μM TFP, exposure to caffeine produced the expected inhibition of PDE activity in both groups. The inhibitory action of 5mM theophylline on PDE activity was considerably increased after preincubation of the cerebella with TFP, but preexposure to TFP did not significantly affect the inhibitory action of IBMX.

Table 1. The effect of methylxanthines on cyclic AMP phosphodiesterase activity of cerebellum from 1-day-old rats.

Additions to Incubation Medium.			PDE Activity.
Incubation 1.	Incubation 2.		(% of intra-assay control)
TFP 100μM	MX	(mM)	
-	Caffeine	10	102 (2.0)
+		10	54 (4.0)
+		5	65.5 (3.6)
+		2.5	81 (4.2)
-	Theophylline	5	74 (4.0)
+		5	57 (4.5)
+		2.5	74 (4.0)
+		1.25	81 (4.2)
-	IBMX	0.25	67 (8.0)
+		0.25	54 (7.0)
+		0.15	71 (5.4)
+		0.15	93 (4.2)

PDE activity was estimated at a final cyclic AMP concentration of 14μM following incubation of cerebellar slices with methylxanthine alone or TFP followed by methylxanthine addition. The values are expressed as a percentage of control, estimated from an aliquot of each preparation without methylxanthine. Values are mean ± SEM for eight observations at each point. Control values, without TFP; 654±10 pmol cyclic AMP hydrolysed, with TFP preincubation; 553±30 pmol hydrolysed.

Table 1. shows the concentration dependence of the inhibition of PDE by the different methylxanthines in the presence of TFP. It can be seen that 0.25mM IBMX, 5mM theophylline and 10mM caffeine are equipotent in terms of their inhibitory activity when the TFP dependent effects are eliminated.

The substrate concentration dependence of the effects of TFP and caffeine are shown in Figure 2. in the form of Lineweaver-Burk plots. Trifluoperazine itself had no significant effects on the kinetic properties of the enzyme (plot B). Caffeine, in the absence of TFP, increased the K_m of the low K_m form of the enzyme (assuming two K_m forms are represented),

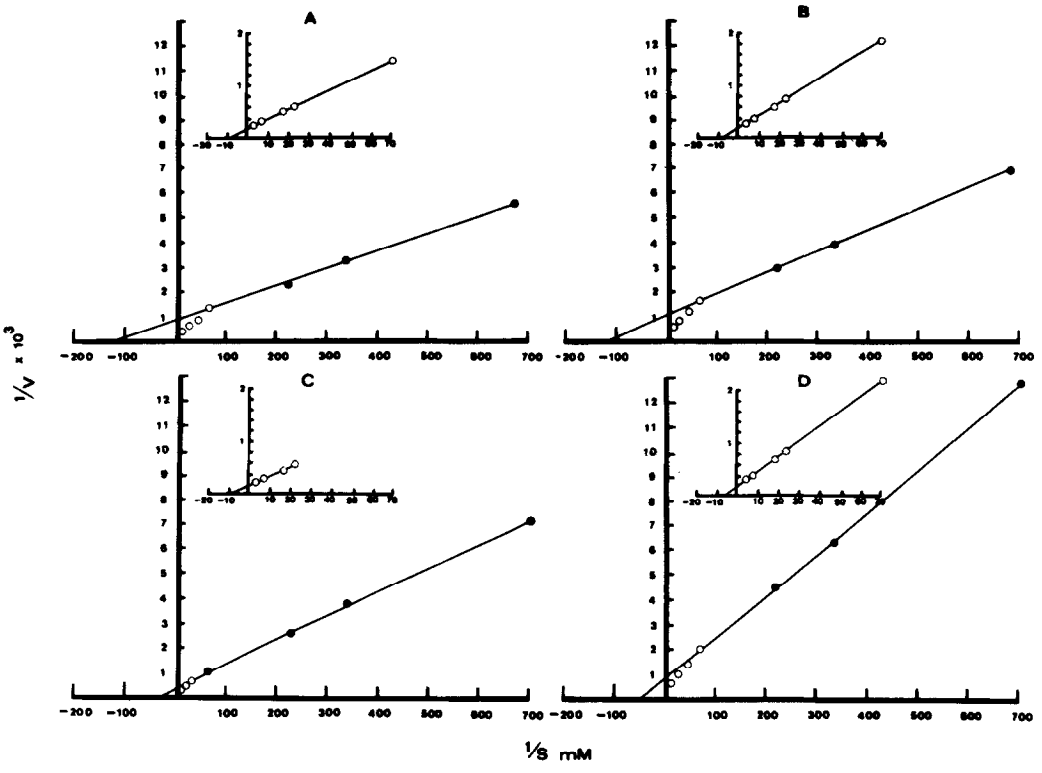

Figure 2. Representative Lineweaver-Burke plots are shown to compare the kinetic properties of the PDE in crude preparations of cerebellum from 1-day-old rats. Plots for a control preparation A, preparation after TFP preincubation B, incubation with caffeine (10mM) C and TFP preincubation followed by caffeine (10mM) D are shown.

V= pmol cyclic AMP hydrolysed per 25mg wet weight per 10 minutes.

Mean values $\pm$ SEM for eight estimations under each set of conditions:

	'Low K_m form'				'High K_m form'			
	K_m (μM)		V_{max}		K_m (μM)		V_{max}	
A.	7.7	(1.8)	1052	(140)	124	(7)	5555	(310)
B.	7.9	(2.1)	1000	(110)	125	(8)	5320	(305)
C.	33.0	(4.1)*	2860	(105)*	110	(10)	5715	(405)
D.	18.2	(2.0)*	1070	(125)	141	(9)	5420	(410)

*Significant difference from control $p < .001$ (Student Distribution).

but also caused a marked increase in the apparent V_{max} of this form of the enzyme (plot C). However, after preexposure to TFP, this effect on the apparent

V_{max} of the low K_m form was prevented, and the inhibition of the PDE activity was apparently due to an increase in the K_m of the low K_m form (plot D) compared to control.

When caffeine (10mM) was added directly to the cytosol prepared from cerebella of 1-day-old animals rather than to incubations of slices of cerebella, the expected reduction of PDE activity (to 67 $\pm$ 3% of control) was seen, even in the absence of preexposure to TFP.

DISCUSSION

The modifications by TFP of the actions of caffeine and theophylline on PDE activity observed in the present study suggests that these two methylxanthines have calmodulin-dependent effects on PDE activity. Studies in many cell systems, reviewed by Means and Dedman (8) and by Cheung (9), suggest that calmodulin-mediated enzymes are regulated by alterations in the cellular distribution of calcium rather than a change in calmodulin content. Thus, the results reported suggest that caffeine and theophylline have important effects on intracellular distribution of calcium in the neonatal rat cerebellum.

This conclusion depends on the assumption that the effects of TFP in tissue are due to prevention of Ca^{2+} dependent activation of calmodulin. The selective binding of TFP to the calcium dependent activator of PDE has been investigated extensively (10). Other potential effects of TFP, such as membrane stabilisation (11) and alteration of glucose uptake (12) should also be considered. However, the minor effect of TFP alone on basal PDE activity indicates it is without major additional effects and that it is acting mainly to prevent methylxanthine-mediated activation of calmodulin.

Experiments in which caffeine was added directly to the cytosol of cerebella from 1-day-old rats support the contention that liberation of Ca^{2+} from intracellular organelles was a factor in the observed actions of caffeine in the intact cerebella. In this situation, even in the absence of pretreatment

with TFP, inhibition of PDE activity was observed. Presumably calmodulin could not be activated because of the lack of intracellular organelles from which calcium could be liberated.

Although elevation of cyclic AMP itself can increase PDE activity (13), it seems unlikely that the observed effects are due to this mechanism, because they are not seen with IBMX, and are blocked by TFP.

The effect of caffeine in the presence of TFP was to inhibit the enzyme activity through increasing the K_m of a low K_m form. In the absence of TFP, the overall activity at the same substrate concentration was increased through an increase in the apparent abundance of a form with hydrolytic ability at low substrate concentration. This is in addition to the effect of caffeine to increase the K_m of the low K_m enzyme. In view of the lack of studies on purified enzyme forms, the crude system used and the possibility of enzyme aggregates with different affinities for cyclic AMP (14) an attempt to reduce the actions of the methylxanthines and TFP to single forms of the enzyme are considered hazardous and unjustified.

In conclusion, interpretation of the actions of methylxanthines on metabolic and hormonal events must take into account direct effects on intracellular calcium-dependent processes as well as an elevation of cyclic necleotides due to PDE inhibition. In the neonatal rat cerebellum, for equivalent PDE inhibitory activity, caffeine is more active than theophylline in affecting calcium distribution and the effect is not seen with IBMX.

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