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Opposing Actions of Methylxanthines and Dibutyryl Cyclic AMP on 1,25 Dihydroxyvitamin D_3 Production and Calcium Fluxes in Isolated Chick Renal Tubules

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In contrast to dibutyryl cyclic AMP, the methylxanthine phosphodiesterase inhibitors theophylline and caffeine were found to inhibit the conversion of 25 hydroxyvitamin D_3 to 1,25 dihydroxyvitamin D_3 in isolated renal tubules from vitamin D deficient chicks. This inhibition occurred at concentrations of methylxanthines which were shown to increase renal tubule cyclic AMP levels. No effect of theophylline or caffeine on 25 hydroxyvitamin D_3 metabolism in isolated chick renal mitochondria was detected. Because of a demonstrated inhibitory action of calcium (10 and 20 μmol/l) on renal mitochondrial conversion of 25 hydroxyvitamin D_3 to 1,25 dihydroxyvitamin D_3, the effect of theophylline and dibutyryl cyclic AMP on cellular calcium-45 efflux and total renal tubule calcium content was estimated. Theophylline 10 mmol/l was found to inhibit renal tubular calcium efflux and to increase total cellular calcium content, while dibutyryl cyclic AMP 1 mmol/l had the reverse effect on both parameters. Divergent actions of the methylxanthines and dibutyryl cyclic AMP on the formation of 1,25 dihydroxyvitamin D_3 and renal tubule calcium efflux and content support the hypothesis that intracellular calcium is an important regulator of renal vitamin D metabolism. The results indicate that observed actions of methylxanthines cannot always be ascribed to cyclic AMP accumulation.

Calcium homeostasis in the face of varying physiological needs and dietary supply is maintained in part by vitamin D. The biologically active metabolite is 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), produced by hydroxylation of circulating 25 hydroxyvitamin D_3, by the enzyme system 25-(OH)-D_3 1-α-hydroxylase, in the renal tubule mitochondrion. This conversion is closely regulated by a number of hormonal and ionic factors in vivo. However control of enzyme activity at the cellular level is poorly understood (1).

Cyclic AMP and dibutyryl cyclic AMP have been shown in various in vitro (2-6) and in vivo (7) systems to enhance 1,25-$(OH)_2D_3$ formation, as have adenylate cyclase agonists in certain conditions (2-7). Calcium is an important regulator of 1-α-hydroxylase, and in vivo (8) and under certain

Abbreviations:
cyclic AMP; Adenosine 3':5' cyclic monophosphate
Hepes; 2-(N-2-hydroxyethyl piperazine-N'-yl)-ethanesulphonic acid
EGTA; Ethylene glycol bis (β-aminoethyl ether) N-N-tetracetic acid

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conditions in vitro (9-16) inhibits 1,25-$(OH)_2D_3$ production. Thus these second messengers appear to possess opposing actions in the physiological control of vitamin D metabolism.

Methylxanthine phosphodiesterase inhibitors are often used to investigate cyclic AMP mediated effects due to their property of raising cyclic AMP levels. This study observes effects of methylxanthines on renal vitamin D metabolism inconsistent with this property and examines the actions of methylxanthines on cellular calcium distribution that may explain this inconsistency.

Materials and Methods

Renal Tubule Preparation

One day old White Leghorn-Australorp cross cockerels were obtained and raised on a soybean meal based vitamin D deficient diet containing 1% added calcium and 1% added phosphorus for 3-4 weeks prior to sacrifice and rapid removal of their kidneys.

An isolated renal tubule suspension was prepared from 2-4 chick kidneys for each experiment by collagenase digestion (Worthington collagenase, Freehold N.J., USA) in tris acetate buffer containing 1.25 mM Ca^+ and 2 mM PO_4 by a modification (17) of described methods (3).

Tubule 1,25 $(OH)_2D_3$ Production

Identical 1.5 ml aliquots of suspension were preincubated for 30 min with the agent under study at 37°C in a shaking water bath, then incubated for a further 30 min with 25-hydroxy[26,(27)-methyl-^{3}H] vitamin D_3 substrate (The Radiochemical Centre, Amersham, U.K.). Final concentration of substrate was 40 nmol/l and specific activity 0.05μCi/vial. Vitamin D metabolites were extracted using methanol:chloroform (2:1 v/v) (18) and separated using normal phase high pressure liquid chromatography (17) with the 1,25 $(OH)_2D_3$ peak identified by coelution with authentic standard (kindly donated by Dr. M. Uskokovic, Hoffman-la Roche, Nutley, N.S. U.S.A.).

Mitochondrial 1,25$(OH)_2D_3$ Production

Mitochondria were prepared from vitamin D deficient chick kidney by standard differential centrifugation techniques. Kidneys were rapidly minced and homogenized in a Potter Elvehjem homogenizer in 10 volumes of an ice cold buffer of sucrose 250 mmol/l Hepes 10 mmol/l, EGTA 1.0 mmol/l, defatted albumin 0.1% pH 7.4. The supernatant from a 400 G x 10 min at 4°C centrifugation was pooled with a second supernatant prepared from further homogenization of the pellet. The combined supernatants were spun at 10,000 G x 10 min at 4°C, and the resulting pellet washed twice in ice cold incubation buffer of KCl 120 mmol/l, HEPES 10 mmol/l, K_2HPO_4 2 mmol/l, $MgCl_2$ 1 mmol/l pH 7.1. Mitochondria prepared this way,when energized, showed satisfactory coupling of oxidative phosphorylation, respiratory control ratios of greater than 4, and energy dependent calcium uptake. This uptake could be inhibited by the specific calcium uptake blocker ruthenium red (19) at 1 μmol/l. 1,25-$(OH)_2D_3$ production in an isolated mitochondial preparation was performed as for the tubule preparation above, in incubation buffer, using sodium succinate 5 mmol/l and rotenone 1 μmol/l as an energy source, and an 8 min incubation time at 37°C.

Cyclic AMP Production

Net cyclic AMP production was measured in experiments identical to those in which 1,25-$(OH)_2D_3$ metabolism was assessed. Tubules and medium were boiled for 3 min to terminate the experiment, then sonicated and centrifuged. The supernatant was assayed for cyclic AMP following acetylation using the radioimmunoassay and antiserum described by Hunt et al (20).

Calcium Efflux

Cellular calcium efflux was studied in an isolated chick tubule suspension, prepared as above, by a desaturation method (21,22). The tubules

were preincubated with ^{45}Ca, 20μCi in 10 ml buffer containing 200μmol/l Ca^{2+} at 37°C for 60 min washed rapidly twice in ice-cold buffer, and resuspended in calcium free buffer containing EGTA 1 mmol/l to prevent calcium recycling. Identical 1 ml aliquots of suspension were incubated at 37°C in a shaking water bath with the agent under study. At various time points, 100μl aliquots of suspension were removed from each vial and layered on to 100μl of oil (di-n-butylphthalate: dinonylphthalate 10:4 v/v, BDH Chemicals Ltd., Poole, U.K.) in Beckman microfuge tubes and immediately centrifuged. The cell pellet passed through the oil layer leaving cell free medium on top. This supernatant was counted in a liquid scintillation spectrometer, and efflux of ^{45}Ca determined by the progressive appearance of tracer in the medium. There was less than 1% contamination of the pellet by extracellular medium using [^{3}H]-sucrose markers.

Total Cell Calcium

Total cell calcium was estimated by the use of a steady state system. Multiple identical 1 ml aliquots of a tubule suspension was prelabeled with ^{45}Ca 2μCi/ml in a multiwell dish in Eagle's modified minimal essential medium pH 7.4 for 6 hours under 5% CO_2 in air at 37°C, to ensure uniform specific activity of tracer in all cell compartments. The medium contained 1.8 mmol/l $CaCl_2$ 1.0 mmol/l NaH_2PO_4, 0.8 mmol/l $MgSO_4$ and 0.1% gelatin. The cells were incubated for further specified time intervals with the agent under study in the continued presence of calcium and tracer, before being washed rapidly three times in ice cold saline with 100μmol/l $LaCl_3$. Each incubation was counted in a liquid scintillation spectrometer, and activity taken to represent cell associated calcium.

Statistical Analysis

Results are expressed as means ± standard error mean. The unpaired student's t test was used for comparisons within experiments.

Results

Theophylline and caffeine caused a dose dependent inhibition of 1,25 $(OH)_2D_3$ production in the isolated tubule system (Fig 1, Panel A). Cyclic AMP accumulated in response to theophylline and caffeine in a dose dependent manner in this system (Fig 1, Panel B), confirming the anticipated inhibition of phosphodiesterase by these agents. In a separate experiment, simultaneous examination of the effects of dibutyryl cyclic AMP, caffeine and theophylline on 1,25 $(OH)_2D_3$ formation showed diametrically opposed effects (table 1).

Dibutyryl cyclic AMP 1 mmol/l added directly enhanced calcium efflux from the tubule cells, particularly early in the incubation. By contrast theophylline 10 mmol/l demonstrated an inhibition of calcium efflux (Fig 2).

Total tubule cell calcium, calculated from cell associated tracer in steady state experiments, was reduced after 50 and 60 min incubation with dibutyryl cyclic AMP 1 mmol/l, but increased after theophylline incubation over the entire time range of the experiment (Fig 3). These results are consistent with the observed effects of these agents on calcium efflux.

1,25-$(OH)_2D_3$ production by an isolated energized mitochondria preparation was not affected by the presence of methylxanthines either in calcium free medium (+ 1 mmol/l EGTA) or with 10μmol/l ionized calcium estimated by the use of calcium-EGTA buffers. The presence of calcium did suppress 1,25-$(OH)_2D_3$ production (Fig. 4) in isolated mitochondria. This suppression was prevented

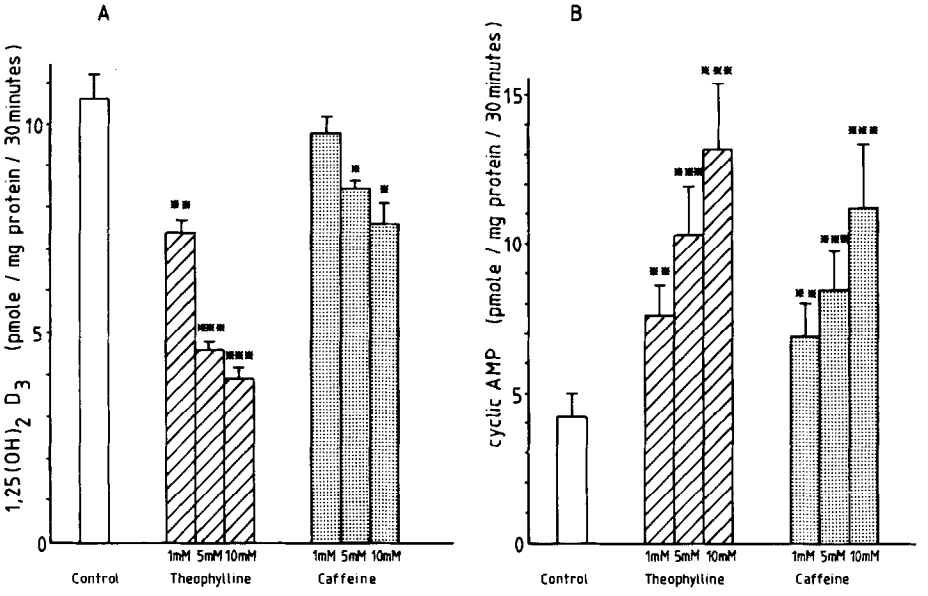

Figure 1A. The effects of theophylline and caffeine on 1,25-$(OH)_2D_3$ formation by vitamin D deficient chick renal tubules.
A tubule suspension was preincubated in 1.5 ml aliquots with varying concentrations of methylxanthines, made up in buffer, at 37°C in a shaking water bath for 30 minutes. Tritiated 25-$(OH)_2D_3$ substrate was added and conversion to 1,25-$(OH)_2D_3$ calculated as described in Methods. A typical experiment showing the mean and SE of four replicates is illustrated.
Figure 1B. Cyclic AMP production by vitamin D deficient chick renal tubules in response to methylxanthines.
Aliquots of 1.0 ml of a tubule suspension were incubated for 30 minutes in concentrations of methylxanthines as indicated. Cyclic AMP accumulation in the medium was measured by radioimmunoassay after termination of the reaction by boiling for 3 minutes and sonication and centrifugation of the cell suspension. Mean and standard error of 4 replicates is shown.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control.

by the prior addition of the specific mitochondrial calcium uptake blocker ruthenium red (Fig. 4).

Theophylline and ruthenium red had no detrimental effect on mitochondrial respiratory function and ruthenium red was found to completely inhibit respiration linked calcium uptake.

Table 1. The effect of preincubation of renal tubules from vitamin D deficient chicks for 30 min with dibutyryl cyclic AMP, or methylxanthines, at the concentrations indicated, on 1,25$(OH)_2D_3$ formation. Results expressed as mean ± SE of 4 replicates, all obtained within a single experiment. a; $p < 0.01$ cf control, b; $p < 0.001$ cf control.

	Formation of 1,25$(OH)_2D_3$ pmole/incubation
Control	25.4 ± 1.6
Dibutyryl cyclic AMP 1mM	31.0 ± 0.7[a]
Caffeine 10mM	8.3 ± 0.6[b]
Theophylline 5mM	6.6 ± 0.6[b]

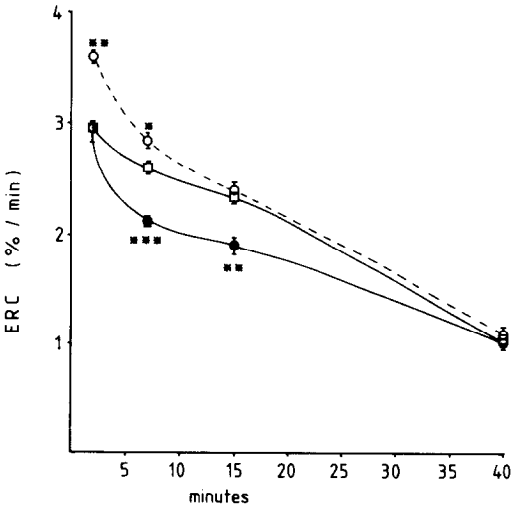

Figure 2. The effect of dibutyryl cyclic AMP and theophylline on vitamin D deficient chick renal tubule calcium efflux. A chick tubule preparation was prelabeled with calcium-45 for 60 minutes at 37°C, washed twice and resuspended in calcium free medium. Identical 1.0 ml aliquots were added to vials containing the agent under study, made up in buffer, and EGTA 1mmol/l at 37°C. Efflux was determined by the accumulation of tracer in the medium as described in Methods. The efflux rate coefficient (ERC) on the ordinate is calculated as the fractional efflux rate of cell calcium, expressed as a percentage, over sequential time intervals during the course of the incubation shown on the abscissa as described by Isaacson & Sandow (21). Each point is the mean ± SE of 4 parallel incubations in this experiment.

□ control, ○ dibutyryl cAMP 1mmol/l, ● theophylline 10 mmol/l
*p < 0.05, **p < 0.01, ***p < 0.001 compared to control.

Discussion

In our isolated chick renal tubule system, dibutyryl cyclic AMP enhanced the production of $1,25(OH)_2D_3$ from $25(OH)D_3$, consistent with previous reports for isolated tubules (2,3,6) cultured chick kidney cells (4), and chick kidney slices (5). In our hands calcium 10μmol/l inhibited $1,25(OH)_2D_3$ production by isolated energized renal mitochondria. This observed inhibition was prevented by the mitochondrial calcium uptake blocker, ruthenium red.

Previous reports of the effect of Ca^{2+} on renal 25-$(OH)D_3$-1α-hydroxylase activity in mitochondria have been conflicting. Some reports showed an inhibitory effect similar to that described in this study (9-13) while others, in which the mitochondria were prepared under conditions of more severe calcium depletion, showed a stimulatory effect of low Ca^{2+} concentrations (24-26).

Caffeine and theophylline, despite raising cyclic AMP levels, suppressed the production of $1,25\text{-}(OH)_2D_3$ in a dose dependent manner in the tubule system but not in an isolated mitochondrial preparation. The possibility of an action of these agents on cell calcium distribution not mediated by cyclic AMP was investigated by studies of cell calcium and calcium efflux. The latter

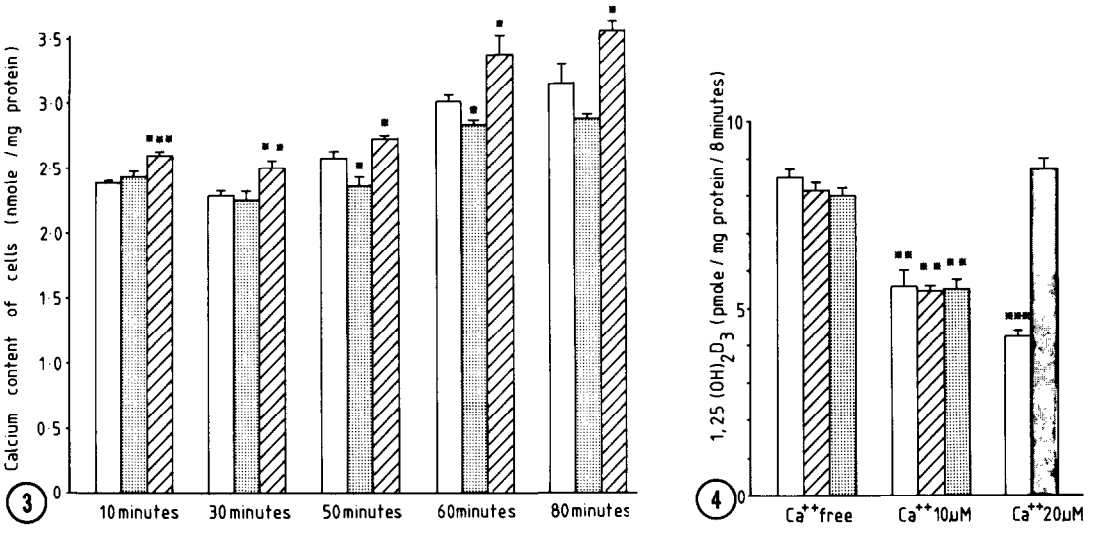

Figure 3. Estimated total renal tubule cell calcium after incubation with dibutyryl cyclic AMP or theophylline.
A chick tubule cell suspension in culture medium, containing $CaCl_2$ 1.8 mmol/l, was prelabelled with calcium-45 for 6 hours to ensure uniform tracer activity in all cell compartments. The cells were then incubated further with dibutyryl cyclic AMP 1 mmol/l or theophylline 10 mmol/l, made up in buffer, in the continuing presence of medium calcium and tracer for the times indicated, before being washed rapidly twice in ice cold saline with 100μmol/l lanthanum chloride. Cell associated tracer was taken to be proportional to total cell calcium. Results expressed as mean ± SE of 4 replicates.

□ control, ▦ db cAMP, ▨ theophylline.
*p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. The effects of ionized calcium, theophylline, caffeine and ruthenium red on isolated mitochondrial production of $1,25\text{-}(OH)_2D_3$.
An isolated mitochondrial preparation from vitamin D deficient chick kidney was incubated with tritiated $25(OH)D_3$ substrate, Na succinate 5mmol/l and rotenone 1μmol/l, theophylline 10mmol/l or caffeine 10mmol/l made up in buffer, and EGTA 1mmol/l with varying amounts of $CaCl_2$, for 8 minutes at 37°C. Ionized calcium was calculated from the dissociation constant of Ca:EGTA under the conditions employed according to Portzehl et al (23). $1,25(OH)_2D_3$ production was measured as for tubule experiments. Results are expressed as mean ± SE of 4 replicates.

□ control, ▨ theophylline 10mmol/l, ▦ caffeine 10mmol/l, ▩ ruthenium red 1μmol/l
**p < 0.01, ***p < 0.001

may reflect primary membrane transport, or an alteration in exchangeable calcium pools (22), while the former indicates new steady state conditions resulting from the test agent. Divergent effects on these parameters were found for dibutyryl cyclic AMP and theophylline.

The inhibition of calcium efflux by theophylline may be invoked to explain its inhibitory effect on renal vitamin D metabolism. Theophylline and caffeine inhibit Ca-Mg ATPase in crude pancreatic islet homogenates (27) (a property not shared by 3-isobutyl-1-methylxanthine or cyclic AMP), thus

presumably inhibiting the membrane calcium pump. Certainly in the islet (28-30) and muscle (21,31) these drugs have been shown to mobilize calcium from microsomes and sarcoplasmic reticulum respectively, perhaps by inhibition of microsomal and sarcoplasmic Ca-MgATPase. The renal tubule cell, like the hepatocyte (32), has a large mitochondrial calcium buffering capacity. Tubule cell mitochondria have high avidity for cytosolic calcium and would be expected to rapidly sequester calcium displaced from other cell stores.

Although in liver (33-38), pancreatic islet (29,30,39) and kidney (22) there is ample data to suggest that cyclic AMP depletes all cell pools, including mitochondrial, of calcium, there is no definite evidence that the stimulatory effect of cyclic AMP on 1,25-$(OH)_2D_3$ production is mediated directly by renal tubule mitochondrial calcium depletion, and other potential effects of cyclic AMP should be remembered. The estimates of total cell calcium following exposure to dibutyryl cyclic AMP in the tubule preparation were consistent with depletion of cellular calcium pools by cyclic AMP.

The opposing actions of dibutyryl cyclic AMP on the one hand and theophylline and caffeine on the other on the formation of 1,25-$(OH)_2D_3$ and on the cellular efflux of calcium and the total cellular content of calcium support the concept that intracellular (presumably intramitochondrial) calcium is an important regulator of the renal 25-$(OH)D_3$-1α-hydroxylase enzyme system, and that it can override the effect of cellular cyclic AMP. The lack of a direct inhibitory effect of theophylline or caffeine on 1α-hydroxylase activity in isolated mitochondria supports the concept that their inhibitory action in whole cells is due to their effects on calcium movements at the cell membrane, and perhaps, in addition, mobilisation of calcium from intracellular stores other than mitochondria.

Theophylline and caffeine are known to be potent competitive antagonists of the actions of adenosine and other purine nucleotides (40). Purinergic receptors are found in many tissues, including kidney (41) and the possibility of an as yet undefined renal action of methylxanthines mediated by the purinergic system cannot be excluded. Similarly, an effect mediated through inhibition of cyclic guanosine-3',5'-monophosphate (cyclic GMP) phosphodiesterase cannot be excluded, as cyclic GMP has recently been shown to inhibit renal 1,25$(OH)_2D_3$ formation (6).

The observation that,in this system,theophylline and caffeine have diametrically opposed actions to cyclic AMP, despite the expected elevation of cyclic AMP content in the cell, highlights the inappropriateness of assuming that observed biological actions of methylxanthines are necessarily mediated by cyclic AMP.

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