THE PRODUCTION OF cIMP BY TOAD BLADDER, AND ITS EFFECTS ON TRANSPORT OF WATER AND SALT

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1. Introduction

In the previous paper [1] it was demonstrated that the toad bladder is capable of breaking down cAMP by an alternative route to that via 5' AMP, it is deaminated to cIMP. The present paper reports some preliminary experiments which demonstrate that cIMP mimics the actions of neurohypophysial hormones on the transport of water and sodium across the intact bladder.

2. Methods

2. . *Experiments on intact bladder*

Toads were pithed, their urinary bladders removed and set up as sacs by the method of Bentley [2] for experiments on water permeability. For experiments on sodium transport the bladder was clamped between two halves of a Perspex chamber and agar gel electrodes used to measure the potential difference and short-circuit current, shown to be a measure of the net transepithelial sodium transport [3].

The low bicarbonate Ringer's solution [4] contained 90 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 5.5 mM glucose.

Water permeability was measured by subtracting the resting water loss over 40 min periods from water loss during experimental 40 min periods. Sodium transport was measured by subtracting the control short circuit current produced by one half of the bladder from that produced by the addition of cyclic nucleotides to the other half of the bladder over a 15 min period.

2.2. *Estimation of binding to 'cAMP-binding' protein*

The method used was the Cooper et al. modification [5] of Gilman's method [6], except that a crude preparation of cyclic binding protein from toad bladder was used instead of protein from rabbit muscle.

Epithelial cells were scraped from bladders and homogenised in ice-cold 0.2 M sucrose, containing 10 mM KCl and 100 mM Tris–HCl, pH 7.4, using an Ultra Turrax homogeniser. The homogenate was centrifuged at 1000 g for 5 min at 4°C, using an 'MSE 18' centrifuge, the supernatant collected and centrifuged at 9000 *g* for 15 min at 4°C, using an 'MSE 65' centrifuge, the supernatant again collected and re-centrifuged at 20000 *g* for 30 min at 4°C. This final supernatant was used for estimation of binding of nucleotides to 'cAMP-binding' protein.

2.3. *The production of cIMP and cAMP from ITP and ATP*

Epithelial cells were scraped from two bladders and homogenised, using a chilled glass hand-homogeniser, in 1 ml of a medium consisting of: 1 mM ATP, 2 mM $MgSO_4$, 3 mM theophylline and 5 mM potassium phosphate buffer, pH 7.2. This homogenate was used for incubations performed at 20°C for 3 min, the tubes were then frozen. This allowed, on thawing, any mucus remaining to be easily removed, along with precipitated protein, by centrifugation at

650 *g* for 5 min. The supernatants obtained were evaporated to dryness, taken up in 0.05 ml of 10% isopropanol and spotted on paper chromatograms.

The following solvent systems were used: n-propanol : conc. NH_4OH : water (6 : 3 : 1); [7] saturated $(NH_4)_2SO_4$: isopropanol : water (79 : 19 : 2); [8] ethanol : 1.0M NH_4 Ac : water (5 : 1 : 1); [9] 2-dimensional chromatography was performed by one of two methods: a) The sample was spotted in one corner of the paper, run in the first solvent, dried, the rough edges cut off, and run at right angles in the second solvent; b) The sample was run in the first solvent, the spots located under ultraviolet light, cut out and sewn on to another paper and re-run in the second solvent system.

A third method of paper chromatography was evolved which satisfactorily separated cIMP from cAMP and both cyclic nucleotides from ITP, ATP, 5'IMP and 5'AMP. The paper was spotted and developed in ethanol : 1.0 M ammonium acetate (6 : 1) but deliberately allowed to over-run. The far end of the paper was cut in a serrated fashion to allow the solvent to drip off, thus minimising 'edge-effects', and a drop of the dye acid fuchsin which runs slightly in advance of cyclic nucleotides was added as a reference. Generally papers were run for 60 hr giving R_f values as follows: 5'IMP 0.028–0.032, 5'AMP 0.032–0.036, cIMP 0.094–0.098, cAMP 0.098–0.103, and inosine 0.109–0.130.

In all chromatograms unlabelled reference substances were added and separated, nucleotides located by ultraviolet light.

Radioactivity was estimated by the method of liquid scintillation counting in the following way. Spots were cut out, and eluted with 0.5 ml of 10% isopropanol for 1 hr in polyethylene counting vials. Scintillation fluid (20 ml) was then added and each tube counted for 10 min; a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3320 was used. For double-isotope counting the method described by Herberg [10] was used.

Estimation of protein was by the method of Lowry et al. [11].

2.4. *The effect of oxytocin on the production of cIMP, cAMP from ITP AND ATP*

The method of Bär et al. [12] was used for preparation of homogenates and incubation techniques.

3. Materials

Standard laboratory chemicals were of 'Analar' grade. Biochemicals were obtained from Sigma Chemical Co., and from Boehringer. Radiochemicals were obtained from The Radiochemical Centre, Amersham; [^{14}C]ATP was purchased as the ammonium salt (Batch no. 16 and 18, specific activity 40–60 mCi/mmol). Oxytocin [^{3}H]ITP was obtained from Dr. B.T. Pickering, Department of Anatomy, University of Bristol; Whatman No. 1 chromatography paper (46 × 56 cm) was used throughout.

4. Results

4.1. *The hydro-osmotic activity of cIMP on toad bladder*

The hydro-osmotic activity of 10 mM cIMP, compared with that of 10 mM cAMP, is shown in table 1. At the concentration used it may be seen that cIMP had an activity similar to that of cAMP.

4.2. *The natriferic activity of cIMP on toad bladder*

cIMP (2 mM) produced a rise of 10.8 ± 4.8 μA and cAMP (2 mM) a rise of 15.6 ± 1.1 μA in short circuit current (n = 11), a measure of net sodium transport by the toad bladder.

Table 1
Hydro-osmotic activity of cIMP on toad bladder.

	N	Water loss (mg/40 min)	*P* for difference from control
Control	9	13	
cIMP	8	365	< 0.05
cAMP	10	298	< 0.001

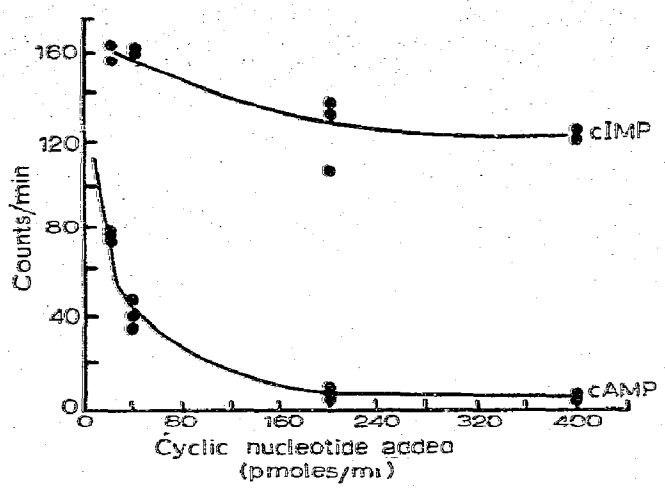

Fig. 1. Displacement of [^{3}H]cAMP from toad bladder mucosal cell cyclic binding protein by cAMP and cIMP.

4.3. *The binding of cIMP to 'cAMP-binding' protein*

The displacement of [^{3}H]cAMP by unlabelled cIMP and cAMP is shown in fig. 1. It may be seen that cIMP has about 10% of the affinity of cAMP for the protein in this crude preparation.

4.4. *The production of cIMP and cAMP from ITP and ATP*

The possibility that ITP, as well as cAMP, might act as a substrate for the formation of cIMP was investigated.

0.1 ml of bladder homogenate or a boiled control was incubated with 0.025 ml of [^{3}H]ITP for 15 min as described in the Methods section and the products separated using 2-dimensional chromatography on a single paper; the first solvent was n-propanol : conc. NH_4OH:water (6:3:1), the second saturated $(NH_4)_2SO_4$:isopropanol:water (19:19:2) to separate cIMP from cAMP. The levels of activity in the cIMP spots in two experiments were estimated as:

Boiled control	164 cpm
	107 cpm
Experimental	362 cpm
	233 cpm

Losses in the latter experiments were greater than in the former, but again demonstrated the production of cIMP from ITP.

There are two possible explanations, either adenyl cyclase can use ITP as a substrate, or there is a distinct enzyme for the reaction, which for convenience will be termed 'inosyl cyclase'.

To distinguish between these possibilities the metabolism of both [^{3}H]ITP and [^{14}C]ATP were studied in the presence of saturation quantities of unlabelled ATP and ITP. 0.1 ml of bladder homogenate or boiled control was incubated with 25 μl of both [^{3}H]ITP and [^{14}C]ATP to which was added either unlabelled ATP or ITP (10 mM) or neither. Incubation was for 15 min at room temperature, as described in the Methods section, and the products were separated by 'over-running' chromatography using ethanol: 1.0 M ammonium acetate (6:1) as the solvent system. The results of three experiments are shown in table 2.

The probable reason for the small production of cyclic nucleotides from ATP compared with that from ITP was that the chemical concentration of [^{14}C]ATP as supplied is several hundred times less than that of [^{3}H]ITP. The difficulties of detection of small quantities of tritium did not permit dilution of the ITP.

Both cIMP and cAMP were formed from either substrate demonstrating that the reaction between the two was freely reversible. Considering the formation from ATP, the addition of excess unlabelled ATP increased the yield of both cAMP and cIMP, indicating that adenyl cyclase was below saturation at the lower level of ATP; when excess ITP was added cyclic nucleotide production fell, indicating that ITP can compete with ATP for adenyl cyclase (table 3).

The addition of excess ITP produced no change in the production of cyclic nucleotides, whilst addition of ATP gave a decrease in production, again indicating the ATP was competing with ITP for the cyclase.

These results provide evidence against the existence of an 'inosyl cyclase' as distinct from adenyl cyclase, but the possibility cannot be totally excluded as the requirements of such an enzyme are unknown.

Table 2
Formation of cAMP and cIMP from [^{14}C]ATP (pmoles/ml/mg protein).

Unlabelled substrate concentration (mM)		Products				cIMP/cAMP	
ATP	ITP	cIMP		cAMP			
0	0	0.58 0.46 0.74	0.59	0.89 0.95 0.81	0.88	0.66 0.46 0.91	0.68
10	0	14.2 30.0 41.0	28.4	16.7 38.3 45.0	33.3	0.85 0.78 0.91	0.85
0	10	0.10 0.08 0.11	0.10	0.12 0.12 0.11	0.12	0.84 0.67 1.00	0.84

4.5. *The effect of oxytocin on the production of cIMP and cAMP from ITP and ATP*

Bär et al. [12] have demonstrated a stimulation of toad bladder adenyl cyclase with oxytocin; for this reason their techniques of homogenisation and incubation were used. In two experiments 0.4 ml of bladder homogenate or boiled control warmed to 37°C, was incubated for 30 min with 0.01 ml of both [^{3}H]ITP and [^{14}C]ATP, with or without enough pure oxytocin to give a final concentration of 10^{-5}M. Separation of the products of incubations was by the technique of 'over-running' paper chromatography. Table 4 shows the results. Oxytocin at this concentration caused a stimulation of cyclic nucleotide production by 2–3 fold, which agrees with the results of Bär et al. [12] for the production of cAMP from ATP. It may also be noted that oxytocin had no apparent effect on the reaction between cAMP and cIMP, the ratio cIMP/cAMP remaining approximately constant.

Table 3
Formation of cIMP and cAMP from [^{3}]ITP.

Unlabelled substrate concentration (mM)		Products (nMoles/ml/mg protein)				cIMP/cAMP	
ATP	ITP	cIMP		cAMP			
0	0	1.46 0.59 0.71	0.92	2.11 0.93 0.93	1.32	0.69 0.64 0.77	0.70
10	0	0.46 0.18 0.22	0.29	0.46 0.16 0.24	0.29	1.00 1.12 0.92	1.01
0	10	1.32 0.86 1.46	1.21	1.26 1.00 1.16	1.14	1.05 0.86 1.26	1.06

Table 4
Effects of oxytocin on production of cIMP and cAMP from ITP or ATP.

Substrate	Oxytocin concentration (M)	Products (nMole/ml/mg protein)				cIMP/cAMP	
		cIMP		cAMP			
ITP	0	0.11 0.08	0.10	0.02 0.02	0.02	5.5 4.0	4.8
ITP	10^{-5}	0.20 0.25	0.23	0.07 0.06	0.07	3.0 4.1	3.6
Ratio $\frac{\text{hormone}}{\text{control}}$		2.3		3.5			
		(pMoles/ml/mg protein)					
ATP	0	1.62 1.23	1.43	0.33 0.35	0.34	4.9 3.5	4.2
ATP	10^{-5}	3.04 3.90	3.47	1.03 0.93	0.98	3.0 4.2	3.6
Ratio $\frac{\text{hormone}}{\text{control}}$		2.4		2.9			

5. Discussion

In several biochemical systems, e.g. lipase activation [13], cIMP has been shown to most closely resemble cAMP in potency. Although the present studies reveal no difference in potency between cAMP and cIMP when applied exogenously to intact toad bladder, care is needed in interpreting these results because: i) the relative rates of entry into the mucosal cells of the nucleotides are unknown; ii) the relative rates of destruction are unknown; iii) it cannot be assumed that the log dose–response lines are parallel and these experiments were done at one concentration only; and iv) the rates of interconversion of the two compounds are not accurately known. This has been demonstrated to be a reversible reaction in the present work, though it is not known whether the deaminase involved is specific for cyclic nucleotides; many deaminases have a degree of specificity. The relative subcellular dispositions of adenyl cyclase, cyclic deaminases and phosphodiesterases are unknown.

No evidence was found for the separate existence of 'inosyl cyclase' however, the stimulatory effect of oxytocin on the toad bladder adenyl cyclase demonstrated by Bär et al. [12] was confirmed, and shown also to occur with the conversion of ITP to cIMP. The deamination reactions were unaffected by oxytocin. These preliminary observations indicate only that cIMP mimics the effects of neurohypophysial hormones on toad bladder in the same way as cAMP. Enzymes exist which convert ATP and cAMP to the corresponding inosine compounds, though the physiological significance of these reactions is not yet clear. It is known however, that although the natriferic and hydro-osmotic actions of neurohypophysial hormones are thought to be mediated by cyclic nucleotides in the cell they are biochemically independent processes, and it seems possible that both cAMP and cIMP may be involved in this mediation.

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