Short communications

Increased concentrations of cyclic 3′,5′-adenosine monophosphate without a physiological response after antidiuretic hormone

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Treatment of the serosal surface of the isolated bladders of toads (*Bufo marinus*) with phospholipase C inhibited the hydro-osmotic response to ADH, but did not prevent the rise in cyclic AMP concentrations associated with hormone action.

Cuthbert & Painter (1971) recently reported that treatment of the serosal surface of frog or toad skin with phospholipase C inhibited the effect of antidiuretic hormone (ADH) on sodium transport and hydro-osmotic flow, without affecting the unstimulated values of these parameters. Since it is believed that ADH acts through cyclic 3′,5′-adenosine monophosphate (cAMP) (Orloff & Handler, 1967) we were interested in the concentrations of nucleotide after treatment of the tissue with phospholipase C. Our results show that enzyme treatment does not prevent the increase in cAMP concentrations after ADH, while the physiological response is inhibited.

Methods.—Experiments were performed on the urinary bladders of toads (*Bufo marinus*). The two lobes of each bladder were tied to separate glass cannulae, one bladder half serving as a control. The bladder sacs were filled with 3 ml of distilled water and then immersed in Ringer solution previously gassed with air. Various treatments of the serosal surfaces of the bladders were achieved by simply including the hormone or enzyme in the bathing solution. Net water flux across the bladder sacs was measured by weighing the sac, contents and cannula every 30 minutes. After the final weighing the bladder halves were quickly removed from the cannulae, lightly blotted, weighed and dropped into 5 ml ice cold 7% TCA. A little washed sand was added and the tissue ground. After centrifugation 2 ml of clear supernatant was taken, extracted 3 times with ether and dried in a vacuum desiccator containing P_2O_5, overnight. One millilitre of 50 mM sodium acetate/acetic acid buffer at pH 4 was added to the dried sample. These solutions were used for the assay of cAMP.

The assay system used for cAMP was that of Gilman (1970), based on the competition between cAMP and ^{3}HcAMP for binding sites in a protein kinase prepared from bovine muscle. The amount of ^{3}HcAMP bound to the protein was determined by filtration through cellulose ester filters (Millipore 0·45 μm). The filters were dissolved in cellusolve and the radioactivity counted in a liquid scintillation spectrometer. The binding reaction was carried out in a volume of 100 μl and at 0°C in sodium acetate/acetic acid buffer (50 mM, pH 4). The reaction mixture consisted of 50 μl of protein kinase plus inhibitor and 50 μl of standard or unknown solutions of cAMP plus ^{3}HcAMP (0·77 pmol/50 μl : 16·3 Ci/mmol). Measurements were made in duplicate and a standard curve was constructed in all experiments. Addition of cAMP to the extracts showed that recovery was almost complete.

The Ringer solution used had the following composition: (mM) NaCl, III; KCl, 1·9; $CaCl_2$, 1·08; NaH_2PO_4, 0·08; $NaHCO_3$, 2·4; glucose, 11·1. After aeration this solution had a pH of 7·6. The following reagents were used: cyclic 3′5′-adenosine monophosphate, A grade; ^{3}H-cyclic 3′5′-adenosine monophosphate, 16 Ci/mmol (Schwarz BioResearch); ADH (Pitressin, Parke Davis & Co); phospholipase C, Type I from *Cl. welchii*, 4 U/mg (Sigma).

Results.—The results of eighteen experiments are shown diagrammatically in Fig. 1. In all experiments toad bladders were used first to measure water flow and then for determination of cAMP. One bladder half always served as the control for the other bladder half of the same animal.

In the first six experiments the effects of ADH on hydro-osomotic flow and on cAMP concentrations were measured. Since the flow measurements took at least 30 min the concentrations of cAMP can be considered as the steady state concentrations of the nucleotide under various con-

ditions. ADH (50 mU/ml) increased water flow from (70±15 μl/bladder half)/30 min to (900±90 μl/bladder half)/30 min, these values being statistically different ($P<0.001$). At the same time the cAMP concentrations increased from 4·0±0·2 pmol/mg wet weight to 7·7±0·4 pmol/mg wet weight, these values also being statistically different ($P<0.001$) (Fig. 1a).

In a second set of six experiments the effects of phospholipase C (100 μg/ml) on non-stimulated water flow and cAMP concentrations were measured. The enzyme was applied to the serosal surface of the bladder sacs for 60 minutes. This treatment had no statistically significant effect on either water flow or cAMP concentrations (Fig. 1b).

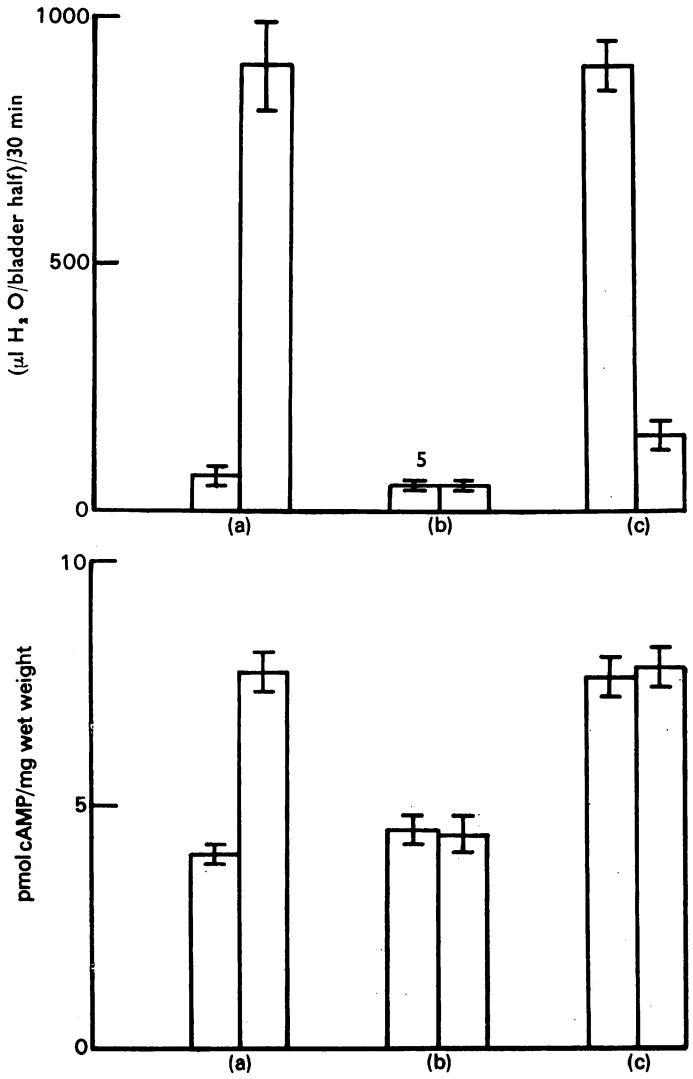

FIG. 1. Hydro-osmotic flow and cAMP concentrations in isolated urinary bladders of toads. The results for the control bladder halves are shown on the left of each pair of columns. Standard errors are shown. Each column represents experiments on six bladder halves except where indicated. (a) Test bladders were treated with ADH (50 mU/ml) for 30 minutes. (b) Test bladders were treated with phospholipase C (100 μg/ml) for 1 hour. (c) Test bladders were treated with phospholipase C (100 μg/ml) for 1 h, followed by ADH (50 mU/ml) for 30 minutes. Control bladders were immersed in Ringer for the same time but were treated with ADH only.

A final set of six experiments measured the effect of phospholipase C (100 μg/ml) on the response to ADH. Test bladders were incubated with the enzyme for 1 h and then transferred to ADH (50 mU/ml). With these conditions the response to ADH on water flow was very significantly reduced ($P<0.001$), while there was no difference in the cAMP concentrations in control and enzyme treated bladder sacs.

Discussion.—This work confirms for the toad bladder the results previously found for frog and toad skin (Cuthbert & Painter, 1971), that phospholipase C inhibits the response to ADH. What is surprising is that, after enzyme treatment, the hormone still causes the same increase in cAMP concentrations. It is reasonable to conclude that adenyl cyclase bound to the serosal membrane remains intact after phospholipase treatment.

There are two possible interpretations of the results. First phospholipase C may affect some receptor for cAMP, or alternatively, the hormone may cause other changes in the serosal membrane necessary for the expression of the response, and which are blocked by the enzyme. If the former interpretation is accepted then the cAMP receptor must be on the outer serosal surface, since it is unlikely that phospholipase C penetrates the cell. In any event it is unlikely that cAMP itself diffuses across the cell and is responsible directly for the change in water permeability of the mucosal face, as suggested by Orloff & Handler (1967), since in these experiments no response was seen even though the cAMP levels were increased. The alternative view that the hormone causes other effects on the serosal face, and that these are affected by phospholipolysis, seems more plausible. Rasmussen (1970) has discussed the possibility of concurrent or cascade second messengers and in particular has suggested the implication of calcium in hormone action. It could be that phospholipolysis removes essential Ca^{2+} binding groups, from which ADH normally displaces calcium. Thorn & Schwartz (1965) have shown that calcium binding by bladders is affected by ADH.

Presumably similar results would have been obtained if sodium transport had been measured rather than water flow, since it has been shown previously that phospholipase C inhibits the increase in short circuit current caused by ADH (Cuthbert & Painter, 1971).

The concentrations of cAMP found in this work were 3 times greater than those reported by Turtle & Kipnis (1967) and 10 times larger than those of Handler, Butcher, Sutherland & Orloff (1965). This may have been the result of the conditions used. The bladders were subject to a transepithelial osmotic gradient whereas in the work cited the bladders were incubated in Ringer solutions. Others have described results in which presumed changes in cAMP levels arose from physical forces (Ripoche, Parisi & Bourguet, 1969). In addition we used a TCA extraction procedure rather than one with HCl. In some preliminary experiments we measured cAMP concentrations in bladders which were not subject to an osmotic gradient and in which we used an HCl extraction procedure. The concentrations of cAMP found were in the range reported by Handler *et al.* (1965) (0.41 ± 0.02 pmol/mg wet weight in four determinations). In the experiments reported here we preferred not to use theophylline to inhibit phosphodiesterase; however, in a few experiments in which theophylline was used the results were qualitatively similar to those described.

Finally, a comment must be made about the specificity of the assay used for cAMP. The protein kinase was selected on its ability to bind cAMP, but Gilman (1970) showed that other nucleotides could also be bound but with lower affinities. Cyclic IMP and cGMP have the highest affinities next to cAMP. However, as Gilman points out, tissue concentrations of cGMP are generally too low to interfere. We have calculated that for our assay conditions if there were equal amounts of cIMP and cAMP present in the extracts the cAMP levels would be overestimated by about 12%. If equal amounts of cGMP and cAMP were present the error would only be 1%. It can be seen from Fig. 1 that even if such extreme amounts of other nucleotides were present the conclusions would be the same.

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