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# Role of cyclic AMP and angiotensin III in the response of toad skin to angiotensin II

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In previous works, it was demonstrated that angiotensin II ($AT_{II}$) produces a significant increase in short-circuit current (SCC) [1] and osmotic water permeability ($P_{osm}$) [2] in the isolated skin of the toad *Bufo arenarum*. Indirect evidence of the involvement of the cyclic AMP (cAMP) system in the hydrosmotic effect of angiotensin II in toad skin was also previously reported [3]. Most of the information about the role of adenylate cyclase [4] and cAMP [5] in response to vasopressin has been obtained using the toad bladder; considerable less data exist concerning amphibian skin [6]. Therefore, experiments were carried out to obtain direct evidence of the influence of $AT_{II}$ on cAMP levels in skin epithelia of the toad *B. arenarum* isolated with collagenase [7]. In addition, in search of a more potent agonist, the possibility of the conversion of $AT_{II}$ to angiotensin III ($AT_{III}$) in toad skin and the relative potency of both peptides were tested. It is known that in some tissues like the adrenal gland [8] $AT_{II}$ is converted to the heptapeptide $AT_{III}$ [(des-aspartic acid[1])-angiotensin II], which interacts with specific receptors, exhibiting greater potency in releasing aldosterone from the adrenal gland.

The effect of $AT_{II}$ on cAMP levels was measured in isolated epithelia from overnight hydrated toads incubated in aerated Ringer, pH 7.4, at room temperature (19–21°) for 5 and 15 min. Paired halves of epithelium were used, one serving as the control. The other was incubated in the presence of oxytocin, $AT_{II}$, 8-Leu-angiotensin II (8-Leu-$AT_{II}$), a competitive inhibitor of $AT_{II}$ in toad skin [9], or combinations thereof.

The incubation was terminated by treatment with 8% trichloroacetic acid. The tissue was immediately homogenized and centrifuged. The protein concentration was determined in an aliquot of the precipitate by the method of Lowry *et al.* [10] and the supernatant passed through columns of Dowex WX 100–200 mesh equilibrated with 0.1 M HCl [11]. The columns were washed with 2 ml of the same solution. Final elution was performed with bidistilled water, and after lyophilization the residue was dissolved in 0.2 ml of 0.05 M Tris-HCl buffer, pH 7.2. The final recovery was about 35–50 per cent. cAMP was measured in 20-$\mu$l aliquots from the same samples, using the method of Gilman [12]; results are expressed in pmoles/mg of protein. The effects of both angiotensins on SCC and $P_{osm}$ in the toad skin were measured by conventional techniques described elsewhere [9].

Table 1 shows that $10^{-8}$ M oxytocin increased cAMP levels significantly at 15 min. Likewise, $AT_{II}$ produced significant increases in cAMP levels in doses of $2.10^{-6}$ and $2.10^{-7}$ M (Tables 1 and 2). The effect was already significant at 5 min, in agreement with the time response curve in the whole skin [2]. Table 1 shows that 8-Leu-$AT_{II}$ had no effect *per se* on cAMP levels, and was able to prevent the effect of $AT_{II}$ (Table 2).

$AT_{II}$ ($10^{-6}$ M) added to the dermal side of the skin increased SCC by $3.2 \pm 1.0$ (S.E.) $\mu A \cdot cm^{-2}$ ($N = 8$, $P < 0.02$), whereas $AT_{III}$ in the paired half increased it by $2.4 \pm 0.9$ ($P < 0.05$). The effects were of nearly the same magnitude (6.6 and 5.6 per cent of basal level, respectively) and did not differ significantly.

In order to test a possible effect of $AT_{III}$ on the sodium outer barrier, given its greater affinity for lipid interaction [13], the heptapeptide was added to the epidermal side, causing no effect on SCC.

Table 1. Effect of oxytocin ($10^{-8}$ M), angiotensin II ($2.10^{-6}$ M) and 8-Leu-angiotensin II ($2.10^{-6}$ M) on cyclic AMP levels (pmoles/mg protein) in isolated toad skin epithelium*

| Hormone | Incubation time (min) | N | Control | Experimental | Difference | P |
|---|---|---|---|---|---|---|
| Oxytocin | 15 | 6 | 2.12 ± 0.52 | 4.24 ± 1.28 | 2.12 ± 0.80 | < 0.05 |
| Angiotensin II | 5 | 6 | 2.25 ± 0.17 | 3.27 ± 0.26 | 1.02 ± 0.16 | < 0.01 |
| | 15 | 7 | 1.37 ± 0.37 | 3.57 ± 0.95 | 2.20 ± 0.83 | < 0.05 |
| 8-Leu-angiotensin II | 15 | 6 | 4.00 ± 1.39 | 4.62 ± 0.94 | 0.62 ± 1.17 | NS |

* Experiments were performed at room temperature in winter for oxytocin and angiotensin II and in spring for 8-Leu angiotensin II. Results are expressed as mean ± S.E., NS = not significant.

Table 2. Inhibitory effect of 8-Leu-angiotensin II ($2.10^{-6}$ M) on the effect of angiotensin II on cyclic AMP levels in toad skin epithelium ($2.10^{-7}$ M)*

| Hormone | Incubation time (min) | N | Control | Experimental | Difference | P |
|---|---|---|---|---|---|---|
| Angiotensin II | 15 | 13 | 3.32 ± 0.60 | 4.70 ± 0.67 | 1.38 ± 0.31 | < 0.001 |
| Angiotensin II + 8-Leu-angiotensin II 5 min before | 15 | 10 | 2.59 ± 0.39 | 2.49 ± 0.30 | 0.10 ± 0.44 | NS |

* Experiments were performed in spring at room temperature. NS = not significant.

$P_{osm}$ was significantly increased by both peptides in doses of $4.10^{-8}$ M added to the dermal side of the skin. The $AT_{II}$-treated skin showed an increase of $15.0 \pm 2.6\ mg \cdot cm^{-2} \cdot hr^{-1}$ ($N = 10$, $P < 0.001$), whereas with $AT_{III}$ the paired half registered an increase of $4.3 \pm 1.1$ ($P < 0.001$). Whereas the weight loss of the paired sacs in the control period was almost identical ($10.4 \pm 0.9$ vs $10.6 \pm 0.8\ mg \cdot cm^{-2} \cdot hr^{-1}$), the response to both peptides differed widely ($P < 0.01$). The time course of the $P_{osm}$ response exhibited a similar profile for both peptides with a peak at 10 min after adding the hormone to the dermal side, $AT_{III}$ showing the lower peak. Experiments were performed using 8-Leu-$AT_{II}$ in order to test if it could also inhibit the effect of $AT_{III}$ on $P_{osm}$. They were performed in winter, since the inhibitory properties of the compound showed seasonal changes. When added in a dose 10-fold higher, the inhibitor reduced the effects of these peptides from $11.7 \pm 2.9$ to $2.4 \pm 1.0\ mg \cdot cm^{-2} \cdot hr^{-1}$ (delta $9.3 \pm 2.7$, $N = 11$, $P < 0.01$) for $4.10^{-8}$ M $AT_{II}$, and from $6.5 \pm 0.9$ to $2.2 \pm 1.1\ mg \cdot cm^{-2} \cdot hr^{-1}$ (delta $4.3 \pm 0.9$, $N = 8$, $P < 0.01$) for a similar dose of $AT_{III}$.

Present data demonstrate that $AT_{II}$ effects in toad skin are not mediated by conversion to $AT_{III}$, this compound being an agonist of lower potency in this tissue. Since both peptides are inhibited by the same competitive inhibitor, the existence of specific receptors for the heptapeptide is ruled out. These data would indicate that receptors present in toad skin are more avid for the octapeptide, as is the case for smooth muscle, rather than for the heptapeptide, as in the adrenal glomerulosa [13].

The role of cAMP in the response of toad skin to $AT_{II}$ is supported by present evidence which confirms previous results obtained by an indirect pharmacological approach. cAMP would be the common agent for hormones having an effect on this parameter in amphibian skin (neurohypophyseal peptides and epinephrine [6]). In addition, the effects of $AT_{II}$, vasopressin and epinephrine on water permeability in toad skin are inhibited by demethylchlortetracycline, an inhibitor of adenylate cyclase and protein kinase [14].

The effects of $AT_{II}$ in other systems would be mediated by cAMP either increasing cAMP levels, as in isolated neurohypophysis [15], or decreasing it, as in the rat aorta and tail artery [16]. However, since no effects of $AT_{II}$ on the cAMP system could be observed in rat kidney cortex slices or colon mucosa [17], mammalian renal adenylate cyclase [18] or rat uterus [19], the physiological significance of the present data remains to be settled.

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