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STIMULATORY EFFECT OF ANGIOTENSIN II ON THE ELECTRIC PROPERTIES OF THE ISOLATED TOAD SKIN

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Abstract—In 1982 we showed that angiotensin II (Agt II) stimulates the bioelectric properties of the isolated toad skin and that this effect is blocked by pretreatment of the skin with indomethacine [J. B. Concha *et al.*, *IRCS Med. Sci.* **10**, 584 (1982)]. Ussing's technique and several inhibitors were used to continue this study on the isolated *Pleurodema thaul* skin. Serosal Agt II produced a dose-dependent increase in electrical parameters: a maximal concentration of 6×10^{-6} M Agt II increased potential difference by 43 ± 7.8% and short-circuit current by 51.5 ± 7.7%. The responses were not affected by either alpha or beta blockers or by atropine. Indomethacine blocked responses to the calcium ionophore A23187 and to Agt II which were similar to each other. Additive effects of Agt II and of the calcium ionophore A23187 were found. No response to Agt II was obtained when Ca^{2+}-free Ringer was used on the serosal side. Calcium channel blockers (nifedipine, verapamil, manganese), pentobarbitone and saralasin blocked the response to Agt II. This pharmacological evidence is in favour of the hypothesis that Agt II activates specific membrane receptors, leading to Ca^{2+} release and formation of prostaglandins which stimulate adenyl cyclase. This increases cAMP secretion, which in turn increases apical membrane permeability to sodium and enhances the active transport system.

The isolated toad skin has been used extensively as a biological model to obtain insight into the mechanism of action of chemical messengers, neurotransmitters, natural and synthetic polypeptides, and numerous drugs.

Many workers have established that the polypeptide angiotensin II (Agt II) is a vascular smooth muscle constrictor which increases the rate of aldosterone secretion [1] in the adrenal cortex. However, there are few reports of a direct effect on sodium transport in amphibian skin and toad bladder epithelium; with the exception of the works of Barbour *et al.* [2] and McAfee and Locke [3] with Agt II amide, our available information comes from the investigations carried out by Coviello and his group of research workers in Argentina [4–6] using synthetic Agt II (Val^5-hypertensin 1-amide).

The effects of Agt II on sodium transport in the whole organism are mediated by the release of aldosterone. Leaf and Bumpus [7], Coviello and Crabbé [4] and Barbour *et al.* [2] reported that Agt II had no direct effect on sodium transport in the isolated toad bladder or toad skin. Later, Coviello *et al.* [8] reported direct stimulation of sodium and water reabsorption in the isolated toad kidney, confirmed in the whole toad in a subsequent work [9]. McAfee and Locke [3] showed that 2 μg/kg Agt II added to the medium bathing the inner (serosal) surface of the isolated *Rana pipiens* skin, increases short-circuit current (SCC) and net ^{22}Na mucosa-to-serosa flux. Similar results were described by Concha *et al.* [10] using (Asp^1-Phe^8) angiotensin II.

Khairallah and Page [11] suggested that the action of the synthetic octapeptide Agt II is mediated by the release of acetylcholine. On the other hand, Feldberg and Lewis [12] and Krasney *et al.* [13] proposed that Agt II (Hypertensin Ciba) promotes catecholamine release.

The present study was undertaken to explore the action of Agt II on the electric properties of the isolated skin of the Chilean toad *Pleurodema thaul* and to examine the effects of antagonists and activators of the toad skin response to Agt II.

Part of this work has been published in a short communication [10].

MATERIALS AND METHODS

The abdominal skin dissected from decapitated and pithed *P. thaul* (5–15 g) toads, kept in tap water 24 hr prior to use, was carefully washed in toad Ringer's solution and mounted between modified lucite Ussing chambers. The composition of the solution was (mM): NaCl, 113; KCl, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 2.3; glucose, 11.0; and phosphate buffered to pH 7.4. The reservoirs on both sides of the skin (exposed surface 0.70 cm^2) were filled with 3 ml of toad Ringer's solution, and constant aeration of the bathing medium was provided. In some experiments Ca^{2+} was omitted from the solution bathing the inner surface of the skin and 0.5 mM ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA) was added. The potential difference (PD) was recorded continuously on a 2-channel Cole–Parmer recorder with non-polarizable calomel electrodes and agar-Ringer bridges. The SCC was measured every 2–5 min through Ag-AgCl wire electrodes connected to the microammeter of a voltage-clamp circuit according to Ussing and Zerahn [14].

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Table 1. Stimulatory effect of increasing concentrations of angiotensin II (Agt II, inner surface) on the electric properties of the isolated toad skin

Angiotensin II	% Increase in PD	% Increase in SCC
1×10^{-6} M	15.00 ± 2.20*	17.50 ± 2.00†
3×10^{-6} M	33.00 ± 2.70‡	39.50 ± 3.80‡
6×10^{-6} M	43.00 ± 7.80‡	51.50 ± 7.70‡

PD = potential difference; SCC = short-circuit current. Results (means ± SEM, N = 7) are expressed as percent increase in basal values. Basal values: PD, 36.40 ± 3.44 mV; SCC, 42.80 ± 3.94 $\mu A/cm^{-2}$.

*–‡ Significantly different from basal values (Student's paired *t*-test): *$P < 0.05$; †$P < 0.01$, and ‡$P < 0.001$.

The following drugs were used: (Asp^1-Phe^8) angiotensin II, indomethacine, dibenzyline, propranolol, atropine, (Sar^1-Ala^8) angiotensin II, calcium ionophore A23187, manganese chloride, pentobarbitone (all from the Sigma Chemical Co.) and verapamil (Knoll Lab.). The organic solvents used in the solubilization of several drugs were ethanol and dimethylsulfoxide in final concentrations of 1.8 and 2.3×10^{-4} M respectively. Aliquots of these solvents were always tested before the drugs were used.

Statistical treatment was performed by means of Student's *t*-test for paired data.

RESULTS

Effect of Agt II on electrical parameters of the isolated toad skin

The electrical response of the skin to Agt II (inner surface) was a transient increase in PD and SCC. No effect was obtained on application of the drug to the mucosal (outer) surface. Since we worked on skins of varying sensitivity, we did not find it easy to construct dose–response curves; however, Table 1 shows such an effect for seven skins. Concentrations of Agt II smaller than 1×10^{-6} M did not evoke response in skins and concentrations larger than 6×10^{-6} M were followed by decreasing responses. Figure 1 illustrates the repetitive effect of 3×10^{-6} M Agt II on the PD and SCC of the isolated skin of *P. thaul*: the maximal effect was reached in 10.3 ± 0.5 min [12] and declined to basal values in 56.0 ± 4.8 min [12] when Agt II was not washed out.

Effects of several agents on the toad skin response to Agt II

Calcium ionophore (A23187) and indomethacine. Exposure of the inner surface of the skin to the calcium ionophore A23187, which increases PD and SCC [15–17], induced an effect similar to that of Agt II. Pretreatment of the inner surface of the skins with indomethacine blocked the responses to A23187 and to Agt II (Table 2). Furthermore, additive effects of both drugs were found: 3×10^{-6} M Agt II and 1×10^{-6} M A23187 given successively in five skins increased SCC from 24.7 ± 1.53 to 37.87 ± 1.81 $\mu A/cm^2$ and to 48.9 ± 1.62 $\mu A/cm^2$ respectively ($P < 0.001$).

Calcium-free Ringer and Ca^{2+} channel blockers. Table 3 shows that the skin response to Agt II was blocked when the normal Ringer's solution bathing the inner surface was replaced by Ca^{2+}-free Ringer's solution and that Ca^{2+} channel blockers such as nifedipine, manganese chloride, pentobarbitone and verapamil inhibited the action of Agt II. It has been shown [18] that pentobarbitone inhibits the effect of A23187 in the toad bladder exposed to normal Ca^{2+} Ringer's solution.

Adrenoceptor and muscarinic blockers. The effect of Agt II was not modified by pretreatment with alpha or beta adrenoceptor blockers (1×10^{-5} M dibenzyline or 1×10^{-5} M propranolol) or by pre-

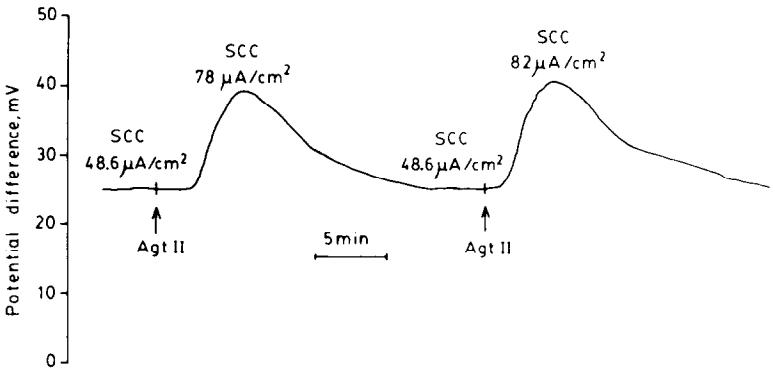

Fig. 1. Repetitive effect of 3×10^{-6} angiotensin II (Agt II, inner surface) on the electric properties of the toad skin. SCC: short-circuit current.

Table 2. Comparative effects of angiotensin II (Agt II) and of calcium ionophore (A23187), inner surface, on the potential difference (PD) and on the short-circuit current (SCC) of the isolated toad skin, before and after application of indomethacine to the inner surface

	PD (mV)	SCC ($\mu A/cm^2$)
Angiotensin II (10^{-6} M)		
Control	34.5 ± 5.0	87.5 ± 14.0
Agt II before	55.0 ± 7.0*	138.0 ± 12.0*
Agt II after indomethacine (10^{-5} M)	28.5 ± 4.0	79.0 ± 10.0
Calcium ionophore A23187 (10^{-6} M)		
Control	30.5 ± 4.5	25.2 ± 4.0
A23187 before	40.0 ± 5.5*	38.5 ± 4.3*
A23187 after indomethacine (10^{-5} M)	31.5 ± 5.0†	30.0 ± 3.0†

Values are means ± SEM, N = 6.
* Significantly different from control (Student's paired *t*-test): $P < 0.05$.
† Not significant.

treatment with 1×10^{-4} M atropine (inner surface, N = 8).

Saralasin. Since, as in other tissues, it seems likely that Agt II acts via specific receptors, skins were pretreated with (Sar^1-Ala^8) Agt II, an inhibitor which binds to Agt II receptors, 15 min before the addition of Agt II. Table 4 shows the blocking effect of this antagonist on the response to Agt II in six skins.

DISCUSSION

Early investigations on Agt II led many authors to consider that the increase in sodium transport across renal tubular epithelium was due to enhancement in the rate of secretion of aldosterone [1]. Although Leaf and Bumpus [7] and Coviello and Crabbé [4] found no response to Agt II in toad bladder or toad skin, respectively, McAfee and Locke [3] demonstrated that Agt II stimulates sodium transport across isolated frog skin and that this effect is not mediated by acetylcholine, epinephrine or norepinephrine as suggested by other authors [11–13]. In 1970, Whittembury and Proverbio [19], working on guinea pig kidneys, postulated the existence of a sodium pump refractory to ouabain, sensitive to ethacrynic acid, potassium independent, and specifically stimulated by Agt II. Munday *et al.* [20] demonstrated effects of Agt II in an electrogenic pump (potassium independent). In contrast, Coviello *et al.* [21] reported that in the isolated toad kidney potassium-free Ringer solution suppresses the effect of Agt II in sodium but not in water reabsorption, suggesting an effect of Agt II in a potassium-dependent sodium pump in amphibians and different sites of action for the effects of Agt II in sodium and water transport.

Parsons and Munday [22] set up the hypothesis that Agt II, in common with other polypetides, releases cAMP, although exogenous cAMP, dibutyryl cAMP and theophyllines are ineffective in renal tubular cells and do not potentiate the response to Agt II.

Cuthbert and Wilson [23] suggested that the transport-stimulating effect of acetylcholine on *Rana*

Table 3. Inhibitory effects of Ca^{2+}-free Ringer and of Ca^{2+} channel blockers on the isolated toad skin response to a maximal concentration of angiotensin II (Agt II, 3×10^{-6} M), inner surface

	% Change in PD		% Change in SCC	
Agent	Control: increase after Agt II	Experimental: decreased Agt II effect after inhibitor	Control: increase after Agt II	Experimental: decreased Agt II effect after inhibitor
Ca^{2+}-free Ringer	46.7 ± 14.0 (32.5 ± 6.4)	88.1 ± 2.5	39.8 ± 13.8 (30.4 ± 5.2)	84.6 ± 4.5
Nifedipine, 10^{-4} M	11.1 ± 5.9 (29.8 ± 4.9)	88.2 ± 0.8	15.9 ± 4.2 (27.6 ± 3.8)	92.5 ± 0.9
$MnCl_2$, 2×10^{-3} M	35.6 ± 5.9 (38.2 ± 6.4)	91.7 ± 6.0	60.9 ± 8.5 (36.7 ± 7.7)	99.8 ± 5.5
Pentobarbitone, 1×10^{-4} M	36.7 ± 3.2 (28.8 ± 4.4)	74.3 ± 3.4	54.6 ± 9.9 (31.7 ± 4.9)	76.4 ± 6.2
Verapamil, 5×10^{-5} M	15.6 ± 3.3 (34.6 ± 5.2)	84.4 ± 2.8	22.8 ± 4.1 (35.1 ± 6.2)	84.4 ± 3.4

Values are means ± SEM, N = 5. PD = potential difference; SCC = short-circuit current. Basal values, expressed in mV (PD) and in $\mu A/cm^2$ (SCC), are indicated in parentheses.

Table 4. Effect of 3×10^{-6} M (Sar^1-Ala^8) angiotensin II (saralasin) on the isolated toad skin response to 3×10^{-6} M angiotensin II (Agt II), inner surface

	PD (mV)		SCC ($\mu A/cm^2$)	
	Control	Agt II	Control	Agt II
Before saralasin	28.0 ± 2.5	44.0 ± 4.0*	19.0 ± 2.3	33.5 ± 2.5*
After saralasin	31.0 ± 3.5	36.0 ± 4.0†	24.5 ± 2.5	26.0 ± 3.0†

Values are means ± SEM, N = 6.
* Significantly different from control (Student's paired *t*-test): $P < 0.01$.
† Not significant.

temporaria skin is due to prostaglandin release. Their hypothesis is based on the fact that indomethacine and mepacrine inhibit prostaglandin synthesis and that both agents block the effect of acetylcholine. Hall *et al.* [24] have shown that PgE_1 increases cAMP levels in *R. temporaria* skin and that this increase precedes the rise in SCC across the skin.

Although species specificity in anurans seems to be suggested in this discussion, further studies are necessary to clarify this point. This work, based on pharmacological evidence, suggests that Agt II releases endogenous prostaglandins from *P. thaul* skin; activation of adenylate cyclase then increases formation of cAMP, leading to a rise in sodium transport, PD and SCC. The transient nature of the response may be due to inactivation of Agt II by proteolytic enzymes. The finding that indomethacine blocks the response to Agt II is in agreement with the possibility of prostaglandin release [23]. The similarity of the effects of A23187 and Agt II could be due to an Agt II receptor-mediated increase in Ca^{2+} permeability since additive effects between Agt II and the ionophore were found. The rise in intracellular Ca^{2+} could then activate phospholipase A_2 leading to the formation of arachidonic acid and subsequent production of prostaglandins, a process described in other structures [24]. Indomethacine inhibits cyclooxygenase and thereby blocks the response to Agt II, since prostaglandins cannot be generated. Evidence in favour of this hypothesis is the finding that Agt II was inactive when normal Ringer's solution was replaced by Ca^{2+}-free Ringer's solution in the inner bathing medium. In addition, Ca^{2+} channel blockers such as nifedipine and verapamil blocked the effect of Agt II. Manganese and pentobarbitone, also Ca^{2+} channel blockers, suppressed the response to Agt II and the effects of A23187 as shown by Wiesman *et al.* [15] in the toad bladder. Thus, it seems likely that both A23187 and Agt II increase Ca^{2+} permeability and release prostaglandins. Moreover, indomethacine also blocked the stimulatory effect of A23187.

If ligand Agt II finds its receptors already bound to the antagonist saralasin, inhibition of the response to Agt II is to be expected.

In summary, we propose that Agt II binds to special receptors on the basolateral membranes of transporting toad skin epithelial cells. This reaction opens up Ca^{2+} channels, initiating prostaglandin synthesis [25–27]; adenylate cyclase is activated and increases intracellular cAMP content. The result is an increase in sodium permeability of the apical membrane; the rise in intracellular sodium will increase the work rate of the sodium pump and thus stimulate sodium transport.

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