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POSSIBLE MECHANISMS OF ACTION OF ANGIOTENSIN II ON HIGH RESISTANCE EPITHELIA

Mecanismos posibles de la acción de la Angiotensina II sobre epitelios de alta resistencia

J.B. CONCHA[1], B.C. NORRIS, G.M. CONTRERAS AND C.S. GONZALEZ*

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. Ussing's technique and evaluation of the electric equivalents of sodium transport 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 $3x10^{-6}$M increased potential difference by 88.7 ± 19.6% and short-circuit current by 103.1 ± 17.2%. The responses were not affected by either alpha or beta blockers or by atropine. Agt II also increased the driving force for sodium (E_{Na}), sodium conductance (G_{Na}) and decreased passive conductance (G_{sh}). Indomethacine blocked responses to calcium ionophore A 23187 and to Agt II wich were similar to each other. 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.

These results favour the hypothesis that Agt II activates specific membrane receptors, leading to Ca^{2+} release, activation of phospholipase A_2 and formation of prostaglandins which stimulate adenyl cyclase. This increases cAMP secretion, which in turn activates protein kinases; phosphorylation of apical membrane proteins then increases permeability to sodium and enhances the active transport system.

RESUMEN

En 1982 demostramos que angiotensina II (Agt II) estimula las propiedades bioeléctricas de la piel aislada de sapo y que este efecto es bloqueado mediante tratamiento previo de la piel con indometacina. Para continuar este estudio en la piel aislada de *Pleurodema thaul*, se usaron la técnica de Ussing y la evaluación de los parámetros eléctricos equivalentes de transporte de sodio.Agt II serosal produjo un aumento dosis-dependiente de los parámetros eléctricos: una concentración máxima de $3x10^{-6}$ M aumentó la diferencia de potencial en 88.7 ± 19.6% y corriente de corto-circuito en 103.1 ± 17.2%. Las respuestas no fueron afectadas por bloqueadores alfa ni beta, ni por atropina. Agt II también aumentó la fuerza impulsora de sodio (E_{Na}), conductancia del sodio (G_{Na}) y disminuyó conductancia pasiva (G_{sh}). Indometacina bloqueó respuestas al ionóforo de calcio A 23187 y a Agt II que fueron similares entre sí. No se obtuvo respuesta a Agt II cuando se usó Ringer sin calcio en el lado serosal. Bloqueado-

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res de canales de calcio (nifedipina, verapamil, manganeso), pentobarbital y saralasin bloquearon la respuesta a Agt II.

Estos resultados apoyan la hipótesis que Agt II activa receptores de membrana específicos, llevando a liberación de Ca^{2+}, activación de fosfolipasa A_2 y formación de prostaglandinas que estimulan adenil ciclasa. Esto aumenta la secreción de AMP cíclico, que a su vez activa protein kinasas; la fosforilación de proteínas de la membrana apical aumenta la permeabilidad al sodio y estimula el sistema de transporte activo.

KEYWORDS: Angiotensin. Toad skin. Resistance epithelia. Sodium transport.

INTRODUCTION

The isolated toad skin has been extensively used 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 and that it increases the rate of aldosterone secretion (Page and Bumpus, 1961) 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, 1964 and McAffee and Locke, 1967 our available information comes from the investigations carried out by Coviello and his group in Argentina (Coviello et al., 1965, 1972a 1972b, 1974, 1975, 1976, 1978).

The effects of Agt II on sodium transport in the whole organism are mediated by the release of aldosterone, which increases transport through a complex mechanism whereby the steroid initiates a rise in mRNA synthesis at nuclear level. The induced mRNA stimulates protein synthesis and the outcome is an increase in passive permeability of the apical cell membrane to sodium (Rossier, B.C., 1978). The increased intracellular sodium stimulates the pump responsible for the active extrusion of sodium.

Leaf and Bumpus, 1960, Coviello and Crabbé, 1965 and Barbour et al. 1964 reported that Agt II had no direct effect on sodium transport in the isolated toad bladder or toad skin. Later, Coviello et al. (1972a, 1972b, 1973, 1974 and 1975) confirmed stimulation of active sodium transport in the skin, bladder and isolated kidney of several amphibians. Proto et al., 1983 showed that Agt II obtained from different species of batrachians increased the short-circuit current in skins of South American frogs. McAffee and Locke, 1967 showed that 2μg/mL. Agt II added to the medium bathing the inner (serosal) surface of isolated *Rana pipiens* skin, increased short-circuit current (SCC) and net ^{22}Na mucosa-to-serosa flux. Similar results were described by Concha et al., 1982.

Khairallah and Page, 1961 suggested that the action of Agt II is mediated by the release of acetylcholine. On the other hand, Feldberg and Lewis, 1964, and Krasney et al., 1966 proposed that Agt II promotes catecholamine release, thus stimulating sodium transport.

The present study was undertaken to explote the action of Agt II on the electric properties of the isolated skin of the Chilean toad *Pleurodema thaul* and to attempt to elucidate a possible mechanism of action applying Isaacson's amiloride test (Isaacson, 1977). This test provides information of changes in: the sodium potential (E_{Na}) of the sodium-potassium pump; sodium conductance (G_{Na}) and shunt conductance (G_{sh}) in addition to the changes observed by direct measurement of transepithelial potential difference (PD), short-circuit current (SCC) and total conductance of the system (G_T). The effects of antagonists, inhibitors and activators of the toad skin response to Agt II were also investigated.

Part of this work has been published in a short communication (17).

MATERIALS AND METHODS

The abdominal skin dissected from decapitated and pithed *Pleurodema thaul* toads 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 1.9; $CaCl_2$ 2.0; $NaHCO_3$ 2.3; glucose 11.0; and phosphate buffered to pH 7.4. The reservoirs on both sides of the skin were filled with 3 mL toad Ringer's solution and constant aeration of the bathing medium was provided. The 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 (21).

Isaacson's amiloride test was performed with a final concentration of $1x10^{-6}$M amiloride. Values of E_{Na}, G_{Na} and G_{sh} were obtained by graphical solution of the equation:

$$SCC/PD = SCC.1/E_{Na} + G_{sh} \quad (1)$$

Determination of G_{sh} and E_{Na} will give the value of G_{Na} since total skin conductance before amiloride treatment is known:

$$G_{Na} = G_T - G_{sh} \quad (2)$$

Amiloride was a gift from Merck, Sharp and Dohme. The following drugs were also used: angiotensin II, indomethacine, dibenzyline, propranolol, atropine, (Sar^1-Ala^8) angiotensin II, calcium ionophore A 23187, manganese chloride and pentobarbitone (Sigma Chemical Co.) and verapamil (Knoll Lab).

Statistical treatment was performed by means of Student's **t** test for paired data (Gray, 1961).

RESULTS

Effects of angiotensin II (Agt II) on electrical parameters of the isolated toad skin

Since we worked on skins of varying sensitivity, it was not easy to construct dose-response curves; however, Fig. 1-A shows these effects for 7 skins. Fig. 1-B illustrates the repetitive effects of $3x10^{-6}$M Agt II (inner surface) on the PD and SCC of the isolated skin of *Pleurodema thaul*. Agt II applied to the outer surface of the skin had no effect.

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

Amiloride. Fig. 2-A represents the changes in PD and in SCC on addition of $1x10^{-6}$M amiloride to the mucosal (outer) surface of the skin, before and after exposure of the inner surface to $3x10^{-6}$M Agt II. Isaacson's equation may be solved graphically from the numerical estimates of PD and SCC before and after the addition of Agt II in the presence of amiloride. Fig. 2-B shows that the slope of the line represents $1/E_{Na}$ and the intercept indicates conductance, when SCC is null. The value of this conductance represents the passive or shunt conductance (G_{sh}). Fig. 2-C illustrates the values of E_{Na}, G_{Na}, G_{sh} and G_T, before and after the effect of Agt II.

Indomethacine. When the skin was pre-treated with $5x10^{-5}$M indomethacine, the response to Agt II was blocked; the upper panel of Fig. 3 shows a representative experiment and the lower panel records the mean values for PD and SCC for 8 skins in the presence of Agt II, before and after exposure to indomethacine.

Adrenoceptor and muscarinic blockers The effect of Agt II was not modified by pre-treatment with alpha or beta adrenoceptor blockers ($1x10^{-5}$M dibenzyline or $1x10^{-5}$M propranolol) in 8 skins, or by pretreatment with $1x10^{-4}$M atropine in 8 skins.

Calcium and Agt II. Exposure of the inner surface of the skin to the calcium ionophore A 23187, which increases PD

and SCC (Wiesman, 1977; Nielsen, 1978; Erlij, 1981 and Balaban, 1979) induced a similar effect to that of Agt II (Fig. 4-A). The 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 (Fig. 1-B). Furthermore, Ca^{2+} channel blockers such as nifedipine, verapamil and manganese inhibit the effect of Agt II (Fig. 4-C, 4-D, 4-E). It has been shown (Wiesman, 1977) that pentobarbitone inhibits the effect of A 23187 in the toad bladder exposed to normal Ca^{2+}-Ringer's solution. Fig. 4-F depicts inhibition of the effect of Agt II by pentobarbitone. Fig. 5 shows that the responses to A 23187 are blocked by indomethacine.

Saralasin, Since, it seems likely that Agt II acts as in other tissues, via specific receptors, skins were pre-treated with (Sar^1-Ala^8) Agt II, an inhibitor which binds to Agt II receptors. Fig. 6 shows the blocking effect of this agent on the response to Agt II in 6 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 (Page, 1961). Although Leaf, 1960 and Coviello and Crabbé, 1966 found no response to Agt II in toad bladder or toad skin respectively, McAffee and Locke, 1967 demonstrated that Agt II stimulated transport across isolated frog skin and that this effect was not mediated by acetylcholine, epinephrine or norepinephrine as suggested by other authors (Khairallah, 1961; Feldberg 1964 and Krasney, 1966). In 1970 Whittembury and Proverbio, 1970 working on guinea pig kidneys, postulated the existance of a sodium pump refractory to ouabain, sensitive to ethacrynic acid, potassium-independent, and specifically stimulated by Agt II. There is a yet no evidence for the existence of such a pump in toad skin, and therefore the increase in sodium transport induced by Agt II is most likely due to a different mechanism of action.

Parsons and Munday, 1972 set up the hipothesis that Agt II, in common with other polypeptides, releases cAMP, although exogenous cAMP, dibutyryl cAMP and theophylline are ineffective in renal tubular cells and do not potentiate the response to Agt II. Coviello et al, 1975 presented evidence which supported the view that the effect of Agt II on the toad skin is mediated by the adenylate cyclase-cyclic AMP system.

Cuthbert and Wilson, 1981 suggested that the transport-stimulating effect of acetylcholine on *Rana 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., 1976 have shown that PgE_1 increases cAMP levels in *Rana temporaria* skin and that this increase preceeds the rise in SCC across the skin.

Our results suggest that Agt II releases endogenous prostaglandins from *Pleurodema thaul* skin; activation of adenylate cyclase then increases formation of cAMP leading to a rise in sodium transport, PD and SCC. Furthermore, sodium potential (E_{Na}) and active conductance (G_{Na}) are enhanced. The finding that indomethacine blocks the response to Agt II is in agreement with the possibility of prostaglandin release (Concha et al, 1982). The similarity of the effects of A 23187 and Agt II could be due to an Agt II receptor-mediated increase in Ca^{2+} permeability. The rise in intracellular Ca^{2+} then activates phospholipase A_2, leading to formation of arachidonic acid which is converted via cyclooxygenase to prostaglandins. This process has been described in other structures (Catt and Dufan, 1981). 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 is inacti-

ve when normal Ringer's solution is replaced by Ca^{2+} free Ringer's solution in the inner bathing medium. In addition, Ca^{2+} channel blockers such as nifedipine and verapamil block the effect of Agt II. Manganese and pentobarbitone, also Ca^{2+} channel blockers, suppress the response to Agt II and the effects of A 23187 as shown in the toad bladder by Wiesman et al., 1977. Thus it seems likely that both A 23187 and Agt II increase Ca^{2+} permeability and release prostaglandins. Moreover, indomethacine also blocks the stimulatory effect of A 23187.

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 (Rasmussen, 1986a and 1986b); adenylate cyclase is activated and increases intracellular cAMP content (Fig. 7). The result is an increase in sodium permeability of the apical membrane due to activation of specific protein kinases by cAMP and phosphorylation of apical membrane proteins. The rise in intracellular sodium will increase the work rate of the sodium pump and thus stimulates sodium transport.

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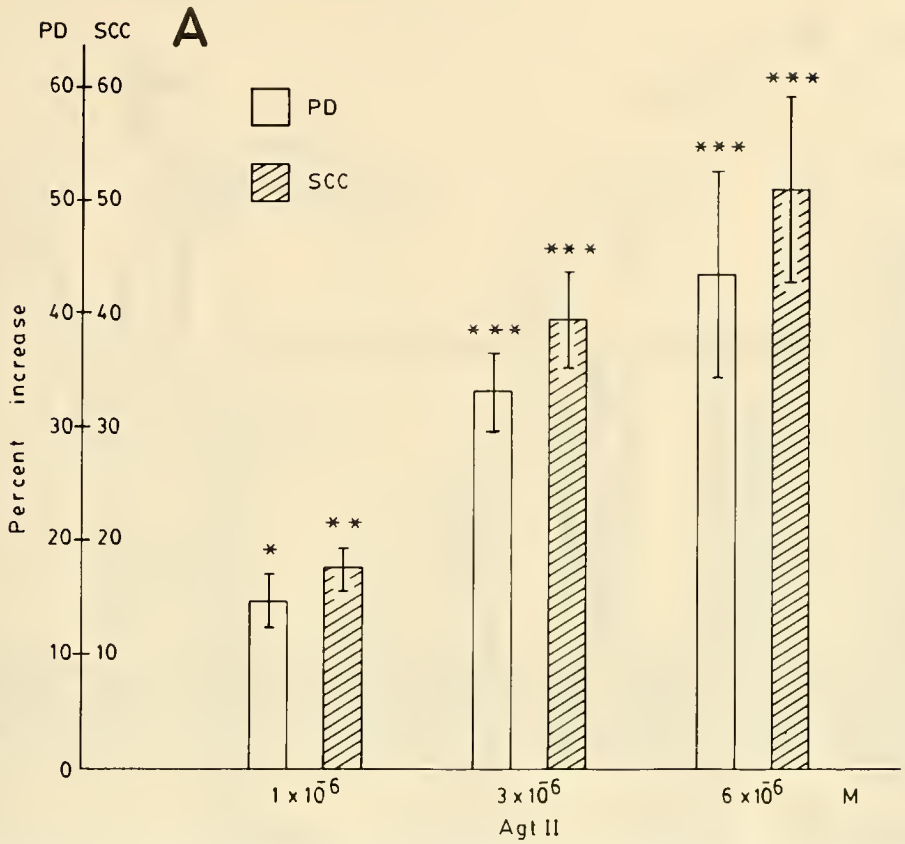


Fig. 1-A. Effect of increasing concentrations of angiotensin II (Agt II) applied in the solution bathing the inner surface, on the electrical properties of the toad skin. PD = potential difference; SCC = short-circuit current. Values are means ± SEM; n = 7. Results are expressed as percent increase in control values. These were: 36.4 ± 3.44 mV and 42.8 ± 3.94 $\mu A/cm^2$. Significance of difference from basal: P* <0.05; ** <0.01; *** <0.001.

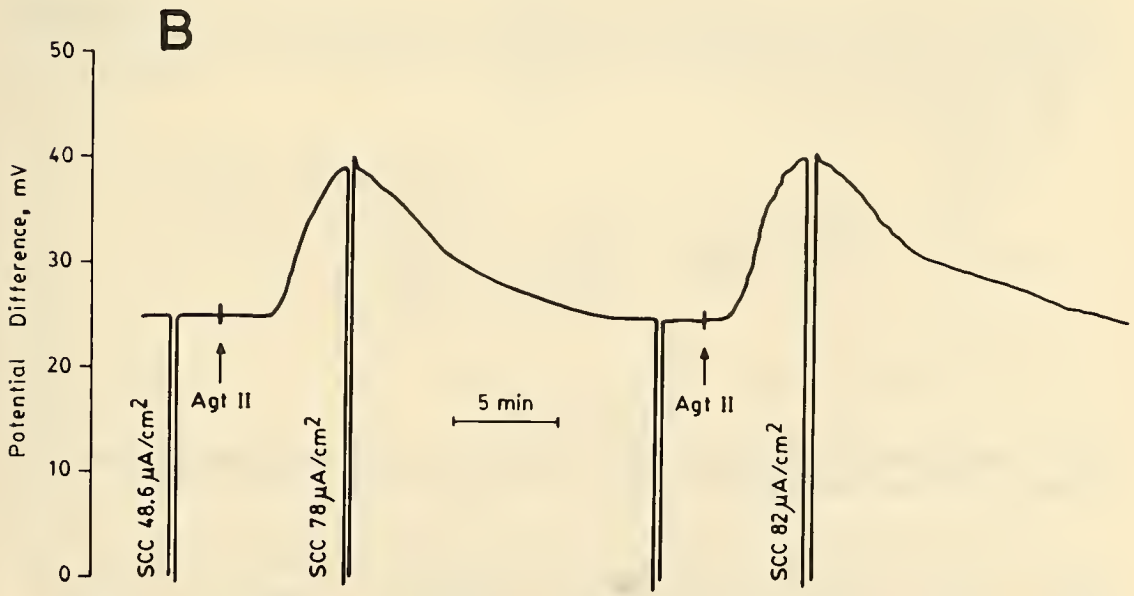


Fig. 1-B.- Repetitive effect of $3x10^{-6}$M Agt II (inner surface) on the electric properties of the toad skin.

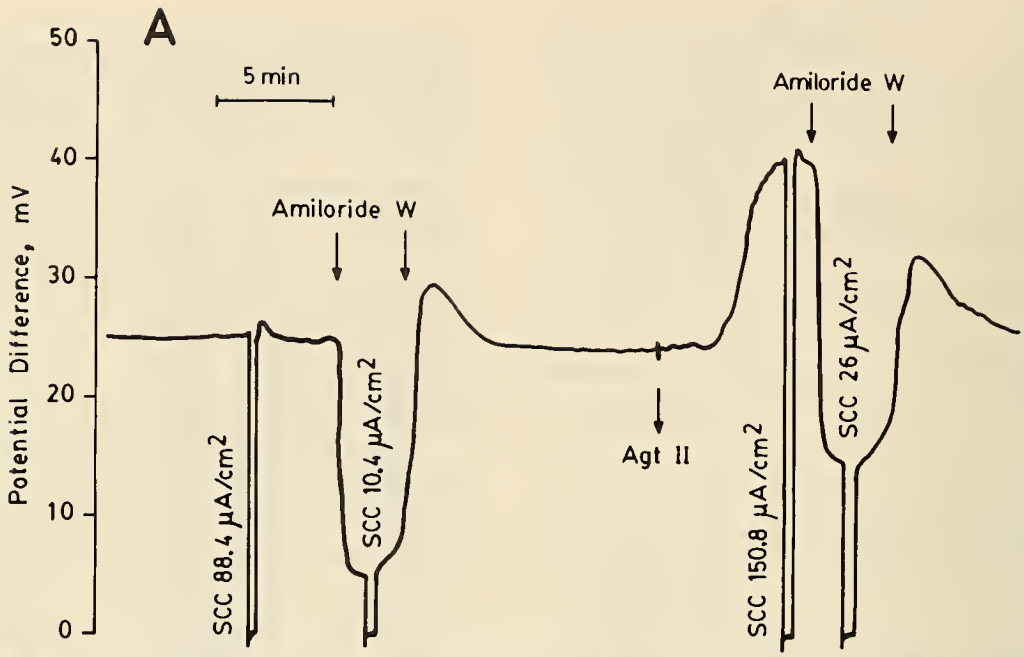
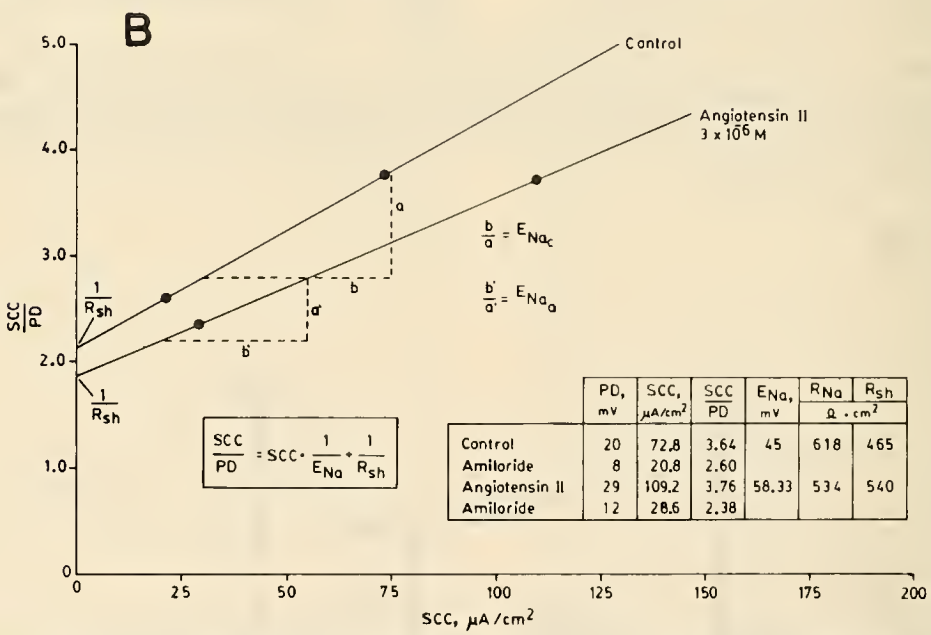


Fig. 2-A. Changes in the electric properties of isolated toad skin after addition of $1x10^{-6}$M amiloride to the outer surface, before and after exposure of the inner surface to $3x10^{-6}$M Angiotensin II. PD = potential difference SCC = short-circuit current. Agt II = angiotensin II. W = 3-fold washout of the inner and outer solutions.



	PD, mV	SCC, µA/cm²	SCC/PD	E_{Na}, mV	R_{Na} Ω·cm²	R_{sh} Ω·cm²
Control	20	72.8	3.64	45	618	465
Amiloride	8	20.8	2.60			
Angiotensin II	29	109.2	3.76	58.33	534	540
Amiloride	12	28.6	2.38			

Fig. 2-B.- Relation between conductance (SCC/PD) and SCC in a toad skin before and after addition of (control) $1x10^{-6}$M amiloride to the outer surface and of angiotensin II to the inner surface.

The values shown for E_{Na} (sodium potential) and R_{sh} (passive conductance) have been derived from the slope and intercept, respectively, of the line drawn through the two points obtained. E_{Na_c} and E_{Na_a} = values of the sodium potential under control conditions and after exposure to Agt II respectively.

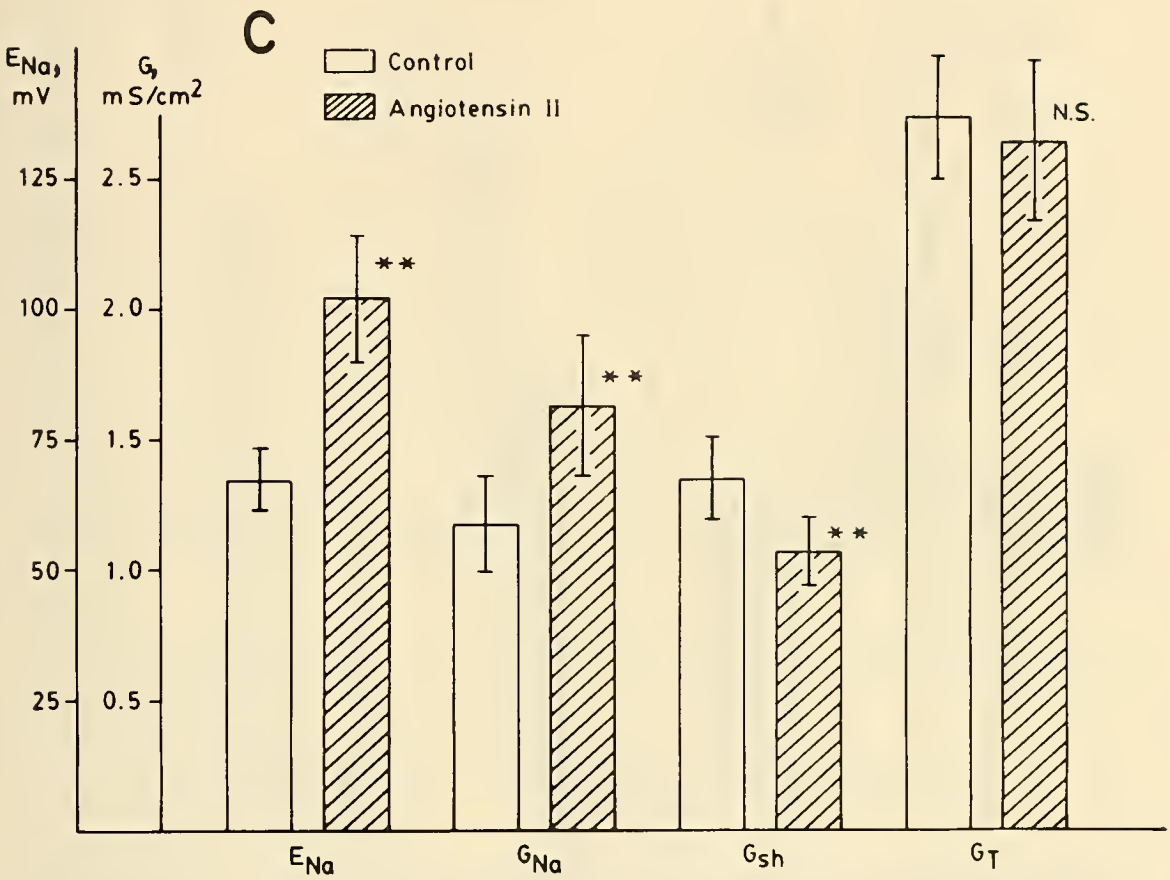


Fig. 2-C.- Effects if Agt II ($3x10^{-6}$M, inner surface) on the isolated toad skin electrical equivalent circuit parameters. E_{Na}= sodium potential; G_{Na}= sodium conductance; G_{sh} = passive conductance; G_T= total conductance. Values are means ± SEM; n = 7. P* < 0.05;** <0.01; NS = not significant.

Fig. 3.- Upper panel.-

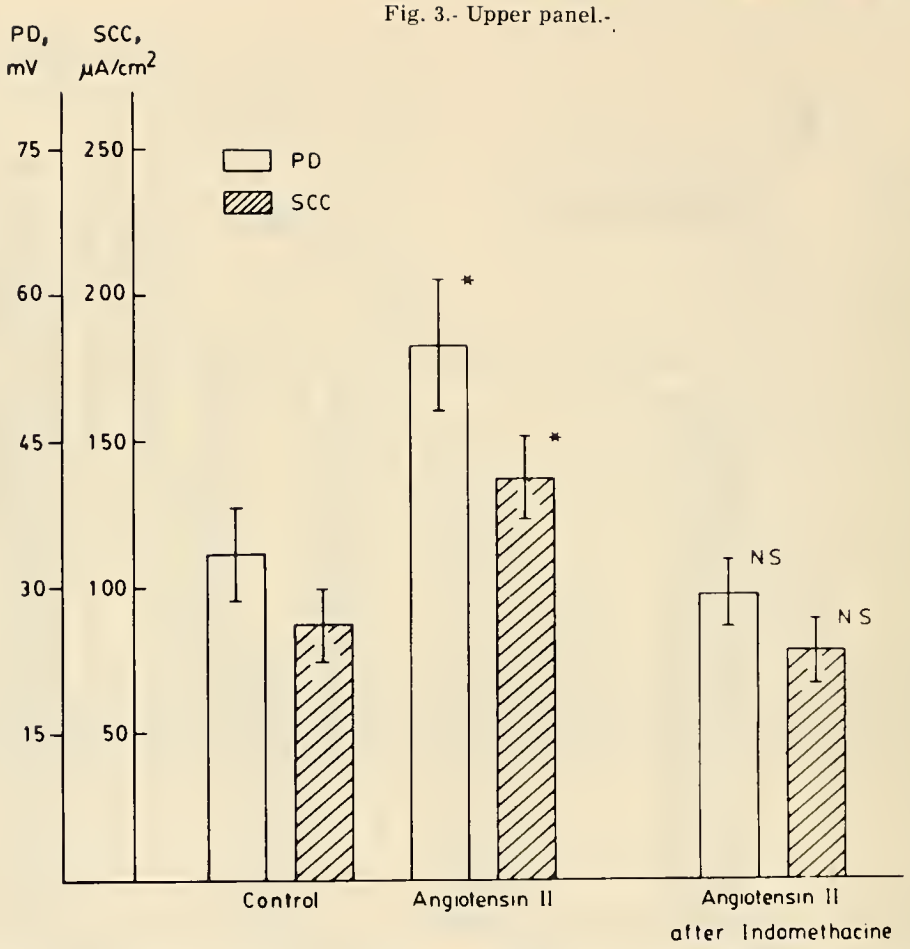
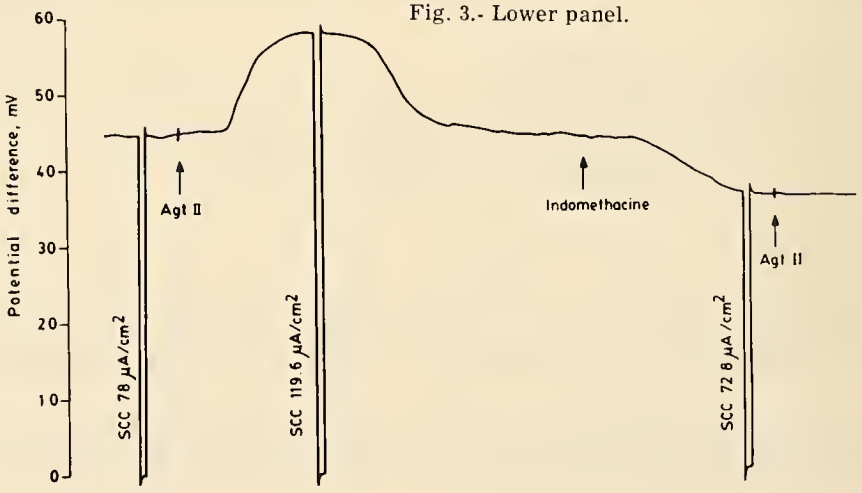


Fig. 3. The blocking effect of $5x10^{-5}$M indomethacine (inner surface) on the isolated toad skin response to $3x10^{-6}$M angiotensin II (Agt II). PD = potential difference; SCC = short-circuit current. The upper panel shows a representative experiment. Statistical results are summarized in the lower panel. Values are means ±SEM. In contrast with control skins, $P^* < 0.01$; NS = not significant.

Fig. 3.- Lower panel.



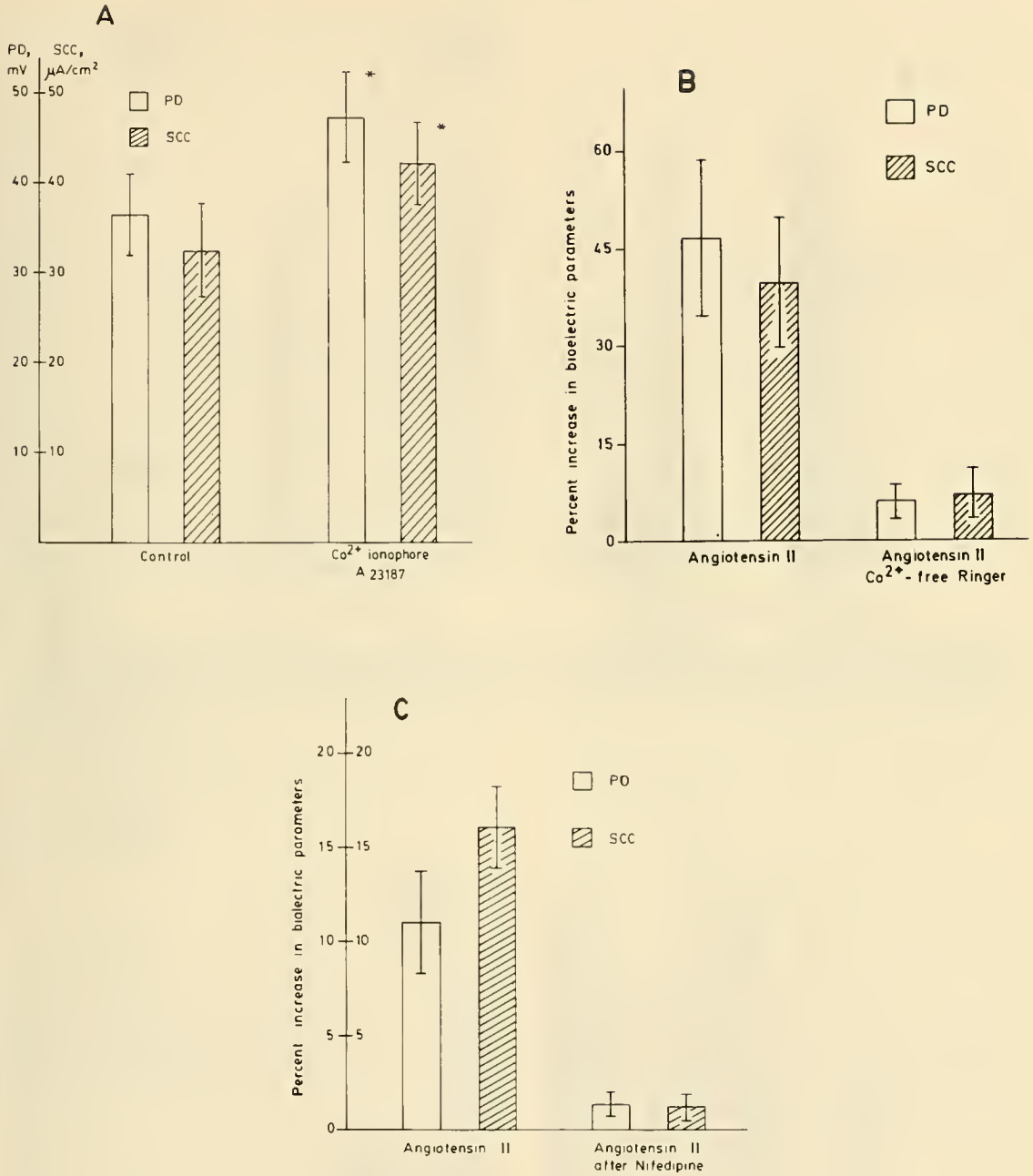


Fig. 4. Effects of the calcium ionophore A 23187, of Ca^{2+}-free Ringer's solution, and of several calcium channel blockers on the isolated toad skin response to $3x10^{-6}$M angiotensin II (Agt II, inner surface). PD = potential difference; SSC = short-circuit current; A 4 µM A 23187. **B** to **F**, results are expressed as percent increase in control values. **B**, Ca^{2+}-free Ringer's solution (basal 32.5 ± 4.6 mV, 30.4 ± 5.2 µA/cm^2); **C**, $1x10^{-4}$M nifedipine (basal 29.8 ± 4.9 mV, 27.6 ± 3.8 µA/cm2).

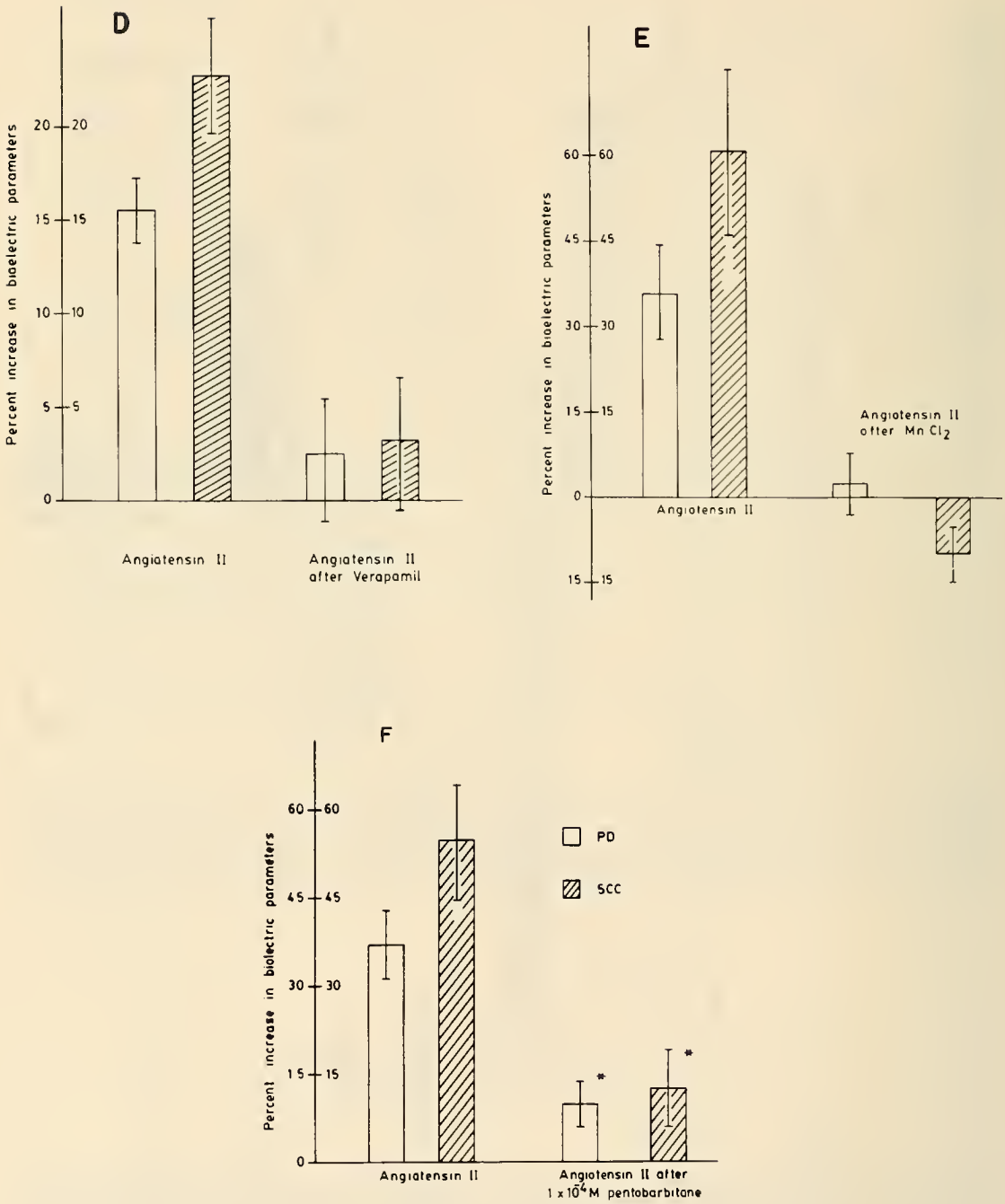


Fig. 4. (Continuation). **D**, 5×10^{-5}M verapamil (basal 34.6 ± 5.2 mV, 35.1 ± 6.2 μ A/cm^2); **E**, 1×10^{-2} manganese chloride (basal 38.2 ± 6.4 mV, 36.7 ± 7.7 μ A/cm^2); **F**, 1×10^{-4}M pentobarbitone (basal 28.8 ± 4.4 mV, 31.7 ± 4.9 μ A/cm^2). Values are means ± **SEM**; n=5. Significance of difference from basal $P^* < 0.01$.

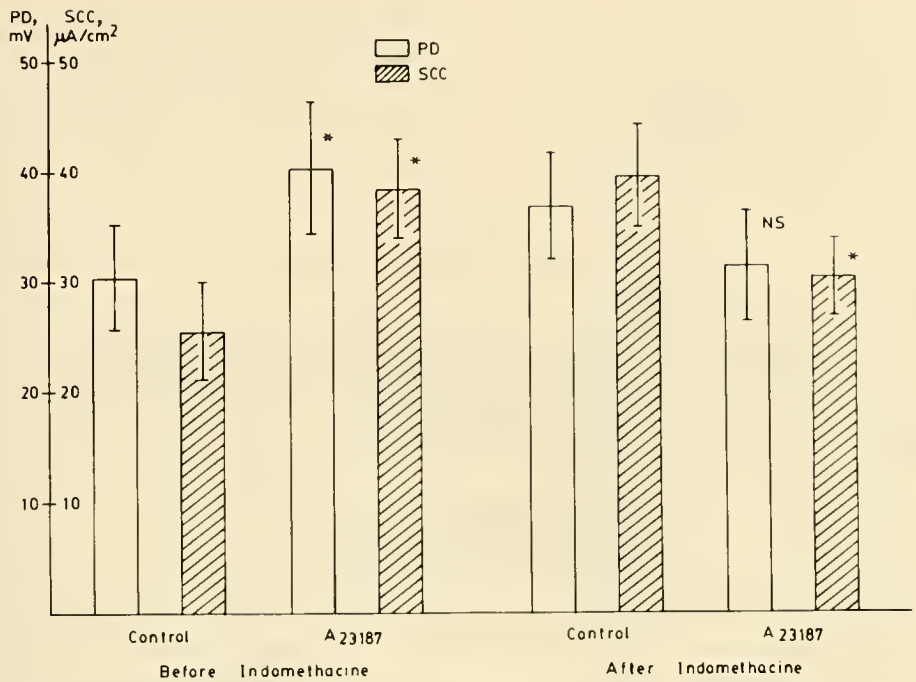


Fig. 5. Effect of $5x10^{-5}$M indomethacine on the isolated toad skin response to A 23187 µM, inner surface. PD = potential difference; SCC = short-circuit current. Values are means ± SEM; n = 6. P* < 0.05; NS = not significant.

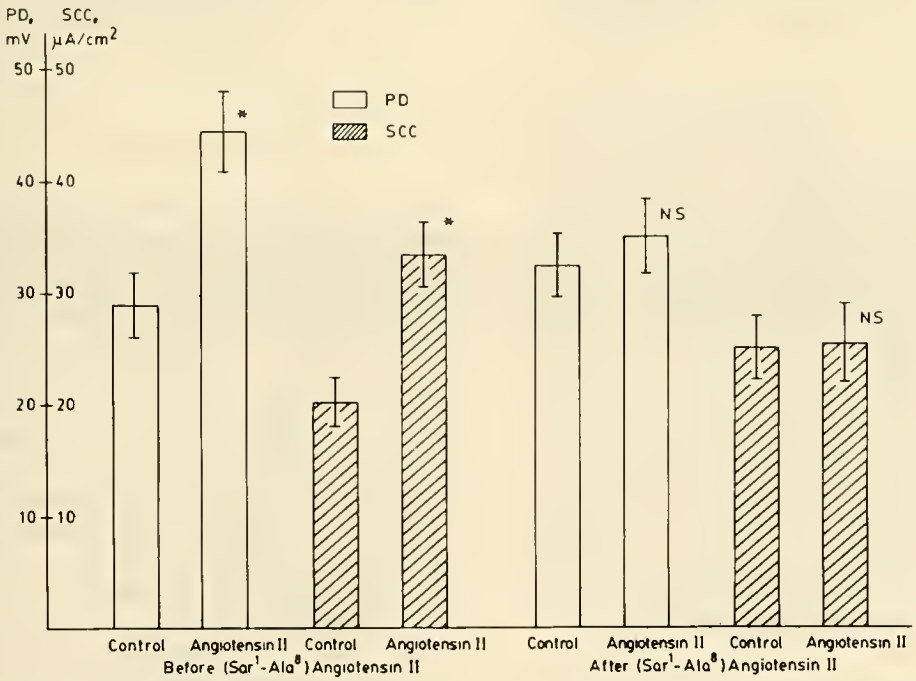


Fig. 6. Effect of $3x10^{-6}$M (Sar^1-Ala^8) angiotensin II, on the isolated toad skin response to $3x10^{-6}$M Agt II, inner surface. PD = potential difference; SCC = short-circuit current. Values are means ± SEM; n = 6, P* < 0.05; NS = not significant.

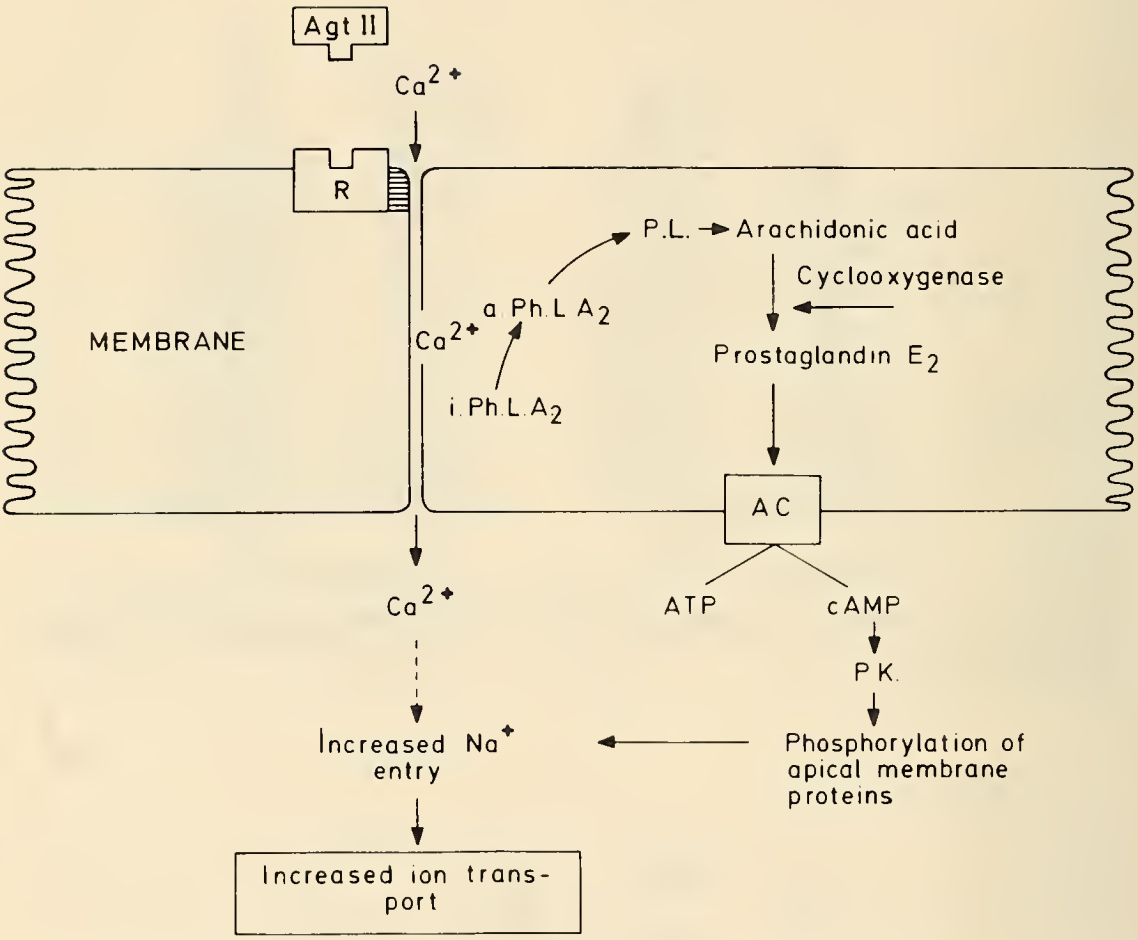


Fig. 7. Proposed model explaining the mechanism of Agt II action on sodium transport in the epithelial cells of amphibian skin. Agt II binds to specific receptors (R) on the basolateral membrane, increasing the intracellular cAMP content. The end result is stimulation of the sodium pump. aPh.L.A_2 = active phosphorylase A_2; iPh.L.A_2 = inactive phosphorylase A_2; P.L. = phospholipids; AC = adenylate cyclase. PK = protein kinases. Further details in text.