J. Membrane Biol. 97, 9–19 (1987)

The Journal of
Membrane Biology

Prostaglandin E_2-Stimulated Glandular Ion and Water Secretion in Isolated Frog Skin (*Rana esculenta*)

Henning F. Bjerregaard and Robert Nielsen
Institute of Biological Chemistry A, University of Copenhagen, DK-2100 Copenhagen 0, Denmark

Summary. Prostaglandins are known to stimulate the active sodium absorption in frog skin. In this paper it is shown that prostaglandin E_2 (PGE_2) stimulates an active secretion of Cl^-, Na^+, and K^+ from the skin glands in *Rana esculenta*. The active Cl secretion is enhanced more than the Na and K secretion. Therefore, in skins where the Na absorption is inhibited by amiloride, the addition of PGE_2 results in an increase in the short-circuit current (SCC). The PGE_2-stimulated Cl secretion could be inhibited by the presence of ouabain or furosemide in the basolateral solution or diphenylamine-2-carboxylate in the apical solution. The PGE_2-stimulated Cl secretion was enhanced by the phosphodiesterase inhibitor, theophylline, indicating that the effect of PGE_2 was caused by an increase in the intracellular cAMP level in the gland cells. The calcium ionophore A23187, which increases the PGE_2 synthesis in frog skin, stimulated the glandular Cl secretion. This secretion could be blocked by the prostaglandin synthesis inhibitor indomethacin, indicating that A23187 acts by increasing the prostaglandin synthesis and not by a direct action of Ca^{2+} ions *per se*. The net water flow (J_w) and the Cl secretion were measured simultaneously under the conditions outlined above. The stimulation, inhibition, and the time-course of the outward-directed J_w were similar to the change observed for the Cl secretion. These results show that PGE_2 stimulates a glandular secretion of Cl and water in frog skin, probably by increasing the cAMP level in the gland cells.

Key Words frog skin glands · prostaglandin E_2 · Cl secretion · water flow

Introduction

It is well established that prostaglandins stimulate the short-circuit current in frog skin. Several reports have shown that this increase in SCC is due to active transepithelial sodium transport (Barry & Hall, 1969; Fassina et al., 1969; Barry et al., 1975; Gerencser, 1978).

PGE_2 has no effect on the Cl^-, K^+, and Ca^{2+} influx and efflux in *Rana temporaria* (Lote et al., 1974). However, we found in *Rana esculenta* that the PGE_2-stimulated SCC could not be accounted for by active sodium transport (Bjerregaard & Nielsen, 1986*a*). The present work was therefore undertaken in order to examine the ionic nature of PGE_2-stimulated SCC.

The results presented in this paper show that prostaglandin E_2, besides stimulating a sodium absorption through the epithelial cells, also stimulates a secretion of salt and water from the glands of the frog skin.

Materials and Methods

The experiments were performed on male and female frogs (*Rana esculenta*), which were kept at room temperature with free access to water; they were fed twice a week with meal worms.

SCC MEASUREMENTS

The isolated abdominal skins were divided into two symmetrical halves so each skin served as its own control. The skins were mounted in Perspex® chambers (area: π cm^2) and bathed in stirred Cl^- Ringer's solution consisting of (mM): Na^+ 115, K^+ 2.5, Ca^{2+} 1, HCO_3^- 2.5, and Cl^- 117, pH = 8.2.

The epithelia were isolated, as described by Johnsen and Nielsen (1982), after a 3-hr incubation of the skins in 0.3 mg collagenase/ml at room temperature. The isolated epithelia were mounted in Perspex chambers (area: 1.5 cm^2) in a modified Ringer's solution (mM) Na^+ 115, K^+ 2.5, Ca^{2+} 1, Mg^{2+} 1, Cl^- 118, CO_3^{2-} 2.5, PO_4^{3-} 1, glucose 5, pH = 7.8.

SCC was measured according to the technique of Ussing and Zerahn (1951), using an automatic voltage clamp that compensated for the current-dependent potential drop between the potential-measuring electrodes. In order to monitor the skin resistance, the skin was clamped at ± 10 mV (5-sec each) every 2 min. The clamp pulses were kept at this low value in order to minimize secondary potential-dependent effects on the transport parameter.

FLUX MEASUREMENTS

In ion flux experiments, one skin half was used for influx measurements and the other for efflux measurements. $^{22}Na^+$, $^{36}Cl^-$, and $^{42}K^+$ were added to the solution, bathing one side of the

Table 1. Comparison of ^{22}Na net fluxes and SCC after stimulation with PGE_2[a]

Period (min)	Incubation	Influx	Efflux	Net flux	SCC
		(n equivalents · min^{-1} · cm^{-2})			
0–30	Control	4.05 ± 0.59	0.57 ± 0.21	3.48 ± 0.62	3.86 ± 0.52
30–60	Control	3.53 ± 0.34	0.53 ± 0.25	3.00 ± 0.40	3.48 ± 0.37
60–90	PGE_2	5.02 ± 0.79	2.41 ± 0.74	2.61 ± 0.48	6.11 ± 1.00[b]
90–120	PGE_2	3.85 ± 0.38	2.99 ± 0.79	0.87 ± 0.80	5.50 ± 0.80[c]
120–150	PGE_2 + amiloride	1.30 ± 0.50	3.25 ± 0.84	−1.95 ± 1.06	2.08 ± 0.40[c]
150–180	PGE_2 + amiloride	1.49 ± 0.33	2.90 ± 0.73	−1.42 ± 0.81	2.01 ± 0.39[c]

[a] Influx and efflux of ^{22}Na was measured in paired frog skin halves for periods of 30 min. After two control periods, 2 μM PGE_2 were added to the inside solution. After another two periods, 0.1 mM amiloride was added to the outside solution. Values are mean ± SE, $n = 4$.

[b] $P < 0.05$.

[c] $P < 0.01$; compared with corresponding net flux.

skins. After a 20-min equilibration period, a 1-ml aliquot was withdrawn from the other side and replaced with fresh solution. The last procedure was repeated with 30-min intervals throughout the experiment, under conditions where the bathing solutions were continuously stirred.

In experiments where the fluxes of Na^+, Cl^-, and K^+ were measured simultaneously, the activity of Cl^- was separated from that of Na^+ and K^+ precipitating chloride with 1 M $AgNO_3$. After centrifugation at 4000 rpm in a table top centrifuge, the supernatant was assayed for $^{42}K^+$ by Cerenkov counting. $^{22}Na^+$ activity was assayed by liquid scintillation after the decay of $^{42}K^+$. The chloride precipitate was dissolved in 1 ml of 0.5 M NaSCN. After centrifugation, the $^{36}Cl^-$ activity in the supernatant was counted by liquid scintillation.

WATER FLOW

SCC and water flow (J_w) were measured simultaneously under conditions where the skin was bathed with isotonic Ringer's solution on both sides. J_w was measured as described by Johnsen and Nielsen (1980) by an improved technique which allowed recordings of J_w with 1-min intervals. In short, the principle is that the outside of the skin is pressed against a stainless steel net with a pressure of 2 cm H_2O. The half-chamber (area: 8 cm^2) containing the outside bathing solution is closed except for an outlet consisting of a capillary tube. The outside bathing solution is allowed to flow into the capillary tube, whereby the light transmission of the tube changes markedly. The transmission is recorded by a detector consisting of a light-emitting diode and a photosensitive transistor. The signal from the detector controls a motor-driven syringe which appropriately adjusted the outside volume in order to keep the position of the meniscus constant. The motor also drives a precision potentiometer which is used as a potential divider, allowing the position of the syringe to be recorded continuously on a pen recorder.

DATA ANALYSIS

The results are expressed as means ± SE. Student's t-test for paired data was used for statistical analysis. A P value of < 0.05 was considered significant.

MATERIALS

PGE_2, indomethacin (both dissolved in ethanol), furosemide, ouabain, and theophylline were obtained from Sigma; A23187 from Calbiochem; collagenase (C. histolyticum), and N-2-0-dibutyryl-cAMP from Boehringer, and diphenylamine-2-carboxylate from Merck. $^{42}K^+$ and $^{36}Cl^-$ were obtained from the Danish Energy Commission, isotope division, Risø and $^{22}Na^+$ from Amersham.

Results

Addition of PGE_2 (2 μM) to the basolateral solution of the frog skin resulted in an increase in the SCC and in a decrease in net Na^+ flux across the skin (Table 1). This was due to the Na^+ efflux, which was enhanced more than the Na^+ influx. Addition of amiloride (0.1 mM) to the apical solution of PGE_2-treated skins resulted in a decrease in Na^+ influx, but the PGE_2-stimulated Na^+ efflux was not affected, so the transepithelial Na^+ flux changed from a net inward- to a net outward-directed Na transport, but SCC did not change direction.

Thus, an active transport of other ions besides Na must take place under these conditions. In order to identify this ion, the effect of PGE_2 was investigated in skins where normal inward-directed Na transport was inhibited by amiloride.

EFFECT OF PGE_2 IN AMILORIDE-TREATED SKINS

The time-course of SCC and transepithelial resistance is shown in Fig. 1. In four skins addition of amiloride resulted in a decrease in SCC from 17.5 ± 2.7 to 0.7 ± 0.3 μA/cm^2. Addition of PGE_2 resulted in a biphasic stimulation of SCC. The SCC reached a maximum level of 6.9 ± 0.5 μA/cm^2 5 to 10 min after addition of PGE_2, and a second maximum of

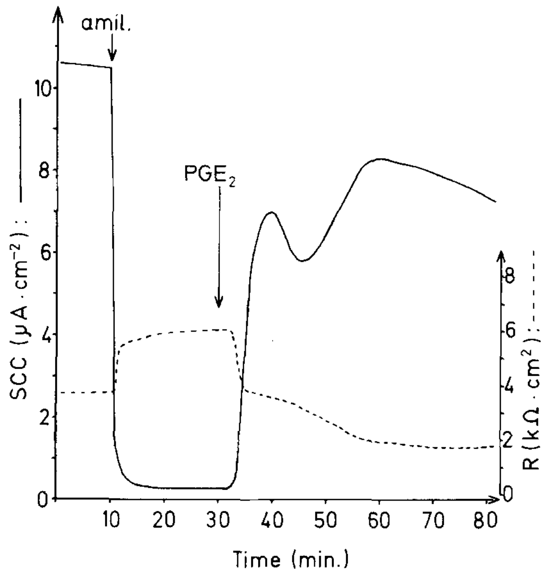

Fig. 1. Time-course of PGE_2-induced change in SCC and resistance in amiloride-treated skin. After addition of 0.1 mM amiloride to the apical solution, 2 μM PGE_2 was added to the basolateral solution. Transepithelial resistance: (--------) SCC: (———)

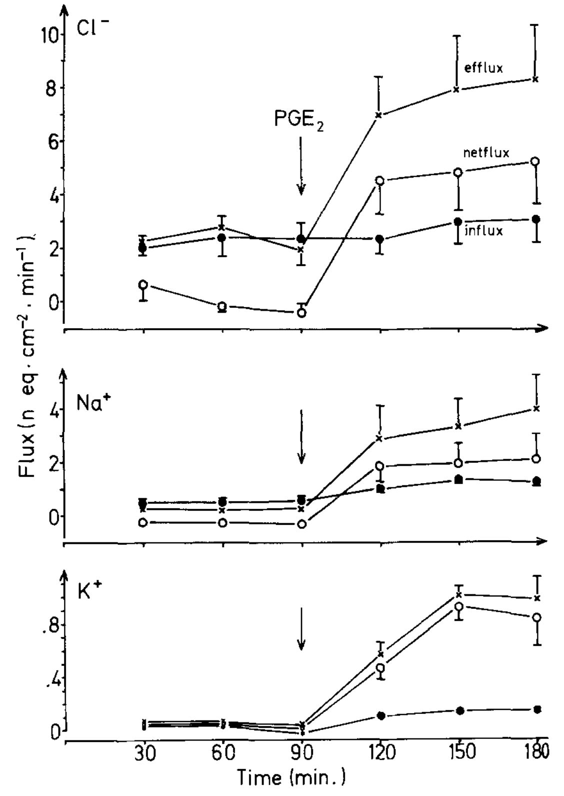

Fig. 2. Effect of PGE_2 on influxes and effluxes of $^{36}Cl^-$, $^{22}Na^+$, and $^{42}K^+$ in amiloride-treated skin. Influx and efflux experiments were carried out simultaneously on symmetrically divided skin halves and performed as described in Materials and Methods. 0.1 mM amiloride was added to the apical solution. After three control periods of 30 min, 2 μM PGE_2 was added to the basolateral solution and the fluxes were measured again in three periods of 30 min. Values are mean + SE; $n = 4$. efflux: (x______x); netflux; (O______O); influx: (●______●);

6.3 ± 0.8 μA/cm^2 was reached after 20 to 30 min. The PGE_2-stimulated SCC in amiloride-treated skin could be maintained for hours.

The time-course of transepithelial resistance change was similar to that observed for SCC (Fig. 1). Addition of amiloride increased the resistance from 2.6 ± 0.1 to 6.7 ± 1.0 kΩ cm^2. After addition of PGE_2 the resistance decreased to 3.1 ± 0.8 kΩ cm^2 ($n = 4$).

PGE_2-STIMULATED Cl^-, K^+, AND Na^+ EFFLUXES

In an attempt to identify the ions responsible for the PGE_2-stimulated SCC, we simultaneously measured the net fluxes of $^{36}Cl^-$, $^{42}K^+$, and $^{22}Na^+$. Figure 2 shows that addition of PGE_2 (2 μM) resulted in large increases in the effluxes of Cl^-, K^+, and Na^+, whereas the influxes of these ions were hardly affected. Because of the large stimulation of the efflux relative to the influx, the net outfluxes of Cl^-, K^+, and Na^+ increased from −0.5 ± 0.4 to 5.6 ± 1.9, −0.03 ± 0.02 to 0.87 ± 0.29 and −0.3 ± 0.1 to 2.7 ± 1.1 neq · min^{-1} · cm^{-2} ($n = 4$), respectively.

The SCC was compared to the total net fluxes of Cl^-, K^+, and Na^+ in these experiments (Table 2). Addition of PGE_2 to amiloride-treated skin resulted in an increase in SCC of 2.12 ± 0.39 neq · min^{-1} · cm^{-2}. In the same period the total net flux of Cl^-, K^+, and Na^+ was increased by 2.20 ± 0.62 neq · min^{-1} · cm^{-2}. These data were not significantly different, demonstrating that the PGE_2-stimulated increase in SCC could be accounted for by the total net fluxes of Cl^-, K^+, and Na^+. Moreover, the results show that the PGE_2-stimulated increase in SCC in amiloride-treated skins was caused by an increase in the chloride efflux.

PGE_2-STIMULATED SCC FROM GLANDS

The PGE_2-stimulated Cl secretion could originate from the glands in the frog skin (as is suggested for the β-adrenergic stimulation of chloride secretion in frog skin (Koefoed-Johnsen et al., 1952; Tomlinson & Wood, 1978; Thompson & Mills, 1983). One way to disclose a PGE_2-stimulated glandular Cl secretion is to remove the glands from the skin.

The epithelium was separated from the dermis by an enzymatic procedure. In histological exami-

Table 2. Comparison of total net fluxes and SCC after stimulation with PGE_2 (2 μM)[a]

Period (min)	Incubation	Net flux			Total	SCC
		Cl^-	Na^+	K^+		
		(n equivalents · min^{-1} · cm^{-2})				
0–30	Control	0.67 ± 0.62	−0.20 ± 0.07	0.03 ± 0.04	0.29 ± 0.53	0.22 ± 0.05
30–60	Control	−0.27 ± 0.14	−0.26 ± 0.05	0.04 ± 0.05	−0.01 ± 0.06	0.19 ± 0.02
60–90	Control	−0.38 ± 0.32	−0.32 ± 0.09	−0.02 ± 0.02	−0.04 ± 0.29	0.16 ± 0.03
90–120	PGE_2	4.54 ± 1.36	1.90 ± 0.88	0.47 ± 0.14	2.18 ± 0.53	2.52 ± 0.53
120–150	PGE_2	4.89 ± 1.58	1.98 ± 0.87	0.93 ± 0.46	1.99 ± 0.32	2.56 ± 0.72
150–180	PGE_2	5.34 ± 1.64	2.18 ± 1.02	0.83 ± 0.23	2.64 ± 0.77	2.62 ± 0.47

[a] Influx and efflux of $^{36}Cl^-$, $^{22}Na^+$ and ^{42}K were measured in symmetrically divided amiloride-treated skin halves as described in Materials and Methods. The net outward directed fluxes are indicated as positive values. SCC compared to the net flux was not significantly different in any period. Values are mean ± SE; $n = 5$.

Table 3. Effect of PGE_2, theophylline and furosemide on $^{36}Cl^-$, $^{42}K^+$ and $^{22}Na^+$ fluxes in amiloride-treated skins[a]

Incubation period (min)		PGE_2 (2μM)		Theophylline (1 mM)		Furosemide (0.5 mM)	
		0–30	30–60	60–90	90–120	120–150	150–180
		(neq · cm^{-2} · min^{-1})		(neq · cm^{-2} · min^{-1})		(neq · cm^{-2} · min^{-1})	
	EF^-	8.68 ± 0.85 (5)	8.20 ± 0.89 (5)	16.75 ± 1.46 (7)	17.42 ± 1.17 (7)	9.94 ± 0.50 (7)	4.58 ± 0.51 (5)
$^{36}Cl^-$	IN^-	2.25 ± 0.53 (3)	2.37 ± 0.55 (3)	5.31 ± 1.18 (3)	6.16 ± 1.01 (3)	4.52 ± 0.94 (3)	3.45 ± 0.32 (3)
	NET	5.26 ± 0.51 (3)	4.82 ± 0.75 (3)	10.06 ± 0.90 (3)	9.52 ± 0.81 (3)	4.64 ± 1.03 (3)	0.92 ± 0.64 (3)
	EF^-	0.70 ± 0.06 (5)	0.65 ± 0.03 (5)	1.27 ± 0.10 (7)	1.18 ± 0.09 (7)	0.46 ± 0.08 (7)	0.14 ± 0.01 (5)
$^{42}K^-$	IN^-	0.05 ± 0.004 (3)	0.08 ± 0.006 (3)	0.10 ± 0.006 (3)	0.11 ± 0.011 (3)	0.14 ± 0.005 (3)	0.14 ± 0.005 (3)
	NET	0.63 ± 0.10 (3)	0.55 ± 0.06 (3)	1.15 ± 0.18 (3)	1.03 ± 0.20 (3)	0.16 ± 0.06 (3)	−0.02 ± 0.02 (3)
	EF^-	3.53 ± 0.32 (5)	3.28 ± 0.31 (5)	8.61 ± 0.70 (7)	8.85 ± 0.84 (7)	4.23 ± 0.46 (7)	2.71 ± 0.48 (5)
$^{22}Na^-$	IN^-	1.00 ± 0.11 (3)	1.15 ± 0.20 (3)	1.78 ± 0.34 (3)	2.25 ± 0.29 (3)	2.59 ± 0.62 (3)	2.18 ± 0.44 (3)
	NET	2.63 ± 0.46 (3)	2.17 ± 0.33 (3)	5.64 ± 0.54 (3)	6.12 ± 0.64 (3)	1.94 ± 0.70 (3)	0.99 ± 0.53 (3)

[a] After addition of amiloride (0.1 mM) to the apical solution, influxes (IN^-) and effluxes (EF^-) of $^{36}Cl^-$, $^{22}Na^+$ and $^{42}K^+$ were measured separately in paired skin halves as described in Materials and Methods under the conditions indicated in the Table. Values are mean ± SE. Number of experiments is given in parentheses.

nation of epithelia from split frog skin we found, as already shown by Thompson and Mills (1981), that the separation procedure had removed the glands, leaving only the neck segment of the gland in the epithelium (Fig. 3).

The first series of experiments was performed to compare the effect of PGE_2 and amiloride on epithelia from split skins with those of intact skin preparations. Addition of PGE_2 (2 μM) to the basolateral solution of epithelia increased the SCC from a control value of 4.22 ± 0.20 to 9.71 ± 0.50 neq · min^{-1} · cm^{-2} ($n = 4$), showing that the separating procedure did not change the ability of the epithelium to be stimulated by PGE_2. Amiloride was then added to the apical solution of the same epithelia and the SCC decreased to a level of 0.43 ± 0.12 neq · min^{-1} · cm^{-2}, which is much lower than the SCC level in PGE_2 and amiloride-treated intact skins, but of the same magnitude as the SCC in control skins treated with amiloride (0.64 ± 0.07 neq · min^{-1} · cm^{-2}, $n = 20$).

In another set of experiments PGE_2 was added to the basolateral solution of amiloride-treated epithelia as shown in Fig. 4. The SCC in five amiloride-treated epithelia did not change after addition of PGE_2.

These results show that in gland-free isolated epithelia PGE_2 was unable to stimulate SCC after amiloride treatment, in contrast to intact skin preparations. This indicates that the PGE_2-induced increase in SCC after addition of amiloride resides in glands of the frog skin.

EFFECT OF SECRETION STIMULATORS AND INHIBITORS

In order to get more information about the mechanism by which PGE_2 stimulates the SCC in ami-

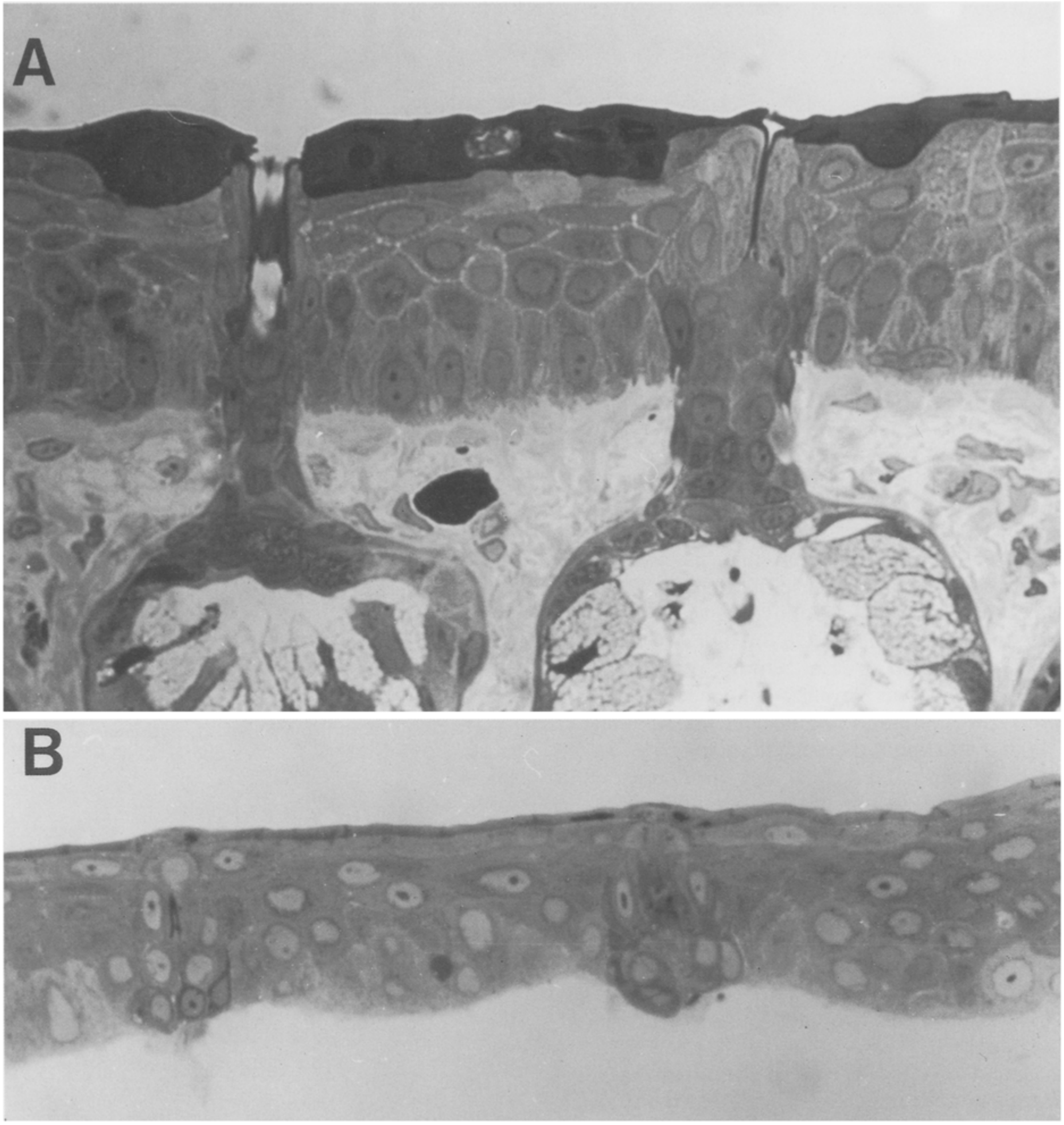

Fig. 3. Light micrographs of frog skin (*A*) and epithelium from split frog skin (*B*) fixed with 1% OsO_4 during short-circuiting. The enzymatic splitting procedure had removed the glands from the skin sections leaving only the duct and the neck segment of the gland in the isolated epithelium (*B*). Light micrographs of methylene blue-stained 1-μ sections. 400×

loride-treated skins, the effect of PGE_2 was investigated in combination with drugs which are known to activate or inhibit glandular secretion. According to the model for glandular Cl secretion proposed by Silva et al. (1977), this secretion could be inhibited by the Na-Cl cotransport inhibitor furosemide or the Na, K-ATPase inhibitor ouabain. The same authors have shown that cAMP and the phosphodiesterase inhibitor theophylline are able to stimulate glandular Cl secretion.

Table 3 shows the effect of PGE_2, theophylline, and furosemide on the ion movement in amiloride-treated skins. Simultaneous measurements of the unidirectional fluxes of chloride, potassium, and sodium show that 1 mM theophylline was able to double the PGE_2-stimulated outward-going net fluxes

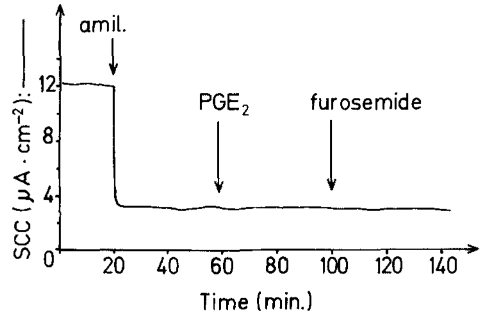

Fig. 4. Effect of PGE_2 and furosemide on SCC in gland-free amiloride-treated epithelia. Amiloride (0.1 mM) was added to the apical solution, PGE_2 (2 µM), and furosemide (0.5 mM) to the basolateral solution at the time indicated in the Figure

for all three ions. The ability of theophylline to stimulate the ion transport and SCC was enhanced when the skins were pretreated with PGE_2 (Fig. 5). In amiloride-treated skins theophylline had only a small effect on SCC (an increase of 0.99 ± 0.27 µA/cm^2), whereas addition of theophylline to skins pretreated with amiloride and PGE_2 resulted in an increase of 3.85 ± 0.60 µA/cm^2. On the other hand, the effect of PGE_2 was strongly enhanced by prior addition of theophylline to amiloride-treated skins, resulting in a maximum increase in SCC of 16.15 ± 4.26 µA/cm^2, compared to a maximum increase in SCC of 6.03 ± 0.46 µA/cm^2 in PGE_2-stimulated amiloride-treated skins. These results show that PGE_2 and theophylline have a synergistic effect on the SCC in amiloride-treated skins.

The effect of the diuretic furosemide was monitored in amiloride-treated skins where the ion transport was stimulated by PGE_2 and theophylline. Addition of furosemide (0.5 mM) to the basolateral solution nearly abolished the net fluxes of Cl^-, K^+, and Na^+ (Table 3).

The effect of ouabain on the PGE_2-stimulated SCC in amiloride-treated skins was tested in two sets of experiments, where ouabain (0.1 mM) was added to the basolateral solution either before or after addition of PGE_2. In skins treated with amiloride and ouabain, the PGE_2-induced stimulation of SCC was nearly abolished, showing an increase from 1.0 ± 0.2 to 1.5 ± 0.3 µA/cm^2, $n = 3$, whereas PGE_2 increased the SCC in amiloride-treated skins from 1.1 ± 0.2 to 5.9 ± 0.5 µA/cm^2, $n = 5$. Addition of ouabain to these skins resulted in a decrease in SCC from 5.9 ± 0.5 to 2.1 ± 0.2 µA/cm^2.

The effect of exogenous cAMP was examined in order to see if cAMP could stimulate the Cl secretion. Addition of 1 mM dibuturyl-cAMP resulted in an increase of 8.8 ± 1.24 µA/cm^2, $n = 5$. This increase could be inhibited by furosemide as seen in Fig. 8.

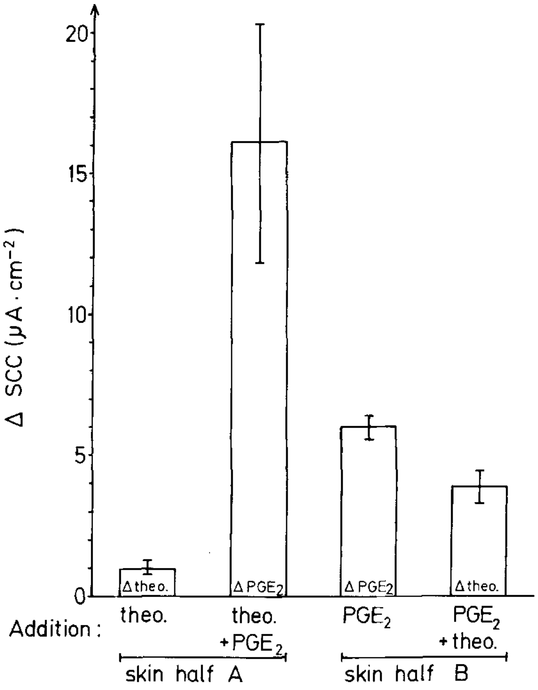

Fig. 5. Effect of theophylline on the PGE_2 response in amiloride-treated skin. Two symmetrical amiloride-treated skin halves (*A* and *B*) were incubated under the conditions noted in the Figure. The increase in SCC (Δ SCC) was calculated as the difference in SCC before addition of the agent and the maximum SCC level after addition of the agent. Values are mean ± SE; $n = 4$

Thus the PGE_2-induced increase in SCC and Cl secretion in amiloride-treated skins could be stimulated by theophylline or exogenous cAMP and inhibited by furosemide or ouabain like the glandular Cl secretion in shark rectal gland. Together these experiments indicate that the PGE_2-induced increase in SCC in amiloride-treated skins is the result of a Cl secretion from the glands in the frog skin.

Diphenylamine-2-carboxylate (DPC) impedes the exit of Cl^- in the thick ascending limb of the loop of Henle as well as in the rectal gland of the shark (Di Stefano et al., 1985). The effect of DPC on the PGE_2-stimulated Cl secretion was investigated by adding 0.35 mM DPC to the apical solution. The net outward-directed fluxes of Cl^-, K^+, and Na^+ were nearly halved by the addition of DPC (Fig. 6). The influx of these ions was not affected by DPC. These results show that DPC is able to inhibit glandular Cl secretion of frog skin.

PGE_2-Stimulated Water Secretion

The net water movement across the skins was measured in order to investigate glandular fluid secre-

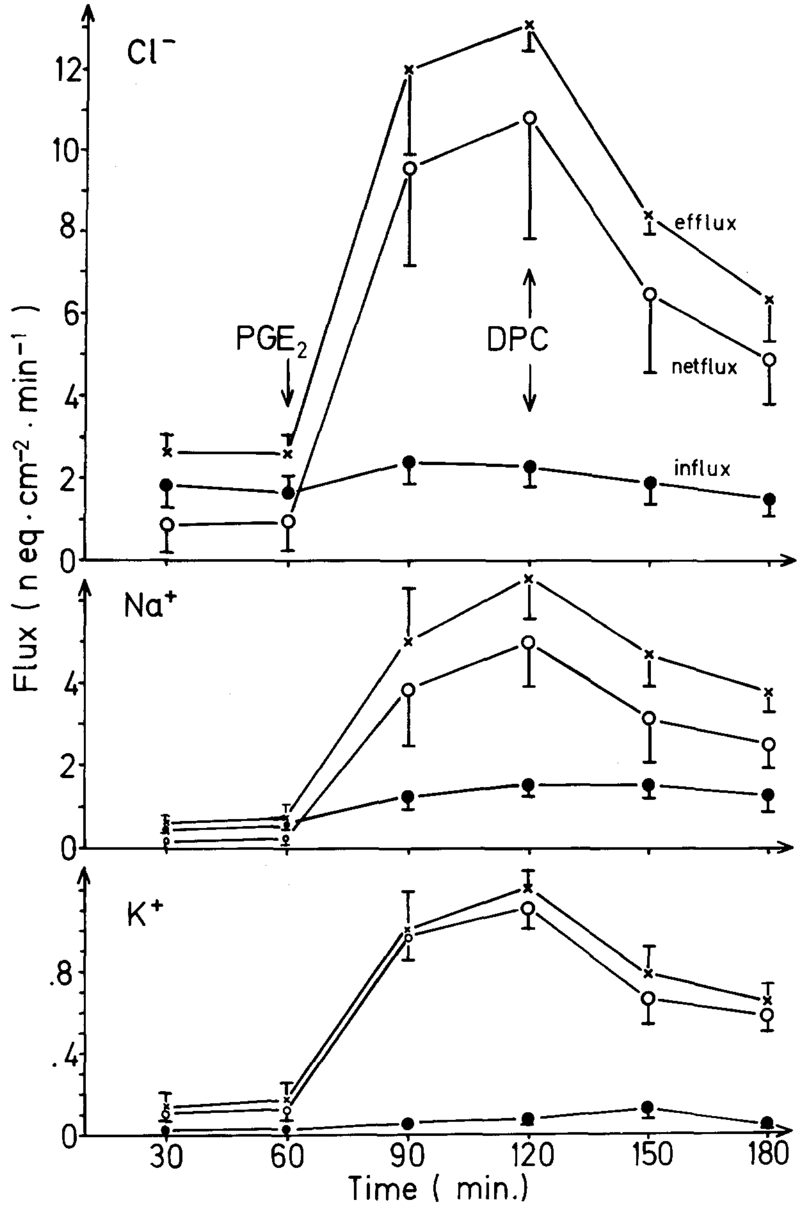

Fig. 6. Effect of diphenylamine-2-carboxylate (DPC) on PGE$_2$-stimulated influxes and effluxes of $^{36}Cl^-$, $^{22}Na^+$ and $^{42}K^+$ in amiloride-treated skin. Influx and efflux experiments were performed as described in Materials and Methods. 2 μM PGE$_2$ was added to the basolateral solution of amiloride-treated skin after two control periods of 30 min. After two additional 30-min periods, DPC (0.35 mM) was added to the apical solution, and the fluxes were observed for the last two periods

tion under conditions which stimulate or inhibit glandular ion secretion.

Figure 7 shows a representative experiment in a series of four, where SCC and net water flow (J_w) were measured simultaneously. Addition of amiloride to the apical solution decreased the inward-directed J_w from 0.77 ± 0.41 to 0.09 ± 0.04 $\mu l \cdot hr^{-1} \cdot cm^{-2}$. After 20 to 30 min with amiloride the inward-directed J_w was 0.60 ± 0.21 $\mu l \cdot hr^{-1} \cdot cm^{-2}$. Addition of PGE$_2$ turned the inward-directed J_w to an outward-directed J_w. The PGE$_2$-stimulated outward-directed J_w was biphasic, where the first transient increase reached a maximum of 8.50 ± 1.61 $\mu l \cdot hr^{-1} \cdot cm^{-2}$, 2 to 5 min after addition. The second maximum of 2.16 ± 0.49 $\mu l \cdot hr^{-1} \cdot cm^{-2}$ was reached after 20 to 30 min of incubation with PGE$_2$. This J_w was stable and could be maintained for hours. The net water flow could be stimulated further by theophylline to a maximum level of 5.51 ± 1.04 $\mu l \cdot hr^{-1} \cdot cm^{-2}$. Addition of furosemide to the PGE$_2$- and theophylline-stimulated skins resulted in a decrease in J_w from 5.23 ± 0.84 to 0.29 ± 0.23 $\mu l \cdot hr^{-1} \cdot cm^{-2}$. Ouabain also inhibited the PGE$_2$ and theophylline-stimulated J_w in amiloride-treated skins to the same degree as furosemide (*unpublished observations*).

The time-course of the J_w change under these different conditions was similar to that observed for SCC (Fig. 7). The change in J_w started 1 to 3 min after the change in SCC, indicating that the glandular ion movement is responsible for the net water flow.

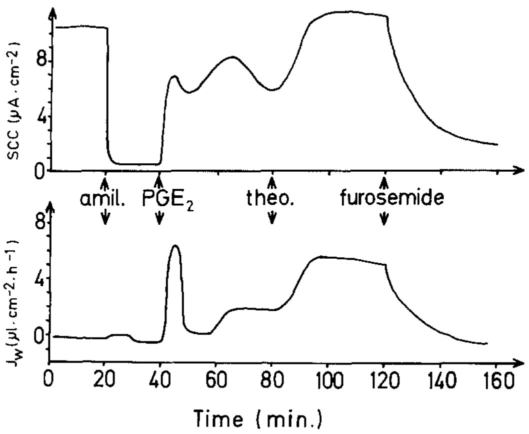

Fig. 7. PGE_2-stimulated SCC and net water flow (J_w) in amiloride-treated skin. SCC and J_w were measured simultaneously with isotonic Ringer's on both sides of the skin. Amiloride (0.1 mM) was added to the apical solution, and PGE_2 (2 µM), theophylline (1 mM), and furosemide (0.5 mM) to the basolateral solution at the time indicated in the Figure

A23187-STIMULATED Cl AND WATER SECRETION

We have shown previously that the calcium ionophore A23187 increases the prostaglandin E_2 synthesis in frog skin (Nielsen & Bjerregaard, 1984).

To test whether or not an A23187-induced PGE_2 synthesis was able to stimulate glandular ion and water secretion, we compared the effect of A23187 in paired amiloride-treated skin halves with or without previous addition of the prostaglandin synthesis inhibitor indomethacin (Fig. 8).

In four control skins, A23187 induced an increase in SCC and J_w of 4.94 ± 0.19 $\mu A/cm^2$ and 5.51 ± 1.03 $\mu l \cdot hr^{-1} \cdot cm^{-2}$, respectively, whereas in the paired indomethacin-treated skins the increase in SCC and J_w was reduced to 0.48 ± 0.28 $\mu A/cm^2$ and 0.28 ± 0.24 $\mu l \cdot hr^{-1} \cdot cm^{-2}$. Indomethacin did not affect the basal or the dibutyryl cAMP-stimulated or the furosemide-inhibited glandular ion and water secretion.

These results suggest that the calcium ionophore A23187 stimulates the glandular ion and water secretion via increased prostaglandin synthesis. Furthermore, this experiment shows that the glandular Cl secretion is not activated by the Ca^{2+} ions *per se,* since the calcium ionophore could not activate the glandular secretion in indomethacin-treated skins.

Discussion

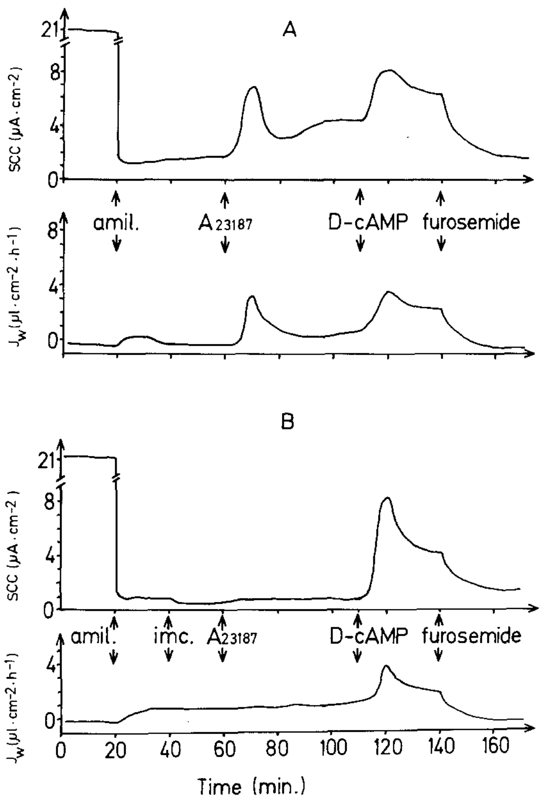

Fig. 8. A23187-stimulated SCC and net water flow (J_w) in amiloride-treated skin. SCC and J_w were measured simultaneously in two symmetrical skin halves (*A* and *B*). Amiloride (0.1 mM) was added to the apical solution, and A23187 (4 µM), 10 µM indomethacin (imc), dibutyryl-cAMP (1 mM), and furosemide (0.5 mM) to the basolateral solution at the time indicated in the Figure

The data presented in this paper show that PGE_2 elicits an increase in SCC in frog skin (*Rana esculenta*) where the sodium absorption is abolished by amiloride (Fig. 1). The increase in SCC was due to an increase in a net outward-directed flux of Cl^-, K^+, and Na^+ (Fig. 2). The net Cl secretion exceeded the net secretion of K^+ and Na^+, yielding a net flux which was equal to the PGE_2-stimulated SCC (Table 2).

In order to locate the cell type responsible for the PGE_2-stimulated Cl secretion, gland-free, isolated epithelia were prepared. The SCC of these epithelia could not be stimulated by PGE_2 in the presence of amiloride (Fig. 4). Thus, the removal of the glands and connective tissue from the frog skin removes also the ability of PGE_2 to induce an active outward-directed Cl transport, but the Na absorption in these epithelia could still be activated by PGE_2. This indicates that the PGE_2-induced increase in SCC after addition of amiloride resides in the glands of the skin. The same method, with gland-free epithelial preparations, has been used to disclose that the β-adrenergic stimulation of the

SCC in amiloride-treated frog skin is the result of a Cl secretion from the glands (Thompson & Mills, 1981; 1983). Further evidence, showing that the Cl secretion in frog skin is due to glandular activity has been reported by Mills et al., 1985. Experiments with electron-microprobe analysis showed that the only significant ion change in isoproterenol-stimulated amiloride-treated skins was an increase in Cl^- and Na^+ in a group of cells at the ductal pole of the acini of the glands.

According to the model proposed by Silva et al. (1977), glandular Cl secretion occurs as follows: Cl^- and Na^+ enter the glandular cells from the blood side through a cotransport mechanism which can be blocked by furosemide. The gradient for inward movement of Na^+ and Cl^- is created by the ouabain-sensitive Na/K pump. Secretory agents are believed to increase, directly or via cAMP, the Cl permeability of the apical membrane of the gland cells. Cl^- ions leave the cells to the lumen by passive diffusion down a favorable electrochemical gradient. The model for glandular Cl secretion in the shark rectal gland has been used to explain the Cl transport in other Cl-secreting systems, such as the rabbit descending colon (Heintz et al., 1983), frog cornea (Zadunaisky et al., 1980), and canine trachea epithelium (Shorofsky et al., 1984).

Several features of the PGE_2-stimulated ion transport in amiloride-treated frog skins presented in this paper are in agreement with the model for glandular Cl secretion. The PGE_2-stimulated SCC and ion transport could be inhibited by addition of furosemide (Fig. 7 and Table 3), ouabain or the Cl channel inhibitor DPC (Fig. 6).

The possibility that PGE_2 stimulates the Cl secretion through a cAMP-mediated increase in the apical Cl permeability was investigated in experiments where the effect of the phosphodiesterase inhibitor, theophylline, and PGE_2, was compared. Inhibition of the decomposition of cAMP by theophylline leads to a small increase in the Cl secretion. Addition of PGE_2 in the presence of theophylline resulted in an increase in Cl secretion which was 2 to 3 times higher than the secretion in skins only treated with PGE_2 (Table 3 and Fig. 5). The same effect was seen in experiments where theophylline was added to skins pretreated with PGE_2. Thus, PGE_2 and theophylline act synergistically, which indicates that PGE_2 might stimulate the adenylate cyclase and thereby increase the cellular cAMP content. In agreement with these results Hall et al. (1976) have shown that PGE_2 increases the cAMP level in frog skin and that this increase in cAMP was facilitated by theophylline. Although, in this context, one should note that the cAMP content was measured in whole skins where different cell types are present. The involvement of cAMP as a possible second messenger in the initiation of Cl secretion was further examined by addition of exogenous dibutyryl cAMP to amiloride-treated skin. In these experiments dibutyryl cAMP induced an increase in SCC which could be inhibited by furosemide. Altogether these results suggest that cAMP is likely to be involved as second messenger in the glandular Cl secretion elicited by PGE_2.

The amphibian antidiuretic hormone, arginine vasotocin, which is known to increase the cAMP content in isolated frog skin epithelia (Johnsen & Nielsen, 1978), could not stimulate the SCC in amiloride-treated skin, whether added before or after addition of theophylline (*unpublished observations*). This observation indicates that the adenylate cyclase in glands does not have receptors for arginine vasotocin.

In addition to the PGE_2-stimulated secretion of Cl^-, PGE_2 stimulated a secretion of Na^+ and K^+ from the inside to the outside of the frog skin. In PGE_2-stimulated amiloride-treated skin the flux ratio (influx/efflux) for Cl^-, Na^+, and K^+ was 0.372, 0.342, and 0.151, respectively. If we assume that the gland cells are short-circuited and bathed with identical solutions on both side, the deviation of the ratio from unity indicates that the secretion of Na and K originates from a primary or secondary active transport mechanism like that suggested for Cl secretion.

With simultaneous measurement of SCC and net water transport in amiloride-treated skin, we were able to correlate the PGE_2-stimulated glandular ion and water secretion. First, PGE_2 elicits a large transient water flow in the outward direction, which could be the result of a glandular discharge evoked by a shrinking action of the myoepithelial cells around the body of the skin gland. This mechanism has been proposed to explain the initial PGE_1-stimulated outward-directed waterflow observed in *Rana temporaria* (Barry et al., 1975). However, in their experiments the net water outflow returned after a period of 6 to 8 min to an inward-directed water flux, associated with a large increase in the active Na transport. In our experiment, where the Na absorption was abolished by amiloride, the water outflow did not reverse but increased to a new steady level which could be maintained for hours. The outward-directed water flow could be stimulated by theophylline or inhibited by furosemide or ouabain, and had a time-course similar to the SCC, indicating that the PGE_2-stimulated ion secretion is responsible for the glandular fluid secretion. Under steady-state secretion conditions, where J_w was 2.16 $\mu l \cdot hr^{-1} \cdot cm^{-2}$, and the net secretion of Cl^-, Na^+, and K^+ was 500.13 $neq \cdot hr^{-1} \cdot cm^{-2}$, thus the fluid-secreted was isosmotic (232 mOsm) to the Ringer's solution (238 mOsm). Watlington and Huf

(1971) have shown that the norepinephrine-stimulated glandular fluids secretion from the body surface of living frogs contains Cl^-, Na^+, K^+, and HCO_3^-, yielding an osmolarity of 180 mOsm. Taking into account the different experimental conditions, this osmolarity of the glandular fluid secreted is in good agreement with our findings.

The experiments presented here indicate that the glandular active transport of Cl both drives the water secretion and is responsible for the SCC. If the glands are perfectly short-circuited, an active transport of Cl from the inside to the outside of the skin is balanced by uptake and liberation of Cl^- from the Ag/AgCl electrode reactions. Thus, the outward-directed active Cl transport is under perfect short-circuited conditions unable to establish the ion gradient necessary for the water secretion. These considerations led us to propose the following model for glandular ion and water secretion in short-circuited frog skin. The glands shift between a closed (not short-circuited) and open (short-circuited) state. In the closed state, the active transport of Cl from the blood side to the glandular lumen creates a potential across the glandular cells which drags Na^+ and maybe K^+ from the blood side to the glandular lumen. This creates a concentration gradient across the glandular cells and drags water from the blood side to the glandular lumen. When the glandular lumen is filled, the glandular duct opens and the content of the glandular lumen is discharged into the solution bathing the outside of the skin. In the open state the glandular cells become short-circuited and the active transport of Cl is measured as a short-circuit current. When the glandular lumen is "empty," the glandular duct becomes closed, and the active transport of Cl produces no short-circuit current, but creates a new concentration gradient across the gland cells. These two extreme situations (the closed and the open state of the glands) are described in order to elucidate the rationale behind the model. When the chloride secretion is activated it is conceivable that the glands are in a sort of intermediate state where the cells are not ideal short-circuited. In this situation the active transport of chloride creates a negative potential (relative to the bathing solutions) in the lumen of the glands. In order to preserve electroneutrality in the glandular lumen sodium would diffuse both from the apical and basolateral solution into the glandular lumen. The increased solute content drags water into the lumen, mainly from the basolateral side because the glandular cells are bathed in this solution. The increased amount of the solution in the glandular lumen, is via the glandular duct secreted into the apical solution.

In frog skin the prostaglandin synthesis can be stimulated by agents that modulate the cellular Ca^{2+} metabolism. We have previously shown that the calmodulin antagonist, trifluoperazine, stimulates the PGE_2 synthesis by a Ca^{2+}-dependent process (Bjerregaard & Nielsen, 1986*b*). The calcium ionophore A23187 stimulates also the prostaglandin synthesis in frog skin (Erlij et al., 1981; 1986; Nielsen & Bjerregaard, 1984). This ability of A23187 was used in order to investigate whether the ionophore could stimulate the glandular Cl and water secretion in amiloride-treated skin via an increase in the prostaglandin synthesis. As shown in Fig. 8, A23187 was able to stimulate both Cl and water secretion. This secretion could be blocked by previous addition of the prostaglandin synthesis inhibitor indomethacin, indicating that the A23187-induced increase in PGE_2 synthesis was responsible for the glandular Cl and water secretion. The secretory functions of both endocrine and exocrine gland cells (Petersen & Maruyama, 1984) and Cl- secreting epithelia like rat colon (Cuthbert, 1985) have been shown to be activated by a rise in intracellular Ca^{2+} levels. Frizzell (1977) reported that A23187 increases the Cl^- secretion by a Ca^{2+}-dependent mechanism in rabbit colon. In this study we have provided evidence for a mechanism where A23187 does not directly stimulate the glandular Cl secretion via an increase in the intracellular Ca^{2+} concentration, since A23187 could not stimulate the Cl secretion in skin where the prostaglandin synthesis was inhibited. The inability of A23187 to stimulate Cl secretion in this situation was not due to an inhibition of the secretion by indomethacin, since addition of dibutyryl cAMP increased the Cl secretion to the same level in control as in indomethacin-treated skin. A possible model for these events consistent with our data is: addition of A23187 increases the intracellular Ca^{2+} concentration. The increase in intracellular Ca^{2+} concentration activates a phospholipase, resulting in a release of arachidonic acid, which is then converted to prostaglandins via the indomethacin-sensitive cyclooxygenase pathway (Feinstein & Sha'afi, 1983). The increased production of prostaglandins resulted in a stimulation of the Cl secretion, probably by an increase in the cellular cAMP content. A similar mechanism has been proposed for the A23187-induced increase in Cl secretion in stripped rabbit colonic mucosa (Smith & McCabe, 1984).

In summary, we find that PGE_2 stimulates a secretion of Cl^-, Na^+, K^+, and fluid in amiloride-treated frog skin. The secretion resides in the skin glands. Theophylline enhanced the PGE_2-stimulated ion secretion, indicating that the effect of

PGE_2 was mediated by cAMP. Experiments with the calcium ionophore A23187 showed that its stimulation of the ion and fluid secretion is not a result of Ca^{2+} *per se*, but is due to an increase in the production of prostaglandins.

This work was supported by the NOVO Foundation, Denmark.

References

Barry, E., Hall, W.J. 1969. Stimulation of sodium movement across frog skin by prostaglandin E_1. *J. Physiol. (London)* **200:**83P

Barry, E., Hall, W.J., Martin, J.D.G. 1975. Prostaglandin E_1 and the movement of salt and water in frog skin (*Rana temporaria*). *Gen. Pharmacol.* **6:**141–150

Bjerregaard, H.F., Nielsen, R. 1986*a*. Prostaglandin E_2-stimulated salt and water excretion in frog skin (*Rana esculenta*). *Renal Physiol.* **9:**91–92

Bjerregaard, H.F., Nielsen, R. 1986*b*. Trifluoperazine stimulated sodium transport by increased prostaglandin E_2 synthesis in isolated frog skin (*Rana esculenta*). *Acta Physiol. Scand.* **127:**75–85

Cuthbert, A.W. 1985. Calcium-dependent chloride secretion in rat colon epithelium. *J. Physiol. (London)* **361:**1–17

Di Stefano, A., Wittner, M., Schlatter, E., Lang, H.J., Englert, H., Greger, R. 1985. Diphenylamine-2-carboxylate, a blocker of the Cl^--conductive pathway in Cl^--transporting epithelia. *Pfluegers Arch.* **405:**S95–S100

Erlij, D., Gersten, L., Sterba, G. 1981. Calcium, prostaglandin and transepithelial sodium transport. *J. Physiol. (London)* **320:**136P

Erlij, D., Gersten, L., Sterba, G., Schoen, H.F. 1986. Role of prostaglandin release in the response of tight epithelia to Ca^{2+} ionophores. *Am. J. Physiol.* **250:**C629–C636

Fassina, G., Carpenedo, F., Santi, R. 1969. Effect of prostaglandin E_1 on isolated short-circuited frog skin. *Life Sci.* **8:**181–187

Feinstein, M.B., Sha'afi, R.I. 1983. Role of calcium in arachidonic acid metabolism and in the actions of arachidonic acid-derived metabolites. *Calcium and Cell Functions* **IV:**337–376

Frizzell, R.A. 1977. Active chloride secretion by rabbit colon: Calcium-dependent stimulation by ionophore A23187. *J. Membrane Biol.* **35:**175–187

Gerencser, G.A. 1978. Effect of prostaglandin E_1 on transmural potential difference and short-circuited current in isolated frog (*Rana catesbeiana*) skin. *Comp. Biochem. Physiol.* **60C:**199–203

Hall, W.J., O'Donoghue, J.P., O'Regang, M.G., Penny, W.J. 1976. Endogenous prostaglandins, adenosine 3′:5′-monophosphate and sodium transport across isolated frog skin. *J. Physiol. (London)* **258:**731–753

Heintze, K., Stewart, C.P., Frizzell, R.A. 1983. Sodium-dependent chloride secretion across rabbit descending colon. *Am. J. Physiol.* **244:**G357–G365

Johnsen, A.H., Nielsen, R. 1978. Effects of the antidiuretic hormone, arginine vasotocin, theophylline, filipine and A23187 on cyclic AMP in isolated frog skin epithelium (*Rana temporaria*). *Acta Physiol. Scand.* **102:**281–289

Johnsen, A.H., Nielsen, R. 1980. Sodium nitropusside induced cGMP accumulation in isolated frog skin epithelium. Effect on cAMP, hydroosmotic and natriferic response to antidiuretic hormone. *Acta Physiol. Scand.* **108:**373–379

Johnsen, A.H., Nielsen, R. 1982. Enhanced sensitivity to stimulation of sodium transport and cyclic AMP by antidiuretic hormone after Ca^{2+} depletion of isolated frog skin epithelium. *J. Membrane Biol.* **69:**137–143

Koefoed-Johnsen, V., Ussing, H.H., Zerahn, K. 1952. The origin of the short-circuit current in the adrenalin-stimulated frog skin. *Acta Physiol. Scand.* **27:**38–48

Lote, C.J., Rider, J.B., Thomas, S. 1974. The effect of prostaglandin E_1 on the short-circuit current and sodium, potassium, chloride and calcium movements across isolated frog (*Rana temporaria*) skin. *Pfluegers Arch.* **352:**145–153

Mills, J.W., Thurau, K., Dörge, A., Rick, R. 1985. Electron microprobe analysis of intracellular electrolytes in resting and isoproterenol-stimulated exocrine glands of frog skin. *J. Membrane Biol.* **86:**211–220

Nielsen, R., Bjerregaard, H.F. 1984. Intracellular Ca^{2+} and prostaglandin as regulator of active transepithelial Na^+ transport in frog skin. First International Congress of Comparative Physiology and Biochemistry, Liège, Belgium. August 27–31:88

Petersen, O.H., Maruyama, Y. 1984. Calcium-activated potassium channels and their role in secretion. *Nature (London)* **307:**693–696

Shorofsky, S.R., Field, M., Fozzard, H.A. 1984. Mechanism of Cl secretion in canine trachea: Changes in intracellular chloride activity with secretion. *J. Membrane Biol.* 81:1–8

Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J.N., Epstein, F.H. 1977. Mechanism of active chloride secretion by shark rectal gland: Role of Na-K-ATPase in chloride transport. *Am. J. Physiol.* **233:**F298–F306

Smith, P.L., McCabe, R.D. 1984. A23187-induced change in colonic K and Cl transport are mediated by separate mechanisms. *Am. J. Physiol.* **247:**G695–G702

Thompson, I.G., Mills, J.W. 1981. Isoproterenol-induced current in glands of frog skin. *Am. J. Physiol.* **241:**C250–C257

Thompson, I.G., Mills, J.W. 1983. Chloride transport in glands of frog skin. *Am. J. Physiol.* **244:**C221–C226

Tomlinson, R.W.S., Wood, A.W. 1978. Effect of amiloride on catecholamine-induced changes in ion transport in short-circuited frog skin. *J. Membrane Biol.* **Special Issue:**135–150

Ussing, H.H., Zerahn, K. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23:**110–127

Watlington, C.O., Huf, E.G. 1971. β-Adrenergic stimulation of frog skin mucous glands: Nonspecific inhibition by adrenergic blocking agents. *Comp. Gen. Pharmacol.* **2:**295–305

Zadunaisky, J.A., Schaeffer, B.E., Cherksey, B. 1980. Chloride active transport, membrane lipids and receptors in the corneal epithelium. *Ann. N.Y. Acad. Sci.* **341:**233–245

Received 10 September 1986; revised 16 January 1987