The Origin of the Short-Circuit Current

in the Isolated Skin of the South

American Frog *Leptodactylus ocellatus*

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ABSTRACT   In isolated skins of *Leptodactylus ocellatus* the short-circuit current is smaller than the sodium net flux and this difference disappears when the skins are bathed in solutions in which the chloride ions have been replaced by sulfate or methylsulfate ions. There is a net movement of chloride ions from outside to inside of the skins in the short-circuit condition with chloride Ringer's solutions bathing the skins. The addition of ouabain to the inside solution markedly reduced not only sodium net flux but also the chloride net influx found. Copper ions added to the outside solutions produced a rise in short-circuit current, as well as the known increase in potential difference. In sodium-free Ringer's (sodium replaced by choline) the orientation of the potential difference across the skins was reversed, the inside being negative instead of positive. The results are interpreted as direct or indirect indications of the presence of a net transfer of chloride ions from outside to inside of these frog skins.

Studies performed on isolated frog skin have shown that the short-circuit current measured in an external circuit, when the spontaneous potential of the skin is abolished, is due only to the net transport of sodium ions from outside to inside of the skin (1). Chloride ions have been shown to behave passively, that is, they do not contribute to the short-circuit current (2).

In "*in vivo*" experiments, there is evidence suggesting the existence of a net chloride movement from outside to inside of the skin, in *Rana* and *Bufo* (3).

The present study of the origin of the short-circuit current was done in isolated skins of the most common local frog, *Leptodactylus ocellatus*, which is a species unrelated to *Rana*, with morphological and physiological characteristics of its own. The evidence to be described indicates the existence of a net chloride transfer from outside to inside of these frog skins "*in vitro*."

METHODS

Frogs of both sexes were kept in moist sinks; when kept in tap or in cold water the survival time in captivity was greatly shortened. The animals were immobilized by pithing down the spinal cord, the abdominal skin was immediately dissected away and soaked for a few minutes in Ringer's solution. Then it was drained and mounted as a membrane between two lucite chambers as described by Ussing and Zerahn (1). Two types of chambers were used, one with an area of 7.1 cm² and another of 3.0 cm². In some cases one frog skin was divided in two symmetrical pieces and each one mounted in separate chambers of the smaller area. In these cases simultaneous experiments were performed with the two pieces and the data were treated as in the case of only one membrane.

The chambers were connected to a Keithley 200B electrometer or to a radiometer 4 millivoltmeter through agar-Ringer bridges and calomel electrodes. The bridges for potential recording were made of fine polyethylene tubing ending very close to the surfaces of the skin. The short-circuiting device was the one developed by Ussing and Zehran (1).

The solutions utilized were Conway's frog Ringer's (4) or a solution containing 115.5 mM sodium chloride, 2.4 mM sodium bicarbonate, 2 mM potassium chloride, and 1 mM calcium gluconate.

These solutions were modified when necessary, substituting all the sodium by choline ions, or all the chloride by sulfate or methylsulfate ions on a molar basis. Due to the hygroscopic nature of the choline chloride, stock solutions of this salt were made and the chloride concentrations determined by microdiffusion analysis (5).

About 1 hour after mounting the skin, the short-circuit current was measured at intervals of 5 to 10 minutes and radioisotopes added to the bathing fluids. Samples were taken every 30 or 60 minutes for flux determinations. A mean short-circuit current for the experiments was obtained by averaging the mean values recorded in each collection period. Means for fluxes were obtained by averaging the values for each individual period of collection of samples. Each experiment was run for a time corresponding to 5 to 11 periods of flux measurement. Fluxes were expressed as microequivalents per hour per square centimeter of skin, and short-circuit current as microamperes per square centimeter of skin.

Radiosodium Fluxes   Unidirectional fluxes of sodium were measured using either Na^{24} or Na^{22}. The radiosodium was added to the inside or outside solution, keeping the total sodium content of the Ringer at the normal level.

The activity of the solutions was from 0.25 to 0.5 μc per ml in all these experiments. Samples of 100 to 500 μl were diluted in tubes containing 2 ml of water and counted in a well scintillation detector.

Simultaneous Measurements of Sodium Influx and Outflux

Sodium influx and outflux were determined in the same skin by the method utilized by Ussing (6), except that here the two sodium isotopes were detected by γ-ray

spectrometry (16). To the inside solution Na^{24} was added at a final concentration of 3 μc per ml to measure outflux, and Na^{22} was added to the outside solution at a final concentration of 0.3 μc per ml to measure influx.

The samples obtained from both sides were first measured for Na^{24} at a pulse height previously determined with a standard solution of Na^{24}. Then the total activity of both Na^{22} and Na^{24} was detected at a preset height determined with a standard mixture of the two isotopes. To evaluate Na^{22} in the samples the amount of Na^{24} first counted was subtracted from the result with the mixture, after corrections for counting efficiency and for decay. Determinations made with standard activities similar to those found in the experiments indicated that the main source of error in the method consisted of differences in the efficiency of Na^{24} detection at the two pulse height levels utilized. This was corrected for by determining the efficiency for Na^{24} with a standard solution at frequent intervals during the counting of the samples at the two pulse height levels.

Radiochloride Fluxes In these experiments Cl^{36} was utilized as an HCl solution neutralized with equimolar amounts of NaOH. Cl^{36} was added to the inside or outside solution at a final concentration of 0.12 to 0.40 μc per ml. Samples of 100 to 500 μl were taken at regular intervals, placed on planchets, dried, and counted with a Geiger-Müller tube.

Corrections for self-absorption were not found necessary with these thin samples.

Simultaneous Measurements of Sodium and Chloride Unidirectional Fluxes

Both sodium and chloride influx and sodium and chloride outflux were determined in one frog skin. The method was to use one piece of the skin for measuring sodium and chloride influx and the other piece for sodium and chloride outflux. For this, Na^{24} and Cl^{36} were added simultaneously at the same final activities as were used for measuring the unidirectional fluxes. Two samples were taken 1 minute apart from the bathing solutions at regular intervals. One was diluted and immediately counted for Na^{24} in the well scintillation detector, and the other was placed on a planchet and counted for Cl^{36} with a Geiger-Müller tube after complete decay of the Na^{24}.

Ouabain, G-strophanthin, was utilized at a final concentration of 10^{-5} M; copper sulfate was added to the outside solution in amounts necessary to obtain a final concentration of 2×10^{-5} M.

RESULTS

The potential difference produced by the frog skins varied between 10 and 40 mv after 1 hour of equilibration in Ringer's solution. The short-circuit currents observed ranged between 30 and 140 $\mu amp/cm^2$, these being extreme values. Summer frogs showed somewhat higher values for potential and current but no definite study of the seasonal variations was made.

Though the skins survived more than 24 hours in the chambers the experiments were done the same day on which the skins were dissected from the animals.

Comparison between Short-Circuit Current and Sodium Fluxes

Measurements of sodium influx and sodium outflux were done in two separate groups of skins. The results for twenty influxes and nine outfluxes for skins with normal Ringer's solutions on both sides are given in Table I. The short-circuit current was smaller than sodium influx. It represented 72.6 per cent of the sodium influx and this difference was statistically significant when analyzed for paired data ($P < 0.01$). The mean outflux was equivalent to 6.1 per cent of the sodium influx.

TABLE I

SODIUM FLUXES AND SHORT-CIRCUIT CURRENTS
IN ISOLATED SKINS OF *LEPTODACTYLUS OCELLATUS*
IN NORMAL RINGER'S SOLUTION

The fluxes were measured separately in different skins.

	No. of experiments	Mean Na flux		Mean short-circuit current	Mean difference
		$\mu eq/hr./cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$
Na influx	20	2.47±0.23*	66.1±6.3	48.1±5.3	18.1±3.0‡
Na efflux	9	0.15±0.03	4.1±0.9	48.0±5.3	
Na net flux		2.32	62.0		

* Standard error of the mean.
‡ The mean difference was statistically significant for paired data ($P < 0.01$).

The average values of the short-circuit current obtained in the two groups of skins were similar and thus justified comparison with the net sodium flux, obtained as the difference between influx and outflux. The results indicated that the short-circuit current was equivalent to 77.4 per cent of the net sodium transport. Thus, in these experiments the short-circuit current could not be explained only on the basis of the sodium net transport.

Measurement of Sodium Influx and Outflux in the Same Skin

Sodium influx was measured with Na^{22} and sodium outflux with Na^{24} simultaneously in the same skin, as described in Methods. The results of the net sodium transport were compared with the short-circuit current and they are presented in Table II. The short-circuit current was between 72.2 and 90.0 per cent of the net sodium influx, and the difference for paired data, between these parameters was statistically significant ($P < 0.01$).

Comparison of Sodium Influx and Short-Circuit Current in Absence of Chloride Ions

A series of experiments was done in which the skins were bathed on both sides by a Ringer solution in which all the chloride had been replaced by the

non-penetrating sulfate or methylsulfate ion. In these cases the potential difference reached values over 100 mv. An increase in the short-circuit current was generally observed as well.

The results of simultaneous measurement of sodium influx and short-circuit current in sulfate Ringer's are given in Table III.

TABLE II

SIMULTANEOUS MEASUREMENT OF SODIUM INFLUX AND OUTFLUX WITH Na^{24} AND Na^{22} IN THE SAME FROG SKIN. COMPARISON BETWEEN NET SODIUM FLUX AND SHORT-CIRCUIT CURRENT FOR SKINS IN NORMAL RINGER'S SOLUTIONS

	Na influx	Na outflux	Net Na flux		Short-circuit current	Mean difference
	$\mu eq/hr./cm^2$	$\mu eq/hr./cm^2$	$\mu eq/hr./cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$
	3.15	0.34	2.81	75.4	65.2	
	4.32	0.24	4.08	109.4	98.6	
	3.03	0.12	2.91	78.0	56.4	
	3.32	0.14	3.18	85.1	72.0	
	1.20	0.12	1.08	28.9	23.4	
Mean	3.00	0.19	2.81	75.4	63.1	12.2 ± 2.6*

* The mean difference for paired data between short-circuit current and sodium net flux was statistically significant ($P < 0.01$).

TABLE III

SODIUM INFLUX AND SHORT-CIRCUIT CURRENT IN ISOLATED SKINS OF *LEPTODACTYLUS OCELLATUS* IN CHLORIDE-FREE RINGER'S

Chloride was replaced by sulfate in the bathing solutions.

No. of experiments	Na influx		Short-circuit current
	$\mu eq/hr./cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$
8	2.28 ± 0.34	60.9 ± 8.4	59.1 ± 9.1

The difference between sodium influx and short-circuit current (1.8 $\mu amp/cm^2$) was not statistically significant ($P < 0.1$) for paired data.

The short-circuit current was equivalent to 96.7 per cent of the sodium influx. Though it should be compared with the net sodium flux and only values for influx were obtained, the efflux is so small as not to make an important contribution to the computation. These results indicate that probably the disagreement between short-circuit current and sodium transport observed in skins in chloride-containing solutions was due to the chloride ions.

Measurements of Chloride Unidirectional Fluxes and Short-Circuit Current

The foregoing results prompted the determination of chloride fluxes in short-circuited skins to ascertain whether there was a net transfer of this ion across the skins.

The results of these experiments performed in two groups of skins, one for influx and the other for outflux determinations, are shown in Table IV. The mean values obtained showed that the influx of chloride was greater than the outflux in short-circuited skins, indicating thus the existence of a net movement of chloride ions from outside to inside of the skin.

TABLE IV

CHLORIDE FLUXES AND SHORT-CIRCUIT
CURRENTS IN SKINS OF *LEPTODACTYLUS OCELLATUS*
IN NORMAL RINGER'S SOLUTION

	No. of experiments	Chloride flux		Short-circuit current
		$\mu eq/hr./cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$
Chloride influx	13	1.86±0.18	49.9±4.7	55.1±5.8
Chloride outflux	9	0.80±0.05	21.3±3.9	47.4±5.5
Chloride net flux		1.06	28.6	

The difference between influx and outflux of chloride (chloride net flux) was statistically significant ($P < 0.01$).

The values of the mean short-circuit current in both groups of experiments were reasonably close and the difference between chloride influx and chloride outflux was statistically significant ($P < 0.01$).

Simultaneous Measurements of Sodium and Chloride Unidirectional Fluxes. Effect of Ouabain

Experiments were devised to determine influx of radiosodium and radiochloride in one-half and outflux of the same ions in the symmetric half of the same skin. In these experiments the short-circuit currents in the two pieces were not exactly the same; differences of 10 per cent or more were usually found. The measurements of the simultaneous ionic fluxes were made only when a difference smaller than 10 per cent was found between the two separate pieces.

The mean values for the net fluxes of sodium and chloride obtained in five experiments of this type are presented in Table V.

Again the short-circuit current was smaller than the sodium net flux and a net chloride transfer from outside to inside was also found. The difference

between sodium transport and short-circuit current was 23.4 μamp per cm^2 and the value of the net chloride transport represented 19.0 μamp per cm^2.

The effect of ouabain was tested by adding the cardiotonic agent to the solution bathing the inside of each of the two pieces of skin.

This was done after four control periods. The ouabain produced a sharp decline of the short-circuit current, with great inhibition of the sodium transport and almost complete abolition of the net chloride flux, as can be observed in Table V. This drug produced a sharp decline in sodium and chloride influxes but did not modify appreciably the outfluxes of these ions.

TABLE V

SIMULTANEOUS MEASUREMENT OF CHLORIDE
AND SODIUM FLUXES AND SHORT-CIRCUIT CURRENTS IN
TWO HALVES OF THE SAME SKIN OF *LEPTODACTYLUS
OCELLATUS* IN NORMAL RINGER'S SOLUTION,
AND THE ACTION OF OUABAIN ON THESE PARAMETERS

		No. of experiments	Net flux		Short-circuit current Average of 10 halves
			$\mu eq/hr./cm^2$	$\mu amp/cm^2$	$\mu amp/cm^2$
	Control		2.84±0.45*	76.1	52.7
Na net flux		5			
	Ouabain		0.91±0.07	24.4	12.4
	Control		0.71±0.13	19.0	
Cl net flux		5			
	Ouabain		0.11±0.02	2.9	

* Standard error of the mean.

Effect of Copper on the Short-Circuit Current

The addition of copper ions to the outside bathing solution produced a rise in the potential difference as previously observed in European frogs (6), but the short-circuit current was also increased in our experiments, as presented in Fig. 1. In six experiments the increase in the short-circuit current after addition of small amounts of copper sulfate and stabilization was 41.6 per cent over the control values. This would indicate that reduction of the permeability for chloride due to copper tends to equalize sodium transport and the short-circuit current.

Potential Difference in Absence of Sodium Ions

Since a net transfer of negative charges was found in the above-mentioned experiments, the possible development of a potential difference due to the chloride net movement was investigated. For this purpose all the sodium

chloride of the Ringer solutions was replaced by choline chloride, on both sides of the skin. Under these conditions a "negative" potential was detected, the inside of the skin being negative instead of positive as is found in sodium Ringer's. This potential difference took from 60 to 90 minutes to reach a maximum, and then gradually disappeared in 2 hours. The magnitude of the negative potential found was between 5 and 35.5 mv and the short-circuit current obtained from it was between 5 and 20 μamp per cm^2 in a series of
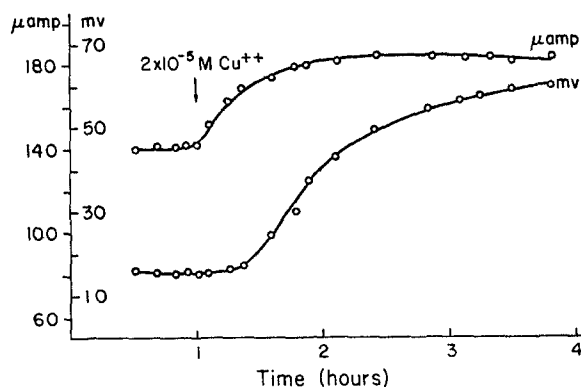
FIGURE 1. Addition of copper ions to the solution bathing the outside of a skin o *Leptodactylus ocellatus*. An increase both of potential difference (mv) and of short-circuit current (μamp) is observed.
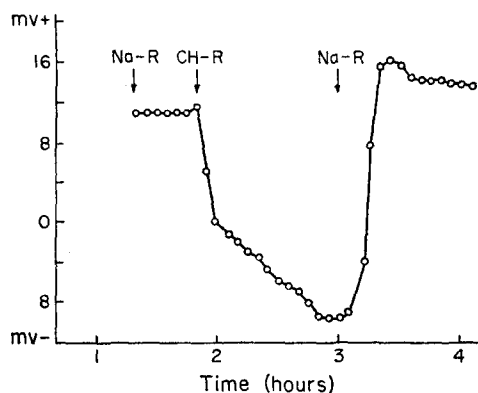
FIGURE 2. Potential difference in the absence of sodium ions. Na-R, sodium Ringer's; Ch-R, choline Ringer's. After equilibrium of the potential in sodium Ringer's, choline Ringer's was placed on both sides of the skin, and a reversal of the potential observed, the inside of the skin being negative instead of positive. With the return to sodium Ringer's, the potential attained again its normal direction and magnitude.

fifteen experiments. Fig. 2 shows a case in which after equilibrium of the potential in normal Ringer's, the solution was changed to choline Ringer's on both sides. In this experiment the negative potential developed, and when the skin was again bathed with normal Ringer's solutions the potential attained again its normal direction and magnitude.

DISCUSSION

The presence of a chloride net transfer from outside to inside in the isolated skin of *Leptodactylus ocellatus* seems to be the main reason for the difference

found between short-circuit current and net sodium flux. Indirect evidence, as the agreement between short-circuit current and sodium influx in the absence of chloride ions and the increase of the short-circuit current during addition of copper ions, also agrees with the main findings. The inhibition by ouabain of both transports confirms previous evidence for sodium in frog skin (8) and for chloride in gastric mucosa (9).

The presence of a negative potential in the absence of sodium ions agrees with predictions to be made from a system behaving like these skins, but its disappearance and the small short-circuit current obtained call for further experimentation to obtain a clearer picture of this situation.

The known passiveness of chloride ions in frog skins, as well as in other systems, contrasts with these results. Outside of the digestive tract, where chloride transport has been found (10, 11), very little is known about the existence of net chloride movements in animal systems. Recently Keynes has shown the presence of a net influx of chloride in freshly isolated axons of the squid (12). Brodsky (13) has found also that the isolated bladder of the turtle shows a negative potential difference in the absence of sodium ions and that most probably this is due to the presence of a chloride pump.

There is an observation of chloride net movement in isolated frog skin under the influence of adrenalin (14) but, in this case the short-circuit current was greater than the net sodium transport and the chloride transport was performed in the outward direction and was attributed to stimulation of the glands of the skin. On the other hand, in *in vivo* experiments a net transport of chloride from outside to inside by the skin of European anurans has been described (3).

It is possible that the mechanism for chloride transport does exist *in vivo* in all kinds of frog skins, but that it shows up *in vitro* only in some species in which it is more potent and lasts longer after the removal of the skin from the animal. This view might be supported by the observation in some long lasting experiments that the value of the chloride influx in the initial periods of the experiments was higher than after 6 hours, with little change in the short-circuit current.

Nevertheless, *Leptodactylus ocellatus*, as well as other South American amphibians, adapt themselves very well to salinities which are higher than can be tolerated by other species (15). This would certainly point to sharp differences among species and could mean that chloride transport is a typical property of these frog skins.

The model proposed by Koefoed-Johnsen and Ussing (7) for the frog skin applies in general to the properties of the frog skins utilized in these studies. The skins are permeable to sodium at the outside surface, and show little sodium permeability at the inside, judging from the small passive outflux of sodium. Though the osmotic and electrical properties of the epithelial cells of the skin of *Leptodactylus ocellatus* may or may not be compatible with the

previously described model (7), more evidence relating directly to this question has to be obtained before these properties can be interpreted in terms of such a model.

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