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CHOLINE AND SODIUM INTERACTIONS IN OUABAIN-TREATED AND ESERINE-TREATED FROG SKINS

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SUMMARY

Previously published results demonstrate that replacement of 80% K^+– Ringer solution by 80% choline–Ringer solution in the solution bathing the epidermal surface of frog skin causes a large rise in short-circuit current and forward Na^+ flux, but leaves backflux unaffected. In the present experiments we have found that a similar replacement in the solution bathing the dermal surface of the skin causes a fall in the short-circuit current and forward Na^+ flux, and does not affect Na^+ backflux. In skins in which active Na^+ movements have been inhibited by preincubation in $5 \cdot 10^{-5}$ M ouabain, the application of choline to the epidermal side of the skin increases forward Na^+ flux. This property is abolished in skins in which active movements of Na^+ have been inhibited by preincubation in 10^{-3} M eserine. In both cases choline causes a fall in short-circuit current.

INTRODUCTION

In a previous paper, MACEY AND KOBLICK[1] reported that choline causes a marked increase in Na^+ influx in short-circuited frog skin, without significantly affecting Na^+ outflux. When choline replaced 80% of the Na^+ in the Ringer solution bathing the epidermal surface of the skin, Na^+ influx against a 5-fold concentration gradient was increased about 40% over the value obtained for a control period in which K^+ replaced Na^+ to the same extent. The net ion movement across the skin, as measured by the short-circuit current, was increased about 50%. About 80% of the increase in short-circuit current can be accounted for by the increase in Na^+ influx; the remainder corresponds to passive choline movement calculated from the choline permeability reported previously.

This enhancement of Na^+ influx by choline might be accounted for by the stimulation of some part of the metabolic chain which provides energy for active Na^+ movements. If this were the case, the effect should be independent of the direction of the choline gradient. Experiments to be reported here will show that reversal of this gradient depresses both short-circuit current and Na^+ influx. More-

over, the effect can be elicited in skins in which active Na^+ transport has been inhibited with ouabain.

USSING[2] has observed that treatments which result in the swelling of the epidermal layer of cells cause an increase in short-circuit current, while those which cause shrinking of these cells have the opposite effect. Under the conditions of our experiments, the observations would be accounted for if the outward facing membranes of the epidermal layer were more permeable to choline than to K^+, and if the reverse were true at the inward facing membranes. Using the technique introduced by MACROBBIE AND USSING[3] we have found that choline applied under the conditions of the experiments reported here, has no effect when present in the epidermal solution and causes swelling when present in the dermal solution*.

The effects of choline on Na^+ movements might be the consequence of competitive, obligatory single file movements in narrow pores, analogous to the movements of K^+ in dinitrophenol-poisoned cephalopod axons observed by HODGKIN AND KEYNES[4]. HLADKY[5] has provided an analysis of such effects from which it is possible to predict the degree of interaction between two or more competing species when the number of sites per pore is known, and when parallel passive movements through non-restrictive pores can be assumed to be absent. MACEY AND OLIVER[6] have studied the transients involved in this process.

METHODS AND MATERIALS

Isolated abdominal skins of *Rana pipiens* were used throughout. These were mounted between lucite half-chambers each containing 5.9 ml of Ringer solution and exposing 2.0 cm^2 of skin to the bathing solutions. Potential was monitored with Ag–AgCl electrodes and read on a Varian G-14 recording potentiometer. Short-circuit currents passed through the skin by a second pair of Ag–AgCl electrodes were measured on a microammeter according to the method of USSING AND ZERAHN[7].

In the experiments reported previously[1], Ringer solution continuously bathed the inside surface of the skin, while short-circuit current and $^{22}Na^+$ flux was measured during 3 approx. 30-min periods. The outside surface of the skin was exposed to a $^{22}Na^+$-containing Ringer solution in which 80% of the stable Na^+ was replaced with K^+ during the first period and to a $^{22}Na^+$–Ringer in which the 80% replacement of stable Na^+ was made with choline during the second period. Conditions during the third period were the same as those during the first. The last period served as a test of the reversibility of the effects observed on application of choline. If no reversal occurred, the experiment was discarded.

In the present study, in addition to experiments of this type, we have determined the effect of these manipulations of epidermal solutions on Na^+ backflux and on short-circuit current and unidirectional Na^+ fluxes in skins preincubated in ouabain and eserine. In addition, the effects of reversal of the gradients were studied.

When inhibitors were used, these were added in equal concentrations to all solutions. Initial incubation in cold solutions was performed under the conditions of Period 1. This was continued until the desired degree of inhibition was attained. At this point, isotope was added to the appropriate solution, and incubation continued

* D. C. KOBLICK AND R. C. LUCAS, unpublished observations.

for another 20 min in order to achieve isotope equilibrium. Samples were taken at the end of isotope equilibration and at the end of each subsequent experimental period. In each case the entire solution into which isotope was moving was removed and a 2.0-ml aliquot taken for counting in a well scintillation counter. Before refilling, the chamber was washed 5 times with the appropriate cold solution. Before each experimental period, the chamber into which modified Ringer solution was to be placed was washed 4 times with the appropriate cold modified solution.

In those experiments in which inhibitors were used, the degree of inhibition was calculated from short-circuit current measurements alone. It should be emphasized that short-circuit current is not necessarily equivalent to net Na^+ flux in these experiments, since the Na^+ gradient and either a K^+ or choline gradient were present at all times.

The inside solution was continuously aerated in all experiments.

RESULTS

The effects of reversal of the direction of K^+ and choline gradients in Periods 1 and 3, and Period 2, respectively, are given in Table I. In initial experiments it was found that replacement of K^+ by choline in dermal solutions was followed by a rapid rise in short-circuit current and forward Na^+ flux, which was in turn followed by an exponential return to a new steady-state value. When the reverse replacement was made the immediate response was a fall in both variables, and was followed by an exponential rise to the new steady-state level. In the experiments reported in Table I, a 30-min equilibration period was interpolated between each solution replacement

TABLE I

EFFECTS OF CHOLINE IN DERMAL SOLUTION ON SHORT-CIRCUIT CURRENT AND UNIDIRECTIONAL Na^+ FLUXES

Outside solution: Ringer during both periods. Inside solution: 80% K^+–Ringer during Period 1 and 80% choline–Ringer during Period 2.

Skin No.	*Short-circuit current*		*Na^+ forward flux*		*Na^+ backflux*	
	*Period 1**	*% Change in Period 2***	*Period 1**	*% Change in Period 2***	*Period 1**	*% Change in Period 2***
4-26A	5.89	−46	6.97	−32		
4-27A	2.54	7	4.07	− 9		
5-3B	2.05	−12	6.00	−18		
5-4B	4.32	−30	7.59	−14		
5-19B	2.45	−46			0.12	33
5-20A	3.61	−36			0.16	−12
5-25A	1.56	2			0.39	8
5-26A	5.55	−30			0.48	−21
Mean ± S.D.	3.50 ± 1.62	−24 ± 21	6.15 ± 1.55	−18 ± 10	0.29 ± 0.13	2 ± 24
P ($\bar{x} > 0$)***		<0.02		<0.05		>0.8

* 10^{-8} mole/min.
** % change relative to Period 1; increases positive.
*** From paired t-test.

TABLE II

EFFECTS OF CHOLINE IN EPIDERMAL SOLUTION ON Na^+ BACKFLUX

Outside solution: 80% K^+–Ringer during Period 1 and 80% choline–Ringer during Period 2. Inside solution: Ringer solution during both periods.

Skin No.	*Short-circuit current**			*Na^+ backflux**		
	Period 1	*Period 2*	*Difference*	*Period 1*	*Period 2*	*Difference*
3-25A	3.17	4.79	1.62	0.20	0.26	0.06
3-25B	1.88	2.90	1.02	0.32	0.24	−0.08
3-29A	4.40	5.12	0.72	0.51	0.60	0.09
4-5A	2.70	5.72	3.02	0.69	0.61	−0.08
4-5B	2.92	3.59	0.67	0.34	0.39	0.05
Mean ± S.D.	3.01 ± 0.91	4.22 ± 1.87	1.18 ± 1.05	0.41 ± 0.19	0.42 ± 0.18	0.01 ± 0.08
$P(\bar{x}_1 = \bar{x}_2)$**			<0.05			>0.8

* 10^{-8} mole/min.
** From paired *t*-test.

TABLE III

EFFECTS OF CHOLINE IN EPIDERMAL SOLUTION ON SHORT-CIRCUIT CURRENT AND UNIDIRECTIONAL Na^+ FLUXES IN OUABAIN-INHIBITED SKINS

Outside solution: 80% K^+–Ringer during Period 1 and 80% choline–Ringer during Period 2. Inside solution: Ringer solution during both periods. $5 \cdot 10^{-5}$ M ouabain was added to all solutions. Skins were initially incubated under Period 1 conditions until the degree of inhibition of the short-circuit current shown in the last column was achieved.

Skin No.	*Short-circuit current*		*Na^+ forward flux*		*Na^+ backflux*		*% Inhibition*
	*Period 1**	*% Change in Period 2***	*Period 1**	*% Change in Period 2***	*Period 1**	*% Change in Period 2***	
10-20	1.05	−100	0.64	52			49
10-21	0.25	−400	0.34	68			92
10-22	0.28	−368	0.27	57			78
1-11	0.17	−306	0.47	−13			91
10-14	0.44	−320	1.04	13			90
6-21A	0.66	+ 42	0.54	106			86
6-18A	1.49	− 5	0.81	10			61
2-11	0.36	−277			1.15	0	81
2-15	0.39	−287			1.18	14	85
2-26	0.22	−295			0.91	16	91
3-1	0.30	−213			0.76	20	90
3-2A	0.28	−321			1.58	28	89
3-2B	0.44	−180			0.87	42	81
Mean ± S.D.	0.48 ± 0.39	−231 ± 140	0.59 ± 0.27	42 ± 41	1.08 ± 0.26	20 ± 14	82 ± 13
$P(\bar{x} > 0)$***		<0.001		<0.05		<0.05	

* 10^{-8} mole/min.
** % change relative to Period 1; increases positive.
*** From paired *t*-test.

and the subsequent sampling period. These experiments show that reversing the choline gradient causes a reduction in short-circuit current and Na^+ influx. No significant change in Na^+ backflux was observed, as in the case in those experiments in which choline was present in the epidermal solution (Table II). The average decrease in Na^+ forward flux is slightly higher than the average decrease in short-circuit current in this instance.

Table III presents the results of experiments performed on skins which had been preincubated in $5 \cdot 10^{-5}$ M ouabain. The degree of inhibition is calculated from the values of short-circuit current before application of the inhibitor and at the beginning of Period 1. It is apparent from a comparison of backflux values from non-inhibited skins with those reported in Table III, that ouabain causes an increase in passive movements of Na^+. When choline was applied to the epidermal surface of ouabain-treated skins, short-circuit current rapidly fell to negative values. It is possible that the ouabain-induced increase in Na^+ backflux was balanced by a similar increase in K^+ movement down its gradient during Period 1. That such movement of K^+ occurred in the presence of ouabain is indicated by the Period 1 values recorded in Table III, where a positive short-circuit current was observed, in spite of the fact that Na^+ backflux was considerably higher than forward flux. This is in contrast to the results of MACROBBIE AND USSING[3], who showed that ouabain decreases the passive K^+ permeability at the inner side of the epidermis. If ouabain did not similarly affect the movement of choline, the increased Na^+ backflux would not have been balanced during Period 2, resulting in the observed negative short-circuit current. The effects of ouabain on passive movements of K^+ and choline are currently under investigation.

In spite of the dramatic fall in short-circuit current, forward Na^+ flux is increased on application of choline just as it is in non-inhibited skins. There is also a statistically significant increase in Na^+ backflux.

The results of parallel experiments in which 10^{-3} M eserine is used to inhibit active Na^+ transport are shown in Table IV. Here again, application of choline caused a decrease in short-circuit current, although this rarely reached negative values, as in the ouabain-inhibited skins. In contrast to the results with ouabain, however, a small, statistically insignificant fall in Na^+ forward flux occurred. The increase in Na^+ backflux is of about the same magnitude as that observed in ouabain-inhibited skins.

DISCUSSION

The fact that the sign of the effects on short-circuit current and forward Na^+ flux produced by choline depends on the direction of the choline gradient, as indicated by a comparison of Table I with the previously published experiments, argues strongly against the idea that these effects are mediated by the action of choline on the metabolism of the skin. The fact that skins in which active Na^+ transport has been abolished by ouabain still show enhancement of forward Na^+ flux in the presence of choline in the outside solution is further evidence that the effect is not a metabolic one.

The previously cited observation of USSING[2] that treatments which cause an increase in the volume of the epithelial layer of cells increase short-circuit current and forward Na^+ flux and that those which cause a decrease in the volume of this layer

TABLE IV

EFFECTS OF CHOLINE IN EPIDERMAL SOLUTION ON SHORT-CIRCUIT CURRENT AND UNIDIRECTIONAL Na^+ FLUXES IN ESERINE-INHIBITED SKINS

Experimental conditions are the same as those specified for the experiments given in Table III, except that the inhibition was performed with 10^{-3} M eserine.

Skin No.	*Short-circuit current*		*Na^+ forward flux*		*Na^+ backflux*		*% Inhibition*
	*Period 1**	*% Change in Period 2***	*Period 1**	*% Change in Period 2***	*Period 1**	*% Change in Period 2***	
1-4	1.58	−64	1.28	−29			79
1-5	1.37	−50	0.84	−5			69
1-6	1.34	−119	0.80	−10			72
1-7	0.76	−30	0.67	1			73
6-28B	1.45	−44	1.38	−14			78
6-30B	1.12	−79	0.73	−30			78
3-5	3.06	−76			1.65	12	51
3-9A	2.47	32			0.56	23	69
3-10A	1.30	−68			0.94	16	56
3-10B	1.83	−57			0.98	20	43
3-11B	1.22	−34			0.69	9	74
3-12A	2.70	20			0.30	30	43
3-12B	1.93	−36			0.84	1	54
2-16A	1.18	−75			1.48	6	88
Mean ± S.D.	1.66 ± 0.65	−48 ± 39	0.95 ± 0.30	−14 ± 13	0.93 ± 0.46	15 ± 9	66 ± 14
$P(\bar{x} > 0)$***		<0.001		>0.05		<0.01	

* 10^{-8} mole/min.
** % change relative to Period 1; increases positive.
*** From paired *t*-test.

of cells have the opposite effect provides a possible explanation for the results reported here. In order that this explanation be tenable, the outward facing membranes of these cells must be sufficiently more permeable to choline than to K^+ so that cell swelling occurs within the 30-min period corresponding to Period 2 in our experiments. We have found that no such swelling in fact occurs when 0.8 K^+–Ringer solution is replaced by 0.8 choline–Ringer solution in the outside solution*. When this replacement is made on the inside of the skin, with normal Ringer bathing the outside, swelling rather than shrinkage occurs. These results indicate that choline permeability in frog skin is more similar to that of K^+ than that of Na^+, the inside boundary of the epithelial layer being freely permeable to it and the outside boundary being relatively impermeable. These permeability relations indicate that the effects produced by choline cannot be explained in terms of swelling and shrinking of the epidermal layer of the skin.

Competitive movements of choline and Na^+ in narrow pores would explain some of the effects reported here. If such pores are sufficiently large to accommodate movement of Na^+ and K^+ in opposite directions little or no coupling between a K^+ gradient and Na^+ movements would be expected, and if the upper limit of the pore diameter is not greater than the sum of the diameters of Na^+ and choline, these ions would be restricted to single file movement, and Na^+ flux would be driven by a

* D. C. KOBLICK AND R. C. LUCAS, unpublished observations.

choline gradient. In non-inhibited skins, effects predicted from this model are verified. An inward directed choline gradient increases short-circuit current and forward Na^+ flux and has no effect on Na^+ backflux (Table II). Reversing the choline gradient causes a decrease in short-circuit current and in forward Na^+ flux (Table I). Again there is no effect on Na^+ backflux. Of the alternative explanations suggested here, only the competitive movement of Na^+ and choline ions in narrow pores is consistent with the asymmetry of the effect.

The experiments performed on skins inhibited with ouabain and eserine reveal a dramatic difference in the mode of action of these two substances. With ouabain-inhibited skins, epidermal application of choline causes a 42% rise in forward Na^+ flux. When eserine is used as an inhibitor, the effect on forward flux observed in normal skins and in ouabain-inhibited skins is completely abolished. With both inhibitors, choline causes a sharp decrease in short-circuit current, and a smaller increase in Na^+ backflux. The fall in short-circuit current would be explained if the inhibitors make the skin leaky to K^+ and Na^+, but not to choline. During Period 1 the increased Na^+ backflux down its concentration gradient would be balanced by passive forward movements of K^+; when choline replaced K^+ during Period 2, the unbalanced Na^+ backflux would result in the large decrease in short-circuit current. KIRSCHNER[8] has shown that ouabain and eserine do increase passive backflux of Na^+. MACROBBIE AND USSING[3] report that ouabain causes a marked decrease in passive ion permeabilities. The fact that Period 1 values in Table III show a positive short-circuit current even though Na^+ backflux is considerably higher than forward Na^+ flux, indicates that ouabain causes an increased passive movement of K^+.

The increase in Na^+ backflux on addition of choline to the epidermal solution observed in inhibited skins is not explained by the narrow pore hypothesis. Since KIRSCHNER[9] has shown that the pump mechanism does not participate to any great extent in the mediation of Na^+ backflux and that the pathway is almost entirely a parallel one, changes in backflux are probably unrelated to the postulated narrow pore interactions which we have invoked to explain the effects of choline on forward Na^+ flux. The fact that ouabain does not abolish the effect of choline on forward Na^+ flux, while eserine does, suggests that the latter has its site of action within these pores, while the former does not.

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