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CHLORIDE FLUX *VIA* A SHUNT PATHWAY IN FROG SKIN: APPARENT EXCHANGE DIFFUSION

LAZARO J. MANDEL* AND PETER F. CURRAN

The Department of Physiology, Yale University School of Medicine, New Haven, Conn. 06510 (U.S.A.)

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SUMMARY

The mode of transepithelial Cl^- passage through frog skin was studied by measuring Cl^- outflux at different external Cl^- concentrations as a function of applied potential. Results indicate that Cl^- outflux is dependent on external Cl^- concentration (*trans*-side), a condition typically interpreted as evidence for the presence of exchange–diffusion. Concurrent measurement of urea outflux (providing a measure of shunt permeability; L. J. Mandel and P. F. Curran, *J. Gen. Physiol.*, 59 (1972) 503) indicated that the changes in Cl^- outflux were solely due to changes in shunt permeability. It is concluded, therefore, that transepithelial Cl^- movements occur primarily through the shunt pathway.

INTRODUCTION

The movement of Cl^- across the skin of most frog species appears to be passive[1], although active Cl^- transport has been observed in *Leptodactylus ocellatus*[2,3]. However, the anionic composition of the bathing solutions affects both the open circuit potential[4,5] and the short-circuit current[5,6] of toad bladder and frog skin, suggesting a degree of coupling between the active transport of Na^+ and passive anionic movement. The nature of this apparent coupling might be better understood if further information about the anionic pathway were available and this study represents an effort to obtain such information regarding the mode of transepithelial Cl^- passage through frog skin. To that end, we studied the effect of external Cl^- concentration on Cl^- outflux as a function of applied potential. Our findings demonstrate that, under several different conditions, virtually all of the Cl^- movement occurs through a shunt pathway[7] that is in parallel with the active Na^+ transport path and that may follow an extracellular route[8]. Some of the initial results suggested that exchange–diffusion played an important role in Cl^- transport, which might suggest a cellular pathway[9] in light of similar observations made for Cl^- in gastric mucosa[10,11], for Na^+ in red blood cells[12], for Na^+ (ref. 13) and Cl^- (ref. 14) in large intestine, and for Na^+ in muscle [15]. However, under further investigation, it became clear that the apparent exchange–diffusion of Cl^- in frog skin could be fully explained in terms of changes in shunt permeability.

* Present address: Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N.C. 27706, U.S.A.

METHODS

The experimental methods used were identical to those previously described[8]. Briefly, the skin of *Rana pipiens* was mounted as a flat sheet (3.14 cm^2) between lucite chambers equipped with solution reservoirs (10 ml each) which were stirred and oxygenated with air. The potential difference (PD) across the skin was measured with calomel electrodes connected to the solution reservoirs *via* agar bridges; current was passed through the skin *via* Ag–AgCl electrodes connected to the solution reservoirs with agar bridges. An automatic voltage clamp that compensated for the resistance of the solution between the agar bridges was used to pass the appropriate current through the skin to maintain a preset PD value. Unidirectional fluxes of Cl^- and urea were determined with $^{36}Cl^-$ and [^{14}C]urea as previously described. The composition of normal NaCl Ringer's solution was 112 mM NaCl, 2.5 mM $KHCO_3$, 1 mM $CaCl_2$, and 2 mM urea. In all experiments, the inside solution was normal Ringer's solution. The NaCl concentration of the outside solution was reduced to 20 mM in some experiments leaving the remaining ionic concentrations constant; this hypotonic solution is denoted dilute 20 mM NaCl Ringer's solution. In some experiments 184 mM sucrose was added to this dilute Ringer's solution to produce a solution isoosmolar with regular Ringer's solution and in others NaCl was replaced with sodium isethionate to yield a solution containing 20 mM Cl^- with an ionic strength and Na^+ concentration the same as normal Ringer's solution.

RESULTS AND DISCUSSION

The unidirectional outflux of Cl^- (J^o_{Cl}) was measured at two different Cl^- concentrations in the outside solution (the *trans*-side) as a function of depolarizing potentials (inside negative). As shown in Table I, the Cl^- outflux is significantly higher when the outside solution is 112 mM NaCl Ringer's solution than when this solution is dilute 20 mM NaCl Ringer's solution or 20 mM NaCl Ringer's solution with sodium isethionate replacement. These results clearly demonstrate that Cl^- outflux is affected by the Cl^- concentration of the *trans*-side at all three measured potentials. Addition of 92 mM sodium isethionate to the dilute 20 mM NaCl solution appears to increase Cl^- outflux somewhat, suggesting that ionic strength of the external solution may have some effect. However, there is a substantial difference in outflux into the

TABLE I

UNIDIRECTIONAL CHLORIDE OUTFLUX

Inside solution was always normal Ringer's solution containing 112 mM NaCl. Fluxes are average values obtained in ten skins for 20 mM NaCl and 112 mM NaCl and in five skins for the solution containing isethionate. Measurements were made at all three PD values in each skin.

Outside solution	J^o_{Cl} ($\mu moles/h/cm^2$)		
PD:	*0*	*−50 mV*	*−100 mV*
20 mM NaCl	0.27 ± 0.06	0.39 ± 0.06	0.76 ± 0.11
20 mM NaCl + 92 mM sodium isethionate	0.44 ± 0.09	0.47 ± 0.08	1.18 ± 0.28
112 mM NaCl	0.71 ± 0.16	1.17 ± 0.15	2.14 ± 0.22

isethionate solution (20 mM Cl^-) and into normal Ringer's solution (112 mM Cl^-) suggesting that the major effect is determined by external Cl^- concentration. This type of behavior in which unidirectional flux of a solute is dependent on its concentration on the "*trans*"-side is typical of membranes exhibiting exchange diffusion. However, if such a mechanism is involved in the present case, there are two rather puzzling points. First, if most of the Cl^- moved through frog skin *via* exchange–diffusion, the primary pathway for Cl^- transport would probably be cellular (as would be the case in carrier-mediated exchange–diffusion[16]), contradicting our previous results[8] that suggested a primary extracellular shunt pathway for transepithelial Cl^- movement. Second, the increase in Cl^- outflux with applied potential would be difficult to reconcile with the traditional notion of electrically neutral exchange–diffusion.

For these reasons, we decided to investigate in more detail the true extent to which exchange–diffusion contributes to Cl^- movement through frog skin. In particular, we felt it essential to examine possible changes in permeability of a shunt pathway (in parallel to the active Na^+ transport path) since previous studies indicated that this permeability could be influenced by external conditions. To do this, we utilized the technique of Mandel and Curran[8] to examine properties of the shunt in the presence of outside solutions of varying Cl^- concentration and ionic strength. This technique employs urea as a tracer for shunt permeability and utilizes the relationship between urea flux and flux of another solute at various applied potentials to evaluate shunt permeability. According to our previous study[8], urea outflux (J_U^o) should be related to Cl^- outflux (J_{Cl}^o) by the following expression (derived from the Nernst–Planck equation with the constant field assumption):

$$\frac{J_{Cl}^0}{J_U^0} = \frac{\alpha_{Cl} c_{Cl}^i}{c_U^i}\left[\frac{-FV/RT}{1-\exp(FV/RT)}\right] \qquad (1)$$

in which $\alpha_{Cl} = P_{Cl}/P_U$ (*i.e.* the ratio of Cl^- and urea permeabilities), c_{Cl}^i and c_U^i are Cl^- and urea concentrations in the inside solution, F is the Faraday, V the applied potential, R the gas constant, and T the absolute temperature.

To test this relationship and evaluate shunt permeability, outfluxes of Cl^- and urea were measured simultaneously as functions of applied PD using different outside solutions. The results immediately suggest that at least a portion of the changes in Cl^- outflux shown in Table I can be explained by changes in permeability of a shunt pathway to Cl^-. For example, at -100 mV, J_U^o was 4.7 ± 0.6 (nmoles/h per cm^2) with dilute 20 mM NaCl outside, 4.9 ± 1.0 with 20 mM NaCl, 92 mM Na isethionate outside and 8.8 ± 1.0 with 112 mM NaCl outside. Thus, the permeability of the shunt path as measured by urea is increased by a factor of nearly 2 when external Cl^- concentration is raised from 20 to 112 mM. A concomitant change in Cl^- permeability would account for part, but not all, of the increase in Cl^- outflux shown in Table I.

Evaluation of Eqn 1 requires a more detailed examination of the relationship between urea and Cl^- fluxes. As shown in Fig. 1, for experiments at -100 mV, there is a linear relationship between Cl^- and urea outfluxes when the external solution is either 112 mM NaCl or 20 mM NaCl. Similar results were obtained at -50 mV and the slopes of the calculated regression lines are given in Table II. The slopes for outfluxes into 112 mM NaCl and 20 mM NaCl differ significantly at -100 mV but

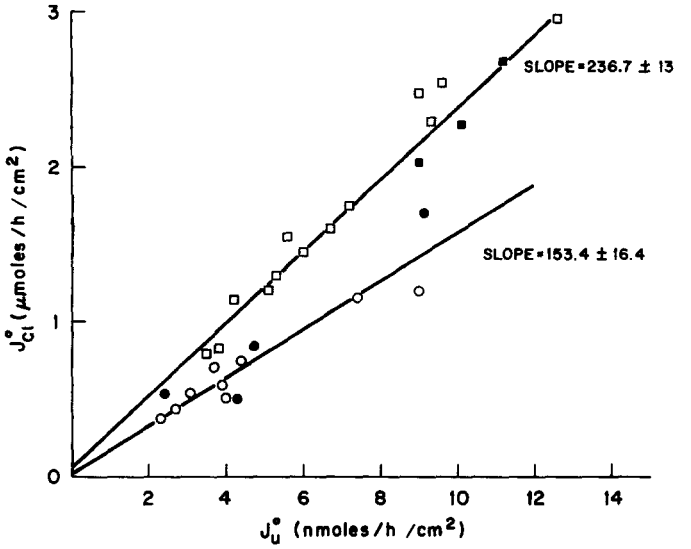

Fig. 1. Unidirectional transepithelial outfluxes of Cl^- *vs* urea at −100 mV. Circles are outfluxes into external solutions with dilute 20 mM NaCl (●, 10^{-4} M ouabain poisoned; ○, unpoisoned). Squares are outfluxes into 112 mM NaCl external solutions (■, 10^{-4} M ouabain poisoned; □, unpoisoned). Each point is the average of two consecutive 20-min flux periods. Slopes were calculated by least squares fit.

TABLE II

LEAST SQUARES SLOPES OF J^o_{Cl} *vs* J^o_U AT DIFFERENT POTENTIALS AND EXTERNAL NaCl CONCENTRATIONS

$[NaCl]_o$	*Slope at −50 mV*	*Slope at −100 mV*
20 mM*	115.5 ± 25.1	153.4 ± 16.4
112 mM**	125 ± 25.6	235.7 ± 13

* $n = 13$.
** $n = 16$.

neither intercept is significantly different from zero. In addition, the slopes appear to be independent of operation of the Na^+ pump since points obtained for ouabain poisoned skins fall on the same lines. The relationship between the slopes for outfluxes into 112 mM NaCl and 20 mM NaCl at −50 mV is unclear because of their large standard errors. All subsequent data analysis was, therefore, performed on the slopes and fluxes at −100 mV.

These results, showing a linear relation between J^o_{Cl} and J^o_U, are similar to those obtained by Mandel and Curran[8], mainly for measurement of inward fluxes. When the skin is bathed in regular Ringer's solution on both sides, $\alpha_{Cl} = 0.95$ at −50 mV and $\alpha_{Cl} = 1.04$ at −100 mV*. Thus, α is independent of PD and Eqn 1 appears

* This value of α_{Cl} is considerably lower than the similar value calculated from the Cl^- influxes *vs* potential by Mandel and Curran[8]. The source of this discrepancy could be in the thicker unstirred layer encountered on the inside than the outside surface of epithelia[17], which could change the actual concentration of ions in the shunt.

suitable to relate the shunt permeabilities of Cl^- and urea. As previously discussed[8], the lack of an effect of ouabain and the applicability of Eqn 1 (which implies a single barrier rather than a series arrangement of two or more barriers) suggest that the transepithelial movements of Cl^- and urea occur through the same shunt pathway.

These results also indicate that the data in Table I can be explained simply in terms of changes in shunt permeability to Cl^-. The values of α_{Cl} obtained with external NaCl concentrations of 112 mM and 20 mM together with the respective average urea fluxes can be used to estimate the ratio of Cl^- permeabilities under these two conditions. At −100 mV, where the data are most accurate, the Cl^- permeability at 112 mM NaCl is 2.9 times the permeability at 20 mM and this is the same as the ratio of the average Cl^- outfluxes under these conditions.

The higher slope of the line relating J^o_{Cl} to J^o_U obtained with 112 mM NaCl than with hypotonic 20 mM NaCl in the external solution is not specifically determined by the external Cl^- concentration but appears instead to be a nonspecific effect of external ionic strength. Addition of 92 mM sodium isethionate to dilute 20 mM NaCl Ringer's solution in the external solution leads to experimental points that fall near the same line as those for 112 mM NaCl (Fig. 2); therefore, Cl^-:Cl^- exchange–diffusion does not seem to play a role in the slope increase observed at the higher external Cl^- concentration. The effect of the added sodium isethionate appears to be due mainly to the change in ionic strength rather than the change in osmolarity since addition of 184 mM sucrose instead of salt to the dilute external solution leads to experimental points that fall nearer the line obtained with the dilute 20 mM NaCl (Fig. 2). This effect of external ionic strength on the J^o_{Cl} *vs* J^o_U slope should be

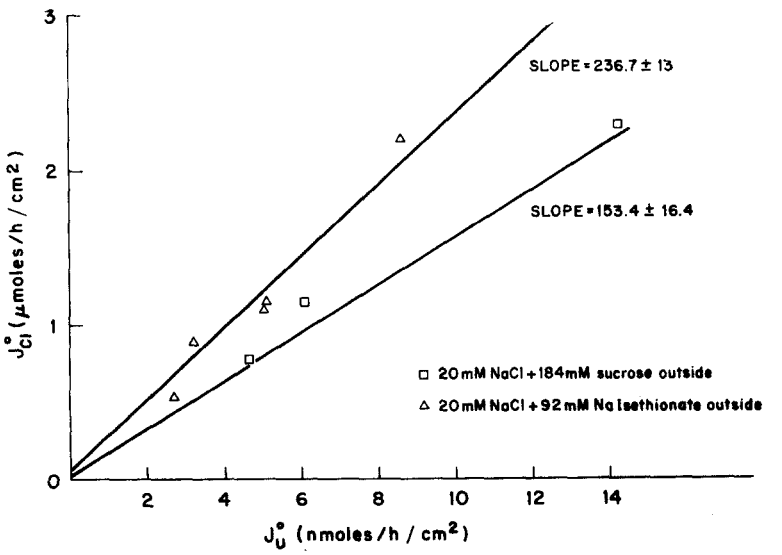

Fig. 2. Effect of ionic strength and hypotonicity on Cl^- *vs* urea outfluxes at −100 mV. Each point is the average of two consecutive 20-min flux periods. The slopes are those calculated in Fig. 1. Addition of 184 mM sucrose to external dilute 20 mM NaCl solution (□) leads to experimental points near the slope obtained at 20 mM NaCl; however, addition of 92 mM sodium isethionate (△) leads to experimental points near the slope obtained at 112 mM NaCl. See text for details.

considered with reference to Eqn 1, since α and/or the voltage-dependent term in brackets could be influenced by ionic strength. The magnitude of α for each solute determines the selectivity of the shunt pathway, a process which may involve the distribution of fixed positive charges or dipoles around a porous structure[8]. The details of this process are unknown and it is, therefore, conceivable that α is affected by ionic strength. On the other hand, if α is assumed to be constant between -50 mV and -100 mV, Eqn 1 predicts the correct values for the slopes (Table II) at 112 mM NaCl but not at 20 mM NaCl. In the latter case, the magnitude of the slope obtained at a clamping potential of -100 mV is such that the effective value of V would be -65 mV and the slope obtained at -50 mV indicates an effective V of -40 mV. Both of these cases represent a deviation from the applicability of Eqn 1 under conditions of low ionic strength. The main assumption in the derivation of Eqn 1 is that the applied potential produces a constant electric field over a single rate determining barrier. This assumption may not be valid under these conditions, since another barrier may become manifest at low ionic strength in series with the one which is rate-determining at 112 mM NaCl. Such a barrier, which may have non-linear resistance characteristics, could be attributed to a structure which offers a higher series resistance and/or causes ionic accumulation or depletion at an interface because of the low ionic strength. The higher solution resistance at 20 mM NaCl could only account for a small portion of this series resistance; however, the external ionic strength may alter the resistance of a structure in series with the shunt pathway causing part of the applied potential to drop across this barrier.

Replacement of 92 mM sodium isethionate for 92 mM NaCl in the external solution at -100 mV, results in the same J^o_{Cl} *vs* J^o_U slope which is obtained with 112 mM NaCl, but the average J^o_U is the same as that obtained at 20 mM NaCl (about half the J^o_U value of that at the higher NaCl concentration). This observation suggests that the permeability of the shunt pathway is selectively dependent on the external Cl^- concentration, in contrast to the slope which does not seem to be Cl^--specific. In our previous study[8], we found that the magnitude of the shunt permeability depends on the external salt concentration without being cation specific; presently, we have demonstrated that this permeability may actually be anion specific.

It is of interest to note that Singer and Civan[5] observed an effect of anions on Na^+ transport activity in toad bladder conforming to a lyotropic series consistent with the presence of fixed positive charges of weak ionic strength in the membrane. On the other hand, the present investigation, suggesting that Cl^- transepithelial movement is through a shunt pathway confirms the findings of Mandel and Curran[6] who suggested that this shunt pathway is probably extracellular and is lined with either fixed positive charges or dipoles with the positive side towards the pore opening. It is tempting to speculate that the similarity in the findings by both groups indicate a close association between the anions passing through the shunt and the active transport of Na^+ but the mechanism that might be involved in such an interaction remains unclear.

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