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THE EFFECT OF *N*-ETHYLMALEIMIDE ON K^+ AND Cl^- TRANSPORT PATHWAYS IN THE LAMPREY ERYTHROCYTE MEMBRANE: ACTIVATION OF K^+/Cl^- COTRANSPORT

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Summary

The effect of the sulphhydryl reagent *N*-ethylmaleimide on the K^+ and Cl^- transport pathways of the lamprey erythrocyte membrane was found to be quite complex. *N*-Ethylmaleimide *inhibited* the Ba^{2+}-sensitive pathway that mediates most of the ouabain-resistant influx of K^+ into the cell under physiological conditions but *stimulated* a Cl^--dependent, Ba^{2+}-resistant K^+ transport pathway that was inhibited by compounds that inhibit Cl^--dependent K^+ transport in the human erythrocyte. *N*-Ethylmaleimide (in most cases) reduced the total influx of Cl^- into the lamprey erythrocyte but (in all cases) introduced a K^+-dependent component into the measured Cl^- uptake; this was explained in terms of *N*-ethylmaleimide having *inhibited* the pathway primarily responsible for Cl^- influx under physiological conditions but having *stimulated* a second, K^+-dependent Cl^- transport pathway. Although the magnitude of the K^+ and Cl^- fluxes stimulated by *N*-ethylmaleimide varied widely between cells from different lampreys, there was, in each individual case, a close similarity between the magnitude of the Cl^--dependent K^+ influx (calculated from the $^{86}Rb^+$ uptake) and the K^+-dependent Cl^- influx; the mean value for the ratio of the former to the latter was 1.01 ± 0.03 ($N=5$). The results are therefore consistent with the sulphhydryl reagent having activated a K^+/Cl^- cotransport system similar to that present in erythrocytes from many mammalian species. This raises the possibility that the lamprey red cell may be a uniquely suitable system in which to study the characteristics of Cl^- transport by this pathway.

Introduction

In the preceding paper it was shown that a small component of the influx of K^+ into erythrocytes from the river lamprey (*Lampetra fluviatilis*) has characteristics similar to those of the K^+/Cl^- cotransport system present in erythrocytes from a number of mammalian species (Kirk, 1991). Although K^+ transport by the mammalian system has been shown to fulfil the criteria for a genuine, coupled K^+ and Cl^- cotransport process (Brugnara *et al.* 1989), the very high Cl^- permeability of the mammalian erythrocyte membrane has precluded the measurement (or

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even the detection) of Cl^- transport *via* this pathway. The Cl^- permeability of the lamprey erythrocyte is many orders of magnitude lower than that of the mammalian erythrocyte (Nikinmaa and Railo, 1987) and this raises the unique possibility of measuring the transport of Cl^- by the putative cotransport pathway in this cell type. The activity of the mammalian K^+/Cl^- cotransport system is normally very low under physiological conditions but increases (*in vitro*) in response to a number of stimuli, the most potent of which is treatment of the cells with the sulphhydryl reagent *N*-ethylmaleimide. The aim of the work reported here was, therefore, to investigate the effects of *N*-ethylmaleimide on both K^+ and Cl^- transport pathways in the lamprey erythrocyte membrane.

Materials and methods

Lampreys

Lampreys (*Lampetra fluviatilis* Linnaeus) were a generous gift from Dr M. Nikinmaa of the Department of Zoology, University of Helsinki, and were captured, transported, maintained and bled as described elsewhere (Kirk, 1991).

Chemicals

N-Ethylmaleimide (NEM) was from Sigma Chemicals (Poole, Dorset). All other chemicals were obtained as described elsewhere (Kirk, 1991).

Erythrocytes

Following collection, the erythrocytes were filtered through a polymer wool column and washed twice in heparinised saline and four times in an ice-cold lamprey Ringer's solution which was constituted using either Cl^- salts (114 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$) or the corresponding NO_3^- salts [114 mmol l^{-1} $NaNO_3$, 1 mmol l^{-1} $Mg(NO_3)_2$, 1 mmol l^{-1} $Ca(NO_3)_2$]. All solutions contained 5 mmol l^{-1} glucose and 10–20 mmol l^{-1} Hepes and were adjusted to pH 7.6 with 10 mol l^{-1} NaOH. In all experiments other than those in which extracellular K^+ was omitted, K^+ was added to suspensions (as either KCl or KNO_3) to give an extracellular concentration ($[K^+]_e$) of 5 mmol l^{-1}. Where appropriate, Ba^{2+} was added to suspensions as either $BaCl_2$ or $Ba(NO_3)_2$.

Influx measurements

$^{86}Rb^+$ and $^{36}Cl^-$ influx measurements were carried out at 11°C using the technique described elsewhere (Kirk, 1991). The influx rates cited were estimated from the uptake of radioisotope during an incubation period that fell within the initial (approximately linear) portion of the influx time course. All experiments were carried out in the presence of ouabain (0.1 mmol l^{-1}), which was added to samples just before transferring them to the incubation bath. Where appropriate, NEM was added to samples at least 15 min prior to the addition of radioisotope. In preliminary experiments it was found that NEM induced a Cl^--independent $^{86}Rb^+$

leak in the membrane when added at concentrations greater than 2 mmol l^{-1}; in the experiments reported here NEM was, therefore, added to samples at no more than 1 mmol l^{-1}.

Statistics

Unless specified otherwise, the results cited represent the mean value ± the standard error of the mean obtained from N separate experiments, where each experiment was carried out using blood from a different lamprey. Differences between mean values were tested for significance using a paired (two-tailed) t-test. In general, the standard errors for the mean influx values obtained under different experimental conditions (e.g. in the presence and absence of a particular reagent) were approximately proportional to the mean value; the flux values (Y, mmol l^{-1} RBC h^{-1}) were, therefore, subjected to a logarithmic transformation of the form $\log(Y+1)$ prior to application of the t-test in order approximately to equalise the variances for the two populations under comparison (Steel and Torrie, 1981).

In those instances in which the results of a single, representative experiment are presented no errors are given; in such cases the standard deviation for the triplicate samples taken from each tube at the end of the flux incubation (in the presence of ouabain and in the absence of other inhibitors) was less than 3 % of the mean value.

Results

$^{86}Rb^+$ influx

Fig. 1 shows the effects of NEM on $^{86}Rb^+$ influx into lamprey erythrocytes in the presence and absence of Ba^{2+}. In Cl^- medium, NEM stimulated a Rb^+ transport pathway which, unlike the pathway responsible for most of the ouabain-resistant K^+ influx into these cells, remained operative in the presence of 2 mmol l^{-1} Ba^{2+}. The magnitude of the NEM-induced K^+ influx (calculated from the $^{86}Rb^+$ uptake) in cells treated with ouabain (0.1 mmol l^{-1}) and Ba^{2+} (2 mmol l^{-1}) varied from 1.5 to 7.0 mmol l^{-1} RBC h^{-1} and averaged 3.5±0.8 mmol l^{-1} RBC h^{-1} (N=6).

The enhanced influx of $^{86}Rb^+$ *via* the pathway activated by NEM was dependent upon the presence of Cl^- in the extracellular solution. For cells suspended in NO_3^- medium there was no significant increase in the Ba^{2+}-resistant $^{86}Rb^+$ uptake following pretreatment with NEM (Fig. 1). There was, however, a 12±4 % (N=3, P<0.10) decrease in the Ba^{2+}-sensitive component of $^{86}Rb^+$ influx, which implies that the Ba^{2+}-sensitive pathway was inhibited by NEM.

The K^+ transport pathway stimulated by NEM was inhibited partially by bumetanide (0.3 mmol l^{-1}) and, more effectively, by the K^+/Cl^- cotransport inhibitor H74 (0.3 mmol l^{-1}; Fig. 2).

$^{36}Cl^-$ *influx*

Fig. 3 shows the effects of H74 and NEM on Cl^- uptake by lamprey erythrocytes. The average Cl^- influx for cells suspended in a normal lamprey Ringer's solution in the absence of inhibitors was $14\pm3\,mmol\,l^{-1}\,RBC\,h^{-1}$ ($N=7$).

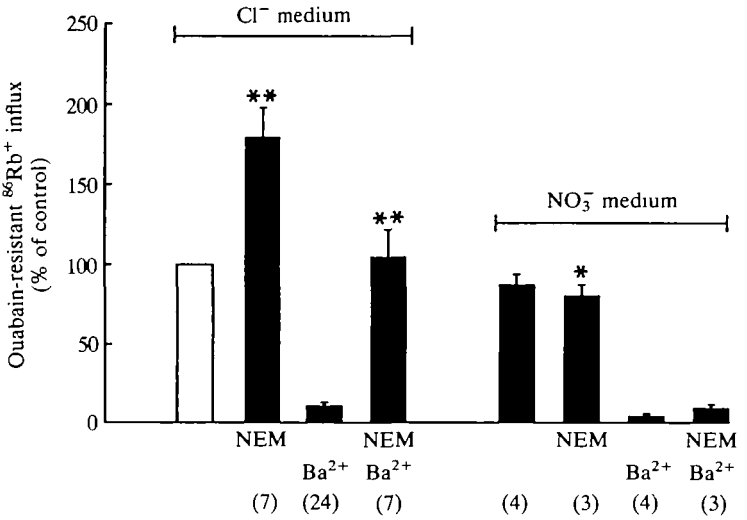

Fig. 1. Effect of *N*-ethylmaleimide (NEM) ($0.3\,mmol\,l^{-1}$) and Ba^{2+} ($2\,mmol\,l^{-1}$) on $^{86}Rb^+$ influx into lamprey erythrocytes suspended in Cl^- and NO_3^- media. $^{86}Rb^+$ influx rates are expressed as a percentage of those measured in the normal lamprey Ringer's solution in the absence of either Ba^{2+} or NEM (open bar). The number of experiments in each case is indicated in parentheses. Asterisks denote a significant difference between the $^{86}Rb^+$ influx in NEM-treated cells and that measured (under otherwise identical conditions) in the absence of NEM (** $P<0.01$; * $P<0.05$). Values are means+S.E.M.

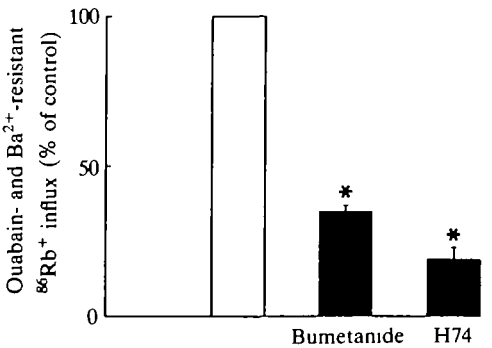

Fig. 2. Effect of bumetanide ($0.3\,mmol\,l^{-1}$; $N=3$) and H74 ($0.3\,mmol\,l^{-1}$; $N=4$) on $^{86}Rb^+$ influx in lamprey erythrocytes pretreated with NEM ($0.3\,mmol\,l^{-1}$). All samples contained ouabain ($0.1\,mmol\,l^{-1}$) and Ba^{2+} ($2\,mmol\,l^{-1}$). $^{86}Rb^+$ influx rates are expressed as a percentage of those measured in the absence of either bumetanide or H74. Asterisks denote a significant difference ($P<0.05$) between the $^{86}Rb^+$ influx measured in the presence and absence of inhibitor. Values are means+S.E.M.

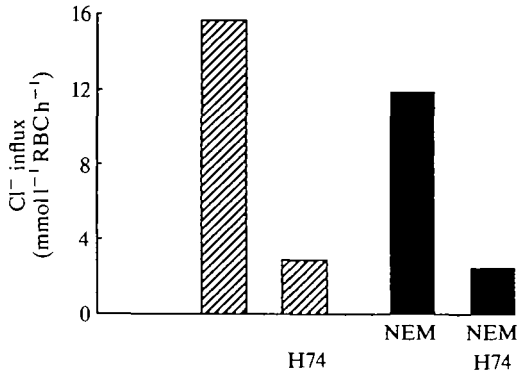

Fig. 3. Effect of H74 (0.3 mmol l^{-1}) on the unidirectional influx of Cl^- into lamprey erythrocytes in the presence (filled bars) or absence (cross-hatched bars) of NEM (0.3 mmol l^{-1}). Cl^- influx rates were calculated from the uptake of $^{36}Cl^-$. The results shown are from a single experiment and are representative of those obtained in at least four similar experiments.

H74 consistently caused a marked inhibition of $^{36}Cl^-$ uptake; in four experiments H74 (0.3 mmol l^{-1}) reduced the $^{36}Cl^-$ uptake by 62±10 % ($P<0.05$) to a mean value of 5.0±1.4 mmol l^{-1} RBC h^{-1}. The results with NEM were more variable. In six out of eight experiments in which cells were pretreated with NEM (0.3–1.0 mmol l^{-1}) there was a substantial reduction (by up to 50 %) in the total $^{36}Cl^-$ uptake. However, in one experiment NEM had little effect on total $^{36}Cl^-$ uptake and in another there was actually a 17 % increase in $^{36}Cl^-$ influx following pretreatment of the cells with NEM. Addition of H74 to cells pretreated with NEM reduced $^{36}Cl^-$ influx to a level that was similar to (and not significantly different from) that measured in cells to which H74 alone had been added; in four such experiments the average Cl^- uptake in the presence of NEM and H74 was 4.5±1.2 mmol l^{-1} RBC h^{-1}, 90±7 % ($P>0.10$) of that in the presence of H74 alone.

The dependence of Cl^- uptake on extracellular K^+ in untreated and NEM-treated cells was investigated and representative results are shown in Fig. 4. Ba^{2+} (2 mmol l^{-1}) was added to all samples to inhibit the membrane conductance to K^+ (Kirk, 1991) and thereby minimise the variation in the membrane potential (E_m) arising from changes in $[K^+]_e$. In the absence of NEM, removal of K^+ from the extracellular solution caused a slight (4±2 %, $N=5$, $P<0.10$) increase in $^{36}Cl^-$ uptake. In cells pretreated with NEM, removal of K^+ significantly reduced Cl^- uptake by 30± 5 % ($N=8$, $P<0.01$), which corresponded to an average decrease of 3.3±0.7 mmol l^{-1} RBC h^{-1} in the unidirectional Cl^- influx.

Coupling of $^{86}Rb^+$ and $^{36}Cl^-$ influx in NEM-treated cells

It is clear from Fig. 1 that pretreatment of lamprey erythrocytes with NEM stimulated a Cl^--dependent K^+ ($^{86}Rb^+$) transport pathway. From Fig. 4 it is evident that NEM pretreatment introduced a K^+-dependent component into the

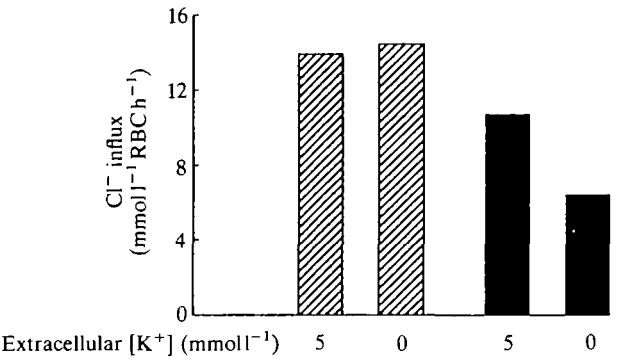

Fig. 4. Effect of extracellular K^+ on Cl^- influx into lamprey erythrocytes suspended in the presence (filled bars) or absence (cross-hatched bars) of NEM (0.3 mmol l^{-1}). Ba^{2+} (2 mmol l^{-1}) was added to all samples to inhibit the $[K^+]_e$-dependence of E_m (Kirk, 1991). Cl^- influx rates were calculated from the uptake of $^{36}Cl^-$. The results are from a single experiment and are representative of those obtained in at least five similar experiments.

Table 1. *Cl^--dependent K^+ influx and K^+-dependent Cl^- influx in NEM-treated cells from five lampreys*

Lamprey	Cl^--dependent K^+ influx (mmol l^{-1} RBC h^{-1})	K^+-dependent Cl^- influx (mmol l^{-1} RBC h^{-1})	K^+ influx/ Cl^- influx
A	3.32	3.26	1.02
B	5.44	6.05	0.90
C	0.90	0.82	1.10
D	0.94	0.89	1.06
E	1.46	1.50	0.97
			Mean 1.01±0.03

K^+ influx was calculated from the measured $^{86}Rb^+$ uptake and Cl^- influx from the measured $^{36}Cl^-$ uptake.

The Cl^--dependent K^+ influx represents the difference between the K^+ influxes measured in Cl^- and NO_3^- media ($[K^+]$=5 mmol l^{-1}, [NEM]=0.3 mmol l^{-1}, $[Ba^{2+}]$=2 mmol l^{-1}).

The K^+-dependent Cl^- influx represents the difference between the Cl^- influxes measured in the presence and absence of 5 mmol l^{-1} K^+ ([NEM]=0.3 mmol l^{-1}, $[Ba^{2+}]$=2 mmol l^{-1}).

NEM, *N*-ethylmaleimide.

measured Cl^- influx. Table 1 shows the magnitudes of the Cl^--dependent K^+ influx and the K^+-dependent Cl^--influx for NEM-treated cells taken from five different lampreys. Although the magnitude of the induced fluxes varied widely between different lampreys, there was, in each individual case, a close similarity between the magnitude of the induced K^+ and Cl^- influxes. The mean value for

the ratio of the Cl^--dependent K^+ influx (calculated from the $^{86}Rb^+$ uptake) to the K^+-dependent Cl^--influx was 1.01±0.03.

Discussion

K^+ transport pathways

The data of Figs 1 and 2 suggest that pretreatment of lamprey erythrocytes with NEM stimulated a Cl^--dependent, Ba^{2+}-resistant K^+ transport pathway that was inhibited partially by bumetanide (0.3 mmol l^{-1}) and almost completely by H74 (0.3 mmol l^{-1}). The properties of this pathway resemble those of the K^+/Cl^- cotransport system that has been described in erythrocytes from many mammalian species (Stewart and Ellory, 1989; Dunham, 1990). K^+ fluxes *via* the mammalian K^+/Cl^- cotransport system are generally small under physiological conditions but increase dramatically in response to treatment of the cells with NEM (Lauf and Theg, 1980; Lauf *et al.* 1984; Stewart and Ellory, 1989). They are abolished by replacement of Cl^- with NO_3^- in the suspending solution and, in human cells, they are inhibited partially by bumetanide (Stewart and Ellory, 1989) and, much more effectively, by H74 (Ellory *et al.* 1990).

In addition to stimulating a Ba^{2+}-resistant $^{86}Rb^+$ flux, NEM inhibited the Ba^{2+}-sensitive pathway that mediates most of the ouabain-resistant K^+ influx into the lamprey erythrocyte (Fig. 1). The characteristics of this pathway are similar to those of a Ca^{2+}-activated K^+ channel (Kirk, 1991) and such channels in other cell types have previously been shown to be inhibited by NEM (Sarkadi *et al.* 1985; Sarkadi and Gardos, 1989).

Cl^- transport pathways

H74 and (in most cases) NEM caused a marked decrease in Cl^- uptake by lamprey erythrocytes, which implies that both compounds inhibited a Cl^- transport pathway in these cells. The inhibitory effects of the two compounds were not additive; Cl^- uptake in the presence of both NEM and H74 was not significantly different from that in the presence of H74 alone (e.g. Fig. 3) and this is consistent with both compounds having inhibited the *same* pathway.

The variability of the effect of NEM on Cl^- uptake (and the observation that in one experiment pretreatment of cells with NEM actually caused an *increase* in Cl^- uptake) might be explained if, as was the case for K^+, NEM inhibited one Cl^- transport pathway and stimulated another, with the relative magnitudes of the Cl^- fluxes *via* these two pathways varying between individual lampreys. Fig. 4 and Table 1 provide evidence in support of this hypothesis. In the absence of NEM (and in the presence of Ba^{2+}), removal of K^+ from the extracellular solution had little effect on Cl^- uptake. However, in cells pretreated with NEM there was a significant reduction of Cl^- uptake on removal of extracellular K^+, which is consistent with the NEM having stimulated a K^+-dependent Cl^- transport system.

Nikinmaa and Railo (1987) have shown previously that the Cl^- permeability of the lamprey erythrocyte membrane is similar to that of a simple lipid bilayer.

However, it is clear from Fig. 3 that well over half of the Cl^- influx was prone to inhibition, which suggests that most of the Cl^- transport across the lamprey erythrocyte membrane was by a facilitated transport system rather than by simple diffusion through the lipid phase. The transport system may have features in common with the volume-activated Cl^- transport pathway of human lymphocytes, which has also been shown to be inhibited by NEM (Sarkadi *et al.* 1985). In human erythrocytes, H74 is a selective inhibitor of the K^+/Cl^- cotransport system; however, it seems unlikely that the H74-sensitive Cl^- influx in lamprey erythrocytes (in the absence of NEM) was attributable to K^+/Cl^- cotransport. The K^+/Cl^- cotransport system is activated by NEM, whereas NEM apparently inhibited the H74-sensitive pathway that mediated most of the $^{36}Cl^-$ influx in the absence of NEM (Fig. 4). Furosemide, a structural analogue of H74, inhibits a number of different Cl^- transport pathways and the same may well be true of H74.

The observation that H74 reduced Cl^- uptake to the same level in NEM-treated cells as it did in untreated cells (e.g. Fig. 3) suggests that, in addition to inhibiting the primary physiological Cl^- transport pathway, H74 effectively inhibited the Cl^- pathway that is postulated to have been stimulated by NEM.

K^+/Cl^- cotransport

The data of Figs 3 and 4 suggest that pretreatment of lamprey erythrocytes with NEM stimulated a K^+-dependent Cl^- transport pathway that was inhibited by H74. This is consistent with the hypothesis that the NEM-induced Cl^- flux was mediated by the K^+/Cl^- cotransport system that was suggested as being responsible for the Cl^--dependent K^+ transport in NEM-treated cells (Fig. 1). If the NEM-induced Cl^--dependent K^+ transport and K^+-dependent Cl^- transport do indeed represent fluxes *via* a common pathway there might be expected to be a close correlation between the magnitudes of these two flux components in NEM-treated cells from individual lampreys. It is clear from Table 1 that this was indeed the case. Despite considerable variability in the absolute magnitudes of the fluxes induced in cells from different lampreys, the ratio of the Cl^--dependent K^+ influx to the K^+-dependent Cl^- influx was, in each individual experiment, close to 1. This is fully consistent with the view that the fluxes were *via* a single pathway. Furthermore, if it can be assumed, first, as has been shown to be the case for the K^+/Cl^- cotransport system of human (Brugnara *et al.* 1985) and sheep (Ellory and Dunham, 1980) erythrocytes, that $^{86}Rb^+$ uptake provides an accurate measure of K^+ influx and, second, that the system does not mediate K^+-independent Cl^-/Cl^- exchange, then the results indicate that the K^+/Cl^- cotransporter operates with a stoichiometry of 1:1.

In summary, the various K^+ and Cl^- transport pathways that are postulated (on the basis of the results presented here and in the preceding paper) to be present in the lamprey erythrocyte membrane are represented schematically in Fig. 5. A significant proportion of the K^+ influx is *via* the Na^+/K^+ pump, which is inhibited by ouabain. Most of the ouabain-resistant K^+ influx is *via* a Ba^{2+}- and amiloride-sensitive pathway, which is inhibited by NEM. Most of the Cl^- influx is *via* a

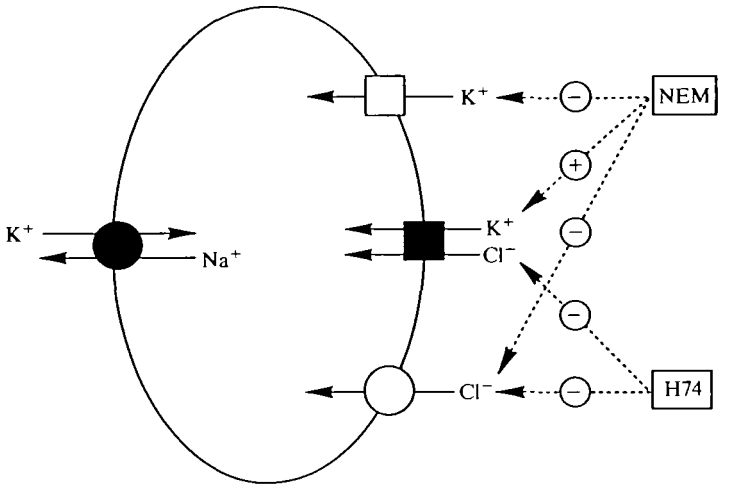

Fig. 5. Schematic representation of a lamprey erythrocyte showing the principal pathways by which K^+ and Cl^- are postulated to enter the cell, as well as the inhibitory (−) or stimulatory (+) effects of NEM and H74 on the pathways. The symbol ● denotes the ouabain-sensitive Na^+/K^+ pump, □ denotes the Ba^{2+}- and amiloride-sensitive K^+ pathway, ○ denotes the principal physiological Cl^- pathway(s) and ■ denotes the K^+/Cl^- cotransport system.

(facilitated transport) pathway, which is inhibited by NEM and by H74. In addition to inhibiting the principal K^+ and Cl^- influx pathways, it is postulated that NEM stimulates a K^+/Cl^- cotransport system which, like that in human erythrocytes, is inhibited by H74.

K^+ transport by the K^+/Cl^- cotransport system in sheep, rabbit and human erythrocytes has been shown to fulfil the criteria for coupled K^+ and Cl^- cotransport (Brugnara *et al.* 1989); however, there has not, as yet, been any measurement of Cl^- transport by this pathway in these cells. Such measurements are made technically different in mammalian erythrocytes by the presence in the membrane of the band 3 protein (capnophorin) which transports Cl^- at a rate many orders of magnitude higher than does the cotransport pathway. In the work reported here, the low Cl^- permeability of the lamprey erythrocyte has enabled the detection of a significant K^+-dependent Cl^- flux and this raises the possibility that the lamprey erythrocyte may be a suitable system in which to study the characteristics of Cl^- transport *via* the K^+/Cl^- cotransport system.

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