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Nature of the cation leak induced in erythrocyte membranes by Kanagawa haemolysin of *Vibrio parahaemolyticus*

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Abstract

Vibrio parahaemolyticus is an important enteric pathogen that produces an exotoxin prepared as Kanagawa haemolysin (KH). Isotope flux techniques were used to analyse toxin action on the basal permeability of human erythrocytes. KH induced a cation leak that was (i) rapid in onset (lag phase < 1 min), (ii) 'pore-like' in terms of kinetic characteristics, and (iii) of high magnitude initially (first 10 min) and then subsequently lower (but still raised with reference to control cells). The susceptibilities of the induced flux pathway to washout in initial and later periods suggested a protracted binding time course for toxin action. Neuraminidase treatment of erythrocytes enhanced both haemolysis and flux induced by KH, suggesting that the affinity of the toxin for the membrane had increased, possibly as a result of additional toxin receptors being unmasked by this enzyme. These results show that KH elevates the basal permeability of human erythrocytes in a complex manner, a process that probably underlies the deleterious effects of this toxin on cellular function.

Keywords: Bacterial toxin; Membrane transport; Hemolysin; Erythrocyte; Neuraminidase; (*V. parahaemolyticus*)

1. Introduction

Vibrio parahaemolyticus is an important cause of diarrhoea [1], especially in Japan and Taiwan where it accounts for 50–70% of cases of food-borne gastroenteritis [2]. The pathogenicity of this organism is thought to depend on its abilities to (i) colonise the intestine, and (ii) produce the thermostable direct haemolysin (TDH) [3]. TDH has been cloned [4] and sequenced [5], and is cytolytic to a variety of cell types [6–10]. Furthermore, TDH causes an increase in short-circuit current across isolated rabbit ileal tissue [11]. Although an understanding of the mechanism of toxin action remains incomplete [12], recent studies using the erythrocyte as a target cell [13–15] suggest that TDH is a pore-forming protein that causes haemolysis by increasing the cation permeability of the cell membrane.

Erythrocytes constitute a useful model system [13–16] to probe toxin mechanisms (e.g. [17,18]). The membrane transport pathways of this cell have been characterised in detail [19,20] allowing the study of toxin effects on the basal 'leak' permeability using transport inhibitors and radio-isotope flux techniques. In this report, a more precise analysis has been performed than previous measurements which have used haemolysis as a measure of toxin action. Cell death appears to be the end result of a complex series of events, some of which are described here.

2. Materials and methods

2.1. Materials

These were from the Sigma Chemical Co. or BDH (both in Poole, Dorset, UK), except for nitrendipine and ^{86}RbCl (10 mCi ml^{-1}) which were from Bayer (Newbury, Berks, UK) and NEN Research, Dupont (UK) (Stevenage, UK), respectively. A standard buffered saline, abbreviated to MBS (concentration in mM): NaCl (145), MOPS (15), glucose (5), pH 7.4 by addition 1 M NaOH was used

Abbreviations: DBP, dibutyl phthalate; HRBC, human red blood cells; Hct, haematocrit; H^{50}, time taken (min) for 50% haemolysis; KH, Kanagawa haemolysin; MBS, MOPS-buffered saline; MOPS, 3-(*N*-morpholino)propane sulfonic acid; PCV, packed cell volume; TDH, thermostable direct haemolysin; Triton X-100, octylphenoxypolyethoxyethanol.

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throughout. Kanagawa haemolysin (KH) is a preparation of purified TDH [15,21,22], and was obtained from Sigma [23] as a lyophilised powder containing approximately

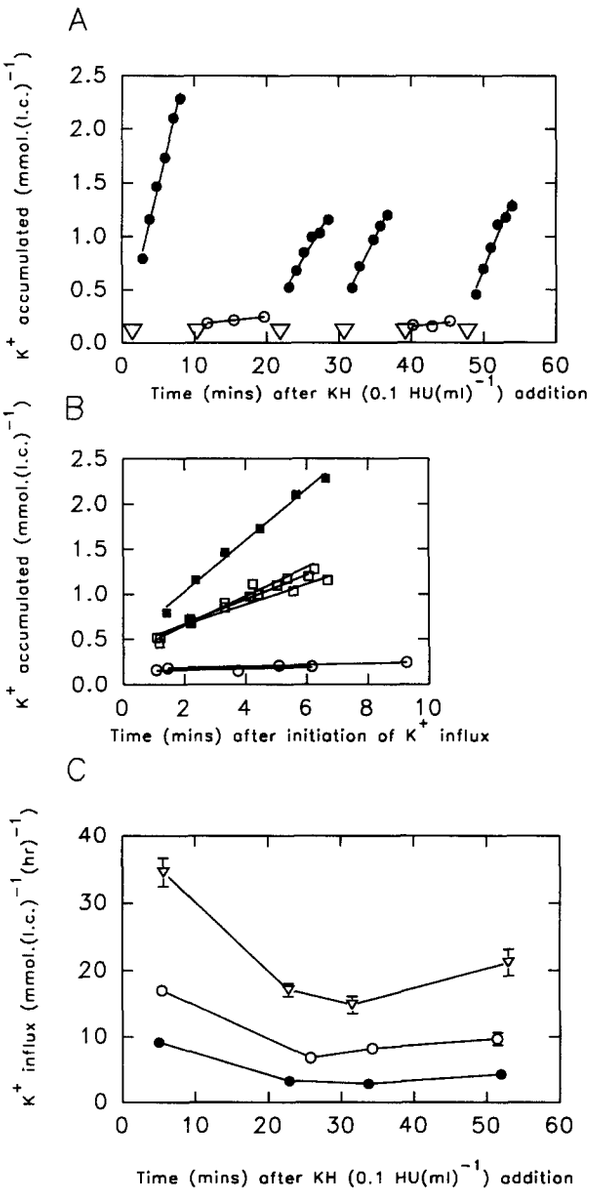

Fig. 1. Extended dynamics of KH-induced inhibitor-insensitive K^+ influx. HRBC were suspended (Hct = 6%) in MBS + sucrose (50 mM) and ouabain, bumetanide and nitrendipine (see Materials and methods). KH (0.1 HU ml^{-1}) was added/not added to the suspension, and immediately transferred (time = 0) to a water bath at 37°C. Subsequently tracer fluxes in cell suspensions (1.17 ml) were initiated with 130 μl K(^{86}Rb)Cl (150 mM) and K^+ uptake ($[K^+]_0$ = 15 mM) was measured over 1–7 min. A: K^+ accumulated plotted against time after KH addition for HRBC treated (●)/untreated (○) by KH after tracer addition at times indicated by ▽. Results of a typical experiment representative of three such experiments. B: analysis of data shown in A; for ease of comparison, results are similarly given as K^+ accumulated, but against time after tracer addition. Results obtained in the first 10 min after KH addition are given as ■; those from subsequent periods are given as □. Results for untreated cells are given as ○. C: results from all three donors are given as K^+ influx calculated by linear regression from data for which typical results are given in A, against the median time of the influx period.

50% protein balanced with Tris/HCl buffer, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and sodium azide (0.05% w/v). One haemolytic unit (HU) is defined by Sigma as that amount causing 50% hemolysis of a 1% erythrocyte suspension in phosphate buffered saline at pH 7.0 after 2 h incubation at 37°C followed by refrigeration for 12–24 h at 4°C. Aliquots of KH (100 HU ml^{-1}) were stored at −22°C.

2.2. Blood

Human blood was obtained either fresh by venepuncture (into heparin or acid citrate dextrose) or as outdated bank blood (Blood Transfusion Centre, John Radcliffe Hospital, Oxford, UK). HRBC were washed (5 ×) by centrifugation (Sorvall RT6000B; 2500 × g, 5 min, 10°C), aspiration (of plasma/buffy coat/MBS) and resuspension in MBS. Haematocrits were obtained (A_{540}) by Drabkin's assay [24].

2.3. Haemolysis

Packed cells were added to MBS containing KH, and haemolysis measured by assaying released haemoglobin [16]. The A_{540} of the supernatant (after centrifugation at 10000 × g, 10 s) was measured, and expressed as percentage total haemolysis. The H^{50} was taken as the time taken for 50% haemolysis to occur [14]. For haemolysis experiments, KH was present at relatively high levels (1 HU ml^{-1}) compared to the K^+ flux experiments (see below) so as to speed up the time course and give significant increases in haemolysis compared to controls (i.e. untreated cells).

2.4. K^+ flux studies

$^{86}Rb^+$ was used as a congener to measure K^+ transport, according to techniques described previously for influx and efflux [25]. Membrane transport inhibitors were present at maximally inhibitory concentrations; ouabain (100 μM; [19]), bumetanide (100 μM; [19]) and nitrendipine (10 μM; [20]) were used to block cation transport by the Na^+/K^+ pump, the Na^+-K^+-$2Cl^-$ cotransporter and the Ca^{2+}-activated K^+ (Gardos) channel respectively. For all these flux experiments, sucrose (50 mM) was used as an osmotic protectant which was confirmed by the lack of haemolysis at the end of the incubation period. The action of KH on K^+ fluxes was more sensitive than on haemolysis, and therefore KH was present at lower doses (0.05–0.1 HU ml^{-1}) than those required for haemolysis experiments.

2.5. Neuraminidase treatment

Washed erythrocytes were suspended (Hct = 30%) in MBS that also contained $CaCl_2$ (8 mM), BSA (0.5% w/v) and 0.42 U ml^{-1} neuraminidase type X (Sigma) from

Clostridium perfringens. Preparations were incubated at 37°C with occasional agitation for 1 h, and then washed twice (3000 × *g*, 5 min) in neuraminidase-free MBS before use [26].

2.6. Data presentation

Data are given as pooled or typical results for $n \geq 3$ experiments, as mean ± SEM for normalised or non-normalised data. Significance values (P) were calculated using Student's unpaired t tests.

3. Results

3.1. Dynamics of pore induction

Fig. 1 shows the magnitude of K^+ influx over an extended time period (~ 55 min), using techniques which circumvented the possible problem of isotope equilibration/backflux by incubating cells with KH for different periods prior to measuring flux. It is clear that during the 10 min subsequent to KH addition, a high K^+ influx was observed. However, flux values obtained over later periods (between 20 and 55 min after toxin addition) though similar to each other, and raised with respect to control cells, were significantly lower than those observed in the first 10 min (Fig. 1B). Although there was a marked donor dependence for the magnitude of induced K^+ influx, the pattern was the same in all experiments (Fig. 1C).

Similar experiments were performed but with the cell suspension being maintained on ice both before and after toxin addition (Fig. 2A, B, C). Immediately after addition of (^{86}Rb)KCl, the aliquot of cell suspension was incubated (37°C, 2 min to allow temperature equilibration), prior to measuring K^+ accumulated at 37°C. Under these conditions (in contrast to Fig. 1), the induced K^+ influx did not decrease with time following addition of toxin. Thus, although toxin may bind at low temperatures [13], it does not appear to induce a flux pathway which progresses through the dynamics characterised in Fig. 1. The dynamics of $^{86}Rb^+$ efflux in response to KH addition are shown for a typical experiment (Fig. 3). A similar pattern was observed: an initial high efflux followed by a phase of reduced efflux (although still increased with reference to controls). These data imply that an effect on membrane potential is unlikely to underly the observed pattern of induced ion movement.

Results shown in Fig. 4 show that KH (0.1 HU ml^{-1}) addition to HRBC at 37°C resulted in the generation of a lesion that allows increased passage of K^+ in a fashion linearly dependent on external K^+ concentration (range 0–30 mM), which is in keeping with the hypothesis that the toxin induces a (non-saturable) pore in the membrane ([13]; see Discussion).

3.2. Nature of the KH-induced pathway

In light of the data indicating a transition from a higher to a lower magnitude of KH-induced cation permeability (Figs. 1 and 3), experiments were performed to investigate the relative susceptibilities of these pathways to washout by centrifugation, aspiration of 0.8 ml supernatant and resuspension (Fig. 5). KH (0.1 HU ml^{-1}) significantly ($P < 0.05$, $n = 3$ for both early and late phases) increased K^+ influx into ouabain, bumetanide and nitrendipine treated HRBC (Fig. 5A) from 0.22 ± 0.036 to 12.21 ± 2.456 (early period; 10 min flux, median time = 11.1 min after KH addition) and 4.22 ± 0.882 (late period; 10 min flux, median time = 39.25 min after KH) mmol · K^+ $(l.c.)^{-1}$ h^{-1}. As noted earlier, there was a marked donor dependence for this effect, which produced large SEMs for

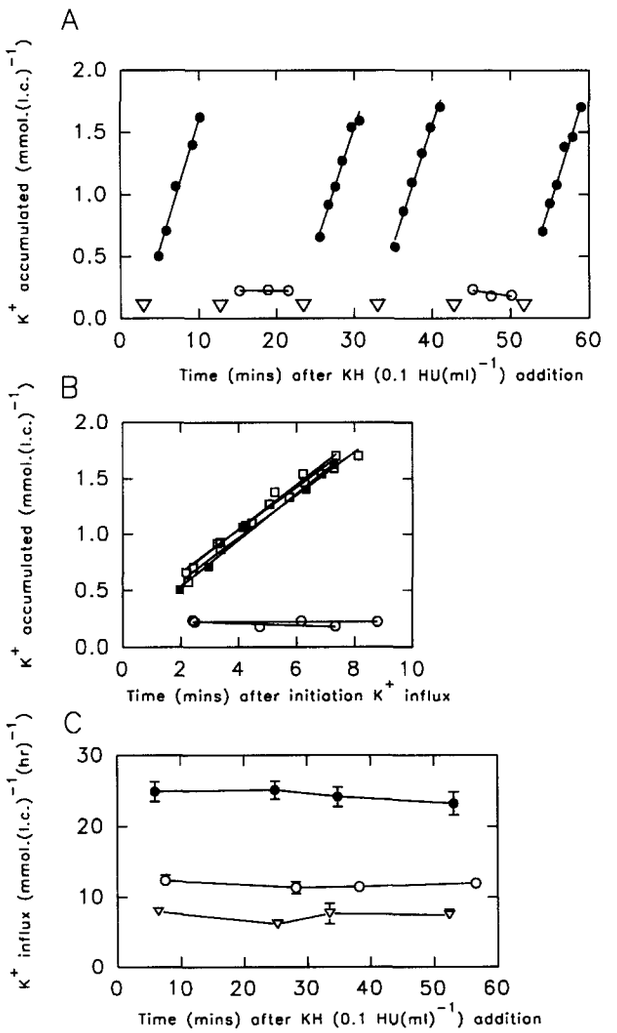

Fig. 2. Dynamics of KH-induced inhibitor-insensitive K^+ influx, when the cells are kept on ice. Details are as for Fig. 1, except the cell suspension was maintained on ice. At the appropriate time, radiotracer was added, and the cell suspensions transferred to 37°C. K^+ uptake was measured over 2–8 min since 2 min was required for temperature equilibration. The symbols used are as for Fig. 1.

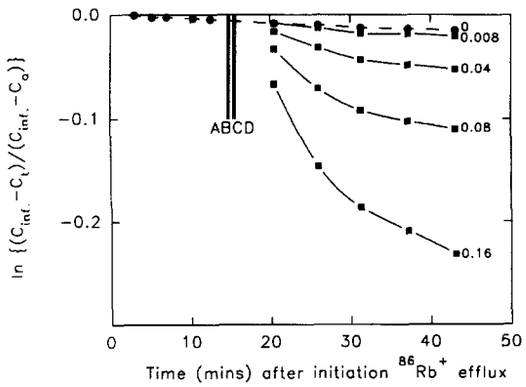

Fig. 3. Dynamics of KH-induced $^{86}Rb^+$ efflux. HRBC were loaded with $^{86}Rb^+$ by overnight suspension (Hct = 80%) in MBS with $^{86}Rb^+$ (10 μCi ml^{-1}) at 8°C, before being washed (centrifugation (10000× g, 10 s)/resuspension) six times in ice-cold MBS with the last resuspension in pre-warmed MBS with sucrose (50 mM) to give a haematocrit of 2%. At the required time, aliquots (1 ml) were centrifuged (10000× g, 10 s) and supernatant (0.75 ml) removed for counting. After the first 5 time points the cell suspension was split five ways and KH added at the indicated dose (HU ml^{-1}).

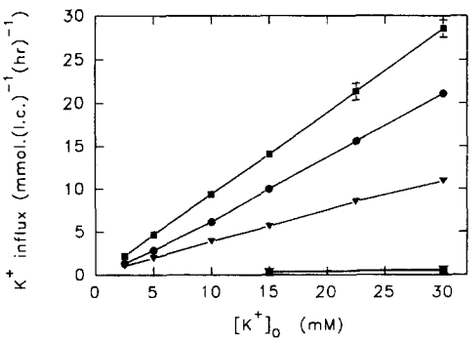

Fig. 4. Dependence of K^+ influx on the extracellular K^+ concentration in KH-treated red cells. HRBC suspended (Hct = 4%) in MBS with sucrose (50 mM) and inhibitors (see Fig. 1) were vortexed either with or without KH (0.1 HU ml^{-1}). Aliquots (0.8 ml) of these cell suspensions were spiked with 200 μl of a solution comprising (K(^{86}Rb)Cl (150 mM)/NaCl (150 mM)), giving a range of extracellular K^+ concentrations. Filled and empty symbols represent data from KH-treated and -untreated HRBC respectively. Each symbol type represents blood from a particular donor.

absolute K^+ values (Fig. 5A); however, the pattern of high and low magnitude phases for toxin induced K^+ influx was confirmed. When results were normalised, the late phase of KH-induced K^+ influx was significantly ($P < 0.001$, $n = 3$) less than the early phase, being reduced to 34.4 ± 0.49% of the early value (Fig. 5B); furthermore the % inhibition of induced K^+ influx by washout was also significantly ($P < 0.005$, $n = 3$) different for early and late phases, being 44.4 ± 3.09 and 10.2 ± 1.55%, respectively (Fig. 5C).

Haemolysis experiments were performed in which HRBC were suspended (Hct = 2%; $t = 0$) in MBS with KH (1 HU ml^{-1}) and incubated as 0.7 ml aliquots. One set was centrifuged (10000 × g, 10 s) and resuspended (no aspiration or replacement) every 10 min during the time course (negative controls (KH-free systems) yielded < 2% haemolysis over an equivalent time course). H^{50} values

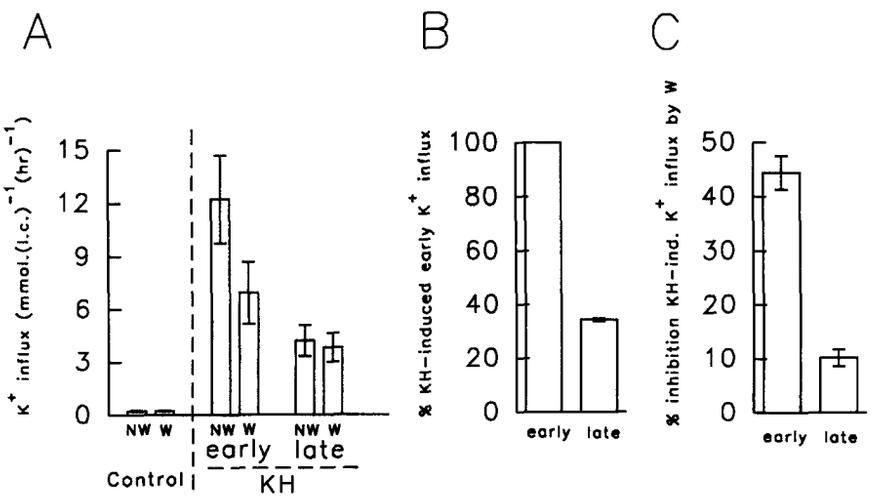

Fig. 5. Sensitivities of KH-induced K^+ influx to washout. HRBC were suspended (Hct = 5%) in MBS with sucrose (50 mM) and inhibitors and prewarmed to 37°C, with, or without KH (0.1 HU ml^{-1}) at time = 0. Aliquots (0.9 ml) were centrifuged (10000 × g, 10 s) at 2 min (early) and 30 min (late) after KH addition. One preparation (W) had 0.8 ml supernatant removed and replaced with KH-free suspending solution (as above) whereas the NW preparation was not subjected to such treatment. The HRBC of these preparations were vortexed back into suspension and incubated at 37°C. K^+ influx was measured over a 10 min flux period for which the median time was 11.10 and 39.25 min for the early and late preparations, respectively. Fluxes were initiated by the addition of 100 μl K(^{86}Rb)Cl (150 mM) ($[K^+]_0 = 15$ mM). W and NW = washed and not washed, respectively. A: K^+ influx for untreated and KH-treated HRBC; B: relative magnitudes of (NW) early and late phase fluxes; and C: percentage inhibition of K^+ influx by washout. Results are from $n = 3$; ± SEM.

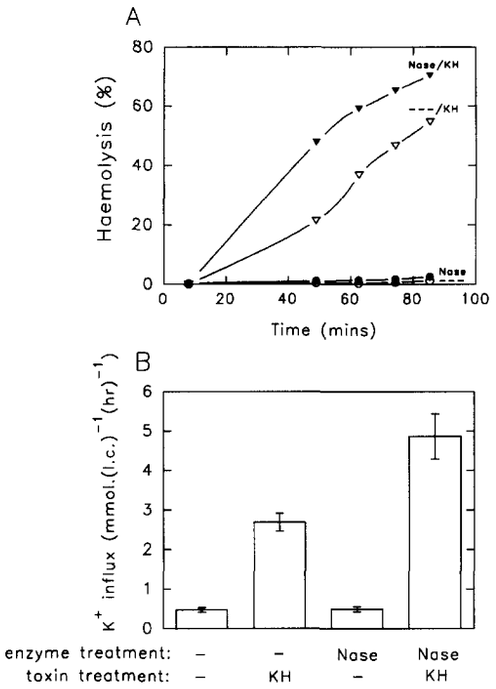

Fig. 6. Effect of neuraminidase treatment on (A) the rate KH-induced haemolysis, and (B) KH-induced K^+ influx. HRBC were treated as detailed in Materials and methods. A: haemolysis time courses (at 37°C) were followed for HRBC suspensions (initial Hct = 2%) in MBS, in the presence or absence of KH (1 HU ml^{-1}). Triangles and circles represent haemolysis in the presence or absence of KH, respectively. Filled and empty symbols represent cells preincubated in the presence/absence of neuraminidase, respectively. B: K^+ influx was measured in HRBC treated with inhibitors and suspended ($2.3 \leq$ Hct (%) ≤ 3.3) in MBS + sucrose (45 mM). Erythrocytes were pretreated for 1 h with BSA and Ca^{2+} (see text) with or without neuraminidase. Fluxes were initiated, in the presence/absence of KH (0.05 HU ml^{-1}), by the addition of 100 μl K(^{86}Rb)Cl (150 mM) to HRBC suspensions ($[K^+]_0 = 15$ mM). Results from one experiment representative of three such identical experiments.

did not differ significantly, being 110.7 ± 26.03 (control) and 111.8 ± 29.84 min (successive centrifugation/resuspension cycles). This suggests that once bound, toxin is not released by centrifugation to reinitiate the high influx phase of its activity.

3.3. Involvement of membrane structures in toxin action

Previous evidence suggested that TDH action is mediated by neuraminidase-sensitive gangliosides in the cell membrane [27,28]. Neuraminidase treatment of HRBC [26] was therefore examined as a prospectively protective treatment against KH. In fact neuraminidase pretreatment increased the rate of haemolysis (Fig. 6A), significantly ($P < 0.001$, $n = 3$) decreasing the H^{50} to 64.8 ± 0.37% of the control value. Moreover, osmotic fragilities (in the absence of toxin) were not found to differ significantly between neuraminidase-treated and untreated cells. The osmolalities just capable of causing 50% haemolysis (initial Hct = 2%) were 132.7 ± 1.30 (neuraminidase-treated) and 132.3 ± 1.59 (control) mosmol kg^{-1}. Neuraminidase treatment caused a significant ($P < 0.05$, $n = 3$) increase in subsequent KH (0.05 HU ml^{-1})-induced K^+ influx (from 2.69 ± 0.225 (control) to 4.86 ± 0.572 (neuraminidase) mmol · K^+ $(l.c.)^{-1}$ h^{-1}) into cells ($2.3 <$ Hct (%) < 3.3) inhibited by ouabain, bumetanide and nitrendipine, whilst having no effect on inhibitor-insensitive influx into cells free from toxin (Fig. 6B).

4. Discussion

These results show that KH causes an increase in the basal cation leak of the red cell membrane. The onset of increased cation flux is rapid (lag phase < 1 min), and the induced pathway is initially (approximately the first 10 min) of high magnitude. However, subsequently (~ 20–55 min after toxin addition), K^+ influx, though still raised compared with control cells, is decreased to a relatively constant level (Fig. 1). As far as the authors are aware, this pattern of induced influx has not been shown for any other toxin, and it is intriguing to speculate as to what type of biophysical process might account for the observed dynamics.

In a previous study, TDH binding (or absorption) was suggested to be temperature-dependent [13]. Sakurai et al. [6] found that some TDH binding did occur at 4°C, a result that was interpreted by Freer [29] as meaning that the binding step is temperature-*independent*, although no such claim was made. Whether or not binding occurs at 4°C, there is no progression through the early to the late phase magnitude influx, when toxin-exposed cells are kept on ice (Fig. 2).

Although band 3 function would almost certainly prevent the induced pathway from altering membrane potential, it was not inconceivable that an increased cation permeability might depolarise the membrane, lessening the electrical driving force favouring cation influx – and hence account for the observed dynamics. However, a similar pattern of flux magnitude was observed in efflux experiments (Fig. 3) suggesting that it is the cation permeability per se that changes with time.

Honda et al. [13] proposed that TDH was a pore-forming protein on the basis of electron micrographic and haemolytic activity. However, pore-like activity remained unsubstantiated in terms of alterations to membrane transport. Fig. 4 shows KH-induced K^+ influx with respect to extracellular K^+ concentration. In the 0–30 mM range there was no evidence of saturation kinetics, supporting the contention that the lesion is 'pore-like'. In the present study we have characterised some aspects of the cation leak induced by KH. It should be remembered that the lesion might be an anion-selective pore, with some cation permeability, rather than a exclusively cation-selective pore. This is worthy of further study, but probably not in erythrocytes because of the very high anion fluxes medi-

ated by the band 3 anion exchanger [19] which would obscure any changes to basal anion permeability arising from the action of KH.

The nature of the different phases (early and late) of induced influx (Figs. 1 and 3) was examined further by assessing the effects of washout (Fig. 5). The late phase (median time = 39.25 min after KH addition) was markedly reduced compared with the early phase (median time = 11.1 min after KH addition). Furthermore, a much higher percentage of the early phase flux was inhibited by washout. It has been proposed in an earlier report [14] that binding of KH occurs over a protracted period, despite the rapidity of onset of increased influx. It is likely therefore that the increased proportion of influx susceptible to washout in the early phase is due to washout not of membrane-bound entities but rather of aqueous KH. Given that it is a permeability change rather than a membrane potential effect that underlies the change of magnitude between early and late phases, and that toxin binds for a protracted period during the early phase, then it is tempting to speculate that prior to the formation of the relatively stable and constant magnitude later phase 'pores', there is a transitory (early) phase that is of relatively increased permeability.

Sakurai et al. [6] speculated that the high pressures encountered in centrifugation might cause bound TDH to be released from the membrane. Since the pattern of KH-induced K^+ influx is such that it is higher initially and lower subsequently, an experiment was performed to test the effect of a centrifugation/resuspension (in the same solution) on haemolytic rate. If bound TDH was released from the membrane by centrifugation, then on resuspension, it would be expected to instigate a lesion of initial phase properties, i.e. mediating a larger cation flux than that occurring during the later phase. Haemolytic activity would therefore be expected to increase. However the treatment had no effect on the rate of KH-induced haemolysis, suggesting that such centrifugation was unlikely to cause release of bound entities, and further strengthening the assertion that the binding phase is longer than 1–2 min.

Takeda et al. [27,28] showed that incubation of TDH with ganglioside preparations decreased the haemolytic activity of the toxin. Furthermore, if the ganglioside mixture was pretreated by neuraminidase then its protective effect was abolished. Further experiments implicated the neuraminidase-sensitive gangliosides G_{D1a} and G_{T1} as the TDH-binding entities important in the TDH–cell interaction. The effect of neuraminidase treatment of target cell (HRBC) on subsequent susceptibility to KH was examined (Fig. 6). The rate of KH-induced haemolysis was increased rather than decreased by this treatment, and appeared to be due to a raised KH-induced leak rather than an increased non-toxin-induced leak or enhanced osmotic fragility. There are two possible explanations for this finding. Neuraminidase could unmask a class of receptor for KH, thereby augmenting the action of KH at a given concentration. Alternatively, the enzyme could 'clean' the membrane surface thereby increasing the accessibility of the toxin for the erythrocyte membrane. If *V. parahaemolyticus* were able to elaborate a protein with neuraminidase-like activity, then leak generation would be an interesting point of synergy in the pathogenic process. However, for 16 pathogenic strains of *V. parahaemolyticus* tested, all were neuraminidase-negative [30]. There is thus no evidence that the bacteria produce neuraminidase and TDH in concert. The red cell is clearly a valuable system for the further assessment of the putative roles of endogenous membrane proteins and glycolipids as receptors for TDH and other toxins. Indeed Yoh et al. [31] have used ^{125}I-labelled TDH to demonstrate specific and saturable binding of toxin to rabbit erythrocytes, finding a rapid toxin-binding phase (< 1 min) at 37°C.

Mutant TDH-derived toxins have been generated by in vitro mutagenesis [32,33]. One mutant toxin (designated R7), with a single amino acid substitution at glycine 67, has been shown to bind to human erythrocytes but not permeablise the membrane [32], suggesting that the steps of binding and leak generation are functionally and structurally separable.

Thus, in the present work, the application of isotope flux techniques to the erythrocyte as a target cell has revealed fundamental features of KH action. Such perturbations of cation transport may indicate pathophysiological alterations which underly the cellular dysfunction associated with *V. parahaemolyticus*.

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