pH-Dependent channel activity of heterologously-expressed main intrinsic protein (MIP) from rat lens

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Abstract Wild-type rat lens main intrinsic protein (MIP) was heterologously expressed in the membrane of *Spodoptera frugiperda* (Sf21) cells using the baculovirus expression system and in mouse erythroid leukaemia cells (MEL C88). Both MEL and Sf21 cell lines expressing wild-type MIP were investigated for the conductance of ions using a whole cell patch clamp technique. An increase in conductance was seen in both expression systems, particularly on lowering the pH to 6.3. In Sf21 cells, addition of antibodies to the NPA1 box resulted in a reduction of current flow. These results suggest that MIP has pH-dependent ion channel activity, which involves the NPA1 box domain. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Baculovirus; Mouse erythroid leukaemia cell; Lens; Patch clamping; Cataract; Eye

1. Introduction

Main intrinsic protein (MIP) is a 26 kDa protein found exclusively in the membranes of lens fibre cells. It was first isolated as the most abundant protein of the fibre cell membrane [1], and since then the cDNA has been cloned and sequenced [2]. Screening of cDNA libraries revealed a wide variety of organisms that possessed homologous membrane protein showing 30–60% sequence similarity with MIP, including the aquaporins (AQP1) and glycerol facilitators. Aquaporins can be further divided into the true aquaporins, which only transport water and the aquaglyceroporins, which transport water and glycerol. The model by Gorin and colleagues proposed that the protein crossed the membrane six times with both the N- and C-termini being cytoplasmic. It was also proposed that the highly conserved NPA (Asn-Pro-Ala) regions were at either side of the membrane. In nearly all cases MIP consists of a two-fold repeat [3] which is thought to have arisen as a result of gene duplication. Each of the repeats contains an NPA box repeat (NPA1 or NPA2), that has been highly conserved throughout evolution, from prokaryotes to vertebrates [3–5]. The first repeat corresponds to the first exon of the *mip* gene whereas the second repeat corresponds to exons 2–4 providing further evidence that the *mip* gene has resulted from gene duplication. Regulation of *mip* expression is thought to involve the transcription factor Sp3 [6]. MIP has been found to undergo selective proteolysis during cataractogenesis and ageing. The product of proteolysis is a 22 kDa protein (MIP22) that has been cleaved at the C-terminus; loss of which appears to affect the regulation of the protein [7,8]. The highest levels of MIP22 are found in the nucleus of the lens, which contains the oldest fibre cells [9]. There is also evidence that the N-terminus undergoes proteolytic cleavage at residues 2–8, 29, 35 and 37 and that truncation of the N- and C-termini was seen in human lenses as young as 7 years [10].

Expression of MIP in oocytes has been shown to increase the osmotic water permeability of the cells by approximately two-fold [11–13]. However this is significantly lower than the 42-fold increase achieved by introduction of AQP1, the 28 kDa homologous water channel of erythrocytes [14]. Comparison of single channel water permeabilities of AQP1 and MIP showed that MIP was again significantly lower than that of AQP1 [14,15], showing that MIP was a poor transporter of water and there may be some other transport function allocated to this protein. It has been suggested that MIP may be involved in the metabolism of glycerol acting as an activator of glycerol kinase [16] by allosteric interaction with the enzyme in a similar fashion to GlpF (the glycerol facilitator protein). Other members of the aquaporin family are also known to be involved in the metabolism of glycerol – GlpF [17] and FPS1, a glycerol facilitator of yeast [18]. The evidence of a current found in planar lipid bilayers incorporating MIP has been found to be substantially greater than that measured in the normal lens [19,20] and although the results in the planar bilayers have been reproducible the relevance to normal lens function still remains uncertain.

The work presented here, using whole cell patch clamping, has allowed channel activity of the heterologously-expressed protein to be evaluated. We have demonstrated the potential importance of the NPA1 box in pH-dependent channel activity of mammalian MIP.

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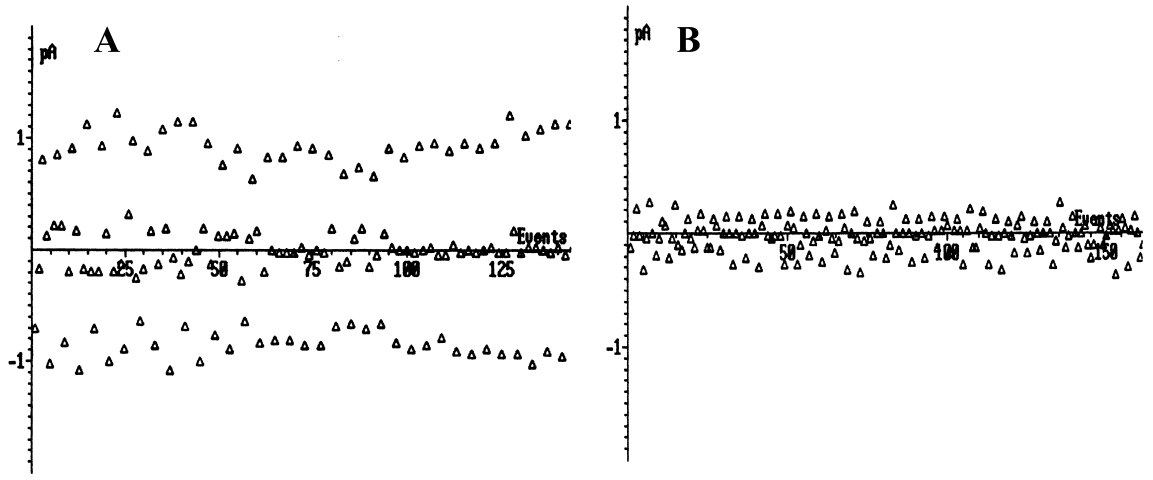

Fig. 1. MELratmip cells whole cell patch clamped with an alternating current of −40, 0, 40 V applied. An increase in current flow can be seen in the cells expressing MIP (in A) compared to the control cells MELnv in B.

2. Materials and methods

2.1. Heterologous expression of rat MIP in murine erythroid leukaemia (MEL) cells and Spodoptera frugiperda (Sf21) insect cells using the baculovirus system

These procedures, and all the molecular biology and electrophoresis, were carried out as previously described [21–27]. pRATMIP was the plasmid containing wild-type rat MIP, while pNV was the control plasmid with no MIP.

2.2. Electrophysiological studies (MEL cells)

Recombinant MEL cells (transfected with pRATMIP and pNV) were induced, immobilised on polylysine (Sigma) coated 22 mm diameter coverslips (Chance Propper Ltd) in 35 mm petri dishes. Electrophysiological studies were carried out using a Leitz Diavert microscope together with a Leitz electrode and remote control. Electrodes with a resistance of 5–10 MΩ were pulled on a two-stage puller (Sutter P30). The whole cell patch clamp technique [28] was used to record channel activity of cells expressing the MIP gene. Cells were bathed in external buffer (120 mM NaCl, 4 mM KCl, 10 mM $CaCl_2$, 11 mM $MgCl_2$, 10 mM HEPES). The pH of the extracellular buffer was either 7.0 or 6.3. Micropipettes contained intracellular buffer (110 mM KCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, pH 6.9). Rabbit anti-MIP (HNPA1) was used in channel blocking (10 μg/ml) in external buffer pH 7.0. Rabbit anti-sheep IgG (10 μg/ml) in the same buffer was used as a control. Voltage and current recordings were obtained using the List L/M-EPC-7 (List Medical, Darmstadt, Germany) patch clamp amplifier, with low pass filtering at 10 kHz. Data were digitised through a HAMEG digital storage scope HM208-1401 Plus and stored on computer. Data analyses were performed using the VCLAMP and PATCH suite of the CED-Electrophysiology Package (V. 6.0).

2.3. Electrophysiological studies (Sf21 cells)

Sf21 cells were seeded onto 22 mm coverslips (Chance and Propper Ltd) in 35 mm dishes at a density of 1×10^6 cells/ml. Cells were infected at a multiplicity of infection (MOI) of 10 with either Ac-MIPN or wild-type virus AcMNPV and incubated for 24 h at 28°C. Electrophysiological studies of changing external pH and channel blocking were carried out as set out above. Lowering of intracellular pH was achieved by replacing NaCl in the external buffer with NH_4Cl. Cells were perfused with external buffer containing NH_4Cl for 10 min, after which the NH_4Cl external buffer was replaced with NaCl buffer. The intracellular pH falls below normal for a prolonged period and resulted in further membrane depolarisation. Results were recorded as set out above. Investigations of chloride channel activity were performed using cells infected as above bathed in an external buffer of 120 mM Na acetate, 4 mM KCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, with a micropipette containing an intracellular buffer of 105 mM *N*-methyl-D-glutamine Cl, 1.2 mM $MgCl_2$, 70 mM D-mannitol, 10 mM HEPES, 1 mM EGTA. Cells were perfused with the low chloride external buffer for 5 min and then the buffer replaced by 120 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, results were recorded as above.

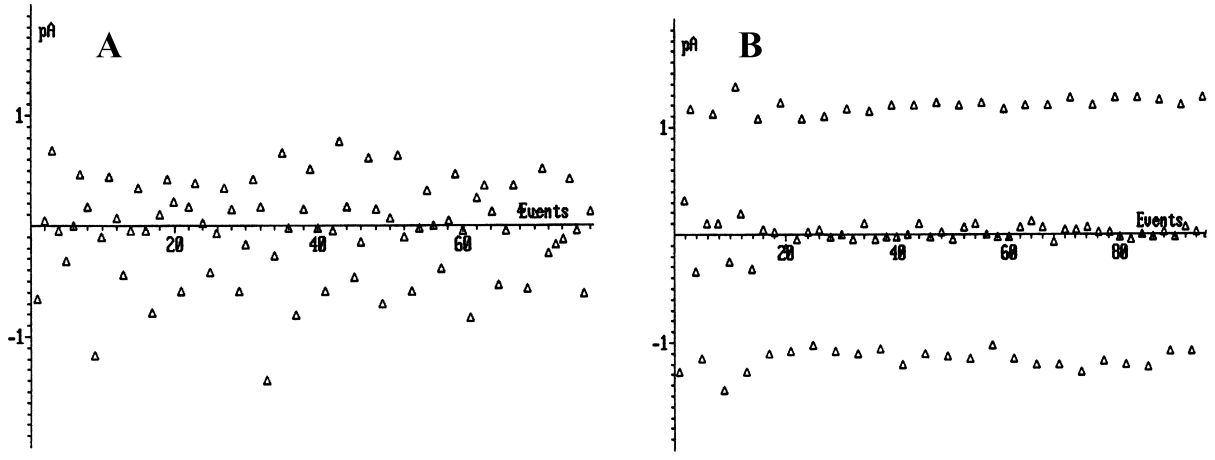

Fig. 2. MELratmip cells whole cell patch clamped with alternating current (−40, 0, 40 V). A: MELratmip cells perfused with external buffer at pH 7. B: MELratmip cells with external buffer change to pH 6.3 showing an increase in conductance.

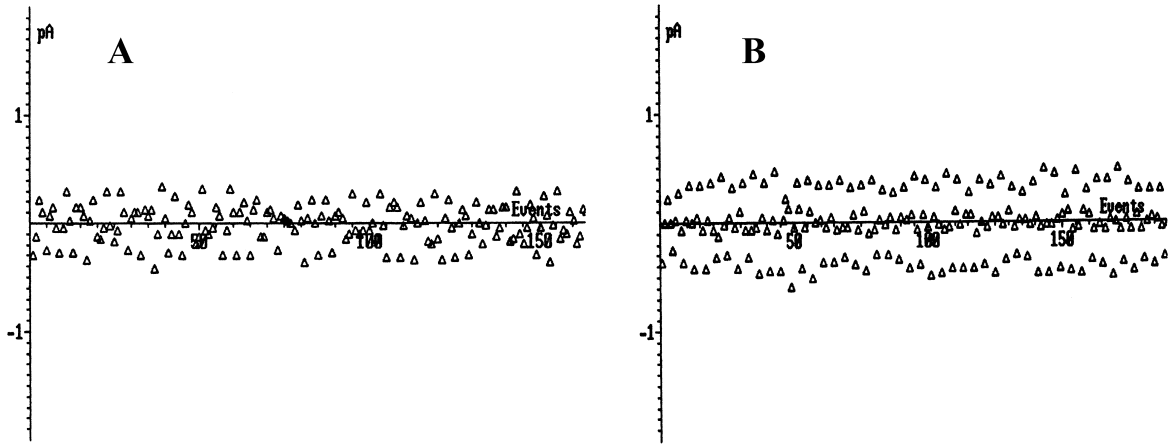

Fig. 3. MELnv cells whole cell patch clamped with an alternating current of −40, 0, 40 V applied. A: MELnv control cells perfused with external buffer at pH 7. B: MELnv control cells perfused with external buffer pH 6.3 showing no increase in current flow on lowering pH.

3. Results

3.1. Patch clamping studies of wild-type MIP in MEL cells

Protein production was induced by the addition of 2% DMSO to growth media and experiments performed 5 days post-induction [21]. Cells were immobilised on polylysine coated coverslips. Cells were whole cell patch clamped, perfused with an external buffer of pH 7 or pH 6.3 and an alternating current applied (−40 V, 0 V, and 40 V). Fig. 1A shows an increased current flow (0.5 pA) in cells expressing MIP compared with the control cells containing vector alone at pH 7 (Fig. 1B), showing that the recombinant protein has increased ion conductance within the cell. When the pH was lowered to 6.3 an increase in current (1.3 pA) was noted in MIP expressing cells, on increasing to pH 7 again the current was reduced to baseline levels (Fig. 2A, B). No changes in current were noted in control cells (Fig. 3A, B). The change in current may be due to a conformational change brought about by the fall in pH that has either increased the size/shape of the channel or changed charges on residues within the pore allowing an increased number of ions to cross the membrane. From the results obtained it would appear that MIP in this expression system has ion channel activity.

3.2. Patch clamping studies of wild-type MIP in Sf21 cells

Preliminary whole cell patch clamping studies were performed on Sf21 cells infected with either wild-type virus (AcMNPV) or recombinant virus (AcMIPN) to investigate

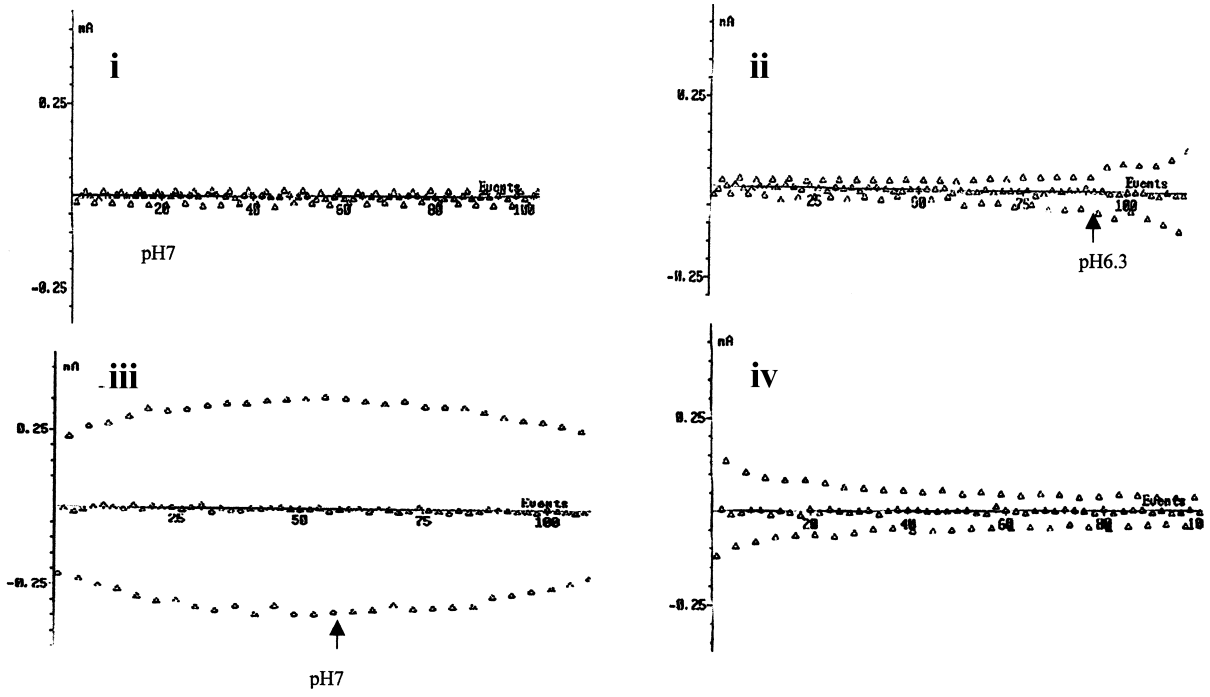

Fig. 4. Shows the effects of lowering pH on Sf21 cells expressing MIP. (i) shows the effect of perfusing the cells with buffer at pH 7, on lowering the pH of the buffer to 6.3 an increase in conductance can be seen (ii, iii). On raising the pH to 7 a decrease in current was seen (iii, iv). Arrows indicate the point at which pH was changed.

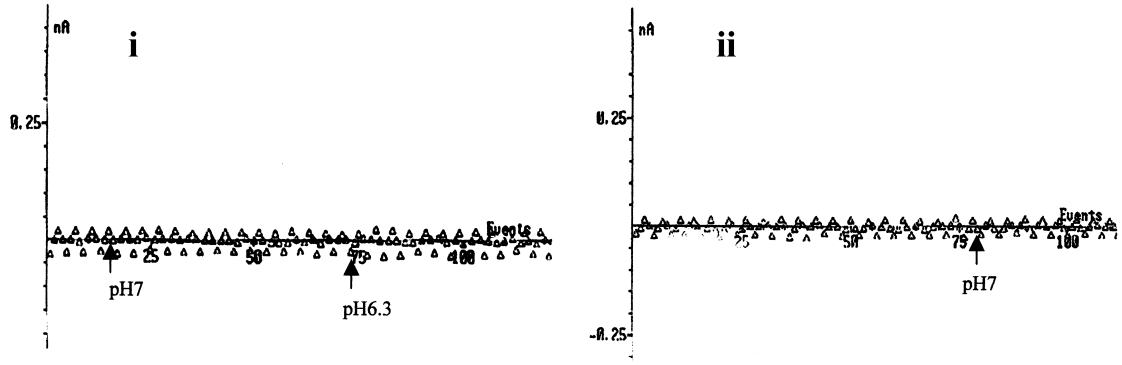

Fig. 5. Shows the effects of changing pH on Sf21 cells infected with wild-type virus. In (i) the pH is lowered with no change in conductance, increasing the pH to 7 has no effect on the level of conductance (ii). An arrow indicates the point at which pH was changed.

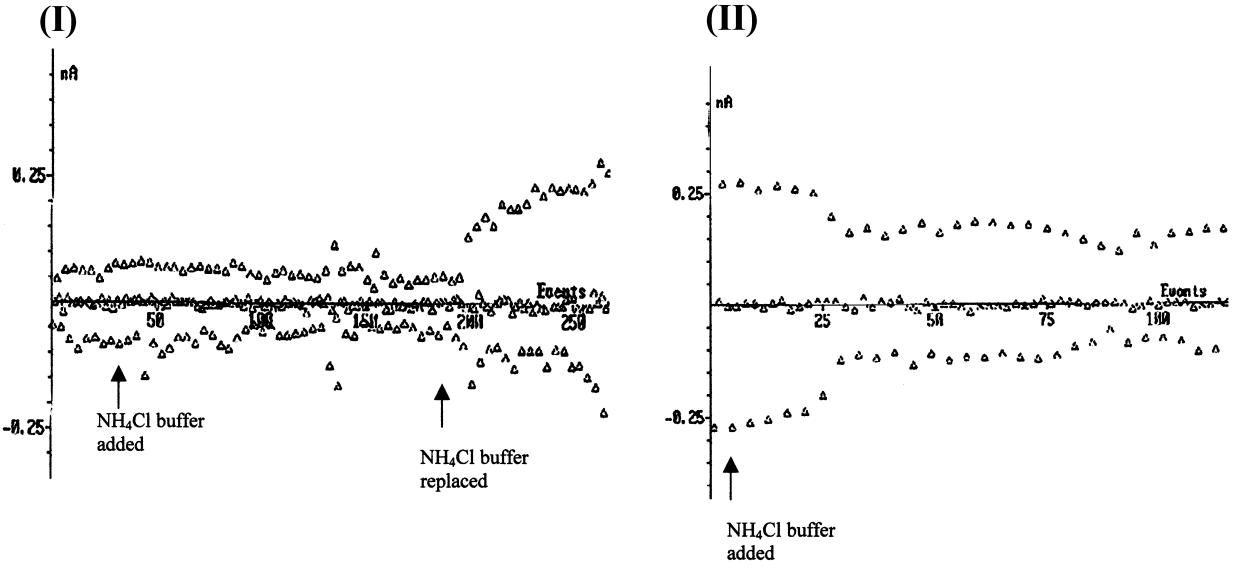

Fig. 6. (I) Sf21 cells expressing MIP were perfused with an external buffer containing NH_4Cl, after incubation the buffer was replaced with one containing no NH_4Cl. On replacing the external buffer the internal pH fell and an increase in conductance was seen. (II) NH_4Cl buffer was again added and a reduction in current was observed.

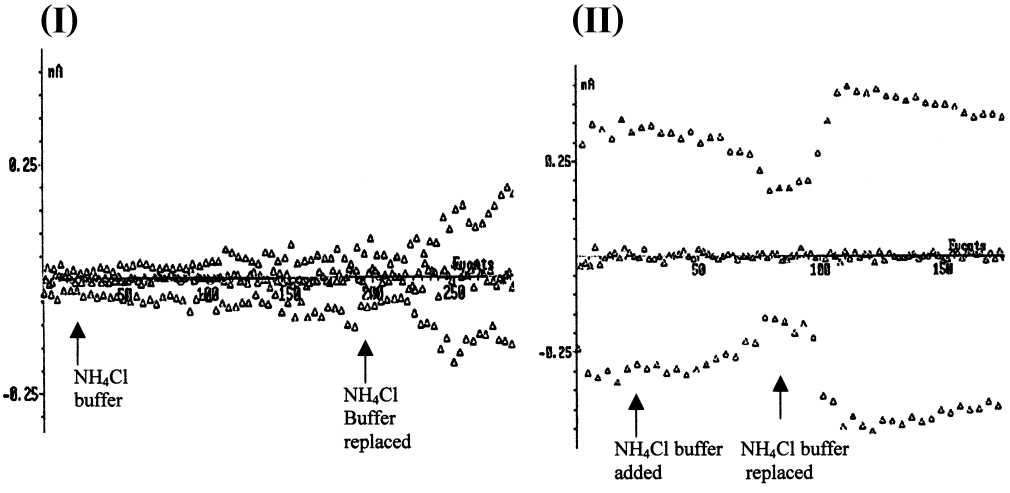

Fig. 7. (I) Sf21 cells infected with wild-type virus were perfused with an external buffer containing NH_4Cl, after incubation the buffer was replaced with one containing no NH_4Cl. On replacing the external buffer the internal pH fell and an increase in conductance was seen. (II) NH_4Cl buffer was again added and a reduction in current was observed, replacing the NH_4Cl buffer resulted in an increase in current.

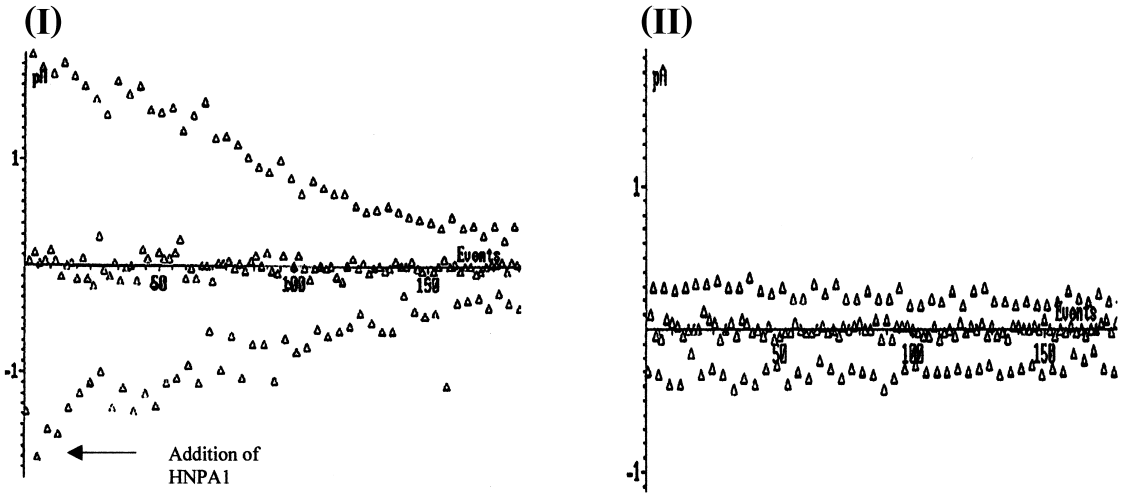

Fig. 8. (I) Sf21 cells expressing MIP perfused with an external buffer containing the antibody HNPA1, following incubation with the antibody a reduction in current was observed. (II) Following incubation with antibody cells were perfused with external buffer at pH 6.3. No increase in current was observed.

the channel activity of the recombinant protein. Sf21 cells were patch clamped as described above, perfused with an external buffer of pH 7 or pH 6.3 and an alternating current applied (−40, 0, 40 V). Figs. 4 and 5 show the affect of lowering pH on Sf21 cells infected with AcMIPN and AcMNPV viruses. Lowering the external pH from 7 to 6.3 resulted in an increase in current from 0 to 0.35 nA in Sf21 cells infected with AcMIPN that could be returned to baseline levels by increasing the external pH to 7. No change in current was noted in control cells. Lowering the internal pH of the cells was achieved by bathing the cells in external buffer containing NH_4Cl, which causes intracellular rise of pH. When the external buffer is replaced by one containing no NH_4Cl and pH 7 the cells pH falls below normal for a prolonged period and results in further membrane depolarisation. Figs. 6 and 7 show the results of intracellular acidification of Sf21 cells, although a change in current can be seen in cells expressing MIP, the same can be seen in control cells. This suggested that Sf21 cells have a channel protein that is activated when the cell becomes acidotic. As there was no evidence that expression of MIP increased the levels of current observed in the cells this would suggest that MIP was not involved in the maintenance of cell pH or activated by low intracellular pH. The increase in current observed when the extracellular pH was lowered suggested that MIP might be activated in response to a fall in external pH. Bassnet [29] found that in lenses large effluxes of chloride ions resulted from treatment with NH_4^+, but these changes were not the result of increased chloride channel permeability but the action of a neutral anion exchange mechanism that is activated by pH.

Addition of HNPA1 to cells expressing MIP resulted in a reduction in current from 1 pA to 0.25 pA (Fig. 8). Cells were washed with external buffer pH 6.3 to remove any unbound antibody and also to re-activate channel activity. On lowering pH, channel activity could not be re-established. When the control antibody was added to MIP expressing cells a reduction in current was again noted however this could be reversed by washing the cells with external buffer of pH 6.3 (Fig. 9). The blockage of channel activity by the antibody HNPA1 suggests that amino acid residues in this region may be involved in the channel activity of MIP. Although non-specific blockage may have occurred due to the large size of the molecule this was taken into account in the control experiment where the IgG molecule was capable of blocking channel activity but on washing could be removed.

4. Discussion

MEL cells expressing wild-type MIP had a higher conductance than control cells and this could be further increased by a reduction in pH. MIP has previously been shown to have ion channel activity in lipid bilayers [30] and in *Xenopus oocytes* [16].

Patch clamping studies of the wild-type protein in Sf21 cells showed that a channel could be activated by lowering the pH from 7 to 6.3, this affect was not seen in cells infected with wild-type virus. Intracellular acidification of cells infected with

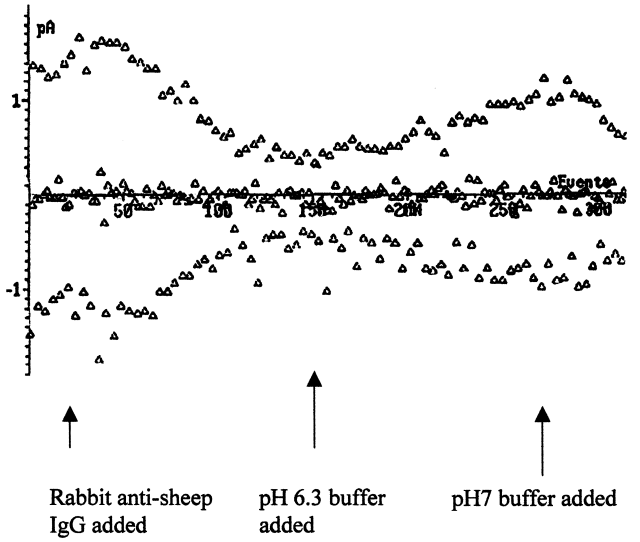

Fig. 9. Sf21 cells expressing MIP perfused with an external buffer containing the control antibody (rabbit anti-sheep IgG), following incubation with the antibody a reduction in current was observed. Cells were then perfused with external buffer of pH 6.3, an increase in current was observed unlike cells expressing MIP. On addition of pH 7 buffer a further reduction in activity was noted.

AcMIPN or AcMNPV showed no difference between control and MIP expressing cells, however there was an increase in conductance in all cells suggesting that Sf21 cells contain an endogenous pH gated ion channel. Channel activity was blocked by the addition of antibody HNPA1 to the external buffer bathing the cells. Following incubation with the antibody channel activity could not be re-established by lowering the pH to 6.3. Control cells expressing MIP and incubated with rabbit anti-sheep IgG were also found to have a reduction in channel activity, however this could be reversed with the addition of external buffer at pH 6.3. Na^+ channels in liposomes containing detergent extracted proteins from pig lenses could be blocked by the addition of anti-MIP antibodies but channels were not blocked by the addition of anti-α-crystallin or anti-vimentin antibodies [30]. Our results suggest that the amino acid residues in the NPA1 region may be involved in channel activity.

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