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PF4, a FMRFamide-related peptide, gates low-conductance Cl^- channels in *Ascaris suum*

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Abstract

Here we describe the actions of the peptide Lys-Pro-Asn-Phe-Ile-Arg-Phe-NH_2, or PF4, on inside-out membrane patches ($n = 164$), recorded from vesicles derived from *Ascaris suum* somatic muscle cells. We observed numerous, small-amplitude Cl^- channels in the membrane patches. The conductance of the Cl^- channels ranged from 1.09 to 7.07 pS, the open probability (P_{open}) ranged from 0.047 ± 0.015 (mean ± S.E.M.) at 0 μM PF4 to 0.156 ± 0.026 at 0.1 μM PF4. The channel mean open time was more variable and prolonged at negative potentials than when the membrane patch was clamped at positive potentials: at 0.03 μM PF4, the mean open time (± S.E.M) at −80 mV was 522 ± 333 ms; at +80 mV, it was 25 ± 7 ms. When patches were isolated from the parent vesicle, there were no changes in channel characteristics, suggesting that the channels function without the involvement of cytoplasmic components. Similarly, the channel characteristics were not affected by the G-protein inhibitor, guanosine-5′-*O*-(2-thiodiphosphate), indicating that the ion channels do not require a G-protein to function. These data indicate that the PF4-activated Cl^- channels function independently of intracellular signal transducers and are, therefore, directly gated by PF4.

Keywords: *(Ascaris)*; Nematode; Cl^- channel; FaRP; PF4

1. Introduction

In the face of increasing incidence of drug resistance among parasitic nematodes (Waller, 1994), there is growing need for anthelmintic drugs with novel mechanisms of action (Thompson et al., 1996). Receptors for FMRFamide-related peptides, or FaRPs, may present a suitable target for new anthelmintics (Geary et al., 1999). The first FaRP to be recognised was originally isolated from a mollusc (Price and Greenberg, 1977). Since then, numerous FaRPs have been identified in invertebrates, including 18 from *Ascaris suum*, the parasitic nematode that was used as an experimental model in this study (for a review, see Davis and Stretton, 1996). A few related peptides have been found in vertebrates, but those that have been isolated differ pharmacologically from the invertebrate FaRPs (for a review, see Davis and Stretton, 1996; Yang and Majane, 1990). This suggests that compounds directed specifically at invertebrate FaRP receptors would be safe to administer to humans, livestock and companion animals.

PF4 is a FaRP that was originally isolated from the free-living nematode, *Panagrellus redivivus* (Maule et al., 1995b). It has the sequence Lys-Pro-Asn-Phe-Ile-Arg-Phe-amide, and is a potent muscle relaxant when applied to tissue preparations made from the parasitic nematode, *A. suum* (Maule et al., 1995a,b; Holden-Dye et al., 1997). The relaxation response to PF4 is dependent on chloride ions and is associated with cell membrane hyperpolarisation (Maule et al., 1995a), with Cl^- channels in the muscle cell membrane being intrinsic to the response (Holden-Dye et al., 1997). PF4 hyperpolarises somatic muscle cell membranes, producing an effect similar in appearance to the response to γ-amino butyric acid (GABA) (Maule et al., 1995b), an inhibitory neurotransmitter in nematodes (Del Castillo et al., 1964). However, despite the fact that PF4 and GABA both open Cl^- channels and cause rapid hyperpolarisation of *Ascaris* somatic muscle cell membrane, they

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do not act on the same receptor (Maule et al., 1995a; Holden-Dye et al., 1997). The work presented here describes the Cl^- channels that open in response to PF4 application and shows that they are distinct from those that open in response to GABA. Furthermore, we present evidence that PF4 is a ligand that directly gates Cl^- channels.

2. Materials and methods

2.1. Sources of Ascaris

A. suum were obtained from three UK collection sites. Intentionally infected pigs raised at Ridgeway Science, Gloucester, England, were slaughtered, the worms collected and sent to us, arriving within 24 h of slaughter. Incidental *Ascaris* infections in pigs slaughtered at Sandyford Abattoir, Paisley, Scotland, and Stevenson and Co., Cullybackey, Northern Ireland, were collected and sent in the same way. After arrival at the laboratory, worms were maintained at 25 °C in Lock's solution that was changed twice daily (composition in mM: NaCl 154, $CaCl_2$ 2.1, KCl 5.6, $NaHCO_3$ 1.8, glucose 5.6).

2.2. Vesicle production

In order to patch clamp the somatic muscle cell membrane, the method employed by Martin et al. (1990) was used to generate vesicles. After transfer to a perspex recording chamber, the vesicles were washed three times with filtered bath solution (composition in mM: CsCl 35, $C_2H_3O_2Cs$ 105, $MgCl_2$ 2, HEPES 10, EGTA 1; pH was adjusted to 7.2 with CsOH).

2.3. Patch pipettes

The patch pipettes were made from Garner USA Glass (type 7052) using a PB-7 Micropipette puller (Narishige, Japan). The pipettes were coated with Sylgard, carefully applied to cover the shank and as much of the tip as possible without damaging or occluding the open end. The tip apertures were fire-polished and the pipettes were filled with filtered pipette solution (composition in mM: CsCl 140, $MgCl_2$ 2, HEPES 10, EGTA 1).

2.4. PF4 and other reagents

PF4 was provided by Pharmacia Animal Health, Kalamazoo, USA, where it was manufactured using standard, solid-phase Fmoc chemistry protocols. PF4 was synthesised on an Applied Biosystems 430A peptide synthesiser (Applied Biosystems, Forrest City, CA, USA) using solid-phase Fmoc chemistry and fast MOC synthesis cycles supplied by Applied Biosystems (Fields et al., 1991). Fmoc-protected amino acids were purchased from Applied Biosystems. The peptide was purified by reverse phase HPLC on a 4×20-cm Delta Pak C18 column (Waters Associates, Milford, MA) using a Waters Delta-Prep 400 chromatographic system, with peptide detection by UV at 218 nm. The gradient employed was 10–40% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 30 ml/min. The identity of PF4 was confirmed by amino acid analysis and mass spectroscopy on a ^{252}Cf plasma desorption time-of-flight instrument (Bio-Ion Model 20, Applied Biosystems).

PF4 was dissolved in distilled water and stored as a 1 mM solution at −20 °C. Some 10-μl aliquots were defrosted and diluted with pipette solution to a final concentration of 0.3–0.003 μM as required. In the absence of data on the in vivo concentration of PF4 (or analogous FaRP) in *A. suum*, this concentration range was based on the effective doses used by other workers (Maule et al., 1995b; Holden-Dye et al., 1997).

All other reagents were obtained from Sigma Aldrich, Poole, UK.

2.5. Patch clamp recordings

The patch pipette was connected to the headstage of an Axon 200B Integrating Patch Clamp (Axon Instruments, USA). Using the classical patch clamp technique (Hamill et al., 1981), a vesicle-attached patch was clamped and channel activity was recorded (digital tape recorder, DTR-1205, Biologic Science Instruments). Experiments were carried out with and without PF4 in the pipette solution to establish a dose–response relationship. Recordings were made from patches in both the vesicle-attached and isolated configurations (Hamill et al., 1981). To investigate G-protein involvement in the response to PF4, a series of recordings were made with the addition of the nonhydrolyzable GDP analogue, guanosine-5′-*O*-(2-thiodiphosphate) (GDP-β-S), to the bath solution. The GDP-β-S was used at a concentration of 100 μM (Eckstein et al., 1979; Green et al., 1994).

2.6. Data collection

Experimental records were selected for digitisation and analysis if they conformed to the following criteria: low electrical noise (trace did not exceed 0.5 pA peak to peak noise) and absence of large amplitude calcium-activated Cl^- channels (Thorn and Martin, 1987). The records were filtered at 1 kHz (Bessel 3-pole active filter, custom-built) and digitised (Digidata 1200 Series Interface, Axon Instruments) prior to analysis. A PC with Clampex 7 software (Axon Instruments) was used to measure the characteristics of the ion channels that opened in response to PF4.

2.7. Data analysis

For each membrane patch, the duration of channel openings and the number of conductance levels were recorded

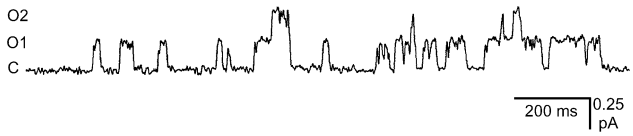

Fig. 1. Channel record from a typical patch clamp experiment in which two channels were active. The recording was made at +120 mV with 0.1 μM PF4 in the patch pipette. The signal was filtered at 1 kHz prior to digitisation, and at 100 Hz before analysis.

over a voltage range within ± 150 mV. From these data, the conductance, reversal potential, probability of channel opening (P_{open}) and the mean open time of the ion channels were calculated. According to the simplest model of ion channel distribution, it was assumed that the channels under observation were distributed evenly over the surface of the muscle cells and, therefore, over the area of any given vesicle. Since the portion of a vesicle that was patch clamped was selected randomly, membrane patches, on average, were representative of the muscle cell membrane from which they were derived. Thus, each patch was assumed to contain the average number of ion channels, although the number of active channels (that is, channels that were recorded to be opening and closing) varied according to the experimental conditions.

Due to the abundance of the channels in the preparation, recordings frequently contained multiple channel openings (see Fig. 1). In order to determine the number of channels active in any patch, it was necessary to recognise the base line (C in Fig. 1), which required that recordings were of sufficient length to give a reasonable likelihood of observing all levels of channel activity. Since each opening is assumed to be independent of others occurring in the same or neighbouring channels, the duration of channel openings is binomially distributed (Colquhoun and Hawkes, 1983). The probability of simultaneous channel opening is given by the following equation:

$$P_{(r)} = \frac{N!}{r!(N-r)!} P_o^r (1-P_o)^{N-r}$$

where $P_{(r)}$ is the probability that r channels are open, N is the total number of channels present, r is the number of channels open at the moment of observation and P_o is the P_{open} of the channel type. Using the equation, the minimum recording period was calculated to reduce the likelihood of underestimating the number of channels present in a membrane patch, assuming that there were numerous channels present in the patch (≤ 10) that had a high P_{open} (≤ 0.5).

The probability of channel opening is the proportion of time that a channel spends in the open state, and is calculated by the following equation:

$$P_{open} = \frac{(1t_1) + (2t_2) + \ldots (nt_n)}{nT}$$

where P_{open} is the probability of channel opening, t_1 is the total time spent at level 1 during the channel recording (O1 in Fig. 1), t_2 is the total time spent at level 2 (O2 in Fig. 1), n is the total number of levels of opening in the recording, T is the length of the recording.

3. Results

Small-amplitude Cl^- channels were observed frequently in recordings made in the presence of PF4 in the patch pipette (Fig. 1). The ion channels showed no evidence of desensitisation throughout a recording period of 10–15 min. In this section, we show that the channel openings did not appear to be second-messenger mediated and that the channel behaviour was consistent with direct gating by PF4. Of the 164 membrane patches that were successfully clamped, 112 showed channel activity on visual inspection; 42% of control patches, 82% of patches exposed to PF4. A total of 77 patches yielded results that were sufficiently clear to digitise and analyse further. The results are expressed as mean ± S.E.M.

3.1. Channel conductance

For each single channel recording, the current amplitude was plotted against transmembrane potential; a typical example is shown in Fig. 2. The current–voltage plots showed that conductance of the ion channels ranged from 1.09 to 7.07 pS ($n = 36$). In the representative inside-out patch recording made in nonsymmetrical Cl^- solutions, shown in Fig. 2, the PF4-induced reversal potential was −28 mV. The mean value of the PF4-induced reversal potential was −24.7 ± 3.9 mV ($n = 36$), which is close to −33 mV, the Cl^- reversal potential predicted by the Nernst equation. The response to PF4 has previously been shown to be dependent on chloride (Maule et al., 1995a), and to involve chloride passage through the somatic muscle cell membrane (Holden-Dye et al., 1997).

The discrepancy between the observed and calculated reversal potential may be explained if the channel is also permeable to acetate ions, according to the Goldman–

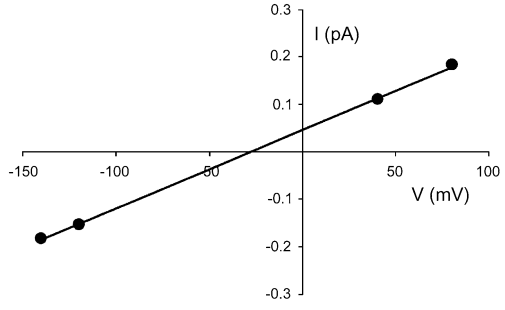

Fig. 2. Plot of current vs. voltage from a typical experiment in which the patch pipette contained 0.1 μM PF4 and current recorded between membrane potentials of −140 and +80 mV. The R^2 value for the line (fitted by linear regression) was 0.99, the conductance (gradient of the line) was 1.65 pS and the reversal potential (abscissa intercept) was −28 mV.

Hodgkin-Katz equation (Hille, 1992). The equation predicts that acetate ions have a relative permeability of 0.13 when compared to chloride ions, thus the presence of caesium acetate in the bath solution would alter the PF4-induced reversal potential. Carboxylate ions, including acetate, have been shown to be permeant in a calcium-activated Cl^- channel of *A. suum* (Valkanov et al., 1994).

3.2. Concentration-dependent behaviour

The low conductance (1–7 pS) channels opened spontaneously, i.e. in the absence of PF4, but both the proportion (%) of patches showing channel activity and the probability of channel opening (P_{open}) were found to increase in a concentration-dependent fashion with addition of peptide (Fig. 3). The Michaelis–Menten equation gave an EC_{50} of 0.001 μM PF4. P_{open} was independent of the potential difference across the patch. In the membrane patches studied, P_{open} ranged from 0.047 ± 0.015 at 0 μM PF4 to 0.156 ± 0.026 at 0.1 μM PF4 ($n = 38$ patches).

There were insufficient data to determine whether mean open time of the channel was related to the concentration of PF4, but channel conductance was independent of dose.

3.3. Mean open time

Fig. 4 shows open time histograms from a representative patch, clamped at positive and negative potentials. In this example, the patch pipette contained 0.003 μm PF4 and the mean open time was 173 ms at −120 mV; 25 ms at +100 mV. In general, the mean open time was more variable and longer at negative potentials than when the membrane patch was clamped at positive potentials. For example, with 0.03 μM PF4 in the pipette solution, mean open times ranged from 522 ± 333 ms at −80 mV to 25 ± 7 ms at +80 mV. The mean open times at −80 mV and +80 mV were significantly different ($P < 0.01$).

Multiple levels of channel opening (such as Fig. 1) were unsuitable for measuring mean open times of individual channels. The mean open time of the small amplitude Cl^- channels was calculated from records with only one level of channel opening. The majority of single channel records (76%, $n = 28$) produced open time histograms that were best fitted with a single exponential curve, signifying that there was one detectable open state for the channels under investigation.

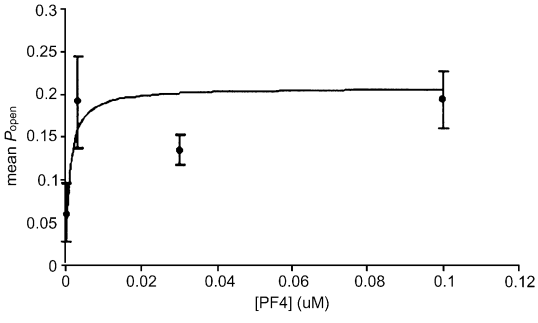

Fig. 3. Relationship between probability of channel opening and concentration of PF4 in the patch pipette. The line illustrates the Michaelis–Menten equation fit to the data points: the EC_{50} was 0.001 μM PF4 and the response was corrected for the P_{open} at zero concentration; $n = 40$ patches.

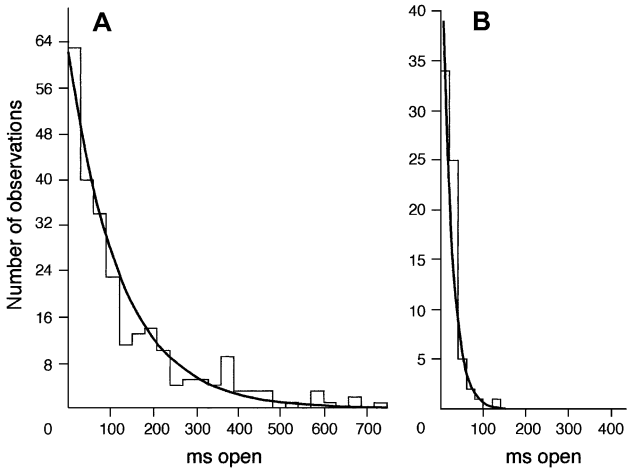

Fig. 4. Histogram of open times from a representative membrane patch (0.003 μM PF4) fitted with a single exponential curve. (A) Channel openings recorded at −120 mV, mean open time 173 ms. (B) Same patch at +100 mV, mean open time 25 ms.

3.4. Involvement of second messengers

Patch isolation had no effect on the channel characteristics. Even after a period of 20 min (to allow intracellular components to disperse), the channels continued to open in a concentration-dependent fashion. There was no significant difference in the P_{open} between vesicle-attached and isolated patches (paired *t*-test, $P > 0.25$, $n = 10$). The lack of effect of isolation indicated that the ion channel function was independent of intracellular constituents. To investigate the possible involvement of membrane delimited G-proteins in the channel opening, the effects of the G-protein inhibitor GDP-β-S were tested. With 100 μM GDP-β-S added to the bath solution, patch activity was monitored before and after patch isolation. In the vesicle-attached configuration, the large, charged molecules of GDP-β-S should not pass through the membrane and act on the intracellular surface, hence, the before-isolation recordings provided a control for the isolated patches. When the patches were isolated from the vesicles and exposed to the GDP-β-S, the P_{open} was unaffected ($P > 0.25$, $n = 4$). These experiments indicate that G-proteins are not involved in modulation of channel opening.

4. Discussion

The Cl^- channels investigated in this study have not previously been described. Other Cl^- channels exist in

nematodes but do not share the characteristics of the small amplitude channels described here. To date, there are no other reports of Cl^- channels that are directly gated by a peptide, in nematodes or other animals.

Direct gating of cation channels by peptides has been shown for two Na^+ channels in mollusc neurones that open in response to FMRFamide (the original FaRP), without second messenger or G-protein involvement (Green et al., 1994). Like the Cl^- channels described here, these directly gated Na^+ channels have low conductances, <10 pS (Cottrell et al., 1990). The protein sequence of one of the FMRFamide-gated Na^+ channels has been identified, and the channel has been cloned (Lingueglia et al., 1995); it is a tetramer (Coscoy et al., 1998). The Cl^- channel that we have characterised in this paper may be structurally related to this FMRFamide-gated Na^+ channel, although determining that will require isolation of the channel-encoding gene.

The vesicle preparation used during this study has been shown to have the same biophysical properties as the somatic muscle cell membrane from which it is derived (Martin et al., 1990). The advantage of using the vesicle preparation is that it forms Giga-seals with the patch pipette more readily than the parent membrane. This facilitated characterisation of the PF4-responsive channels. The channels were very abundant; multiple levels of channel activity, indicating the presence of more than one channel in a patch, were present in 64% of the patch clamp recordings (49 out of 77 patches showed multiple levels of channel activity). The profusion of channels may compensate for the low conductance of individual channels, hence, the response to PF4 at the whole cell level is a marked change in input conductance (Maule et al., 1995a; Holden-Dye et al., 1997). The high probability of channel opening and the long mean open times would also tend to counterbalance the small channel conductance, combining to produce a large response when PF4 is applied to intact cells, even at low concentrations.

The occurrence of spontaneous channel opening in the absence of PF4 was noted in 42% of membrane patches, although seldom was more than one channel active in these patches. This observation is consistent with the ubiquity of the channels and their high probability of opening. Spontaneously opening $GABA_A$-activated Cl^- channels have also been observed in cultured preparations of mouse neurones (Hamill et al., 1983; MacDonald et al., 1989). Like the Cl^- channels in the present study, the $GABA_A$-activated channels were noted to open randomly in the abscence of ligand, and to show increased P_{open} in the presence of ligand. An explanation for the spontaneous channel activity might be the presence of endogenous ligand in the vesicle preparation (Hamill et al., 1983).

At 1.1–7.1 pS, the channel conductance is low by comparison with other Cl^- channels that have previously been described. This characteristic also indicates that the channel investigated here is different from others characterised to date in *A. suum*. The nematode Ca^+-activated Cl^- channel has a conductance of 200 pS (Thorn and Martin, 1987), the GABA-activated Cl^- channel a conductance of 22 pS (Martin, 1985) and the glutamate-gated Cl^- channel a conductance of 21 pS (Adelsberger et al., 1997).

The function of this low amplitude Cl^- channel in vivo is unknown. Other Cl^- channels in *A. suum* appear to be involved in maintaining the resting membrane potential (Thorn and Martin, 1987); in excretion of volatile fatty acids, the waste products of anaerobic metabolism, (Dixon et al., 1993; Robertson and Martin 1996; Valkanov et al., 1994; Valkanov and Martin, 1995); or in muscle control (Adelsberger et al., 1997). Though the in vitro effect of PF4 application on the Cl^- channels studied here is associated with muscle contraction (Maule et al., 1995a,b; Holden-Dye et al., 1997), the function of these channels in vivo has yet to be elucidated.

The low conductance Cl^- channels showed no signs of receptor desensitisation (Katz and Thesleff, 1957) or channel run-down (Becq, 1996); they continued to respond to PF4 in the pipette solution even after 30-min exposure. This observation indicates that the mechanism of channel opening is robust and unlikely to involve complex, energy-expensive intracellular processes. Data collected after isolation of membrane patches from the parent vesicle also provide strong evidence that the Cl^- channel under investigation can function independently of cytoplasmic contents. Channel opening did not require cytoplasmic constituents, suggesting that intracellular signal transduction mechanisms are not involved. Possible involvement of membrane bound or membrane delimited G-proteins (Hille, 1994) in channel opening was also investigated. Exposure of the intracellular face of the membrane patches to GDP-β-S did not affect the number of channels opening in the patch, indicating that G-proteins were not integral to channel opening.

The lack of evidence of desensitisation reported here is at odds with a previous study. This work has shown that PF4-induced relaxation of muscle strips from *A. suum* is transient, which is consistent with physiological desensitisation of the PF4 effect (Maule et al., 1995a). This apparent contradiction may be due to the disparity in concentration of PF4 (>1 μM in the previous study, <1 μM in the study presented here). The difference in tissue preparation (whole muscle strips in contrast to cleaned cell membranes) may also account for the different responses seen in each case.

There was some variation in channel characteristics that corresponded to the worm collection site; *Ascaris* from Paisley showed higher P_{open} and conductance than those from Gloucester or Belfast, which suggests that there are channel subtypes within the *A. suum* population. This observation was unexpected but unsurprising, given the pleiomorphic nature of the nematode genome. Studies have shown that allelic variation in parasitic nematodes is higher than that seen in other species (Blouin et al., 1995). The availability of alternative alleles allows rapid adaptation to environmental changes; an example of this is the development of drug resistance among parasitic nematodes (see

Waller, 1994 for a review). Posttranslational events could further contribute to variability of target sites, such as occurs in glutamate-gated Cl^- channels in *C. elegans*, where alternative splicing generates distinct isoforms of the ion channel (Dent et al., 1997). In nematode populations exposed to anthelmintic selection pressure, resistant worms have different channel characteristics to those found in susceptible individuals; ion channels exist as distinct subtypes (Robertson et al., 1999, 2000).

The results reported here show that the PF4 activated Cl^- channel functions independently of intracellular signal transducers and is, therefore, directly gated by PF4. There is a precedent for a peptide to directly gate ion channels. Two Na^+ channels in mollusc neurones open in response to FMRFamide without second messenger or G-protein involvement (Green et al., 1994). However, this is the first report of a Cl^- channel that is directly gated by a peptide. It is conceivable that this channel may be involved in modulating the excitability of the somatic muscle cell. Altered extrasynaptic levels of peptide (PF4) could increase or decrease the responsiveness of muscle to the classical neurotransmitters. Regardless of function, the ubiquity of this channel and the large whole-cell responses to PF4 reported in earlier studies make a strong case for considering the *Ascaris* PF4-gated Cl^- channel an attractive target for anthelmintic drug discovery.

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