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Irreversible and reversible pore formation by polymeric alkylpyridinium salts (poly-APS) from the sponge *Reniera sarai*

[1]D. McClelland, [1]R.M. Evans, [2]I. Abidin, [1]S. Sharma, [1]F.Z. Choudhry, [3]M. Jaspars, [4]K. Sepčić & *,[1]R.H. Scott

[1]Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD; [2]Biophysics Department, Faculty of Medicine, Akdeniz University, 07070 Arapsuyu, Antalya, Turkey; [3]Marine Natural Products Laboratory, Department of Chemistry, University of Aberdeen, Old Aberdeen AB24 3UE and [4]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

1 In this study, we investigated the electrophysiological actions of a high molecular weight fraction, predominantly containing two polymeric 1,3-alkylpyridinium salts (poly-APS) of 5.5 and ~19 kDa isolated from the marine sponge *Reniera sarai*. The biological properties of poly-APS are of particular interest because this preparation may be used to deliver macromolecules into the intracellular environment without producing long-term damage to cells. Poly-APS (50–0.05 $\mu g\,ml^{-1}$) was applied to cultured dorsal root ganglion neurones or HEK 293 cells and changes in cell membrane properties were measured using whole-cell patch-clamp recording and fura-2 Ca^{2+} imaging.

2 Poly-APS (50 $\mu g\,ml^{-1}$) evoked irreversible depolarisations in membrane potential and reductions in input resistance. However, doses of 5 $\mu g\,ml^{-1}$ and less produced reversible effects on these cell membrane characteristics and on Ca^{2+} permeability.

3 At 0.05 $\mu g\,ml^{-1}$, poly-APS could evoke robust transient increases in Ca^{2+} permeability without damaging the neurones or subsequently attenuating Ca^{2+} entry through voltage-activated channels.

4 Bathing cells in NaCl-based extracellular medium containing 1.5 mM zinc attenuated the irreversible and reversible effects of poly-APS on membrane properties (membrane potential, input resistance and whole-cell currents). In both DRG neurones and HEK 293 cells, zinc attenuated Ca^{2+} entry evoked by poly-APS. These effects of zinc were only observed if zinc was continually present during poly-APS application. However, zinc failed to attenuate the actions of poly-APS if it was applied after the sponge toxin preparation had evoked changes in membrane properties.

5 In conclusion, the pore-forming preparation poly-APS can have dose-dependent interactions with cell membranes and at low doses these can be reversible. Additionally, the interactions between poly-APS and cell membranes could be attenuated by zinc.
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Keywords: Pore former; sponge toxin; polymeric alkylpyridinium salt; halitoxins; intracellular calcium; sensory neurone

Abbreviations: APS, alkylpyridinium salts; DRG, dorsal root ganglion; poly-APS, polymeric 1,3-alkylpyridinium salts

Introduction

Over the last 500–700 million years, sessile marine sponges have developed a variety of spectacular strategies to survive. In addition to being able to regenerate and take on a single-cell existence, sponges have an extensive armoury of chemical defences, which prevent overpredation, facilitate establishment of a sponge colony and control colonisation of the surfaces of sponges by other marine organis ms (Sepčić, 2000). Of the many distinct chemical weapons produced by sponges, a number of them are novel alkylpyridinium salts (APS) that have interesting biological properties that may be exploited. Halitoxins (1,3-APS oligomers) were originally identified from sponges of the Haploscerid genera such as *Haliclona*, *Amphimedon and Callyspongia* (Schmitz *et al*., 1978; Berlinck *et al*., 1996; Sepčić *et al*., 1997; Scott *et al*., 2000). Diverse biological activities have been identified for different 1,3-APS oligomer preparations. These include cytotoxicity (Schmitz *et al*., 1978; Malovrh *et al*., 1999), neurotoxicity and inhibition of action potentials (Berlinck *et al*., 1996), stimulation of transmitter release (Jaffe *et al*., 1993), inhibition of K^+ conductances (Sevcik *et al*., 1986) and anticholinesterase activity (Sepčić *et al*., 1997). At least some of these actions observed with 1,3-APS oligomers relate to the pore-forming or membrane lesion effects of these compounds. We have previously isolated and characterised the chemical and biological properties of some natural pore-forming 1,3-APS oligomers in a fraction with a mean molecular weight of 5 kDa (Scott *et al*., 2000). These compounds, isolated from the sponge *Callyspongia ridleyi*, were stable in aqueous solution and we found that a cocktail of natural 1,3-APS oligomers depolarised cultured sensory neurones by forming pores permeable to cations. When applied to artificial bilayers, halitoxins evoked channel-like events with unitary conductances between 145 and 2280 pS. Additionally, the 1,3-APS oligomers could release Ca^{2+} from intracellular stores possibly

*Author for correspondence; E-mail: rod.scott@abdn.ac.uk
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by gaining access to the intracellular environment and then subsequently producing pores in the membranes of intracellular Ca^{2+} stores (Scott *et al.*, 2000).

In this study, we have investigated the actions of a cocktail of mainly two larger polymeric 1,3-alkylpyridinium salts (poly-APS) with molecular weights of 5.5 and ~19 kDa (Sepčić *et al.*, 1997). Extracellular zinc and other divalent ions have been shown to protect erythrocytes from membrane damage by a variety of agents including pore-forming α-toxin (Bashford *et al.*, 1989). We have also studied the attenuation of poly-APS actions by zinc, which has previously been shown to decrease the haemolytic actions of this toxin preparation, with an IC_{50} of 0.3 mM (Malovrh *et al.*, 1999).

Methods

Sponge toxin preparations

Poly-APS (Figure 1a) preparation was purified from the marine sponge *Reniera* (=*Haliclona*) *sarai* Pulitzer-Finali (Haliclonidae) as previously described, and it contained a mixture of two polymers with molecular weights of 5.5 and ~19 kDa. In Figure 1a, $n=29$ and 99; where n denotes the number of repeating monomeric units that are combined to make the polymers in the poly-APS preparation (Sepčić *et al.*, 1997). The Halitoxin preparation was supplied by Dr M. Jaspars and had a mean molecular weight of ~5 kDa (Scott *et al.*, 2000). Test solutions containing poly-APS were prepared from a stock solution containing 5 mg ml^{-1} poly-APS in distilled water.

Cell culture

Primary cultures of dorsal root ganglion (DRG) neurones were prepared following enzymatic and mechanical dissociation of DRG from decapitated 2-day-old Sprague–Dawley rats. The sensory neurones were plated on laminin–polyornithine-coated coverslips and bathed in F14 culture medium (Imperial Laboratories, Andover, England) supplemented with 10% horse serum (Gibco, Paisley, Scotland), penicillin (5000 IU ml^{-1}), streptomycin (5000 mg ml^{-1}), $NaHCO_3$ (14 mM) and nerve growth factor (20 ng ml^{-1}). The cultures were maintained for up to 2 weeks at 37°C in humidified air with 5% CO_2, and re-fed with fresh culture medium every 5–7 days.

HEK 293 cells were maintained in culture as previously reported (McFarlane *et al.*, 2002) and were generously provided by Steven Tucker.

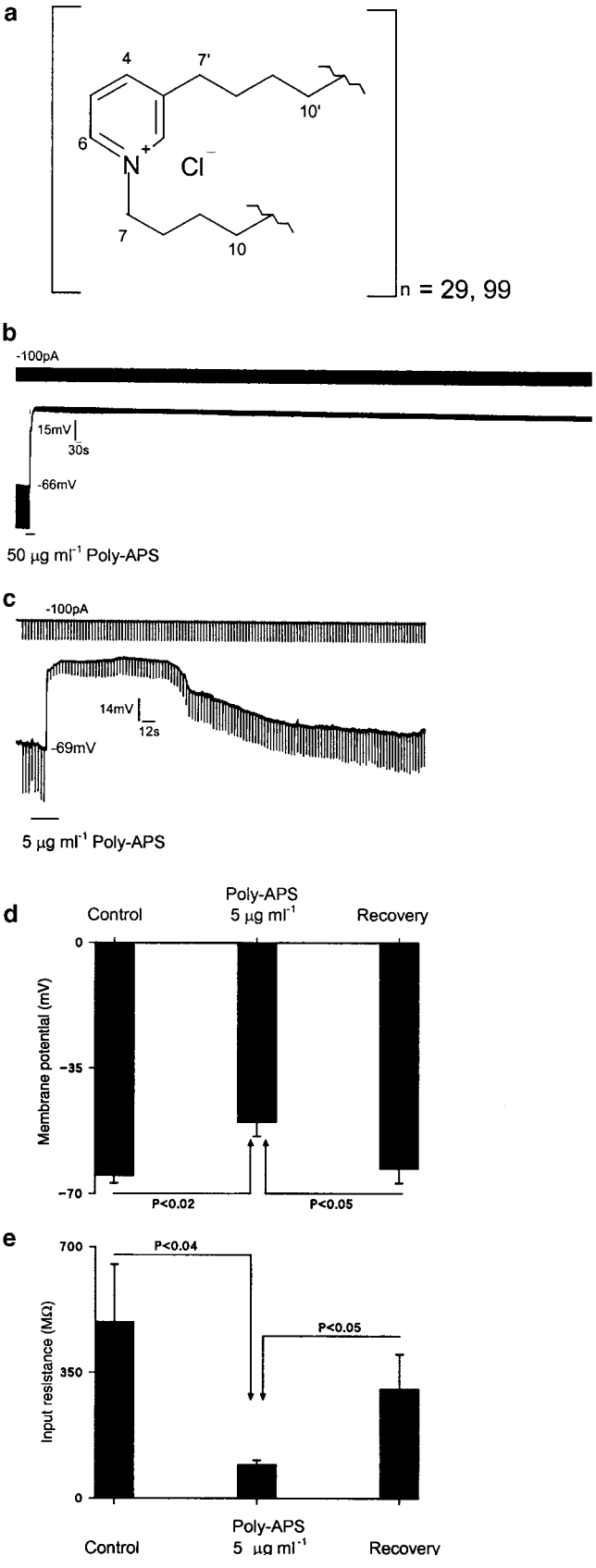

Figure 1 (a) Chemical structure of poly-APS from the marine sponge *R. sarai*. The preparation used in this study was a mixture of polymers composed of repeating units. For poly-APS $n=29$ and 99, where n denotes the number of repeating monomeric units that make the polymers. (b) Trace showing a long condensed recording of the irreversible actions of poly-APS (50 μg ml^{-1}) on membrane potential and electrotonic potentials. The −100 pA hyperpolarising current commands and the resulting electrotonic potentials activated every 3 s are not individually resolved in this trace that shows about 45 min of recording. (c) Reversible actions of poly-APS (5 μg ml^{-1}) on membrane potential and electrotonic potentials (used to calculate input resistance). Records were obtained from cultured DRG neurones. Bar charts showing the reversible actions of 5 μg ml^{-1} poly-APS on (d) membrane potential ($n=8$) and (e) input resistance ($n=8$). Poly-APS was applied to cultured DRG neurones for 20 s.

Electrophysiology and calcium imaging

All experiments were conducted at room temperature (approximately 23°C). The whole-cell recording technique (Hamill *et al*., 1981) was used to study the actions of poly-APS on membrane potential, input resistance (evaluated from 100–300 ms electrotonic potentials evoked by −50 to −200 pA current commands) and holding current. Whole-cell recordings were made using an Axoclamp-2A switching voltage clamp amplifier operated at a sampling rate of 15–20 kHz. Low resistance (4–10 MΩ) borosilicate glass patch pipettes were fabricated using a Kopf model 730, needle-pipette puller. The neurones were bathed in a NaCl-based extracellular solution containing in mM: NaCl, 130; KCl, 3.0; $CaCl_2$, 2.0; $MgCl_2$, 0.6; $NaHCO_3$ 1.0, HEPES 10.0, glucose 5.0. NaCl-based extracellular solution with zinc was made up separately and contained 1.5 mM zinc (zinc atomic absorption standard solution, Sigma, Poole, Dorset, England). The pH and osmolarity of extracellular solutions were adjusted to 7.4 and 310–320 mOsmol l^{-1} with NaOH and sucrose, respectively. The patch pipette solution contained in mM: KCl, 140; EGTA, 5; $CaCl_2$, 0.1; $MgCl_2$, 2.0; HEPES, 10.0; ATP, 2.0; and the pH and osmolarity were adjusted to 7.2 with Tris and 310–315 mOsmol l^{-1} with sucrose. For voltage-clamp recordings, neurones were held at −90 mV and linear current–voltage relationships were generated with 100 ms voltage step commands to potentials between −170 and −70 mV. HEK 293 cells were held at −70 mV and linear current–voltage relationships were generated with 100 ms voltage step commands to potentials between −150 and +10 mV. The poly-APS preparation was applied to the extracellular environment by low-pressure ejection *via* a blunt micropipette (tip diameter about 10 μm) positioned approximately 100 μm from the neurone being recorded. The cells were maintained in a bath and were not continually perfused, drug concentrations declined after pressure ejection as a result of diffusion.

The electrophysiological data were stored on digital audio tape (DAT) using a DTR-1200 DAT recorder (Biologic) and subsequently analysed using Cambridge Electronic Design voltage clamp software (version 6). For monitoring changes in membrane potential or holding current, continuous records were obtained on a chart recorder (Gould 2200 s pen recorder).

Cultured DRG neurones and HEK 293 cells were incubated for 1 h in NaCl-based extracellular solution containing 10 μM fura-2AM (Sigma, 1 mM stock in dimethylformamide) and the effects of poly-APS and halitoxin preparations on intracellular Ca^{2+} were evaluated using fluorescence ratiometric imaging as previously described (Scott *et al*., 2000).

All data are given as mean ± standard error of the mean (s.e.m.), and statistical significance was determined using the Student's two-tailed *t*-test, paired or independent where appropriate and *P*-values are reported in the text.

Results

Irreversible and reversible actions of poly-APS on electrophysiological properties of cultured DRG neurones

Extracellular application of 50 μg ml^{-1} poly-APS for approximately 20 s resulted in an irreversible collapse in membrane potential and a dramatic fall in input resistance in all DRG neurones studied (Figure 1b). The mean membrane potential was depolarised from −65 ± 1 to −9 ± 3 mV ($n = 17$; $P < 0.0001$) and this was associated with a reduction in input resistance from 350 ± 79 to 56 ± 27 MΩ ($n = 17$; $P < 0.0001$). No recovery was seen 120 min after removal of the perfusion pipette containing poly-APS. At concentrations of 5 and 0.5 μg ml^{-1}, poly-APS evoked reversible depolarisations and associated decreases in input resistance (Figure 1c). As previously shown for halitoxins (Scott *et al*., 2000), poly-APS showed a degree of dose-dependent action when the sponge toxins were applied for approximately 20 s. The mean percentage reductions in input resistance were 84, 67 and 17% for 50, 5, and 0.5 μg ml^{-1} poly-APS, respectively. Recovery periods varied but were seen within 20 min of removing the perfusion pipette containing poly-APS. In response to 5 μg ml^{-1} poly-APS, six out of eight neurones showed at least partial recovery of membrane potential and input resistance after toxin application (Figure 1d, e).

Reversible actions of poly-APS on cell membrane calcium permeability

Consistent with the electrophysiological results, Ca^{2+} imaging experiments on cultured DRG neurones showed that poly-APS evoked irreversible (Figure 2a) and reversible (Figure 2b) increases in intracellular Ca^{2+}. Although the responses were very variable, a dose-dependent trend was clearly apparent. Poly-APS (5 μg ml^{-1}) mainly produced responses that only recovered partially or were irreversible, while 0.05 μg ml^{-1} poly-APS evoked predominantly transient responses with four out of 45 neurones failing to respond (Table 1). No significant differences were observed when comparing the mean amplitudes of irreversible and reversible rises in intracellular Ca^{2+} and the mean amplitude of events evoked by 5, 0.5 and 0.05 μg ml^{-1} poly-APS. This relates partially to the considerable variability of responses, for example, the maximum and minimum changes in fluorescence ratio produced by 5 μg ml^{-1} poly-APS were 5.42 and 0.48. Even in neighbouring neurones during the same experiment, great differences in responses were seen. Additionally, the poly-APS preparation was applied for 2–2.5 min, which was significantly longer than the periods for which the toxins were applied for in the electrophysiological experiments. The longer period of poly-APS application in the imaging experiments was because bath application by gravity-fed perfusion takes longer than low-pressure ejection. Additionally, drug equilibration takes longer in a population of cells than it does in a single cell. One benefit of using cultured DRG neurones in this study was that these neurones express voltage-activated Ca^{2+} channels, which can be used to assess the viability of neurones after exposure to poly-APS. KCl (30 mM) can be briefly applied to excitable cells, including cultured DRG neurones, to depolarise the cells and produce consistent transient Ca^{2+} influx through voltage-activated channels (Sutton *et al*., 2002). The experimental trace in Figure 2c shows that Ca^{2+} transients evoked by a depolarising pulse of extracellular solution containing KCl (30 mM) could be produced before and after application of 0.05 μg ml^{-1} poly-APS. Poly-APS (0.5 μg ml^{-1}) and halitoxins (5 μg ml^{-1}; Scott *et al*., 2000) were applied to neurones in a single experiment to compare pore-forming properties of equivalent estimated concentrations of the two toxin preparations that differ in their molecular weights. The basal fluorescence ratio value was

1.13±0.05; on application of poly-APS and transient Ca^{2+} entry, the mean ratio increased to 2.75±0.36 and subsequent application of halitoxins produced a larger irreversible increase to 5.62±0.43 ($n=11$; $P<0.01$). This experiment indicates that in a single preparation of DRG neurones, distinct responses to chemically related poly-APS and halitoxin sponge toxin preparations can be observed. Consistent with this study, the halitoxin preparation, at a range of doses, was found to produce sustained responses that persisted after washing (Scott *et al*., 2000).

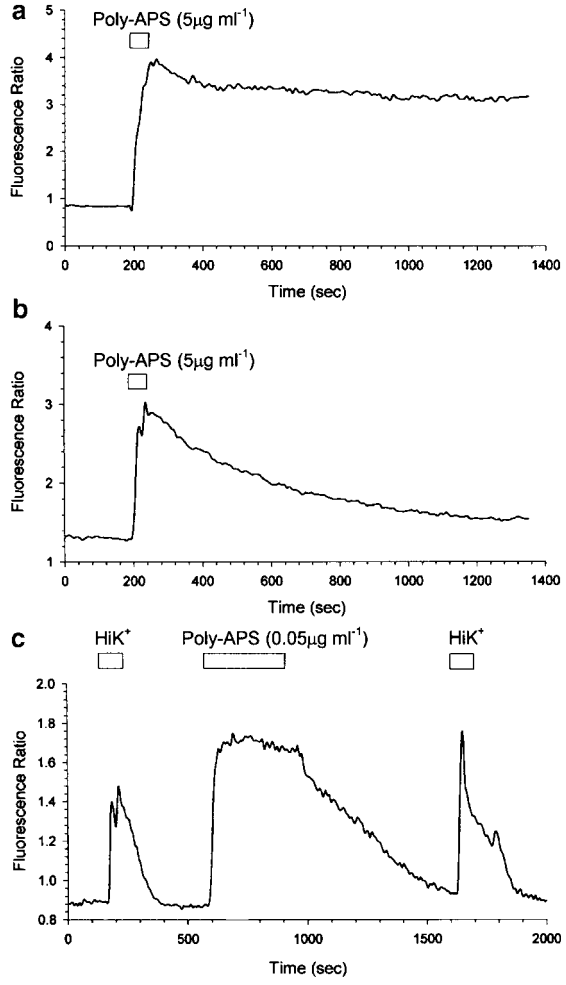

Figure 2 Changes in intracellular Ca^{2+} in cultured DRG neurones produced by poly-APS. (a) Record of an irreversible rise in intracellular Ca^{2+} evoked by 5 μg ml^{-1} poly-APS. (b) Record from a neurone in the same preparation as in (a) showing a reversible rise in intracellular Ca^{2+} evoked by 5 μg ml^{-1} poly-APS. (c) Trace from a single neurone showing the reversible action of 0.05 μg ml^{-1} poly-APS. This record also shows that Ca^{2+} transients evoked by application of NaCl-based extracellular solution containing 30 mM KCl (HiK^+) were not attenuated by exposure of the neurone to 0.05 μg ml^{-1} poly-APS.

Actions of zinc on pore formation by poly-APS

The haemolytic actions of poly-APS have been found to be attenuated by zinc, most likely in the ionic form Zn^{2+} (Malovrh *et al*., 1999). So, we evaluated the protective properties of zinc (Zn^{2+}) after pore formation by poly-APS or when applied before poly-APS and then continually throughout the experiment. Experiments were carried out on the same cultures of DRG neurones to allow comparisons between results obtained using the two protocols. Using the first experimental protocol, poly-APS (50 μg ml^{-1}) reduced the membrane potential from −66±2 to −13±6 mV ($P<0.0001$), but during subsequent application of extracellular solution containing 1.5 mM zinc the membrane potential did not significantly recover and had a value of −17±6 mV ($n=8$). A similar pattern of results was obtained for input resistance measurements that had values of 252±52, 13±3 MΩ (significance compared to control $P<0.005$) and 19±7 MΩ ($n=8$), under control conditions, during application of 50 μg ml^{-1} poly-APS and during application of zinc. It was clear from this experiment that once poly-APS had produced pores or lesions in the cell membrane, zinc failed to attenuate the conductances (Figure 3a). However, using the second experimental protocol that involved continually bathing neurones with extracellular solution containing 1.5 mM zinc resulted in significant inhibition of poly-APS actions (Figure 3b and c). In the presence of zinc, the resting membrane potential was −47±7 mV, application of poly-APS (50 μg ml^{-1}) with zinc significantly reduced the membrane potential to −27±6 mV ($n=7$; $P<0.007$). Similarly, in the presence of zinc, the input resistance was 261±34 MΩ, application of poly-APS (50 μg ml^{-1}) with zinc significantly reduced the input resistance to 114±62 MΩ ($n=7$; $P<0.05$). Figure 3c shows a bar chart of data normalised with respect to the resting values to illustrate the significant attenuation of poly-APS effects when zinc was present prior to and during application of the toxin.

Experiments were then conducted under voltage-clamp conditions to remove the influence of voltage-activated

Table 1 Reversible and irreversible rises in intracellular Ca^{2+} evoked by poly-APS as detected using the Ca^{2+}-sensitive ratiometric dye fura-2

Poly-APS concentration (μg ml^{-1})	*Irreversible (I) or reversible (R) responses*	*Amplitude of response in ratio units±s.e.m.*	*Number of DRG neurones*	*Percentage of DRG neurones*
5[a]	I	2.54±0.28	15	29
5[a]	R	2.18±0.45	9	17
0.5	I	3.16	1	3
0.5	R	2.58±0.51	31	97
0.05	I	—	0	0
0.05	R	2.53±0.4	41	100

Reversible is defined as a transient event that showed recovery of 75% or more. Irreversible is defined as a sustained event that only decayed by 10% or less during washing.
[a]28 neurones (54%) showed partial recovery and were not included in this analysis.

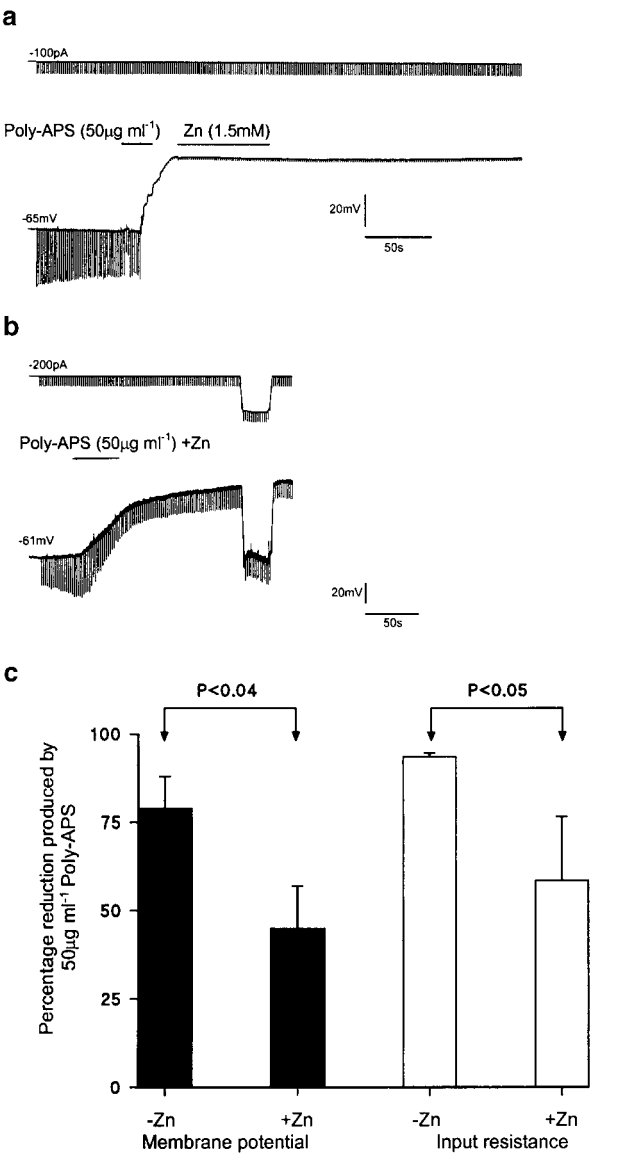

Figure 3 Zinc pretreatment attenuated electrophysiological responses to poly-APS ($50\,\mu g\,ml^{-1}$). (a) Record showing the lack of effect of acute application of NaCl-based extracellular solution containing 1.5 mM zinc applied after poly-APS had evoked changes in membrane potential and input resistance. (b) Recording showing a slowed and reduced response to poly-APS when they were applied in the continual presence of NaCl-based extracellular solution containing 1.5 mM zinc. Constant current injection was applied at the end of the recording to hyperpolarise the membrane potential back to the resting level. (c) Bar chart showing the significant influence of the continued presence of zinc on poly-APS-evoked changes in membrane potential ($n=9$ and 7) and input resistance ($n=8$ and 7).

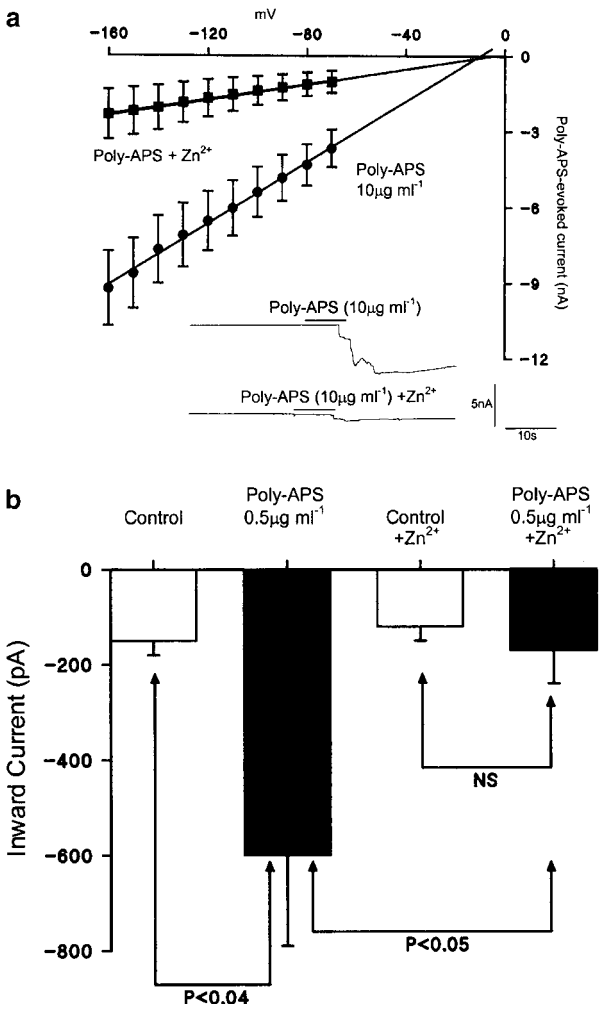

Figure 4 Under voltage clamp, zinc attenuated poly-APS-evoked currents. (a) Current–voltage relationships for the poly-APS-evoked currents in the presence ($n=6$) and absence of zinc ($n=6$). Linear $I-V$ relationships were obtained between -160 and -70 mV after leakage subtraction (the r^2 values from linear regressions were 0.996 and 0.995 for poly-APS applied alone and in the continual presence of zinc, respectively). Inset traces show currents evoked at -90 mV by poly-APS applied in the absence and presence of zinc. (b) Bar chart showing the inhibition of poly-APS ($0.5\,\mu g\,ml^{-1}$)-evoked inward currents by zinc. Control data show the mean holding currents required to clamp the neurones at -90 mV, zinc had no significant effect on the mean holding current. Poly-APS in the absence of zinc evoked a significant inward current ($n=5$); however, no significant inward current was produced by poly-APS in the presence of zinc ($n=6$).

channels. Neurones were held at -90 mV and currents were activated by poly-APS ($10\,\mu g\,ml^{-1}$) in the presence and absence of 1.5 mM zinc. Current–voltage relationships were generated between -160 and -70 mV and subtracted to produce difference current–voltage relationships for the poly-APS-evoked currents (Figure 4a). The results with $10\,\mu g\,ml^{-1}$ poly-APS showed that although zinc significantly reduced the action of the toxin its effects were not abolished, so the concentration of the poly-APS preparation was reduced. The presence of 1.5 mM zinc prevented significant activation of inward currents evoked by $0.5\,\mu g\,ml^{-1}$ poly-APS. In the absence of zinc, the holding current required to clamp the cell membrane at -90 mV increased from -150 ± 30 to -600 ± 190 pA ($n=5$; $P<0.04$) during application of $0.5\,\mu g\,ml^{-1}$ poly-APS. In contrast, with 1.5 mM zinc continually present the holding current was not significantly increased by poly-APS ($0.5\,\mu g\,ml^{-1}$) from a resting level -120 ± 30 to -170 ± 70 pA ($n=6$; NS). The mean amplitudes of currents evoked by poly-APS in the presence and absence of zinc were also significantly different ($P<0.05$; Figure 4b).

Imaging experiments were then carried out to determine whether zinc would attenuate the rise in Ca^{2+} evoked by poly-APS. Protocols were again designed to assess the ability of zinc

to inhibit rises in Ca^{2+} produced by poly-APS, when applied before or after poration of the cell membrane (Figure 5). We were unable to characterise clearly the effects of zinc when applied after poly-APS because of the slow partial recovery of the responses that occurred during washing (Figure 5a). Application of zinc itself produced a variable but significant increase in fluorescence ratios above basal levels and poly-APS ($5\,\mu g\,ml^{-1}$) applied with zinc subsequently evoked a sustained rise in fluorescence ratio (Figure 5b). However, these responses in the presence of zinc were significantly smaller than the responses evoked by poly-APS in the absence of zinc (Figure 5a–c). The changes in the mean fluorescence ratios evoked by poly-APS in the absence and presence of zinc were significantly different and had values of 3.46 ± 0.18 ($n = 46$) and 1.2 ± 0.05 ($n = 13$; $P < 0.0001$), respectively. These experiments were conducted on two populations of neurones. However, it was hoped that by lowering the poly-APS concentration it would be possible to record poly-APS responses in the absence and presence of zinc in the same neurones. Preliminary observations showed that repeatable responses to 0.5 and $0.05\,\mu g\,ml^{-1}$ poly-APS could be obtained from some neurones. To address the variability of the responses, the second response was normalised with respect to the first response for each DRG neurone. The mean normalised second responses to 0.5 and $0.05\,\mu g\,ml^{-1}$ poly-APS were 0.85 ± 0.1 ($n = 41$) and 1.39 ± 0.82 ($n = 11$), respectively. Application of zinc alone after $0.5\,\mu g\,ml^{-1}$ was problematic, because the resulting changes in fluorescence ratios were larger than the first poly-APS responses in 15 out of 23 neurones (Figure 6a). Analysis of the whole population of neurones indicated that a very significant effect ($P < 0.003$) was seen with zinc that masked any additional response to poly-APS. This prevented analysis of the second poly-APS in all but five cells where the zinc response was less than 60% of the initial poly-APS response. In these five neurones, the second response in the presence of zinc was significantly reduced ($P < 0.03$) compared with the normalised second response in the absence of zinc. Clearer data were obtained with $0.05\,\mu g\,ml^{-1}$ poly-APS. The mean value for the second response to $0.05\,\mu g\,ml^{-1}$ poly-APS reflected some larger responses to the second application of sponge toxin (Figure 6a and b) and the mean response to zinc was only 70% ($n = 18$) of the first poly-APS response. Under these conditions, the second response to $0.05\,\mu g\,ml^{-1}$ poly-APS in the presence of zinc was greatly reduced ($n = 18$, $P < 0.04$; Figure 6a and c). Out of 18 neurones, 10 showed no increase in fluorescence ratio in response to $0.05\,\mu g\,ml^{-1}$ poly-APS in the presence of zinc.

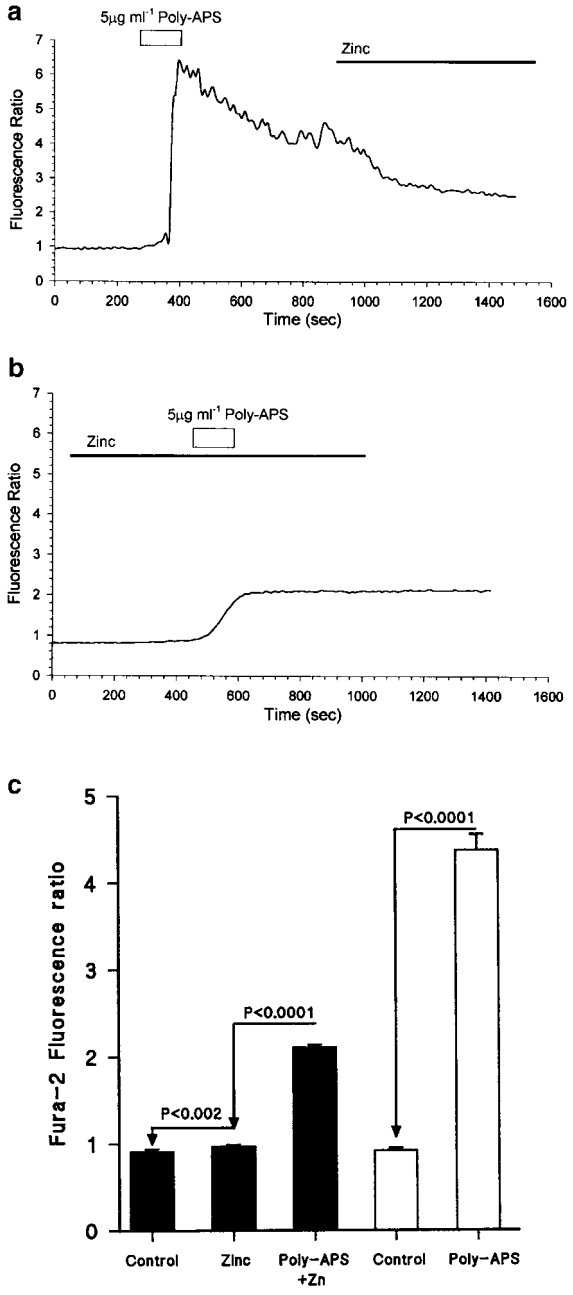

Figure 5 Poly-APS ($5\,\mu g\,ml^{-1}$)-evoked Ca^{2+} transients were attenuated by simultaneous application of zinc. (a) Record showing an increase in intracellular Ca^{2+} evoked by $5\,\mu g\,ml^{-1}$ poly-APS. Note that zinc was applied but that it was difficult to determine its actions due to the decay of the fluorescence ratio during washing. Irreversible responses were not attenuated by acute application of zinc. (b) Record showing the sustained but relatively modest rise in fluorescence ratio observed when the poly-APS preparation was applied in the continual presence of zinc. (c) Bar chart showing the mean imaging data derived from applying poly-APS ($5\,\mu g\,ml^{-1}$) in the presence of zinc (filled bars, $n = 13$) and absence of zinc (open bars, $n = 46$). Zinc alone produced a significant but very modest increase in fluorescence ratio but subsequently attenuated the response to poly-APS.

The responses to zinc indicate that it was likely to pass through residual pores in the membrane produced during the first application of poly-APS and resulted in slow sustained increases in fluorescence ratio. This is supported by the fact that the zinc responses were larger following application of $0.5\,\mu g\,ml^{-1}$ poly-APS than $0.05\,\mu g\,ml^{-1}$ poly-APS. In spite of this difficulty, preliminary experiments were also carried out using a protocol in which zinc was applied prior to the first application of poly-APS and then washed off before a second application of poly-APS. In three out of 22 cells, it was clearly possible to detect a larger response to $0.5\,\mu g\,ml^{-1}$ poly-APS in the absence of zinc than in its presence (Figure 7a). However, the sustained response to zinc made this experiment difficult to assess because of the poly-APS response being masked by zinc, and zinc remaining in the intracellular environment in majority of the neurones (Figure 7b).

As previously observed for halitoxin (Scott *et al.*, 2000), poly-APS ($0.5\,\mu g\,ml^{-1}$) evoked transient increases in intracellular Ca^{2+} when perfused with Ca^{2+}-free extracellular

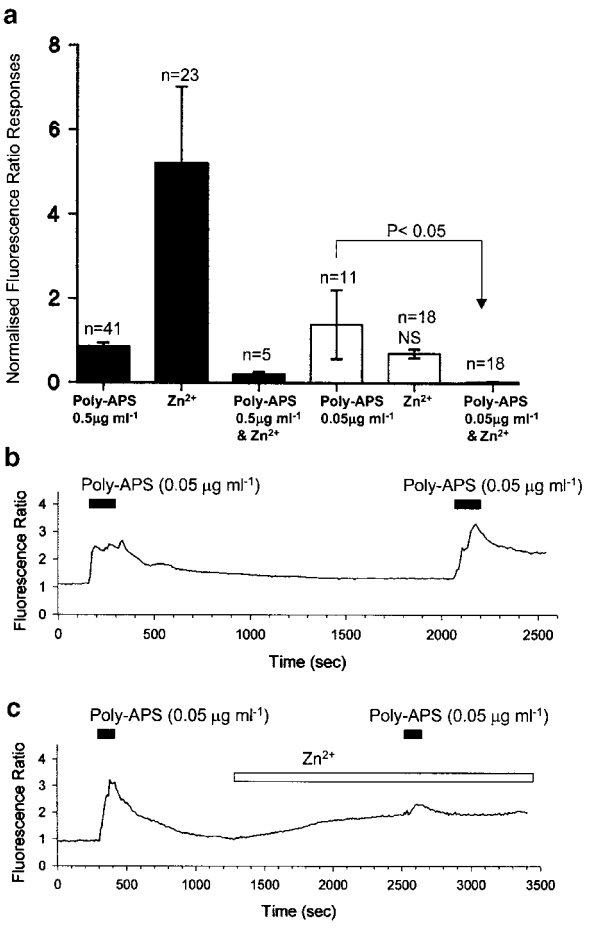

Figure 6 Transient responses in intracellular Ca^{2+} evoked by 0.5 and 0.05 $\mu g\,ml^{-1}$ poly-APS were attenuated by zinc. (a) Bar chart of normalised fluorescence ratio responses evoked by a second application of 0.5 $\mu g\,ml^{-1}$ poly-APS, zinc applied after 0.5 $\mu g\,ml^{-1}$ poly-APS and 0.5 $\mu g\,ml^{-1}$ poly-APS applied in the presence of zinc (filled bars). Open bars show data for a second application of 0.05 $\mu g\,ml^{-1}$ poly-APS, zinc applied after 0.05 $\mu g\,ml^{-1}$ poly-APS and 0.05 $\mu g\,ml^{-1}$ poly-APS applied in the presence of zinc. All data were normalised with respect to the fluorescence change produced by the first application of poly-APS in the same neurone. (b) Mean record of fluorescence ratio values produced in five DRG neurones by two applications of poly-APS (0.05 $\mu g\,ml^{-1}$). (c) Mean record of fluorescence ratio values produced in four DRG neurones by application of poly-APS (0.05 $\mu g\,ml^{-1}$), recording medium containing 1.5 mM zinc (open bar) and poly-APS (0.05 $\mu g\,ml^{-1}$) applied with zinc. Application of zinc caused a sustained rise in fluorescence. However, application of poly-APS (0.05 $\mu g\,ml^{-1}$) with zinc evoked only a modest further increase in fluorescence ratio.

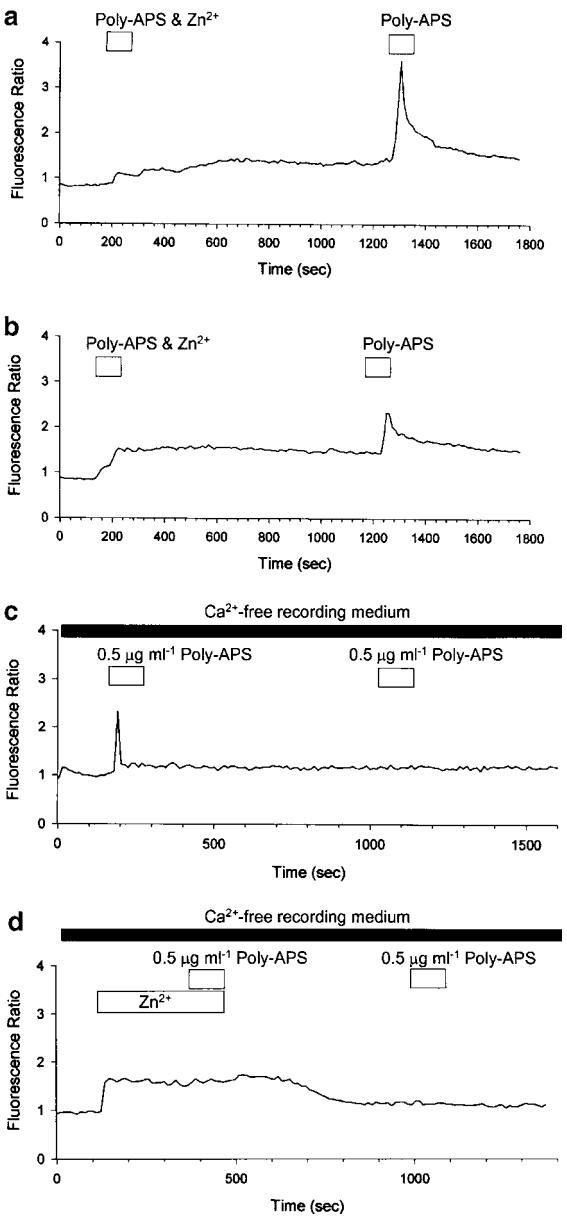

Figure 7 Fluorescence ratio changes produced by zinc and poly-APS. (a) Single record showing a response to simultaneous application of poly-APS (0.05 $\mu g\,ml^{-1}$) and 1.5 mM zinc and then a second larger response to poly-APS (0.05 $\mu g\,ml^{-1}$) applied alone. (b) A single and more typical record than in (a) showing a sustained response to simultaneous application of poly-APS (0.05 $\mu g\,ml^{-1}$) and 1.5 mM zinc and then a similar second response to poly-APS (0.05 $\mu g\,ml^{-1}$) applied alone. Note that the lack of recovery from zinc presents a problem with data interpretation and suggests that zinc remains in the intracellular environment. (c) Trace showing that under Ca^{2+}-free conditions poly-APS (0.5 $\mu g\,ml^{-1}$) evoked a single response. (d) Trace showing that zinc evoked a response under Ca^{2+}-free conditions but that application of 0.5 $\mu g\,ml^{-1}$ subsequently failed to evoke a change in fluorescence ratio either in the presence or absence of zinc.

recording medium (Figure 7c). This single response is believed to be due to permeabilisation of intracellular Ca^{2+} stores by pore-forming sponge toxins. No second response can be elicited from cells bathed in Ca^{2+}-free recording medium because the stores are unable to refill. In this study, poly-APS evoked an increase in fluorescence ratio of 0.78 ± 0.11 ($n = 10$) in Ca^{2+}-free conditions. Application of zinc in Ca^{2+}-free conditions evoked a similar increase in fluorescence ratio of 0.65 ± 0.03 ($n = 23$), but poly-APS subsequently failed to produce any response (Figure 7d). It is not clear whether the failure of poly-APS to produce a response under these conditions is related to depletion of intracellular Ca^{2+} stores produced by zinc or to inhibition of poly-APS pore formation by zinc. However, these experiments do raise the issue that the Ca^{2+} responses evoked by poly-APS are a complex series of events that include Ca^{2+} influx from the extracellular environment through pores in the cell membrane, Ca^{2+}-induced Ca^{2+} release from stores and pore formation in the membranes of intracellular Ca^{2+} stores. The inhibitory effects of zinc in the Ca^{2+} imaging experiments appear to involve

direct block of poly-APS pore formation in the cell membrane and under some recording conditions masking of poly-APS responses.

Actions of zinc on pore formation by poly-APS in HEK 293 cells

One real difficulty of assessing rises in intracellular Ca^{2+} evoked by poly-APS in cultured DRG neurones is that in addition to Ca^{2+} entry through pores formed by poly-APS, rises in intracellular Ca^{2+} will also be produced by activation of voltage-gated Ca^{2+} channels and mobilisation of Ca^{2+} from stores. Although it should be noted that repeatable responses to low doses of poly-APS were obtained, we may be underestimating or overestimating the inhibitory effects of zinc, given the other pathways for raising intracellular Ca^{2+}. For this reason, experiments were conducted on HEK 293 cells that did not express significant numbers of voltage-gated Ca^{2+} channels. The mean fura-2 fluorescence ratios under control conditions and in the presence of extracellular solution containing 30 mM KCl were 1.17 ± 0.05 and 1.24 ± 0.05 ($n = 18$). Poly-APS ($0.5\,\mu g\,ml^{-1}$) evoked repeatable rises in intracellular Ca^{2+}, with the second response being significantly larger than the first response (Figure 8a). The mean fluorescence ratios for the first and second applications of poly-APS were 1.89 ± 0.15 and 2.34 ± 0.283 ($n = 36$; $P < 0.01$). This may reflect some poly-APS remaining in the cell membrane between applications of the sponge toxin preparation. Experiments were then carried out to assess the influence of zinc on the Ca^{2+} entry produced by poly-APS. Application of 1.5 mM zinc significantly reduced the poly-APS response in cells that previously did not respond significantly to brief exposure to 30 mM KCl (Figure 8b). Although a slow zinc-type response was observed, in the majority of cells no distinct transient increase in fluorescence ratio was produced when poly-APS and zinc were applied together. In this set of experiments, the mean fluorescence ratio recorded in the presence of poly-APS ($0.5\,\mu g\,ml^{-1}$) was 1.80 ± 0.06 and this value was reduced to 1.51 ± 0.04 ($n = 8$; $P < 0.0001$) in the same cells when poly-APS was applied in the presence of zinc. A clear indication of the effect of zinc was obtained when data were normalised with respect to the first poly-APS response. Figure 8c shows that in the absence of zinc, the second normalised response was increased but that zinc alone and poly-APS with zinc produced significantly smaller changes in fluorescence ratio.

Whole-cell patch-clamp recordings were also made from HEK 293 cells. HEK 293 cells were held at −70 mV and current–voltage relationships activated between clamp poten-

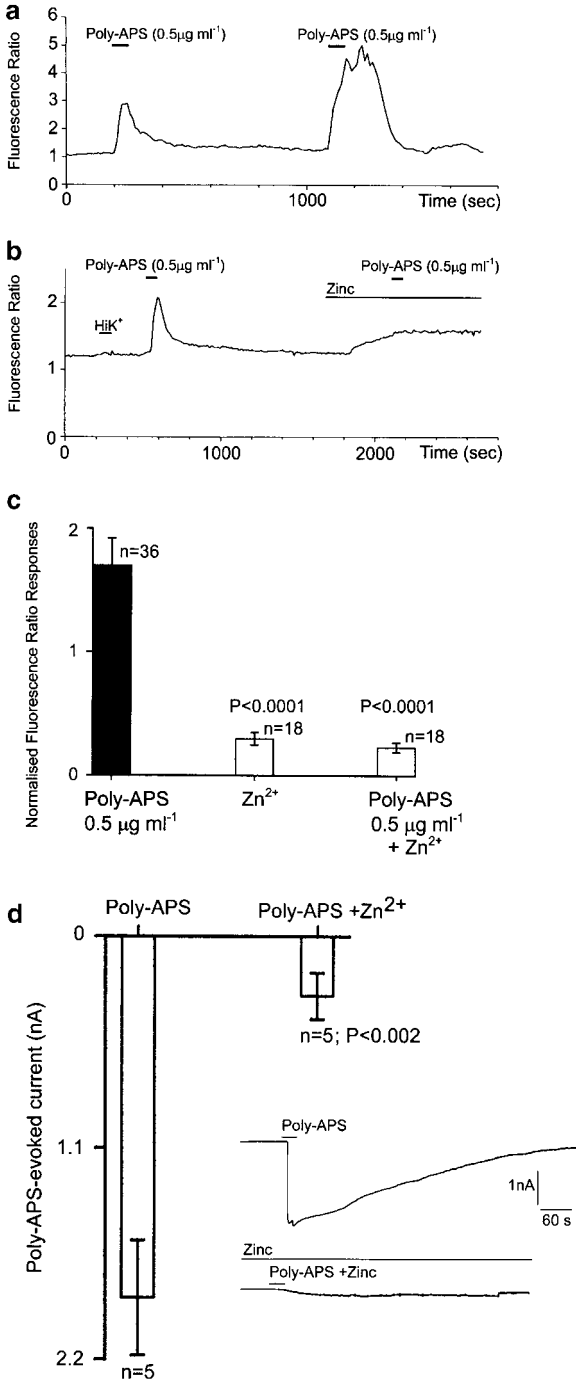

Figure 8 Zinc attenuated poly-APS-evoked Ca^{2+} transients in HEK 293 cells. (a) Example record from a HEK 293 cell showing that these cells responded to two applications of poly-APS ($0.5\,\mu g\,ml^{-1}$). The second responses to poly-APS were equal to or larger than the first responses in the majority of cells (26 out of 36 cells). (b) Example record from a HEK 293 cell showing that these cells did not respond to extracellular solution containing a depolarising concentration of 30 mM KCl (Hi K^+) and therefore did not express significant numbers of voltage-activated Ca^{2+} channels. However, this cell responded to the first application of poly-APS, but showed only a modest increase in fluorescence ratio when poly-APS ($0.5\,\mu g\,ml^{-1}$) was applied for a second time but with zinc present. (c) Bar chart showing the mean normalised fluorescence ratio response values for the second response to poly-APS ($0.5\,\mu g\,ml^{-1}$; filled bar), the responses to zinc (1.5 mM) applied after poly-APS and a second response to poly-APS applied in the presence of zinc. The responses were normalised with respect to the first response to poly-APS. The mean response to poly-APS was significantly smaller when zinc was present compared with responses recorded with poly-APS alone. (d) Bar chart showing whole-cell patch-clamp data from HEK 293 cells held at −70 mV. Data are the mean inward currents evoked by poly-APS ($0.5\,\mu g\,ml^{-1}$) applied alone and in conjunction with zinc. Inset records from two HEK 293 cells held at −70 mV showing inward currents evoked by $0.5\,\mu g\,ml^{-1}$ poly-APS and by $0.5\,\mu g\,ml^{-1}$ poly-APS applied in the continual presence of 1.5 mM zinc. The presence of zinc had no effect on the basal current required to hold cells at −70 mV.

tials of −150 and +10 mV. The current–voltage relationships were linear and the mean reversal potential for the poly-APS-evoked current was −14±5 mV ($n=5$). At a holding potential of −70 mV, the presence of 1.5 mM zinc inhibited the poly-APS (0.5 μg ml^{-1})-evoked current by 84% ($n=5$). Consistent with the data from DRG neurones, these experiments on HEK 293 cells showed that inward currents evoked by poly-APS (0.5 μg ml^{-1}) were significantly attenuated by continually bathing cells with extracellular medium containing zinc (1.5 mM; Figure 8d).

Discussion

1,3-APS compounds have distinct but comparable properties with cationic surfactants, but variation in the extent of polymerisation of the 1,3-APS oligomers appears to modulate the biological activity in an unpredictable manner. In this study, we have identified reversible as well as irreversible effects of poly-APS on cell membrane properties. Previously, the related, but lower molecular weight, preparation of halitoxins evoked irreversible pore formation and associated changes in cell membrane input conductance (Scott *et al*., 2000). There are several possible explanations for the reversible changes in membrane potential, input resistance, currents and intracellular Ca^{2+} seen with poly-APS. Firstly, the larger degree of polymerisation in poly-APS (Sepčić *et al*., 1997) may result in less stable interactions in the cell membranes and allow washout of the larger pore-forming sponge toxins with a resulting temporary pore formation. Secondly, and perhaps more likely, is the possibility that the larger poly-APS molecules may be sufficiently flexible that lipids can rearrange themselves after pore formation and thus block the ion conducting pathways through the cell membrane. This may occur due to 'hydrophobic collapse' when the toxin is in the membrane. In such a circumstance, alkyl chains may surround all the pyridinium groups so that they are compatible with the membrane. The ability of poly-APS to form noncovalently bound aggregates with a mean hydrodynamic radius of 23 nm (Sepčić *et al*., 1997) and its cationic charge density and hydrophobicity (Narita *et al*., 2001) may be the key factors in transient pore formation. Repeatable poration could be obtained and entry of Ca^{2+} *via* voltage-gated channels remains intact after application of low doses of poly-APS, indicating that at least at low concentrations cytotoxic damage does not occur as a result of pore formation. It was surprising that voltage-gated Ca^{2+} channels still function after a period of poration, given the sensitivity of Ca^{2+} channels to intracellular Ca^{2+}-induced inactivation.

Poly-APS produced highly variable Ca^{2+} transients, which could reflect the nature of Ca^{2+} stores within DRG neurones and variable expression of voltage-activated Ca^{2+} channels. Poly-APS evoked membrane potential depolarisation, which would activate endogenous Ca^{2+} channels. Entry of Ca^{2+} through both poly-APS pores and native channels would mobilise Ca^{2+} from stores through Ca^{2+}-induced Ca^{2+} release. In this study, we also present evidence that poly-APS directly causes release of Ca^{2+} from stores. Variability in responses to KCl-evoked depolarisation has been reported in DRG neurones, but single neurones can respond consistently to repeated stimulation with KCl (Sutton *et al*., 2002) and poly-APS. Interestingly, crude organic extracts containing bioactive pyridinium alkaloids from the sponge *Amphimedon viridis* showed selective antibacterial activity. Some marine bacterial strains that possibly have symbiotic relations with the reef sponges were resistant to the toxin extracts (Kelman *et al*., 2001). Poly-APS has also been found not to have antibacterial activity against terrestrial and pathogenic Gram positive and Gram negative bacteria (Sepčić, K., Turk, T., Maček, P., unpublished observations). Little comparative data have been obtained on the actions of sponge toxins on diverse pro- and eukaryotic cell types. However, our findings raise the possibility that variable sensitivity of cells and organisms to poly-APS is based on their intrinsic membrane composition and properties. The variability in the responses to poly-APS seen in the DRG neurone cultures could, in part, reflect the heterogeneous population of neurones in this preparation, with sensory neurones varying in their biophysical and pharmacological characteristics.

In the second part of this study, we clearly demonstrated that zinc, if continually present during application of poly-APS, could attenuate the effects of the sponge toxin preparation on membrane potential, input resistance, whole-cell currents and Ca^{2+} permeability. It is not clear how zinc blocked the formation of pores or lesions by poly-APS. This could involve an interaction between zinc and the cell membrane to prevent access of the toxin to sites on the membrane. The Shai–Matsuzaki–Huang model for actions of antimicrobial peptides (Zasloff, 2002) may provide a mechanism for pore formation by poly-APS. The initial stage in the model involves pore-forming molecules carpeting the outer membrane leaflet and this may be prevented by extracellular Zn^{2+} binding to negative charges on the membrane. The affinity of zinc could be much greater than that for pyridinium compounds, and hence as seen in this study premixed poly-APS with zinc results in reduced poration. Thus, zinc might prevent the further stages in pore formation which are, integration of the pore former into the membrane, thinning of the outer leaflet, phase transition and 'wormhole' formation (Zasloff, 2002) with the poly-APS molecules finally spanning the membrane producing a conductance pathway. Alternatively, zinc may interact with poly-APS directly to disrupt its biological activity. Previous work on erythrocytes has suggested that Zn^{2+} and other divalent cations (Hg^{2+}) close pores produced by poly-APS but not lysis caused by hypo-osmotic shock (Malovrh *et al*., 1999). This has not been demonstrated in this study, although as suggested above, the interactions of poly-APS with membranes may vary and thus influence the protective actions of zinc, and the mechanism of erythrocyte lysis is made distinct by the osmotic influence of haemoglobin.

Fura-2 was used to measure changes in intracellular Ca^{2+} produced following pore formation by poly-APS and significant reductions in fluorescence ratios were seen when zinc was applied. However, several features of the Ca^{2+} imaging experiments require further consideration. Firstly, fura-2 has also proved useful in measuring small increases in intracellular zinc (Cheng & Reynolds, 1998) because of its high zinc sensitivity (K_D values ~2 nM), which is about 100 times greater than that for Ca^{2+} (Grynkiewicz *et al*., 1985). The sustained responses consistently observed with zinc present seem to reflect fura-2 detecting zinc rather than Ca^{2+}. The sustained nature of these events indicate that unlike Ca^{2+}, zinc was not readily removed from the intracellular compartment. This suggests that zinc can pass through the poly-APS-evoked

pores, but that Ca^{2+} entry was attenuated to a greater extent than the data indicate. Combined with the electrophysiological data, the imaging studies with fura-2 indicate that zinc reduced levels of poration and may also inhibit Ca^{2+} entry through the pores that are formed by poly-APS. However, we cannot rule out zinc interactions with fura-2 competing significantly to reduce Ca^{2+} detection. Secondly, zinc has been shown to inhibit voltage-activated neuronal conductances, including those carried by Ca^{2+} (Büsselberg *et al*., 1994), which may contribute to the poly-APS responses and this could result in an overestimation of the inhibitory action of zinc on poly-APS effects. However, data have also been obtained from HEK 293 cells under voltage clamp and these data are consistent with zinc inhibiting poly-APS-evoked pore formation independently of voltage-activated Ca^{2+} channels.

In conclusion, poly-APS can reversibly form pores in the membranes of DRG neurones and HEK 293 cells, and applying extracellular zinc with the preparation of sponge toxins can attenuate these actions. The survival of cells even after dramatic but temporary changes in membrane potential and input conductances raises the possibility that transient pore formation by low doses of poly-APS can be used to deliver materials to the intracellular environment, without cell damage. Preliminary studies have indicated that the poly-APS sponge toxin preparations can be used as a transfection reagent to deliver cDNA into cells (Tucker, McClelland, Sepčić, MacEwan, Scott, unpublished data). Work is now underway to assess intracellular application of proteins. Throughout this paper, the term 'pore' has been used to describe the openings in the cell membranes produced by poly-APS. This seems appropriate when considering the transient changes in conductance observed and channel-like events in artificial lipid bilayers have previously been reported with halitoxins (Scott *et al*., 2000). However, lesion may better describe the irreversible events consistent with membrane damage produced with high concentrations of sponge toxins.

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