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The influence of alkyl pyridinium sponge toxins on membrane properties, cytotoxicity, transfection and protein expression in mammalian cells

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Abstract

The ability of two alkyl pyridinium sponge toxin preparations (poly-APS and halitoxin) to form transient pores/lesions in cell membranes and allow transfection of plasmid cDNA have been investigated using HEK 293 cells. Poly-APS and halitoxin preparations caused a collapse in membrane potential, reductions in input resistance and increased Ca^{2+} permeability. At least partial recovery was observed after poly-APS application but recovery was more rarely seen with halitoxin. The transfection with plasmid cDNAs for an enhanced green fluorescent protein (EGFP) and human tumour necrosis factor receptor 2 (TNFR2) was assessed for both toxin preparations and compared with lipofectamine. Stable transfection was achieved with poly-APS although it was less efficient than lipofectamine. These results show that viable cells transfected with alien cDNA can be obtained using novel transient pore-forming alkyl pyridinium sponge toxins and a simple pre-incubation protocol. This provides the first proof of principle that pore-forming alkyl pyridinium compounds can be used to deliver cDNA to the intracellular environment without permanently compromising the plasma membrane.

Keywords: Transfection; Pore formation; Sponge toxin; cDNA; Protein expression

1. Introduction

Sponges produce a variety of toxins that have an array of functions including providing protection from predators and invasion by microorganisms [1]. Some preparations of these toxins that contain cocktails of polymeric alkyl pyridinium salts have been found to produce hemolysis and neurotoxicity [2–5]. These actions may be attributed to the formation of lesions or pores in cell membranes [6,7], an effect that can also be produced in artificial lipid bilayers [6]. Two preparations, poly-APS and halitoxin, both derived from sponges and containing a cocktail of different size polymeric alkylpyridinium salts (Fig. 1A), evoked increases in intracellular Ca^{2+}, collapsed membrane potentials and reduced input resistance. The halitoxin preparation, which contains a large number of alkyl pyridinium compounds (mean molecular weight ~ 5 kDa) produced predominantly irreversible effects on cultured dorsal root ganglion (DRG) neurones and F-11 cells [6]. However, poly-APS that contains two polymeric 1,3-alkylpyridinium salts (poly-APS) of 5.5 and ~ 19 kDa can readily produce at low concentrations (≤ 5 μg/ml) transient pore formation [7]. In aqueous solutions, poly-APS form noncovalently bound aggregates with a mean hydrodynamic radius of 23 ± 2 nm [1]. The radius of the pores formed within these aggregates has been estimated on bovine erythrocytes by the use of osmotic protectants, and calculated with the Renkin equation to be about 2.9 nm [5].

The combination of the pore size and the transient nature of the pore formation raised the possibility that poly-APS could be used to introduce macromolecules such as DNA into cells. Previously, pore properties and potential mechanisms of passive DNA flow have been investigated in a model system [8]. Furthermore, single-stranded polynucleotide RNA and DNA molecules have been shown to traverse lipid bilayers treated with *Staphylococcus aureus* α-hemolysin, which creates membrane pores of 2.6-nm

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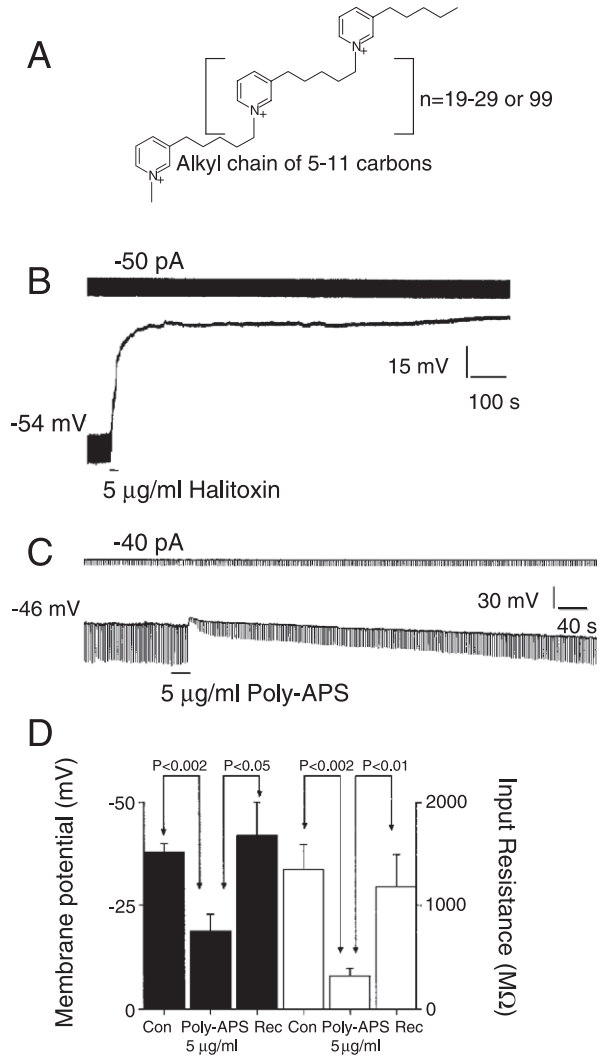


Fig. 1. Structure and electrophysiological actions of halitoxin and poly-APS preparations on HEK 293 cells. (A) General structure of an alkylpyridinium salt present in the halitoxin and poly-APS sponge toxin preparations. The *n* values define the number of monomeric units, indicated by square brackets, forming the polymers. (B) Record showing the irreversible collapse of membrane potential and input resistance evoked by 20-s application of 5 μg/ml halitoxin. Top trace 50 pA hyperpolarising current commands evoked every 3 s; bottom trace showing the membrane potential and electrotonic potentials. (C) Record showing the reversible reductions in membrane potential and input resistance evoked by 20-s application of 5 μg/ml poly-APS. Top trace shows 40 pA hyperpolarising current commands evoked every 3 s; bottom trace shows the membrane potential and electrotonic potentials. (D) Bar graph showing the effects of poly-APS (5 μg/ml) on membrane potential (filled bars) and input resistance (open bars); mean values are presented under control conditions (con; $n=9$), in the presence of toxin ($n=9$) and at 10-min recovery (rec; $n=6$).

diameter. The passage of these nucleotide molecules across the bilayer was signalled by fluctuations in channel conductances as individual molecules passed through, and allowed molecular characterisation [9,10]. A number of other diverse molecules have been similarly shown to create perforations in lipid bilayers including perforin [11,12], complement [12] and alamethicin [13], but as yet none have been shown to provide passage for DNA. More conventional approaches to DNA transfer across lipid bilayers and into intact cells include lipid-micelle-mediated transfection, e.g. lipofectamine [14,15], electroporation [16] and microinjection [17], but these approaches yield variable results and can be difficult to control. So there is scope for new improved methods for DNA transfer.

The aim of this study was to determine whether a simple incubation protocol using poly-APS could enable the introduction of large pieces of super-coiled DNA into living HEK 293 cells without irreparable cell damage. This could provide a novel and reproducible approach to allow both transient and stable transfection of HEK 293 cells with alien DNA, which to our knowledge has never before been achieved with pore-forming molecules of this nature.

2. Materials and methods

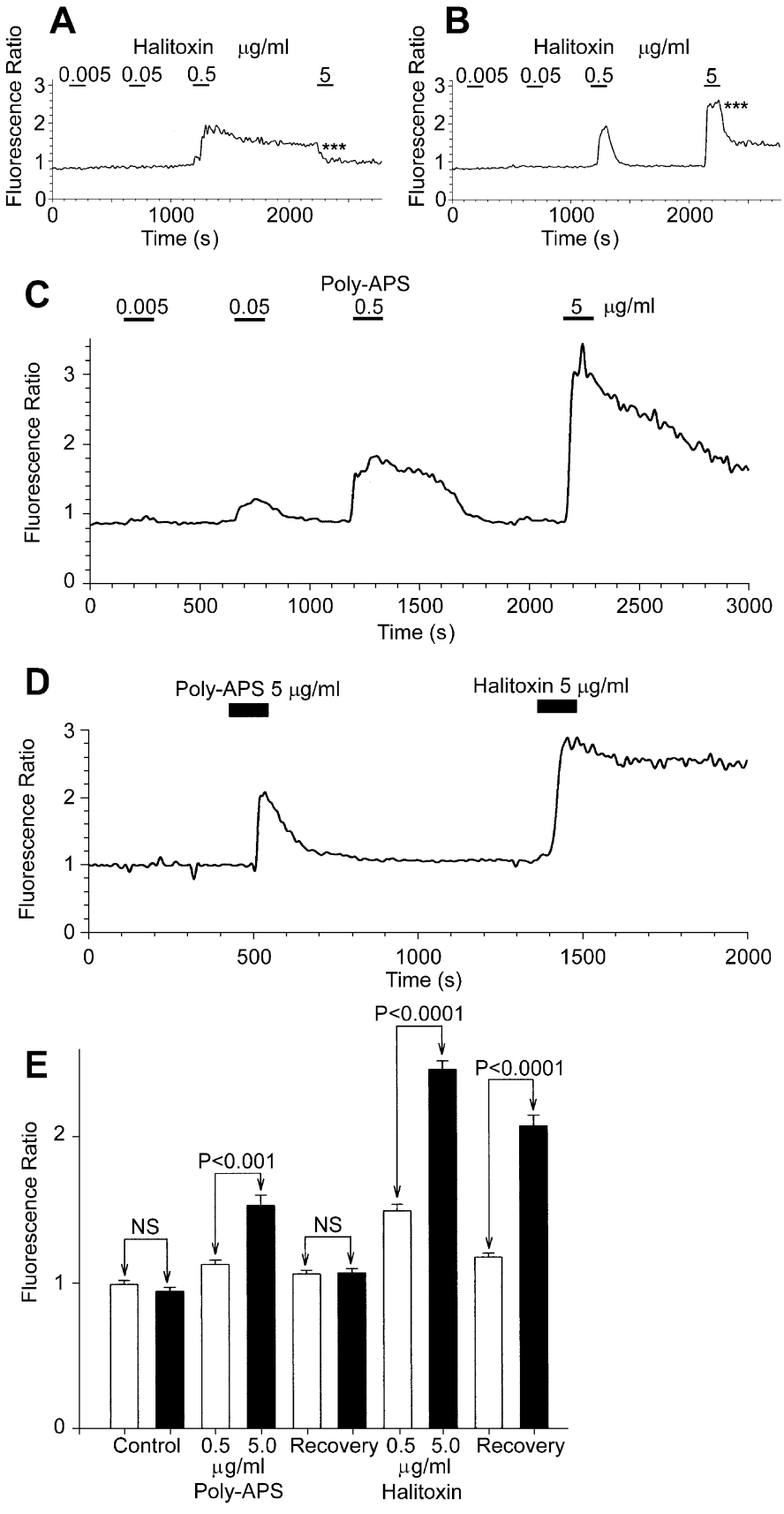
2.1. Sponge toxin preparations

Poly-APS were purified from the marine sponge *Reniera* (=*Haliclona*) *sarai* Pulitzer–Finali (Haliclonidae) as previously described [18]. The halitoxin preparation was isolated from *Callyspongia ridleyi* as previously described [6]. Stock solutions containing 5 mg/ml of either of the toxin preparations were diluted to produce test solutions containing either poly-APS or halitoxins.

2.2. Cell culture

HEK 293 cells (human embryonic kidney cell line) were maintained in culture as previously reported [19]. Briefly, cells were cultured in Eagle's minimum essential medium (EMEM), supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 u/ml penicillin, 50 μg/ml streptomycin and 1% nonessential amino acids. In preparation for transfection experiments, cells were seeded at a density of 0.5 million cells/well in six-well plates; the same seeding density was used for confocal and electrophysiology/calcium imaging

Fig. 2. Actions of halitoxin and poly-APS preparations on intracellular Ca^{2+} in HEK 293 cells loaded with fura-2. (A)–(B) are two example traces showing in (A) a single sustained response and in (B) a transient response to 0.5 μg/ml halitoxin. In both cases no responses were obtained with 0.005 and 0.05 μg/ml halitoxin and *** denotes the points of dye loss and cell damage produced by 5 μg/ml halitoxin. (C) Single example trace showing the dose-dependent actions of poly-APS on fura-2 fluorescence ratio indicative of a rise intracellular Ca^{2+}. (D) Single example trace showing transient and sustained responses to poly-APS (5 μg/ml) and halitoxin (5 μg/ml), respectively. (E) Bar chart showing the mean peak fluorescence values for experiments carried out to investigate the actions of 0.5 μg/ml poly-APS and halitoxin (open bars) and 5 μg/ml poly-APS and halitoxin (filled bars).


A
Halitoxin μg/ml
0.005 0.05 0.5 5
***
Fluorescence Ratio
Time (s)
B
Halitoxin μg/ml
0.005 0.05 0.5 5
***
Fluorescence Ratio
Time (s)
C
Poly-APS
0.005 0.05 0.5 5 μg/ml
Fluorescence Ratio
Time (s)
D
Poly-APS 5 μg/ml
Halitoxin 5 μg/ml
Fluorescence Ratio
Time (s)
E
P<0.0001
P<0.0001
P<0.001
NS
NS
Fluorescence Ratio
Control 0.5 5.0 Recovery 0.5 5.0 Recovery
μg/ml
Poly-APS
μg/ml
Halitoxin

experiments but in 35-mm dishes. This culture was carried out 24 h prior to experimentation.

2.3. Electrophysiology and Ca^{2+} imaging

Electrophysiological and Ca^{2+} imaging experiments were conducted at room temperature (approximately 23 °C). The whole-cell recording technique was used as previously [6,7] to study the actions of poly-APS and halitoxin preparations on membrane potential and input resistance (evaluated from 300–700 ms electrotonic potentials evoked by −30 to −100 pA current commands). Whole cell recordings were made using an Axoclamp-2A switching voltage clamp amplifier. Low resistance (4–7 MΩ) borosilicate glass patch pipettes were fabricated using a Kopf model 730, needle/pipette puller. The HEK 293 cells 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. The pH and osmolarity of extracellular solutions were adjusted to 7.4 and 310–320 mosM/l 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 mosM/l with sucrose. Poly-APS and halitoxin were applied to the extracellular environment by low-pressure ejection for 20 s via a blunt micropipette (tip diameter about 10 μm) positioned approximately 100 μm from the HEK 293 cell 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 2200s pen recorder).

Cultured 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 [6]. All data are given as mean ± standard error of the mean (S.E.) and statistical significance was determined, using the Student's two-tailed *t* test, paired or independent where appropriate.

2.4. DNA transfer

Plasmid cDNAs used were pEGFP-C1 (Clontech), an enhanced green fluorescent protein (EGFP) cDNA vector under control of a constitutively active SV40 promoter [20,21], human tumour necrosis factor receptor 2 (TNFR2) cDNA (provided by Werner Lesslauer, Basel, Switzerland) and pBABE hygromycin resistance cDNA [19].

Control transfections were carried out using optimized lipofectamine (Invitrogen Life Technologies) lipid-micelle-mediated transfection protocol as previously reported [19], which incubates cells with 1-μg cDNA and lipofectamine in the absence of serum for 3 h prior to reintroduction to serum-containing medium. A standard toxin transfection protocol was developed and optimized throughout the passage of this work (see below). The protocol involved a 5-min serum-free cell incubation with 0.5 μg/ml of a sponge toxin preparation, followed by addition of 2.5-μg cDNA, after a further 3-h incubation medium was replaced by standard serum-containing medium. Lipofectamine and poly-APS stable transfections involved co-transfection with 1.0- or 2.5-μg cDNA, respectively (either pEGFP or TNFR2 cDNA), and 1.0 μg of pBABE cDNA. Colonies of stably transfected cells were selected in EMEM containing 100 μg/ml hygromycin B. Once established, colonies were harvested using cloning discs and trypsin-EDTA and seeded into larger vessels until sufficient cells were available for experimentation [19]. In order to reduce variability, cDNA was introduced into 1-ml preparations at a volume of 10 μl. A similar protocol was used to assess the potential of the cationic surfactants cetypyridinium chloride (CPC) and cetyltrimethylammonium bromide (CTAB) (0.5 μg/ml) as transfection reagents.

2.5. Crystal violet cytotoxicity assay

Adherent cells were fixed in paraformaldehyde and stained in crystal violet dye as previously described [22]; subsequent elution and spectrophotometric analysis quantified the amount of intact cells capable of harboring dye following transfection.

2.6. GFP detection

Cellular expression of GFP was evaluated using a fluorescence/visible light microscope set-up to directly assess the percentage of total cells fluorescing. Alternatively, cells were imaged and photographed using a Bio-Rad microradiance confocal system.

2.7. FACS analysis

HEK 293 cells stably overexpressing TNFR2 were dissociated from their culture vessels with 3-ml cell dissociation solution (trypsin-free) and assessed for TNFR1 and TNFR2 expression with specific mouse monoclonal antibodies (htr-9 and utr-1, respectively), as previously described [23]. Secondary labeling with flourescein-isothiocyanate (FITC) anti-mouse IgG allowed FACS detection.

2.8. Confocal analysis

HEK 293 TNFR2 clones were plated into triplicate 35-mm dishes and fixed in ice-cold methanol. Individual dishes were probed with no antibody, TNFR1- or TNFR2-specific mouse monoclonal antibodies (htr-9 and utr-1, respectively) and then secondary labeled with FITC anti-mouse IgG, as previously reported [24]. Cellular FITC labeling was then assessed using a Bio-Rad micro-radiance confocal system, and was indicative of TNF receptor expression levels.

3. Results

3.1. Characterisation of pore formation

The actions of poly-APS and the halitoxin preparations on membrane potential, input resistance and Ca^{2+} permeability of HEK 293 cells were evaluated. As previously observed in cultured DRG neurones, a 20 s application of halitoxin (0.5 and 5.0 μg/ml) predominantly resulted in irreversible effects on the electrophysiological properties of HEK 293 cells (Fig. 1B). Halitoxin (0.5 μg/ml) reduced membrane potential from -45 ± 5 to -16 ± 5 mV ($n=6$; $P<0.003$), and partial but significant recovery to -26 ± 4 mV ($n=6$; $P<0.02$) was observed 10–20 min after toxin application. Halitoxin (5 μg/ml) reduced membrane potential from -46 ± 3 to -3 ± 1 mV ($n=5$; $P<0.003$) with no significant recovery. Similar trends were observed when input resistance was determined from the electrotonic potentials evoked by hyperpolarising current commands. For example, the resting input resistance was reduced from 903 ± 313 to 315 ± 206 MΩ ($n=6$; $P<0.04$) by 0.5 μg/ml halitoxin but no significant partial recovery was observed. There was also evidence that the effects on membrane potential were dose-dependent with 0.5 and 5 μg/ml halitoxin reducing membrane potentials by $64 \pm 10\%$ ($n=6$) and $94 \pm 3\%$ ($n=5$; $P<0.03$), respectively. In contrast, the actions of poly-APS applied for 20 s were predominantly reversible both at 5 μg/ml ($n=6$ out of 9 cells, Fig. 1C and D) and at 0.5 μg/ml ($n=6$ out of 9 cells). The dose dependency was less clear with poly-APS; both 5 and 0.5 μg/ml toxin preparation gave equivalent electrophysiological responses to those obtained with 0.5 μg/ml halitoxin. Poly-APS at 5 and 0.5 μg/ml reduced the mean membrane potential from control values of -38 ± 2 mV ($n=9$) and -44 ± 2 mV ($n=8$) to -19 ± 4 mV ($n=9$; $P<0.0015$) and -21 ± 5 mV ($n=8$; $P<0.0001$), respectively. These actions were associated with reductions in input resistance. Poly-APS at 5 and 0.5 μg/ml reduced the mean input resistance from control values of 1350 ± 246 MΩ ($n=9$) and 1027 ± 363 MΩ ($n=8$) to 325 ± 73 MΩ ($n=9$; $P<0.0012$) and 179 ± 41 MΩ ($n=8$; $P<0.05$), respectively. The majority of cells showed recovery from the actions of poly-APS at both concentrations, with 6 of 9 cells at least partially recovering from 5 μg/ml poly-APS and 6 of 8 cells recovering from 0.5 μg/ml poly-APS.

Experiments using the ratiometric dye fura-2 also indicated that both halitoxin and poly-APS evoked large

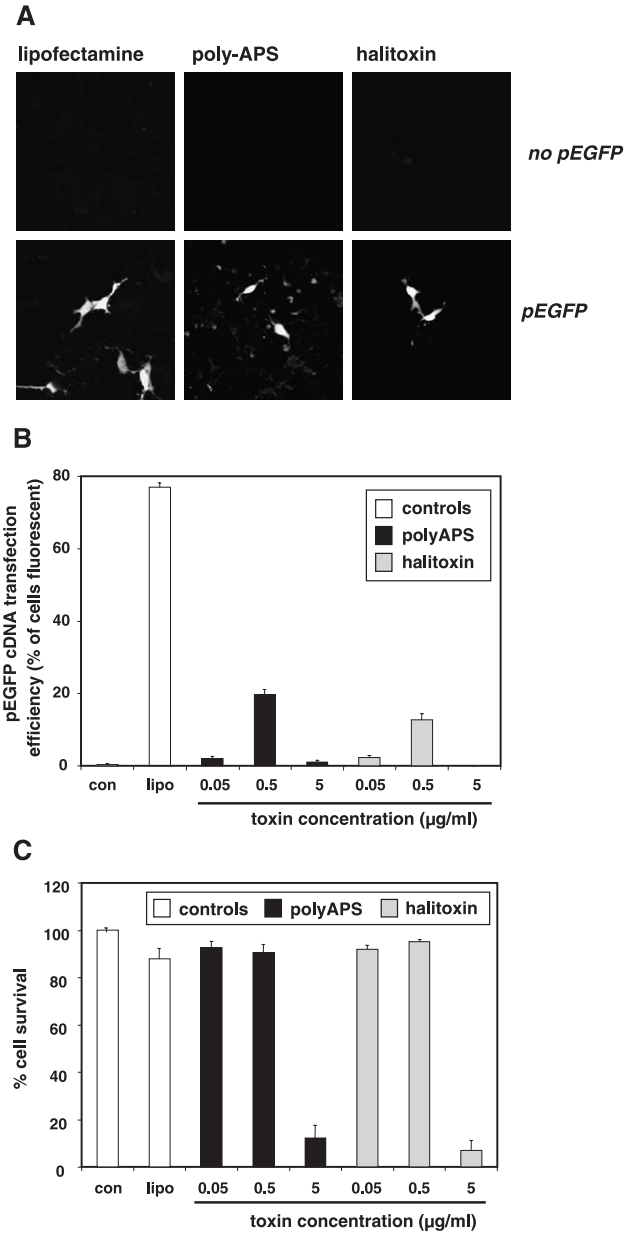


Fig. 3. Transient transfection of HEK 293 cells with pEGFP cDNA using sponge toxins. (A) HEK 293 cells treated with lipofectamine, 0.5 μg/ml poly-APS or 0.5 μg/ml halitoxin in the absence (upper panels) or presence (lower panels) of pEGFP. Confocal images of fluorescent cells captured 48 h post-transfection; images are representative of at least three other independent experimental repeats. (B) HEK 293 cells transfected with pEGFP using lipofectamine or 0.05, 0.5 and 5.0 μg/ml poly-APS/halitoxin were assessed for percentage of total cells fluorescent 48 h post-transfection. Data shown are means ± S.E., $n=3$. (C) HEK 293 cells transfected and assessed as in (B) were subjected to crystal violet staining 48 h post-transfection to quantify intact and adherent cells. Data expressed as percent of surviving cells relative to cDNA-only control, shown as means ± S.E., $n=3$.

changes in intracellular Ca^{2+}. Initially, a protocol was used to examine the dose-dependent effects of the sponge toxins. Halitoxin or poly-APS were applied for 140 s at doses of 0.005, 0.05, 0.5 and 5.0 μg/ml with variable wash periods between each application. Few cells responded to the sponge toxins when they were applied for 140 s at doses of 0.005 μg/ml, 10 of 115 cells for halitoxin and 2 of 90 cells for poly-APS. So for our protocol, 0.005 μg/ml appears to be the threshold concentration for a detectable Ca^{2+} transient evoked by sponge toxin preparations in HEK 293 cells. The halitoxin preparation produced some anomalous results because cell lysis and dye loss were usually observed with 0.5 or 5 μg/ml when the cells had been exposed previously to the two or three lower doses of toxin

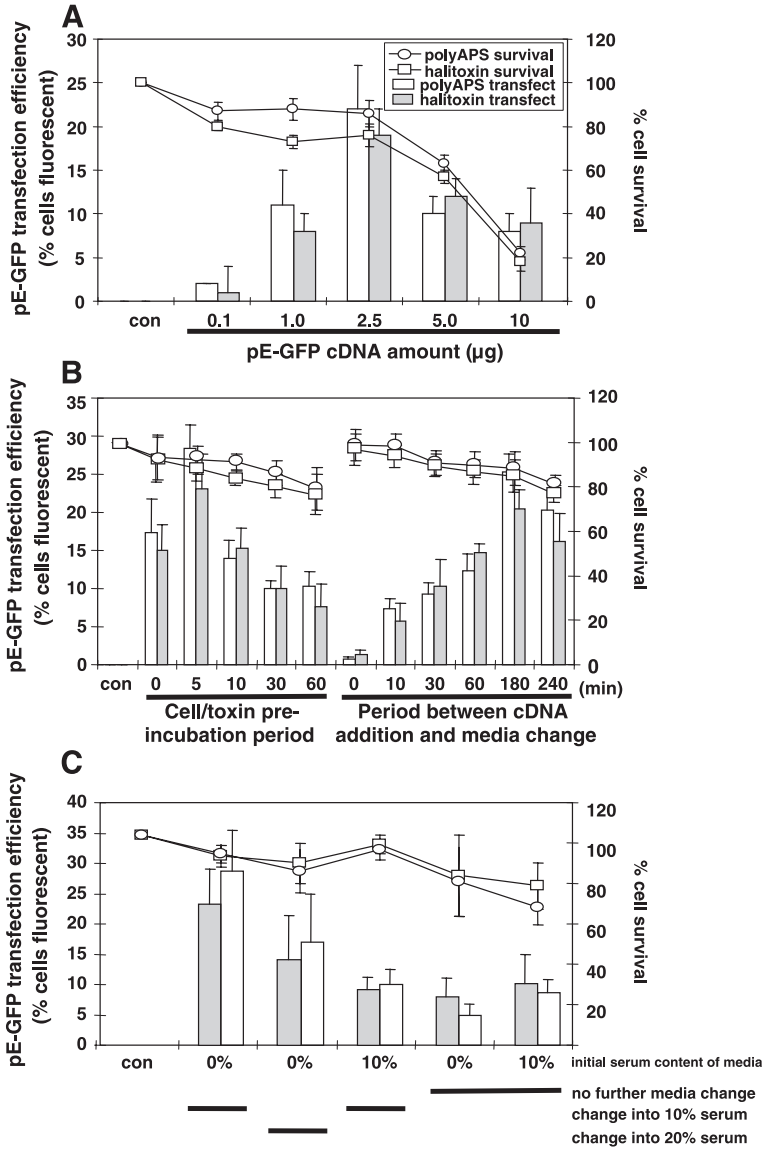


Fig. 4. Optimisation of toxin transfection protocol. (A) HEK 293 cells were transfected with varying quantities of pE-GFP cDNA for 3 h using 0.5 μg/ml polyAPS or halitoxin. To control for cDNA volume, pE-GFP was diluted such that a 10-μl addition was required to achieve the desired cDNA quantity. Fluorescent measurements were followed by crystal violet cytotoxicity assays 48 h post-transfection, and are shown as bars and lines, respectively (white bars, circles: polyAPS; grey bars: squares halitoxin). All data show mean ± S.E., $n=3$. (B) HEK 293 cells were transfected with 2.5-μg pE-GFP following variable pre-incubation period with 0.5 μg/ml polyAPS or halitoxin. Alternatively, HEK 293 cells were transfected with 2.5-μg pE-GFP following 5-min pre-incubation with 0.5 μg/ml polyAPS or halitoxin and the period between cDNA addition and media change varied. Fluorescent measurements and crystal violet cytotoxicity assays were performed 48 h post-transfection and are shown as bars and lines, respectively (white bars, circles: polyAPS; grey bars: squares halitoxin). All data show mean ± S.E., $n=3$. (C) HEK 293 cells were incubated for 180 min with 2.5-μg pE-GFP in either serum-free media or serum-containing (10%) media following 5-min pre-incubation with 0.5 μg/ml polyAPS or halitoxin. Subsequently, cDNA and the sponge toxin preparation were removed and cells were maintained in media containing either 10% or 20% serum or the cells remained in the transfection medium. Fluorescent measurements and crystal violet cytotoxicity assays were performed 48 h post-transfection and are shown as bars and lines, respectively (white bars, circles: polyAPS; grey bars: squares halitoxin). All data show mean ± S.E., $n=3$.

(Fig. 2A and B). The mean peak changes in fluorescence ratios observed were 0.2 ± 0.03 ($n = 10$), 0.43 ± 0.03 ($n = 80$) and 1.09 ± 0.04 ($n = 114$) with 0.005, 0.05 and 0.5 μg/ml halitoxin, respectively. These data were obtained from cells that responded to usually single applications of halitoxin and do not show the true dose-dependency of the toxins actions but reflect varying threshold sensitivities to the toxins. However, at least in some cells transient changes in intracellular Ca^{2+} were observed with 0.5 μg/ml halitoxin (Fig. 2B). Dose-dependent and predominantly reversible responses were obtained with poly-APS (0.005–5 μg/ml; Fig. 2C). The mean peak changes in fluorescence ratios observed were 0.002 ± 0.002 ($n = 90$), 0.46 ± 0.05 ($n = 90$), 0.76 ± 0.06 ($n = 90$) and 1.17 ± 0.05 ($n = 89$) with 0.005, 0.05, 0.5 and 5.0 μg/ml poly-APS, respectively. Varied threshold sensitivities of different HEK 293 cells to the sponge toxins were observed so that some cells did not respond to the lower concentrations. Out of 90 cells, only 2 responded to 0.005 μg/ml poly-APS, 63 cells responded to 0.05 μg/ml poly-APS and 83 cells responded to 0.5 μg/ml μg/ml poly-APS. All the cells responded to the highest toxin concentration (5 μg/ml) tested with one cell not surviving the application of 0.5 μg/ml poly-APS. Separately applying single doses of each toxin clearly showed the different amplitudes and natures of responses produced by the sponge toxins (Fig. 2D and E). All cells studied responded to 0.5 and 5.0 μg/ml toxins, so these doses were used in subsequent experiments.

3.2. Optimisation of transient cDNA transfection

The transient, reversible nature of the pores formed by the sponge toxins prompted further study to investigate whether these transient membrane lesions were sufficient to conduct plasmid cDNA across the lipid bilayer. Qualitative assessment of HEK 293 cells incubated with 0.5 μg/ml poly-APS or halitoxin and 2.5-μg pEGFP indicated successful transfection, as a number of fluorescent cells were observed (Fig. 3A, lower panels). This was in contrast to cells incubated with toxin alone (Fig. 3A, upper panels) or pEGFP alone (data not shown), which displayed no fluorescent cells. Use of a commercial transfection reagent (lipofectamine) to transfect HEK 293 cells (Fig. 3A, left-hand panels) provided a positive control for both the pEGFP cDNA and the imaging of transfected fluorescent cells. Extensive optimisation experiments were required to maximise transfection of HEK 293 cells using sponge toxins and to provide a standardised, repeatable protocol. Such experiments investigated the influence on transfection efficiency of a number of variable factors and assessed both percentage of cells transfected and cell survival. A range of cDNA concentration experiments indicated that 2.5-μg pEGFP maximises transfection efficiency without inducing intolerable toxicity (Fig. 4A). The use of lesser quantities of cDNA, while being far less toxic, displayed low transfection efficiency, with greater quantities producing the opposite (high toxicity, moderate transfection efficiency) (Fig. 4A).

Experiments focussing on the duration of incubation with toxin/cDNA revealed that a 5-min incubation with toxin prior to cDNA addition produced higher levels of transfection compared to simultaneous addition or longer periods of toxin pre-incubation (Fig. 4B, left). Furthermore, a period of 180-min incubation was optimal following addition of cDNA, with shorter periods reducing transfection efficiency and longer periods proving more toxic (Fig. 4B, left right). Serum has a profound effect on lipid-mediated cDNA transfection [15]; we investigated whether similar effects would be seen with toxin-mediated transfection. Serum-free conditions proved most effective for toxin-mediated transfection, without raising toxicity levels (Fig. 4C); in addition, a return to standard 10% serum-containing medium following transfection was more beneficial than raising serum levels to 20% or above (Fig. 4C).

Quantification of pEGFP transfection indicated 0.5 μg/ml poly-APS/halitoxin achieved higher levels of transfection than either 0.05 or 5.0 μg/ml (Fig. 3B). Comparable levels of transfection were achieved with both toxins, although at 0.5 μg/ml, poly-APS achieves marginally higher transfection success (Fig. 3B). Interestingly, these concentration-driven toxin activities coincide with the electrophysiological toxin profiles presented in Figs. 1 and 2. Although these transfection levels are fourfold lower than lipofectamine transfection efficiency, they are raised considerably above cDNA-only control levels (consistently 0). Analysis of the lethality of these toxins suggests that at both 0.05 and 0.5 μg/ml, neither of the toxins induce cell death above the tolerable level of death seen in lipofectamine-mediated transfection (88–95% survival of HEK 293 cells) (Fig. 3C). However, approximately 10–12-fold more death occurred with 5.0 μg/ml poly-APS and halitoxin (12% and 6% survival, respectively) (Fig. 3C), suggesting a dose-dependent sponge toxin toxicity, with cells tolerant up to 0.5 μg/ml toxin. Furthermore, this very high toxicity explains the low proportion of fluorescent cells (Fig. 3B), with the toxin killing the cells as opposed to transfecting them at 5.0 μg/ml. This compliments data presented in Fig. 2 concerning dose-dependent reduction in pore reversibility, which may be a factor contributing heavily to toxicity.

Incubation of cells in the presence of monomeric pyridinium surfactants CPC and CTAB failed to achieve any pEGFP transfection in HEK 293 cells (data not shown). This indicates that transfection cannot be attributed purely to surfactant properties but involves distinct mechanisms associated with the chemistry of 3-alkyl pyridinium polymers.

3.3. Stable transfection

Stable transfection of HEK 293 cells using 0.5 μg/ml poly-APS further illustrates this toxin as a potentially

useful transfection tool. Following selection of stable colonies of HEK 293 cells in hygromycin B, a large number of colonies remained in six-well plates transfected using lipofectamine or poly-APS, in contrast to control cells which had no hygromycin B-resistant colonies (Table 1), indicative of hygromycin B lethality to normal cells. In

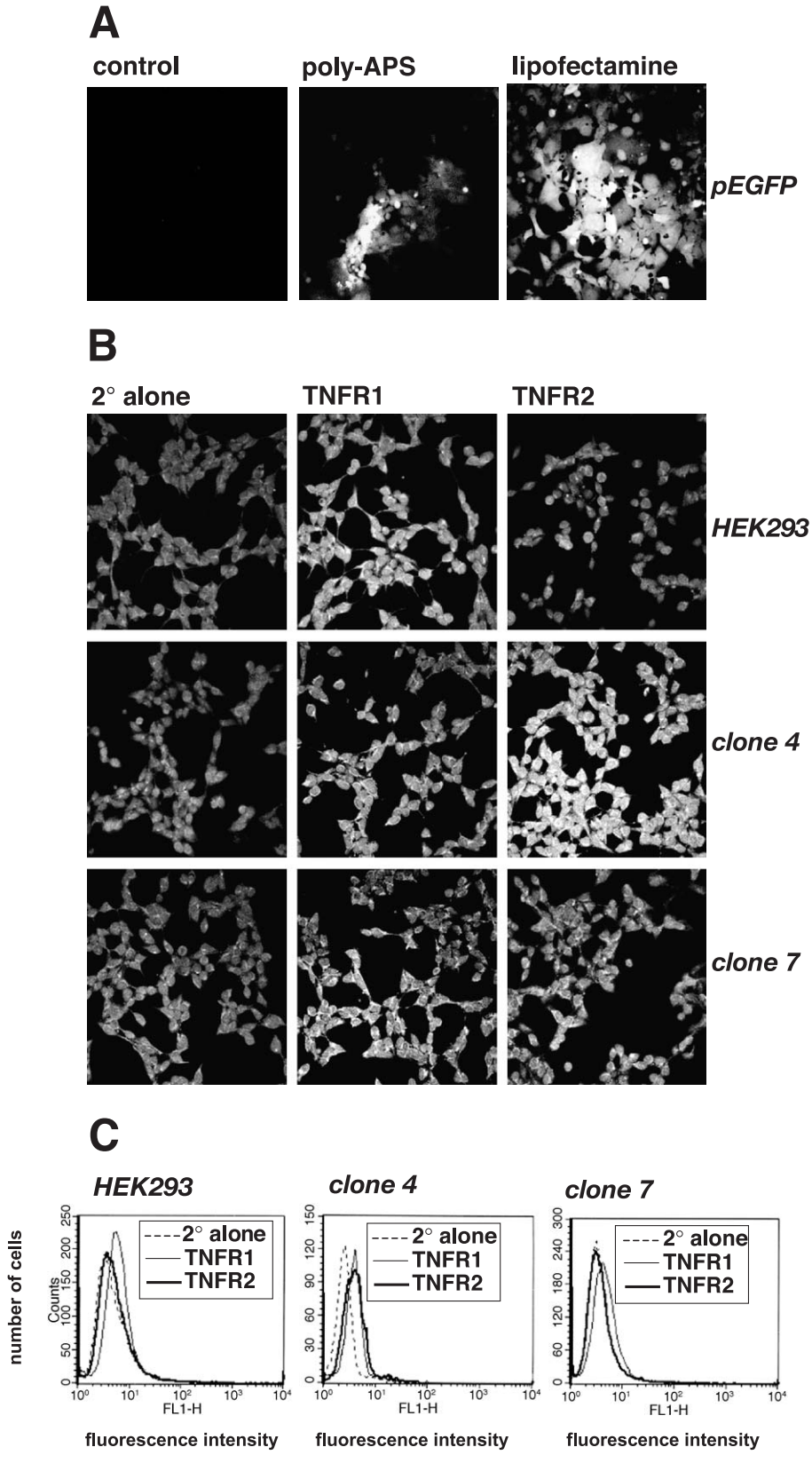


Table 1
Comparison of lipofectamine and poly-APS transfected stable colony number

Vehicle	Mean colony number
Stable transfection	
None	0
Lipofectamine	18.5 ± 4.5
Poly-APS	7.1 ± 1.4
Poly-APS stable clones cDNA	
pEGFP	6.2. ± 2.0
TNFR2	8.0 ± 2.0

Colonies of hygromycin B selected HEK 293 cells stably transfected with pEGFP or TNFR2 cDNAs using poly-APS or lipofectamine were counted in culture wells prior to harvesting. Upper panel shows comparison of lipofectamine and poly-APS colony numbers, $n = 5$ and $n = 10$, respectively. Lower panels split poly APS stable data into either pEGFP or TNFR2 expressing colonies, $n = 5$ in both cases.

addition, lipofectamine is more effective than poly-APS at stably transfecting HEK 293 cells (2.5-fold more colonies in lipofectamine plates), further evidence that a lower, yet still relevant level of transfection is achieved using poly-APS than commercial reagents (Table 1 and Fig. 3B). Moreover, confocal visualisation of pEGFP stably transfected cells suggests larger numbers of positive cells in lipofectamine wells, compared to poly-APS-transfected wells, although again poly-APS shows considerably larger numbers of stably transfected cells compared to controls (Fig. 5A).

The type of cDNA being co-introduced to the cell along with pBABE has no bearing on the success of stable expression, with poly-APS-mediated stable transfection of pEGFP and TNFR2 cDNA producing comparable numbers of stable colonies of HEK 293 cells (Table 1). Analysis and comparison of TNF-receptor levels in untransfected HEK 293 cells and two TNFR2 stably transfected clones show relatively low and comparable FITC labelling of TNFR1 in all three cell types (Fig. 5B), consistent with wild-type TNFR1 expression. In contrast, only clone 4 shows raised TNFR2 labelling, indicating successful stable transfection of TNFR2 cDNA. Clone 7 does not show an enhanced TNFR2 expression level, with very low FITC labelling similar to control cells (Fig. 5B), suggesting only pBABE and not TNFR2 cDNA has been successfully transfected—resulting in expression of only the hygromycin resistant phenotype. Study of the same cells using FACS shows a similar rightward shift in fluorescence intensity for all three cell types when labelled with TNFR1-specific antibodies compared to secondary antibody alone (Fig. 5C), indicating similar levels of TNFR1 in the three cell types. However, no TNFR2 shift is visible in control or clone 7 cells with TNFR2 histogram lines overlying secondary antibody alone lines (Fig. 5C), suggesting little or no TNFR2 expression. In clone 4 a considerable increase in fluorescence intensity is apparent in TNFR2-labelled samples, consistent with enhanced TNFR2 expression (Fig. 5C). Thus clone 4 stably co-expresses both hygromycin-resistant and TNFR2-enhanced phenotypes, following initial transfection with poly-APS, as proved by both confocal and FACS analysis.

4. Discussion

Consistent with findings in cultured dorsal root ganglion neurones and F-11 cells, halitoxin [6] and poly-APS preparations [7] produced conductance increases and raised intracellular Ca^{2+} in a manner consistent with pore or lesion formation in the cell membranes of HEK 293 cells. As previously observed, the poly-APS appeared to be less effective at doses of 0.5 and 5 μg/ml but produced more reversible responses than the halitoxin preparation. It seems likely that the degree of polymerisation influences how readily the responses reverse; however, both sponge toxin preparations contain two or more distinct alkyl pyridinium compounds. In addition to size of alkyl pyridinium compounds, issues of aggregation [18] and lipid membrane constituents are likely influences on pore formation and cell recovery and even between cells from a cell line considerable variability in responses were observed. Monomeric pyridinium compounds like CPC and CTAB were unable to allow cDNA transfer consistent with the importance of the polymeric structure of halitoxins and poly-APS.

The transfer of cDNA into HEK 293 cells and subsequent protein expression indicated that both sponge toxin preparations at 0.5 μg/ml were able to provide pores for cDNA transfer without cytotoxicity. Clearly the longer sponge toxin incubation periods used in the transfection

Fig. 5. Stable transfection of HEK 293 cells with pEGFP and TNFR2 cDNAs using poly-APS. (A) HEK 293 cells co-transfected with pEGFP and pBABE cDNA using no transfection vehicle (left-hand panel), 0.5 μg/ml poly-APS (centre panel) or lipofectamine (right-hand panel). Following hygromycin B selection, cells were imaged under confocal microscope and photographed. Photographs representative of three other independent experimental repeats. (B) HEK 293 cells co-transfected with TNFR2 and pBABE cDNA using 0.5 μg/ml poly-APS were selected with hygromycin B and individual stable clones probed with no antibody (left-hand panels), TNFR1- (centre panels) or TNFR2-specific mouse monoclonal antibodies (right-hand panels). Subsequent secondary labelling with FITC anti-mouse IgG allowed confocal visualisation. Shown here are untransfected HEK 293 cells (upper panels), a positive TNFR2 clone (clone 4) (middle panels) and a negative TNFR2 clone (clone 7) (lower panels). Photographs are representative of three other independent experiments. (C) Poly-APS transfected stable TNFR2 clones were dissociated, probed with no antibody, TNFR1- or TNFR2-specific mouse monoclonal antibodies and secondary labelled with FITC anti-mouse IgG. Subsequent FACS analysis allowed quantitation and comparison of TNFR expression between untransfected HEK 293 cells (left-hand panel), a TNFR2 positive clone (clone 4) (centre panel) and a TNFR2 negative clone (clone 7) (right-hand panel). Histograms of fluorescence intensity (arbitrary units) against number of cells (cell counts) are representative of several other repeat experiments.

studies gave rise to greater toxicity at 5.0 μg/ml than was observed with electrophysiology and Ca^{2+} imaging. The optimised protocol required pre-incubation with the sponge toxins and pore formation prior to addition of cDNA, suggesting that in solution toxin and cDNA interactions attenuated pore formation and/or cDNA transfer. Similar effects were seen with serum present, which also reduced transfection efficiency achieved with poly-APS. Poly-APS has strong interactions with serum proteins and, consistent with our study, incubation of poly-APS with highly diluted serum can prevent the hemolytic activity of poly-APS. Comparison between the methods using lipofectamine and sponge toxin preparations shows that although presently lipofectamine has higher efficiency, poly-APS has some clear advantages including high stability, good water solubility, and the sponge toxins provide a simpler and more consistent method that could be of benefit to in vivo studies. These encouraging results with alkyl pyridinium compounds enabling low efficiency transfection may be further improved when individual defined molecules are used in place of cocktails of alkyl pyridinium compounds present in the sponge toxin preparations. Our data using halitoxin and poly-APS preparations suggest that larger alkyl pyridinium compounds with greater degrees of polymerisation may provide transient pores that allow enhanced cDNA transfer across the cell membrane but enable cell recovery. The negative results with CPC and CTAB and the requirement for pre-incubation with sponge toxin preparations suggest that the alkyl pyridinium compounds form pores to deliver cDNA to the intracellular environment. This is in contrast to the actions of polycationic reagents that achieve transfection by ionic attraction, membrane association and endocytosis [25].

Experiments were conducted to compare and optimise the cell survival and transfection of EGFP, TNF receptor 2 and pBABE (hygromycin resistance) cDNAs after exposure to the alkyl pyridinium compounds or lipofectamine. The use of such functional assays herein provides evidence that the transfection reagents and protocols we have devised allow for fully functional protein expression with no detriment to the transfection, incorporation, transcription and translation processes. Exploitation of alkyl pyridinium compounds allows both transient and stable transfection and, although the sponge products were less efficient than lipofectamine, they are simpler to use, highly chemically stable and show good water solubility. This work is a significant advance because of the novel use of transient pore-forming molecules to achieve transfection, and we believe that these novel sponge toxins provide the first successful approach to delivering double-stranded cDNA to cells via transient membrane spanning pores. In nature, and in addition to defence, sponge toxins may provide perhaps a unique environment surrounding sponges that afford a novel natural mechanism for the exchange of genetic material between marine organisms.

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