IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF A CLASS ON NON-SELECTIVE CATION CHANNELS IN *SACCHAROMYCES CEREVISIAE*

SCOTT ANTHONY WILLIAM CARTER

School of Agriculture, Food and Wine

The University of Adelaide

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5. Identification of a protein family with voltage dependent non-selective cation channel activity

5.1. Introduction

5.1.1. viNSCCs and cation flux in plants

5.1.1.1. viNSCCs and their role in Na^+ and NH_4^+ uptake in plants

Salinity is a general term relating to all salts found in soil, where the most relevant salt for a majority of cropping systems is NaCl (Munns, 2008). Salinity can impact the fitness of plants through effecting changes in the osmotic environment and through ionic toxicity (Munns, 2005; Munns, 2008; Amtmann, 1999). Most plants readily take up Na^+ from the soil solution, however the primary mechanism for this flux remains poorly understood. Once Na^+ enters the symplastic pathway in the root, there is often a rapid transfer and accumulation of Na^+ in the shoots (Essah et al., 2003). In most instances this results in a positive correlation between Na^+ accumulation in the shoots and Na^+ toxicity symptoms (Amtmann, 1999; Essah et al., 2003). Initial uptake of Na^+ and its subsequent transfer within the cell requires the flow of Na^+ across cellular membranes. Many proteins facilitating flow of Na^+ across plant membranes have been described previously (Chapter 1). However, physiological evidence suggests there is a separate mechanism, catalysed by an until recently, unknown protein, responsible for larger net Na^+ flux events into plant cells (Essah et al., 2003; Maathuis, 2006; Wang et al., 2006; Cheeseman, 1982; Wang, 2007). The molecular identity of this transport phenomenon remains unknown. A likely candidate may be a member of the viNSCC class of trans-membrane channel proteins (Demidchik, 2002a). This suggestion has been supported through the use of electrophysiological techniques to determine that Na^+ fluxes observed in plant cells

appear to be channel mediated and non selective both of which are consistent with observed net fluxes *in planta* (Davenport and Tester, 2000; Demidchik, 2002a; Essah et al., 2003). The non-selective nature of this channel means it also influences the flux of other physiologically relevant cations, an important example of which is NH_4^+.

Flux of NH_4^+ in plant systems is biphasic in nature. For external NH_4^+ concentrations of approximately 5 mM and less, the high affinity uptake system (HATS) is the primary mechanism for NH_4^+ uptake into roots. HATS flux follows a saturable uptake kinetics profile, concurrent with Michaelis – Menten saturable uptake kinetics. Proteins within the Mep / AMT / Rh protein superfamily have been identified to account for HATS flux in yeast and plants (Marini, 1994; Ninnemann, 1994; Yuan et al., 2007). At higher external concentrations of NH_4^+, a low affinity uptake system (LATS) becomes the dominant mechanism (Wang et al., 1993; Kronzucker, 1996). NH_4^+ flux through the LATS follows an arithmetically linear uptake with unsaturable kinetics (Wang et al., 1993). There have been no proteins identified to account for this flux. viNSCCs are considered good candidates for the low affinity flux of NH_4^+ across biological membranes in plants (Loque and von Wiren, 2004).

5.1.1.2. PQ loop repeat proteins as candidate viNSCCs

Two members of the yeast PQ Loop membrane protein family were identified using a bioinformatic screen that compared unannotated putative membrane transport proteins common in both yeast and plants (Chapter 3). Both PQ loop repeat proteins were partially characterised for both NH_4^+ and Na^+ transport using a yeast-based heterologous expression system. In this chapter, data showing the transport activities of these proteins gathered through heterologous expression in *Xenopus laevis* oocytes.

The results strongly suggest PQ loop repeat proteins behave as non-selective cation channels capable of both Na^+ and NH_4^+ transport.

5.2. Results

5.2.1. Characterisation of fluxes mediated by PQ Loop proteins YDR352w and YOL092w expressed in *Xenopus laevis* oocytes.

Characterisation of transporters and channels using electrophysiology typically involves analysis of species selectivity and affinity and identification of putative blockers. Characteristics of flux facilitators can also be defined, in part, through the analysis of their responses in relation to classical Nernstian modelling. The Nernst equation is a mathematical relationship that can be used to determine equilibrium potentials of chemical systems. This can be applied to predict electrical potentials, such as the reversal potential (E_{rev}) across biological membranes in ideal models based on the activity co-efficient on either side of the membrane and the potential applied. Reversal potential can be defined as the potential at which, in a specific system being observed, ionic flux is zero (i.e. an equilibrium is reached and maintained). Shifts in E_{rev} can be used to indicate the nature of a species moving across the membrane, according to the Nernst equation, E_{rev} will typically shift closer to the predicted E_{rev} of the species that accounts for the majority of the flux.

The Nernst Equation

$E_a = RT/zF \cdot \ln([a_o]/\ln[a_i])$

Where:

Ea = The electrical potential across the membrane due to the concentration gradient of the ionic species a.

R = The universal gas constant

T = The temperature of the system (°K)

z = the number of electric charges carried by a mole of species a

F = Faraday constant

$[a_o]$ = The activity of species a on the outside of the membrane.

$[a_i]$ = The activity of species a on the inside of the membrane.

YDR352w and YOL092w were investigated using such methods. In this study, *X. laevis* oocytes were injected with either YDR352w or YOL092w cRNA and compared to water injected controls for their ability to transport Na^+, MA^+, NH_4^+, K^+, choline$^+$ and Ca^{2+}. Of the cations tested, Na^+ proved to generate data most useful for further characterisations. Native Na^+ currents in water-injected controls were relatively small while expression of YDR352W and YOL092w elicited large Na^+ induced currents (Figures 5-1 A & B and Figure 5-2). To ensure the currents observed were due to the influx of Na^+ and not efflux of Cl^- or the efflux of Cl^-, a series of buffers with differing NaCl concentrations was tested. As the external NaCl concentration increased, the reversal potential (E_{rev}) became less negative (Figure 5-1 A &B) which is consistent with the influx of a cation (Na^+) as opposed to the efflux of

an anion (Cl^-). At the physiologically relevant potential of -130 mV, conductance was greater under these conditions for oocytes expressing YDR352w or YOL092w compared to those injected with H_2O (Figure 5-1 C). In oocytes expressing YDR352w or YOL092w, conductance increased with increasing Na^+ concentration (Figure 5-1, B). Observed E_{rev} was different to E_{Na+} for oocytes injected with H_2O or either of YDR352w or YOL092w (Figure 5-1, D). This is unsurprising as E_{rev} is not solely due to the influence of Na^+ flux. Nevertheless, observed E_{rev} for oocytes injected with YDR352w or YOL092w was closer to the predicted E_{Na+} than for H_2O injected oocytes, suggesting Na^+ flux is exerting a greater influence on membrane conductance in oocytes expressing YDR352w or YOL092w rather than water-injected control oocytes.

Significantly higher currents were recorded in both inward and outward directions when compared to the water injected control for all concentrations of Na^+ examined (Figures 5-1 and 5-2). Whereas inward current has been established as being the result of primarily Na^+ influx, the outward currents observed at positive membrane potential may not be due to only Na^+ efflux. As K^+ is the dominant mono-valent cation within *X. laevis* oocytes (Weber, 1999) it is therefore likely that the observed flux is due to K^+ movement. Similar increased outward flux is seen in YDR352w injected oocytes when voltage is clamped at positive potentials in the base buffer (Figures 8-1 and 8-2). This is consistent with PQ loop repeat proteins facilitating bi-directional flux of cations.

5.2.2. Relationship between K^+ and Na^+ flux through YDR352w and YOL092w

K^+ is often the dominant cation transported in both yeast and *Xenopus* oocyte expression systems (Weber, 1999). It is was therefore important to investigate the potential influence of native K^+ transport systems on the flux recorded by expression of these PQ loop repeat proteins. TEA^+ was used in its role as a K^+ channel blocker both to reduce native K^+ transport systems and to determine possible interactions with the proteins being investigated. The addition of 10 mM TEA^+ to a bath solution containing 90 mM NaCl did not alter the general trends of flux but in fact increased the magnitude of currents (Figures 5-3 and 5-4). The reversal potentials do not significantly change with the addition of TEA^+, suggesting TEA^+ itself is not being carried. It is possible that any difference in E_{rev} is eclipsed by the presence of much higher Na^+ and internal K^+ concentrations.

5.2.3. Investigation of potential anionic flux through YDR352w and YOL092w

The influence of Cl^- on observed currents was investigated through the substitution of NaCl with Na_2SO_4 (Figures 5-5 and 5-6). Currents were unchanged for oocytes expressing YDR352w, suggesting the gross currents were not influenced by external Cl^- concentrations. Currents for YOL092w were decreased with the substitution of Cl^- for SO_4^{2-}. This may be due either to a decrease in a Cl^- induced effect or as an oocyte response to added SO_4^{2-}.

5.2.4. Non-selectivity of YDR352w and YOL092w to mono-valent cations

Analysis of cation selectivity suggests YDR352w and YOL092w have poor discrimination between mono-valent cations. Flux of choline$^+$, Na^+, K^+ and NH_4^+ is increased in oocytes expressing YDR352w or YOL092w (Figures 5-7 and 5-8). Control oocytes showed a high degree of K^+ flux, most likely masking any potential K^+ flux through the PQ loop repeat proteins examined (Figure 5-7, D & I and Figure 5-8; D, E and F). Further investigation, possibly using known K^+ channel blockers or lower K^+ concentrations would assist in resolving potential K^+ flux. The presence of NH_4^+ in the bathing buffer induced a large bi-directional current and a negative E_{rev} shift for oocytes expressing YDR352w, indicating the flux of this cation is catalysed by the presence of this protein (Figure 5-7, E and Figure 5-8, J and K). The presence of choline$^+$ also increased bi-directional current in oocytes expressing YDR352w or YOL092w (Figure 5-7, B & G and Figure 5-8, G, H & I), suggesting these proteins also facilitate the flux of choline$^+$ across *Xenopus laevis* membranes.

5.2.5. Influence of Ca^{2+} on Na^+ flux catalysed by YDR352w and YOL092w

To investigate the possibility that YDR352w and/or YOL092w were responsible for the Ca^{2+}-sensitive NSC1-like cation flux, we investigated the effect of differing Ca^{2+} concentrations on the flux of Na^+ in oocytes (Figures 5-9 and 5-10). A decrease of approximately 50% of inward and outward current was recorded with the addition of 2 mM $CaCl_2$ and a further 20% reduction with the addition of 10 mM $CaCl_2$. This strongly suggests that Ca^{2+} acts to block the cation channel characteristics of YDR352w and YOL092w in a manner similar to that observed with NSC1.

5.3. Discussion

5.3.1. YDR352w and YOL092w elicit responses concurrent with recorded viNSCCs

Expression of YDR352w and YOL092w cRNA in *Xenopus* significantly increased current flow in oocytes in Na^+ containing bath solutions. Current amplitude was influenced by Na^+ concentration with greater current observed at higher Na^+ concentrations (Figure 5-5). The greater the Na^+ concentration the further the E_{rev} moved positive. This confirms the current recorded was the result of Na^+ flux as opposed to Cl^-. Confirmation of this trait was achieved through the substitution of 100 mM NaCl with 50 mM Na_2SO_4 (Figures 5-5 and 5-6) revealing little change in E_{rev}.

5.3.2. Differences in the physiology of YDR352w and YOL092w

YDR352w and YOL092w induced similar physiology when over expressed in yeast and when expressed in *Xenopus* oocytes. Subtle differences are evident between them, however, suggesting discreet roles for each protein. In particular, these proteins show differences when the membrane is hyperpolarised. The current / voltage relationship of YDR352w and YOL092w shows that in a Ca^{2+} free Na^+ bath solution currents are reasonably linear to each other across the range of potentials investigated (Figures 5-1 and 5-2). YOL092w shows a loss of linearity at potentials negative of -70 mV, however, mirroring the response shown in water injected control oocytes. Examples of viNSCCs showing these traits exist in the literature. viNSCCs with linear (YDR352w like), cation conductances at hyperpolarised potentials have been described in many systems, including *Arabidopsis* (Demidchik and Tester, 2002c), in

soybean peribacteroid membranes (Tyerman et al., 1995) and in yeast (Bihler, 1998). Responses similar to those observed for YOL092w have been described for cation flux in bean cotyledon protoplasts (Zheng et al., 2004), where a linear I/V relationship due to K^+ flux was observed membrane potential positive of approximately -100 mV but conductance was reduced at potentials less than this. These differences could be indicative of separate roles for these proteins play within the yeast cell.

TEA^+ has been shown to enhance low affinity Na+ flux in plants (Wang et al., 2006)(Malagoli et al., 2008). The observed impact of TEA^+ on Na^+ fluxes in oocytes expressing YDR352w and YOL092w may be related to this. These data suggest that either TEA^+ is being carried by YOL092w and YDR352w or that TEA^+ is acting like an agonist to the observed traces, increasing their capacity to carry cations, in this case mainly Na^+. The existence of a receptor-like system working in conjunction with these proteins, as is observed in classical agonist / receptor relationships, cannot be determined from these data. The possibility of currents being carried by TEA^+ is intriguing given the structural similarities between TEA^+, MA^+ and NH_4^+ and observed MA^+ and NH_4^+ flux characteristics (Figures 5-3, 5-4, 5-7 and 5-8, Figures 8-1 and 8-2). A TEA^+ inducible inward current has been recorded in *Xenopus* oocytes that may also play a component role in the flux observed (Huang et al., 1995), where increasing external concentrations of TEA^+ act to increase outward current, suggesting the inward movement of cations. Currents described through the addition of TEA^+ are at the most approximately 20 nA, however, which would be difficult to resolve on the scale which the PQ loop repeat protein data is presented.

5.3.3. Time dependence of currents elicited by YDR352w and YOL092w

The currents observed between YDR352w and YOL092w showed different degrees of time dependence. In general, currents elicited by YDR352w did not show time dependence (Figures 5-2, 5-4, 5-6, 5-8 and 5-10). Some time dependence was observed at low membrane potentials when SO_4^{2-} was used as the Na^+ coupled anion (Figure 5-6). However, this is not seen when Cl^- is the counter ion and may be an artefact of the presence of SO_4^{2-} in the bath solution or possibly the influence of the differing activity coefficients possessed by the different counter ions. Possible effects of different counter ions on cellular electrochemistry also need to be taken into consideration. For example, the presence of SO_4^{2-} may induce H^+ flux that is absent when Cl^- is the counter ion (Kochian, 1989). YOL092w elicited currents that were mostly independent of time, except when 100 mM Na^+ was present in the bath solution (Figure 2). Under these conditions, currents obtained at potentials negative of -90 mV show a time dependent decrease until a steady state is reached after approximately 1.5 seconds. Current measurements were taken as described methods (5.4.1.2).

5.3.4. Potential Ca^{2+} flux through viNSCCs

Changes in Ca^{2+} activity influenced the current due to Na^+ flux and slightly altered E_{rev}. A slight positive shift of E_{rev} with increasing Ca^{2+} concentration in the bath solution suggests that Ca^{2+} may be transported through these proteins. The observed activation of the Ca^{2+} activated Cl^- conductance in oocytes expressing YDR352w (Figures 8-1 and 8-2) also suggests Ca^{2+} influx is increased by this protein.

5.3.5. The role of PQ loop repeat proteins in biological systems

These data are consistent with the hypothesis that the *S. cerevisiae* PQ loop repeat proteins YDR352w and YOL092w catalyse the non-selective flux of mono-valent cations across biological membranes. Evidence of cation flux through physiologically similar proteins such as these exists within many biological systems (Hagiwara et al., 1992; Bihler, 1998; Amtmann, 1999; Davenport and Tester, 2000; Demidchik and Tester, 2002c; Essah et al., 2003). Proteins with similar sequences to the PQ loop repeat proteins characterised here are also evident in many biological systems (Figure 6-1). It is likely that at least a sub group of these PQ loop repeat proteins behave as viNSCCs in their native organism.

5.4. Materials and Methods

5.4.1. Heterologous expression of PQ loop repeat proteins in the *Xenopus laevis* oocyte expression system

5.4.1.1. Synthesis of cRNA and injection into *X. laevis* oocytes

Oocytes of *Xenopus laevis* were prepared as per standard protocols (Zhou et al., 2007) with the use of a Calcium Frog Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, 0.6 mM $CaCl_2$, 8% (v/v) horse serum, 0.1 mg / ml Tetracycline, 1000 U / ml Penicillin and 0.1 mg / ml Streptomycin). A pDONR (Invitrogen) vector containing either YDR352w or YOL092w, cloned from genomic DNA of the *S. cerevisiae* strain 31019b, was used to recombine the insert of interest into the vector pGEMHE (M. Shelden, unpublished data, Figure 5-11) using the LR clonase reaction (Invitrogen). The pGEMHE vector containing the gene of interest was sequenced to confirm orientation and then digested overnight with *Sph* 1 (NEB) to linearise the DNA. The mMessage mMachine 5´ capped RNA transcription kit (Ambion) was used according to the manufacturer's protocol to synthesise cRNA for the gene of interest. cRNA concentration was normalised to 1 µg / µL and 46 nL injected into each oocyte using a micro injector (Drummond 'Nanoject II' automatic nanolitre injector, Drummond Scientific, Broomall, PA, USA). Control oocytes were injected with 46 nL of nuclease free H_2O. Injected oocytes were incubated at 16°C in Calcium Frog Ringers solution for 3 days prior to use.

5.4.1.2. Electrophysiology of *S. cerevisiae* PQ loop repeat proteins expressed in *Xenopus laevis* oocytes.

Unless otherwise specified, oocyte voltage clamping occurred at 25°C in a base buffer consisting of 5 mM MES / Tris at pH 7.0, with ions of interest added previously. Mannitol was used to keep osmolarity at 200 mOsm. Signal was amplified using a Gene Clamp 500 voltage clamp amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) and displayed using Clampex 8.2 (Axon Instruments). Oocytes were impaled with glass capillaries filled with 3 M KCl. Electrodes responsible for maintaining the voltage clamp and current flow were bathed in this 3 M KCl solution. The voltage protocol used is detailed in Figure 5-12. Currents used in the generation of I/V curves were taken as the average of current from 1.2 to 1.6 seconds into the voltage clamp as these best represented differences observed between each of the samples investigated.

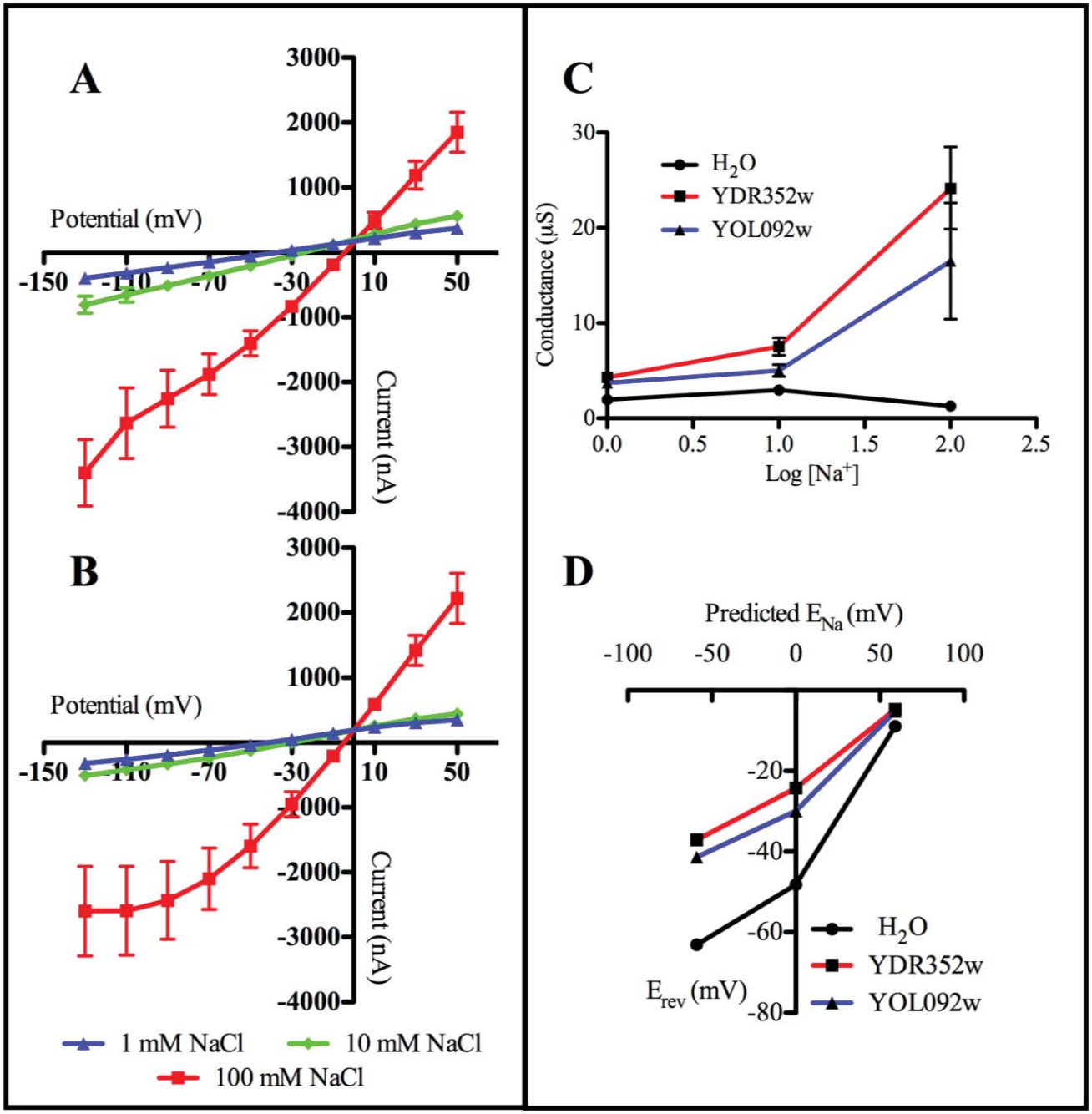


Figure 5-1: Comparison of the current / voltage relationships of *Xenopus laevis* oocytes expressing *S. cerevisiae* PQ loop repeat proteins as a function of external Na^+ concentration.

I/V relationship between oocytes injected with H_2O, YDR352w cRNA or YOL092w cRNA and bathed in buffers containing different Na^+ concentrations. Buffers are

mannitol / MES based as described in the methods of this chapter with 100 mM NaCl, 10 mM NaCl or 1 mM NaCl for YDR352w (A) or YOL092w (B). Data presented is the mean ± SE (n = 5 oocytes). Conductance as a function of log Na^+ concentration (C) taken from these data at -130 mV show increased current through oocytes injected with YDR352w or YOL092w is much higher than for H_2O injected controls. The movement of E_{rev} towards E_{Na+} for oocytes expressing YDR352w or YOL092w (D) suggests Na^+ flux is influencing E_{rev}. E_{rev} was calculated by the linear regression of two points either side of the Y-axis and the subsequent determination of intercept. Predicted E_{Na} was calculated using the Nernst equation and assuming cytosolic Na^+ concentration of 10 mM (Webber, 1999).

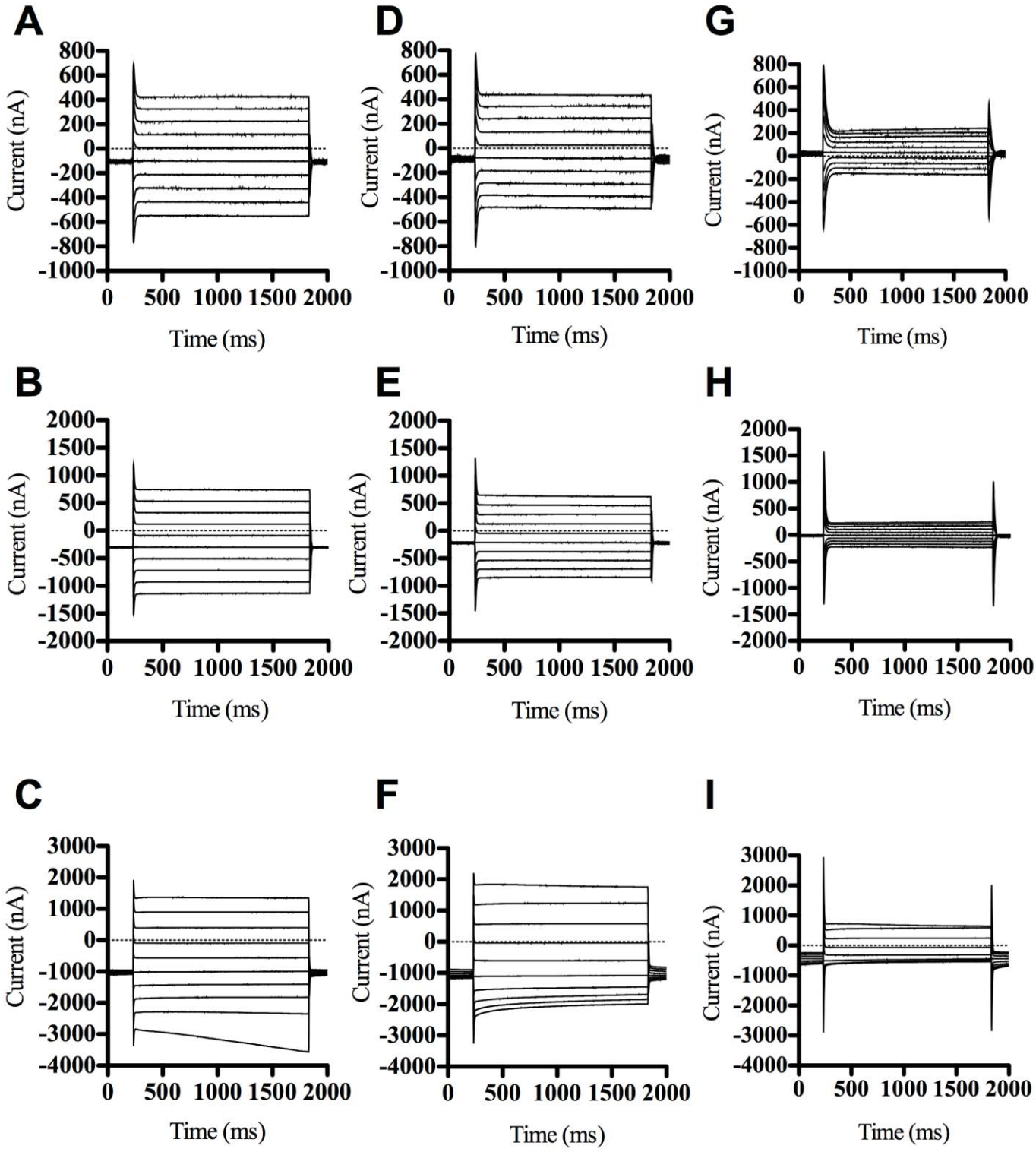

A
D
G
B
E
H
C
F
I
Current (nA)
Time (ms)
0
500
1000
1500
2000

Figure 5-2: Comparison of currents induced in *Xenopus laevis* oocytes expressing YDR352w or YOL092w with those injected with H_2O over various Na^+ concentrations.

Representative time dependant current profiles of oocytes injected with YDR352w cRNA (A, B, C), YOL092w cRNA (D, E, F) or H_2O (G, H, I). Oocytes were bathed in base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) with 1 mM NaCl (A, D, G), 10 mM NaCl (B, E, H) or 100 mM NaCl (C, F, I) added.

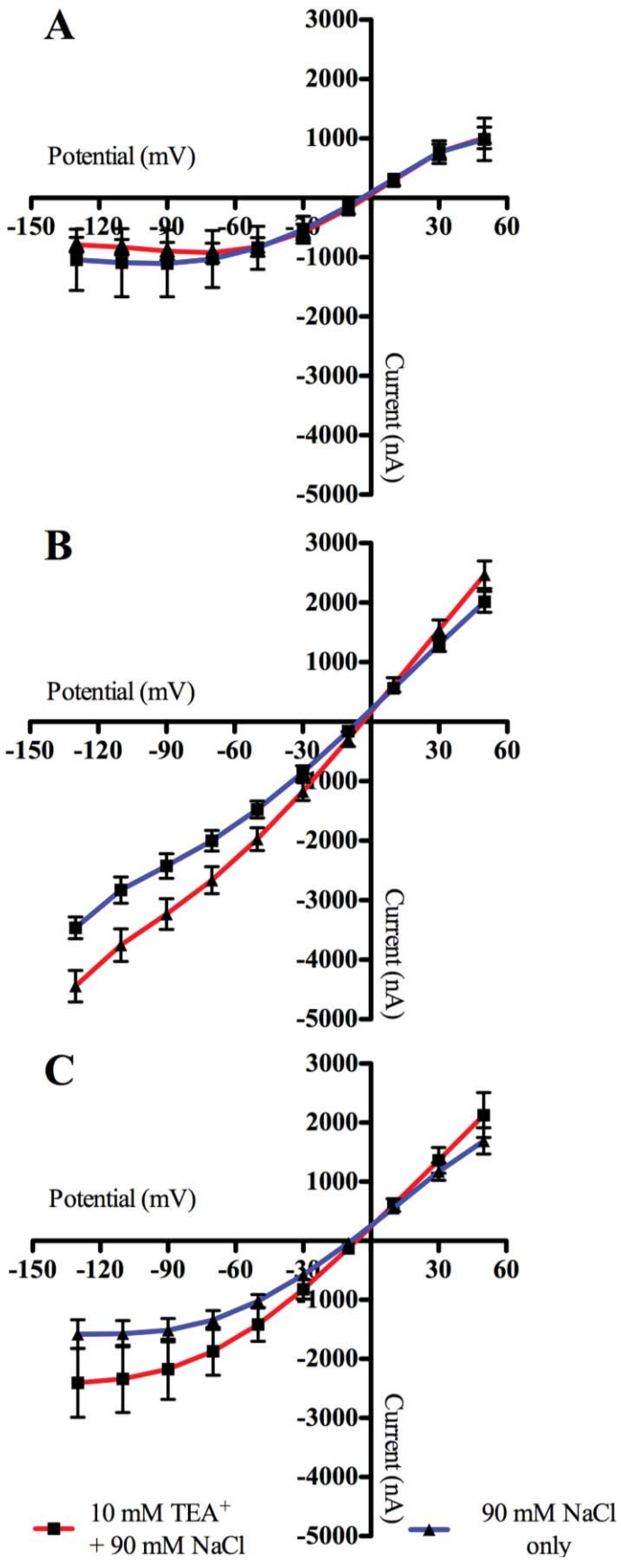

A
B
C
Potential (mV)
Current (nA)
3000
2000
1000
-1000
-2000
-3000
-4000
-5000
-150
-120
-90
-60
-30
30
60
10 mM TEA+
+ 90 mM NaCl
90 mM NaCl
only

Figure 5-3: The effect of the K^+ channel blocker TEA^+ on Na^+ flux through YDR352w and YOL092w.

The presence of TEA^+ (10 mM TEA-OH, 90 mM NaCl, 5 mM MES/Tris pH 7.0) showed less current compared to those oocytes in a 90 mM NaCl alone (90 mM NaCl, 5 mM MES/Tris) for oocytes injected with H_2O (A) but those injected with YDR352w (B) or YOL092w (C) cRNA elicited a greater current in the presence of TEA^+ compared to the TEA^+ free control. This may be due to flux of TEA^+ through these proteins or the result of TEA^+ acting as an agonist to native NSCCs. Data presented is the mean ± SE (n = 5 oocytes).

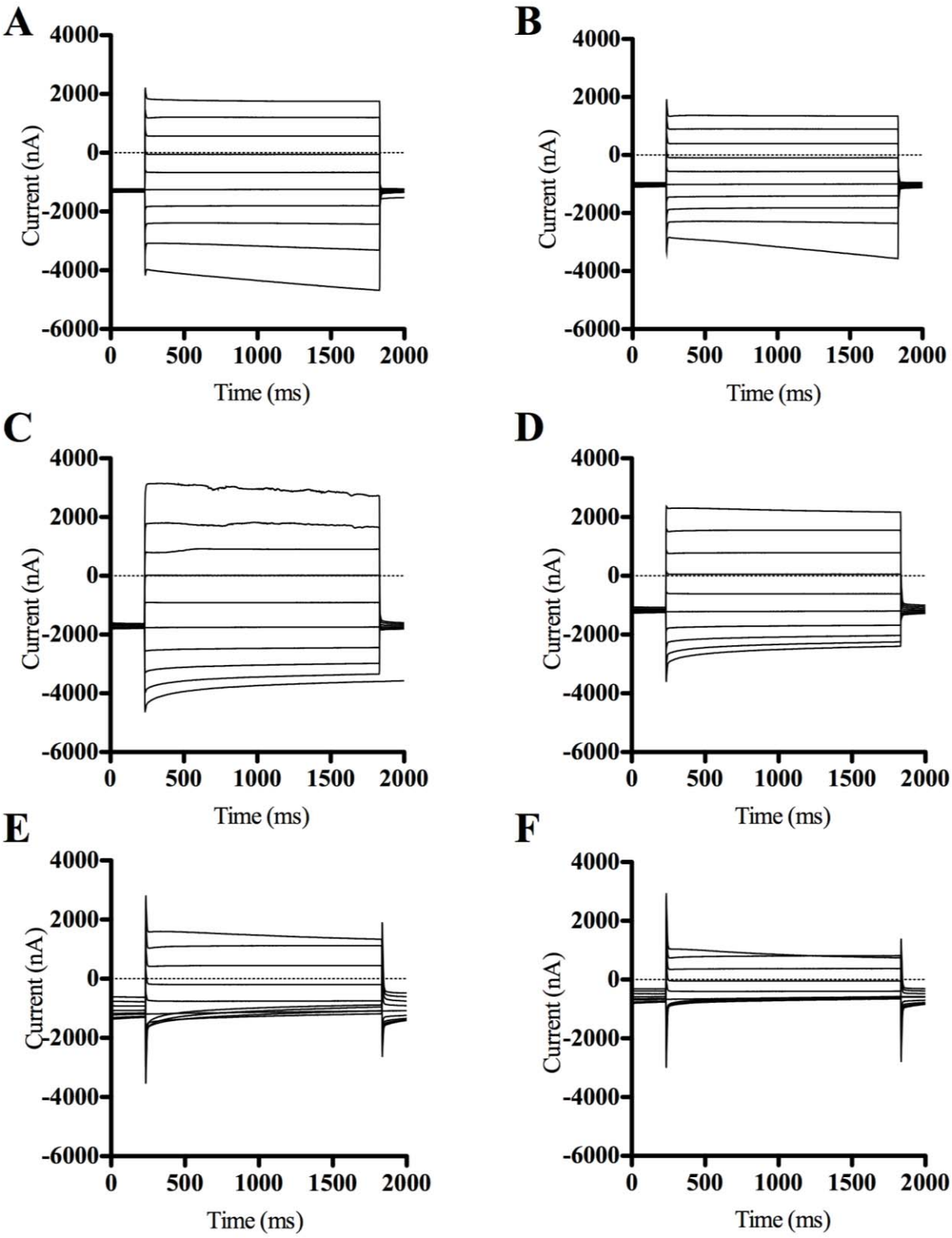

A
B
C
D
E
F
Current (nA)
Time (ms)
4000
2000
0
-2000
-4000
-6000
0
500
1000
1500
2000

Figure 5-4: The effect of TEA^+ on Na^+ induced currents in *Xenopus laevis* oocytes expressing YDR352w or YOL092w with those injected with H_2O.

Representative time dependant current profiles of oocytes injected with YDR352w cRNA (A, B), YOL092w cRNA (C, D) or H2O (E, F). Oocytes were bathed in base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) with 90 mM NaCl and 10 mM TEA^+ (A, C, E) or 90 mM NaCl and 20 mM Mannitol (B, D, F).

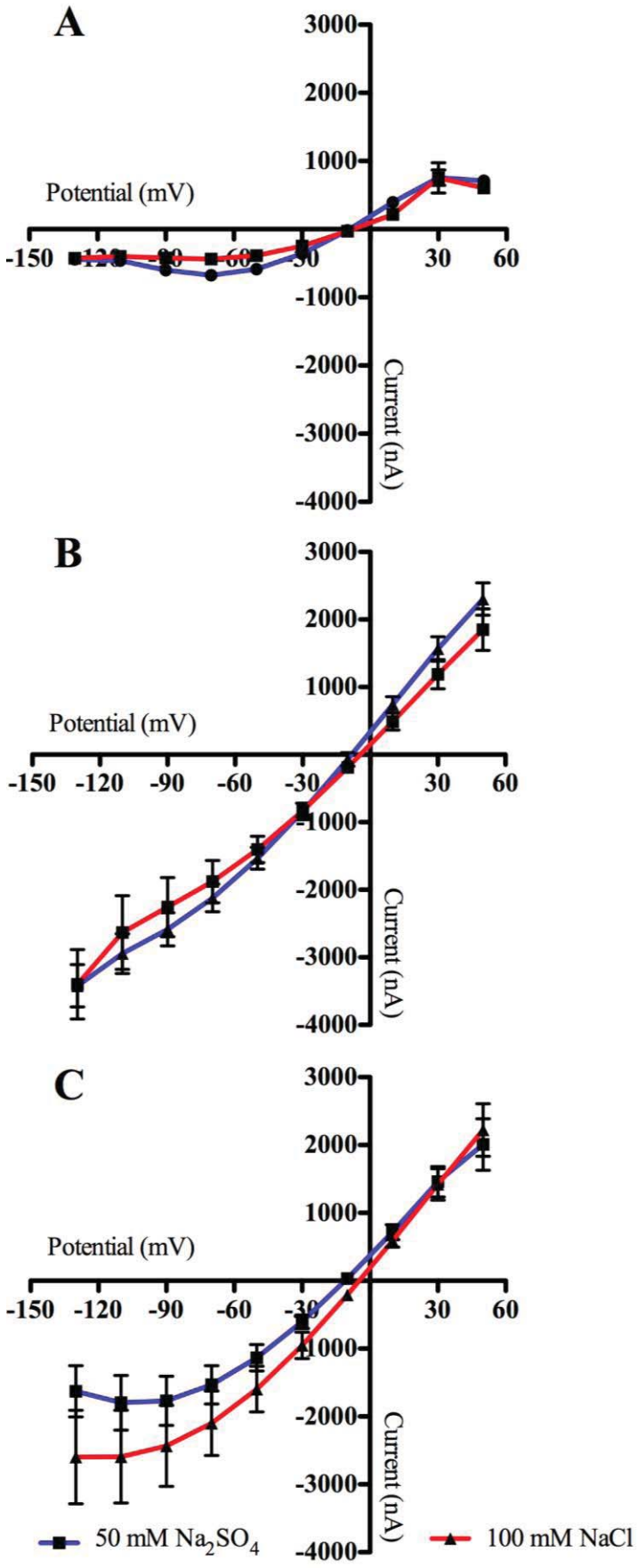

A
B
C
3000
2000
1000
-1000
-2000
-3000
-4000
Potential (mV)
-150
-120
-90
-60
-30
30
60
Current (nA)
50 mM Na_2SO_4
100 mM NaCl

Figure 5-5: The influence of anions on Na^+ based current in *Xenopus laevis* oocytes injected with cRNA from YDR352w or YOL092w.

The Cl^- anion was substituted with SO_4^{2-} to determine whether cation flux was the source of observed currents. Base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) had either 100 mM NaCl or 50 mM Na_2SO_4 added and I/V relationships determined for oocytes injected with H_2O (A), YDR352w (B) or YOL092w (C). Data presented is mean ± SE (n = 5 oocytes).

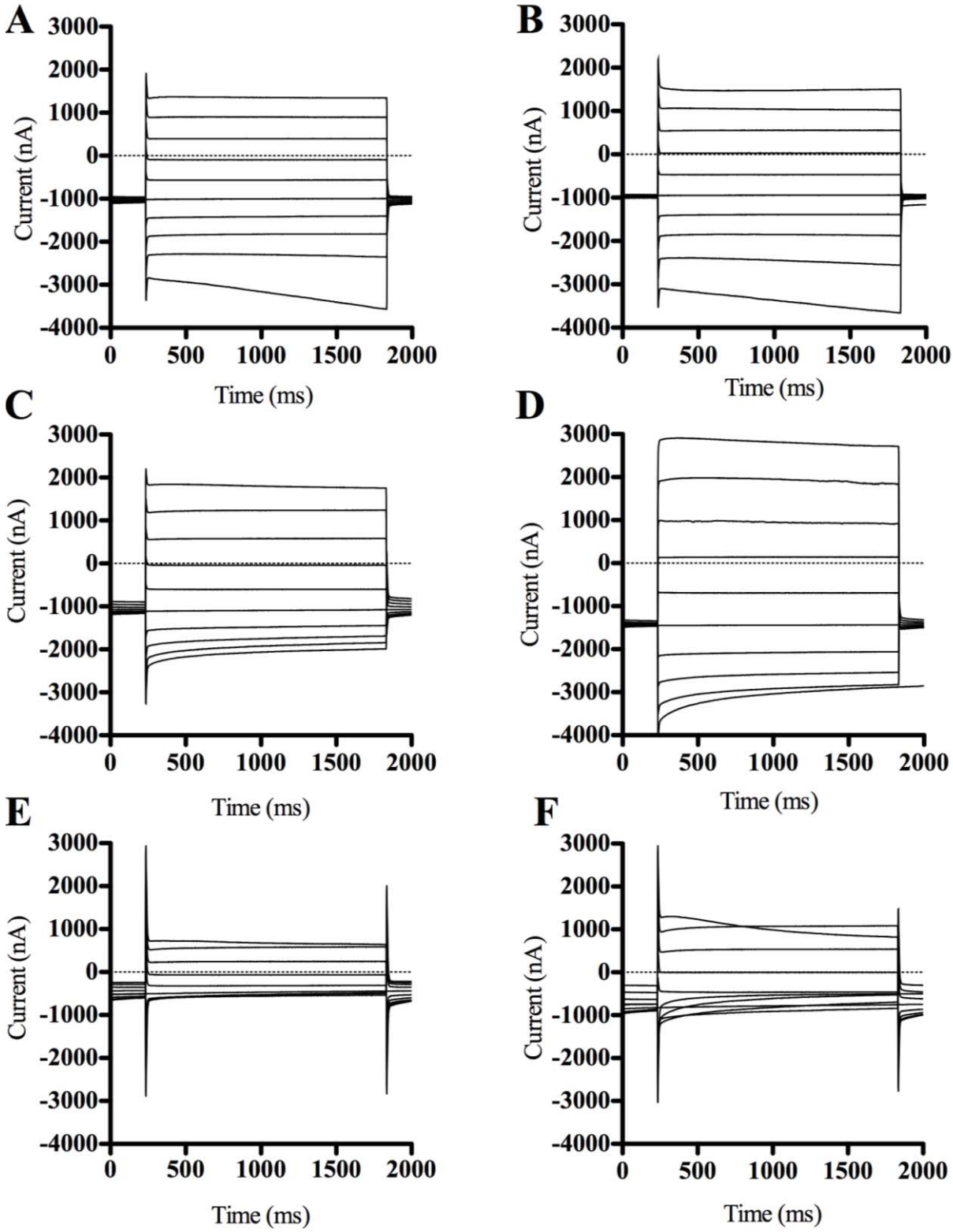
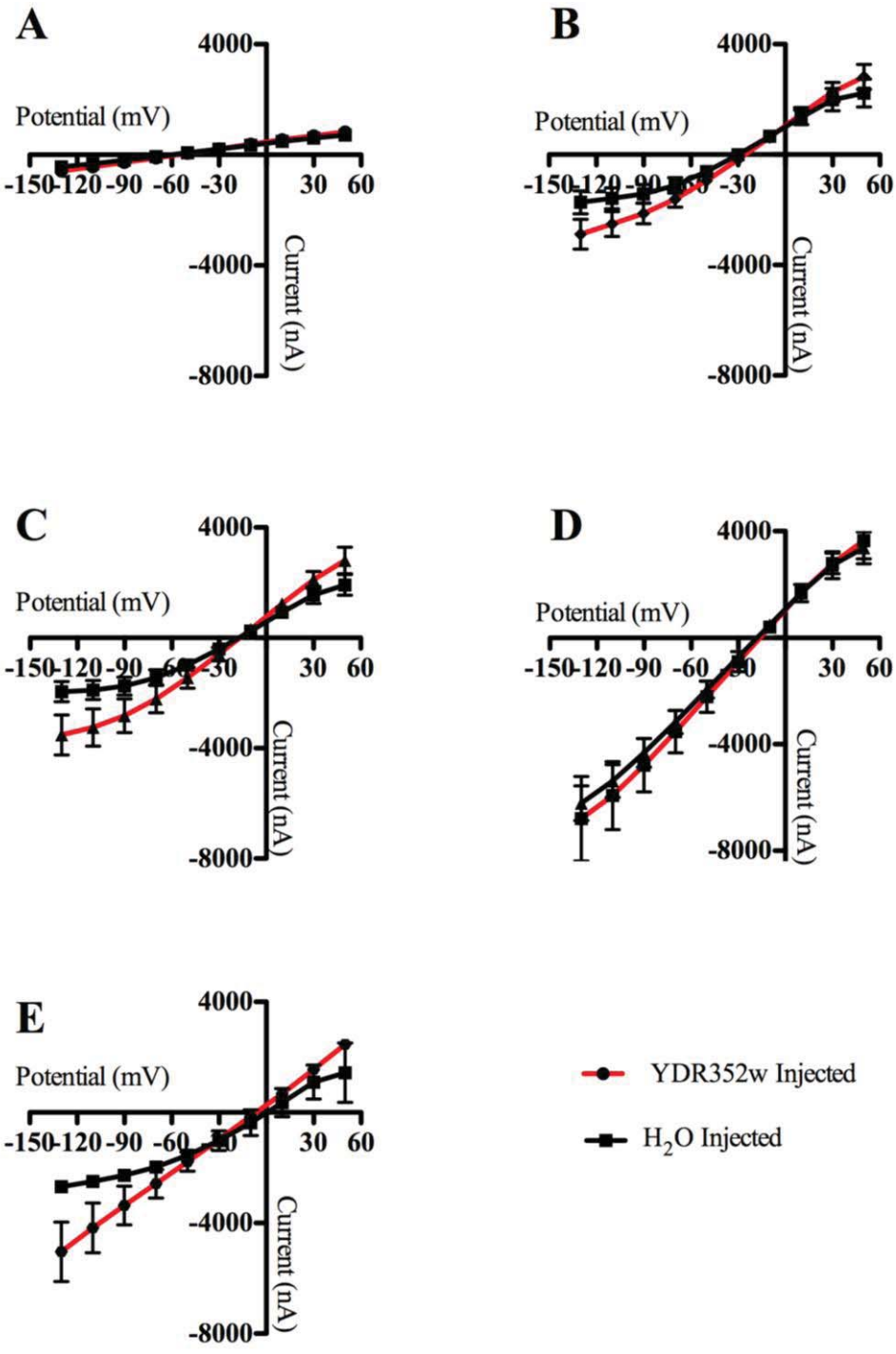

A
B
C
D
E
F
Current (nA)
Time (ms)
3000
2000
1000
0
-1000
-2000
-3000
-4000
0
500
1000
1500
2000

Figure 5-6: Representative currents elicited from *Xenopus laevis* oocytes injected with H_2O or cRNA of YDR352w or YOL092w in the presence of 100 mM Cl^- or 50 mM SO_4^{2-}.

Representative time dependant current profiles of oocytes injected with YDR352w cRNA (A, B), YOL092w cRNA (C, D) or H_2O (E, F). Oocytes were bathed in base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) with 100 mM NaCl (A, C, E) or 50 mM Na_2SO_4 (B, D, F).


A
B
C
D
E
4000
-4000
-8000
Potential (mV)
Current (nA)
-150 -120 -90 -60 -30 30 60
YDR352w Injected
H2O Injected

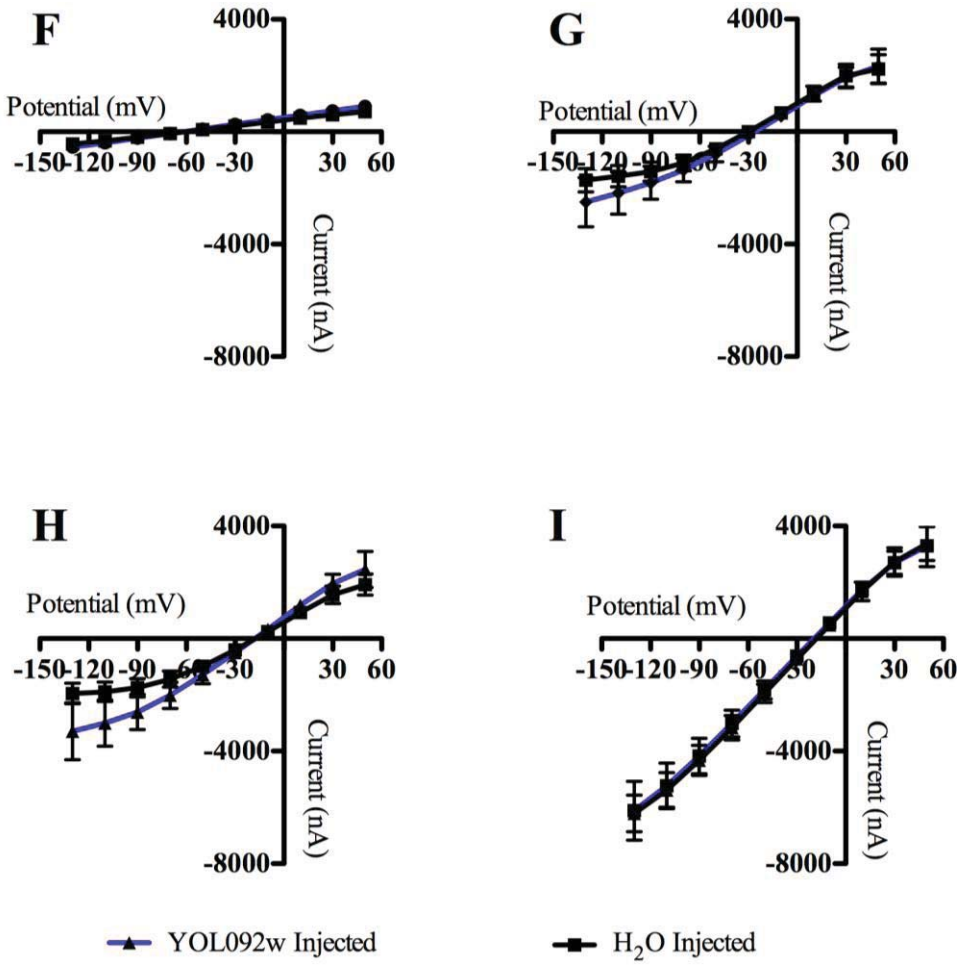


Figure 5-7: Catalysis of cation flux as a function of YDR352w and YOL092w expression in *Xenopus laevis* oocytes.

Oocytes of *Xenopus laevis* were bathed in solutions containing a base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) and one of a variety of cations as their Cl^- salts. Oocytes were injected with H_2O, YDR352w cRNA (A to E) or YOL092w cRNA (F to I). Data relative to H_2O injected controls is presented for 200 mM Mannitol (A & F; n = 5), 100 mM Choline-Cl (B & G; n = 5); 100 mM NaCl (C & H; n = 5); 100 mM KCl (D & I; n = 2) and 100 mM NH_4Cl (E, n = 2). All data is mean ± SE.

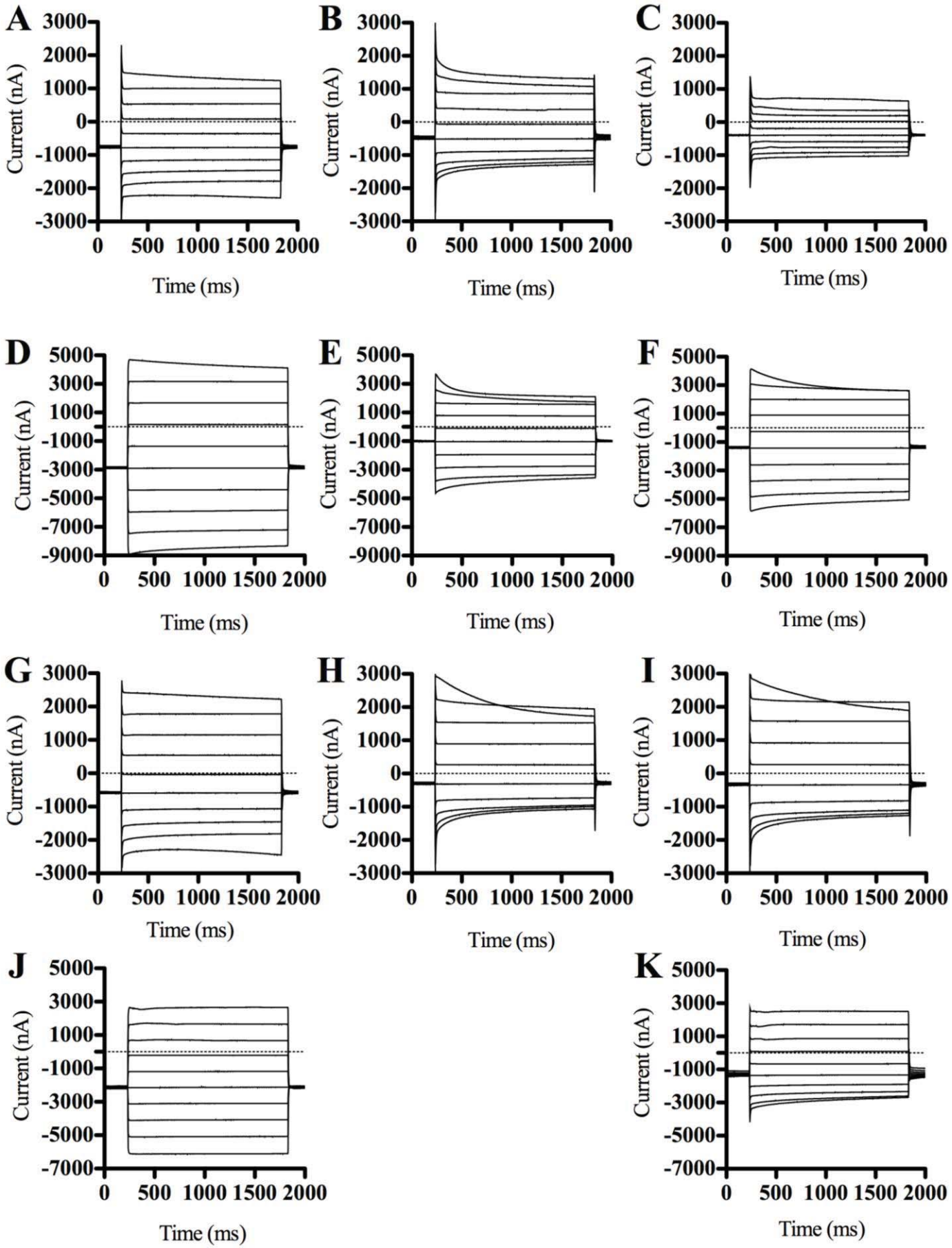

A
B
C
D
E
F
G
H
I
J
K
Current (nA)
Time (ms)
0
500
1000
1500
2000
3000
2000
1000
0
-1000
-2000
-3000
5000
3000
1000
-1000
-3000
-5000
-7000
-9000

Figure 5-8: Representative currents elicited from *Xenopus laevis* oocytes injected with H_2O or cRNA of YDR352w or YOL092w in the presence of varied cations.

Representative time dependant current profiles of oocytes injected with YDR352w cRNA (A, D, G, J), YOL092w cRNA (B, E, H) or H_2O (C, F, I, K). Oocytes were bathed in base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) with 100 mM NaCl (A, B, C); 100 mM KCl (D, E, F); 100 mM Choline-Cl (G, H, I) or 100 mM NH_4Cl (J, K).

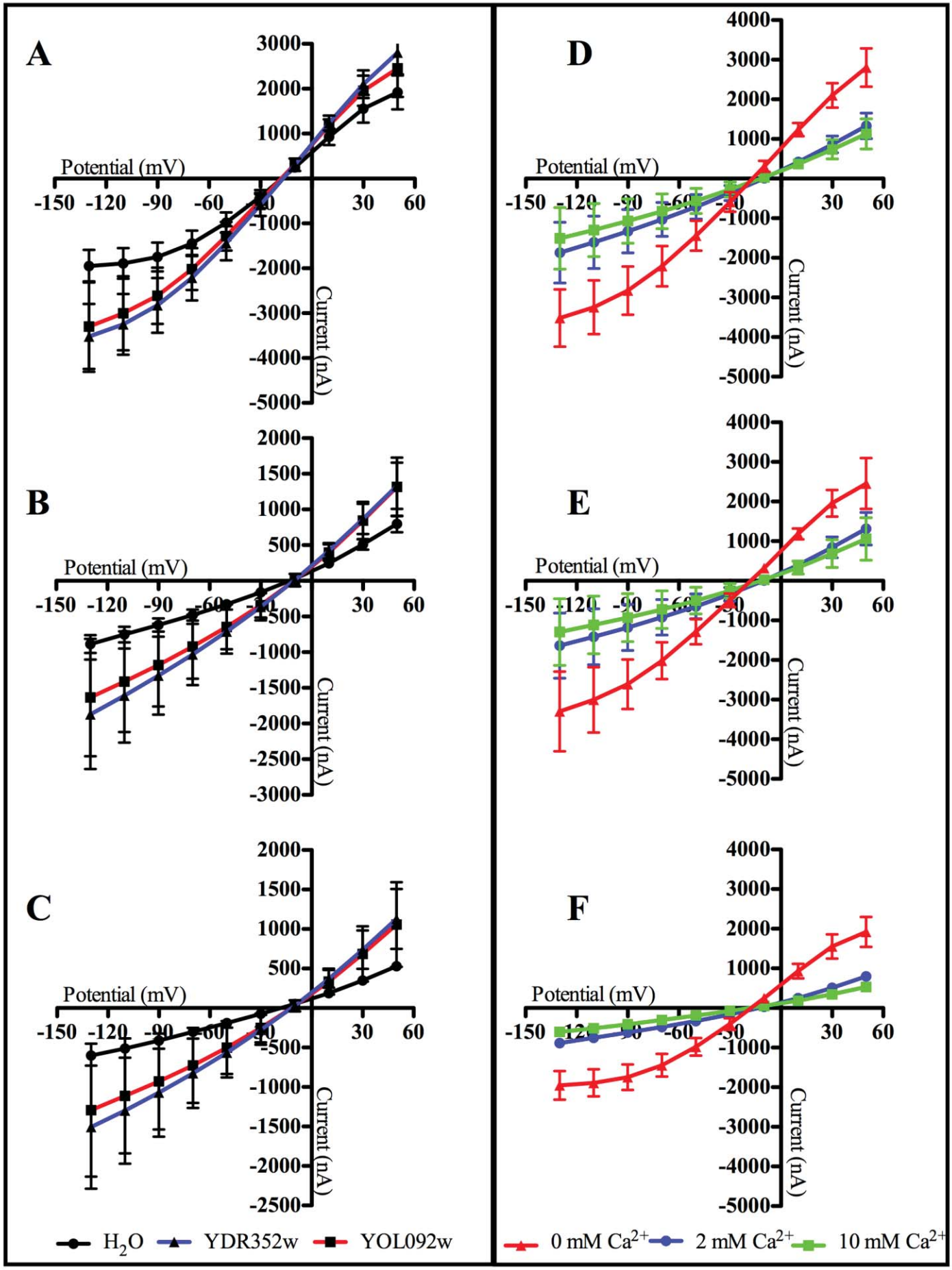

A
B
C
D
E
F
Potential (mV)
Current (nA)
-150 -120 -90 -60 30 60
H2O
YDR352w
YOL092w
0 mM Ca2+
2 mM Ca2+
10 mM Ca2+

Figure 5-9: Influence of bathing solution Ca^{2+} concentrations on the conductance of Na^{+} elicited current in *Xenopus laevis* oocytes injected with H_2O, YDR352w cRNA or YOL092w cRNA.

Oocytes were bathed in a base buffer (200 mM Mannitol, 5 mM MES/Tris pH 7.0) with the addition of 100 mM NaCl. Currents were measured across oocyte membranes in the presence of 0 mM (A), 2 mM (B) or 10 mM (C) added $CaCl_2$. Data has been rearranged (D, E and F) to highlight the effect of Ca^{2+} on current measured in YDR352w (D), YOL092w (E) or H2O (F) injected oocytes. A degree of native oocyte Na^{+} flux is apparent but oocytes injected with YDR352w (D) and YOL092w (E) show a greater Na^{+} flux induced current and a greater degree of Ca^{2+} mediated blockage than the water injected control (F).

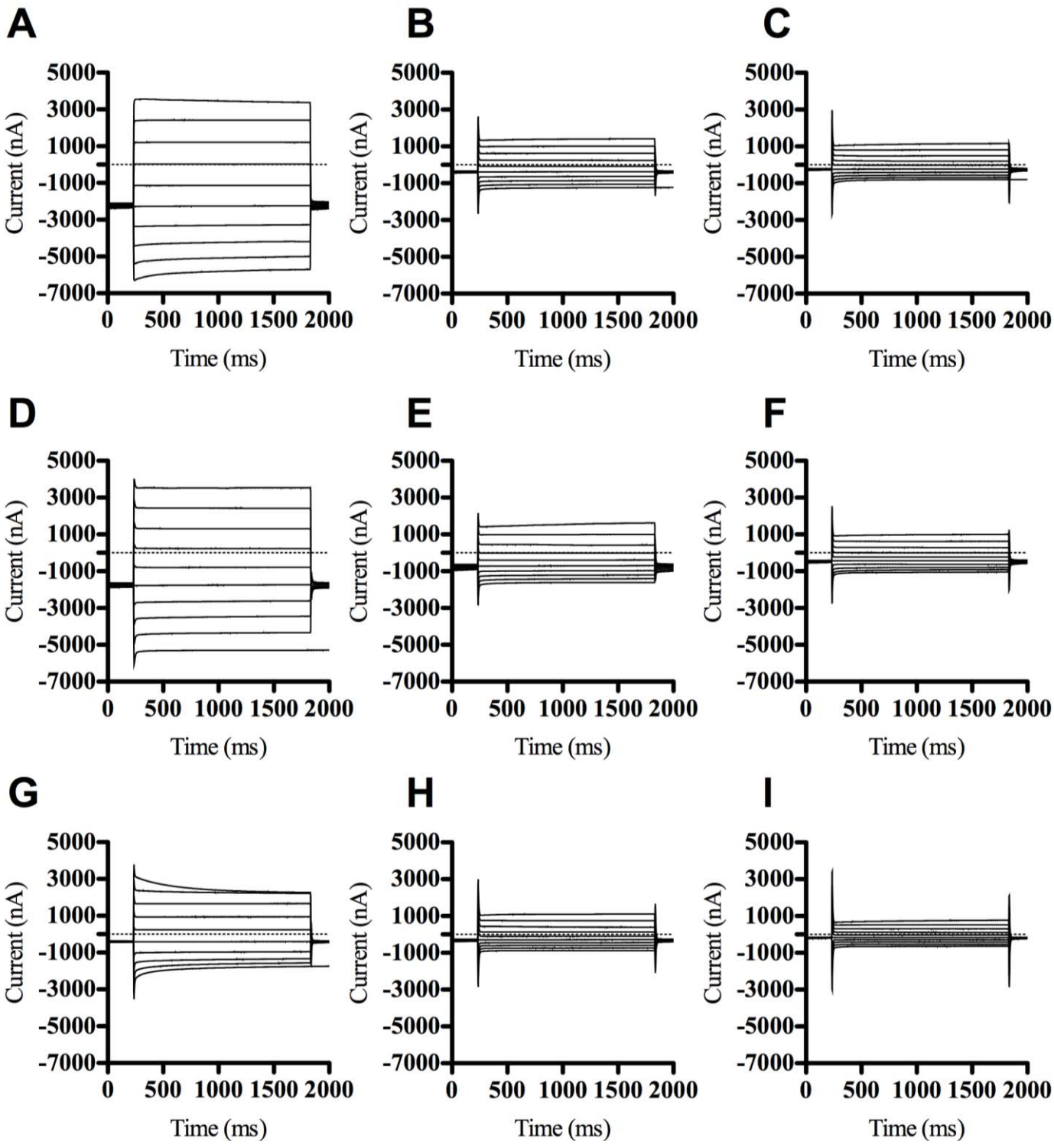

A
B
C
D
E
F
G
H
I
Current (nA)
5000
3000
1000
-1000
-3000
-5000
-7000
0
500
1000
1500
2000
Time (ms)

Figure 5-10: Representative currents elicited from *Xenopus laevis* oocytes injected with H_2O or cRNA of YDR352w or YOL092w in the presence of 100 mM NaCl and varied Ca^{2+} concentrations.

Representative time dependant current profiles of oocytes injected with YDR352w cRNA (A, B, C), YOL092w cRNA (D, E, F) or H_2O (G, H, I). Oocytes were bathed in base buffer (Appendix, section 1) with 100 mM NaCl (A, D, G); 100 mM NaCl and 2 mM $CaCl_2$ (B, E, H) or 100 mM NaCl and 10 mM $CaCl_2$ (C, F, I).

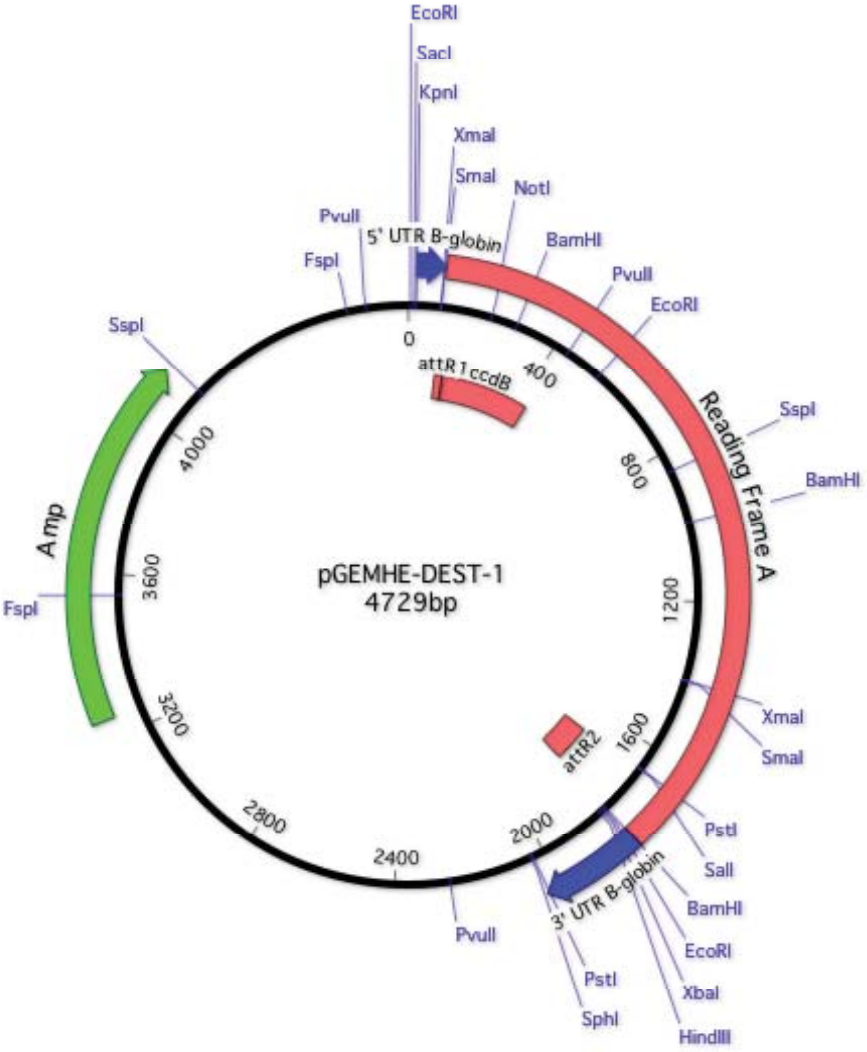


Figure 5-11: Vector map of pGEMHE-DEST

pGEMHE (Invitrogen) was modified by M. Shelden (unpublished data) to incorporate an attR1 and attR2 flanked gateway reading frame. This vector was subsequently used to create cRNA from gateway-adapted cDNA of genes of interest, allowing expression in *Xenopus laevis* oocytes.

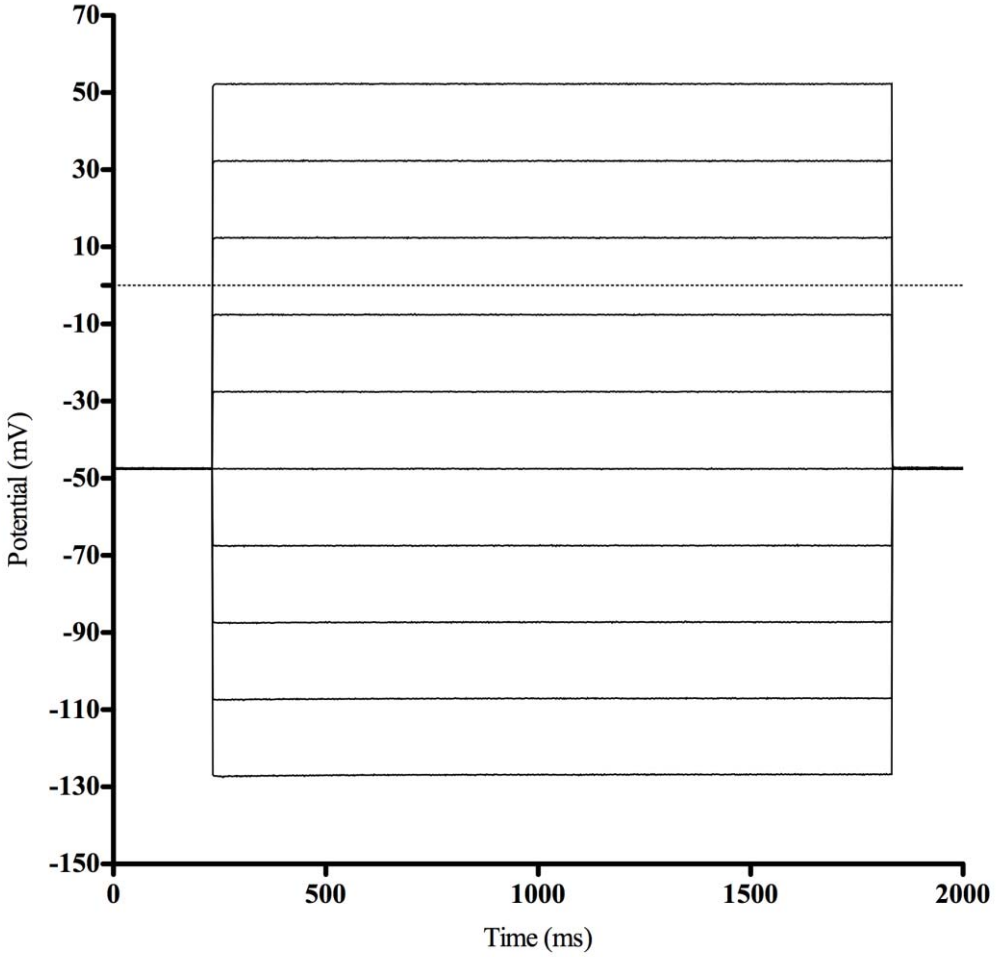


Figure 5-12: Voltage protocol employed for all voltage clamp data presented in this thesis.