J Membrane Biol (2011) 241:21–29
DOI 10.1007/s00232-011-9359-5

Penetratin Peptide Potentiates Endogenous Calcium-Activated Chloride Currents in *Xenopus* oocytes

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Received: 1 November 2010 / Accepted: 16 March 2011 / Published online: 27 March 2011

Abstract Calcium-activated chloride currents (CaCCs) are required for epithelial electrolyte and fluid secretion, fertilization, sensory transduction and excitability of neurons and smooth muscle. Defolliculated *Xenopus* oocytes express a robust CaCC formed by a heterologous group of proteins including transmembrane protein 16A (TME M16A) and bestrophins. Penetratin, a 17-amino acid peptide, potentiated endogenous oocyte CaCCs by ~50-fold at 10 μM, recorded using a two-electrode voltage clamp. CaCC potentiation was rapid and dose-dependent (EC_{50} = 3.2 μM). Penetratin-potentiated currents reversed at −18 mV and were dependent on the extracellular divalent cations present, showing positive regulation by Ca^{2+} and Mg^{2+} but effective block by Zn^{2+} (IC_{50} = 5.9 μM). Extracellular Cd^{2+}, Cu^{2+} and Ba^{2+} resulted in bimodal responses: CaCC inhibition at low but potentiation at high concentrations. Intracellular BAPTA injection, which prevents activation of CaCCs, and the Cl^- channel blockers niflumic acid and DIDS significantly reduced potentiation. In contrast, the K^+ channel blockers Cs^+, TEA, tertiapin-Q and halothane had no significant effect. This pharmacological profile is consistent with penetratin potentiation of zinc-sensitive CaCCs that are activated by influx of extracellular Ca^{2+}. These findings may stimulate basic research on CaCCs in native cells and may lead to development of novel therapeutics targeting disorders caused by insufficient chloride secretion.

Keywords Calcium-activated chloride channel · Chloride secretion · Penetratin · TMEM16A · *Xenopus* oocyte · Zinc

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Introduction

Penetratin is a highly positively charged (cationic) and hydrophilic 17-amino acid peptide derived from the third α-helix of the homeodomain of Antennapedia, a *Drosophila* homeoprotein (Derossi et al. 1994, 1998). Penetratin has a protein transduction domain (PTD), which allows it to translocate through the plasma and nuclear membranes of various cell types without apparent disruption (Derossi et al. 1994, 1998; Richard et al. 2003). Due to this property, penetratin and other cell-penetrating peptides with PTDs have been used in the delivery of polar bioactive compounds (e.g., drugs, peptides, proteins, nanoparticles, nucleic acids and antisense oligonucleotides) into cells as an alternative to gene delivery and therapy by transfection (Derossi et al. 1998; Jarver et al. 2010). Membrane translocation involves a series of events, initiated by positively charged penetratin attachment to an anionic cell surface, followed by the association of these complexes with lipid rafts, which then triggers macropinocytosis (Richard et al. 2003). However, recent studies suggest that the membrane repair response serves to mask the pore-forming effect of penetratin, raising the possibility that uptake may occur through rapid and temporary membrane disturbance (Palm-Apergi et al. 2009). The membrane repair response is a fast (within seconds) resealing mechanism triggered in cells with injured plasma membrane because of extracellular Ca^{2+} influx (Palm-Apergi et al. 2009). Penetratin has been shown to raise intracellular Ca^{2+} concentrations in cultured HeLa and CHO-K1 cells (Palm-Apergi et al. 2009). However, the mechanisms in these processes remain unclear.

While we were trying to translocate peptides across the *Xenopus* oocyte membranes, we soon realized that penetratin rapidly changes membrane conductance. We noticed

that extracellular application of penetratin potentiated an endogenous outwardly rectifying current resembling the calcium-activated chloride channel (CaCC), which was first described and characterized in *Xenopus* oocytes in the early 1980s (Barish 1983; Miledi 1982). Defolliculated *Xenopus* oocytes express relatively few endogenous ion channels, including a robust CaCC at extremely high levels (0.5 mA/cm^2), which is important for generating the fertilization potential after fusion with a sperm cell to prevent polyspermia (Dascal 1987; Hartzell et al. 2005). Recently, it has been shown that TMEM16A is responsible for the CaCC in oocytes (Schroeder et al. 2008). A significant limitation in studying CaCCs has been a lack of potent or selective modulators (Davis et al. 2010; Hartzell et al. 2005; Manoury et al. 2010; Schreiber et al. 2010; Schroeder et al. 2008; Verkman and Galietta 2009). Identification of penetratin as a potentiator of endogenous CaCCs is thus likely to stimulate research on CaCCs in native cells.

Methods

All animal experiments were performed in accordance with ethical guidelines of the University of Queensland Animal Ethics Committee and the National Health and Medical Research Council of Australia.

Preparation of *Xenopus* oocytes and Two-Electrode Voltage Clamp

The procedures for preparation and recording from oocytes were modified from previous studies (Kanjhan et al. 2003, 2005). Adult female *Xenopus laevis* frogs were anesthetized by immersion in dechlorinated water containing 0.13% (w/v) MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; ICN Biomedicals, Irvine, CA). Ovarian lobes were excised and placed in frog Ringer solution (in mM: NaCl 115, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and HEPES 10, pH 7.2 adjusted with NaOH, 285 mOsm). Oocytes were transferred to a tube containing 15 ml of collagenase type 1A (2 mg/ml; Sigma, St. Louis, MO) in Ca^{2+}-free Ringer medium (in mM: NaCl 82.5, KCl 2.0, $MgCl_2$ 1.0 and HEPES 5.0, pH 7.5 with NaOH) and gently rocked for 2–3 h. Separated oocytes were washed initially in Ca^{2+}-free Ringer and subsequently in ND96 (in mM: NaCl 96, KCl 2.0, $MgCl_2$ 1.0, $CaCl_2$ 1.8 and HEPES 5.0, pH 7.5 with NaOH) with 5 mM pyruvic acid (Sigma) and gentamycin (0.05 mg/ml; Serva, Heidelberg, Germany) to remove collagenase. Mature oocytes (stages V and VI) free of any follicular remains were transferred into sterile dishes with Ringer solution containing gentamycin sulfate and stored at 19°C for a minimum of 24 h prior to recording. Some oocytes were injected with 23 nl of 50 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA) solution with a microinjector (World Precision Instruments, Sarasota FL) ~10–20 min prior to recording, to obtain a final intracellular concentration of ~1 mM (Kuruma and Hartzell 1999; Kuruma et al. 2000). Control oocytes were injected with 23 nl of MilliQ water (Millipore, Bedford, MA).

The voltage and current recording microelectrodes were pulled with a horizontal puller (P-87; Sutter Instrument, Novato, CA) from filamented borosilicate glass capillaries (GC150TF-10; Harvard Apparatus, Edenbridge, UK) and filled with 3 M KCl (R = 0.1–0.6 MΩ). Oocytes were placed in a gravity-fed continuous-flow chamber (60 µl volume) and superfused at a rate of 2 ml/min with frog Ringer solution, allowing rapid exchange of fluids (~2 s). Penetratin was also applied at 2 ml/min during acute experiments (i.e., up to 30-s applications), such as during dose–response measurements. During prolonged applications of penetratin, the flow rate was slowed to 0.2 ml/min after the initial 10–30 s regular flow. Membrane currents evoked by voltage steps were recorded from oocytes using a two-electrode voltage-clamp virtual ground at a holding potential ($V_{HOLD} = -40$ mV) controlled by a Gene Clamp 500B amplifier (Molecular Devices, Sunnyvale, CA). All recordings were carried out at 21–23°C. Electrophysiological data were acquired at a sampling rate of 5 kHz, low-pass-filtered at 2 kHz and stored on a computer using a digitizer interface and software (Digidata 1322A and PClamp 8.2, Molecular Devices). The data were analyzed off-line using Clampfit 9.0 software (Molecular Devices). SigmaPlot software (Systat Software, San Jose, CA) was used for statistical analysis and to fit concentration–response curves by nonlinear regression. Data are presented as mean ± standard deviation (SD), and significance was accepted at $P < 0.05$ using Student's *t*-test.

Peptides

Penetratin (CRQIKIWFPNRRMKWKK), Tat4 (YARAA ARQARA) and penetratin-conjugated peptides (Coulson et al. 2000) were commercially synthesized and purified (Auspep, Melbourne, Australia).

Results

Bath application of penetratin (10 µM) resulted in depolarization of oocyte mean resting membrane potential from −46 ± 7 mV in control to −21 ± 6 mV after application ($n = 12$). Penetratin application changed the holding current, resulting either in outward currents at positive holding

potentials or in inward currents at negative potentials (Fig. 1a). For example, 10 μM penetratin resulted in a sustained inward current of −821 ± 348 nA at −40 mV holding potential ($n = 8$). Penetratin's effect on resting membrane potential and holding current was rapid, reaching steady state within a few seconds of bath application, and displayed limited desensitization (Fig. 1a). For example, we observed ~20% desensitization after 20–30 min of application at 10 μM ($n = 5$). Returning to normal extracellular solution (washout) abolished penetratin potentiation within a few minutes (Fig. 1a). The rapid onset and washout of penetratin effects are consistent with an extracellular site of action. Furthermore, microinjection of 50 nl of 50 μM penetratin (~2.5 μM final concentration) into the oocytes immediately prior to recording did not potentiate currents evoked by a voltage step to +80 mV from a holding potential of −40 mV ($n = 4$, 146 ± 23 nA) compared to measurements in uninjected control oocytes from the same frog ($n = 10$, 128 ± 19 nA), ruling out the possibility of an intracellular site of action following membrane translocation.

We next tested the effects of penetratin on endogenous currents evoked by 1-s-long voltage steps (Fig. 1b). The outwardly rectifying currents evoked by depolarization from a holding potential of −40 mV are CaCCs that activate upon voltage-dependent influx of Ca^{2+} (Fig. 1b), which is the only known robust current in oocytes activated at these voltages (Barish 1983; Kuruma and Hartzell 1999; Kuruma et al. 2000; Miledi 1982; Schroeder et al. 2008). Penetratin indeed strongly potentiated these instantaneous outwardly rectifying currents resembling oocyte CaCCs (Fig. 1c, d). Penetratin-potentiated currents evoked by voltage steps or ramps (10 mV/50 ms) had a reversal potential (zero current) of −18 ± 5 mV ($n = 12$). Peak steady-state current amplitudes were measured "peak to peak" for currents evoked by voltage steps to −80 and 80 mV from a holding potential of −40 mV. Peak currents evoked under control conditions varied from 50 to 650 nA (mean 235 ± 118 nA, $n = 15$) (Fig. 1b). Penetratin (10 μM) potentiated the peak currents significantly, ranging from 7.1 to 18.3 μA (11.8 ± 2.9 μA, $n = 9$; $P < 0.001$), giving a mean potentiation of ~50-fold (range 23- to 128-fold). In three of nine cells that had smaller control currents (≤120 nA), potentiation exceeded 100-fold.

Potentiation of outwardly rectifying currents by penetratin was dose-dependent (Fig. 1e, f). The EC_{50} value calculated from a concentration–response curve fit to normalized peak currents by nonlinear regression was 3.2 μM (Fig. 1f). The threshold concentration for potentiation occurred at ~0.1–0.2 μM ($n = 8$), and maximal potentiation of outwardly rectifying currents up to >100-fold of control was reached at ~30 μM ($n = 6$). Application of high doses (100 μM, $n = 6$) did not further increase potentiation and resulted in reduction of peak currents and gradual loss of rectification (Fig. 1e, thick line pointed with arrows) when applied for longer than 1 min ($n = 3$); longer applications of lower concentrations of penetratin did not show these effects ($n = 12$).

Next, we tested the sensitivity of penetratin-potentiated current to divalent cations to determine the involvement of Ca^{2+} influx. We found that the penetratin-potentiated current was strongly dependent on the extracellular divalent cations. Addition of extracellular Zn^{2+} (≥10 μM) reversibly blocked penetratin (1 μM)-potentiated currents within seconds ($n = 7$, $P < 0.001$) (Fig. 2a). The IC_{50} value for Zn^{2+} block of penetratin-potentiated current was 5.9 μM (Fig. 2b). In contrast, increasing extracellular Ca^{2+} from 1.8 to 2 mM reversibly enhanced penetratin (1 μM) potentiation of currents within seconds by 81 ± 8% ($n = 5$, $P < 0.01$) (Fig. 2c). Increasing extracellular Ca^{2+} (1.8 mM) to 3 mM further increased penetratin-potentiated current and resulted in the gradual loss of outward rectification of penetratin-potentiated current, which became linear after ~5 min (Fig. 2c). Increasing extracellular Mg^{2+} (1 mM) to 3 mM also further increased penetratin-potentiated current. Zn^{2+} block of the penetratin-potentiated current was also dependent on the extracellular Ca^{2+} and Mg^{2+} concentration as increasing either then required higher Zn^{2+} concentrations for a complete block ($n = 6$). Extracellular Cd^{2+} (100 μM, $n = 5$), Cu^{2+} (10 μM, $n = 6$) and Ba^{2+} (200 μM, $n = 6$) (Fig. 3a) also significantly blocked 1 μM penetratin-potentiated current by 85 ± 9%, 75 ± 7% and 60 ± 6%, respectively. However, higher concentrations of Cd^{2+} (≥2 mM), Cu^{2+} (≥100 μM) and Ba^{2+} (≥2 mM) either were less effective at blocking the penetratin effect or resulted in paradoxical increases in penetratin-potentiated current ($n = 4$ each) (Fig. 3a).

We then tested various K^+ channel blockers to find out whether K^+ channels had any contribution to the penetratin-mediated potentiation. In contrast to divalent cations, K^+ channel blockers had no significant effect on penetratin-potentiated current. Extracellular Cs^+ (3–6 mM, $n = 5$) (Fig. 3b), a blocker of neuronal I_h and K^+ currents; tetraethylammonium (TEA; 5 mM, $n = 5$) (Fig. 3b), a blocker of voltage-dependent K^+ channels; 100–500 nM tertiapin-Q ($n = 7$, not shown), a blocker of inwardly rectifying K^+ channels and Ca^{2+}-activated K^+ channels; and halothane (10 mM, $n = 5$; not shown), a blocker of two-pore domain K^+ leak currents had no significant effects on penetratin-potentiated current.

We then tested the effects of compounds that have been reported to block or prevent activation of native CaCCs that are activated by Ca^{2+} influx. Firstly, BAPTA, a Ca^{2+} buffer that chelates intracellular Ca^{2+}, was microinjected into oocytes to prevent activation of CaCCs. Indeed, intracellular application of BAPTA prevented penetratin (10 μM)

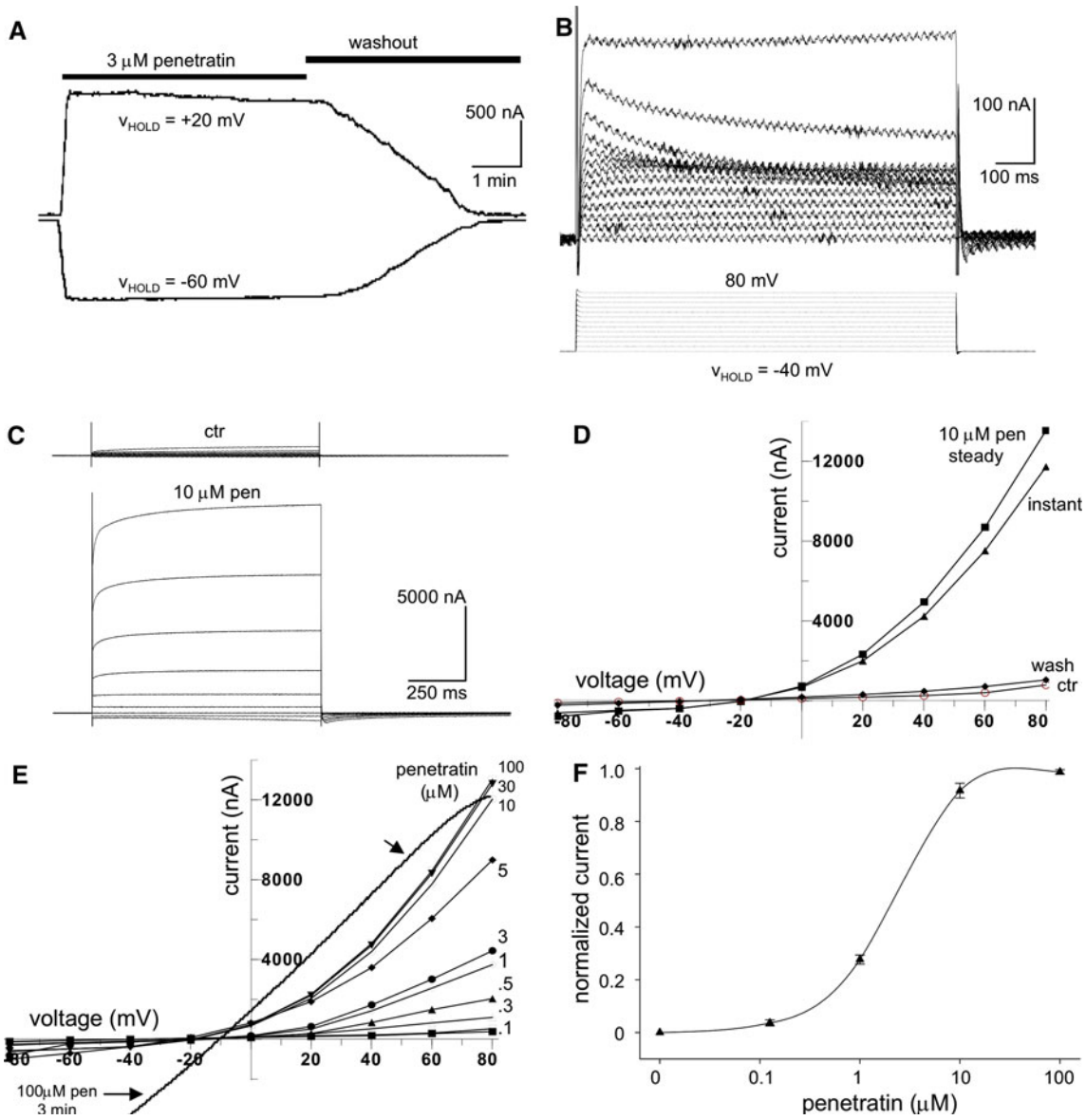

Fig. 1 Penetratin potentiates endogenous ionic currents in *Xenopus* oocytes. **a** Changes in holding current traces in response to bath application of 3 μM penetratin for 5 min at holding potentials of +20 and −60 mV. Recovery of holding current was observed within several minutes upon washout. **b** Oocyte CaCC is characterized by outwardly rectifying currents activated by depolarization-activated Ca^{2+} influx. Outwardly rectifying CaCCs were activated by 1-s-long depolarizing voltage steps at 10-mV intervals in a *Xenopus* oocyte clamped at −40 mV holding potential. **c** Penetratin potentiation of outwardly rectifying currents evoked by voltage steps (1 s duration, −80 to +80 mV at 20-mV intervals, $V_{HOLD} = -40$ mV) started 5 s after bath application of penetratin. Current traces are shown before (*ctr*) and during bath application of 10 μM penetratin. **d** Current–voltage plots for instantaneous (*filled triangles*) and steady-state (*filled squares*) currents shown in **a**, control (*ctr*, *circles*) and following 5-min washout (*filled diamonds*). Note that the penetratin-potentiated instantaneous and steady-state currents are both outwardly rectifying. The instantaneous currents were measured at the start of the voltage step after the initial transient, where they reached their plateau at ∼20 ms after the onset. The steady state was taken at the end of a 1-s-long step. **e** Current–voltage plots of the currents evoked by voltage steps, before (*filled square*) and after penetratin at increasing concentrations (0.1–100 μM). Data were obtained from the same oocyte, and each concentration was applied for 30 s. Prolonged application (3 min) of penetratin at maximal dose resulted in loss of outward rectification (*thick line indicated by arrows*). **f** Penetratin concentration–response curve fitted for normalized peak currents obtained by measuring "peak to peak" currents evoked by voltage steps to −80 and 80 mV from a holding potential of −40 mV ($n = 6$ each concentration)

potentiation of currents by 78 ± 9% ($n = 6$, $P < 0.01$) (Fig. 4a) compared to control oocytes injected with the same amount of MilliQ water. Secondly, a nonsteroidal anti-inflammatory chloride channel blocker, niflumic acid (NFA, 100 μM), significantly reduced penetratin (1 μM)-potentiated current by 76 ± 9% ($P < 0.01$, $n = 4$) (Fig. 4b).

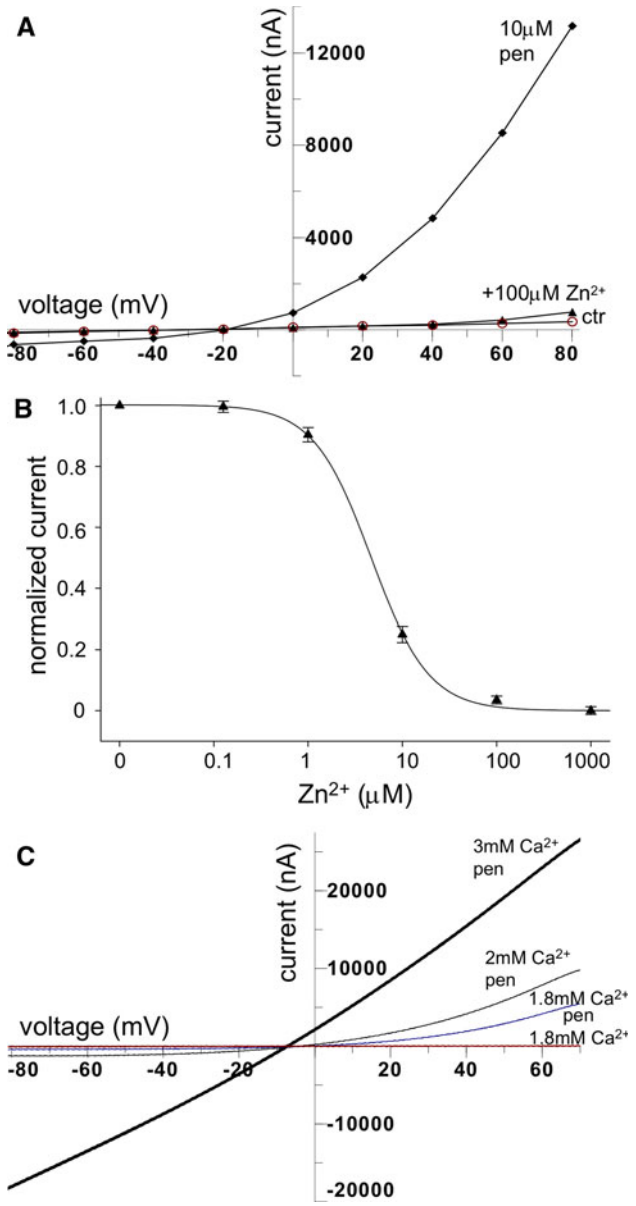

Fig. 2 Effects of extracellular Zn^{2+} on penetratin-potentiated currents. **a** Extracellular Zn^{2+} (100 μM, *filled triangles*) block of penetratin (10 μM, *filled diamonds*)-potentiated currents evoked by voltage steps. Note that bath application of Zn^{2+} completely blocked penetratin-potentiated currents to control levels (*circles*). **b** Concentration–response curve for Zn^{2+} block of penetratin (1 μM)-potentiated normalized peak currents obtained by measuring "peak to peak" currents evoked by voltage steps to −80 and 80 mV from a holding potential of −40 mV ($n = 7$ each concentration). **c** Raising extracellular Ca^{2+} concentration from 1.8 to 2 mM significantly increased the penetratin (5 μM)-potentiated currents. Further increasing the bath Ca^{2+} concentration to 3 mM resulted in gradual loss of the outward rectification, and current responses became linear ~5 min later. Control displays the current responses prior to penetratin (1.8 mM Ca^{2+}). Current responses were evoked by voltage ramps (10 mV/50 ms, −80 to +80 mV, $V_{HOLD} = -40$ mV)

Finally, a voltage-dependent blocker of CaCCs, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS, 100 μM) also significantly inhibited penetratin-potentiated outward currents by 71 ± 8% ($P < 0.01$, $n = 7$) (Fig. 4c). The reversibility of the blockers was not tested. Together, the above results are consistent with penetratin potentiation of a zinc-sensitive CaCC.

We then performed additional control experiments to test the specificity of penetratin potentiation of endogenous CaCCs in oocytes. Another cationic PTD-containing peptide, *trans*-activator of transcription (Tat, 0.1–10 μM), had no effect on CaCCs in defolliculated oocytes ($n = 5$, not shown). Thus, it is unlikely that penetratin potentiation is due to the presence of a PTD. Penetratin potentiation, washout and block by Zn^{2+} remained similar when penetratin was conjugated to 29- or 35-amino acid long fragments of the P75 receptor, alone or with a lipid (palm) (Coulson et al. 2000) ($n = 11$). These results rule out the possibility that penetratin potentiation of endogenous oocyte CaCCs is due to a nonspecific effect of a short peptide on the membrane. Finally, we did not detect any significant effect on voltage-dependent currents or change to the resting membrane potential with similar doses of penetratin applied up to 10 min in cultured newborn mouse dorsal root ganglion (DRG) neurons of small to large size, prepared as described previously (Kanjhan et al. 2005) ($n = 10$, not shown). These results together suggest that penetratin's effects are likely to be cell type-specific.

Discussion

This study describes a dose-dependent and high-efficacy (23- to 128-fold increase by 10 μM penetratin) potentiation of endogenous CaCC by penetratin in defolliculated *Xenopus* oocytes. Oocytes are arguably ideal for the study of endogenous CaCCs, due to the expression of a robust and easily distinguishable CaCC in the absence of most other ion channels, such as Ca^{2+}-activated K^+ channels (Dascal 1987; Hartzell et al. 2005). The endogenous oocyte CaCC is activated by rising intracellular Ca^{2+} following activation of a voltage-dependent cation channel that is primarily permeable to both Ca^{2+} and Mg^{2+} ions (Hartzell et al. 2005; Kanjhan et al. 2005; Kuruma and Hartzell 1999; Kuruma et al. 2000; Marin et al. 2010; Schroeder et al. 2008).

Penetratin-potentiated currents in oocytes display the following characteristics, which strongly indicate that the current is an endogenous CaCC: (1) voltage-dependent Ca^{2+} influx leading to outwardly rectifying currents at submaximal intracellular Ca^{2+} and becoming linear with

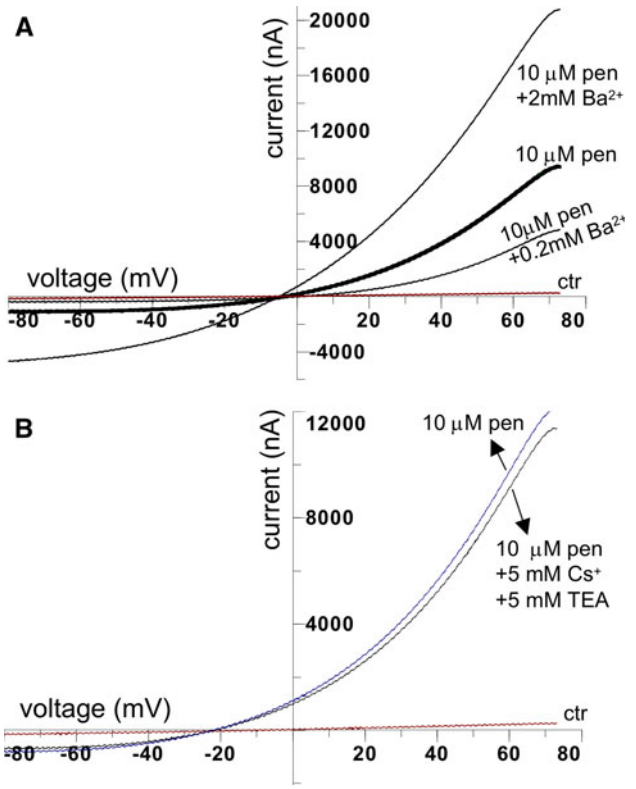

Fig. 3 Effects of other divalent cations and K^+ channel blockers on penetratin-potentiated currents. **a** Extracellular application of Ba^{2+} reduced penetratin-potentiated current at 0.2 mM concentration; however, Ba^{2+} at 2 mM further potentiated the penetratin effect. Current responses were evoked by voltage ramps (10 mV/50 ms, −80 to +80 mV, $V_{HOLD} = -40$ mV). **b** Extracellular application of K^+ channel blockers Cs^+ (5 mM) and TEA (5 mM) was ineffective at blocking penetratin-potentiated currents evoked by voltage ramps

increasing Ca^{2+} (Barish 1983; Kuruma and Hartzell 1999; Kuruma et al. 2000; Miledi 1982; Schroeder et al. 2008); (2) positive modulation by increases in extracellular Ca^{2+} and Mg^{2+} (Hartzell et al. 2005; Kuruma and Hartzell 1999; Kuruma et al. 2000; Schroeder et al. 2008); (3) a reversal potential of −18 mV close to E_{Cl} of −24 mV in oocytes (Barish 1983); (4) block by low concentrations of extracellular Zn^{2+} (Hartzell and Qu 2003); (5) bimodal responses to extracellular Cd^{2+}, Cu^{2+} and Ba^{2+}, characterized by inhibition at low concentrations and potentiation at high concentrations (Hartzell et al. 2005; Kanjhan et al. 2005; Kuruma and Hartzell 1999; Kuruma et al. 2000; Schroeder et al. 2008); (6) significant inhibition by known compounds that block or prevent activation of CaCCs, including intracellular BAPTA (Kuruma and Hartzell 1999; Kuruma et al. 2000) and extracellular DIDS (Schroeder et al. 2008) and NFA (White and Aylwin 1990). In contrast, blockers of various K^+ channels such as Cs^+, TEA, tertiapin-Q and halothane had no significant effect on penetratin-potentiated current.

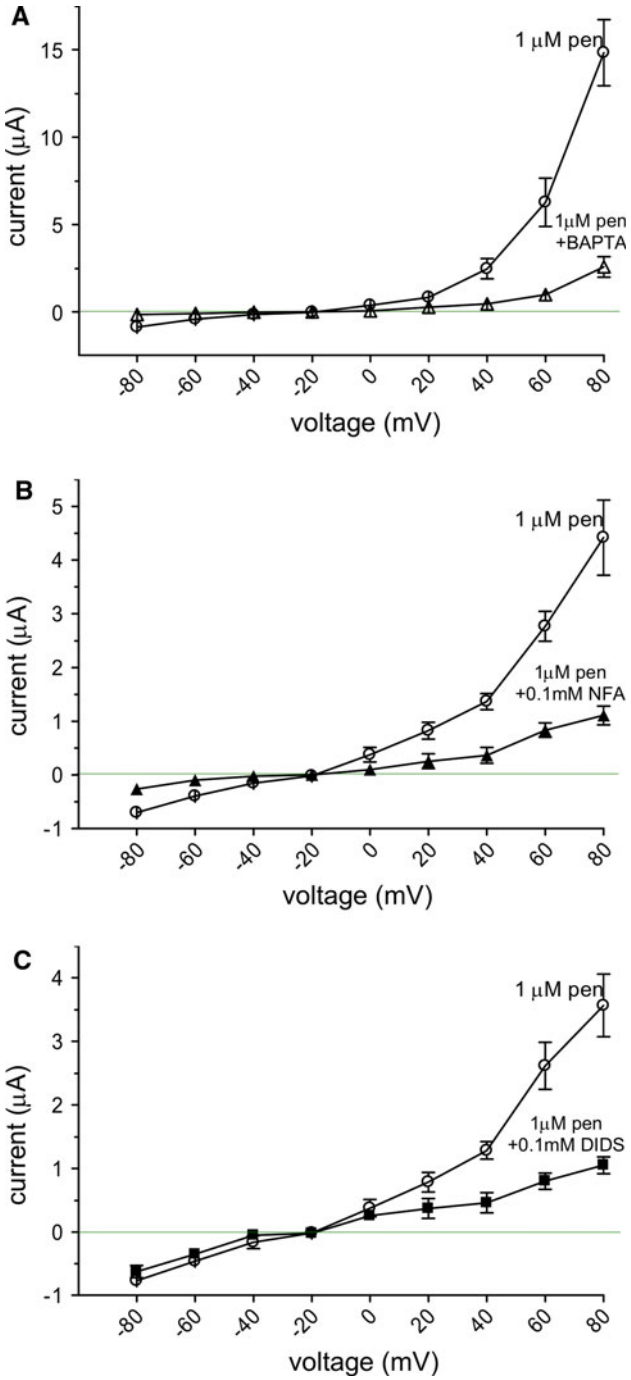

Fig. 4 Effects of CaCC blockers and Ca^{2+} chelators on penetratin potentiation. **a** In oocytes that were microinjected with 23 nl of 50 mM BAPTA prior to recording (*triangles*) to prevent CaCC activation, penetratin (10 μM)-potentiated currents were largely reduced compared to H_2O-injected control oocytes (*circles*, $n = 6$ each group). Current responses evoked by voltage steps (1 s duration: −80 mV to +80 mV at 20-mV intervals, $V_{HOLD} = -40$ mV). **b** Current–voltage plots of mean ± SD values illustrating significant block by extracellular NFA (0.1 mM, *filled triangles*, $n = 4$, $V_{HOLD} = -40$ mV) of penetratin-potentiated currents (1 μM penetratin, *circles*). **c** Plots of mean ± SD values showing significant block by DIDS (0.1 mM, *filled squares*, $n = 7$, $V_{HOLD} = -40$ mV) of penetratin-potentiated outwardly rectifying currents (1 μM penetratin, *circles*)

We cannot exclude the possibility of a contribution from hyperpolarization-activated nonselective cation currents, which can lead to appreciable Ca^{2+} inflow and subsequent activation of CaCCs in oocytes (Kuruma et al. 2000; Marin et al. 2010). Indeed, we have seen strong penetratin potentiation of hyperpolarization (−120 to −160 mV)-activated currents; however, due to their mixed cationic nature, the data were not included. We instead focused on depolarization-activated outwardly rectifying currents to minimize any contribution from the nonselective cation currents.

Although we show that penetratin potentiation of oocyte CaCCs requires Ca^{2+} influx from the extracellular side, it is also likely that this influx is boosted significantly by release of Ca^{2+} from internal stores (Hartzell et al. 2005; Kuruma and Hartzell 1999; Marin et al. 2010). Such a contribution from internal Ca^{2+} stores is supported by our observation that the activation/deactivation is faster and greater at high doses of penetratin (>5 μM).

The present study does not pinpoint the exact site(s) of penetratin action or the mechanisms of penetratin-mediated Ca^{2+} influx required for potentiation. Ca^{2+} influx via penetratin-mediated formation of pores through rapid membrane disturbances, as shown in cultured HeLa and CHO-K1 cells (Palm-Apergi et al. 2009), remains a possibility. However, membrane disturbances were brief and temporary in HeLa and CHO-K1 cells (Palm-Apergi et al. 2009), differing from our prolonged (up to 30 min) recordings of penetratin potentiation in oocytes (see Fig 1a). Furthermore, penetratin potentiation is unlikely to be due to a nonspecific membrane disturbance or leak as we have seen no effect of penetratin on cultured DRG neurons, which are known to express Ca^{2+}-activated K^+ (Kanjhan et al. 2005) and CaCCs (Hartzell et al. 2005; Yang et al. 2008), which should have been activated if there was a Ca^{2+} influx. A subset of DRG cells may express CaCCs (Hartzell et al. 2005), but CaCC is reported to be expressed by both small- and large-diameter DRG neurons (Yang et al. 2008). We sampled both small and large DRG cells and did not see any change in currents evoked by voltage steps. This finding suggests that it is unlikely that penetratin potentiates a voltage-dependent Ca^{2+} influx, which is expected to subsequently activate Cl^- and/or K^+ currents in DRG neurons. Our findings suggest that penetratin-mediated responses may be cell type- or membrane type-specific, and an agonistic or allosteric modulatory role for penetratin remains a possibility. Further research is needed to determine whether the potentiating effect of penetratin on CaCCs is unique to oocytes, which are specialized to generate fertilization potential by utilizing CaCCs upon sperm penetration, or common among other cells expressing CaCCs, such as epithelial cells lining airways and the gastrointestinal tract.

The consequences of endogenous CaCC activation are cell type-specific and include chloride secretion in epithelial cells, transduction of olfactory stimuli in olfactory receptor neurons, smooth muscle contraction and prevention of polyspermia in oocytes (Galietta 2009; Hartzell et al. 2005, 2009; Verkman and Galietta 2009). However, the molecular identity and functional roles of CaCCs in many cell types are poorly understood, due to a lack of specific blockers and modulators (Davis et al. 2010; Galietta 2009; Hartzell et al. 2005, 2009; Manoury et al. 2010; Schreiber et al. 2010; Schroeder et al. 2008; Verkman and Galietta 2009). If available, inhibitors of CaCCs may be useful in treating hypertension (Davis et al. 2010; Manoury et al. 2010) and respiratory problems related to airways and various cancers (Galietta 2009; Hartzell et al. 2005, 2009; Schreiber et al. 2010; Verkman and Galietta 2009). Here, we show that penetratin potentiation of the oocyte CaCC was blocked by extracellular Zn^{2+}at low concentrations. Zn^{2+} block of some CaCCs and other chloride conductances has previously been shown (Hartzell and Qu 2003; Mathie et al. 2006; Piper and Large 2004). Therefore, Zn^{2+} block of CaCCs may have a limited value as Zn^{2+} is also known to modulate the activity of other ion channels, including purinergic receptors (Kanjhan et al. 2003; Mathie et al. 2006). Interestingly, a recent study reported that extracellular Zn^{2+} regulates purinergic Ca^{2+} influx at varying extracellular pH values and Na^+ concentrations in human airway epithelial cells (Hargitai et al. 2010).

Although the oocyte CaCC was physiologically and pharmacologically characterized many years ago, its molecular identity remained a mystery until recently. Early cloning studies identified bestrophins in *Xenopus* oocytes (Qu et al. 2003); however, bestrophins alone lack some typical biophysical properties of classical CaCCs described in native cells (Hartzell et al. 2005; Schroeder et al. 2008). The gene encoding oocyte CaCC was recently cloned and identified as transmembrane protein 16A (TMEM16A, also known as anoctamin-1, ANO1) (Schroeder et al. 2008), an ortholog of human and mouse TMEM16A containing eight PTDs (Caputo et al. 2008; Hartzell et al. 2009; Yang et al. 2008). Subsequently, TMEM16A was identified in many other cell types including epithelia, smooth muscle and neurons (Davis et al. 2010; Manoury et al. 2010; Rock et al. 2009; Schreiber et al. 2010). The pharmacological and biophysical properties of CaCCs (TMEM16A) in oocytes are shared by CaCCs expressed in many other cell types including sensory neurons, secretory epithelial cells and smooth muscle cells in blood vessels, airways and gut (Davis et al. 2010; Galietta 2009; Hartzell et al. 2005; Manoury et al. 2010; Rock et al. 2009; Schreiber et al. 2010). However, it is not yet clear how TMEM16A is modulated by intracellular Ca^{2+} as its sequencing has not

revealed any obvious Ca^{2+} binding domains (Galietta 2009; Hartzell et al. 2009; Yang et al. 2008).

Mice deficient in the *TMEM16A* gene fail to thrive, die postnatally and display markedly reduced CaCCs (Yang et al. 2008) and pathology similar to that of cystic fibrosis (Ousingsawat et al. 2009; Rock et al. 2009; Schreiber et al. 2010). There are currently two activators of CaCCs in clinical trials for the treatment of cystic fibrosis, both of which act indirectly by elevating cytoplasmic Ca^{2+}: Moli1901, a 19-amino acid polycyclic bacterial peptide that elevates intracellular Ca^{2+} by interacting with membrane phospholipids, and denufosol tetrasodium, a $P2Y_2$ purinoceptor agonist (Verkman and Galietta 2009). Although our results are preliminary, it is tempting to speculate that strong potentiation of CaCCs by penetratin may be a promising prospect for a novel treatment of CaCC-related diseases such as cystic fibrosis.

This is the first study to show the action of penetratin on ion channels, and such effects should always be considered when penetratin is used as a delivery vehicle for therapeutic agents. Given that CaCC in oocytes is primarily involved in generation of fertilization potential, possible effects of penetratin particularly on the fertilization process following in vivo delivery, need to be carefully examined.

Acknowledgements We are grateful to David Adams for generously providing oocytes and voltage-clamp equipment and for his constructive comments on the manuscript. Elizabeth Coulson is thanked for kindly supplying peptides. Joseph Lynch is thanked for his critical comments on the manuscript. This work was supported by grants from the National Health and Medical Research Council of Australia to MCB.

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