Ca$^{2+}$-dependent regulation of a non-selective cation channel from *Aplysia* bag cell neurones

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Ca$^{2+}$-activated, non-selective cation channels feature prominently in the regulation of neuronal excitability, yet the mechanism of their Ca$^{2+}$ activation is poorly defined. In the bag cell neurones of *Aplysia californica*, opening of a voltage-gated, non-selective cation channel initiates a long-lasting afterdischarge that induces egg-laying behaviour. The present study used single-channel recording to investigate Ca$^{2+}$ activation in this cation channel. Perfusion of Ca$^{2+}$ onto the cytoplasmic face of channels in excised, inside-out patches yielded a Ca$^{2+}$ activation EC$_{50}$ of 10 μM with a Hill coefficient of 0.66. Increasing Ca$^{2+}$ from 100 nM to 10 μM caused an apparent hyperpolarizing shift in the open probability ($P_o$) versus voltage curve. Beyond 10 μM Ca$^{2+}$, additional changes in voltage dependence were not evident. Perfusion of Ba$^{2+}$ onto the cytoplasmic face did not alter $P_o$; moreover, in outside-out recordings, $P_o$ was decreased by replacing external Ca$^{2+}$ with Ba$^{2+}$ as a charge carrier, suggesting Ca$^{2+}$ influx through the channel may provide positive feedback. The lack of Ba$^{2+}$ sensitivity implicated calmodulin in Ca$^{2+}$ activation. Consistent with this, the application to the cytoplasmic face of calmodulin antagonists, calmidazolium and calmodulin-binding domain, reduced $P_o$, whereas exogenous calmodulin increased $P_o$. Overall, the data indicated that the cation channel is activated by Ca$^{2+}$ through closely associated calmodulin. Bag cell neurone intracellular Ca$^{2+}$ rises markedly at the onset of the afterdischarge, which would enhance channel opening and promote bursting to elicit reproduction. Cation channels are essential to nervous system function in many organisms, and closely associated calmodulin may represent a widespread mechanism for their Ca$^{2+}$ sensitivity.

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Found in the nervous systems of all species examined, Ca$^{2+}$-activated, non-selective cation channels set excitability and firing rates (Partridge et al. 1979; Swandulla & Lux, 1985; Morisset & Nagy, 1999; Egorov et al. 2002), tune information processing (Hall & Delaney, 2000; Van den Abbeele et al. 1994), and play roles in neuropathology (Fraser & MacVicar, 1996; Chen et al. 1997; Smith et al. 2003). These channels are non-selective to monovalent cations, may be Ca$^{2+}$ permeable or voltage dependent, and typically show activation at ~1 μM intracellular Ca$^{2+}$, but can respond over the nanomolar to millimolar range (Yellen, 1982; Partridge & Swandulla, 1987, 1988; Razani-Boroujerdi & Partridge, 1993; Cho et al. 2003; Liman, 2003; Liu & Liman, 2003; Prawitt et al. 2003; Guinamard et al. 2004).

Particularly in the nervous system, the means by which Ca$^{2+}$-activated cation channels transduce Ca$^{2+}$ is not fully understood. The ubiquitous Ca$^{2+}$-binding protein, calmodulin, mediates the Ca$^{2+}$-dependent activation, inactivation, or facilitation of many ion channel species (Saimi & Kung, 2002; Xia et al. 1998; Levitan, 1999; Michikawa et al. 1999; Zuhlke et al. 1999). In addition, the effect of Ca$^{2+}$ on ligand-gated cation channels, such as cyclic nucleotide-gated (CNG) channels (Liu et al. 1994; Bradley et al. 2004) and NMDA receptors (Krupp et al. 1999; Rycroft & Gibb, 2004), is due to closely associated calmodulin. Moreover, calmodulin is the Ca$^{2+}$ sensor for a non-neuronal, transient receptor potential/melastatin (TRPM) cation channel, expressed in heart, pancreas, kidney, and intestine (Launay et al. 2002; Nilius et al. 2005). However, for native, neuronal, steady-state, Ca$^{2+}$-activated cation channels, the mechanism of Ca$^{2+}$ sensitivity remains unknown.

The present study examines the Ca$^{2+}$-dependent activation and modulation of a non-selective cation channel from the bag cell neurones of the marine snail, *Aplysia californica*. This channel provides the depolarizing drive for the afterdischarge, a prolonged burst that initiates...
egg-laying behaviour (Kupfermann, 1967; Kupfermann & Kandel, 1970; Pinsker & Dudek, 1977; Conn & Kaczmarek, 1989). The afterdischarge is characterized by ~30 min of action potential firing, with a concomitant release and influx of Ca$^{2+}$ that results in the neurohaemal secretion of egg-laying hormone (Fink et al. 1988; Fisher et al. 1994). Upon termination of the afterdischarge, a lengthy refractory period ensues, during which a second burst cannot be elicited (Conn & Kaczmarek, 1989). Previously, Wilson et al. (1996) found that elevating Ca$^{2+}$, from nanomolar to micromolar levels, at the cytoplasmic face of patches excised from bag cell neurones increased cation channel activity; however, the extent or the mechanisms of Ca$^{2+}$ activation were not examined. We now provide evidence to suggest that closely associated calmodulin serves as the cation channel Ca$^{2+}$ sensor. Linking the cation channel and calmodulin provides a means to translate a change in intracellular Ca$^{2+}$ into a change in excitability, which, in a system like the bag cell neurones, is essential for triggering behaviour. As a whole, calmodulin may act in this capacity for neuronal cation channels throughout the metazoa.

Methods

Animals and cell culture

Adult *Aplysia californica* weighing 150–300 g were obtained from Marinus Inc. (Long Beach, CA, USA). Animals were housed in an ~300 l aquarium containing continuously circulating, aerated sea water (Instant Ocean; Aquarium Systems, Mentor, OH, USA or Kent sea salt; Kent Marine, Acworth, GA, USA) at 14–16°C on a 12 : 12 h light : dark cycle, and fed Romaine lettuce five times a week.

For primary cultures of isolated bag cell neurones, animals were anaesthetized by an injection of isotonic MgCl$_2$ (50% of body weight), the abdominal ganglion removed and treated with neutral protease (13.33 mg ml$^{-1}$; Roche Diagnostics, Indianapolis, IN, USA) for 18 h at 20–22°C, dissolved in tissue culture artificial sea water (tcASW) (composition (mm): 460 NaCl, 10.4 KCl, 11 CaCl$_2$, 55 MgCl$_2$, 15 Heps, 1 mg ml$^{-1}$ glucose, 100 U ml$^{-1}$ penicillin, and 0.1 mg ml$^{-1}$ streptomycin, pH 7.8 with NaOH). The ganglion was then transferred to fresh tcASW for 1 h, after which time the bag cell neurone clusters were dissected from their surrounding connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurones were dispersed in tcASW onto 35 mm × 10 mm polystyrene tissue culture dishes (Corning, Corning, NY, USA). Cultures were maintained in tcASW in a 14°C incubator, and used for experimentation within 1–3 days. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH, USA), or Sigma-Aldrich (St Louis, MO, USA).

Excised, patch-clamp recording

Single cation channel current was measured using an EPC-8 amplifier (HEKA Electronics, Mahone Bay, NS, Canada), and primarily the excised, inside-out patch-clamp method. Microelectrodes were pulled from 1.5 mm internal diameter, borosilicate glass capillaries (TW 150 F-4; World Precision Instruments, Sarasaota, FL, USA) and were fire polished to a resistance of 2–8 MΩ when filled with normal artificial sea water (nASW) (composition as per tcASW but lacking glucose, penicillin, and streptomycin). To lower the root mean squared noise of the current signal, microelectrode capacitance was reduced by coating the shank and half of the shoulder with dental wax (Heraeus Kulzer, South Bend, IN, USA) under a dissecting microscope. Following excision, the cytoplasmic face was bathed with artificial intracellular saline (composition (mm): 500 potassium aspartate, 70 KCl, 1.2 MgCl$_2$, 10 Heps, 11 glucose, 5 EGTA, 10 reduced glutathione, pH adjusted to 7.3 with KOH). In the majority of experiments, CaCl$_2$ was added for a free Ca$^{2+}$ concentration of 1 μM. Some experiments were performed using intracellular saline with Ca$^{2+}$ or Ba$^{2+}$ concentrations ranging from 100 nm to 300 μM. Experiments involving Ba$^{2+}$ required the substitution of CaCl$_2$ with BaCl$_2$. In all cases, the added and free Ca$^{2+}$ and Ba$^{2+}$ concentrations were calculated using the WebMaxC program (http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm). In one set of experiments, single-channel current was measured using the excised, outside-out patch-clamp technique. Microelectrodes were filled with artificial intracellular saline containing 1 μM or 300 μM free Ca$^{2+}$. The extracellular face of excised, outside-out patches was exposed to nASW containing 11 mm Ca$^{2+}$ or 11 mm Ba$^{2+}$. In all cases, current was low-pass filtered at 1 kHz using the EPC-8 Bessel filter and acquired at a sampling rate of 10 kHz using an IBM-compatible personal computer, a Digidata 1300 analog-to-digital converter (Axon Instruments, Union City, CA, USA), and the Clampex acquisition program of pCLAMP (version 8.0; Axon Instruments). Data were gathered at room temperature (~22°C) in 1–3 min intervals, typically while holding the patch at ~60 mV.

Patch perfusion array, drug application and reagents

An eight-barrel perfusion array was constructed by tightly aligning borosilicate square tubing (outer diameter: 0.75 mm, internal diameter: 0.5 mm; VitroCom Inc., Mountain Lakes, NJ, USA) attached to one another using superglue. The section of the array that was submerged into the bath did not come in contact with superglue. The barrels at the opposite end of the array were fitted with silicone tubing (outer diameter: 3.3 mm, internal...
Each of these perfusion lines was connected to a 5 ml syringe. Prior to experimentation, the entire perfusion system was rinsed with Sigmacote (SL-2; Sigma-Aldrich) and allowed to dry for at least 24 h. Gravitational flow was controlled by an alligator clip over the tubing and setting the level of the syringes to a fixed height. When the clip was released, the result was a flow of ~1 ml min⁻¹. Any greater flow rate disturbed the patch and led to mechanical-based noise or seal failure. The perfusion system allowed patches to be moved from the mouth of one barrel to the next, permitting an almost instantaneous change in solutions at the face of the channel. During perfusion, the culture dish was gently drained as required using a plastic Pasteur pipette.

Drugs were made up as concentrated stock solutions and frozen at −30°C. They were introduced to the patch at the indicated working concentration, either with the perfusion array or by pipetting a small volume of stock solution into the culture dish. In the latter case, care was taken to pipette the stock near the side of the dish and as far away as possible from the patch at the tip of the microelectrode. In experiments examining the interplay of Ca²⁺ concentration and voltage dependence, TEA (Sigma-Aldrich) was added to nASW in the pipette to a final concentration of 20 mM in order to reduce outward currents through Ca²⁺-activated K⁺ channels. At or approaching 0 mV, these currents interfered with resolving inward current through the cation channel. Three calmodulin pharmacological inhibitors were employed to test the role of calmodulin as the cation channel Ca²⁺ sensor. Calmidazolium chloride (Calbiochem, San Diego, CA, USA) was dissolved in 100% ethanol for a stock solution of 14.5 mM. Calmidazolium (10 μM final) or its ethanol control (0.07% final) were perfused onto the cytoplasmic face of excised, inside-out patches. Similarly, N-((6-aminohexyl)-1-naphthalenesulphonamide HCl (W-5) (Calbiochem) was dissolved in 100% ethanol for a stock solution of 70 mM. W-5 (100 μM final) or its ethanol control (0.15% final) were also perfused onto the cytoplasmic face of excised, inside-out patches. Calmodulin-binding domain (CBD) (Calbiochem) was dissolved in sterile, double-distilled H₂O for a stock solution of 2.2 mM. For experiments, a small volume of CBD, 17 μl (50 μM final), was pipetted into a centre-well organ culture dish (VWR, Mississauga, ON, Canada) containing 750 μl of intracellular saline. Recording of cation channel current from excised, inside-out patches began following a short diffusion period of ~1 min.

Calmodulin purification

Calmodulin was purified using repeated chromatography on phenyl-Sepharose from an Escherichia coli strain (BL21) stably transformed with the expression plasmid (pCAM) coding for wild-type bovine calmodulin (accession number P62157). A sample of frozen E. coli cell stock was a gift from Dr A. S. Mak (Department of Biochemistry, Queen’s University, Kingston, Ontario, Canada). The sample was cultured in 80 ml of LB broth with 100 μg ml⁻¹ ampicillin (Fisher) and grown overnight at 37°C with shaking at 250 r.p.m. The following day, 20 ml of the overnight culture was inoculated into 11 of LB broth with 100 μg ml⁻¹ ampicillin and grown at 37°C, shaking at 250 r.p.m. until an OD₆₀₀ of 0.8–1.1 was reached. The bacteria were then induced with 1 mM isopropyl-β-D-thiogalactoside (Fisher) at 30°C for 4 h. Cells were then spun down for 25 min at 600g at 4°C, the supernatant discarded, and the pellet frozen at −80°C overnight. On ice, cells were resuspended in 50 ml of buffer A (containing: 25 mM Tris-HCl pH 7.5, 1 mM DTT (Fisher), 0.02% NaN₃, and 0.1 mg ml⁻¹ phenylmethylsulphonylfluoride (PMSF) (Sigma-Aldrich). Cells were ruptured by sonicating the solution 5–10 times at 30 s intervals separated by 1 min rest periods, until the solution was visibly clearer and less viscous. The solution was then centrifuged at 17 500g (JA-20) for 40 min at 4°C, after which time the pellet was discarded and the supernatant centrifuged at 110 000g (Ti45) for 1 h at 4°C. CaCl₂ was added to a concentration of 2.5 mM to expose the hydrophobic regions of calmodulin, and stored overnight at 4°C. A disposable chromatography column (732 1010; Biorad, Hercules, CA, USA) was packed with 5 ml phenyl-Sepharose beads (Amersham, Piscataway, NJ, USA) and pre-equilibrated with 25 ml of H₂O and 25 ml of buffer B (containing: 25 mM Tris-HCl (pH 7.5) and 2.5 mM CaCl₂). The column was capped with parafilm and stored overnight at 4°C. At room temperature, the column was rinsed with 5 ml of buffer B. Extracts were mixed with phenyl-Sepharose beads in the column and left to sit for 30 min with gentle mixing every 5 min. The solution was then centrifuged at 50 ml of buffer B, then washed with 100 ml of buffer C (containing: 50% buffer B and 0.5 mM NaCl), and eluted with buffer E (containing: 25 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, 5 mM EGTA). Three different elutions of 5 ml were collected in separate tubes and tested for protein content using a protein assay kit (500-0006; Biorad). The second elution tube contained protein and was added to a 5 kDa cut-off ultrafree centricron (UFV2BCC10-5K; Millipore, Nepean, ON, Canada) to concentrate the calmodulin. The centricron was centrifuged at 400g at 4°C until 1 ml of buffer E remained, at which time it was rinsed with 5 ml of intracellular saline containing 10 μM free Ca²⁺. This step was repeated, and centrifugation continued until 1 ml of calmodulin in intracellular saline remained in the centricron. The absorbance of calmodulin (at 280 nm) was then tested with spectrophotometry, and combined with the extinction...
coefficient of calmodulin (peptide property calculator; http://www.basic.northwestern.edu/biotools/proteincalc.html) to calculate calmodulin concentration. Aliquots (260 μM stock, 25 μl) were prepared and kept at −80°C prior to experimentation. During experiments, the stock calmodulin was diluted down to a final concentration of 3 μM by pipetting into a bath containing an excised, inside-out patch bathed in intracellular saline with 10 μM free Ca2+. As a control, aliquots of stock calmodulin were boiled for 10 min prior to application.

As a test of calmodulin protein stability, a SDS-PAGE gel was run. From a working stock of 7.36 mg ml−1, 1.5 μl (11 μg), 7.5 μl (55 μg), or 15 μl (110 μg) was added to a sample buffer (containing: 62.5 mM Tris–HCl pH 6.8 at 25°C, 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue and 42 mM DTT totalling a final volume of 30 μl. Samples and standards were boiled for about 5 min, and 20 μl of each loaded into a lane and allowed to run for ∼4 h. By comparison to a broad-range protein marker (7701-S; New England Biolabs, Ipswich, MA, USA), the gel indicated that the purified protein was calmodulin (∼17 000 kDa) and that no degradation had occurred.

Data analysis

To determine channel open probability (P_o), events lists were made from data files using the half-amplitude threshold criterion (Colquhoun & Sigworth, 1995) of the Fetchan analysis program of pCLAMP. Fetchan was also used to generate all-points histograms for determining channel amplitude. For display in figures, all data were filtered to a final cut-off frequency of 500 Hz using the Fetchan digital Gaussian filter. The Pstat analysis program of pCLAMP was used to read events lists and determine P_o, either automatically or manually, using the formula:

\[
P_o = (1 \times t_1 + 2 \times t_2 + \ldots n \times t_n)/(N \times t_{\text{tot}})
\]

where \(t\) = the amount of time that \(n\) channels are open, \(N\) = the number of channels in the patch, and \(t_{\text{tot}}\) = the time interval over which \(P_o\) is measured. The number of channels in the patch was determined by counting the number of unitary current levels, particularly at more positive voltages (typically −20 mV). Pstat was also used to determine the mean open- and closed-state current level by fitting all-points histograms with Gaussian functions using the least-squares method and a simplex search. Channel current amplitude was then calculated by subtracting the mean closed current level from the mean open current level at a given voltage.

The concentration–response curve was constructed using \(P_o\) values obtained at each concentration of Ca2+ or Ba2+, which were normalized by dividing by the \(P_o\) at 300 μM Ca2+, averaged, and plotted versus divalent concentration using Origin (version 7; OriginLab Corporation, Northampton, MA, USA). The Ca2+ concentration–response curve was then fitted with a Hill function to yield the EC50 and Hill coefficient. The Ba2+ concentration–response curve could only be fitted by linear regression. To make \(P_o\) versus voltage relationships, \(P_o\) was first normalized to \(P_o\) at 0 mV and then plotted against patch holding potential using Origin. This relationship was then fitted with a Boltzmann function to derive the half-maximal voltage (V0.5) and the slope factor (k), which is the change in voltage required to move the \(P_o\) e-fold. Channel current versus voltage relationships were produced in Origin by plotting channel-current amplitude against patch-holding potential, and single-channel conductance was then determined by linear regression. Predicted reversal potential was extrapolated from the Origin linear regression fit.

Statistical analysis

Data are presented as the mean ± s.e.m. throughout. Statistical analysis was performed using Instat (version 3; Graphpad Software, San Diego, CA, USA). Student’s t test (two-tailed or one-tailed, and paired or unpaired) was used to test whether the mean differed between two groups. A standard ANOVA with Dunnett’s multiple comparisons test was used to test for differences between multiple means. The test for linear trend was used to determine if there was statistically significant linear trend in a series of multiple means. Analysis of outside-out recordings was based on a Ba2+-induced percentage change in \(P_o\) or current amplitude. The \(P_o\) and current amplitude with Ca2+ at the extracellular face was considered to have a mean of zero, and the data with Ba2+ at the extracellular face were compared for a difference from that mean of zero using a two-tailed, one-sample t test. In all cases, data were considered significantly different if the \(P\) value was < 0.05.

Results

The bag cell neurone cation channel is activated by Ca2+

Cation channels were identified in excised, inside-out patches from cultured bag cell neurones by their conductance (25–30 pS; ∼2 pA at −60 mV), voltage dependence of opening (an increase in \(P_o\) with depolarization), and absence of voltage-dependent inactivation. Many cation channels are regulated by Ca2+, yet few Ca2+ concentration–response curves for neuronal cation channels are published. We perfused the cytoplasmic face of cation channel-containing patches with intracellular saline that had a free Ca2+ concentration
Ca\(^{2+}\) dependence of a neuronal cation channel

The voltage dependence of cation channel \(P_o\) is modulated by Ca\(^{2+}\)

The voltage dependence of cation channel \(P_o\) is known to be modulated by Ca\(^{2+}\) in hamster vomeronasal sensory neurones (Liman, 2003) and membrane vesicles from placenta (Gonzalez-Perrett et al. 2002). Wilson et al. (1996) examined the effect of Ca\(^{2+}\) on the voltage dependence of the bag cell neurone cation channel, and suggested that increasing the cytoplasmic face Ca\(^{2+}\) from 100 nM to 1 \(\mu\)M caused a hyperpolarizing shift in the \(P_o\) versus voltage curve. The present study examined the voltage dependence of bag cell neurone cation channel \(P_o\) as a function of 100 nM, 10 \(\mu\)M, or 300 \(\mu\)M cytoplasmic face Ca\(^{2+}\). The voltage dependence of the cation channel was maintained at all three Ca\(^{2+}\) levels such that \(P_o\) increased with depolarizing potentials. Figure 2A shows an example of this effect at 300 \(\mu\)M Ca\(^{2+}\). When held at potentials positive to 0 mV, the cation channel current did not

\[ P_o = \frac{1}{1 + \left(\frac{[Ca^{2+}]}{EC_{50}}\right)^{n_H}} \]

where \(n_H\) is the Hill coefficient, \(EC_{50}\) is the concentration of Ca\(^{2+}\) required to activate half of the channels, and \([Ca^{2+}]\) is the free Ca\(^{2+}\) concentration in the cytoplasm. The voltage dependence of cation channel \(P_o\) was examined using excised, inside-out patches of hamster vomeronasal sensory neurones. The patch was perfused with intracellular saline containing 300 \(\mu\)M Ca\(^{2+}\), and the voltage dependence of channel activity was measured using a two-electrode voltage clamp technique. The voltage dependence of channel activity was determined by measuring the relative opening probability \(P_o\) as a function of the cytoplasmic face Ca\(^{2+}\) concentration, ranging from 100 nM to 300 \(\mu\)M (Fig. 1A). Upon excision, the patch was always initially perfused with intracellular saline containing 300 \(\mu\)M Ca\(^{2+}\); subsequently, as many concentrations from within the range as possible were then delivered. Individual \(P_o\) values from a total of 24 patches exposed to some or all of the concentration range were normalized to 300 \(\mu\)M Ca\(^{2+}\) and plotted versus Ca\(^{2+}\) concentration (Fig. 1B). Cation channel activity was maximal at 300 \(\mu\)M Ca\(^{2+}\) and above (data not shown), and gradually diminished upon exposure to decreasing concentrations of free Ca\(^{2+}\) to a minimum, but still detectable level, in 100 nM Ca\(^{2+}\). The concentration–response curve was fitted with a Hill function, yielding an \(EC_{50}\) of 10 ± 5 \(\mu\)M Ca\(^{2+}\) and a Hill coefficient of 0.66 ± 0.1. The response of the channel to the concentration of Ca\(^{2+}\) was independent of the order in which the Ca\(^{2+}\) concentrations were presented after the initial 300 \(\mu\)M Ca\(^{2+}\). For a given channel, the \(P_o\) upon initial exposure to 300 \(\mu\)M Ca\(^{2+}\) was very similar to a repeated exposure, even after application of any of the lower concentrations and vice versa (Fig. 1C). Thus, cation channel activity was reversible and highly dependent on cytoplasmic face Ca\(^{2+}\) concentration, confirming its Ca\(^{2+}\)-dependent activation.

Figure 1. Concentration-dependent effects of Ca\(^{2+}\) on the cation channel

**A**. Sample traces of cation channel activity recorded from an excised, inside-out patch held at –60 mV. Cation channel activity, seen as unitary inward current deflections of ~2 pA, steadily increases as the cytoplasmic face is perfused with intracellular saline containing 100 nM to 300 \(\mu\)M Ca\(^{2+}\). The closed state is at the top of the trace and designated by C, while the open states are at the bottom and designated by O, O, and O. B. Concentration–response of cation channel \(P_o\) exposed to a range of Ca\(^{2+}\) concentrations. All patches were exposed to 300 \(\mu\)M Ca\(^{2+}\) (n = 24) with the remaining concentrations consisting of: 100 nM (n = 6), 300 nM (n = 6), 1 \(\mu\)M (n = 9), 3 \(\mu\)M (n = 9), 10 \(\mu\)M (n = 13), 30 \(\mu\)M (n = 11) and 100 \(\mu\)M (n = 11). In three of these 24 cases, the patch was exposed to the entire concentration range (100 nM to 300 \(\mu\)M). \(P_o\) was normalized to \(P_o\) at 300 \(\mu\)M Ca\(^{2+}\). Data points fitted with a Hill function yield an \(EC_{50}\) of 10 ± 5 \(\mu\)M and a Hill coefficient of 0.66 ± 0.1. C. Sample traces of cation channel activity recorded from an excised, inside-out patch held at –60 mV. Upon exchange of the intracellular saline perfusing the cytoplasmic face from one containing 300 \(\mu\)M Ca\(^{2+}\) to one with 100 nM, the activity decreases. However, this effect is completely reversible and the \(P_o\) reverts back to the prior level upon return to 300 \(\mu\)M Ca\(^{2+}\).
reverse, but became unresolvable. This lack of reversal may be due to block by cytoplasmic face Mg\(^{2+}\), as found in astrocyte (Chen & Simard, 2001) and TRPC5 (Obukhov & Nowycky, 2005) cation channels. Thus, despite not being an ideal standardization, \(P_o\) was normalized to \(P_o\) at 0 mV, which in turn revealed an apparent hyperpolarizing shift in voltage dependence without a change in sensitivity at 10 \(\mu\)M (\(n=6\)) or 300 \(\mu\)M Ca\(^{2+}\) (\(n=9\)) as compared to 100 nM Ca\(^{2+}\) (\(n=5\)) (Fig. 2B). The \(V_{0.5}\) increased from \(-12 \pm 0.8\) mV in 100 nM Ca\(^{2+}\) to \(-30 \pm 0.5\) mV or \(-27 \pm 0.2\) mV in 10 \(\mu\)M or 300 \(\mu\)M Ca\(^{2+}\), without an appreciable change in the \(k\) (15 \(\pm\) 0.7 versus 17 \(\pm\) 0.4 versus 16 \(\pm\) 0.2 in 100 nM versus 10 \(\mu\)M versus 300 \(\mu\)M Ca\(^{2+}\), respectively). Overall, channel activity not only increased upon exposure to increasing Ca\(^{2+}\) levels, but as the membrane was depolarized, the increase in channel activity was further enhanced by the presence of 10 \(\mu\)M or 300 \(\mu\)M Ca\(^{2+}\). Not surprisingly, linear regression analysis of current–voltage relationships in the three Ca\(^{2+}\) concentrations showed no change in the conductance or, as established by extrapolating beyond 0 mV, the reversal potential (data not shown).

**The bag cell neurone cation channel is not sensitive to Ba\(^{2+}\)**

The Ca\(^{2+}\) sensitivity of ion channels is mediated by Ca\(^{2+}\) binding directly to the channel or a channel-associated Ca\(^{2+}\) sensor (Hille, 2001; Levitan & Kazcmarek, 2001). The most versatile and ubiquitous Ca\(^{2+}\) sensor is calmodulin (Berridge et al. 2000), and it has been implicated in mediating the Ca\(^{2+}\) sensitivity of numerous ion channels (Levitan, 1999). To initially test this, we used a method first carried out on small-conductance Ca\(^{2+}\)-activated K\(^+\) channels (Cao & Houamed, 1999); namely, Ba\(^{2+}\) was substituted for Ca\(^{2+}\) and perfused on the cytoplasmic face of the cation channel. The rationale is based on Ba\(^{2+}\) binding/activating calmodulin poorly because it is a weak substitute for Ca\(^{2+}\) at the EF-hand motifs (Haiech et al. 1981; Chao et al. 1984; Wang, 1985; Ozawa et al. 1999). Therefore, if calmodulin mediates Ca\(^{2+}\) sensitivity of the cation channel, replacing Ca\(^{2+}\) with Ba\(^{2+}\) would result in a failure to activate the channel in a concentration-dependent fashion. Cation channel-containing patches were initially exposed to intracellular saline containing 300 \(\mu\)M Ca\(^{2+}\), and then subsequently perfused with intracellular saline containing Ba\(^{2+}\) ranging in concentration from 100 nM to 300 \(\mu\)M (\(n=6\)). Cation channel \(P_o\) was dramatically reduced upon exposure to Ba\(^{2+}\) and remained relatively unchanged throughout the concentration range (Fig. 3). Overall, the cation channel showed very little, if any, sensitivity to Ba\(^{2+}\), suggesting a possible involvement of calmodulin as the sensor mediating Ca\(^{2+}\) dependence of channel activity.

**Ba\(^{2+}\) as a charge carrier reduces cation channel activity**

For voltage-gated Ca\(^{2+}\) channels, Ca\(^{2+}\) entry in turn inactivates the channel through associated calmodulin, and this can be prevented by substituting Ba\(^{2+}\) for Ca\(^{2+}\) as a charge carrier (Zuhlke et al. 1999). To examine if Ca\(^{2+}\) entry through the cation channel also serves as a regulator, patches were excised from bag cell neurones in

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**Figure 2. Interaction between Ca\(^{2+}\) and voltage dependence of the cation channel**

A, sample traces of cation channel activity recorded from an excised, inside-out patch perfused with intracellular saline containing 300 \(\mu\)M Ca\(^{2+}\) and held at the indicated voltages. As the membrane potential is depolarized, channel activity increases. B, mean cation channel \(P_o\) normalized to \(P_o\) at 0 mV for each respective Ca\(^{2+}\) concentration and plotted against voltage. As given in the inset, points fitted with a Boltzmann function yield values for half-maximal activation (\(V_{0.5}\)) and slope factor (\(k\)). When Ca\(^{2+}\) is elevated from 100 nM to 10 \(\mu\)M, the voltage dependence shifts in the hyperpolarizing direction (\(V_{0.5}= -12 \pm 0.8\) mV versus \(-30 \pm 0.5\) mV) without an appreciable alteration in sensitivity (\(k= 15 \pm 0.7\) versus 17 \(\pm\) 0.4); however, a further increase in Ca\(^{2+}\) to 300 \(\mu\)M does not cause any obvious additional change (\(V_{0.5}= -27 \pm 0.2\) mV; \(k= 16 \pm 0.2\)).
the outside-out configuration, and the extracellular face of the channel was perfused with nASW containing 11 mM Ca\(^{2+}\) followed by ASW with Ba\(^{2+}\) substituted for Ca\(^{2+}\). The pipette, which bathed the cytoplasmic face, contained intracellular saline with either 1 \(\mu\)M or 300 \(\mu\)M Ca\(^{2+}\) \((n = 4\) and 4\) (Fig. 4A and B). In agreement with Ba\(^{2+}\) permeating the cation channel as a superior charge carrier, following perfusion with Ba\(^{2+}\) the current amplitude increased by \(\sim 18\%\) with 1 \(\mu\)M Ca\(^{2+}\), and \(\sim 15\%\) with 300 \(\mu\)M Ca\(^{2+}\) in the pipette (Fig. 4C). Wilson \textit{et al.} (1996) reported that the relative monovalent cation permeability of the channel was K\(^{+}\) \(\approx \) Na\(^{+}\) \(\gg\) Tris \(\approx\) NMDG; moreover, consistent with Ca\(^{2+}\) permeability and our data on enhanced current amplitude under external Ba\(^{2+}\), they showed that replacing external Ca\(^{2+}\) with Ba\(^{2+}\) caused the conductance to increase from 29 pS to 36 pS. However, Wilson \textit{et al.} (1996) did not report at all regarding the effect of external Ba\(^{2+}\) on \(P_o\). We found that Ba\(^{2+}\) perfusion produced an \(\sim 70\%\) and \(\sim 50\%\) decrease in \(P_o\) with 1 \(\mu\)M and 300 \(\mu\)M Ca\(^{2+}\) in the pipette, respectively (Fig. 4C). This drop in \(P_o\) is probably due to a loss of Ca\(^{2+}\) activation. Changes to both current amplitude and \(P_o\) with either 1 \(\mu\)M or 300 \(\mu\)M Ca\(^{2+}\) in the pipette were statistically significant, and suggest that Ca\(^{2+}\) influx through the channel itself may be a source of Ca\(^{2+}\) activation.

Pharmacological block of calmodulin reduces cation channel activity

In those cases where calmodulin is the sensor mediating Ca\(^{2+}\)-dependent channel regulation of ion channels, calmodulin antagonists often inhibit the effects of Ca\(^{2+}\) on channel function (Krupp \textit{et al.} 1999; Michikawa \textit{et al.} 1999; Bobkov \& Ache, 2003; Moreau \textit{et al.} 2005). Calmidazolium chloride is a potent, specific, and widely effective organic antagonist that inhibits many calmodulin-activated proteins (Van Belle, 1981; Gietzen \textit{et al.} 1982; DeRiemer \textit{et al.} 1985). When cation channels excised from bag cell neurones were perfused with intracellular saline containing 10 \(\mu\)M calmidazolium and 1 \(\mu\)M Ca\(^{2+}\), the \(P_o\) decreased \((n = 5)\) (Fig. 5A). The antagonizing effect of calmidazolium on cation channel activity was also apparent at a cytoplasmic face Ca\(^{2+}\) concentration of 10 \(\mu\)M or 300 \(\mu\)M \((n = 6\) and 6\) (Fig. 5B and C). When the cytoplasmic face of excised patches was exposed to ethanol, the vehicle for calmidazolium, cation channel activity decreased by no more than 30\%, regardless of the Ca\(^{2+}\) concentration \((n = 5, 5\) and 5\) (Fig. 5D). Compared to parallel ethanol controls, 10 \(\mu\)M calmidazolium significantly reduced channel \(P_o\) by \(\sim 60\%\) and 70\% in 1 \(\mu\)M and 10 \(\mu\)M, respectively (Fig. 5E). The concentration dependence of calmidazolium was demonstrated by applying additional concentrations in the presence of 300 \(\mu\)M Ca\(^{2+}\). At 3 \(\mu\)M and 30 \(\mu\)M \((n = 4\) and 4\), calmidazolium caused lower \((\sim 45\%)\) and higher \((\sim 90\%)\) reductions in channel \(P_o\) than that produced by 10 \(\mu\)M \((\sim 75\%)\) (Fig. 5E).

DeRiemer \textit{et al.} (1985) found that 50 \(\mu\)M of the chloride-deficient calmodulin antagonist W-5 had no effect on the initiation of an afterdischarge in intact bag cell neurone clusters. In keeping with that prior negative result, when 100 \(\mu\)M W-5 was tested on the cation channel

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**Figure 3. Lack of effect of Ba\(^{2+}\) on cation channel activity**

\(A\), sample traces of cation channel activity recorded at \(\sim 60\) mV from an excised, inside-out patch. The cytoplasmic face was perfused with intracellular saline with 300 \(\mu\)M Ca\(^{2+}\) followed by intracellular saline with Ba\(^{2+}\) substituted for Ca\(^{2+}\) at concentrations ranging from 100 nM to 300 \(\mu\)M. The Ba\(^{2+}\) intracellular saline contained no added Ca\(^{2+}\). Upon exchange to Ba\(^{2+}\), the \(P_o\) decreases and remains unchanged regardless of the concentration of subsequently applied Ba\(^{2+}\). \(B\), summary of mean cation channel \(P_o\) after Ba\(^{2+}\) perfusion compared to that of Ca\(^{2+}\). \(P_o\) was normalized to \(P_o\) at 300 \(\mu\)M Ca\(^{2+}\) (perfused onto each patch at the start of the experiment prior to the introduction of Ba\(^{2+}\) \((G, n = 6)\). The points could only be fitted with a linear regression function. For comparison, the concentration–response curve for Ca\(^{2+}\) from Fig. 1B is replotted here (●).

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it did not reduce \(P_o\) during perfusion with intracellular saline containing 300 \(\mu\)M Ca\(^{2+}\) \((n = 5)\) (Fig. 5E). Due to its ineffectiveness at 300 \(\mu\)M Ca\(^{2+}\), W-5 was not tested with intracellular saline containing 1 \(\mu\)M or 10 \(\mu\)M Ca\(^{2+}\).

A synthetic peptide inhibitor of calmodulin, known as CBD and corresponding to residues 290–309 of the Ca\(^{2+}\)–calmodulin-dependent kinase regulatory domain, is highly effective at impairing calmodulin-mediated Ca\(^{2+}\) processes (Payne et al. 1988). This peptide is a very specific calmodulin antagonist and a reliable indicator of calmodulin being involved in a process. The cytoplasmic face of excised, inside-out patches containing Ca\(^{2+}\)–cation channels was exposed to 50 \(\mu\)M CBD added to intracellular saline containing 1 \(\mu\)M, 10 \(\mu\)M and 300 \(\mu\)M Ca\(^{2+}\) \((n = 7, 6 \text{ and } 6)\) (Fig. 6A–C). CBD was manually added to the bath and allowed to diffuse for 1 min prior to a 5 min recording period. For controls, water (the vehicle) was initially employed, and it produced no change in cation channel activity \((n = 2, \text{ data not shown})\). Subsequently, timed controls were used for analysis and can be described

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**Figure 4. Extracellular Ba\(^{2+}\) decreases cation channel \(P_o\) and increases amplitude**

A, sample traces recorded from an excised, outside-out patch held at –60 mV. The cytoplasmic face is exposed to intracellular saline (in the pipette) containing 300 \(\mu\)M Ca\(^{2+}\), and upon excision the extracellular face is perfused with ASW (in the bath) containing 11 \(\mu\)M Ca\(^{2+}\) or 11 \(\mu\)M Ba\(^{2+}\). Upper panel, replacement of Ca\(^{2+}\) with Ba\(^{2+}\) at the extracellular face results in both an increased channel amplitude and a lowering of \(P_o\). The closed and two open current levels are indicated by dotted lines. The 2 s time base applies. Lower panel, 100 ms portion of cation channel activity during both Ca\(^{2+}\) and Ba\(^{2+}\) perfusion taken from the upper panel at times indicated by arrows. The dotted lines again show the increase in current amplitude when Ba\(^{2+}\) replaces Ca\(^{2+}\). The 25 ms time base applies.

B, all-points histograms from the outside-out patch shown in A. The closed (C) and two open (\(O_1, O_2\)) current levels are denoted just to the right of their corresponding peaks. An increase in current amplitude with Ba\(^{2+}\) versus Ca\(^{2+}\) application is evident by the left shift in both of the open state peaks. \(C\), summary of Ba\(^{2+}\)-induced percentage changes in \(P_o\) and current amplitude from outside-out patches. Ba\(^{2+}\)-induced percentage changes are compared to a mean of zero using a two-tailed, one-sample \(t\) test. When recorded with intracellular saline containing either 1 \(\mu\)M or 300 \(\mu\)M Ca\(^{2+}\), the \(P_o\) and current amplitude (amp) are significantly reduced and enhanced, respectively, by the perfusion of Ba\(^{2+}\) (*\(P < 0.05\) or **\(P < 0.01\)). For this and all subsequent bar graphs, the \(n\) values are given within, just below, or just above a given bar.
as the percentage change in cation channel activity between the first and second half of the control period prior to the addition of CBD. Compared to timed controls, 50 μM CBD caused a significant decline in cation channel $P_o$ of $\sim$20%, 40%, and 65% in 1 μM, 10 μM, and 300 μM Ca$^{2+}$, respectively (Fig. 6D). Collectively, the antagonizing effects of pharmacological blockers of calmodulin support the role of calmodulin in Ca$^{2+}$-dependent cation channel activation.

**Purified bovine calmodulin increases cation channel activity**

The application of exogenous calmodulin to Ca$^{2+}$-sensitive ion channels is a widely practised method to assess possible modulation by this protein (Liu et al. 1994; Zhang et al. 1998; Krupp et al. 1999; Zuhlke et al. 1999; Rycroft & Gibb, 2004; Bradley et al. 2004). Excised, inside-out patches containing cation channels were bathed in 10 μM intracellular Ca$^{2+}$ and exposed to 3 μM of either intact or boiled/denatured purified bovine calmodulin. Calmodulin was used in the presence of a Ca$^{2+}$ concentration that led to a 50% activation of the cation channel, i.e. 10 μM. Exogenous calmodulin caused a significant, $\sim$60% increase in cation channel $P_o$ (Fig. 7A and C), whereas when the protein was heat-inactivated it induced a moderate, $\sim$20% reduction in $P_o$ (Fig. 7B and C). Overall, 10 μM Ca$^{2+}$ was sufficient to activate 3 μM of added calmodulin to cause an increase in bag cell neurone cation channel activity.

**Discussion**

Ca$^{2+}$-activated, non-selective cation channels are essential to the control of neuronal excitability and the generation of depolarizing afterpotentials, plateau potentials, or bursting (Kramer & Zucker, 1985; Swandulla & Lux, 1985; Partridge & Swandulla, 1988; Partridge et al. 1994; Zhang et al. 1995; Congar et al. 1997; Haj-Dahmene & Andrade, 1997; Rekling & Feldman, 1997; Morisset & Nagy, 1999; Egorov et al. 2002). In *Aplysia* bag cell neurones, a cation channel provides the depolarizing drive for an afterdischarge that triggers egg-laying behaviour (Conn & Kaczmarek, 1989; Wilson et al. 1996). A prior report found that elevating cytoplasmic face Ca$^{2+}$ from

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1 μM or 10 μM Ca$^{2+}$ ($P < 0.05$). An ANOVA followed by Dunnett’s multiple comparisons test shows that 10 and 30 μM calmidazolium produce significant and concentration-dependent decreases in $P_o$ in the presence of 300 μM Ca$^{2+}$ ($P < 0.05$ or $**P < 0.01$). A test for linear trend amoung the 300 μM Ca$^{2+}$ points also shows that there is a significant linear trend, i.e. a negative slope with increasing calmidazolium concentration ($P < 0.005$). In 300 μM Ca$^{2+}$, a one-tailed, unpaired t test finds no significant (n.s.) difference between W-5-treated patches and ethanol controls.
100 nm to 1 μM increased channel activity threefold (Wilson et al. 1996). We now show that the cation channel is indeed Ca$^{2+}$ activated, through what appears to be a close, physical association with calmodulin.

Ca$^{2+}$ activation is a defining characteristic of many cation channels. First described in cardiac Purkinje fibre (Kass et al. 1978), this process has been studied at the single-channel level in various cells, including cardiomyocytes (Colquhoun et al. 1981; Guinamard et al. 2004), epithelia (Miyashita et al. 2001), kidney cells (Gonzalez-Perrett et al. 2002), hair cells (Van den Abbeele et al. 1994) neuroblastoma (Yellen, 1982), sensory neurones (Razani-Boroujerdi & Partridge, 1993; Cho et al. 2003; Liman, 2003) and Helix neurones (Partridge & Swandulla, 1987). Ca$^{2+}$ activation of the bag cell neurone channel has an $EC_{50}$ of 10 μM and peaks at 300 μM. Although cytoplasmic Ca$^{2+}$ rarely exceeds 1 μM in spiking bag cell neurones (Fisher et al. 1994; Magoski et al. 2000), evidence from squid shows that, at the plasma membrane, the concentration in a Ca$^{2+}$ channel microdomain can be 200–300 μM (Llinas et al. 1992). Also, depending on the distance from the pore, the nanodomain for single Ca$^{2+}$ channels is 5–50 μM (Smith & Augustine, 1988; Neher, 1998). Thus, Ca$^{2+}$ channels opened during the after-discharge would provide a substantial, near-membrane concentration of Ca$^{2+}$ to the cation channel. In addition, release from intracellular stores would be a source of Ca$^{2+}$ for cation channel activation (Fink et al. 1988; Magoski et al. 2000).

Both Wilson et al. (1996) and the present study provide evidence that the bag cell neurone cation channel is Ca$^{2+}$ permeable. We also now show that substituting Ba$^{2+}$ for Ca$^{2+}$ as an extracellular charge carrier decreased channel $P_e$. Given that Ba$^{2+}$ activates the channel poorly, this suggests that Ca$^{2+}$ influx through the cation channel itself provides a degree of stimulation. In general, for those Ca$^{2+}$-activated, non-selective cation channels that are Ca$^{2+}$ permeable, the intrinsically higher Ca$^{2+}$ concentrations at the channel mouth may act as a regulator (Lan et al. 1996; Zitt et al. 1997; Strubing et al. 2001; Lambers et al. 2004). The result of Ba$^{2+}$ permeation reducing bag cell neurone cation channel activity is, to our knowledge, some of the first evidence supporting such a mechanism. Conversely, a number of additional cation channels, such as TRPM4 and TRPM5, show little or no Ca$^{2+}$ permeability and require other sources of Ca$^{2+}$ influx or release for activation (Yellen, 1982; Chen & Simard, 2001; Miyashita et al. 2001; Launay et al. 2002; Cho et al. 2003; Liman, 2003; Prawitt et al. 2003; Guinamard et al. 2004).

Comparing the $EC_{50}$ and Hill coefficient values of the bag cell neurone channel (10 μM and 0.66) to other cation channels reveals both similarities and differences, e.g. 460 nm and 0.49 in chick sensory neurones (Razani-Boroujerdi & Partridge, 1993), 510 μM and 2.1 in vomeronasal neurones (Liman, 2003), 774 μM and 0.97 in rat sensory neurones (Cho et al. 2003), or $\sim$10 μM in cochlear hair cells (Van den Abbeele et al. 1994). The $EC_{50}$ of the non-neuronal TRPM4b has been reported as 400 nm (Launay et al. 2002) or 15 μM (Nilius et al. 2005), while TRPM5, which probably plays a role in taste

Figure 6. Calmodulin-binding domain inhibitor peptide (CBD) decreases cation channel $P_e$

A–C, sample traces of cation channel activity, before and after addition of 50 μM CBD, recorded from excised, inside-out patches held at −60 mV and bathed with intracellular saline containing 1 μM, 10 μM, or 300 μM Ca$^{2+}$. In each case, the delivery of CBD markedly lowers the $P_e$. D, a summary of the mean percentage change in $P_e$ between timed controls and CBD-treated preparations. Timed controls are expressed as the percentage change between the first and second half of the control period. Comparisons using a one-tailed, paired t test show that the reduction in $P_e$ during the CBD treatment period produces means that are significantly different from timed controls at 1 μM or 10 μM Ca$^{2+}$ (*$P < 0.05$) and 300 μM Ca$^{2+}$ (**$P < 0.01$).
transduction, has an EC\textsubscript{50} of either 840 nM or 21 \mu M, with a Hill coefficient of either 5.0 or 2.4 (Prawitt et al. 2003; Liu & Liman, 2003). A significant proportion of these EC\textsubscript{50} values are, like the bag cell neurone cation channel, in the micromolar range. In addition, the Hill coefficient of less than one for either the bag cell or chick sensory neurone channels suggests negative cooperativity. Perhaps initial Ca\textsuperscript{2+} binding lowers the affinity for additional binding, or Ca\textsuperscript{2+} may partially block the channels at higher concentrations. A Hill coefficient of 1.0, such as for rat sensory neurones, indicates competitive Ca\textsuperscript{2+} binding to a single site on the channel. Regarding the affinity of Ca\textsuperscript{2+} for calmodulin alone, in the absence of an effector protein such as a channel, Haiech et al. (1981) reported EC\textsubscript{50}s of \(~4\) and \(~7\) \mu M in 100 and 200 mM KCl, respectively, for \({}^{45}\text{Ca}^{2+}\) binding to sheep calmodulin.

Some cation channels are voltage dependent, including those from cardiomyocytes (Guinamard et al. 2004), epithelia (Miyashita et al. 2001), superchiasmatic neurones (Kononenko et al. 2004), pyramidal neurones (Alzheimer, 1994), and vomeronasal neurones (Liman, 2003). For the bag cell neurone cation channel, activity is steadily enhanced at potentials positive to \(-60\) mV; moreover, when Ca\textsuperscript{2+} is increased from 100 nM to 10 \mu M or 300 \mu M, the V\textsubscript{0.5} shifts from \(-12\) mV to \(-30\) mV, with little change in k (\(~16\) throughout). This is consistent with Wilson et al. (1996), who intimated that the P\textsubscript{o} versus voltage curve shifted to the left when Ca\textsuperscript{2+} was increased from 100 nM to 1 \mu M. An interdependence of Ca\textsuperscript{2+} and voltage activation has also been observed for cation channels from vomeronasal neurones (Liman, 2003), astrocytes (Chen & Simard, 2001), and endothelia (Csanady & Adam-Vizi, 2003), although for TRPM4b the V\textsubscript{0.5} and k (+32 mV and 9) do not change overall when Ca\textsuperscript{2+} is altered (Nilius et al. 2003). In the case of the bag cell neurone cation channel, Ca\textsuperscript{2+} may not only act as a ligand by increasing P\textsubscript{o} in a concentration-dependent manner, but could further augment activity by moving voltage dependence to more hyperpolarized potentials. During the afterdischarge, action potential firing occurs from a membrane potential of \(~20\) to \(~40\) mV (Conn & Kaczmarek, 1989), a range that readily accommodates the V\textsubscript{0.5} (\(~30\) mV) of the cation channel in high Ca\textsuperscript{2+}.

However, such postulations are made knowing that the high-Ca\textsuperscript{2+}-induced shift may be ostensible, given that P\textsubscript{o} values in the present study were determined only for a limited range of voltages.

If cation channel Ca\textsuperscript{2+} activation is due to a channel-associated Ca\textsuperscript{2+} sensor, rather than Ca\textsuperscript{2+} binding directly to the channel, calmodulin is a good candidate. A ubiquitous Ca\textsuperscript{2+} sensor, calmodulin, mediates the Ca\textsuperscript{2+}-dependent properties of numerous ion channels, including gating of small- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Xia et al. 1998; Levitan, 1999), activation of ryanodine receptors (Saimi & Kung, 2002), inactivation and facilitation of L-type Ca\textsuperscript{2+} channels (Zuhlke et al. 1999), as well as inhibition of CNG channels (Liu et al. 1994; Bradley et al. 2004), NMDA receptors (Zhang et al. 1998; Rycroft & Gibb, 2004), and IP\textsubscript{3} receptors (Michikawa et al. 1999). For the bag cell neurone cation channel, the Po shows no concentration response to Ba\textsuperscript{2+}, a metal that binds/activates calmodulin poorly (Haiech et al. 1981; Chao et al. 1984; Wang, 1985; Ozawa et al. 1999). Thus, as with Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels

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**Figure 7. Exogenous calmodulin increases cation channel P\textsubscript{o}**

A, sample traces of cation channel activity recorded from an excised, inside-out patch held at \(-60\) mV and bathed in intracellular saline containing 10 \mu M Ca\textsuperscript{2+}, before and after addition of 3 \mu M purified bovine calmodulin. The introduction of calmodulin robustly elevates cation channel P\textsubscript{o}. B, sample traces of cation channel activity recorded from an excised, inside-out patch held at \(-60\) mV and bathed in intracellular saline containing 10 \mu M Ca\textsuperscript{2+} before and after addition of 3 \mu M boiled calmodulin. Heat inactivation renders calmodulin modestly inhibitory. C, a comparison of the mean percentage change in P\textsubscript{o} between excised inside-out patches treated with 3 \mu M calmodulin versus boiled calmodulin. A two-tailed, unpaired t test shows that the increase in cation channel P\textsubscript{o} is significantly different following calmodulin administration compared to patches treated with boiled protein (*P < 0.05).

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(Cao & Houamed, 1999) and Ca\(^{2+}\) channels (Zuhlke et al., 1999), the failure of Ba\(^{2+}\) to mimic Ca\(^{2+}\) suggests that calmodulin may be the cation channel Ca\(^{2+}\) sensor.

Calmodulin inhibitors have also been used to implicate calmodulin in channel regulation, e.g. 5–100 \(\mu\)M camidazolium inhibits Ca\(^{2+}\)-dependent facilitation and inactivation of Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (Moreau et al., 2005), as well as the Ca\(^{2+}\) sensitivity of a Na\(^{+}\)-activated cation channel in lobster olfactory neurones (Bobkov & Ache, 2003) and the Drosophila TRPL cation channel expressed in oocytes (Lan et al., 1996). Moreover, 10 \(\mu\)M camidazolium strongly inhibits both Aplysia CNS Ca\(^{2+}\)-calmodulin-dependent kinase and the ability of bag cell neurones to fire an afterdischarge (DeRiemer et al., 1984, 1985). Depending on the added calmodulin concentration, camidazolium maximally inhibits brain phosphodiesterase and blood cell Ca\(^{2+}\)-ATPase in the range of 100 nM to 3 \(\mu\)M (Van Belle, 1981). For phosphorylase kinase, a protein that incorporates calmodulin as a subunit, maximal inhibition requires more than 10 \(\mu\)M (Van Belle, 1981). Cation channel activity after exposure to 10 \(\mu\)M camidazolium in 1 \(\mu\)M, 10 \(\mu\)M and 300 \(\mu\)M Ca\(^{2+}\), was reminiscent of that in 100 nM Ca\(^{2+}\). Regarding CBD, it was first shown to inhibit Ca\(^{2+}\)-calmodulin-dependent kinase II and phosphodiesterase by preventing calmodulin–enzyme binding (Payne et al., 1988). In addition, 20–25 \(\mu\)M CBD inhibits Ca\(^{2+}\)-dependent inactivation of NMDA (Krupp et al., 1999) or IP\(_3\) receptors (Michikawa et al., 1999). Accordingly, we found that 50 \(\mu\)M CBD reduced bag cell neurone cation channel activity at 1 \(\mu\)M, 10 \(\mu\)M and 300 \(\mu\)M Ca\(^{2+}\). This again indicates that, rather than Ca\(^{2+}\) binding directly to the channel, closely associated calmodulin acts as a Ca\(^{2+}\) sensor.

A role for calmodulin in cation channel Ca\(^{2+}\) sensitivity was further confirmed by our observation that 3 \(\mu\)M bovine calmodulin, in 10 \(\mu\)M Ca\(^{2+}\), increased \(P_o\). Bovine calmodulin readily activates various Aplysia proteins, including Ca\(^{2+}\)-calmodulin-dependent kinase (\(~500\) nM calmodulin with 500 \(\mu\)M Ca\(^{2+}\)) (DeRiemer et al., 1984), adenylyl cyclase (3 \(\mu\)M calmodulin with 3 \(\mu\)M Ca\(^{2+}\)) (Abrams et al., 1991), and twitchin (100 nM calmodulin with 1 mM Ca\(^{2+}\)) (Heierhorst et al., 1994). The effectiveness of exogenous calmodulin appears to depend on the combined protein and Ca\(^{2+}\) concentration. This is also apparent for exogenous calmodulin–mediated inhibition of CNG channels (500 nM calmodulin with 100 nM Ca\(^{2+}\)) (Bradley et al., 2004), NMDA receptors (100 nM calmodulin with 100 \(\mu\)M Ca\(^{2+}\)) (Krupp et al., 1999), and IP\(_3\) receptors (20 \(\mu\)M calmodulin with 200 \(\mu\)M Ca\(^{2+}\)) (Michikawa et al., 1999), as well as enhancement of non-neuronal TRPM4b (10 \(\mu\)M calmodulin with 100 \(\mu\)M Ca\(^{2+}\)) (Nilius et al., 2005) and TRPM5 (10 \(\mu\)M calmodulin with 5–7 \(\mu\)M Ca\(^{2+}\)) (Ordaz et al., 2005). Interestingly, when any of the TRPM2, TRPM4b, TRPV5, or TRPV6

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**Figure 8. A complex of interchanging proteins regulates the cation channel during the afterdischarge**

A, at rest, calmodulin (CaM) is associated with the cation channel, but activity is minimal due in the presence of low intracellular Ca\(^{2+}\). Channel \(P_o\) is indicated by the width of the ‘pore’ in the illustration. B, following synaptic stimulation, an increase in intracellular Ca\(^{2+}\) activates the cation channel over the short term, as the afterdischarge begins (Wilson et al., 1996; present study). C, later in the afterdischarge, Ca\(^{2+}\) continues to activate the cation channel along with maintained upregulation provided by the simultaneous association of stimulatory PKC (Wilson et al., 1998; Magoski et al., 2002). D, upon termination of the afterdischarge, intracellular Ca\(^{2+}\) falls with the cessation of action potential firing. Either as a consequence of refractoriness, or as a mechanism contributing to refractoriness, inhibitory PKA is exchanged with PKC to downregulate the cation channel (Magoski, 2004; Magoski & Kaczmarek, 2005). During the refractory period, cation channel activity and bag cell neurone excitability are maintained at lowered levels, thus preventing the generation of additional afterdischarges which could disrupt ongoing egg-laying behaviour.
cation channels were coexpressed with a calmodulin mutant whose Ca²⁺-binding sites are impaired, the current was decreased (Lammers et al. 2004; Nilius et al. 2005; Tong et al. 2006). For both the bag cell neurone cation channel and others, exogenous calmodulin may displace endogenous calmodulin and/or bind to unoccupied calmodulin-binding sites.

In the bag cell neurones, intracellular Ca²⁺ rises rapidly upon synaptic stimulation, possibly due to release from intracellular stores (Fink et al. 1988). When the afterdischarge begins, there is a second elevation due to Ca²⁺ that are in a complex with the cation channel. In addition to Ca²⁺ activation, the P₀ of the cation channel is also increased by closely associated protein kinase C (PKC) (Wilson et al. 1998; Magoski et al. 2002) or decreased by closely associated protein kinase A (PKA) (Magoski, 2004). These kinases can reconfigure depending on bag cell neurone excitability or activity levels. During the afterdischarge, stimulatory PKC is channel associated, while inhibitory PKA is present through the refractory period (Magoski & Kaczmarek, 2005). Calmodulin, which presumably is always present, would allow Ca²⁺ to influence cation channel activity in a graded fashion during the afterdischarge, with PKC maintaining spiking, and PKA then contributing to refractoriness (Fig. 8). Overall, this complex of regulatory proteins determines cation channel function and fundamentally affects species propagation.

In summary, we provide the first evidence that Ca²⁺ activation of a native, neuronal cation channel is mediated by closely associated calmodulin. Given the similarities between the bag cell neurone channel and other cation channels, calmodulin probably mediates Ca²⁺ sensitivity in many versions of this conductance. This crucial aspect of gating is central to the influence of these channels over neuronal excitability, activity and behaviour.

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