Cyclic AMP prevents activation of a swelling-induced chloride-sensitive conductance in chick heart cells

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1. Changes in myocardial cell volume and whole-cell currents were measured simultaneously during hyposmotically induced cell swelling. In the conventional patch clamp configuration, hyposmotic challenge caused myocytes to swell continuously and was associated with the development of a sustained, swelling-induced chloride conductance (I_{Cl}). In contrast, perforated patch-clamped myocytes demonstrated regulatory volume decreases (RVD) during hyposmotic challenge, and I_{Cl} was not generated.

2. The swelling-induced I_{Cl} in conventionally patch-clamped myocytes was inhibited by application of forskolin (15 μM) and was prevented when the pipette filling solution contained cAMP (10 μM) and isobutylmethylxanthine (IBMX, 1 mM). I_{Cl} could also be prevented by inhibition of protein phosphatase activity, using okadaic acid (100 nM). Conversely, a swelling-induced current could be generated in myocytes under perforated patch clamp by inhibition of protein kinase A, using the antagonist Rp-cAMPS (10 μM). These data demonstrate that cAMP-dependent protein phosphorylation is both necessary and sufficient to prevent development of I_{Cl} during cell swelling.

3. Unlike other chloride currents described previously in heart muscle, generation of the novel swelling-induced I_{Cl} requires dephosphorylation of a cAMP-dependent protein phosphorylation site; hence it can be prevented by stimulation of cAMP-dependent protein phosphorylation or by inhibition of protein phosphatase activity.

Conventional whole-cell voltage clamp experiments in cardiac cells have identified and characterized a Cl^- current which is activated during hyposmotic swelling (Sorota, 1992; Tseng, 1992; Zhang, Rasmusson, Hall & Lieberman, 1993), and a similar current has been recorded in the same cells during isosmotic swelling (positive pressure injection; Hagiwara, Masuda, Shoda & Irisawa, 1992; Zhang & Lieberman, 1993). However, it is difficult to interpret any putative role for this swelling-induced Cl^- current in the maintenance and regulation of cell volume from such experiments, because the regulatory processes underlying volume homeostasis appear to be compromised using the conventional whole-cell patch clamp configuration. In such experiments, cells fail to demonstrate the classic regulatory volume decrease (RVD) otherwise observed in intact cardiac cells (Desai, Jacob, Lieberman & Rosenthal, 1986; Roos, 1986; Rasmusson, Davis & Lieberman, 1993) and the loss of volume regulation may be a consequence of the conventional whole-cell patch clamp technique (in which the composition of the sarcoplasm is effectively fixed by the buffering capacity of the pipette filling solution for physiological ions, and intracellular signals can be diluted in the relatively large volume of this solution). In this study, we have used the nystatin-perforated patch clamp technique to allow simultaneous measurement of volume and current responses with minimum disturbance of the intracellular milieu. This modification of the conventional whole-cell patch clamp technique allows electrical access to the cell interior with only limited dialysis of the cytoplasm (Horn & Marty, 1988). Monovalent ions pass freely through the nystatin pores, but divalent ions and soluble intracellular components (e.g. amino acids, energy-generating systems and second messengers) are not permeable and therefore remain undisturbed in the cytoplasm. Results from our experiments show that, under these more physiological recording conditions, activation of the swelling-induced Cl^- conductance is prevented. We have identified the mechanism preventing activation as an intracellular transduction pathway involving cAMP-dependent protein kinase A; this is the first demonstration of phosphorylation-mediated inhibition of a Cl^- current in heart. Furthermore, although the Cl^- current is absent in perforated patch-clamped cells, these cells do undergo volume regulation in response to hyposmotic challenge;

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hence our results also reveal that the swelling-induced Cl^- current cannot contribute to the regulation of cardiac cell volume. Preliminary results of this study have been presented in abstract form (Hall, Zhang & Lieberman, 1993).

METHODS

Cell preparation

Nine or eleven-day-old chick embryos were extirpated aseptically from the eggs and decapitated immediately; the hearts were rapidly removed and minced. Single heart cells were isolated by enzymatic dissociation in the absence of antibiotics, using the method described previously by Jacob, Lieberman & Liu (1987), with minor modifications. Briefly, the hearts were removed under sterile conditions, minced and serially digested in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (composition (mM): 137 NaCl, 0·4 Na_2HPO_4, 4·2 $NaHCO_3$, 5·4 KH_2PO_4, 5·4 KCl, 5·6 dextrose) containing 0·05% trypsin at 37 °C. The cell suspension was subsequently transferred to a standard modified saline G solution containing 10% fetal bovine serum (FBS), filtered through a 60 μm Nitex filter (Tetko Inc., New York, USA) then diluted in culture medium (60% medium 199, 5% FBS, 2% chick embryo extract, 33% Earle's balanced salt solution) to a final concentration of $0·3 \times 10^6$ or $0·5 \times 10^6$ cells per 35 mm petri dish. The preparations were incubated overnight at 37 °C. The culture dishes were transferred to a specially designed heated stage on an inverted microscope (Nikon Diaphot-TMD, Tokyo, Japan), and the cells were superfused at a rate of ~2 ml min^{-1} with a Hepes-buffered saline solution (HBSS, see below for composition) at 37 °C. Single spherical myocytes were used for electrophysiological recording and simultaneous monitoring of cell volume.

Electrophysiological recording

Patch pipettes were fabricated from borosilicate glass capillary tubing (7052, Garner Glass Co, Claremont, CA, USA) and were fire polished just prior to use. Pipette resistance was 5–10 MΩ when filled with pipette solution (see below for composition). Slight positive pressure was applied to the back of the pipette as it passed through the air–fluid interface; this pressure was then released before the pipette was aligned with a suitable cell and the pipette tip was brought into contact with the cell surface. Application of gentle negative pressure (−5 to −30 cmH_2O) promoted formation of a high resistance seal (> 10 GΩ) between the pipette and the membrane. For conventional whole-cell recording, a brief pulse of strong suction was applied to rupture the membrane beneath the pipette tip, allowing access to the cell interior. In the perforated patch configuration, the slight negative pressure was released after formation of the gigaohm seal, and the capacity transients associated with a 10 mV applied pulse were monitored over a period of 10 min, as the ionophore was incorporated into the patch of membrane beneath the pipette tip and the series resistance fell. At least 10 min was allowed following gigaohm seal formation, to achieve equilibration of Cl^- across the perforated patch (Korn, Bolden & Horn, 1991). Further details of the preparation of nystatin-containing solutions are given below.

Whole-cell currents were recorded using a Dagan 8900 patch clamp amplifier (Dagan Corp., Minneapolis, MN, USA). Currents were low-pass filtered at 2 kHz and stored digitally using an Axolab-1 data aquisition system (Axon Instruments, Inc. Burlingame, CA, USA). Voltage clamp protocols were generated using pCLAMP software (Version 5.5; Axon Instruments Inc.), and the same software package was used for acquisition and subsequent analysis of the data. The membrane potential was clamped at −40 mV, close to the membrane potential measured during cell swelling (Zhang *et al.* 1993). Currents were elicited using ramp voltage protocols. A depolarizing voltage ramp was first applied from the holding potential to +60 mV, to inactivate voltage-dependent Na^+ and Ca^{2+} currents, followed by a hyperpolarizing voltage ramp over the range +60 to −90 mV at a rate of −0·5 V s^{-1}. These voltage ramps were repeated at 10 or 15 s intervals to monitor the changes in whole-cell current over the course of the experiment. Cell membrane capacitance and the series resistance were not compensated. Cell membrane capacitance was estimated from the integral of the transient current response to a 5 mV hyperpolarizing clamp step, and all whole-cell currents were normalized to this value (5–10 pF). Series resistance (< 30 MΩ in all experiments) was estimated by fitting a single exponential function to the same transient current and dividing the time constant of this function by the cell membrane capacitance.

Cell volume measurement

Cell volume was determined by video microscopy, using the JAVA software system (Jandel Scientific, Corte Medera, CA, USA). The circumference of the cell was traced from its video image at 1 min intervals throughout the experiment, and mean cell diameter was determined from this measurement. No changes in cell diameter were observed after seal formation or during current recording under control isosmotic conditions. Cell volume was then calculated, assuming a spherical geometry. Changes in cell volume determined in this way have been shown to correlate well with measured changes in the cell water content (Rasmusson *et al.* 1993). Changes in cell volume were expressed relative to the control cell volume in isosmotic solution just prior to the hyposmotic intervention, so that each cell was its own control.

Solutions

Isosmotic HBSS had the following composition (mM): 121·5 NaCl, 21·5 sodium aspartate, 5·4 KCl, 0·8 NaH_2PO_4, 10 Hepes (pH 7·4), 0·8 $MgSO_4$, 1·0 $CaCl_2$, 5·6 dextrose. The final osmolarity of HBSS was ~300 mosmol l^{-1}, to prevent cell swelling on removal from the culture medium and during control electrophysiological recording. Solutions were rendered hyposmotic, without altering $[Cl^-]$, by omission of sodium aspartate to give a final osmolarity of ~270 mosmol l^{-1}. This degree of hyposmotic challenge gave consistent volume and current changes which were qualitatively similar to the responses generated by more severe hyposmotic challenges, without causing degeneration of the gigaohm seal often associated with cell swelling in more dilute solutions. K^+ currents were blocked by replacement of KCl with TEA-Cl, or by inclusion of 1 mM $BaCl_2$ in the solution; similar results were obtained in both cases. Solution osmolarities were measured using a vapour pressure osmometer (model 5100B, Wescor Inc., Logan, UT, USA).

The K^+-free pipette solution for whole-cell patch clamp recording contained (mM): 30 CsCl, 110 caesium aspartate, 2·0 $MgCl_2$, 0·5 $CaCl_2$, 1·0 EGTA, 10 Hepes (pH 7·2), 5·0 Na_2ATP. The same pipette solution, without added ATP, was used for the perforated patch experiments. Nystatin solution (5 mg in 0·1 ml dimethyl sulphoxide (DMSO)) was prepared fresh each week and stored at −4 °C. Nystatin (2 μl of the stock solution) was added to 0·5 ml filling solution and sonicated for 30 s to ensure complete dispersion of the nystatin; final concentration of nystatin was

~5000 units ml^{-1}. The maximum final concentration of DMSO in the pipette filling solution was 0·1%. This pipette filling solution was stored on ice and used within 3 h of preparation.

Drugs and reagents

Cyclic AMP was obtained from Sigma Chemical Co. and dissolved in distilled water to a stock concentration of 10 mM. Forskolin and dideoxyforskolin were also obtained from Sigma, and were dissolved in DMSO to a stock concentration of 50 mM. Sulphonated analogues of cAMP (Rp-cAMPS (Rp diasteriomer of adenosine 3′,5′-cyclic monophosphorothioate) and Rp-8-Br-cAMPS (Rp diasteriomer of 8-bromoadenosine 3′,5′-cyclic monophosphorothioate) were obtained from either Research Biochemicals Inc. (Natick, MA, USA) or Biolog, Life Sciences Institute (La Jolla, CA, USA) and were dissolved in water to a stock concentration of 10 mM. Okadaic acid was obtained from LC Laboratories (Woburn, MA, USA) and dissolved in DMSO to give a stock solution of 0·1 mM which was stored frozen at −20 °C. All other chemicals and reagents were obtained from Sigma.

Data analysis and statistics

All data are presented as digitized current records from single experiments or as the arithmetic mean ± S.E.M. (n), where n represents the number of cells. Statistical analysis was made using Student's t test (2-tailed) for paired or unpaired data where appropriate; the null hypothesis was rejected when $P < 0.05$.

RESULTS

Whole-cell current measurements during cell swelling

Embryonic chick cardiac myocytes have been shown to undergo volume regulation when the osmolarity of the extracellular solution is reduced (Desai *et al.* 1986; Roos, 1986; Rasmussen *et al.* 1993). However, this regulatory volume decrease (RVD) was not apparent under conventional whole-cell voltage clamp; rather, in this

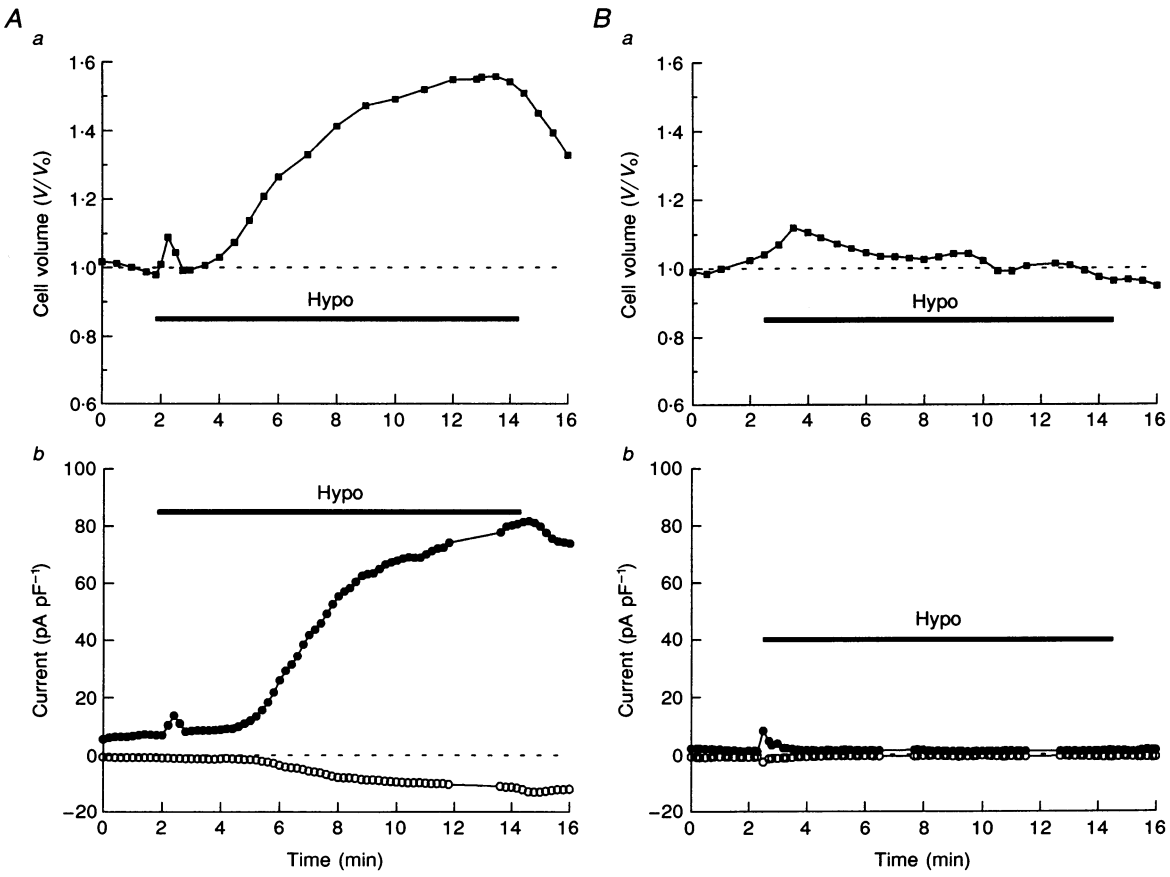

Figure 1. Volume and current responses during hyposmotic challenge are influenced by the patch clamp recording configuration

Changes in cell volume and whole-cell currents during hyposmotic challenge in cardiac myocytes under conventional whole-cell patch clamp (*A*) and nystatin-perforated patch clamp (*B*) are plotted as a function of time. Cell volume is expressed as a proportion of the volume under control isosmotic conditions (V/V_o, ■). Whole-cell current magnitude is normalized to the membrane capacitance (pA pF^{-1}) and measured at the holding potential (V_h, −40 mV, ○) and a test potential (V_t, +60 mV, ●). Temperature, 37 °C; K^+-free filling solution. Extracellular solution was rendered hyposmotic by omission of sodium aspartate (Hypo); [Cl^-] remained constant (control solution ~300 mosmol l^{-1}, hyposmotic solution ~270 mosmol l^{-1}; see Methods). These conventions apply to all figures and the responses illustrated are representative of typical responses observed in these experiments.

experimental configuration, myocytes continued to swell throughout the duration of the hyposmotic challenge (osmolarity reduced from 300 to 270 mosmol l^{-1}; Fig. 1*A a*). The possible explanations for this continued swelling in whole-cell voltage clamp experiments have been discussed previously (Zhang *et al.* 1993). Simultaneous measurements of the whole-cell current revealed that cell swelling initially activated a small, transient current 'spike' which was followed by a second, sustained phase of current activation appearing within 2–3 min (Fig. 1*A b*). The amplitude of this second current did not reach steady state but continued to increase in hyposmotic solution, reaching ~25 times control amplitude at +60 mV after 5–10 min. The second phase sustained current has been identified previously as a Cl^--selective current (I_{Cl}) (Zhang *et al.* 1993); this observation is consistent with the results of similar experiments in canine atrial and ventricular myocytes (Sorota, 1992; Tseng, 1992).

Since RVD was not apparent in the conventional voltage clamp experiments, we exploited the nystatin-perforated patch clamp technique to measure whole-cell currents with minimal disturbance to the sarcoplasm. These experiments addressed the possibility that intracellular signal transduction pathways are involved in modulating the swelling-induced current and the mechanisms underlying volume regulation. Parallel experiments revealed that the volume and current responses to cell swelling were influenced by the experimental configuration. With the perforated patch clamp, cells underwent volume regulation during the hyposmotic challenge (Fig. 1*B a*). Peak cell volume was 114 ± 6·2% of control ($n = 12$; $P < 0{\cdot}001$) and was reached after 1–3 min in hyposmotic solution. A pronounced RVD was apparent when cells were exposed to hyposmotic solution for > 3 min, indicating the activity of a volume regulatory process. This RVD was comparable to that observed in individual myocytes and spherical aggregates of cells not under voltage clamp of any kind (Desai *et al.* 1986; Roos, 1986; Rasmusson *et al.* 1993).

Simultaneous electrophysiological recording from the same cells showed that the small, transient current 'spike' (I_{swell}) was activated rapidly, but the second phase I_{Cl} was not observed ($n = 15$). The time course of the current response to hyposmotic challenge recorded from a single chick cardiac myocyte, with the perforated patch clamp, is shown in Fig. 1*B b*; the magnitude of the currents at +60 mV and −40 mV are plotted together with the changes in volume of the same cell. When the extracellular solution was rendered hyposmotic, I_{swell} was activated transiently; following the peak, current amplitude returned to control levels within 3 min and no further current developed while the cells remained in hyposmotic solution for up to 12 min. The slower onset of the large I_{Cl} associated with cell swelling in the conventional patch clamp experiments was absent in the perforated patch configuration and return to isosmotic solution had no further effect on basal currents ($n = 15$). The current–voltage relationships measured under the two patch clamp configurations are compared in Fig. 2; these currents were recorded during the experiments shown in Fig. 1. Under conventional whole-cell clamp, hyposmotic cell swelling is associated with generation of I_{Cl}; in this example, the reversal potential (E_{rev}) = −18 mV and the magnitude of the difference current (Hypo − Control) is ~70 pA pF^{-1} at +60 mV (Fig. 2*A*). Under perforated patch clamp, however, it is

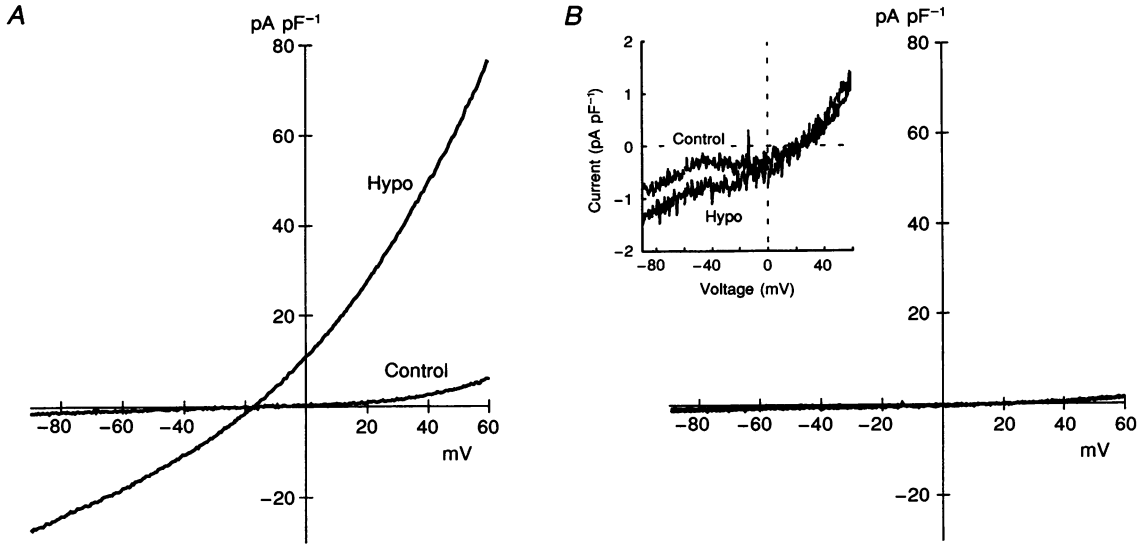

Figure 2. *I–V* relationships for the whole-cell currents under control conditions and during hyposmotic challenge recorded using conventional (*A*) and perforated patch clamp (*B*)

Current records were obtained during hyperpolarizing ramp changes in membrane potential in isosmotic solution (Control) and after 8 min in hyposmotic solution (Hypo); these *I–V* relationships are taken from the experiment shown in Fig. 1. The inset in panel *B* shows the same records on a magnified current scale.

apparent that after the initial 'spike', the I–V relationship of the current was very similar to the control I–V curve of the basal current (Fig. 2*B*). The time course and magnitude of the transient swelling-induced current measured with perforated patch was comparable to the initial current 'spike' that was routinely observed using conventional whole-cell recording prior to the onset of I_{Cl}; the nature of this transient I_{swell} is discussed further below.

Modulation of the swelling-induced I_{Cl}

The absence of I_{Cl} using the perforated patch clamp recording technique suggests that mechanisms in the intact cell limit or prevent activation of I_{Cl} during cell swelling. It is possible that such control mechanisms are attenuated or disrupted during cellular dialysis associated with conventional whole-cell clamp; loss of some second messenger component could explain the generation of I_{Cl} in these experiments. We investigated the possibility that the swelling-induced I_{Cl} is prevented in intact cells by a transduction pathway involving cAMP, since this pathway is known to modulate the activity of other Cl^- currents in heart cells (Bahinski, Nairn, Greengard & Gadsby, 1989).

The swelling-induced I_{Cl} measured in myocytes using the conventional whole-cell recording technique was attenuated by bath application of forskolin (15 μM) during hyposmotic challenge ($n = 6$, Fig. 3*A*), but cell swelling was unaffected. In all experiments using conventional whole-cell voltage clamp, the cells continued to swell throughout the hyposmotic challenge, despite interventions which altered the swelling-induced current. The swelling-activated I_{Cl} was also prevented or attenuated by application of forskolin prior to hyposmotic challenge, although this intervention had no effect on the basal current under control isosmotic conditions ($n = 4$). Forskolin has been shown previously to produce a significant increase in the cAMP content of the cells under both iso- and hyposmotic conditions (Zhang, Smith, Lobaugh, Hall & Lieberman,

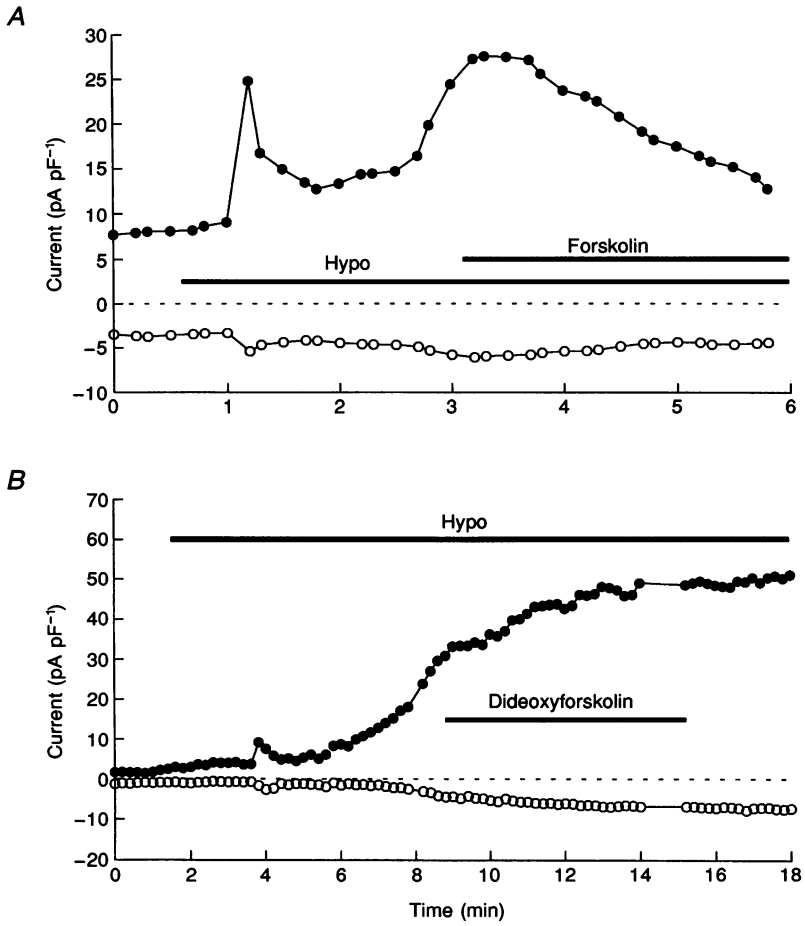

Figure 3. Forskolin inhibits the swelling-induced I_{Cl} by activation of adenylyl cyclase

The effects of forskolin (15 μM; *A*) and dideoxyforskolin (15 μM; *B*) on the swelling-activated I_{Cl} in myocytes under conventional whole-cell patch clamp are plotted against time during hyposmotic challenge. Forskolin and dideoxyforskolin were applied extracellularly in the hyposmotic bathing solution. Whole-cell currents were measured at +60 mV (V_t, ●) and −40 mV (V_h, ○). Temperature, 37 °C; K^+-free filling solution. As stated in the text, the myocyte volume continued to increase throughout the duration of the hyposmotic challenge, independent of the activity of I_{Cl}.

1992) and was associated with an increase in the magnitude of the voltage-dependent L-type Ca^{2+} current, which was fully and rapidly reversible on removal of forskolin. To ensure an effective concentration of cAMP in the cell, we used an apparent supramaximal concentration of forskolin (15 μM) to overcome the possible diluting effects of dialysis of cAMP into the pipette filling solution, together with the increase in cell volume during hyposmotic challenge. Dideoxyforskolin (15 μM), an analogue of forskolin which does not activate adenylyl cyclase, had no effect on the current activity when applied extracellularly during cell swelling ($n = 4$; Fig. 3*B*), indicating that inhibition of I_{Cl} was mediated by the generation of cAMP rather than a direct pharmacological action of forskolin on the ion channels.

Based on the observation that increasing [cAMP] by activation of adenylyl cyclase inhibited I_{Cl} associated with cell swelling, we propose that maintenance of sarcoplasmic [cAMP] by supplementation of the patch pipette filling solution should also prevent activation of I_{Cl} in dialysed cells. Figure 4 illustrates the time course of the current response in a conventional patch clamp experiment when the pipette filling solution contained cAMP (10 μM) and isobutylmethylxanthine (IBMX; 1 mM) to maintain sarcoplasmic cAMP levels. Under these conditions, the profile of the current response was the same as the current observed using the perforated patch technique, i.e. the initial transient I_{swell} was generated, but the second phase I_{Cl} did not develop during hyposmotic challenge (cf. Fig. 1*Bb*); this same response was observed in four out of four cells. These data demonstrate that the I_{Cl} associated with cardiac cell swelling can be inhibited by maintainance of sarcoplasmic [cAMP]. As stated above, cAMP-mediated inhibition of the swelling-induced I_{Cl} had no effect on the volume responses in all these experiments; the cells continued to swell throughout exposure to hyposmotic solution, regardless of the current activity.

Cyclic AMP acts as an important mediator in protein kinase A (PKA)-dependent protein phosphorylation by promoting dissociation of PKA into its catalytic and regulatory subunits; we proposed that a subsequent kinase-mediated phosphorylation step might be important in regulating the swelling-induced I_{Cl}. We tested this hypothesis by using diastereoisomers of sulphonated cAMP as antagonists of cAMP-dependent PKA activation. In perforated patch experiments, bath application of Rp-cAMPS (10 μM; $n = 5$) or its analogue Rp-8-Br-cAMPS (10 μM; $n = 2$) had no effect on the basal current, but revealed a swelling-induced current during the hyposmotic challenge. Figure 5*B* illustrates the time course of the whole-cell current response when activation of cAMP-dependent PKA was prevented by Rp-8-Br-cAMPS; the profile of the current response is very similar to that observed in conventional patch clamp experiments, i.e. the initial transient I_{swell} was followed by a second sustained phase of current activity (cf. Fig. 1*Ab*). The same results were obtained with both analogues; after 4–6 min hyposmotic challenge, the outward current at +60 mV was 572 ± 209% ($n = 7$, $P < 0{\cdot}05$) larger than under isosmotic conditions. Current amplitude returned to the control level on return to isosmotic solution, once Rp-cAMPS was removed from the extracellular solution. Furthermore, in the presence of Rp-cAMPS, cells continued to swell during hyposmotic challenge (Fig. 5*A*), i.e. volume regulation was compromised in the absence of PKA-dependent phosphorylation. Bath application of Rp-cAMPS

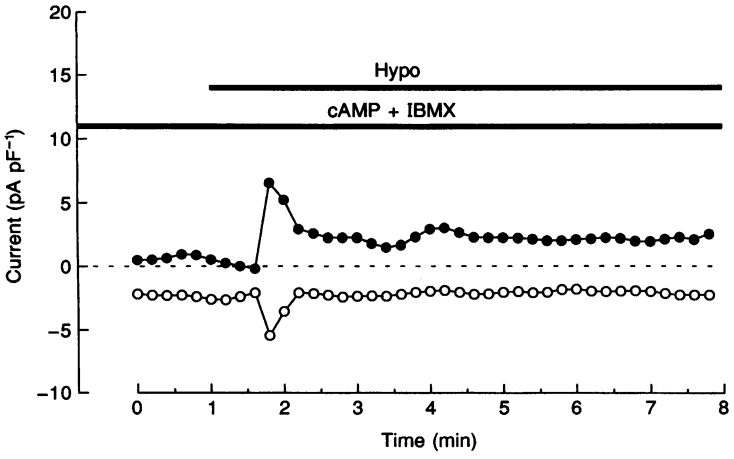

Figure 4. The swelling-induced I_{Cl} is prevented by inclusion of cAMP and IBMX in the patch pipette

The whole-cell current response with cAMP (10 μM) and IBMX (1 mM) in the patch pipette filling solution (conventional whole-cell patch clamp) is plotted against time during hyposmotic challenge. Whole-cell currents were measured at +60 mV (V_t, ●) and −40 mV (V_h, ○). Temperature, 37 °C; K^+-free filling solution. As stated in the text, the myocyte volume continued to increase throughout the duration of the hyposmotic challenge, despite inhibition of I_{Cl}.

also restored the current blocked by forskolin in dialysed cells ($n = 3$; Fig. 6), demonstrating that cAMP is acting through the same PKA pathway in these cells. None of the interventions used to alter the cAMP-mediated PKA activity (i.e. cAMP and IBMX, forskolin, Rp-cAMPS) had any significant effect on the basal currents under control conditions. This suggests a specific stimulus associated with cell swelling which can activate I_{Cl}, rather than simply a fall in [cAMP] or inhibition of PKA activity.

The current–voltage relationship of the current revealed during PKA inhibition is shown in Fig. 7 and the data indicate that this current may be the same as the I_{Cl} measured under conventional voltage clamp (cf. Fig. 2*A*). Like the swelling-induced I_{Cl} identified previously (Zhang *et al.* 1993), the current revealed by Rp-cAMPS showed outward rectification at the whole-cell level, but the current generated under these conditions reversed near 0 mV ($n = 5/7$). The positive shift of reversal potential away from the equilibrium potential for Cl^- (E_{Cl}) implies that the channels may not be exclusively selective for Cl^-, or that the Cl^--selective nature of the current may be emphasized when the ionic composition of the sarcoplasm is tightly controlled in conventional patch clamp experiments. Alternatively, I_{Cl} may be contaminated by another stretch-activated conductance in these cells (Bustamante, Ruknudin & Sachs, 1991). Removal of extracellular Cl^- (Cl^- salts replaced with methylsulphonate and SO_4^{2-} salts) caused attenuation of the current and E_{rev} shifted in the depolarizing direction ($n = 2$; Fig. 7), demonstrating that Cl^- is the predominant charge carrier for the swelling-induced current revealed by PKA inhibition.

The data indicate that PKA-mediated protein phosphorylation is essential to prevent the generation of I_{Cl} during cell swelling. We found that inclusion of okadaic acid (100 nM) in the pipette solution also prevented the swelling-induced I_{Cl} during a 10 min period of hyposmotic

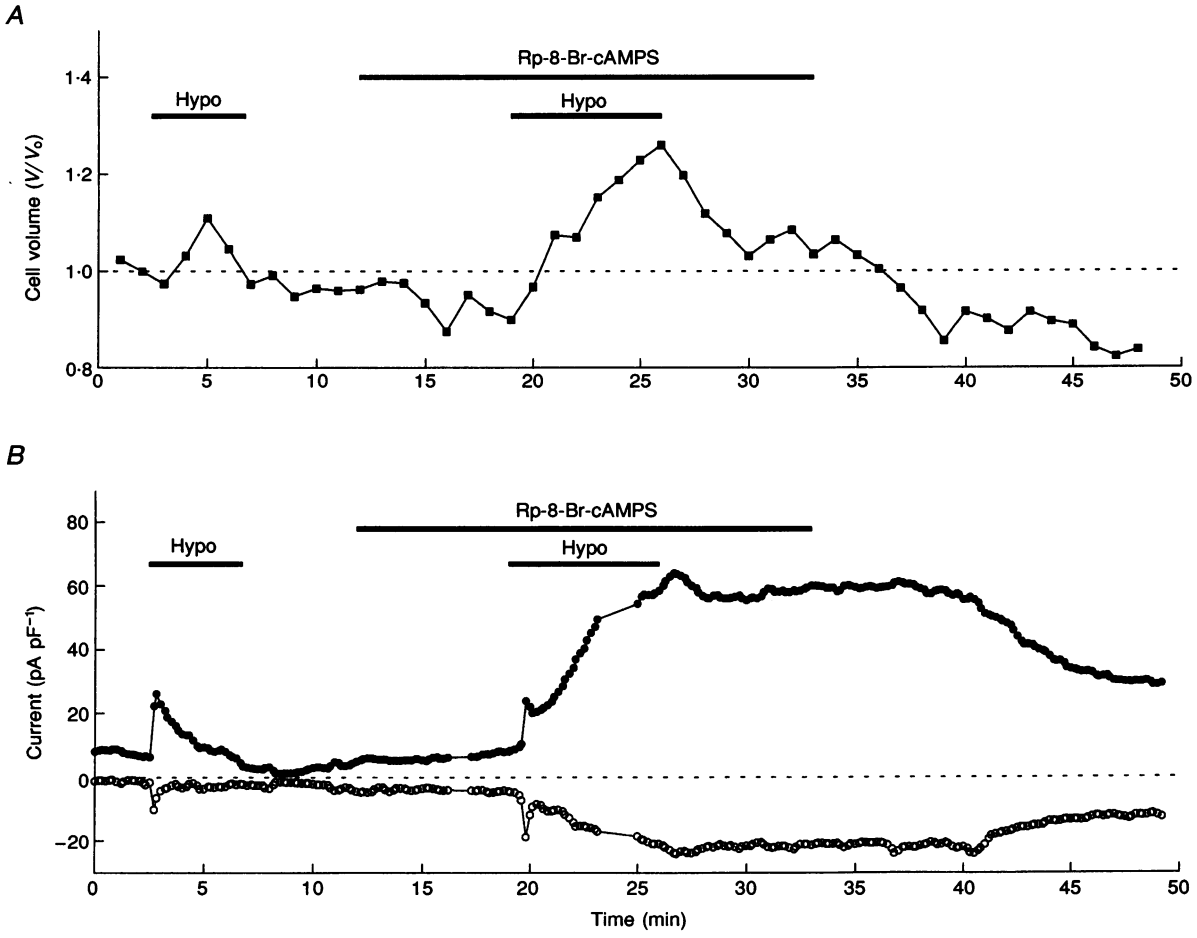

Figure 5. Inhibition of PKA activity reveals a swelling-induced current during hyposmotic challenge

The volume (*A*) and current (*B*) responses in a myocyte under perforated patch clamp are plotted against time during hyposmotic challenge, in the presence of 10 μM Rp-8-Br-cAMPS in the bathing solution. Cell volume is expressed as a proportion of the volume under control isosmotic conditions (■). Whole-cell currents were measured at +60 mV (V_t, ●) and −40 mV (V_h, ○). Temperature, 37 °C; K^+-free filling solution.

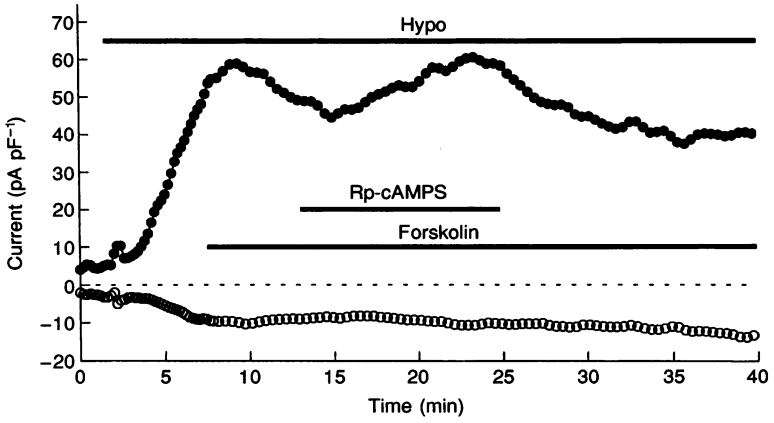

Figure 6. Rp-cAMPS restores the current blocked by forskolin

The whole-cell current response (conventional whole-cell patch clamp) is plotted against time during hyposmotic challenge, in the presence of forskolin (10 μM) and Rp-cAMPS (10 μM) in the bathing solution. Inhibition of I_{Cl} by forskolin is reversed by inhibition of PKA. Whole-cell currents were measured at +60 mV (V_t, ●) and −40 mV (V_h, ○). Temperature, 37 °C; K^+-free filling solution. As stated in the text, the myocyte continued to swell throughout the duration of the hyposmotic challenge.

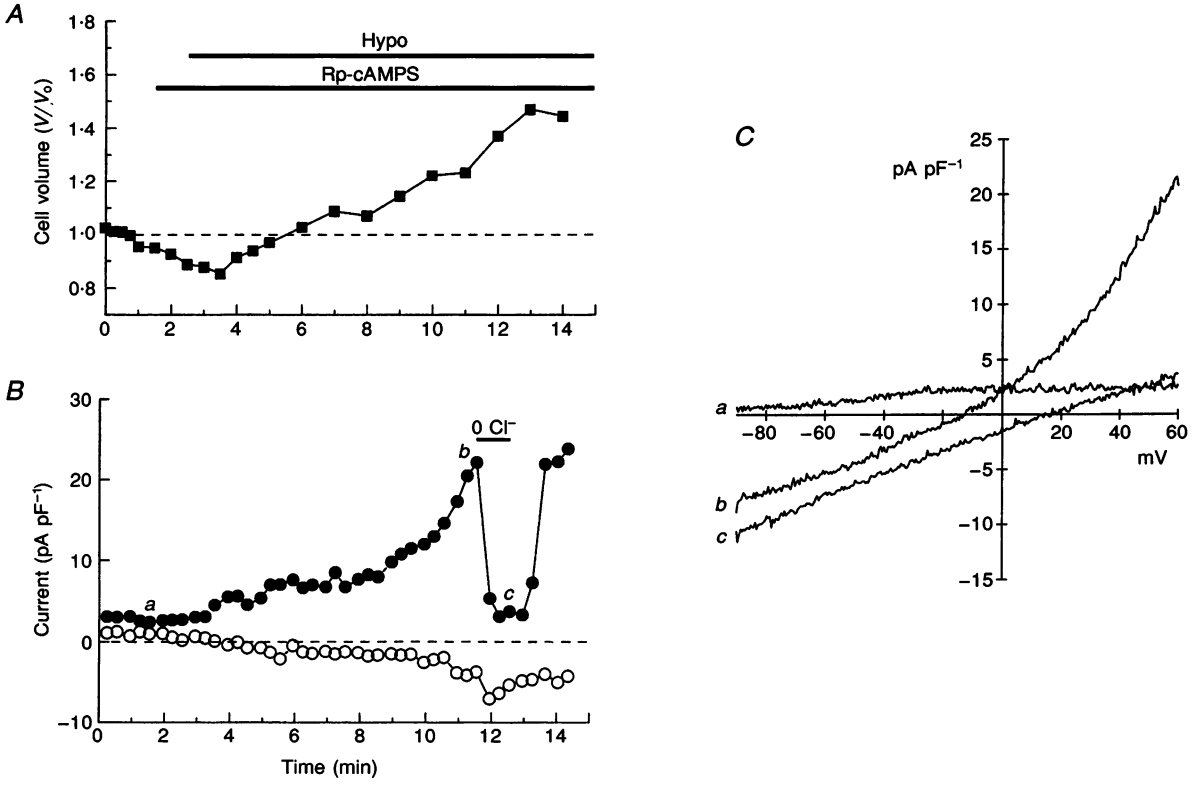

Figure 7. The swelling-induced current revealed during PKA inhibition is attenuated by removal of $[Cl^-]_o$

The volume (*A*) and current (*B*) responses during hyposmotic challenge in a myocyte (perforated patch) treated with 10 μM Rp-cAMPS in the bathing solution are plotted against time, while $[Cl^-]_o$ is removed. Cell volume is expressed as a proportion of the volume under control isosmotic conditions (■). Whole-cell current magnitude is normalized to the membrane capacitance (pA pF^{-1}) and measured at the holding potential (−40 mV, ○) and a test potential (+60 mV, ●). Temperature, 37 °C; K^+-free filling solution. *C*, the current–voltage relationship of the swelling-induced current revealed by Rp-cAMPS, in the presence and absence of $[Cl^-]_o$, recorded at the time points indicated in panel *B*. *a*, control + Rp-cAMPS; *b*, Hypo + Rp-cAMPS; *c*, 0 Cl^- Hypo + Rp-cAMPS.

challenge ($n = 4$; Fig. 8; cf. Fig. 1*Bb*). Okadaic acid (100 nM) is sufficient to block the activity of protein phosphatase IIa completely and attenuate protein phosphatase I activity (Cohen, Klumpp & Schelling, 1989); under these conditions, dephosphorylation is inhibited, and proteins are maintained in a phosphorylated state. Hence, our data suggest that the phosphorylation–dephosphorylation balance is disturbed during conventional patch clamp recording, either by attenuation of protein kinase activity or, alternatively, by activation of protein phosphatase activity.

The transient swelling-induced current

The characteristics of the initial transient swelling-induced current (I_{swell}) recorded using both patch clamp techniques were distinct from those of the later swelling-induced I_{Cl} measured in dialysed cells. In perforated patch experiments, the transient I_{swell} showed minor outward rectification at the whole-cell level and reversed at +15 mV ($n = 11/12$). The initial 'spike' current recorded from dialysed cells also reversed at +15 mV ($n = 6$), in comparison to the later current which reversed near the Cl^- equilibrium potential (−25 mV; Zhang *et al.* 1993). These findings, together with the observation that I_{swell} is not abolished in the absence of Cl^-_o (S. K. Hall, unpublished data), indicate that the initial I_{swell} probably represents a change in membrane conductance which is different from that involved in the generation of I_{Cl}. Since the peak of I_{swell} was observed before the peak of the volume increase, it is possible that this transient current acts as a signal in the cascade of events underlying the RVD; this intriguing hypothesis is currently under investigation.

DISCUSSION

Regulation of the swelling-induced I_{Cl}

Complementary use of the conventional and perforated patch clamp techniques revealed that the activity of the swelling-induced I_{Cl} can be prevented by a cAMP-dependent protein phosphorylation pathway involving PKA. Our data show that generation of I_{Cl} in conventional voltage clamp experiments is due to loss of regulation of the current by a cAMP-dependent signalling pathway. Dialysis of the sarcoplasm appears to remove some component of the second messenger pathway stimulating PKA activity. Although inhibition of the current is restored when intracellular [cAMP] is maintained, this does not necessarily mean that cAMP itself is dialysed out of the cell, only that some component necessary for its production has been removed. Furthermore, inhibition of protein phosphatases also prevents the generation of I_{Cl} in dialysed cells, and inhibition of PKA activity in intact cells is sufficient to prevent the inherent inhibition of I_{Cl} during cell swelling. These findings indicate a dynamic balance between kinase and phosphatase activity under physiological conditions, so that inhibition of the phosphorylation pathway allows dephosphorylation mechanisms to predominate, resulting in the generation of I_{Cl} during cell swelling. Disturbing the cAMP cascade *per se* is not sufficient to alter the basal time-independent currents; only the current generated during cell swelling is altered. These observations indicate that some event associated with cell swelling is a prerequisite for activation of I_{Cl}, rather than simply a fall in intracellular [cAMP] as the cell swells. Our hypothesis suggests that this swelling stimulus, or a transduction mechanism associated with the primary swelling event, is

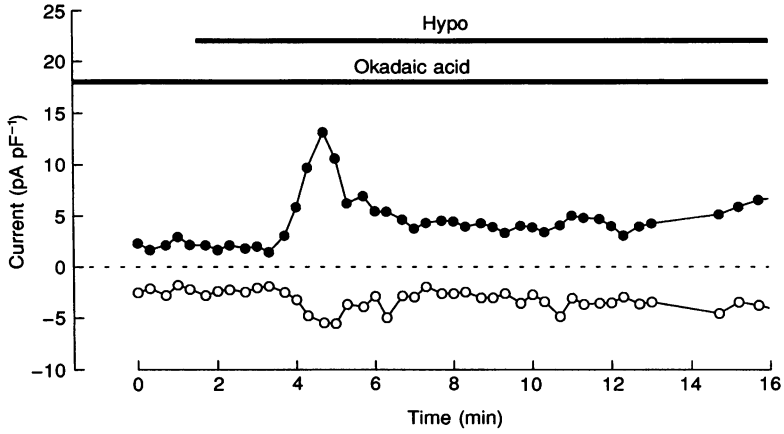

Figure 8. Okadaic acid prevents the generation of the swelling-induced I_{Cl}

The whole-cell current response with okadaic acid (100 nM) in the patch pipette filling solution (conventional whole-cell patch clamp) is plotted against time, during hyposmotic challenge. Whole-cell currents were measured at +60 mV (V_t, ●) and −40 mV (V_h, ○). Temperature, 37 °C; K^+-free filling solution. As stated in the text, the myocyte continued to swell throughout the duration of the hyposmotic challenge in this experimental configuration, despite inhibition of I_{Cl}.

effective only when a protein involved in activation of the Cl^- current is in a dephosphorylated state; this protein may be the Cl^- channel itself.

In intact cells, PKA-mediated phosphorylation prevents activation of I_{Cl} during cell swelling; thus the evidence supports a current which is maintained in an inactive state by a cAMP-dependent pathway. Electrophysiological and molecular biological studies have revealed several distinct populations of Cl^- channels in cardiac muscle (for review see Ackerman & Clapham, 1993). In some cases, specific signalling mechanisms have been associated with channel activity, although the exact channel identity remains obscure. Experiments have described Cl^- currents which are activated by cAMP-dependent protein kinase A (Harvey & Hume, 1989; Bahinski *et al.* 1989; Matsuoka *et al.* 1990; Hume & Harvey, 1991), protein kinase C (Walsh, 1991), intracellular Ca^{2+} (Zygmunt & Gibbons, 1991, 1992) and purinergic agonists such as ATP (Matsuura & Ehara, 1992). The swelling-induced Cl^- current appears functionally distinct from other cardiac Cl^- currents, since it is activated only when cAMP-dependent PKA activity is compromised, i.e. generation of this novel current requires dephosphorylation of a cAMP-dependent protein phosphorylation site. Although a distinct Cl^- current activated by cAMP has been well documented (e.g. Liu, Stimers & Lieberman, 1994), no such currents were apparent under the conditions of our experiments. Furthermore, the cAMP-stimulated I_{Cl} and the swelling-induced I_{Cl} display distinct electrophysiological and pharmacological characteristics (Vandenberg, Yoshida, Kirk & Powell, 1994). The cAMP-mediated inactivation of I_{Cl} observed in this study may be unique to the I_{Cl} associated with cell swelling, and may indicate that swelling activates a specific subpopulation of Cl^- channels in heart cells.

This is the first demonstration of diverse effects of cAMP-mediated protein phosphorylation on Cl^- currents, although an analogous situation exists for Ca^{2+}-dependent K^+ currents. Subpopulations of Ca^{2+}-activated K^+ channels have been described in rat brain which can be either activated or inhibited by cAMP-dependent modulation (Reinhart, Chung, Martin, Brautigan & Levitan, 1991). In a rat pituitary cell line, PKA-mediated phosphorylation attenuates the whole-cell K^+ current by promoting transformation of the large conductance K^+ channel to a less active state, i.e. a state which has a reduced probability of opening; this effect can be reversed by stimulation of protein phosphatase activity (White, Schonbrunn & Armstrong, 1991). It is reasonable to suggest that the activity of the swelling-induced Cl^- channel is regulated by a similar mechanism. The lack of swelling-induced current in the perforated patch experiments could be explained if the channels through which this current flows are maintained in a phosphorylated state (i.e. low opening probability) under physiological conditions.

The role of I_{Cl} during cell swelling

The initial observation that I_{Cl} is active only in dialysed cells, which fail to undergo RVD, could imply either that the current requires a continuous swelling stimulus or, alternatively, that the current contributes to the loss of volume regulation. However, it is apparent from our results that although generation of current is associated with loss of RVD, conventionally patch-clamped cells continue to swell throughout the hyposmotic challenge even under conditions where I_{Cl} is prevented (the possible reasons for this phenomenon have been discussed previously (Zhang *et al.* 1993)). Since the volume and current responses can be dissociated, these findings demonstrate that I_{Cl} is unlikely to contribute directly to changes in cardiac cell volume and, therefore, cannot be solely responsible for short-term volume recovery. Furthermore, the observed failure of cell volume regulation in dialysed cells indicates that physiologically relevant mechanisms of cell volume regulation, such as amino acid loss (Rasmusson *et al.* 1993), must also be compromised in this experimental configuration. This finding should be borne in mind in the design of all electrophysiological experiments to investigate modulation of cell function by signal transduction pathways. The results of our study indicate that the swelling-induced I_{Cl} is suppressed under normal physiological conditions. A mechanism to prevent generation of I_{Cl} during cardiac cell swelling would be important in attenuating the potentially arrhythmic effects of any such current. The presence of a swelling-induced I_{Cl} in metabolically compromised cells (with low levels of cAMP) could, however, offer an additional mechanism to attenuate swelling and thereby limit damage in ischaemic regions of the myocardium.

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