

# Glucagon activates $\text{Ca}^{2+}$ and $\text{Cl}^-$ channels in rat hepatocytes

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Glucagon is one of the major hormonal regulators of glucose metabolism, counteracting the hepatic effects of insulin when the concentration of glucose in the bloodstream falls below a certain level. Glucagon also regulates bile flow, hepatocellular volume and membrane potential of hepatocytes. It is clear that changes in cell volume and membrane potential cannot occur without significant ion fluxes across the plasma membrane. The effects of glucagon on membrane currents in hepatocytes, however, are not well understood. Here we show, by patch-clamping of rat hepatocytes, that glucagon activates two types of currents: a small inwardly rectifying  $\text{Ca}^{2+}$  current with characteristics similar to those of the store-operated  $\text{Ca}^{2+}$  current and a larger outwardly rectifying  $\text{Cl}^-$  current similar to that activated by cell swelling. We show that the mechanism of glucagon action on membrane conductance involves phospholipase C and adenylyl cyclase. Contribution of the adenylyl cyclase-dependent pathway to activation of the currents depended on Epac (exchange protein directly activated by cAMP), but not on protein kinase A. The activation of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  channels is likely to play a key role in the mechanisms by which glucagon regulates hepatocyte metabolism and volume.

(Resubmitted 15 March 2006; accepted 30 March 2006; first published online 31 March 2006)

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Stimulation of hepatic glucose output when the concentration of glucose in the blood is low is one of the main physiological roles of glucagon. It is well established that, acting through G-protein-coupled receptors on the hepatocyte plasma membrane, glucagon activates glycogenolysis and gluconeogenesis, while inhibiting glycolysis and glycogenesis, thus providing a mechanism to counteract the effects of insulin (for review see Jiang & Zhang, 2003). In addition to its role in regulating hepatic glucose output, glucagon is known to enhance bile flow and increase the bicarbonate concentration in bile in humans and other animal species (Alvaro *et al.* 1995; Tanaka *et al.* 2003) and to regulate hepatocellular volume (Lang *et al.* 1998). These processes are likely to involve significant glucagon-induced ion movements across the plasma membrane (Graf & Haussinger, 1996). Early electrophysiological studies suggested that glucagon causes membrane hyperpolarization in rat hepatocytes (Graf & Petersen, 1978; Friedmann & Dambach, 1980). However, knowledge of effects of glucagon on ion fluxes across the hepatocyte plasma membrane is limited.

It is universally accepted that the primary pathway of glucagon action involves the activation of  $G_{\alpha s}$ , leading to activation of adenylyl cyclase, an increase in intracellular levels of cAMP, and activation of protein

kinase A (PKA; Jiang & Zhang, 2003). The contribution of a second pathway which involves activation of  $G_q$  and phospholipase C (PLC), resulting in accumulation of inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and increased cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), remains contentious (Morand *et al.* 1988; Mine *et al.* 1993; Burcelin *et al.* 1996). Several studies have shown that glucagon can have effects independent of increases of the intracellular concentration of cAMP and that these effects are likely to be mediated by elevations in  $[\text{Ca}^{2+}]_i$  (Birnbaum & Fain, 1977; Heyworth & Houslay, 1983; Heyworth *et al.* 1983). While the observation that glucagon stimulates production of  $\text{IP}_3$  in rat hepatocytes supports this notion, the levels of  $\text{IP}_3$  produced by stimulation of hepatocytes with glucagon are significantly lower than those produced by stimulation with vasopressin (Wakelam *et al.* 1986). The results of some studies have suggested that glucagon might increase  $[\text{Ca}^{2+}]_i$  through a cAMP-dependent mechanism (Combettes *et al.* 1986; Kass *et al.* 1994) and that  $\text{Ca}^{2+}$  is essential for regulation of glycogenolysis by cAMP (Kraus-Friedmann & Feng, 1996). Thus, while there is reasonable evidence to indicate that glucagon increases  $\text{IP}_3$  and  $[\text{Ca}^{2+}]_i$  in hepatocytes, and that  $\text{Ca}^{2+}$  is required for some of the actions of glucagon, the 'Ca<sup>2+</sup> pathway' in the mechanism of glucagon action is not well understood.

In the present work we investigate the effects of glucagon on membrane conductance of rat hepatocytes using the patch-clamping technique in whole-cell and perforated-patch configurations. It was found that in rat hepatocytes glucagon activates  $\text{Ca}^{2+}$  current with characteristics similar to those of the store-operated  $\text{Ca}^{2+}$  current ( $I_{\text{SOC}}$ ) activated by ATP and vasopressin in these cells (Rychkov *et al.* 2005) and  $\text{Cl}^-$  current similar to that activated by cell swelling (Moore *et al.* 2002). The results show that the mechanism of the glucagon response involves adenylyl cyclase and phospholipase C. Interestingly, the adenylyl cyclase and cAMP-dependent pathway did not involve PKA, the main target for cAMP, but involved Epac (exchange protein directly activated by cAMP; de Rooij *et al.* 1998). This is the first demonstration that glucagon activates  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  currents in rat hepatocytes.

## Methods

### Cells

For the preparation of isolated primary hepatocytes, fed male hooded Wistar rats were anaesthetized with an intraperitoneal injection of Nembutal (pentobarbitone,  $60 \text{ mg kg}^{-1}$  body mass) and surgically prepared for liver perfusion. After cannulation of the portal vein, the liver was perfused *in situ* for 15 min in the presence of collagenase, and hepatocytes isolated as previously described (Berry *et al.* 1991). After Nembutal injection the animals did not recover consciousness, and were killed by cannulation of the liver. Isolated hepatocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Australia, Mt Waverley, VIC, Australia) supplemented with penicillin ( $100 \text{ units ml}^{-1}$ ), streptomycin ( $100 \mu\text{g ml}^{-1}$ ), 10 mM Hepes (pH 7.4), 1% BSA (Sigma, St Louis, MO, USA), 100 nM insulin (Sigma) and  $1 \mu\text{M}$  dexamethasone (Sigma) and plated on glass coverslips at  $37^\circ\text{C}$  in 5% (v/v)  $\text{CO}_2$  in air for 4 h. Culture medium was replaced after 4 h with DMEM supplemented with penicillin ( $100 \text{ units ml}^{-1}$ ), streptomycin ( $100 \mu\text{g ml}^{-1}$ ), 10% (v/v) fetal bovine serum (FBS; Trace, Melbourne, Australia), 2 mM L-glutamine (Sigma) and 0.1 mM non-essential amino acid solution (Invitrogen Australia), and cells were incubated further for 24–48 h before patch clamping.

Rats used in the present study were housed in a 12 h daylight climate-controlled environment; nevertheless, during summer time (December–March in the southern hemisphere) hepatocytes from most preparations generally were less amenable for patch-clamping studies. The reasons for the observed seasonal variation were not investigated, and the results presented here were obtained during the cooler months of the year.

### Electrophysiology

Whole-cell patch clamping was performed at room temperature using a computer-based patch-clamp amplifier (EPC-9, HEKA Electronics, Lambrecht Germany) and PULSE software (HEKA Electronics). The control bath solution contained (mM): NaCl, 140; CsCl, 4;  $\text{CaCl}_2$ , 10;  $\text{MgCl}_2$ , 2; glucose, 10; and Hepes, 10; adjusted to pH 7.4 with NaOH. Solutions containing lower  $\text{Ca}^{2+}$  concentration were prepared by replacing  $\text{CaCl}_2$  in the control solution with  $\text{MgCl}_2$  on an equimolar basis. Bath solution with low  $\text{Na}^+$  was prepared by replacing 140 mM of NaCl in the control solution with 140 mM NMDG chloride. Bath solution with low  $\text{Cl}^-$  was prepared by replacing 140 mM NaCl with 140 mM sodium glutamate. Isotonic bath solution for studies of volume-regulated  $\text{Cl}^-$  channels was prepared by replacing 50 mM NaCl in control solution with 100 mM sucrose, while in the hypotonic solution sucrose was omitted. In experiments investigating  $\text{Ba}^{2+}$  conductance in rat hepatocytes, 130 mM NaCl and 10 mM  $\text{CaCl}_2$  in the control bath solution were substituted by 100 mM  $\text{BaCl}_2$ . The pipette solution used in whole-cell experiments contained (mM): CsCl, 15; caesium glutamate, 135;  $\text{CaCl}_2$ , 0.5; MgATP, 6; EGTA, 1; and Hepes, 10; adjusted to pH 7.2 with NaOH. In some experiments, 10 mM EGTA was used in the above pipette solution with no  $\text{Ca}^{2+}$  added. The pipette solution used in perforated-patch experiments contained (mM): CsCl, 15; caesium glutamate, 140;  $\text{CaCl}_2$ , 1;  $\text{MgCl}_2$ , 1; Hepes, 10; and  $100 \mu\text{g ml}^{-1}$  amphotericin B (solubilized form, Sigma); adjusted to pH 7.2 with NaOH. A stock solution of amphotericin B ( $50 \text{ mg ml}^{-1}$ ) was prepared in DMSO every week and stored at  $4^\circ\text{C}$ . An aliquot of stock solution was added to the pipette solution to give a final amphotericin B concentration of  $100 \mu\text{g ml}^{-1}$ . Pipette solution with amphotericin B was sonicated for 20 s, and used within 2 h. Patch pipettes were pulled from borosilicate glass and fire polished; pipette resistance ranged between 3 and 4 M $\Omega$ . Series resistance in whole-cell configuration did not exceed 20 M $\Omega$  and was 50–70% compensated. In perforated-patch experiments the recording started when the series resistance dropped below 20 M $\Omega$ . In order to monitor changes in the amplitude of the membrane currents, voltage ramps between  $-138$  and  $+102$  mV were applied every 2 s. Acquired currents were filtered at 2.7 kHz and sampled at 10 kHz. Liquid junction potential for the solutions used varied between  $-16$  and  $-20$  mV (estimated by JPCalc; Barry, 1994), and the correction of  $-18$  mV was applied to all voltages shown. The holding potential was  $-18$  mV throughout. Cell capacitance of hepatocytes used in this study varied between 35 and 90 pF and was compensated for automatically by the EPC-9 amplifier. The results are expressed as the means  $\pm$  S.E.M.

## Chemicals

Unless specified, all compounds were supplied by Sigma (St Louis, MO, USA). Stock solution of glucagon was prepared in 5% acetic acid and freshly diluted in bath solution as required. H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride), NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid (RBI, Natick, MA, USA), Rp-cAMPS (*R<sub>p</sub>*-adenosine-3',5'-cyclic monophosphothioate), des-His<sup>1</sup>-[Glu<sup>9</sup>]glucagon amide, U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidinedione) were all diluted in DMSO and then prepared for use in bath solution so that the final concentration of DMSO did not exceed 0.1%. Stock solutions of cAMP, 8-pCPT-2'-*O*-Me-cAMP (8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate), and 6-Bnz-cAMP (*N*<sup>6</sup>-benzoyladeniosine-3',5'-cyclic monophosphate; BIOLOG Life Science Institute, Bremen, Germany) were prepared in H<sub>2</sub>O and the required amounts were added to the appropriate pipette solution prior to use.

## Data analysis

To extract the maximal amplitude of the inward current after full development in response to glucagon ( $I_{\max}$ ) and the time to half-maximal activation of the current ( $\tau_{50}$ ), the amplitude of the inward current at -118 mV was plotted against time and fitted by the following equation:

$$I(t) = I_{\min} + \frac{I_{\max} - I_{\min}}{1 + \exp((\tau_{50} - t)/k)} \quad (1)$$

where  $I_{\min}$  is the amplitude of the baseline current before addition of glucagon and  $k$  is the slope factor. Slope factor  $k$  is used as a measure of the rate of the current development (smaller  $k$  means a steeper curve and a higher rate).

The Goldman-Hodgkin-Katz equation of the form:

$$\Delta E_{\text{rev}} = 58.4 \log \frac{[\text{Cl}^-]_o^a}{[\text{Cl}^-]_o^b + \frac{P_{\text{Glut}}}{P_{\text{Cl}}} [\text{Glut}^-]_o^b} \quad (2)$$

has been used to calculate the permeability of glutamate relative to Cl<sup>-</sup> ( $P_{\text{Glut}}/P_{\text{Cl}}$ ); where  $\Delta E_{\text{rev}}$  was the shift of the reversal potential when 168 mM of Cl<sup>-</sup> in the external solution ( $[\text{Cl}^-]_o^a$ ) was replaced with 140 mM of glutamate ( $[\text{Glut}^-]_o^b$ ), and  $[\text{Cl}^-]_o^b$  was the remaining Cl<sup>-</sup> concentration.

## Results

### Glucagon activates an outwardly rectifying current in rat hepatocytes

All the experiments presented in this work were performed in whole-cell and then repeated in perforated-patch configuration to ensure that the intracellular perfusion in whole-cell mode did not interfere with the mechanisms of glucagon response. In the perforated-patch configuration, the background conductance present before the addition of glucagon did not change throughout the experiment, whereas in whole-cell configuration the background conductance was smaller and decreased with time. Nevertheless, qualitatively, the results obtained by both techniques were the same.

Glucagon applied to the bath (1–250 nM) activated an outwardly rectifying membrane current in rat hepatocytes. Results for perforated-patch and whole-cell configurations are shown in Fig. 1A and B. The development of the current started after a 20–40 s delay, and fit of eqn (1) to the experimental data in Fig. 1A and B showed that time to the half-maximal activation for both the outward and the inward current was significantly shorter ( $P < 0.05$ ) in the perforated-patch ( $110 \pm 4$  and  $130 \pm 4$  s, respectively;  $n = 5$ ) compared to the whole-cell experiments ( $187 \pm 6$  and  $220 \pm 7$  s, respectively;  $n = 15$ ). The inward current developed more slowly than the outward, and this difference was clearer when  $I$ - $V$  plots obtained at different time points of the glucagon response were compared (Fig. 1C). These results suggested that the current activated by glucagon was carried by at least two different types of ion channels. To ensure that the effect of glucagon on membrane conductance was due to specific receptor binding, a selective antagonist of the glucagon receptor, des-His<sup>1</sup>-[Glu<sup>9</sup>]glucagon amide, was used (Post *et al.* 1993). When added alone to rat hepatocytes, the antagonist did not activate any measurable membrane conductance (Fig. 1D), while pretreatment of hepatocytes with the antagonist prevented activation of any current by glucagon (Fig. 1E).

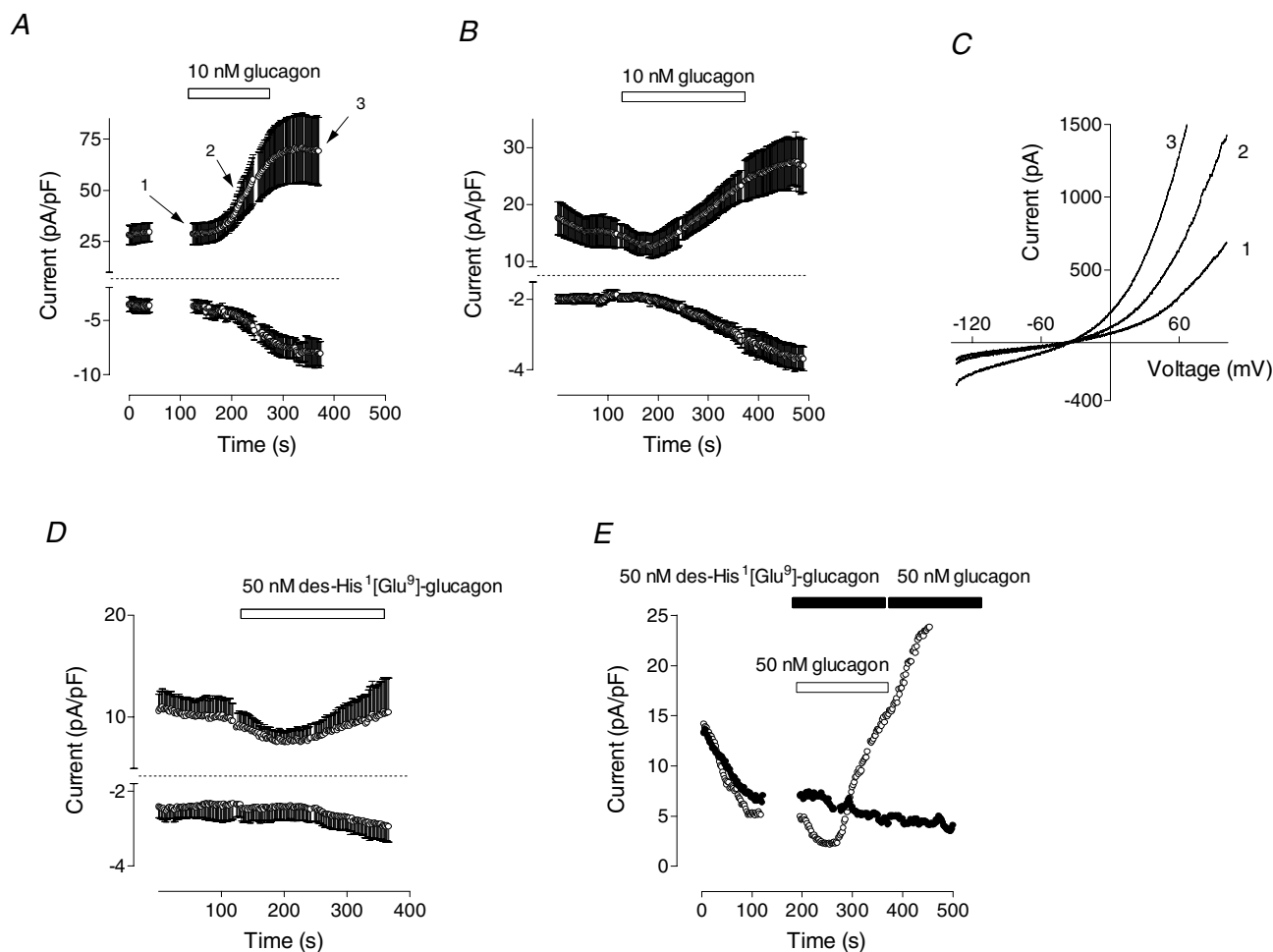
After full activation by glucagon, the amplitude of the currents declined with time to the baseline levels (5–15 min), irrespective of whether or not glucagon was present in the bath (not shown). A second application of glucagon, even after a 20 min wash, failed to activate any current in most cells. In a small minority of cells (~5%) where current was activated a second time, it was significantly smaller than the one activated by the first application of glucagon. Similar results were obtained in perforated-patch mode, which eliminates possible wash-out of the intracellular components as an explanation for the failure of the second application of glucagon to activate the current, and suggests desensitization of the glucagon receptor.

## Ca<sup>2+</sup> and Cl<sup>-</sup> channels are activated by glucagon

Ion substitution was used to investigate the nature of channels underlying the response to glucagon. Since the internal solution contained Cs<sup>+</sup> in place of all K<sup>+</sup>, the outward current could be carried either by Cs<sup>+</sup> passing out through non-selective cation channels or by Cl<sup>-</sup> moving in through anion channels. Reduction of the Cl<sup>-</sup> concentration in the external solution from 168 to 28 mM resulted in a drastic reduction of the outward current and a shift of the reversal potential to more positive values (Fig. 2A), suggesting that most of the outward conductance activated by glucagon was due to the inward

movement of Cl<sup>-</sup>. Replacement of 140 mM NaCl in the control bath solution with 140 mM sodium glutamate resulted in an average shift in the current reversal potential of  $34 \pm 3$  mV ( $n = 4$ ), which corresponds to permeability of glutamate relative to Cl<sup>-</sup> ( $P_{\text{Glut}}/P_{\text{Cl}}$ ) of  $\sim 0.11$  (eqn (2)). A non-selective anion channel blocker, NPPB ( $50 \mu\text{M}$ ), completely blocked outward current activated by glucagon (not shown), which confirms the notion that this current is carried through Cl<sup>-</sup> channels.

Since the pipette solution contained 16 mM Cl<sup>-</sup>, some part of the inward current activated by glucagon was carried by Cl<sup>-</sup> moving out of the cell. The contribution of cations to the inward current was determined by



**Figure 1.** Effect of glucagon on membrane conductance in rat hepatocytes

*A* and *B* show the time course of the development of the inward and outward current in response to glucagon (10 nM) in perforated-patch (*A*,  $n = 5$ ) and whole-cell experiments (*B*,  $n = 15$ ). Current amplitudes were taken from the responses to the voltage ramps at  $-118$  and  $+82$  mV for the inward and the outward current, respectively, normalized to the cell capacitance and plotted against time. Application of glucagon is indicated by a horizontal bar. *C*, examples of the  $I$ - $V$  plots obtained in response to voltage ramps between  $-138$  and  $+102$  mV before application of glucagon (trace 1, see *A*), at the beginning of the glucagon response (trace 2), and when the currents are fully developed (trace 3). *D*, lack of effect of the glucagon receptor antagonist des-His<sup>1</sup>[Glu<sup>9</sup>]-glucagon amide (50 nM) on membrane conductance ( $n = 8$ ). *E*, inhibition of the glucagon response (50 nM) by des-His<sup>1</sup>[Glu<sup>9</sup>]-glucagon amide (50 nM; ●; representative cell,  $n = 5$ ). For comparison, a response to glucagon (50 nM) is shown in a representative cell from the same preparation (○).

substitution of  $\text{Na}^+$  or  $\text{Ca}^{2+}$  in the bath solution. Replacement of  $\text{Na}^+$  in the external solution with  $\text{NMDG}^+$  had little effect on the inward current activated by glucagon (Fig. 2B). Although there was some reduction in inward current amplitude when  $\text{Na}^+$  was replaced by the larger cation, the absolute magnitude of that reduction was the same before and after application of glucagon, suggesting that glucagon does not activate  $\text{Na}^+$  channels or non-selective cation channels (Fig. 2B).

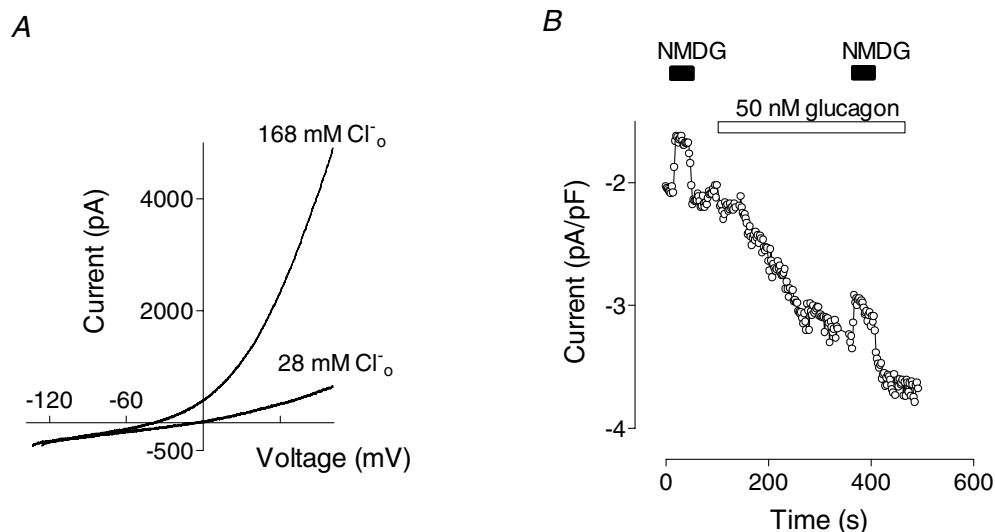
Replacement of  $\text{Ca}^{2+}$  in the external solution with an equimolar amount of  $\text{Mg}^{2+}$  resulted in a decline of both inward and outward currents (Fig. 3A). Upon readmission of  $\text{Ca}^{2+}$ , there was rapid increase in the inward current amplitude of about  $1 \text{ pA pF}^{-1}$  (Fig. 3A, indicated by arrow), while outward current increased gradually. These results suggested that there is a  $\text{Ca}^{2+}$  component in the inward current, and that activation of  $\text{Cl}^-$  current required the presence of  $\text{Ca}^{2+}$ . This  $\text{Ca}^{2+}$  component of the inward current could be clearly identified when  $\text{Cl}^-$  conductance activated by glucagon declined to a baseline level. Removal of  $\text{Ca}^{2+}$  at this point resulted in a decrease of the inward current by  $\sim 1 \text{ pA pF}^{-1}$ , while the outward current remained mainly unchanged (Fig. 3B).

Application of  $1 \mu\text{M}$   $\text{La}^{3+}$  in the bath also led to inhibition of both the inward and the outward current (Fig. 3C). More detailed analysis of the time course of the  $\text{La}^{3+}$  block showed that the onset of the effect on the inward current was almost instantaneous, and that the inward current amplitude diminished in two phases: fast and slow (Fig. 4A). In contrast, block of the outward current occurred with a 5–10 s delay and followed a

single exponential function. These results provide further evidence that glucagon activates two types of channels, namely  $\text{Ca}^{2+}$  and  $\text{Cl}^-$ , and suggest that the inward current induced by glucagon is largely due to activation of  $\text{Ca}^{2+}$  channels, while activation of the  $\text{Cl}^-$  channels is  $\text{Ca}^{2+}$  dependent. The presence of a glucagon-activated  $\text{Ca}^{2+}$  current is also consistent with the observation that 10% of all cells that responded developed only the inward current after addition of glucagon (not shown). In these cells, the outward current either did not change or declined.

Since inhibition of the outward current by  $\text{La}^{3+}$  was 10–15 s later in onset than the block of the inward current, it was possible to obtain relatively 'pure' currents by subtracting  $I$ - $V$  plots obtained at different times during  $\text{La}^{3+}$  application (Fig. 4A and B). The  $I$ - $V$  plot for the current that was very rapidly blocked by  $\text{La}^{3+}$  could be obtained by subtracting the current trace recorded 10 s after  $\text{La}^{3+}$  application (when most of the inward current was already blocked whilst the outward current was still relatively unchanged; Fig. 4A, second mark; Fig. 4B, trace 2) from the trace that was recorded before  $\text{La}^{3+}$  application (Fig. 4A, first mark; Fig. 4B, trace 1). Current that was very rapidly blocked by  $\text{La}^{3+}$  had an inwardly rectifying  $I$ - $V$  plot and no clear reversal potential up to  $+100 \text{ mV}$  (Fig. 4C). Under the conditions of the experiment such a current can only be attributed to  $\text{Ca}^{2+}$ . This  $\text{Ca}^{2+}$  current also showed fast inactivation during voltage steps to potentials more negative than  $-80 \text{ mV}$  (Fig. 4D).

The second component of the current that was blocked by  $\text{La}^{3+}$  was obtained by subtracting  $I$ - $V$  plots recorded 2 min after  $\text{La}^{3+}$  application (Fig. 4A, third mark;



**Figure 2.** Effects of ion substitution on glucagon-activated conductance

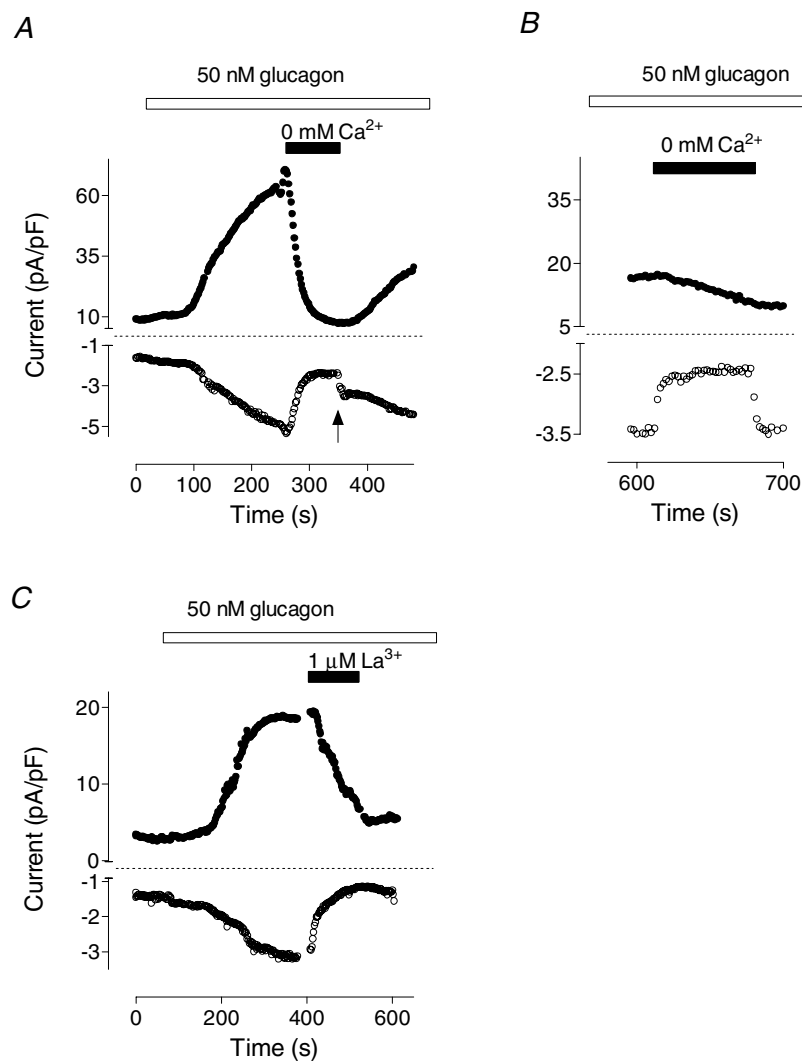
A, effect of  $\text{Cl}^-$  replacement in the external solution with an equimolar amount of glutamate on the outward current shown in a representative cell ( $n = 5$ ). B, effect of replacement of  $\text{Na}^+$  in the external solution with  $\text{NMDG}^+$  on inward current shown in a representative cell ( $n = 4$ ).  $\text{NMDG}^+$  was perfused both before and after development of the glucagon response.

Fig. 4B, trace 3) from that recorded after 10 s (Fig. 4A, second mark; Fig. 4B, trace 2). It had an outwardly rectifying  $I$ - $V$  plot and a reversal potential of  $-43 \pm 3$  mV ( $n = 4$ ; Fig. 4E). This reversal potential corresponds to a  $\text{Cl}^-$ -selective channel with  $P_{\text{Glut}}/P_{\text{Cl}}$  of  $\sim 0.11$ .

The  $\text{Ca}^{2+}$  current activated by glucagon was characterized further by using  $\text{Ba}^{2+}$  as  $\text{Ca}^{2+}$  substitute. Barium permeates all types of  $\text{Ca}^{2+}$  channels to a different extent (Yue & Marban, 1990); however, it induces specific changes in conductance of SOCs in rat hepatoma cell line (H4IIE) cells Rychkov *et al.* 2001) and  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  (CRAC) channels rat basophilic leukaemia cells in (RBL) and mast cells (Hoth, 1995). Application of the solution containing 100 mM  $\text{Ba}^{2+}$  to a cell with a fully developed glucagon response resulted in almost instantaneous increase of the inward current amplitude followed by its decline (Fig. 5A). Upon  $\text{Ba}^{2+}$  removal, the inward current decreased further before slowly recovering. The outward current in the presence of  $\text{Ba}^{2+}$  declined after some delay and did not recover after removal of  $\text{Ba}^{2+}$  (Fig. 5A). It has been shown previously

that depletion of  $\text{Ca}^{2+}$  stores in rat hepatocytes using  $\text{IP}_3$ , thapsigargin or vasopressin activates  $I_{\text{SOC}}$  with characteristics similar to that of  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  current ( $I_{\text{CRAC}}$ ) of RBL and mast cells (Rychkov *et al.* 2005). When  $\text{Ba}^{2+}$  was applied to hepatocytes with  $I_{\text{SOC}}$  activated by intracellular perfusion with  $\text{IP}_3$  (Fig. 5B) or by application of  $1 \mu\text{M}$  thapsigargin to the bath (Fig. 5C), the change in inward current amplitude was similar to that of the inward current activated by glucagon. If applied before the depletion of the stores (Fig. 5C) or before glucagon (not shown,  $n = 7$ ), 100 mM  $\text{Ba}^{2+}$  decreased background currents. These results provide further evidence that the  $\text{Ca}^{2+}$  current activated by glucagon is the same as  $I_{\text{SOC}}$  activated by  $\text{IP}_3$ - or thapsigargin-induced release of  $\text{Ca}^{2+}$  from stores. They also show that the depletion of  $\text{Ca}^{2+}$  stores and activation of  $\text{Ca}^{2+}$  entry *per se* is insufficient to activate  $\text{Cl}^-$  current in rat hepatocytes.

Chloride conductance activated by glucagon was sensitive to the osmolarity of the external medium. Addition of 100 mM sucrose to the control bath solution completely inhibited  $\text{Cl}^-$  current activated by glucagon



**Figure 3. Effects of removal of extracellular  $\text{Ca}^{2+}$  and addition of  $\text{La}^{3+}$  on the currents activated by glucagon**

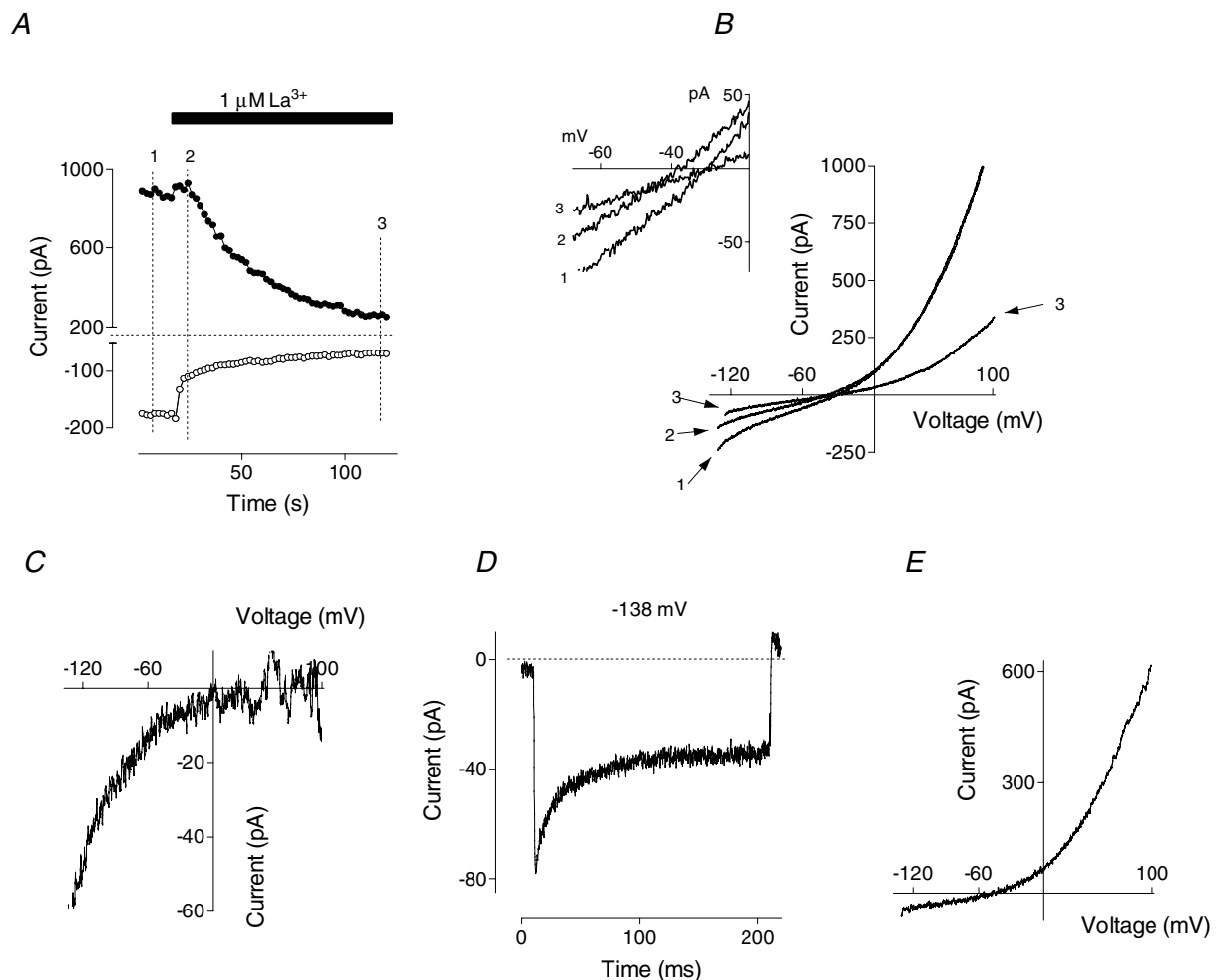
A and B show the effect of removal of  $\text{Ca}^{2+}$  from the external solution on both the inward and outward currents at early (A) and late stages (B) of the glucagon response shown in a representative cell ( $n = 5$ ).  $\text{CaCl}_2$  was replaced with  $\text{MgCl}_2$ . Arrow shows the point when  $\text{Ca}^{2+}$  was added back to the bath. C, inhibition of the glucagon response by  $\text{La}^{3+}$  shown in a representative cell ( $n = 8$ ).

(Fig. 6A). Currents activated by glucagon and recorded in response to voltage steps showed inactivation at potentials above 80 mV (Fig. 6B), while background currents showed no such inactivation (Fig. 6C). Chloride current similar to that activated by glucagon could be activated by cell swelling (Fig. 7). This is shown in Fig. 7A, where reduction of the osmolarity by removing 100 mM sucrose from isotonic bath solution (denoted as 'hypo' in the figure) resulted in development of large outwardly rectifying conductance. Restoration of osmolarity to normal inhibited this conductance within several minutes (Fig. 7A, 'control'). As with glucagon-activated current, swelling-activated current recorded in response to voltage steps showed inactivation at potentials above 80 mV (Fig. 7B). The reversal potential of the  $I-V$  plot was

$-40 \pm 2$  mV ( $n = 3$ ) in the control solution, which shifted to more positive potentials by  $35 \pm 2$  mV ( $n = 3$ ) when 140 mM NaCl in the external solution was replaced with 140 mM sodium glutamate (Fig. 7C). Removal of  $\text{Ca}^{2+}$  from the external solution had no effect on the development of the swelling-activated  $\text{Cl}^-$  current, and did not alter the amplitude after the current had fully developed (not shown).

#### Activation of membrane currents by glucagon depends on PLC

Activation of  $\text{Ca}^{2+}$  entry in non-excitable cells is usually associated with activation of PLC by G-protein-coupled receptors or tyrosine kinase-coupled receptors (Berridge,



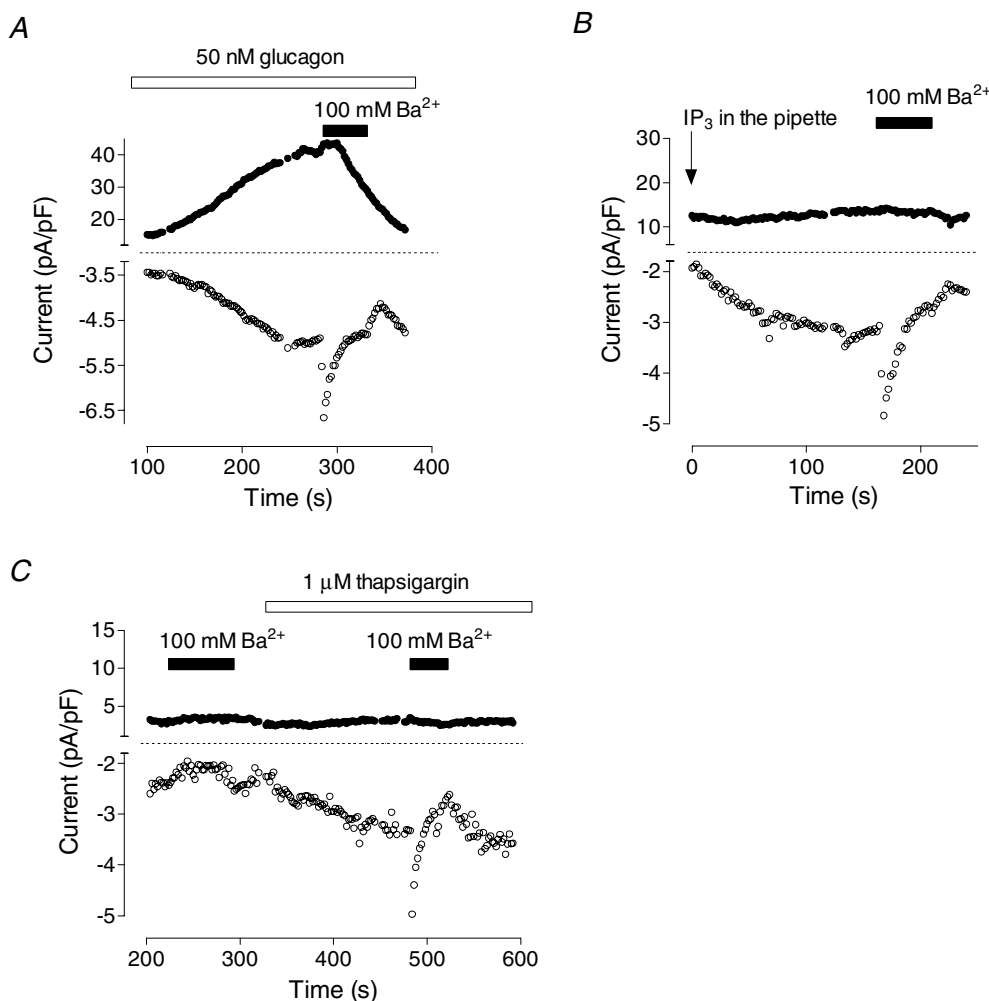
**Figure 4.** Separation of the currents inhibited by  $\text{La}^{3+}$

A, time course of  $\text{La}^{3+}$  block (results similar to those in Fig. 3C are shown using an expanded time scale). Marks 1, 2 and 3 correspond to time points before application of  $\text{La}^{3+}$ , just after application of  $\text{La}^{3+}$  when only inward current is blocked, and after the complete development of the block, respectively. B, the  $I-V$  plots of the currents recorded before addition of  $\text{La}^{3+}$  (trace 1), at 10 s (trace 2) and at 90 s (trace 3) after  $\text{La}^{3+}$  addition (cf. corresponding numbers in A). Inset shows currents around the reversal potential. C, the  $I-V$  plot of the current blocked by  $\text{La}^{3+}$  within first 5–10 s (obtained by subtraction of trace 2 from trace 1 shown in B). D, the same current as in C, but recorded in response to a voltage step to  $-138$  mV. E, the  $I-V$  plot of the current inhibited by  $\text{La}^{3+}$  between 10 and 120 s of application (obtained by subtraction of trace 3 from trace 2 shown in B).

1993). While glucagon has been shown to activate PLC, PLC is not considered the main target for glucagon in liver (Wakelam *et al.* 1986; Jiang & Zhang, 2003). An inhibitor of PLC, U73122 (Bleasdale *et al.* 1990), added at  $4 \mu\text{M}$  in the bath for 2 min, completely abolished the response of rat hepatocytes to glucagon (Fig. 8A), inhibiting development of both  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  currents. The inactive analogue, U73343, applied in the bath for up to 30 min at a concentration of  $10 \mu\text{M}$ , had no effect on the glucagon response (Fig. 8B). U73122 also completely inhibited  $\text{Cl}^-$  current when applied to the bath after full development of that current in response to glucagon (Fig. 8C). Since U73122 could potentially have direct

effects on ion channels, we investigated its effects on the types of channels that are most likely to represent those activated by glucagon. Neither the  $\text{Cl}^-$  current activated by cell swelling nor the  $I_{\text{SOC}}$  activated by depletion of the  $\text{Ca}^{2+}$  stores by intracellular perfusion with  $\text{IP}_3$  was affected by  $4 \mu\text{M}$  U73122 applied to the bath (Fig. 8D and E).

Another commonly used inhibitor of PLC, edelfosine (Horowitz *et al.* 2005), was also tested. However, addition of  $10 \mu\text{M}$  edelfosine to the bath solution caused a rapid increase in non-specific leakage in hepatocytes within 5 min of incubation ( $n=20$ ), indicating that it is not possible to use edelfosine in patch-clamping studies of rat hepatocytes.



**Figure 5. Activation of  $\text{Ba}^{2+}$ -permeable channels in rat hepatocytes by glucagon and depletion of intracellular  $\text{Ca}^{2+}$  stores**

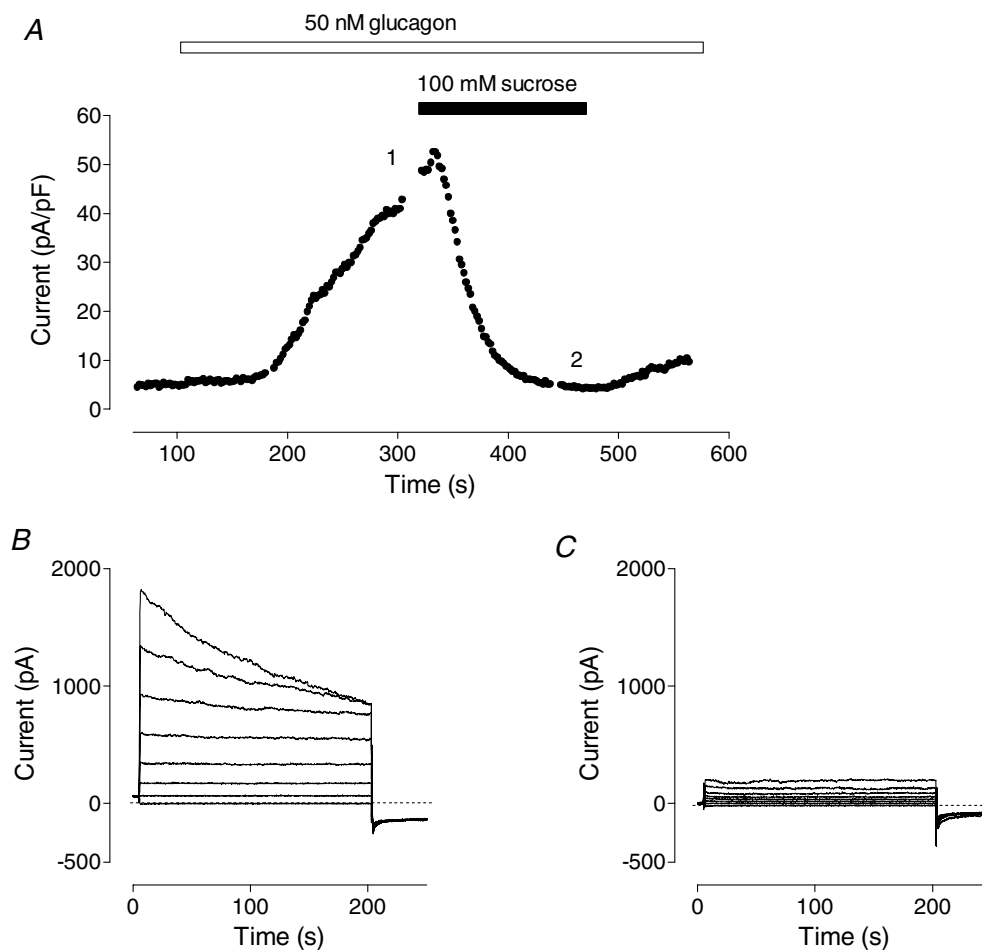
A, effect of replacement of the control external solution with solution containing  $100 \text{ mM Ba}^{2+}$  on the currents activated by glucagon (representative cell,  $n=6$ ). B, effect of  $100 \text{ mM Ba}^{2+}$  on store-operated  $\text{Ca}^{2+}$  current activated by intracellular perfusion with  $20 \mu\text{M IP}_3$  (representative cell,  $n=9$ ). C, effect of  $100 \text{ mM Ba}^{2+}$  on store-operated  $\text{Ca}^{2+}$  current activated by  $1 \mu\text{M}$  thapsigargin (representative cell,  $n=3$ ). Note absence of the effect when  $\text{Ba}^{2+}$  was applied before thapsigargin.



### Activation of the $\text{Cl}^-$ current does not depend on PKA, but requires cAMP

Activation of adenylyl cyclase followed by PKA-dependent phosphorylation is considered the main pathway for glucagon regulation of liver function (Jiang & Zhang, 2003). To investigate the role of PKA in the development of membrane currents activated by glucagon, a competitive inhibitor of the activation of PKA, Rp-cAMPS (Alvaro *et al.* 1995), was applied at saturating concentrations through a patch pipette. This had no effect on membrane conductance of rat hepatocytes, and it did not inhibit the ability of glucagon to activate either  $\text{Cl}^-$  or  $\text{Ca}^{2+}$  currents (Fig. 9A). Similarly, another inhibitor of PKA, H-89 (Alvaro *et al.* 1995), also applied at a saturating concentration, had no effect on the development of the response to glucagon (Fig. 9B). These results suggest that the pathways involving PKA are not involved in the mechanism of activation of either  $\text{Ca}^{2+}$  or  $\text{Cl}^-$  conductance by glucagon.

The results presented in Fig. 9 did not exclude the possibility that cAMP might still be involved in the glucagon response through a PKA-independent mechanism. Pre-incubation of hepatocytes with adenylyl cyclase inhibitor SQ22536 (0.5 mM in the bath for 5 min; Fabbri *et al.* 1991) inhibited the amplitude of  $\text{Cl}^-$  current developed in response to glucagon by  $\sim 80\%$  (Fig. 10A). This suggested that activation of adenylyl cyclase by the glucagon receptor and subsequent increase in intracellular cAMP level may be responsible for activation of the  $\text{Cl}^-$  conductance. Inclusion of  $100 \mu\text{M}$  cAMP in the pipette solution resulted in gradual increase of the  $\text{Cl}^-$  conductance after achieving the whole-cell configuration (Fig. 10B). Properties of the  $\text{Cl}^-$  current activated by cAMP (selectivity and rectification) were similar to those of the  $\text{Cl}^-$  current activated by glucagon or cell swelling (not shown). To elucidate the pathway of cAMP modulation of the volume-regulated  $\text{Cl}^-$  conductance, we used cAMP analogues that specifically activate either PKA or Epac



**Figure 6.  $\text{Cl}^-$  conductance activated by glucagon is inhibited by hepatocyte shrinkage**

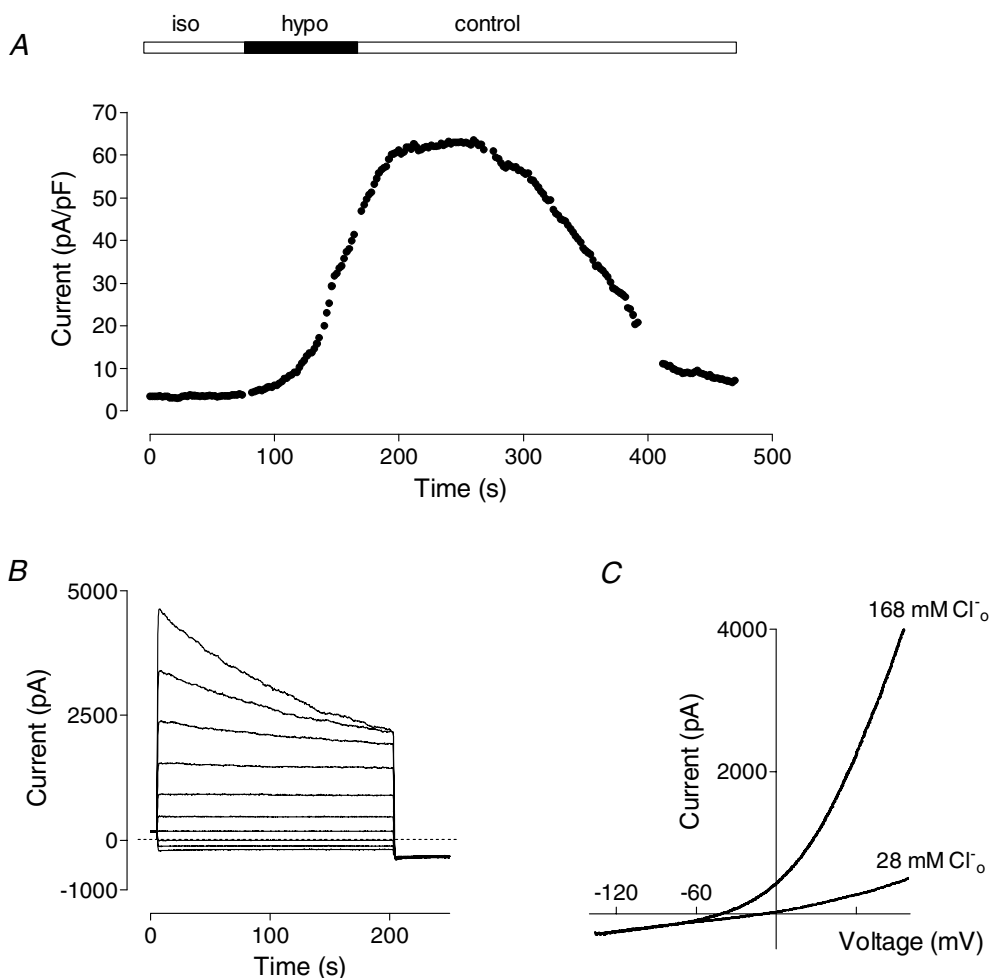
A, effect of addition of 100 mM sucrose to the external solution on the  $\text{Cl}^-$  currents activated by glucagon. B and C show membrane currents recorded in response to voltage steps ranging between  $-98$  and  $+82$  mV in 20 mV increments in isotonic and hypertonic solutions, respectively. Results shown in a representative cell ( $n = 4$ ).

(de Rooij *et al.* 1998). An analogue of cAMP selectively activating PKA, 6-Bnz-cAMP ( $200 \mu\text{M}$  in the pipette solution), had no effect on the  $\text{Cl}^-$  conductance, while a specific activator of Epac, 8-pCPT-2'-O-Me-cAMP ( $200 \mu\text{M}$  in the pipette solution), activated a  $\text{Cl}^-$  conductance of similar amplitude and the same properties as that activated by cAMP (Fig. 10B). Presence of the strong  $\text{Ca}^{2+}$  buffer in the pipette solution ( $10 \text{ mM}$  EGTA) significantly attenuated activation of  $\text{Cl}^-$  conductance by cAMP (Fig. 10C).

## Discussion

In this work, we present the first detailed studies of the effects of glucagon on membrane conductance of primary

rat hepatocytes. The main result is that glucagon initiates the activation of a  $\text{Ca}^{2+}$  current with characteristics similar to those of the  $\text{Ca}^{2+}$  current activated by depletion of  $\text{Ca}^{2+}$  stores, and a  $\text{Cl}^-$  current similar to that activated by cell swelling. These two glucagon-activated channels are likely to play a significant role in the glucagon-mediated effects in liver, particularly in the regulation of transcellular ion movements and the regulation of cell volume. Since hepatocytes express  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels (Duszynski *et al.* 1995), activation of  $\text{Ca}^{2+}$  entry is likely to increase  $\text{K}^+$  efflux and cause membrane hyperpolarization. The activation by glucagon of both a  $\text{Cl}^-$  and a  $\text{K}^+$  conductance of comparable size would lead to a significant efflux of both  $\text{Cl}^-$  and  $\text{K}^+$ , causing cell shrinkage. Both membrane hyperpolarization and shrinkage in the presence of glucagon have been previously



**Figure 7. Hepatocyte swelling activates  $\text{Cl}^-$  current similar to that activated by glucagon**

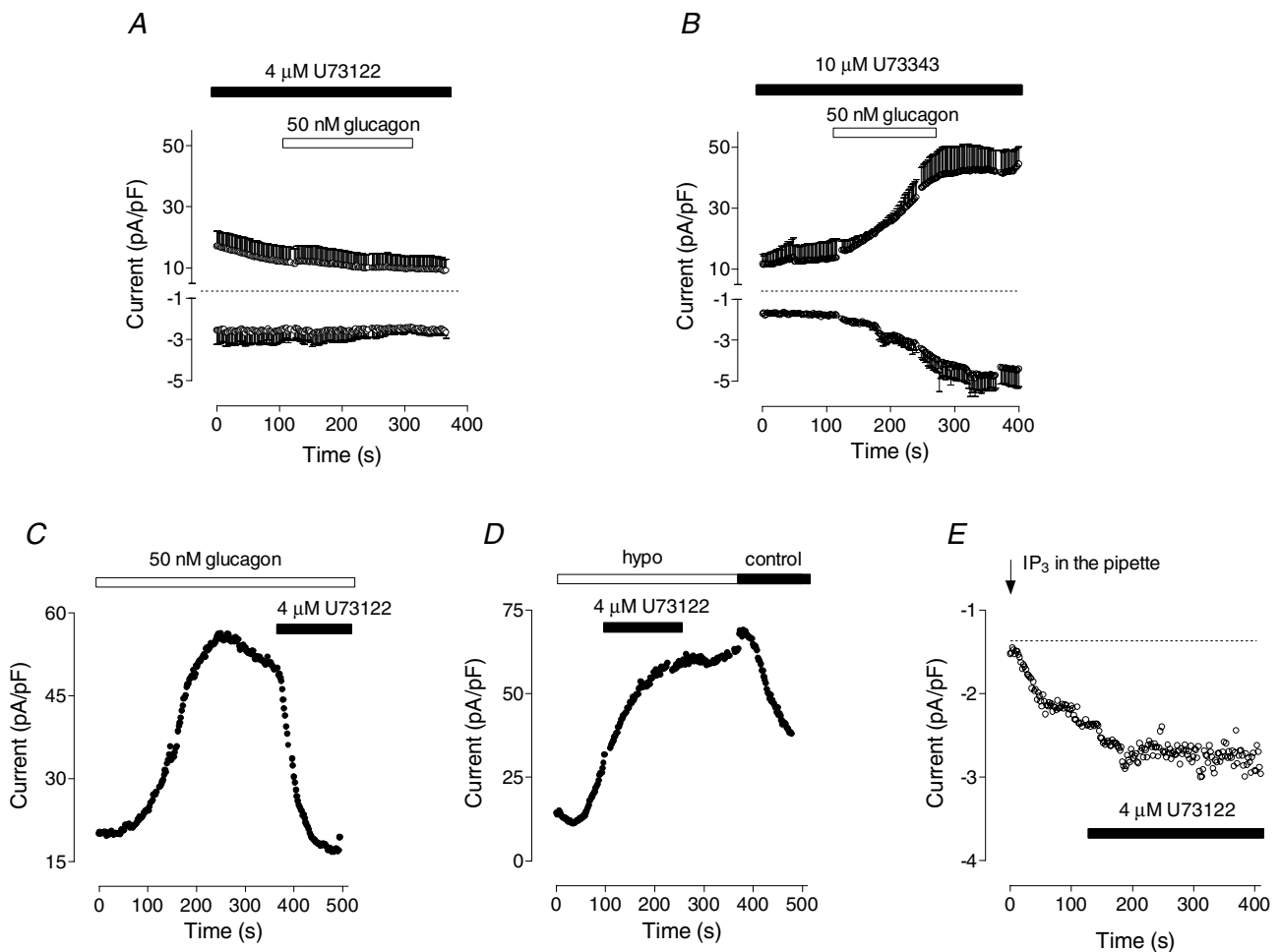
A, time course of  $\text{Cl}^-$  current development in hypotonic solution and inhibition upon return to control bath solution. Initially, cell was perfused with isotonic solution in which  $50 \text{ mM}$   $\text{NaCl}$  was replaced with  $100 \text{ mM}$  sucrose, which subsequently was changed to hypotonic solution lacking  $100 \text{ mM}$  sucrose (see Methods; representative cell,  $n = 4$ ). B, membrane currents activated by swelling recorded in control solution in response to voltage steps ranging between  $-98$  and  $+82 \text{ mV}$  in  $20 \text{ mV}$  increments. C,  $I-V$  plots of the membrane currents activated by swelling in control solution and in solution in which  $140 \text{ mM}$   $\text{NaCl}$  was replaced with  $140 \text{ mM}$  sodium glutamate.

shown in rat hepatocytes (Gaussin *et al.* 1992; Lang *et al.* 1998). Activation of  $\text{Cl}^-$  channels may also be essential for the glucagon-induced bile flow and enrichment of bile fluid with bicarbonate (Alvaro *et al.* 1995), providing the escape route for  $\text{Cl}^-$  accumulated in hepatocytes due to activation of the  $\text{Cl}^-$ - $\text{HClO}_3^-$  exchanger.

### Identity of $\text{Ca}^{2+}$ channels

The only type of  $\text{Ca}^{2+}$ -selective channel identified so far in primary rat hepatocytes by patch-clamping is the store-operated  $\text{Ca}^{2+}$  channel (Rychkov *et al.* 2005). Although comprehensive characterization of the  $\text{Ca}^{2+}$  current activated by glucagon in rat hepatocytes is problematic because it is masked by a much larger  $\text{Cl}^-$  current, features of this current uncovered in the present

study were similar to those of  $I_{\text{SOC}}$  previously described in rat hepatocytes and H4IIE liver cells (Litjens *et al.* 2004; Rychkov *et al.* 2005). The store-operated  $\text{Ca}^{2+}$  current activated in hepatocytes by vasopressin, ATP, intracellular  $\text{IP}_3$  and thapsigargin (Rychkov *et al.* 2005) and the  $\text{Ca}^{2+}$  current activated by glucagon reported here have identical inwardly rectifying  $I$ - $V$  plots, are blocked by the same concentrations of  $\text{La}^{3+}$ , both have an average amplitude of about  $1 \text{ pA pF}^{-1}$ , and, more importantly, both have the same kinetics of fast inactivation at negative potentials. Moreover, a transient increase of the inward current, observed when  $100 \text{ mM Ba}^{2+}$  was applied to the bath when  $\text{Ca}^{2+}$  current had already been activated, exhibited an amplitude and time course similar to those of the inward current activated by depletion of intracellular  $\text{Ca}^{2+}$  stores by either  $\text{IP}_3$  or thapsigargin. Such changes in conductance



**Figure 8. Effect of the PLC inhibitor U73122 on the glucagon response**

A, U73122 ( $4 \mu\text{M}$ ) prevents development of the glucagon response ( $50 \text{ nM}$ ;  $n = 4$ ). B, U73343, which is an inactive analogue of U73122, had no effect on the glucagon response. C, U73122 ( $4 \mu\text{M}$ ) inhibits  $\text{Cl}^-$  current activated by glucagon (representative cell,  $n = 4$ ). D, U73122 ( $4 \mu\text{M}$ ) has no effect on  $\text{Cl}^-$  current activated by cell swelling (representative cell,  $n = 3$ ). E, U73122 ( $4 \mu\text{M}$ ) has no effect on  $\text{Ca}^{2+}$  current activated by intracellular perfusion with  $20 \mu\text{M}$   $\text{IP}_3$  (representative cell,  $n = 3$ ).

in the presence of  $\text{Ba}^{2+}$  have previously been shown for  $I_{\text{CRAC}}$  in mast cells, RBL cells and Jurkat cells (Hoth, 1995), and for  $I_{\text{SOC}}$  in H4IIE liver cells (Rychkov *et al.* 2001).

Although all discernable properties of  $\text{Ca}^{2+}$  current activated by glucagon point to the conclusion that it is  $I_{\text{SOC}}$ , the evidence that glucagon depletes  $\text{Ca}^{2+}$  stores in rat hepatocytes is scarce (Combettes *et al.* 1986), so the store dependence of this current is yet to be established.

Nevertheless, it is well known that glucagon potentiates  $\text{Ca}^{2+}$  inflow to hepatocytes induced by low concentrations of  $\text{Ca}^{2+}$ -mobilizing hormones (e.g. vasopressin and phenylephrine) or thapsigargin in hepatocytes, and affects  $\text{Ca}^{2+}$  distribution inside the cell (Morgan *et al.* 1983, 1984; Combettes *et al.* 1986; Somogyi *et al.* 1992; Bygrave & Benedetti, 1993; Fernando *et al.* 1998). Furthermore, glucagon has been shown to produce small amounts of  $\text{IP}_3$  in hepatocytes (Wakelam *et al.* 1986) and to deplete  $\text{Ca}^{2+}$  stores in baby hamster kidney cells (BHK) expressing ectopic glucagon receptors (Hansen *et al.* 1998).

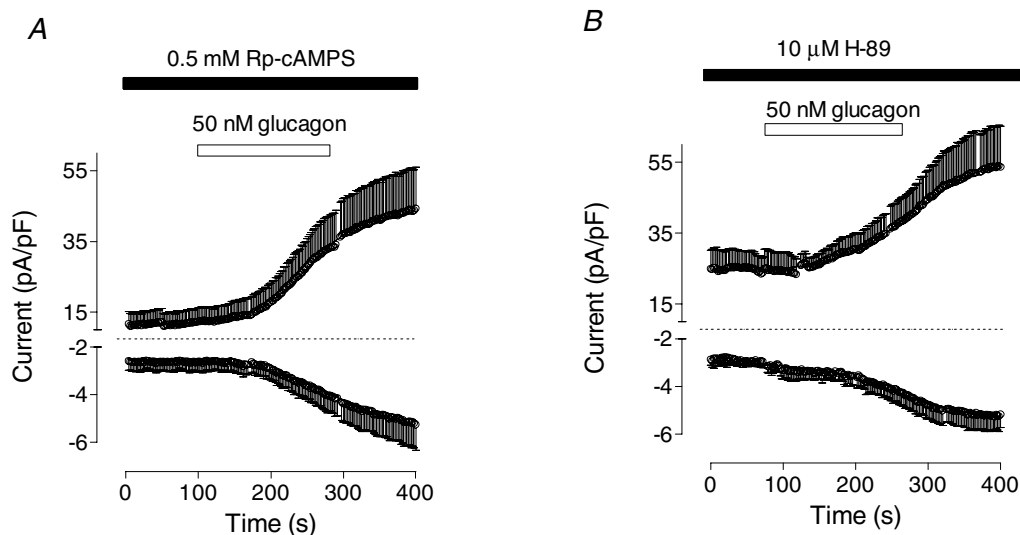
### Identity of $\text{Cl}^-$ channels

Two types of  $\text{Cl}^-$  currents have previously been characterized in rat hepatoma cell line (HTC) liver cells, widely used as model cell line for primary hepatocytes. These are  $\text{Cl}^-$  currents activated by cell swelling, which show inactivation at potentials above +80 mV (Bodily *et al.* 1997; Feranchak *et al.* 2000), and  $\text{Cl}^-$  currents activated by G-proteins, phosphoinositide-3 kinase and  $\text{Ca}^{2+}$ , which show clear activation at positive potentials (Kilic & Fitz, 2002). In the present study,  $\text{Cl}^-$  channels activated by glucagon showed inactivation at potentials above +80 mV

similar to that found in  $\text{Cl}^-$  channels activated by cell swelling. Chloride current could also be suppressed by increasing osmolarity of the external solution. Both the  $\text{Cl}^-$  current activated by glucagon and the  $\text{Cl}^-$  current activated by cell swelling had similar outward rectification and the same selectivity for  $\text{Cl}^-$  over glutamate. Both currents could also be inhibited by NPPB (Meng & Weinman, 1996). Taking all the above together, it is safe to conclude that glucagon activates the same  $\text{Cl}^-$  channels that are activated by hepatocellular swelling.

### Signalling pathways involved in the response to glucagon

In addition to the generation of cAMP induced by the coupling of glucagon receptors to adenylyl cyclase, it is likely that glucagon increases hepatocyte  $\text{IP}_3$ . Thus stimulation by glucagon results in accumulation of small amounts of  $\text{IP}_3$  in hepatocyte plasma membrane preparations, suggesting that it activates PLC (Wakelam *et al.* 1986). Since the concentrations of  $\text{IP}_3$  produced by glucagon are significantly smaller than those produced by the 'classical'  $\text{Ca}^{2+}$ -mobilizing hormone vasopressin, it has been difficult to ascertain the role of a PLC-dependent mechanism in physiological effects of glucagon. It became even more doubtful after some studies reported that  $\text{Ca}^{2+}$ -mobilizing properties of glucagon are mediated by cAMP and not  $\text{IP}_3$  (Kass *et al.* 1994). Although heterologous expression of glucagon receptor in BHK cells showed unequivocally that glucagon does activate PLC and release  $\text{Ca}^{2+}$  from intracellular  $\text{Ca}^{2+}$  stores by producing  $\text{IP}_3$  (Hansen *et al.* 1998), the contribution



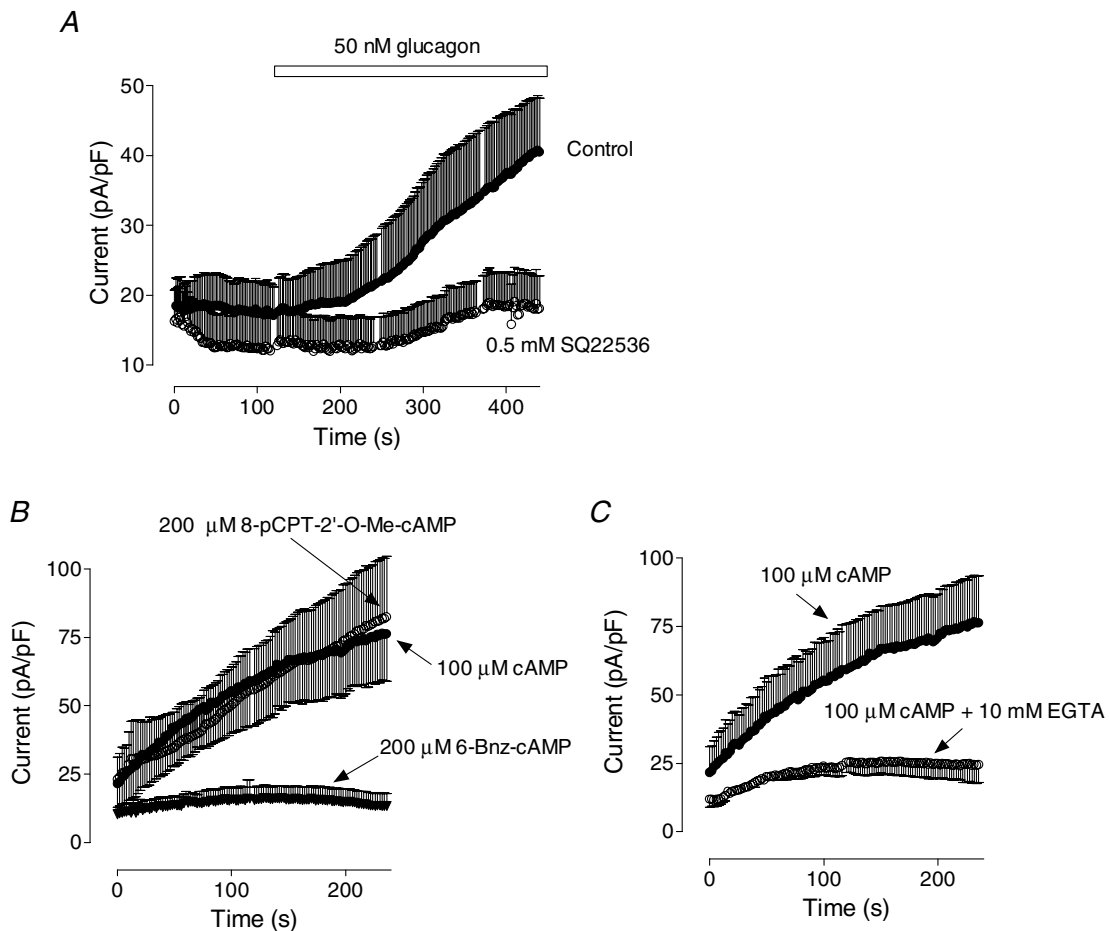
**Figure 9. Inhibition of PKA-dependent pathway has no effect on glucagon-activated conductance**  
 A, effect of Rp-cAMPS (500  $\mu\text{M}$  in the pipette solution) on glucagon response (50 nM;  $n = 3$ ). B, effect of PKA inhibitor, H-89 (10  $\mu\text{M}$ ), on glucagon response (50 nM;  $n = 3$ ). Cells were incubated for 20 min with H-89 before application of glucagon.

of this signalling pathway to the physiological effects of glucagon in hepatocytes remained unresolved, since receptors over-expressed in a heterologous system might not behave in the same way as in their native environment.

The results of the present investigation suggest that activation of PLC by glucagon is a key element in the signalling pathway that leads to activation of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  channels in hepatocytes. It is conceivable that a  $\text{Ca}^{2+}$  current that has properties similar to those of an  $I_{\text{SOC}}$  would be activated by  $\text{IP}_3$  produced by PLC; however, it is possible that another mechanism which includes regulation of channel activity by PLC substrate  $\text{PIP}_2$  is involved (Suh & Hille, 2005). Inability of the PLC inhibitor U73122 to block either swelling-activated  $\text{Cl}^-$  current or  $I_{\text{SOC}}$ , which are similar to those activated by glucagon, argues against direct regulation of these channels

by  $\text{PIP}_2$ . Nevertheless, although the results of the present investigation do implicate PLC in the glucagon response and suggest that it activates a current similar to  $I_{\text{SOC}}$ , the precise mechanism of PLC involvement in the activation of the current is yet to be established.

Since depletion of  $\text{Ca}^{2+}$  stores by  $\text{IP}_3$ - or PLC-coupled hormones, such as ATP and vasopressin, only activates  $I_{\text{SOC}}$  but not the  $\text{Cl}^-$  current (Rychkov *et al.* 2005; also see Fig. 5), it is clear that activation of PLC *per se* does not necessarily lead to activation of  $\text{Cl}^-$  current in rat hepatocytes, and it is likely that other enzymes are involved. It is also clear that increase of intracellular  $\text{Ca}^{2+}$  concentration is insufficient to activate this  $\text{Cl}^-$  current, since only glucagon activates it, but not intracellular  $\text{IP}_3$ , vasopressin, ATP or thapsigargin, which activate  $\text{Ca}^{2+}$  current of similar amplitudes. Nevertheless, several lines of evidence suggest that there is an essential  $\text{Ca}^{2+}$ -dependent



**Figure 10. cAMP activates  $\text{Cl}^-$  conductance in hepatocytes by activating Epac**

A, inhibition of the glucagon response by the inhibitor of adenylyl cyclase SQ22536 ( $n = 4$ ). SQ22536 (0.5 mM) was applied to the bath solution for at least 5 min prior to addition of glucagon. B, effects of cAMP (100  $\mu\text{M}$  in the pipette solution) and its analogues 8-pCPT-2'-O-Me-cAMP (200  $\mu\text{M}$  in the pipette solution) and 6-Bnz-cAMP (200  $\mu\text{M}$  in the pipette solution) on the outward conductance of rat hepatocytes. C, dependence of the cAMP-activated  $\text{Cl}^-$  conductance on intracellular  $\text{Ca}^{2+}$ . Calcium concentration was either weakly buffered to  $\sim 120$  nM by 1 mM EGTA, or strongly buffered to  $\sim 10$  nM by 10 mM EGTA.

step in activation of  $\text{Cl}^-$  current. It is inhibited when  $\text{Ca}^{2+}$  is replaced with either  $\text{Mg}^{2+}$  or  $\text{Ba}^{2+}$ , or when the  $\text{Ca}^{2+}$  current is inhibited by  $\text{La}^{3+}$ . In all cases, decrease in the  $\text{Ca}^{2+}$  current precedes the decline in the  $\text{Cl}^-$  current and, whilst the  $\text{Ca}^{2+}$  current is restored by reintroducing  $\text{Ca}^{2+}$  or washing out  $\text{La}^{3+}$ ,  $\text{Cl}^-$  current is never restored to its initial size. Thus it can be concluded that  $\text{Ca}^{2+}$  plays the role of an important cofactor in the mechanism of  $\text{Cl}^-$  current activation by glucagon, but it does not activate the channel itself. Independence of this  $\text{Cl}^-$  channel of  $\text{Ca}^{2+}$  is obvious when it is activated by cell swelling. While the  $\text{Cl}^-$  current in this case is similar to that activated by glucagon, the mechanism of activation is different, and removal of extracellular  $\text{Ca}^{2+}$  has no effect.

Since neither activation of PLC nor increase in  $[\text{Ca}^{2+}]_i$  alone was able to activate  $\text{Cl}^-$  currents, it was logical to suggest that a cAMP-mediated signalling pathway might be involved in the mechanism of activation of the  $\text{Cl}^-$  conductance by glucagon. Indeed, it has been shown previously that in rat hepatocytes cAMP modulates volume-regulated  $\text{Cl}^-$  current similar to that activated by glucagon here (Meng & Weinman, 1996). Inhibition of the glucagon-activated  $\text{Cl}^-$  current by the inhibitor of adenylyl cyclase SQ22536 supported the notion that cAMP is involved in the mechanism of glucagon response, which was further strengthened by the fact that cAMP alone, applied through the patch pipette, activated  $\text{Cl}^-$  conductance similar to that activated by glucagon. The size of the  $\text{Cl}^-$  current activated by cAMP was significantly smaller if intracellular  $\text{Ca}^{2+}$  was strongly buffered to  $\sim 10 \text{ nM}$  by  $10 \text{ mM}$  EGTA in the pipette, which suggests that at least some of the  $\text{Ca}^{2+}$  dependence of the  $\text{Cl}^-$  current activated by glucagon is due to  $\text{Ca}^{2+}$  dependence of the cAMP-dependent pathway.

Protein kinase A, which is considered the main target for cAMP, did not seem to have any role in glucagon response, since inhibitors of PKA, H-89 and Rp-cAMPS, had no effect on the glucagon-activated currents, and a specific activator of PKA, 6-Bnz-cAMP, unlike cAMP, did not cause any change in membrane conductance of rat hepatocytes. Another analogue of cAMP, 8-pCPT-2'-O-Me-cAMP, that specifically activates Epac (de Rooij *et al.* 1998), fully mimicked the effect of cAMP on the  $\text{Cl}^-$  conductance, providing first evidence that activation of Epac by cAMP may be involved in the membrane response to glucagon.

In conclusion, these results provide explanation for the previously known effects of glucagon on hepatocellular volume and bile flow, and show for the first time that both signal transduction pathways associated with the glucagon receptor (the adenylyl cyclase-dependent pathway and the PLC-dependent pathway) are involved in the mechanism by which glucagon activates  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  channels in rat hepatocytes.

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## Acknowledgements

We would like to thank Rachael Hughes for excellent technical assistance. The Australian Research Council and National Health and Medical Research Council, Australia supported this work.