Modulation of hepatocellular swelling-activated K⁺ currents by phosphoinositide pathway-dependent protein kinase C

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Modulation of hepatocellular swelling-activated K⁺ currents by phosphoinositide pathway-dependent protein kinase C. Am J Physiol Cell Physiol 291: C93–C103, 2006. First published February 1, 2006; doi:10.1152/ajpcell.00602.2005.—K⁺ channels participate in the regulatory volume decrease (RVD) accompanying hepatocellular nutrient uptake and bile formation. We recently identified KCNQ1 as a molecular candidate for a significant fraction of the hepatocellular swelling-activated K⁺ current (Iₖᵥᵒˡ). We have shown that the KCNQ1 inhibitor chromanol 293B significantly inhibited RVD-associated K⁺ flux in isolated perfused rat liver and used patch-clamp techniques to define the signaling pathway linking swelling to Iₖᵥᵒˡ activation. Patch-electrode dialysis of hepatocytes with solutions that maintain or increase phosphatidylinositol 4,5-bisphosphate (PIP₂) increased Iₖᵥᵒˡ, whereas conditions that decrease cellular PIP₂ decreased Iₖᵥᵒˡ. GTP and AIF₃ stimulated Iₖᵥᵒˡ development, suggesting a role for G proteins and phospholipase C (PLC). Supporting this, the PLC blocker U-73122 decreased Iₖᵥᵒˡ and inhibited the stimulatory response to PIP₂ or GTP. Protein kinase C (PKC) (PKC) is involved, because K⁺ current was enhanced by 1-oleoyl-2-acetyl-sn-glycerol and inhibited after chronic PKC stimulation with phorbol 12-myristate 13-acetate (PMA) or the PKC inhibitor GF 109203X. Both Iₖᵥᵒˡ and the accompanying membrane capacitance increase were blocked by cytochalasin D or GF 109203X. Acute PMA did not eliminate the cytochalasin D inhibition, suggesting that PKC-mediated Iₖᵥᵒˡ activation involves the cytoskeleton. Under isometric conditions, a slowly developing K⁺ current similar to Iₖᵥᵒˡ was activated by PIP₂, lipid phosphatase inhibitors to counter PIP₂ depletion, a PLC-coupled α₁-adrenoceptor agonist, or PKC activators and was depressed by PKC inhibition, suggesting that hypotonicity is one of a set of stimuli that can activate Iₖᵥᵒˡ through a PIP₂/PKC-dependent pathway. The results indicate that PIP₂ indirectly activates hepatocellular KCNQ1-like channels via cytoskeletal rearrangement involving PKC activation.

KCNQ1: patch clamp; phosphatidylinositol 4,5-bisphosphate; regulatory volume decrease

Liver cell volume maintenance in the face of nutrient, bile acid, and xenobiotic uptake is mediated by a regulatory volume decrease (RVD) involving K⁺ and anion efflux. Swelling-activated K⁺ efflux and the ensuing choleretic can be induced by exposure of the liver or isolated hepatocytes to hypotonic solutions (4, 27, 43, 44). However, little is known about how the molecular candidate(s) responsible for passive K⁺ flux or the signaling pathways involved in RVD-induced bile formation. Work in our laboratory (27) recently defined conditions for assaying a swelling-activated K⁺ current (Iₖᵥᵒˡ) in rat hepatocytes and identified KCNQ1/KCNE3 as molecular candidates for Iₖᵥᵒˡ. KCNQ1 belongs to the voltage-dependent, outwardly rectifying KCNQ channel family. Functionally, it conducts K⁺ current to increase anion secretion in intestinal cells (38) and is involved in gastric acid secretion in parietal cells (12).

In heterologous expression systems, KCNQ1 is activated by plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) (15, 29, 30, 36, 49), likely through a direct electrostatic interaction with positively charged amino acids in this protein (18, 36). However, it is not yet clear whether this stimulatory effect of PIP₂ on KCNQ1 is valid for native KCNQ1-like channels. For instance, exogenously applied PIP₂ inhibited KCNQ1-like currents in guinea pig atrial myocytes (6). Cardiac KCNQ1 channels also are activated by other factors, including protein kinase C (PKC) (41, 48) and cell swelling (13, 25). Hepatocellular swelling-activated K⁺ efflux involves cytoskeletal changes mediated by p38MAPK (44) and Src (1) signaling pathways, although further details of the signaling pathway linking volume increase to K⁺ channel activation remain unclear. In this respect, we recently found that hepatocellular Iₖᵥᵒˡ required cytoplasmic Mg-ATP (27). Mg-ATP is not necessary for KCNQ channel activity (39), probably through PIP₂ synthesis catalyzed by phosphatidylinositol (PI) 4-kinase and PI5-kinase (17). It is not clear, however, whether PIP₂ is responsible for activation of KCNQ1 by cell swelling.

Although strong evidence suggests that PIP₂ functions as an activator of resting KCNQ1 channels, its effect on swelling-activated KCNQ1 is not yet known. Neither can we exclude the possibility that phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-mediated PIP₂ hydrolysis, such as diacylglycerol or inositol 1,4,5-trisphosphate exert such a role. Interestingly, PLC is activated by hepatocellular swelling (1, 32). The present study was therefore designed to explore the role of the PIP₂/PLC signaling pathway in the modulation of resting and swelling-activated KCNQ1-like K⁺ currents in short-term cultured rat hepatocytes and to determine whether these channels contribute to RVD-induced whole organ K⁺ flux in the intact liver. Our results demonstrate that KCNQ1 channels indeed participate in the RVD-induced K⁺ efflux in the intact liver. Furthermore, PIP₂ indirectly regulates Iₖᵥᵒˡ through a PLC-dependent process involving PKC activation and cytoskeletal rearrangement.

Materials and Methods

Animals and materials. Female Sprague-Dawley rats (200–225 g) were obtained from Charles River (Montreal, QC) and housed under a 12:12-h light-dark regime with access to water and rat chow ad

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libitum. All experimental protocols were approved by the Queen’s University/Canadian Council on Animal Care. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or British Drug Houses (Toronto, ON, Canada) and were of the highest grade available. Chromanol 293B [trans-6-cyano-4-(N-ethyl-sulfonyl-N-methylamino)-3-hydroxy-2,2-dimethylchromane] was a gift from Aventis Pharma (Frankfurt, Germany).

**Rate liver perfusion and whole organ K⁺ flux.** Livers from rats weighing 200–210 g were perfused via the portal vein with Krebs-Henseleit bicarbonate-buffered saline, using a single-pass, constant-flow perfusion system (21). The saline solution was warmed to 37°C, saturated with 95% O₂-5% CO₂ (vol/vol), and perfused at a flow rate of 2.7 ± 0.4 ml.min⁻¹.g⁻¹ liver⁻¹. Tissue viability was achieved by maintaining portal pressure (average 15–17 cmH₂O), O₂ supply, temperature, and buffer pH (7.35–7.40) throughout the perfusion. The Krebs-Henseleit solution contained (in mM) 116.8 NaCl, 25 NaHCO₃, 1.4 NaH₂PO₄, 1 CaCl₂, 1.2 MgSO₄, and 0.9 M K⁺ to increase the driving force for K⁺ efflux. Livers were perfused for 30 min with isotonic Krebs before the introduction of hypotonic Krebs solution, in which the concentration of NaCl was reduced by 40 mM. The established inhibitor of KCNQ1 channels, chromanol 293B, or the vehicle (dimethyl sulfoxide; DMSO) was perfused for 2 min during the hypotonic challenge. Chromanol 293B (1 mM) in DMSO was diluted to 50 μM in the Krebs perfusate.

Effluent perfusate K⁺ was monitored continuously during liver perfusion with a K⁺-selective electrode (Ionplus; Orion Products, Thermo Electron, Beverly, MA). The electrode was calibrated before the start of the perfusion with two standard K⁺ solutions (0.5 and 5 mM K⁺), and set in-line immediately downstream of the hepatic vein. Effluent perfusate K⁺ was monitored and acquired for off-line analysis with the use of a CB-405 connector box and Datacan V acquisition and analysis software from Sable Systems International (Las Vegas, NV). Net K⁺ flux was calculated as the difference between the influent concentration and the effluent and expressed as micromoles per gram of liver per 10-min hypotonic challenge.

**Isolation and culture of rat hepatocytes.** Hepatocytes were isolated using an enzymatic dissociation procedure as described previously (20). Livers were perfused via the portal vein with nominally Ca²⁺-free Krebs solution containing 0.35 mg/ml collagenase (Liberase; Roche Biochemicals, Montreal, QC, Canada) for 7–10 min. After the enzymatic treatment, hepatocytes were dissociated and cultured on glass coverslips in a humidified atmosphere of 95% air-5% CO₂ at 37°C. The culture medium contained DMEM salts plus 0.15% saturated with 95% O₂-5% CO₂ (vol/vol), and perfused at a flow rate of 2.7 ± 0.4 ml.min⁻¹.g⁻¹ liver⁻¹. Tissue viability was achieved by maintaining portal pressure (average 15–17 cmH₂O), O₂ supply, temperature, and buffer pH (7.35–7.40) throughout the perfusion. The Krebs-Henseleit solution contained (in mM) 140 Na-gluconate, 5 K-gluconate, 1.4 NaH₂PO₄, 1 CaCl₂, 1.2 MgSO₄, and 0.9 M K⁺ to increase the driving force for K⁺ efflux. Livers were perfused for 30 min with isotonic Krebs before the introduction of hypotonic Krebs solution, in which the concentration of NaCl was reduced by 40 mM. The established inhibitor of KCNQ1 channels, chromanol 293B, or the vehicle (dimethyl sulfoxide; DMSO) was perfused for 2 min during the hypotonic challenge. Chromanol 293B (1 mM) in DMSO was diluted to 50 μM in the Krebs perfusate. Effluent perfusate K⁺ was monitored continuously during liver perfusion with a K⁺-selective electrode (Ionplus; Orion Products, Thermo Electron, Beverly, MA). The electrode was calibrated before the start of the perfusion with two standard K⁺ solutions (0.5 and 5 mM K⁺), and set in-line immediately downstream of the hepatic vein. Effluent perfusate K⁺ was monitored and acquired for off-line analysis with the use of a CB-405 connector box and Datacan V acquisition and analysis software from Sable Systems International (Las Vegas, NV). Net K⁺ flux was calculated as the difference between the influent concentration and the effluent and expressed as micromoles per gram of liver per 10-min hypotonic challenge.

**Current and capacitance recording.** Single hepatocytes were voltage-clamped using the whole cell configuration of the patch-clamp technique with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and Clampex 7 software as described previously (20). Patch pipettes were made from borosilicate glass (no. 1756A; World Precision Instruments, Sarasota, FL) and had a resistance of 2–4 MΩ when filled and immersed in K⁺-containing solutions. All experiments were performed at 20°C. Membrane capacitance was monitored using Membrane Test software (Clampex 7). Briefly, pipette capacitance was cancelled in cell-attached configuration before membrane rupture. The time course of capacitance development was monitored from a holding potential of 0 mV by repetitively applying voltage steps of 0.1 s in duration to 20 mV. The whole cell membrane capacitance was calculated from the output as described by Lindau and Neher (28). The sampled data were plotted using Origin 6.0 software (Origin Lab, Northampton, MA). The time course of swelling-induced current development was monitored at 0 mV. Currents were mainly K⁺ currents under this condition (27). Whole cell currents were digitized (Digidata 1200B) at 5 kHz, and sampled data were analyzed using Origin 6.0 software. To minimize swelling-activated anion currents, we carried out whole cell recordings using low Cl⁻-containing pipette and bath solutions, and 0.1 mM 4,4'-disothioctanoylstatibene-2,2'-disulfonic acid (Toronto Biochemicals, Toronto, ON), a blocker of volume-activated Cl⁻ channels in rat hepatocytes (26, 31), was included in the bath solution. Cells were bathed in a solution containing (in mM) 140 Na-glucuronate, 5 K-glucuronate, 5 glucose, 5 HEPES, 1 CaCl₂, and 1 MgCl₂ (pH adjusted to 7.4 with NaOH). The isotonic pipette solution contained (in mM) 1 MgCl₂, 1 CaCl₂, 5 HEPES, 4 ATP, 11 EGTA, and 140 K-glucuronate, or 96 mM K-glucuronate when FVPP (a mixture of 4 KF, 3 Na₃VO₄, and 10 K₃PO₄) was added (pH adjusted to 7.2 with KOH). Either 20 or 80 mM rafinose was included in the pipette solution to induce hypotonic stress to activate volume-sensitive K⁺ currents (27). The concentrations of free Ca²⁺ and Mg²⁺ in the pipette solution were calculated to be about 13 nM and 37 μM, respectively (27). Chromanol 293B, phorbol 12-myristate 13-acetate (PMA), cytochalasin D, aminoalkyl-bisindolylmaleimide I (GF 109203X), phenylephrine, or phentolamine was added to the bath solution. Unless otherwise noted, wortmannin, 2-(4-morpholinyl)-8 phenyl-4H-1-benzopyran-4-one (LY-294002), ML-7, PIP₂, U-73122, U-73343, neomycin, AIC₁, poly-i-lysine, diacylglycerol (DAG) analog 1-octanol-2-acetyl-sn-glycero (OAG), arachidonic acid (AA), GTP, PMA, or 100 μM AlF₄⁻ (a mixture of 100 μM AlCl₃ and 10 mM NaF) was added to the pipette solution and was present throughout the recording period. In some experiments, cytochalasin D, PMA, and GF 109203X were added to the culture medium. Chromanol 293B, cytochalasin D, phenylephrine, phentolamine, wortmannin, LY-294002, ML-7, U-73122, U-73343, AA, GF 109203X, and PMA were prepared as 5–100 mM stock solutions in DMSO. In preliminary experiments, we confirmed that DMSO alone did not have any appreciable effect on K⁺ currents at concentrations of up to 0.5%. Neomycin, AIC₁, GTP, AlF₄⁻, and poly-i-lysine were stored at 10–100 mM stock solutions in distilled water. PIP₂ was dissolved in CCH₄·CH₃OH·H₂O (5:5:1, vol/vol/vol) and diluted to 10 μM in the pipette solution before use.

**Data analysis.** Time courses for each experimental condition in which the pattern of time courses was significantly different from that of controls were generated from pooled data from three rats or at least four cells and are presented as means ± SE (patch-clamp data) or means ± SD (perfusion studies) with the number of experiments stated. Histograms were generated from the pooled current or capacitance values reached after 20 min of dialysis with the pipette solution following the initiation of whole cell recording. Statistical comparisons were made using either Student’s paired or unpaired t-tests as appropriate, and differences were considered to be significant at P < 0.05.

**RESULTS**

**KCNQ1 is involved in RVD-induced K⁺ flux in intact liver.** A previous study in our laboratory (27) showed that the swelling-induced K⁺ current Iₚ,K⁺ was partially inhibited by chromanol 293B, a KCNQ1 inhibitor (3). To address the possibility that KCNQ1 also plays a role during swelling-induced RVD in vivo, we examined the effect of chromanol 293B on the K⁺ release during hypotonic challenge in isolated perfused rat livers. Reduction of perfusate osmolarity by removing 40 mM NaCl led within 2 min to a transient increase of the effluent perfusate K⁺ concentration, which peaked within 5 min (Fig. 1A). When 293B (73 ± 9.2 μM) was perfused just before the peak (3 min after the onset of hypotonic exposure), the rate of K⁺ release was decreased at all sampling times and baseline was reached about 1 min earlier than in the control perfusions. As shown in Fig. 1B, under control conditions, net K⁺ flux during the 10-min hypotonic
Reduced the volume regulatory K\(^+\)/H\(_{11001}\) activated a slowly developing K\(^+\) current, \(I_C\), (Fig. 2A) that reached 978 ± 165 pA at 20 min of dialysis (Fig. 2C; \(n = 8\), \(P < 0.01\)), indicating that rat hepatocytes express a high phosphatase activity. Addition of the KCNQ channel inhibitor chromanol 293B (30 \(\mu\)M) to the bath 5–15 min before whole cell attainment significantly inhibited PIP\(_2\)- and FVPP-activated currents at 20 min to 163 ± 52 pA (\(n = 3\), \(P < 0.05\)) and 347 ± 85 pA, respectively (Fig. 2, A and C; \(n = 4\), \(P < 0.01\)). Both PIP\(_2\)- and FVPP-activated currents responded to changes in the K\(^+\) equilibrium potential. These results indicate that a significant fraction of both currents was produced by KCNQ1. Under hypotonic conditions induced by dialysis with 80 mM raffinose (27), 10 \(\mu\)M PIP\(_2\) accelerated the development of

\[\text{HYPOTONIC} \]

\[\text{K}^+\text{ release (\text{mmol/min/g})} \]

\[\text{Time (min)} \]

\[\text{Control} \quad \text{+ 293B} \]

\[\begin{array}{c}
\text{A}
\end{array} \]

\[\text{B} \]

\[\text{Net K}^+\text{ release (\text{mmol/g/min})} \]

\[\text{Control} \quad \text{+ 293B} \]

\[\begin{array}{c}
\text{A}
\end{array} \]

\[\text{Isotonic} \quad \text{Hypotonic} \]

\[\text{Current (\text{pA})} \]

\[\text{Time (min)} \]

\[\text{Control} \quad \text{PIP}_2 \quad \text{FVPP} \quad \text{PIP}_2, \text{FVPP} \quad \text{+ 293B} \quad \text{+ 293B} \quad \text{+ FVPP} \quad \text{+ FVPP} \]

\[\text{C} \]

\[\text{Isotonic} \quad \text{Hypotonic} \]

\[\text{Current (\text{pA})} \]

\[\text{Time (min)} \]

\[\text{Control} \quad \text{PIP}_2 \quad \text{+ 293B} \quad \text{+ FVPP} \quad \text{PIP}_2 \quad \text{+ 293B, FVPP} \quad \text{+ FVPP} \quad \text{+ FVPP, 293B} \]

\[\begin{array}{c}
\text{A}
\end{array} \]

\[\text{Fig. 1. Effect of chromanol 293B on hypotonically induced K}^+\text{ efflux in the perfused rat liver. A: mean time course (±SD) of net K}^+\text{ flux in perfused rat livers before, during, and after a 10-min hypotonic challenge (reduction of perfusate osmolarity by removal of 40 mM NaCl). After 3 min of hypotonic solution, DMSO (control) or chromanol 293B (+293B) was infused for 2 min (33–35 min, open horizontal bar). B: net K}^+\text{ release during the hypotonic period in the absence and presence of 293B. The area under the net K}^+\text{ release curve between 30 and 40 min was calculated for each animal, and the mean ± SD is plotted (3 perfusions per condition).}^*P < 0.05\text{ compared with control group.} \]

\[\text{Fig. 2. Potentiation of resting K}^+\text{ current (}I_{\text{K}}\text{) and swelling-activated K}^+\text{ current (}I_{\text{KVol}}\text{) by internal dialysis with 10 \(\mu\)M phosphatidylinositol 4,5-bisphosphate (PIP}_2\text{) or FVPP (a mixture of 4 KF, 3 Na}_3\text{VO}_4\text{, and 10 K}_4\text{P}_2\text{O}_7\text{). A: mean time courses (±SE) of changes in }I_{\text{K}}\text{ at 0 mV in hepatocytes whole cell dialyzed with control solution containing either 10 \(\mu\)M PIP}_2\text{, 10 \(\mu\)M PIP}_2\text{ plus perfusion with 30 \(\mu\)M 293B, or FVPP. B: mean time courses of changes in }I_{\text{KVol}}\text{ at 0 mV in hepatocytes dialyzed with solution containing 80 mM raffinose in the absence or presence of 10 \(\mu\)M PIP}_2\text{. C: mean current (±SE)}; \text{obtained 20 min after attainment of the whole cell configuration from cells dialyzed with isotonic solution in the absence (isotonic) or presence of 10 \(\mu\)M PIP}_2\text{ or FVPP. Results are from 3–7 cells per condition.}^*P < 0.05; **P < 0.01; ***P < 0.005\text{ compared with control group.} \]

**PIP\(_2\) activates \(I_{\text{KVol}}\).** Earlier work in our laboratory (27) identified KCNQ1 as a candidate for \(I_{\text{KVol}}\) in hepatocytes. PIP\(_2\) activates KCNQ1 in heterologous expression systems (15, 29, 30, 36, 49) but inhibits endogenous KCNQ-like currents in guinea pig atrial myocytes (6). To determine whether PIP\(_2\) modulates hepatocellular K\(^+\) currents under iso- and hypotonic conditions, the latter of which would induce a significant KCNQ-like current, we dialyzed short-term cultured rat hepatocytes with a solution containing 10 \(\mu\)M PIP\(_2\) in the absence or presence of 80 mM raffinose. Under isotonic conditions, outward K\(^+\) currents at 0 mV decayed from 137 ± 63 pA (\(n = 7\)) immediately after attainment of the whole cell configuration to 12 ± 3 pA at 20 min of dialysis with control intracellular solution (Fig. 2, A and C), indicating slow loss of a channel-activating substance. Inclusion of PIP\(_2\) in the pipette solution activated a slowly developing K\(^+\) current, \(I_K\) (Fig. 2A), that reached 416 ± 92 pA at 20 min of dialysis (Fig. 2C; \(n = 4\), \(P < 0.01\)). Our preliminary experiments showed that 10 \(\mu\)M represents a close-to-saturating PIP\(_2\) concentration, because 50 and 100 \(\mu\)M PIP\(_2\) generated the same current amplitude, whereas at <1 \(\mu\)M, PIP\(_2\) did not affect hepatocellular K\(^+\) currents (data not shown). Intracellular dialysis with FVPP, a mixture of phosphatase inhibitors (10) that activates KCNQ2/3 (49) and other channels (18) by decreasing the rate of PIP\(_2\) depletion, also stimulated a similar current (Fig. 2A) that reached 978 ± 165 pA at 20 min of dialysis (Fig. 2C; \(n = 8\), \(P < 0.01\)).
IP$_2$ resynthesis by PI4/5-kinase is necessary for $I_{K_{Vol}}$ activation. We next examined whether $I_{K_{Vol}}$ activation involved PI4/5-kinase-catalyzed synthesis of IP$_2$. At micromolar concentrations, wortmannin inhibits the activity of most PI kinases (33), resulting in a significant decrease of IP$_2$ content (33, 49). In rat hepatocytes, pretreatment with 10 μM wortmannin reduced resting IP$_2$ content by 25% and eliminated recovery from vasopressin-stimulated IP$_2$ metabolism (34). We found that addition of 10 μM wortmannin to the pipette solution containing 80 mM raffinose significantly inhibited the development of $I_{K_{Vol}}$ (Fig. 3A), reaching 252 ± 49 pA at 20 min of dialysis (Fig. 3B; n = 5, P < 0.05). Inclusion of 10 μM IP$_2$ in the pipette solution reduced the inhibitory effect of 10 μM wortmannin, resulting in 437 ± 32 pA of current at 20 min, not significantly different from the hypotonic control (Fig. 3B; n = 4).

Wortmannin at micromolar concentrations also blocks myosin light chain kinase (MLCK) and PI3-kinase (35, 39). If the effect of 10 μM wortmannin reflected an involvement of MLCK rather than PI4-kinase, the MLCK inhibitor ML-7 should also attenuate the development of $I_{K_{Vol}}$ in a similar manner as wortmannin. This was not the case. ML-7 (10 μM) significantly stimulated the development of $I_{K_{Vol}}$, reaching 715 ± 65 pA at 20 min (Fig. 3B). Despite this, 10 μM wortmannin still suppressed $I_{K_{Vol}}$ by 45% in the presence of ML-7 (data not shown). The pronounced inhibition of $I_{K_{Vol}}$ seen with 10 μM wortmannin thus does not appear to result from MLCK inhibition.

At submicromolar concentrations (e.g., 50 nM), wortmannin inhibits PI3- but not PI4-kinase (35). Micromolar LY-294002 also specifically blocks PI3-kinase activity (42). Pretreatment of rat hepatocytes (46) or human hepatoma cells (9) with either 50 nM wortmannin or 10 μM LY-294002 significantly inhibited PI3-kinase activity induced by hypotonic solutions. Inclusion of 50 nM wortmannin in the pipette solution significantly stimulated $I_{K_{Vol}}$ (Fig. 3A), so that at 20 min of dialysis, mean current at 0 mV reached 767 ± 73 pA (Fig. 3B). Similarly, dialysis with 10 μM LY-294002 accelerated $I_{K_{Vol}}$ development (919 ± 132 pA at 20 min; Fig. 3B) compared with the hypotonic control. These results confirm and extend those obtained using the intact liver in which net K$^+$ efflux in response to hypotonic challenge was not affected by nanomolar wortmannin or LY-294002 (44). The combined results suggest that the decrease in $I_{K_{Vol}}$ in the presence of 10 μM wortmannin was primarily mediated by reducing IP$_2$ synthesis by inhibition of PI4-kinase, although additional nonspecific effects cannot be excluded.

IP$_2$ chelators do not inhibit $I_{K_{Vol}}$. Plasma membrane IP$_2$ can directly modulate many ion channels and transporters by an electrostatic interaction with these proteins (18). To further address the possibility that IP$_2$ regulates $I_{K_{Vol}}$ indirectly, as opposed to a direct electrostatic effect at the level of the swelling-activated K$^+$ channel, we examined the effect on $I_{K_{Vol}}$ of internal application of the following polyvalent cation chelators of IP$_2$: neomycin (10 μM), AlCl$_3$ (10 μM), or poly-L-lysine (30 μg/ml). All of these substances interfere with the electrostatic interaction of IP$_2$ and channels (6, 49). Neither AlCl$_3$ nor neomycin significantly affected $I_{K_{Vol}}$ over a 30-min dialysis, reaching 550 ± 120 and 604 ± 123 pA, respectively, at 20 min (Fig. 4A; n = 6, P < 0.05). Surprisingly, poly-L-lysine significantly increased $I_{K_{Vol}}$ by about 2.5-fold to 1,258 ± 159 pA at 20 min (Fig. 4, A and B; n = 8, P < 0.05). Poly-L-lysine activates PLC in rat hepatocytes (14), raising the possibility that poly-L-lysine stimulates $I_{K_{Vol}}$ through PLC activation. To test this possibility, we added 10 μM U-73122 to the pipette solution containing poly-L-lysine. U-73122 significantly decreased poly-L-lysine-stimulated $I_{K_{Vol}}$ by 44% to 821 ± 81 pA at 20 min of dialysis (Fig. 4, A and B; n = 6, P < 0.05). These results suggest that IP$_2$ stimulation of $I_{K_{Vol}}$ does not occur through a direct electrostatic interaction with this channel; rather, this occurs through a process involving IP$_2$ metabolism.

PLC activation is required for $I_{K_{Vol}}$. Cellular hydrolysis of IP$_2$ is mainly carried out by PLC and PI3-kinase. Hepatocellular PLC is activated by cell swelling (32). We hypothesized that inhibition of the swelling-activated PLC and consequent inhibition of IP$_2$ metabolism should inhibit the development

![Fig. 3. Effects of lipid and myosin light chain kinases inhibitors on $I_{K_{Vol}}$ development. A: mean time courses (±SE) of changes in $I_{K_{Vol}}$ at 0 mV in hepatocytes dialyzed with a solution containing 80 mM raffinose in the absence or presence of 10 or 0.05 μM wortmannin. B: mean current (±SE) obtained 20 min after whole cell configuration from cells dialyzed with the raffinose solution in the absence (hypotonic) or presence of 10 μM wortmannin [± WMN (10)], 10 μM wortmannin and 10 μM IP$_2$ [± PI3], 10 μM ML-7 [± ML-7], 0.05 μM wortmannin [± WMN (0.05)], or 10 μM LY-294002 [± LY294002]. Results are from 4–8 cells per condition. *P < 0.05 compared with hypotonic group.](http://ajpcell.physiology.org/10.2306/ajpcell.291.4.715)
of $I_{K_{VOL}}$ in rat hepatocytes. To test this idea, we dialedized cells with the PLC inhibitor U-73122 in the presence of 80 mM raffinose. U-73122 (10 μM) reduced the development of $I_{K_{VOL}}$ (Fig. 5A) to 154 ± 41 pA at 20 min of whole cell recording (Fig. 5B). Inclusion of 10 μM PIP2 in the pipette solution containing 10 μM U-73122 increased $I_{K_{VOL}}$ to 344 ± 80 pA, about 42% of the control (Fig. 5, A and B; $n = 5$, $P < 0.05$), suggesting that U-73122 reduced $I_{K_{VOL}}$ by inhibiting PIP2 metabolism and that the latter could be partially recovered by exogenously applied PIP2. Inhibition by U-73122 was specifically due to PLC blockade, because U-73343, a structural analog of U-73122 lacking PLC inhibitory activity, did not significantly affect $I_{K_{VOL}}$ (Fig. 5B). These results show that decreasing the rate of PIP2 hydrolysis catalyzed by PLC does not enhance but, rather, reduces $I_{K_{VOL}}$, implying that PIP2 regulates $I_{K_{VOL}}$ through a PLC-dependent process.

Enhancement of PLC activity stimulates $I_K$ and $I_{K_{VOL}}$. To further test the proposal that increased PLC activity favors the development of $I_{K_{VOL}}$, we assessed the effect of PLC-linked G protein activation, either directly or via the G protein-coupled $\alpha_1$-adrenergic receptor. Initially, we recorded the effects of G protein activators on resting $I_K$. This resulted in the development of a slowly developing outward current (Fig. 6A) that reached 465 ± 63 pA at 20 min of dialysis (Fig. 6A; $n = 7$, $P < 0.01$). This GTP-enhanced $I_K$ was inhibited by the intracellular application of 10 μM U-73122 (Fig. 6A), decreasing $I_K$ to 195 ± 39 pA at 20 min after initiation of whole cell recording (Fig. 6C; $n = 4$, $P < 0.05$), implying that PLC activation by G proteins mediates $I_K$. Although GDP (0.1 mM) also significantly increased $I_K$ from basal to 144 ± 30 pA, this was significantly less than the response to the same concentration of GTP (Fig. 6C; $n = 4$, $P < 0.05$). We previously reported that the $\alpha_1$-adrenoceptor agonist phenylephrine stimulated $I_K$ to 774 ± 83 pA at 20 min of dialysis (Fig. 6C; $n = 4$, $P < 0.05$). Incubation of the cells with phenolamine (10 μM), an $\alpha_1$-adrenoceptor antagonist, before phenylephrine inhibited the agonist-induced response by 70% (260 ± 33 pA at 20 min) (Fig. 6C; $n = 7$, $P < 0.05$), indicating that

Fig. 5. U-73122 but not U-73343 inhibits $I_{K_{VOL}}$ development. A: mean time courses (±SE) of changes in $I_{K_{VOL}}$ at 0 mV in hepatocytes dialyzed with a solution containing 80 mM raffinose in the absence (○) or presence of 10 μM U-73122 (■) or 10 μM U-73122 and 10 μM PIP2 (□). B: mean current (±SE) obtained 20 min after whole cell configuration from cells dialyzed with the raffinose solution in the absence (hypotonic) or presence of 10 μM U-73122 (+ U-73122), 10 μM U-73122 and 10 μM PIP2 (+ PIP2), or 10 μM U-73343 (+ U73343). Results are from 6–7 cells per condition. *$P < 0.05$ compared with hypotonic group.

Fig. 4. Effect of PIP2 chelators on $I_{K_{VOL}}$. A: mean current (±SE) obtained 20 min after whole cell configuration from cells dialyzed with raffinose solution in the absence (hypotonic) or presence of 10 μM AlCl3 (+ AlCl3), 10 μM neomycin (+ Neomycin), 30 μg/ml poly-L-lysine (+ poly-lysine), or 30 μg/ml poly-L-lysine and 10 μM U-73122 (+ U73122). Results are from 6–8 cells per condition. *$P < 0.05$ compared with hypotonic group. B: mean time courses (±SE) of changes in $I_{K_{VOL}}$ at 0 mV in hepatocytes dialyzed with a solution containing 80 mM raffinose in the absence (○) or presence of 30 μg/ml poly-L-lysine (■), or 30 μg/ml poly-L-lysine and 10 μM U-73122 (□).
phenylephrine stimulated $I_K$ via its specific $\alpha_1$-adrenoceptor activity. Furthermore, the $\alpha_1$-adrenergic response was decreased to 421 $\pm$ 25 pA when the cells were dialyzed with U-73122 (Fig. 6C; $n = 4$, $P < 0.05$). These results suggest that PLC activation by G proteins and G protein-coupled receptors stimulates hepatocellular $I_K$.

The effect of G protein-mediated PLC activation on $I_{\text{KVol}}$ was investigated in rat hepatocytes dialyzed with 80 mM raffinose. Intracellular application of GTP (0.1 mM) significantly enhanced the rate and extent of $I_{\text{KVol}}$ activation (Fig. 6B), reaching 885 $\pm$ 83 pA at 20 min of dialysis (Fig. 6C; $n = 10$, $P < 0.05$). Dialysis with the same concentration of GDP did not significantly affect the time course of $I_{\text{KVol}}$ development (559 $\pm$ 110 pA at 20 min) (Fig. 6C; $n = 7$, $P < 0.05$). In addition, GTP-stimulated $I_{\text{KVol}}$ was further increased to 1,197 $\pm$ 81 pA at 20 min by the intracellular application of AlF$_4^-$ (0.1 mM) (Fig. 6C; $n = 7$, $P < 0.05$), which directly reacts with G proteins to increase enzyme activity (2). These results show that enhancement of G protein activity contributes to the development of $I_{\text{KVol}}$ in the rat hepatocyte. To assess whether PLC is involved in GTP-activated $I_{\text{KVol}}$, we included U-73122 in the pipette solution containing GTP. U-73122 decreased the GTP-activated $I_{\text{KVol}}$ to control levels (560 $\pm$ 68 pA at 20 min of dialysis) (Fig. 6, B and C; $n = 6$). These results indicate that enhancement of PLC activity by G proteins contributes to $I_{\text{KVol}}$ in rat hepatocytes.

DAergic-dependent PKC is involved in $I_K$ and $I_{\text{KVol}}$ activation. PLC hydrolyzes PIP$_2$ to DAG and inositol 1,4,5-trisphosphate (IP$_3$). Earlier observations in our laboratory (27) showed that increased cytosolic Ca$^{2+}$ is not involved in $I_{\text{KVol}}$, suggesting that IP$_3$ does not play a major role in channel activation. DAG activates PKC and hence may activate $I_K$ and $I_{\text{KVol}}$ via generation of downstream products of PLC activity. Because DAG can alter the gating of some cation channels, including KCNQ1 (23), we dialyzed rat hepatocytes with the water-soluble DAG analog OAG to determine whether DAG stimulates $I_K$ and $I_{\text{KVol}}$. OAG (10 $\mu$M) increased $I_K$ to 459 $\pm$ 70 pA at 20 min (Fig. 7; $n = 5$, $P < 0.01$), indicating that OAG can activate KCNQ1-like channels in the rat hepatocytes. To test the possibility that OAG directly associates with and activates the channel, we dialyzed hepatocytes in the isotonic bath solution with OAG in the absence of ATP to eliminate any residual kinase activity. Under these conditions, outward current at 20 min (98 $\pm$ 13 pA) was significantly lower than that recorded in the presence of OAG and ATP (Fig. 7; $n = 4$, $P < 0.01$). These results suggest that OAG activates $I_K$ in an ATP-dependent process that does not involve direct association of OAG with
the channel. To determine whether the stimulatory effect of OAG on $I_k$ was due to the activation of PKC, we pretreated cells with GF 109203X, a highly selective inhibitor of multiple PKC subtypes (40). The addition of GF 109203X (2 μM) to the bath solution inhibited the OAG-induced increase in $I_k$ (139 ± 26 pA; Fig. 7; n = 4, P < 0.05). These results suggest that the OAG stimulation of $I_k$ may be a consequence of PKC activation.

In contrast to $I_k$, neither 10 nor 50 μM OAG significantly affected $I_{K_{\text{vol}}}$ induced by 80 mM raffinose (420 ± 33 or 594 ± 84 pA, respectively, at 20 min; Fig. 7; n = 6 and 5). This raised the possibility that cell swelling induced with 80 mM raffinose is associated with significant, perhaps saturating, endogenous DAG synthetic activity so that exogenous activators have no additional effect. To test this proposal, we applied 20 mM raffinose, rather than 80 mM, to decrease the level of cell swelling. At 20 mM, raffinose increased $I_{K_{\text{vol}}}$ to 340 ± 77 at 20 min of dialysis, or 70% of the response to 80 mM raffinose (Fig. 7; n = 4, P < 0.05). OAG further increased the 20 mM raffinose-induced $I_{K_{\text{vol}}}$ to 525 ± 20 pA at 20 min of dialysis (Fig. 7; n = 4, P < 0.05).

AA, a product of DAG lipase-catalyzed hydrolysis of DAG, is involved in the activation of *Drosophila* light-sensitive transient receptor potential channels (5). To establish whether DAG activates the hepatocellular $I_{K_{\text{vol}}}$ in a similar process, we added 10 μM AA to pipette solutions containing 80 mM raffinose. $I_{K_{\text{vol}}}$ induced by either 80 (Fig. 7; n = 5, P < 0.05) or 20 mM raffinose (data not shown) was not significantly affected by AA, indicating that AA is not necessary for $I_{K_{\text{vol}}}$ development in rat hepatocytes.

Involvement of PKC in the OAG-induced stimulation of $I_k$ and $I_{K_{\text{vol}}}$ was further evaluated with an activator and an inhibitor of PKC. Intracellular application of the PKC activator PMA (10 μM) slowly activated $I_k$ with a time course mimicking that observed with OAG. Pretreating the hepatocytes for at least 20 min with GF 109203X (2 μM) significantly inhibited the PKA response (Fig. 8A). PMA-stimulated $I_k$ (421 ± 70 pA at 20 min; n = 4, P < 0.01 compared with isoprenaline control) was decreased to 105 ± 19 pA at 20 min (n = 4, P < 0.05) in GF 109203X-treated cells (Fig. 8C). PKC is activated and transferred from the cytosol to the plasma membrane by cell swelling and metabolic stress in hepatoma cells and a cholangiocarcinoma line (37, 45). To test whether PKC mediates cell swelling-induced $I_{K_{\text{vol}}}$, we included 10 μM PMA in the pipette solution with 80 mM raffinose. This acute exposure to PMA did not significantly affect $I_{K_{\text{vol}}}$ (502 ± 27 pA at 20 min; Fig. 8C; n = 6), similar to the observations by Grunnet et al. (13) in *Xenopus* oocytes.

PKC is activated by acute exposure to micromolar concentrations of PKC, whereas longer exposures (18 h) inactivate the kinase (37). Furthermore, chronic exposure to PMA decreased cell swelling- and metabolic stress-induced PKC translocation in hepatoma and cholangiocarcinoma cells (37, 45). We used this method to confirm the involvement of PKC in $I_{K_{\text{vol}}}$ development in rat hepatocytes. Cells were pretreated with PMA (0.1 μM) or its vehicle, DMSO, for at least 18 h before whole cell recording. Pretreatment of cells with PMA did not have any significant effect on $I_k$ (data not shown). In contrast, PMA significantly attenuated $I_{K_{\text{vol}}}$ (Fig. 8B), attaining only 45% (221 ± 42 pA; Fig. 8C; n = 8, P < 0.05) of the control amplitude at 20 min of dialysis. Exposure to GF 109203X had a similar inhibitory effect on $I_{K_{\text{vol}}}$ as the 18-h PMA incubation, with mean current reaching 252 ± 31 pA at 20 min (Fig. 8C; n = 4, P < 0.05 relative to hypotonic control). These data show that PKC plays an important role in the development of $I_{K_{\text{vol}}}$.

$I_{K_{\text{vol}}}$ development involves cytoskeleton and PKC. As shown earlier, $I_{K_{\text{vol}}}$ in rat hepatocytes develops slowly over minutes of dialysis with 80 mM raffinose, suggesting that channel recruitment to the plasma membrane occurs in response to an as yet only partially defined series of events. To investigate whether the rat hepatocyte $I_{K_{\text{vol}}}$ involves a vesicular-to-plasma membrane flux of membrane constituents containing $K^+$ channels, we measured outward $K^+$ current at 0 mV (Fig. 9A) and cell capacitance as a monitor of plasma membrane area (Fig. 9B). Pooled data from these experiments at 20 min of dialysis are illustrated in Fig. 9C. Under isotonic conditions membrane capacitance slowly declined about 4 pF over 20 min from 23 ± 0.9 to 18 ± 1.6 pF (Fig. 9B). Conversely, membrane capacitance...
PKC is involved in the recruitment of a volume-sensitive vesicular pool to a readily releasable state in a human cholangiocarcinoma cell line (11), and Ca\(^{2+}\)-independent PKC isoforms mediate trafficking of the bile salt export protein to the canalicular membrane in HepG2 cells and rat hepatocytes (24). Stimulation of PKC activity by acute exposure to 10 \(\mu\)M PMA did not significantly affect capacitance (25 \(\pm\) 0.8 pF at 20 min; Fig. 9C; \(n = 6\)), similar to the observations by Gatof et al. (11) in cholangiocytes. However, both chronic exposure to 0.1 \(\mu\)M PMA and perfusion with GF 109203X reduced raffinose-induced capacitance and current increase (PMA, 22 \(\pm\) 0.5 and 221 \(\pm\) 42 pA; GF 109203X, 22 \(\pm\) 0.8 and 252 \(\pm\) 31 pA at 20 min; Fig. 9C; \(P < 0.05\) relative to hypotonic control), indicating that PKC is involved in channel recruitment.

Grunnet et al. (13) reported that activation of KCNQ1 by hypotonic solutions was inhibited by the microfilament polymerization blocker cytochalasin D. Pretreatment of the cultured cells with cytochalasin D (2 \(\mu\)M) for 2–6 h significantly depressed \(I_{K\text{Vol}}\) (Fig. 9A) to a mean current of 251 \(\pm\) 49 pA at 20 min (Fig. 9C; \(n = 4\), \(P < 0.05\)). Cytochalasin D also blocked the raffinose-induced capacitance increase (Fig. 9B), reaching 21 \(\pm\) 0.7 pF at 20 min (Fig. 9C), which was not significantly different from isotonic conditions. Acute exposure of hepatocytes to 10 \(\mu\)M PMA did not rescue the cells from the inhibitory effects of cytochalasin D (22 \(\pm\) 0.6 and 241 \(\pm\) 56 pA at 20 min; Fig. 9C). These results suggest that the swelling-induced development of \(I_{K\text{Vol}}\) involves an F-actin-dependent recruitment process in addition to PKC-dependent activation.

**DISCUSSION**

The present study identified PIP\(_2\) as a second messenger in the intracellular pathway leading to the activation of KCNQ1-like currents by cell swelling in short-term cultured rat hepatocytes. Our results suggest that PIP\(_2\) (maintained through PI kinase synthetic activities) hydrolysis mediated by PLC sustains the PKC activity requisite for the development of \(I_{K\text{Vol}}\) (Fig. 10). This conclusion is based on the observations that tance did not significantly change from 25 \(\pm\) 0.6 pF over 20 min of dialysis with 80 mM raffinose. Thus hypotonic conditions caused a 4-pF increase in cell capacitance relative to isotonic solutions.

![Fig. 9. Temporal relationship between \(I_{K\text{Vol}}\) development and membrane capacitance change in response to hypotonic solutions. Mean time courses \((\pm\)SE) of changes in current at 0 mV (A) and membrane capacitance (B) of hepatocytes dialyzed with isotonic (C) or hypotonic solutions (80 mM raffinose) after incubation for 6–9 h in the absence (○) or presence of 2 \(\mu\)M cytochalasin D (●). C: mean current and capacitance \((\pm\)SE) obtained 20 min after whole cell configuration for cells dialyzed with either isotonic or hypotonic solutions. Some cells dialyzed with raffinose were bathed in extracellular solution containing 10 \(\mu\)M PMA (10 PMA) or 2 \(\mu\)M GF 109203X (GF) or were pretreated (18 h) with 0.1 \(\mu\)M wortmannin but not by 50 nM wortmannin or LY-294002. PIP\(_2\), in turn, contains the PKC activity requisite for the development of \(I_{K\text{Vol}}\).

![Fig. 10. Model showing proposed relationship between the PIP\(_2\) signaling pathway and hepatocellular swelling-activated KCNQ1-like channel activation. Hepatocellular swelling in response to nutrient/bile salt accumulation is associated with the activation of phosphatidylinositol 4/5-kinases (PI4/5K) to synthesize PIP\(_2\) from phosphatidylinositol (PI). This synthesis is inhibited by 10 \(\mu\)M wortmannin but not by 50 nM wortmannin or LY-294002. PIP\(_2\), in turn, is hydrolyzed by U-73122-sensitive PLC. PLC can also be activated by \(G_q\) protein (via dialysis with GTP or AlF\(_3\) or activation of \(G_q\) protein-coupled receptor stimulation by, for example, phenylephrine). PLC activation leads to generation of inositol 1,4,5-trisphosphate (IP\(_3\)) and DAG and activation of PKC. PKC activity is stimulated by acute exposure to 10 \(\mu\)M PMA but inhibited by chronic treatment with 0.1 \(\mu\)M PMA or GF 109203X. PKC is proposed to activate chromanol 293B-sensitive KCNQ1-like channels by either direct phosphorylation of the channel at multiple potential sites, indicated schematically by the single “P,” or indirectly by supporting trafficking of channel-containing vesicles to the plasma membrane assisted by the F-actin cytoskeleton. The latter process is inhibited by cytochalasin D. The resulting increase in KCNQ1-like channel activity results in increased \(K^+\) efflux, which, accompanied by anion efflux, leads to regulatory volume decrease (RVD).
stimulation of PLC and PKC activities increased the amplitude and rate of development of \( I_{K_{Vol}} \), whereas manipulations that would depress PLC and/or PKC inhibited \( I_{K_{Vol}} \). Additional results from membrane capacitance measurement and whole organ \( K^+ \) flux studies indicate that cytoskeletal reorganization, potentially mediating a channel recruitment process, is requisite for PKC activation of hepatocellular \( I_{K_{Vol}} \) and that KCNQ1 contributes significantly to this \( K^+ \) flux in the intact liver.

\( \textit{PIP}_2 \) is necessary for \( I_{K_{Vol}} \). \( \textit{PIP}_2 \) is reported to either stimulate human KCNQ1 when heterologously expressed (15, 30, 36, 49) or inhibit KCNQ1-like currents in guinea pig atrial myocytes (6). The present results demonstrate that an increase in cellular \( \textit{PIP}_2 \) concentration stimulates both resting and swelling-activated KCNQ1-like \( K^+ \) currents in primary cultures of rat hepatocytes. Specifically, procedures designed to increase intracellular \( \textit{PIP}_2 \) increased both \( I_k \) and \( I_{K_{Vol}} \) (dialysis with \( \textit{PIP}_2 \) or inhibitors of lipid phosphatases or PI3-kinase) (Figs. 2 and 3), whereas conditions that decrease cellular \( \textit{PIP}_2 \) synthesis (inhibition of PI4-kinase) decreased \( I_{K_{Vol}} \) (Fig. 3). PI4-kinase-mediated \( \textit{PIP}_2 \) synthesis is reported to be necessary for KCNQ2 and KCNQ3 activities (39, 49), in addition to other cation channels and transporters (18). Supporting the proposal that \( \textit{PIP}_2 \) synthesis is also required for hepatocellular \( I_{K_{Vol}} \) activation, we showed that these currents are blocked by micromolar but not nanomolar concentrations of wortmannin (Fig. 3). A 15-min exposure of intact rat hepatocytes to 10 \( \mu \text{M} \) wortmannin depresses steady-state plasma membrane \( \textit{PIP}_2 \) by \( \sim 35\% \) (34). The inhibitory effects of micromolar wortmannin on \( I_{K_{Vol}} \) do not reflect inhibition of PI3-kinase or MLCK, because blockers of these enzymes, LY-294002, 50 \( \mu \text{M} \) wortmannin, or ML-7, did not mimic the effect of 10 \( \mu \text{M} \) wortmannin (Fig. 3). In addition, inhibition of \( I_{K_{Vol}} \) by 10 \( \mu \text{M} \) wortmannin was attenuated by intracellular application of \( \textit{PIP}_2 \) (Fig. 3). The combined results suggest that PI4-kinase-catalyzed \( \textit{PIP}_2 \) synthesis supports the development of \( I_{K_{Vol}} \). An explanation for the noted difference in \( \textit{PIP}_2 \) effects on KCNQ1 in hepatocytes and multiple heterologous expression systems compared with guinea pig atrial myocytes (6) may reside in a species and/or cell type in which the channel is expressed.

\( \textit{PIP}_2 \) activates \( I_{K_{Vol}} \) indirectly through a PLC-sensitive process. Because \( \textit{PIP}_2 \) can be hydrolyzed by PLC, \( I_{K_{Vol}} \) activation by \( \textit{PIP}_2 \) may occur through either a direct electrostatic interaction with the hepatocellular KCNQ-like protein or a product of its hydrolysis by PLC. Our results (Fig. 4) show that \( I_{K_{Vol}} \) was not inhibited by molecules (neomycin, \( \text{AlCl}_3 \), poly-L-lysine) that interfere with the electrostatic interaction of \( \textit{PIP}_2 \) with KCNQ/KCNE channels (6, 49). Furthermore, plasma membrane \( \textit{PIP}_2 \) concentration was not significantly affected by G protein-coupled receptor-linked PLC activity in both rat hepatocytes due to the resynthesis of \( \textit{PIP}_2 \) by PI4-kinase (34) or tobacco pollen cells exposed to hypotonic solutions (50). Therefore, it seems unlikely that \( \textit{PIP}_2 \), at least as a signaling molecule, plays a direct role in the activation of \( I_{K_{Vol}} \). It is noted that these experiments do not exclude the possibility that \( \textit{PIP}_2 \) may be tightly bound to the \( I_{K_{Vol}} \) channels, making them insensitive to both \( \textit{PIP}_2 \) chelation during whole cell recording and \( \textit{PIP}_2 \) concentration fluctuations in vivo.

Hypotonic swelling of rat hepatoma cells is associated with U-73122-sensitive PLC activation (32). We found that U-73122, but not its inactive isomer U-73343, inhibited \( I_{K_{Vol}} \) development in primary cultures of rat hepatocytes (Fig. 5). In addition, U-73122 inhibited both \( \textit{PIP}_2 \)-stimulated resting and swelling-induced \( K^+ \) currents (Fig. 5). These results imply that a threshold concentration of \( \textit{PIP}_2 \) is required to sustain PLC activity to activate KCNQ1-like channels. Supporting the proposal, GTP stimulated the rate and extent of \( I_{K_{Vol}} \) development; this was enhanced with \( \text{AlF}_4^- \), and these activities were attenuated by U-73122 (Fig. 6). In resting cells, a slowly developing \( K^+ \) current similar to \( I_{K_{Vol}} \) was activated by dialysis with 0.1 mM GTP or the PLC-coupled adrenoceptor agonist phenylephrine (Fig. 6), supporting our hypothesis that this conductance appears in response to conditions that stimulate PLC activity.

\( \textit{PIP}_2 \) stimulates hepatocellular \( K^+ \) currents through a PKC-dependent process. Activation of PLC results in accumulation of IP3 and the PKC activator DAG in rat hepatocytes. IP3 (and increased cytosolic Ca\(^{2+}\)) is not responsible for RVD-induced \( K^+ \) flux in the perfused rat liver (43) or activating \( I_{K_{Vol}} \) in isolated rat hepatocytes (27). Although DAG can be generated by PLA\(_2\), this pathway is not likely involved in \( I_{K_{Vol}} \) development, because inhibition of this phospholipase does not affect RVD-induced \( K^+ \) efflux from the perfused rat liver (43). AA and other DAG metabolites also are not involved in activating \( I_{K_{Vol}} \) in rat hepatocytes or RVD-induced \( K^+ \) flux in the perfused rat liver (43). Several of our observations support an important role for PKC in the process of \( K^+ \) current activation in rat hepatocytes. First, the DAG analog OAG stimulated \( K^+ \) currents in isotonically bathed cells (\( I_k \)) in an ATP-dependent and PKC inhibitor-sensitive manner and enhanced \( I_{K_{Vol}} \) induced by weakly hypotonic solutions (Fig. 7). Second, acute exposure to the PKC activator PMA activated \( I_k \), and activation was inhibited by pretreatment with GF 109203X (Fig. 8). And last, long-term treatment of cells with PMA to downregulate endogenous PKC activity and GF 109203X attenuated hypotonically induced \( I_{K_{Vol}} \) (Fig. 8). This PKC is likely to be a Ca\(^{2+}\)-insensitive subtype, because human cardiac KCNQ1 was activated by Ca\(^{2+}\)-insensitive PKC (48), and the development of \( I_{K_{Vol}} \) in rat hepatocytes (27) and RVD-induced \( K^+ \) flux in rat liver (43) are Ca\(^{2+}\)-independent.

The events underlying PKC induction of hepatocellular \( I_{K_{Vol}} \) may include either a direct activation via channel phosphorylation or indirect activation by way of F-actin disassembly and recruitment of channels to the plasma membrane. Supporting the first possibility, KCNQ1 can be directly activated by PKC protein, because KCNQ1 has conserved PKC phosphorylation sites in its COOH terminus (23, 41), OAG stimulates human KCNQ1/KCNE1 (23), and PMA activated the resting \( I_k \) in the present study (Fig. 8). Nevertheless, changes in the structure of the F-actin cytoskeleton are a prerequisite for swelling-induced ion channel activity (1, 7, 8, 13), probably through a process of vesicle-mediated insertion and retrieval of proteins (7). This process is enhanced by PMA (11) and impaired by mutation of PKC activity (16), implying that PKC is a critical regulatory element for efficient cytoskeleton-mediated membrane protein sorting. Our results further suggest that PKC-induced \( I_{K_{Vol}} \) activation is dependent on recruitment of channels to the plasma membrane, because PMA was unable to eliminate the inhibitory effect of cytochalasin D on the development of \( I_{K_{Vol}} \) and the swelling-induced membrane capacitance increase (Fig. 9).

\textbf{Physiological implications.} Several types of \( K^+ \) channels have been proposed as the molecular entities mediating the
swelling-activated K⁺ current associated with RVD in different tissue, and work in our laboratory recently identified KCNQ1 as a significant participant of this current in isolated rat hepatocytes (27). In the present study, we extended earlier results by demonstrating that KCNQ1 channels provide a significant fraction of the K⁺ flux associated with RVD in the intact perfused rat liver (Fig. 1). To our knowledge, this is the first report that provides evidence for the role of KCNQ1 in RVD-induced K⁺ flux in vivo. RVD and whole organ K⁺ flux have been correlated with an increase in bile flow and bile acid excretion (4). RVD dysfunction induced by K⁺ flux disruption is associated with cholestasis (4), toxin-induced hepatocellular injury (45), and ischemia related to hypoperfusion or organ preservation (22). We speculate that disturbance of the normal development of the hepatocellular volume-regulated K⁺ current may play a fundamental role in the pathophysiological consequences of liver dysfunction.

In summary, we have presented functional evidence for KCNQ1-like channels as significant contributors to RVD-induced K⁺ efflux in the intact perfused rat liver. Pharmacological and electrophysiological evidence reported supports the view that PIP₂ indirectly mediates the development of hepatocellular IₖVol through a PKC-dependent process and that PKC stimulation of IₖVol requires cytoskeletal rearrangement resulting in increased numbers of functional K⁺ channels in the plasma membrane (Fig. 10). Recent evidence demonstrates that the normal trafficking to the plasma membrane of KCNQ1 is disrupted in channels bearing mutations associated with disease in nonhepatic tissue (47). The molecular identification of KCNQ1/KCNE3 in rat and human liver (27) and characterization of the signaling pathways involved in IₖVol activation reported should permit discovery of ways in which to manipulate channel activity and ameliorate specific liver dysfunctions.

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