**Mg^{2+}**- and MgATP-inhibited and Ca^{2+}/calmodulin-sensitive TRPM7-like current in hepatoma and hepatocytes

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Mishra R, Rao V, Ta R, Shobeiri N, Hill CE. Mg^{2+} and MgATP-inhibited and Ca^{2+}/calmodulin-sensitive TRPM7-like current in hepatoma and hepatocytes. Am J Physiol Gastrointest Liver Physiol 297: G687–G694, 2009. First published August 6, 2009; doi:10.1152/ajpgi.90683.2008.—Although understood to be ubiquitously expressed, the functional identification and significance of Mg^{2+}-inhibited, nonselective cation currents has been established in only a few cell types. Here we identified an outwardly rectifying nonselective cation current in quiescent rat hepatocytes and the proliferating and polarized rat hepatoma, WIF-B. Under whole-cell recording conditions in which cells were bathed and dialyzed with Na-glucuronate solutions, the latter Ca^{2+} and Mg^{2+} free, current reversed close to 0 mV, was time independent, and was greater than 10 times higher at +120 mV compared with +120 mV. Outward current at +120 mV developed slowly, from 17.7 ± 10.3 pA/pF at patch rupture to 106.6 ± 15.6 pA/pF at 12 min in WIF-B cells, and 4.9 ± 2.7 to 20.6 ± 5.6 pA/pF in rat hepatocytes. The nonspecific TRP channel inhibitor, 2-aminoethoxyphenylborate (2-APB), inhibited current (IC_{50} = 72 ± 13 µM) and caused apoptotic cell death in WIF-B cells. Rat hepatocyte survival was more resistant to 2-APB. Dialysis of WIF-B cells with physiological concentrations of Mg^{2+} and Mg-ATP, but not ATP alone, inhibited current development, suggesting that Trpm7 rather than Trpm6 underlies this current. RT-PCR demonstrated that both Trpm6 and Trpm7 are expressed at similar levels in both cell types, suggesting that the functional differences noted are not transcription dependent. Intracellular Ca^{2+} (IC_{50} = 125 ± 35 nM) also inhibited current development, and this could be partially relieved by the calmodulin and Ca^{2+}/calmodulin-dependent kinase inhibitors W-7, staurosporine, KN-93, or calmodulin kinase II (CaMKII) inhibitory peptide. To summarize, our results show that in addition to their homologous expression results in ATP-dependent inhibition in the absence of Mg^{2+} (37). Similarly, internal Ca^{2+} inhibited heterologously expressed TRPM7 activity (25), whereas a TRPM7-like current in microglia was not significantly affected by intracellular Ca^{2+} (16). It is not yet clear how to account for these different observations of these complex chanzymes. TRPM7 subunits can assemble with TRPM6 subunits to form conducting channels having different properties than the homologous protein. For example, 2-APB inhibits TRPM7 channels (IC_{50} ~ 50 µM) (24, 31), although at mM concentrations it is reported to stimulate (24). In contrast, TRPM6 expression with or without TRPM7 results in inversion of the inhibitory response to 2-APB to a stimulatory one (24). Thus 2-APB may be used as a gauge of the relative expression of TRPM6 and TRPM7 and of the cellular role of these channels. Here we identified an outwardly rectifying, Mg^{2+}- and Mg-ATP-inhibited Trpm7-like cation current in both terminally differentiated rat hepatocytes and the proliferated rat hepatoma-human skin fibroblast cross WIF-B. The current is depressed by Ca^{2+} in a calmodulin- and calmodulin kinase II (CaMKII)-dependent manner. Compared with quiescent hepatocytes, the proliferating WIF-B cells expressed more Trpm7-like channel activity and underwent a higher rate of apoptosis in the presence of 2-APB. These results are discussed in terms of a vital role for Trpm7 in hepatocellular survival during conditions supporting proliferation.

Nonspecific cation currents inhibited by cytosolic Mg^{2+} were first recorded from Jurkat T lymphocytes (18), sequenced in 2001 (26, 32, 34) and classified as transient receptor potential melanin (TRPM) members six and seven. TRPM7 and the closely related TRPM6 channels are thought to play diverse roles in vital physiological functions including Mg^{2+} homeostasis (17, 36) and Ca^{2+} influx, volume (4, 28) and intracellular pH sensing (21), cell survival and proliferation (2, 3, 13, 17, 26, 35, 39), and pathophysiological situations such as anoxic cell death and ischemia-reperfusion injury (1, 41). Although a role for TRPC1 in stimulus-induced Ca^{2+} influx in the major liver epithelial cell, the hepatocyte, has been suggested (5), and both TRPM6 and TRPM7 are expressed in relatively high amounts in liver tissue (7, 9, 22), Mg^{2+}-sensitive nonselective cation currents have not been demonstrated in these cells. Nevertheless, 2-aminoethoxyphenylborate (2-APB), albeit a relatively nonspecific inhibitor of TRPM and TRPC channels and other transporters, inhibited bile generation in perfused rat livers (10), and reduced hepatic store-operated Ca^{2+} current and ischemia-reperfusion injury (15, 27). TRPM7 currents are outwardly rectifying and exhibit very small inward currents under divalent-free intracellular recording conditions due to block of inward current by external Mg^{2+} (26, 34). These currents develop over the course of minutes following the initiation of intracellular dialysis due to the dilution of cytosolic Mg^{2+}. Dialysis with Mg^{2+}-containing solutions also induces a slow inactivation, or “rundown,” of TRPM7 current [IC_{50} 0.1–0.7 mM (26, 31, 40)], TRPM6 and TRPM7 channels are also variously reported to be modulated, or not, by intracellular Mg-ATP (6, 20, 26). On the one hand, ATP was initially reported to activate TRPM7 (34). Further studies provided evidence that this was likely due to chelation of Mg^{2+} by ATP (11, 20, 40). Conversely, in other hands Mg-ATP, at millimolar concentrations, inhibited TRPM7 currents (6, 26). Inhibition by Mg-ATP involved Mg^{2+}, such that higher Mg^{2+} concentrations increased the sensitivity to Mg-ATP (6, 26). TRPM6 modulation presents a different picture as homologous expression results in ATP-dependent inhibition in the absence of Mg^{2+} (37). Similarly, internal Ca^{2+} inhibited heterologously expressed TRPM7 activity (25), whereas a TRPM7-like current in microglia was not significantly affected by intracellular Ca^{2+} (16). It is not yet clear how to account for these different observations of these complex chanzymes.
MATERIALS AND METHODS

Ethical approval. Female Sprague-Dawley rats (200–225 g body wt) were obtained from Charles River (Montreal, QC) and maintained on a 12-h light-dark regime with access to water and rat chow ad libitum according to the Canada Council on Animal Care as implemented by the Queen’s University Animal Care Committee. The latter approved all animal protocols. Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg) prior to liver perfusion.

Materials. WIF-B cells were gifts from A. Hubbard (Johns Hopkins University). Fetal calf serum and Tag DNA polymerase were supplied by Gibco/Invitrogen (Burlington, ON, Canada). Trizol and other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) or British Drug Houses (Toronto, ON, Canada). Other chemicals were purchased from Sigma-Aldrich Chemicals (Montreal, QC, Canada). 2-Aminophenylborate was from Tocris Bioscience (Ellisville, Missouri, ON, Canada) and Roche Biochemicals (Montreal, QC, Canada). Trizol and other chemicals were purchased from Gibco/Invitrogen (Burlington, ON, Canada). 2-Aminophenylborate was from Tocris Bioscience (Ellisville, Missouri, ON, Canada) and Roche Biochemicals (Montreal, QC, Canada). 2-Aminophenylborate was from Tocris Bioscience (Ellisville, Missouri, ON, Canada) and Roche Biochemicals (Montreal, QC, Canada). 2-Aminophenylborate was from Tocris Bioscience (Ellisville, Missouri, ON, Canada) and Roche Biochemicals (Montreal, QC, Canada).

Cell isolation and culture. WIF-B cells were maintained in Coon’s F12 medium with further addition of 0.15% NaHCO3, 10 μM hypoxanthine, 40 nM aminopterin, 1.6 μM thymidine, 100 U/100 μg penicillin-streptomycin and 5% FCS, according to the protocols provided by A. Hubbard (http://www.bs.jhmi.edu/wifb/). Following passaging every 7 days, single-cell suspensions were plated onto glass coverslips containing the same medium except that 5% calf serum was used instead of FCS, and cells were patch clamped within 48 h.

Rat hepatocytes were isolated from a collagenase (0.023 mg/ml Liberase, Roche Biochemicals, or 0.5 mg/ml CSL-1, Worthington Biochemical, Lakewood, NJ) perfusion technique (14) and plated at 1 × 105 cells/ml on glass coverslips. Cells were cultured in DMEM solution containing 0.15% NaHCO3, 10 mM HEPES, 10% (vol/vol) fetal calf serum, 2 mM glutamine, 5 μg/ml insulin, 1 μM dexamethasone, and 100 U/100 μg penicillin-streptomycin, pH 7.4 in a humidified atmosphere of 95:5% air-CO2 at 37°C. Electrophysiological experiments were carried out on cells cultured between 12 and 72 h.

Electrophysiological recording. Whole-cell currents were measured in WIF-B cells and short-term cultured rat hepatocytes by the patch-clamp technique. Extracellular solutions contained, in mM, 5 K-glucuronate, 140 Na-glucuronate, 1 CaCl2, 1 MgCl2, 5 glucose, and 10 HEPES, pH 7.4. Standard intracellular (patch pipette) solution contained 140 Na-glucuronate, 4 NaCl, 1 EGTA, 1 EDTA, and 10 HEPES, pH 7.2. In some experiments Na2ATP and/or MgCl2 or CaCl2 were included in the patch solution. Glucuronate salts were reduced appropriately to maintain constant osmolarity.

Patch-clamp recordings were made at room temperature in the whole cell and cell-attached, single-channel configurations by using an Axopatch 200A amplifier (Axon Instruments, Foster City) and Clampex 7 software as described previously (14). Patch pipettes were made from borosilicate glass (Warner Instrument, Hamden, CT, no. G8516ST-4) with a resistance of 4–5 MΩ when filled and immersed in Na-glucuronate-containing solutions. Whole-cell currents were digitized (Digidata 1200B) at 5 kHz. Sampled data were analyzed using Origin 7.5 software (OriginLab, Northampton, MA). To monitor the development of currents, voltage ramps from −150 to +150 mV and 400-ms duration were applied 30 s after the whole cell configuration was attained and once each successive minute for 12 min. Time-dependent properties of the currents were measured by applying voltage steps from −150 mV and increased by 15 mV. Mean data were pooled after normalization of individual records to whole cell capacitance, determined through the amplifier circuitry in response to a 5 mV step from a holding potential of 0 mV and expressed as mean pA/pF ± SD. The capacitance transients were compensated by greater than 90 and 98% in, respectively, rat hepatocytes and WIF-B cells. Representative mean whole cell capacitance of rat hepatocytes (30.5 ± 4.7, n = 35) was significantly larger than that of WIF-B cells (23.5 ± 5.1, n = 35). Data are presented as representative recordings or as mean ± SD of n observations, in which n is the number of cells recorded from.

Reverse transcription and polymerase chain reaction. RT-PCR was conducted on total RNA extracted from two different WIF-B cultures and two separate rat hepatocyte preparations according to the manufacturer’s directions. Gene-specific forward and reverse oligonucleotide primers were designed from unique sequences in different exons of the rat TRPM6 (sense CCAACACAGGTGGAAGCTGC, antisense TGCGT- GAGTGGACGACCG, NCBI accession no. NC_005100) and TRPM7 (sense AAAACATGGTGTAGTGCCTCT, antisense ACCACACAA- CACATGGAACGG, NCBI accession no. NC_005102) genes. 18S rRNA (sense ACCACATCAAGGAGGCG, antisense ACCA- GACTTGCCCTCAATG, NCBI accession no. X01117) was amplified in the same samples to monitor template amount in each reaction. 20 μl PCR reactions were carried out containing cDNA synthesized from 25–100 ng WIF-B or rat hepatocyte RNA, 0.5 U Tag DNA polymerase, 1.5 mM MgCl2, 0.2 μM dNTPs, and 0.5 μM primers. PCR products were cloned into an A/U cloning vector (pDrive, Qiagen) and sequenced by the automated dideoxy method for identity confirmation.

Hoechst 33342 staining. WIF-B cells and rat hepatocytes were cultured in 96-well tissue culture plates, the latter plated at 2 × 105 cells per well. WIF-B cells were grown to ~70% confluency and harvested for 24 h prior to incubating the cells with 2-APB and 1% (vol/vol) DMSO. Hoechst 33342 (5 μg/ml) was added to each well, and the cells were incubated at 37°C for 30 min prior to observation by use of an inverted fluorescence microscope. Images were collected by using a Pro Series 128 camera and ImagePro (Media Cybernetics, Silver Spring, MD) software. Three different cell preparations or passages of cells were imaged and representative images are illustrated.

Carboxyfluorescein (CF) accumulation and analysis. WIF-B cells and rat hepatocytes were cultured in 96-well tissue culture plates as above. After 24 h the medium was replaced with PBS containing 0.5% (vol/vol) Triton X-100 plus or minus (blank) 10 μM 5-carboxyfluorescein diacetate and incubated at 37°C for 30 min. Triton X-100 facilitated CF release and quantitation without affecting esterase activity. CF generation was measured at 492 nm, where CFDA has undetectable absorbance. Data were corrected for blank readings and means (± SD) from three different cell preparations or passages of cells were plotted as functions of the 2-APB concentration. In preliminary experiments we demonstrated that CF generation (i.e., esterase activity) in WIF-B cells was proportional to cell number.

RESULTS

WIF-B cells and rat hepatocytes exhibit time-independent, outwardly rectifying cation currents. To establish the presence of active TRP family nonspecific cation channels in the liver parenchyma, we recorded currents in rat hepatocytes and WIF-B cells in response to voltage steps between −150 and +135 mV, from a holding potential of 0 mV. Under standard whole cell recording conditions in which cells were bathed and dialyzed with Na-glucuronate-rich solutions, the latter Mg2+ and Ca2+ free, currents in WIF-B cells at 12 min of dialysis reversed at 0 mV and showed no time dependence (Fig. 1A, left). The current-voltage relationship, as depicted by observing current in response to a voltage ramp from −150 to +150 mV, also at 12 min dialysis, illustrates the outwardly rectifying characteristic of this cation current (Fig. 1A, right). Identical currents, with respect to time independence and outward rectification, were observed in rat hepatocytes (not shown). In hepatocytes the outwardly rectifying current at ±120 mV developed slowly over dialysis, reaching a steady state 8–10 min following patch rupture (Fig. 1B, triangles). Mean outward current in rat hepatocytes increased from 4.9 ± 2.7 pA/pF at patch rupture
20.6 ± 5.6 pA/pF (n = 6) at 12 min whereas inward current at
−120 mV (−1.5 ± 0.9 pA/pF) did not change significantly
over the time course of the dialysis. Although WIF-B cells
showed a similar pattern of current development, the amplitude
of the outward currents was more than five times higher than
those recorded from the rat hepatocytes (Fig. 1B, circles).

**2-APB inhibits outward currents.** Although 2-APB is a
relatively nonspecific inhibitor of TRP channels, each of these
proteins exhibits different sensitivity to this compound. Cells
were dialyzed with divalent-free intracellular solution and
bathed in divalent-containing Na-glutamate solution containing a range of
2-APB concentrations (10 μM to 2 mM). Voltage ramps were
applied every minute for 12 min, and current at +120 mV and
12 min dialysis (−4.5 ± 1.6 pA/pF) did not change significantly over the same
time course.

**2-APB reduces viability of WIF-B cells and rat hepatocytes.**
Proliferating cells appear to require Trpm7 expression to sur-
vive (2, 3, 13, 17, 26, 35, 39). Here we tested the capacity of
proliferating WIF-B cells and quiescent rat hepatocytes to
survive under conditions in which Trpm7 is inhibited by a wide
range of 2-APB concentrations for 24 h. Cell viability was
assessed by measuring the amount of carboxyfluorescein
(CF) generated from the esterase substrate, CFDA. Both cell
types were sensitive to 2-APB, with increasing inhibitor
concentration associated with a reduction in cellular esterase
activity (Fig. 3). The rat hepatocytes (dashed line) were less
sensitive to 2-APB, as reflected in the higher baseline (open symbols) and
−120 mV (open symbols) extracted from voltage ramps applied
30 s and successive minutes following breakthrough into the whole cell configuration.

![Fig. 1. Slowly developing time-independent and out-
wardly rectifying cation currents in WIF-B cells and
rat hepatocytes. A: currents were recorded from a
representative WIF-B cell dialyzed for 12 min with
divalent-free Na-gluconate intracellular solution and
bathed in divalent-containing Na-glucenate solution.
Currents were generated in response to 2-s voltage
steps incremented 15 mV between −150 and +135
mV (left) or a 400-ms voltage ramp from −150 to
+150 mV (right). B: average whole cell currents from
12 WIF-B cells (circles) and rat hepatocytes (triangles)
(± SD) at +120 (solid symbols) and −120 mV
(open symbols) extracted from voltage ramps applied
30 s and successive minutes following breakthrough
into the whole cell configuration.**
representative WIF-B (left) or rat hepatocyte (right) cultures exposed to 35, 178, or 1,000 μM 2-APB. Magnification is ×100, with inset images magnified ×400. With 35 μM 2-APB nuclei in both cell types are weakly and uniformly stained (top); 178 μM 2-APB caused distinct differences in the staining pattern of the two cell types (middle) with the majority of WIF-B cell nuclei exhibiting marginated DNA and shrunken nuclei. In contrast, the hepatocyte nuclei are much less shrunken, although some condensation and margination of DNA is evident. Yet further divergence between the two cell types is visible when the cells were incubated with 1,000 μM 2-APB (bottom). The WIF-B cells are greatly reduced in number, restricted to small patches of cells in which all nuclei were brightly and almost homogeneously stained, indicative of massive DNA fragmentation. Conversely, the number of hepatocytes remaining attached to the substratum was not significantly different from control. The data were not significantly different from control and confirm its similarity with Trpm6/Trpm7 channels.

Mg<sup>2+</sup> inhibits outward current in WIF-B cells and sensitizes the channels to Mg-ATP. To assess whether dilution of cytosolic Mg<sup>2+</sup> by the dialysis solution could account for the slow increase in outward current, we measured mean current at +120 mV and 12 min dialysis with solutions containing 0.1, 0.5, or 1 mM Mg<sup>2+</sup> and compared these with control currents (106.6 ± 15.1 pA/pF, n = 12) observed in cells exposed to divalent-free solutions (Fig. 5). Inclusion of 2.04 mM MgCl<sub>2</sub> in the pipette solution (Mg<sup>2+</sup> ~1 mM) caused almost complete inhibition of current development, decreasing the mean current to 4.1 ± 2.2 pA/pF (n = 6), and 0.1 or 0.5 mM Mg<sup>2+</sup> (respectively 1.068 or 1.52 mM MgCl<sub>2</sub>) inhibited current development by close to 50% (0.1 mM, 63.4 ± 8.3 pA/pF, n = 6; 0.5 mM, 55.8 ± 9.7 pA/pF, n = 4). To establish whether Mg-ATP modulates this current, we dialyzed WIF-B cells with a solution containing 0.5 mM Mg<sup>2+</sup> and 3.7 mM Mg-ATP (5.28 mM MgCl<sub>2</sub> and 4.6 mM Na<sub>2</sub>ATP and Na-glucuronate reduced to 125 mM to maintain osmolarity). Mean current at 12 min of dialysis was significantly inhibited by 58%. The inhibition by Mg-ATP was sensitive to the cytosolic Mg<sup>2+</sup> concentration, since in the presence of 0.1 mM Mg<sup>2+</sup> neither 3.7 mM Mg-ATP (4.8 mM MgCl<sub>2</sub> and 8 mM Na<sub>2</sub>ATP) nor 7.3 Mg-ATP (8 mM MgCl<sub>2</sub> and 15 mM Na<sub>2</sub>ATP) significantly inhibited current development. In these experiments isosmotic conditions were maintained by reducing the dialysate Na-glucuronate concentration to 116 and 105 mM, respectively. Currents measured with 8 mM Na<sub>2</sub>ATP in the absence of Mg<sup>2+</sup> (104.3 ± 3.9 pA/pF, n = 4) were not significantly different from control currents. The combined results establish the Mg<sup>2+</sup> and Mg-ATP sensitivity of the current and confirm its similarity with Trpm6/Trpm7 channels.

Both Trpm7 and Trpm6 are expressed in rat hepatocytes and WIF-B cells. Although both TRPM6 and TRPM7 expression are documented in human, mouse, and zebrafish liver (7, 9, 22), neither mRNA nor protein expression has been studied in isolated hepatocytes of any species. Here we show expression of both Trpm6 and Trpm7 channel transcripts using RT-PCR of total RNA isolated from three different cultures of WIF-B cells and three separate preparations of rat hepatocytes (Fig. 6). 18S rRNA was used as the loading control. Both preparations show the same relative distribution of Trpm6 and Trpm7 PCR products. These results suggest that the Mg<sup>2+</sup>-sensitive outwardly rectifying current could be carried by either of the two channels or a heterotetramer of the two proteins. Furthermore, the activity differential observed between WIF-B cells and rat hepatocytes (Fig. 1B) cannot be explained in terms of differences in message level.

Ca<sup>2+</sup> inhibits outward current development via a calmodulin-dependent kinase. Trpm7 channels are thought to contribute to Ca<sup>2+</sup> influx in many cell types. To determine whether increased cytosolic Ca<sup>2+</sup> might provide a negative feedback signal to channel activity we dialyzed the cells with Mg<sup>2+</sup>-free, EDTA- and EGTA-buffered solutions containing 10 nM to 1 μM Ca<sup>2+</sup>. Normalized currents measured from individual cells 12 min after attaining the whole cell configuration are plotted as functions of the Ca<sup>2+</sup> concentration and compared with cells dialyzed with divalent-free solution (Fig. 7). The data were not significantly different from control and confirm its similarity with Trpm6/Trpm7 channels.

Fig. 2. Inhibition of cation currents by 2-aminoethoxyphenylborate (2-APB). Cells were bathed in divalent-containing Na-glucuronate solution containing 10 μM to 2 mM 2-APB and dialyzed for 12 min with divalent-free Na-glucuronate intracellular solution. Each symbol represents data from 4–7 cells. The data were fitted to a sigmoid function with the maximum set at 106.5 pA/pF and an IC<sub>50</sub> of 72 ± 13 μM 2-APB.

Fig. 3. Differential effect of 2-APB on viability of WIF-B cells and rat hepatocytes. The 24-h cultured rat hepatocytes (dashed line) and day 5 postpassage WIF-B cells (solid line) were incubated with 35–1,000 μM 2-APB for 24 h, followed by Triton X-100 and 10 μM CFDA. Carboxyfluorescein (CF) generation was normalized to that observed at 35 μM 2-APB. Mean of triplicate WIF-B passages or hepatocyte preparations (± SD) are shown. Pooled data were fitted to a sigmoid function having maximum, minimum, and IC<sub>50</sub> values of 1.03 ± 0.08, 0.01 ± 0.05, and 160 ± 16 μM 2-APB (WIF-B) and 1.02 ± 0.02, 0.12 ± 0.04, and 229 ± 15 μM 2-APB (rat hepatocytes), respectively.
fitted to a sigmoid function with the minimum and maximum values set at, respectively, 0 and 106.6 pA/pF. Under these constraints the IC₅₀ was 125 ± 35 nM and the slope 0.67 ± 0.18, indicating that the WIF-B current is sensitive to physiological levels of Ca²⁺.

Ca²⁺ might regulate channel activity directly or through a signaling pathway. Dialysis with 1 μM Ca²⁺ and the calmodulin inhibitor W-7 (50 μM) enabled development of significantly larger currents (61.7 ± 16.1 pA/pF, n = 4 vs. 14.2 ± 2.2 pA/pF, n = 7 in the absence of W-7) over the 12-min recording time (Fig. 8A, left). To assess whether Ca²⁺-bound calmodulin associates with a channel subunit or inhibits channel activity via a Ca²⁺/calmodulin-dependent kinase reaction, we dialyzed the cells with 1 μM Ca²⁺ and 200 nM staurosporine to inhibit general kinase activity. Under these conditions current inhibition by Ca²⁺ was significantly alleviated (43.4 ± 12.3 pA/pF, n = 4 at 12 min) (Fig. 8A, left).

Ca²⁺/calmodulin-dependent kinases include CaMKII, which could inhibit current activity through a phosphorylation reaction. We tested this possibility by dialyzing the cells with 1 μM Ca²⁺ and the specific CaMKII inhibitor CaMKII inhibitory peptide (2 μM; AIP) or by preincubating and dialysis with 1 μM KN-93. Mean data for each of these conditions (Fig. 8A, right) show that both KN-93 and AIP significantly increased the currents at 12 min to, respectively, 42.5 ± 8.6 pA/pF (n = 4) and 66.9 ± 19.4 pA/pF (n = 4). The combined results indicate that Ca²⁺ inhibition of the TRPM7-like current is at least partially mediated by increased CaMKII-like current. Figure 8B summarizes the mean results at 12 min of dialysis with or without the different agents.

**DISCUSSION**

TRPM6 and TRPM7 channels are expressed in a wide range of cell types including human liver (9). Here we refined their molecular expression to the hepatocyte and show that both terminally differentiated rat hepatocytes and WIF-B cells, a polarized rat hepatoma-human skin fibroblast cross, express Mg²⁺-inhibited, nonspecific, and outwardly rectifying cation currents identical to “MIC”/“MagNuM” currents first de-
scribed in T lymphocytes, HEK293, and rat basophilic leukemia cells (26, 34) and more recently in terminally differentiated cardiomyocytes, microglia, and neurons (11, 16, 38). Our functional and expression data suggest that homologous Trpm7 channels, rather than heterologous Trpm6/Trpm7 channels, are responsible for the whole cell currents in these cells. In addressing the physiological role of these channels in the liver, our results show that proliferating hepatoma cells (WIF-B) express larger Trpm7-like currents and undergo significantly greater apoptosis in response to channel blockade compared with nondividing rat hepatocytes. We also present novel evidence that the channels underlying the Trpm7-like currents are inhibited by increases in Ca$^{2+}$ concentration expected to occur in response to cell stimulation or injury and that this action is mediated by a calmodulin-dependent kinase. These results are discussed in terms of a role for Trpm7 in supporting proliferative growth in the liver.

**Trpm7 channels underlie the whole cell current in hepatocytes.** The WIF-B and hepatocyte currents exhibit identical properties to cloned TRPM7, the latter of which conducts outward Na$^{+}$ or K$^{+}$ equally, is time independent and strongly outwardly rectifying in the presence of physiological extracellular Mg$^{2+}$, and activates over minutes in response to dialysis with divalent-free solution (see Ref. 30). Perhaps the most defining property of this current is its sensitivity to millimolar concentrations of intracellular Mg$^{2+}$. All of these properties, however, are also shared by the closely related channel TRPM6. We therefore looked to two additional traits of the WIF-B current in our search to further define the underlying channels. First, TRPM6 and TRPM6/TRPM7 hybrid channels are both activated by 2-APB whereas the WIF-B current was half maximally inhibited at 78 μM, similar to the native Jurkat cell or heterologously expressed TRPM7 (24, 31). And second, contrary to our data and earlier reports on TRPM7 (20), ATP inhibits heterologously expressed TRPM6 channels (37). So although we observed both Trpm6 and Trpm7 expression in rat liver, we did not see any evidence for heteromeric channels at the functional level. The simplest explanation for this is that the Trpm6 message we observed by standard RT-PCR is not translated, or, if it is, the amount of protein expressed functionally at the plasma membrane is much smaller than that for Trpm7. In support of this contention, TRPM7 mRNA is expressed at 10–100 times that of TRPM6 in both human and mouse liver (9, 22).

**Mg$^{2+}$ sensitizes Trpm7-like currents in WIF-B cells to Mg-ATP.** Mg-ATP has been variously reported to inhibit (6, 26) or to have no effect on (20) TRPM7 channels. Here we demonstrated the ineffectiveness of Mg-ATP, both at a physiological concentration (3.7 mM) and at a concentration previously reported to be inhibitory for TRPM7 (see Ref. 30). Instead, Mg-ATP inhibited the WIF-B current half maximally at 78 μM, similar to the native Jurkat cell or heterologously expressed TRPM7 (24, 31). Second, contrary to our data and earlier reports on TRPM7 (20), ATP inhibits heterologously expressed TRPM6 channels (37). So although we observed both Trpm6 and Trpm7 expression in rat liver, we did not see any evidence for heteromeric channels at the functional level. The simplest explanation for this is that the Trpm6 message we observed by standard RT-PCR is not translated, or, if it is, the amount of protein expressed functionally at the plasma membrane is much smaller than that for Trpm7. In support of this contention, TRPM7 mRNA is expressed at 10–100 times that of TRPM6 in both human and mouse liver (9, 22).
The presence of increased Mg$^{2+}$ identified an increased sensitivity to Mg-ATP blockade in the inhibition of TRPM7 currents and clearly shown to significantly inhibit TRPM7 currents (7.3 mM, Ref. 6), against the development of the WIF-B currents. However, these data were collected in the presence of "low" or subphysiological Mg$^{2+}$ (0.1 mM). Similarly, Kozak and Cahalan (20), who also reported an insignificant effect of 5 mM Mg-ATP on TRPM7 currents, conducted their experiments with less than 0.3 mM Mg$^{2+}$. DeMeeuse et al. (6) systematically characterized the inhibition of TRPM7 currents by Mg-ATP and clearly identified an increased sensitivity to Mg-ATP blockade in the presence of increased Mg$^{2+}$. We confirmed this association for the WIF-B current by showing that 3.7 mM Mg-ATP significantly inhibited current development when Mg$^{2+}$ was increased to 0.5 mM. Hence the liver cell current carries the added property of Mg$^{2+}$ and Mg-ATP sensitivity earlier ascribed to TRPM7 channels.

Physiological role in liver. TRPM7 appears to present differing survival influences on dividing and nondividing cells since knockdown or 2-APB exposure decreases proliferation in dividing cells (2, 3, 13, 17, 19, 23, 26, 35, 39), yet knockdown increased survival in injured terminally differentiated neurons (1, 41). It is noted that Trpm7 knockdown did not affect the survival of healthy neurons (1). These studies suggest that functional Trpm7 is expressed at relatively lower levels in normal, nondividing cells compared with those having the potential to proliferate and that, whereas proliferation is dependent on Trpm7 expression, survival of normal healthy terminally differentiated cells is less so. Here we compared two closely related cell types, one of which normally proliferates (WIF-B rat hepatoma-fibroblast cross) and one which does not (primary hepatocytes from rat liver). Our results demonstrate that rat hepatocytes, compared with WIF-B cells, have significantly lower Trpm7-like channel activity and lower sensitivity to the Trpm7 inhibitor 2-APB. Thus these results support the proposal that this ubiquitously expressed channel has quite different functions in related cell types having different proliferative capacities.

Increased apoptosis in proliferating cells (12), as exemplified by WIF-B cells, and in nonproliferating hepatocytes in the presence of protein phosphatase inhibitors (8), is associated with CaMKII activation. We showed here that the calmodulin-CaMKII association inhibitor, W-7, and both nonspecific protein kinase and CaMKII inhibitors relieved inhibition of the WIF-B current by Ca$^{2+}$, suggesting that the latter is mediated by a calmodulin-CaMKII process. In support of this, the Trpm7-like current was inhibited by Ca$^{2+}$ concentrations (IC$_{50}$ 0.13 μM) similar to those that activate CaMKII [EC$_{50}$ 0.5–1 μM (33)]. Although our results do not identify whether CaMKII directly phosphorylates Trpm7, we identified a CaMKII target sequence within the COOH-terminal domain of this protein. This sequence, 1264-ITRELS, contains the Hyd X R (NB) X S/T sequence specific to CaMKII, as opposed to other serine/threonine kinases (29). Lastly, inspection of rat Trpm7 did not reveal a consensus sequence for a calmodulin binding site, supporting our contention that calmodulin and Ca$^{2+}$ act indirectly (through a kinase) rather than via a direct interaction with the channel.

In conclusion, this study revealed the presence of functional Trpm7-like channels in rat hepatocytes and WIF-B cells that appear to support survival in the latter but not in the former. We also describe novel data demonstrating that the Trpm7-like channel is influenced by cytosolic Ca$^{2+}$ via a CaMKII-dependent process. These data provide a basis for both investigating the molecular nature of Trpm7 regulation and linking this to its proposed role in cell survival.

GRANTS

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