The ϵ -Isoform of PKC Mediates the Hypertonic Activation of Cation Channels in Confluent Monolayers of Rat Hepatocytes

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Key Words

Rat • Liver • Cell volume • Volume regulation • Cation channel • PKC • Isoform

Abstract

We were interested whether PKC α , δ , ϵ or ζ is the isoform actually employed in the activation of hypertonicity-induced cation channels (HICCs) in primary cultures of rat hepatocytes. Quantitative SDS-page and Western-blot experiments revealed that PKC α , δ and ϵ were stimulated by Indolactam V (as a DAG substitute for activation of c and nPKCs) but that only PKC δ and ϵ did respond to hypertonic stress. Furthermore, chelation of intracellular Ca⁺⁺ by BAPTA-AM did not alter HICC activation in cable-analysis experiments whereas Indolactam V as well as V8 (an Indolactam derivative specific for PKC δ and ϵ) activated HICC currents under isotonic conditions. Finally, by use of Rottlerin (as an inhibitor exhibiting a slight preference for PKC δ over ϵ) PKC ϵ could be identified as the most likely isoform responsible for the activation of the HICC.

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Introduction

In addition to the mere maintenance of homeostasis, the mechanisms of cell volume regulation are employed in a variety of most relevant physiological processes. Among these is the locomotion of cells, the coordination of apical and basal-lateral transport in epithelia, the triggering of cell metabolism and gene expression and, most notably, the control of proliferation as well as apoptosis [1-5].

Whenever analyzed in a quantitative fashion, hypertonicity-induced cation channels (HICCs) were found to be the main mechanism of the regulatory volume increase (RVI) of a shrunken cell (see [6, 7] for review). This also holds for confluent monolayers of rat hepatocytes, from which a relative contribution of the HICC, NHE1 and NKCC1 to hypertonicity-induced Na⁺ import and RVI of 4: 1: 1 was reported [8]. In this system, HICC activation has recently been shown to employ focal adhesion signalling, G proteins, PLC, PKC and finally the exocytotic insertion of channels into the plasma membrane [9, 10].

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No information is available so far, however, as for the actual PKC isoform in charge of this process.

Here, the PKC isoform mediating the activation of HICCs in rat hepatocytes monolayers was defined by means of quantitative Western-blot analysis, with cable-analysis and current-clamp recordings, as well as by use of effective blockers and activators. It is found that hypertonic stress activates the *novel* PKC isoforms δ and ϵ and that, most likely, it is PKC ϵ that is actually responsible for the activation of the HICC.

Materials and Methods

Cell Culture

Harvesting of rat hepatocytes and preparation of confluent monolayers were the same as described previously [8, 11, 12].

SDS-Page and Western-Blot Analysis

For preparation of the cytosolic and the particulate fraction, cells were exposed to the respective experimental buffer solution (see below) at 4°C that was supplemented with the protease inhibitors aprotinin (15 µg/ml), leupeptin (20 µg/ml), pepstatin (5 µg/ml), chymostatin (5 µg/ml) as well as trypsin-inhibitor (500 µg/ml); they were scraped off from their support, lysed by a 3-times freezing / thawing procedure with liquid N₂ and finally homogenized. Both fractions were then separated by a 1 h centrifugation at 100,000x g and 4°C. The pellet (particulate fraction) was solubilized in 500 µl of 1 % Triton X-100 in the same buffers.

Proteins were separated by discontinuous SDS-PAGE on 4 to 12 % acrylamide gels with the Nu-PAGE® Bis-Tris Electrophoresis System (Invitrogen, Karlsruhe, Germany). Samples were diluted 4 : 5 in 5x sample buffer (50 mmol/l Tris, 10 % SDS, 0.02 % bromphenol blue, 10 % glycerol, 2.5 % β -mercaptoethanol). The SDS running buffer was 50 mmol/l MES, 50 mmol/l Tris, 1 mmol/l EDTA, 0.1 % SDS, pH 7.3. As a molecular weight marker, the SeeBlue® Plus2 prestained standard from Invitrogen was used.

Proteins separated by SDS-PAGE were transferred to PVDF membranes (Bio-Rad, Munich, Germany) by electroblotting. After equilibration of the gel, methanol-activated PVDF membranes, blotting pads and filters in transfer buffer (96 mmol/l glycine, 12 mmol/l Tris-HCl, 20 % (v/v) methanol, pH 7.8 to 8.4) were placed in the XCell II™ Blot Module according to the instructions of the manufacturer (Invitrogen). Transfer was achieved at a constant current of 180 mA for 40 min.

Unspecific binding was blocked with 5% non-fat dry milk powder plus 1% BSA in TBST buffer (8 g/l NaCl, 0.2 g/l KCl, 20 mmol/l Tris-HCl, pH 7.5, 0.1 % Tween 20) for 1 h at room temperature or over night at 8°C. Anti-rat IgGs raised in rabbit were used as primary antibodies at a dilution of 1000x; anti-rabbit IgG from goat (HRP conjugated; diluted 10,000x) was the secondary antibody. All antibodies were purchased from Sigma,

Deisenhofen, Germany. Primary antibodies were directed against the V5-regions of each kinase that are highly isoform-specific.

The protein bands obtained were scanned on a Gel Doc 1000 unit (Bio-Rad) and data were analyzed by use of the Molecular Analyst software, version 1.5.

Electrophysiology

Cable-analysis and current-clamp recordings with low Cl⁻ pulses are intracellular microelectrode techniques and have been described in detail previously [8-12]. Briefly, in a cable-analysis experiment, current pulses are injected into a cell and the resultant changes in membrane voltage (ΔV_m) are recorded with a second microelectrode at 35, 100, 200 and 400 µm from the point of current injection. These measurements are done consecutively on four sheets of monolayers cut from one single Petri dish. Data are fitted to the form.

$$\Delta V_m = A \cdot K_0(x/\lambda)$$

where A and λ are constants, K_0 is a zero-order Bessel function and x is the distance between both electrodes [13;14]. From A and λ , the resistance that determines current spread within the monolayer through electric cell-to-cell coupling (R_x) as well as the specific membrane resistance (R_z) can be computed according to

$$R_x = (2 \cdot \pi \cdot A) / I_0$$

and

$$R_z = (2 \cdot \pi \cdot A \cdot \lambda^2) / I_0$$

respectively, where I_0 is the total current applied.

In a Cl⁻ substitution experiment, extracellular Cl⁻ is step-like reduced in exchange for a non-permeable substitute and from the resultant depolarisation of membrane voltage (and on the basis of the Nernst-equation) the relative Cl⁻ conductance of the cell membrane can be calculated.

Because of the high degree of electric cell-to-cell coupling, patch-clamp experiments on confluent monolayers of rat hepatocytes are not feasible [8, 11, 12, 15].

Solutions

The isotonic control solution (300 mosmol/l, pH 7.4) contained (in mmol/l): NaCl, 144; KCl, 2.7; MgCl₂, 1.1; CaCl₂, 1.8; NaH₂PO₄, 0.4; HEPES, 2.5; Na-HEPES, 2.5; glucose, 5.6. Hypertonicity was achieved by the addition of 100 mosmol/l sucrose and the osmolality of solutions was routinely checked (Knauer, Berlin, Germany). In the cable-analysis measurements, 0.5 mmol/l quinine was used to block K⁺ conductance [8, 11, 12]. In the Cl⁻ substitution experiments, Cl⁻ was 100-fold reduced in exchange for gluconate. For the latter recordings, a custom-made flowing junction was used as the reference electrode to avoid liquid junction potentials. Experiments were performed at 36.5 ± 0.5°C.

All chemicals were of the highest purity available. BAPTA-AM, indolactam V and rottlerin were purchased from

Fig. 1. Activation of various PKC isoforms in confluent monolayers of rat hepatocytes. A, typical Western-blots on PKC α , δ , ϵ and ζ from the particulate and cytosolic fraction of cells, under isotonic conditions ("300"), after 5 min of hypertonic stress ("400") and following 5 min of (isotonic) exposure to 5 μ mol/l indolactam V ("indo"). B to E, summary of results obtained for PKC α , δ , ϵ and ζ , as indicated. *, ** and ***, significantly different from the isotonic control (set at 100%) with $p < 0.05$, 0.01 and 0.001, respectively ($n = 8$ to 10, for each experimental condition).

Sigma Calbiochem, Deisenhofen, Germany. V8 was synthesised in-house.

Statistical Analysis

Data are presented as means $\pm$ SEM with n denoting the number of preparations tested. For the statistical analysis, Student's t -tests for paired and unpaired data were applied as appropriate.

Results and Discussion

As could be shown by others, rat hepatocytes express the "classical" cPKC isoform α (supposed to be activated by the DAG substitute indolactam V [16] and Ca^{++}), the "novel" nPKCs δ and ϵ (activated by indolactam V but insensitive to Ca^{++}) as well as the "atypical" aPKC ζ (being insensitive to both, indolactam V and Ca^{++}) [17-22]. Here, we were first testing for the putative activation of these PKC isoforms in rat hepatocyte monolayers upon hypertonic stress, i.e. at 5 min after osmolality was increased from 300 to 400 mosmol/kg- H_2O . To this end, a series of quantitative SDS-page and Western-blot experiments was performed. These measurements were done with reference to 5 μ mol/l indolactam V, as a positive (isotonic) control. As is exemplified in Figure 1A and summarized in Figure 1B to E, indolactam V increased the amounts of PKC α , PKC δ and PKC ϵ in the particulate fraction of cells to $130.9 \pm 11.1\%$ ($p < 0.05$), $226.8 \pm 34.3\%$ ($p < 0.05$) and $213.5 \pm 23.2\%$ ($p < 0.01$) of the control level, respectively. In parallel, the above PKC isoforms were reduced to $75.5 \pm 7.2\%$ ($p < 0.05$), $56.6 \pm 4.5\%$ ($p < 0.001$) and $72.3 \pm 8.5\%$ ($p < 0.05$) of control, in the cytosolic fraction. As expected, indolactam V had no significant effect on the aPKC ζ which was $135.8 \pm 21.7\%$ ($p = 0.15$, n.s.) and $114.0 \pm 6.8\%$ ($p = 0.08$, n.s.) in the particulate and in the cytosolic fraction of cells, respectively. Concerning hypertonic stress, there were no effects on PKC α detectable,

Fig. 2. Cable-analysis on the activation of cation channels in rat hepatocyte monolayers. A, for the time indicated, osmolality was increased from 300 to 400 mosmol/kg-H₂O. Note the pronounced decrease of specific membrane resistance (R_z). B, same experiments as in A but in the continuous presence of BAPTA-AM (as an intracellular Ca⁺⁺ chelator). C and D, effects of indolactam V and its derivative V8 on R_z , applied under isotonic conditions, both. $n = 6$ to 8 , for each experimental condition. See text for details.

Fig. 3. Indolactam V does not change the Cl⁻ conductance in rat hepatocyte monolayers. A, typical current-clamp recording under control conditions. For the times indicated, osmolality was increased from 300 to 400 mosmol/kg-H₂O and Cl⁻ was 100-fold reduced in exchange for gluconate. B, summary of results. Hypertonic stress (400 mosmol/l) leads to a slight reduction of the apparent Cl⁻ conductance of hepatocyte monolayers (t_{Cl}) that is very similar in the absence (left) and in the presence (right) of 5 μ mol/l indolactam V (see text for details; $n = 5$ for each experimental condition).

that equalled $99.4 \pm 21.6\%$ and $90.5 \pm 10.2\%$ of the reference value, in the particulate and in the cytosolic fraction of cells. In sharp contrast, however, there was a pronounced increase in the particulate portion of PKC δ and PKC ϵ to $130.1 \pm 11.4\%$ and $133.7 \pm 13.1\%$ of control, respectively, at 400 mosmol/kg-H₂O (with $p < 0.05$, both). As with indolactam V, there were no significant effects on the particulate portion of PKC ζ , that equalled $126.9 \pm 15.9\%$ ($p = 0.14$, n.s.) of the reference level, under hypertonic conditions.

Fig. 4. Effects of rottlerin on the activation of PKC δ . A, typical Western-blots, under control conditions and with 5 or 20 $\mu\text{mol/l}$ rottlerin as indicated. B and C, summary of the results obtained. Note, the complete block of the hypertonic activation of PKC δ at 5 $\mu\text{mol/l}$ rottlerin, already. # and ##, significantly different from the control experiments (Figure 1C) at $p < 0.05$ and 0.01, respectively ($n = 8$ to 10, for each experimental condition).

The additional bands observed at a slightly higher molecular weight than PKCs α and ζ only occurred in the cytosolic fraction and may reflect the pronounced auto and/or transphosphorylation of the enzymes as it was reported by others [23, 24].

These data suggest that PKCs δ and ϵ may be employed in the activation of the HICC in confluent rat

Fig. 5. Effects of rottlerin on the activation of PKC ϵ . A, typical Western-blots, under control conditions and with 5 or 20 $\mu\text{mol/l}$ rottlerin as indicated. B and C, summary of the results obtained. Note the block of the hypertonic activation of PKC ϵ at 20, but not 5 $\mu\text{mol/l}$ rottlerin. # and ##, significantly different from the control experiments (Figure 1D) at $p < 0.05$ and 0.01, respectively ($n = 8$ to 10, for each experimental condition).

hepatocytes and, at the same time, they render an actual contribution of isoforms α and ζ to the process rather unlikely. To test for this hypothesis on a functional level, cable-analysis experiments were performed. These measurements were done in the presence of 0.5 mmol/l quinine to exclude possible effects of hypertonic stress on cell membrane K^+ conductance [8, 10-12]. As depicted

Fig. 6. Effects of rottlerin on the hypertonic activation of cation channels. A and B, cable-analysis experiments performed in the presence of 5 and 20 $\mu\text{mol/l}$ of the compound as indicated. For comparison, the data obtained under control conditions are also depicted (diamonds; c.f. to Figure 2A). $n = 6$, for both experimental conditions.

in Figure 2A, changing osmolarity from 300 to 400 mosmol/kg- H_2O significantly reduced the specific membrane resistance (R_z) of rat hepatocyte monolayers that, during the last 5 min of hypertonic stress, equalled $83.7 \pm 0.5\%$ of the control level ($p < 0.001$). As was reported in detail previously, this change of R_z is actually reflecting the activation of the HICC [8, 10-12]. Next, a series of recordings with the same protocol but in the continuous presence of 40 $\mu\text{mol/l}$ of the Ca^{++} chelator BAPTA (in its membrane permeable AM-form) was conducted that, in rat hepatocytes, was shown to efficiently clamp cell Ca^{++} to its baseline value [25-27]. Under these conditions, hypertonic stress led to a membrane response that was virtually identical to the one obtained in the control experiments including a new steady-state of $84.3 \pm 0.8\%$

of the isotonic reference value (Figure 2B; $p = 0.57$, n.s.). This result renders a (Ca^{++} activated) cPKC, namely PKC α as the mediator of HICC activation highly unlikely. On the other hand, as shown in Figure 2C, adding 5 $\mu\text{mol/l}$ indolactam V to the isotonic control solution led to a slowly developing decrease of R_z that, in the last 5 min of the experimental time frame, equalled $81.0 \pm 0.9\%$ of the reference level. In combination, these data strongly suggest that it is some type of nPKC, i.e. PKC δ or PKC ϵ that is actually employed in the activation of the HICC. In line with this, addition of 200 nmol/l V8 exhibited effects on R_z that are very similar to those of indolactam V with a final reduction to $85.3 \pm 0.9\%$ of control (see Figure 2D). The compound is an indolactam derivative that has recently been reported to be specific for nPKCs, with a slight preference for PKC δ over PKC ϵ [28, 29].

Although, in the cable-analysis experiments, K^+ conductance was blocked by quinine (to achieve a quantitative monitoring of HICC activation) it may be argued that the observed decrease of R_z could, at least in part, reflect the activation of Cl^- channels rather than of HICCs - both, upon hypertonic stress as well as following the addition of indolactam V. To elucidate this point, ion substitution experiments were performed in which Cl^- was 100-fold reduced in exchange for the impermeant anion gluconate. From the resultant depolarization of membrane voltage (and on the basis of the Nernst equation), the relative Cl^- conductance (t_{Cl}) of the hepatocyte cell membrane can be computed [12, 14]. As exemplified in Figure 3A and summarized in Figure 3B, however, hypertonic stress rather *reduced* t_{Cl} from 0.39 ± 0.03 to 0.31 ± 0.05 ($p < 0.01$), i.e. to some 80% of the isotonic control level. Since ion substitution experiments are known to yield the *relative* conductance of cell membranes for a particular ion rather than absolute values, this decrease of t_{Cl} is very likely to be the mirror image of the activation of the HICC. In the presence of 5 $\mu\text{mol/l}$ indolactam V, t_{Cl} was 0.37 ± 0.01 , under isotonic conditions, and it was reduced to 0.34 ± 0.02 and 0.29 ± 0.03 , at 5 and 10 min of hypertonicity, respectively (with $p < 0.05$, each, the latter being equivalent to 78% of control; Figure 3B). From these data, it is concluded that the observed decrease of R_z in the cable-analysis experiments reported here do, in fact, quantitatively reflect the activation of the HICC and that indolactam V does not change Cl^- conductance.

Rottlerin (also called mallotoxin) is an effective inhibitor of PKC δ with IC_{50} values of some 3 to 6 $\mu\text{mol/l}$ and with a considerably reduced inhibitory activity on

PKC ϵ [30-32]. Here, 5 and 20 $\mu\text{mol/l}$ rottlerin were used to differentiate between the actual role that PKC δ and ϵ do play in the activation of the HICC. As shown in Figure 4, already 5 $\mu\text{mol/l}$ of the compound were sufficient to completely block the hypertonic activation of PKC δ with no additional effect of 20 $\mu\text{mol/l}$ detectable at all; with $94.8 \pm 6.9\%$ and $94.7 \pm 4.8\%$ of the isotonic values, respectively, both series of measurements were significantly different from the control recordings (with $p < 0.05$, each; see Figure 1A, C). Quite in contrast, 5 $\mu\text{mol/l}$ rottlerin appeared to have little if any effect on the hypertonic activation of PKC ϵ (Figure 5A, B) that, with 117.4 ± 5.9 of the isotonic value, was statistically undistinguishable from the control experiments (see Figure 1A, D). With 20 $\mu\text{mol/l}$ rottlerin, however, PKC ϵ in the particulate fraction equalled $94 \pm 7.1\%$ of the isotonic value which is significantly different from the control recordings with $p < 0.01$ (Figures 5C and Figure 1A, D).

Next, the effects of rottlerin on HICC activation were tested. In the continuous presence of 5 $\mu\text{mol/l}$ of the compound, hypertonic stress reduced R_z to $89.1 \pm 0.7\%$ of the isotonic reference ($p < 0.001$; Figure 6A). Whereas this reduction was *per se* significantly smaller than the one obtained in the control recordings (with $p < 0.001$; c.f. to Figure 2A) the response of R_z still amounts to some 70% of the maximal effect. With 20 $\mu\text{mol/l}$ rottlerin, hypertonicity-induced changes of membrane conductance were no longer detectable; under these conditions, R_z equalled $98.8 \pm 1.5\%$ which is virtually identical to the

isotonic reference ($p = 0.44$), but significantly different to the experiments with 5 $\mu\text{mol/l}$ rottlerin as well as to the control recordings (with $p < 0.001$, each; Figure 6B). Considering the complete block of PKC δ with 5 $\mu\text{mol/l}$ rottlerin already and given the sensitivity profile of PKC ϵ for the compound that is very similar to the one observed for HICC activation, this renders the latter isoform the most likely candidate actually in charge of channel activation.

Conclusions

In conclusion, we observe the activation of PKC δ and ϵ - but not PKC α and ζ - in monolayers of rat hepatocytes under hypertonic stress. Activation of hypertonicity-induced cation channels (HICCs) does not appear to employ changes in cell Ca^{++} but, under isotonic conditions, HICC activation can be mimicked by indolactam V as well as by the PKC δ and ϵ specific indolactam derivative V8. Finally, by use of the PKC δ blocker rottlerin, PKC ϵ could be identified as the actual isoform in charge of HICC activation.

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