Signalling Events Employed in the Hypertonic Activation of Cation Channels in HeLa Cells

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Key Words

HeLa cell • Volume regulation • Cation channel • Cell signalling • Kinases

Abstract

In the present study, the signalling network behind the hypertonic activation of cation channels in HeLa cells was analysed by use of various inhibitors. Channel activation was monitored in whole-cell patch-clamp recordings, whereas the role of the channel in cell volume regulation was determined by electronic cell sizing. It is found that channel activation and volume control probably employs tyrosine kinases, G-proteins, PLC, PKC and p38MAP kinase, and that the process appears to depend on an intact actin skeleton. In contrast, RhoA, PI 3-kinase, ERK 1/2, JNK 1/2 as well as the exocytotic insertion of channels into the plasma membrane are likely not part of the signalling machinery.

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Introduction

It became quite evident in recent years that, in addition to the mere maintenance of homeostasis, mechanisms of cell volume regulation are employed in a variety of most relevant physiological processes. Among these are 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].

If one considers the rates of ion transport through channels that are some 4 to 5 orders of magnitudes higher than those achieved by carriers, it is quite obvious that any modulation of ion channel activity may serve as a fast and efficient mechanism of osmolyte transport and cell volume regulation. Accordingly, whenever analyzed in a quantitative manner, 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). Despite the paramount significance of HICCs for cell volume control and functioning, however, very little is known so far as for the intracellular signalling machinery employed in hypertonic channel regulation.

In the present study, the signalling network of HICC activation was examined in the human cervix carcinoma cell-line HeLa. To this end, inhibitors of various cell signalling pathways were applied and their putative effects on HICC activation were monitored in whole-cell patch-clamp recordings. In parallel, the effects of these compounds on the process of RVI were determined by Coulter-counter techniques. Together, both approaches suggest that the activation of HICCs is mediated via tyrosine kinases, G-proteins, PLC, PKC as well as p38MAP kinase, and that an intact actin skeleton may play a permissive role in this process. In contrast, PI3 kinase, RhoA, MAP kinases ERK 1/2 and JNK 1/2, as well as the process of exocytosis are probably not employed. The data presented here provide a guide-post for the future detailed analysis of the signalling machinery of HICC activation in the HeLa model system.

Materials and Methods

Cell culture

Human cervix HeLa cells were grown as monolayers in minimum essential medium plus 10 % fetal bovine serum, 100 µg/ml streptomycin and 40 IU/ml penicillin under 95% air - 5% CO₂. For the experiments, cells were mechanically detached from the plastic bottom of the culture flasks with a jet of culture medium.

Electrophysiology

Membrane-currents were recorded from single detached HeLa cells in the fast whole-cell mode of the patch-clamp technique. Pipette resistance was in the range of 2 to 4 MΩ; offsets, series resistance and capacitive transients were compensated on the patch-clamp amplifier (Axopatch 200A; Molecular Devices, Union City, CA, USA). Currents were filtered at 1 kHz and digitized at 5 kHz (DigiData 1200B; Molecular Devices). Data acquisition and analysis was done with the pCLAMP 8.0 software (Molecular Devices). In the experiments, voltage ramps from -80 to +20 mV and of 1 s duration were applied every 10 s; holding voltage was -30 mV.

The bath solution (pH 7.5) contained (in mM): NaCl, 94; Na-gluconate, 6; MgCl₂, 1; CaCl₂, 1; TEA-Cl, 2; HEPES, 10. Osmolarity was adjusted to 340 (isotonic) and 450 mosM (hypertonic) by adding mannitol under osmometric control (OM802; Vogel, Giessen, Germany).

The pipette solution (pH 7.3, 300 mosM) contained: NaCl, 26; Na-gluconate, 69; MgCl₂, 1; TEA-Cl, 2; Na₂-ATP, 2; Na₂-GTP, 0.5; HEPES, 10; EGTA, 1. With these ion gradients, the equilibrium potential for Na⁺ and Cl⁻ (as the only permeant ions) is set at 0 and -30 mV, respectively, which allows the determination of HICC currents in HeLa cells at a high level of accuracy [8]. With a physiological K⁺ gradient present as well, HICC activation was virtually identical to the one reported here (not shown) but computation of HICC currents was much more

Fig. 1. Activation of hypertonicity-induced cation channels (HICCs) and regulatory volume increase (RVI) in HeLa cells. (A) Typical whole-cell recording at $V_h = E_{Cl} = -30$ mV with 1 s voltage ramps (from -80 to +20 mV; see (B)) applied every 10 s. Note the increase in current and membrane conductance under hypertonic stress (340 → 450 mosM = HPR). For the time indicated, 300 µM Flufenamate (FLU), a blocker of the HICC was added to the superfusate. (B) Current-voltage relations obtained under isotonic conditions (ISO), at the time of maximal HICC activation (HPR) and after addition of Flufenamate (HPR-FLU). (C) Hypertonic cell shrinkage and RVI determined under control conditions (CTR, $n = 10$) and in the presence of Flufenamate (FLU, $n = 10$). Typical sets of Coulter-counter recordings. RVI values were computed with reference to the maximal amount of cell shrinkage observed at 15 min of hypertonic stress.

difficult to achieve.

Determination of cell volumes

Changes of HeLa cell volumes were quantified by electronic cell sizing on a Coulter-type analyzer (CDA-500, Sysmex, Japan), as was described previously [8]. Experimental solutions contained: NaCl, 95; KCl, 4.5; MgCl₂, 1; CaCl₂, 1; NaHCO₃, 10; HEPES, 5. Osmolarities were adjusted to 300 mosM (isotonic) and 450 mosM (hypertonic) by addition of mannitol.

Statistical analysis

Data are presented as means $\pm$ SEM with n denoting the number of experiments. Student's t -tests for paired and unpaired data were applied as appropriate. *, ** and *** stands for $p < 0.05$, 0.01 and 0.001, respectively.

Results and Discussion

HICC activation and RVI

Changing extracellular osmolarity from 340 to 450 mosM led to an increase of Na^+ currents (that were determined at $V_h = E_{\text{Cl}} = -30$ mV) from -0.29 ± 0.09 to -3.91 ± 0.35 pA/pF, i.e. by -3.62 ± 0.39 pA/pF ($n = 11$, $p < 0.001$; see Figures 1a, b and 2a). The effect coincided with a shift in zero-current voltage from -19.3 ± 2.6 mV to -2.4 ± 0.6 mV ($p < 0.001$) which is very close to $E_{\text{Na}} = 0$ mV. Furthermore, addition of 300 μM flufenamate, an established blocker of the HICC in HeLa cells [8] decreased the hypertonicity-induced current by $88.2 \pm 4.3\%$ ($n = 4$, $p < 0.001$; Figure 1a, b).

As was determined by Coulter-counter techniques, increasing osmolarity from 300 to 450 mosM led to an initial shrinkage of cells to $77.6 \pm 1.2\%$ of the control value (Figure 1c). Thereafter, cell volume was increasing again to $86.6 \pm 1.7\%$ at 120 min of hypertonic stress. Referred to the initial amount of cell shrinkage, this is equivalent to an actual RVI value of $42.7 \pm 1.6\%$ ($n = 10$, $p < 0.001$; Figures 1c and 2b). In the presence of 300 μM flufenamate, RVI was reduced to $2.2 \pm 0.6\%$ ($n = 10$, $p < 0.001$). These data give further support to the significance of HICCs in the RVI of HeLa cells [8].

RhoA and the actin cytoskeleton

In the first screen, we were testing for a possible role of the actin cytoskeleton and RhoA in the activation process of HICCs. To this end, H-1152 was employed as an inhibitor of Rho kinase (ROCK), which is a target downstream to RhoA [9, 10]. Swinholide A was used as a stabilizer of F-actin [11, 12], whereas latrunculin B was applied to sever actin filaments ([10, 11, 13]; see Table I for concentration of compounds and protocols of incubation). As shown in Figure 2a, H-1152 and Swinholide A had no detectable effect on HICC currents, which equalled -3.39 ± 0.99 pA/pF ($n = 7$) and -4.02 ± 0.79 pA/pF ($n = 6$), with these compounds. Latrunculin B, in contrast, reduced HICC activation by more than half, i.e. to -1.65 ± 0.43 pA/pF ($n = 8$, $p < 0.01$). These data suggest a permissive role of an intact actin cytoskeleton in HICC activation but they render an actual contribution of RhoA and the formation of stress fibres to the process rather

Fig. 2. HICC currents (A) and RVI (B) under control conditions (CTR, $n = 11$ and 10), in the presence of the ROCK inhibitor H-1152 (100 nM, $n = 7$ and 10), the stabilizer of F-actin Swinholide A (SWIN A, 2 nM, $n = 6$ and 10) and Latrunculin B (LAT-B, 20 nM, $n = 8$ and 10) known to sever F-actin filaments. In (B), data obtained in the presence of Amiloride (100 μM , as a blocker of NHE1) and Flufenamate (as blocker of HICCs) is given as reference values. See text for details.

unlikely. This notion is further supported by the Coulter-counter recordings, where RVI equalled $40.4 \pm 1.5\%$ and $44.3 \pm 1.6\%$ in the presence of H-1152 and Swinholide A, respectively ($n = 10$, n.s., for each condition), but where it was significantly reduced to 30.2 ± 1.3 , with latrunculin B ($n = 10$, $p < 0.001$; Figure 2b).

In addition to the control recordings with flufenamate, a series of Coulter-counter experiments was performed in the presence of 100 μM amiloride as a further point of reference. Under these conditions, i.e. with NHE1 almost completely blocked [14–16] but with no effect on HICCs to be expected [8], RVI equalled $22.2 \pm 1.4\%$ (see Figure 2b).

Fig. 3. HICC currents (A) and RVI (B) under control conditions (CTR) and in the presence of Genistein (GEN, 50 μ M, $n = 6$ and 10) as an inhibitor of tyrosine kinases, pertussis toxin (PTX, 100 ng/ml, $n = 10$ and 10) as a blocker of G-proteins, and U73122 (5 μ M, $n = 5$ and 10) and Bis-indolyl-maleimide-I (BIM-I, 200 nM, $n = 7$ and 10) as inhibitors of PLC and PKC, respectively. U73343 (5 μ M, $n = 5$ and 10) was used as the negative control for U73122).

Fig. 4. HICC currents (A) and RVI (B) under control conditions (CTR) and in the presence of Wortmannin (WRTM, 100 nM, $n = 6$ and 10) and LY294002 (20 μ M, $n = 6$ and 10) as inhibitors of PI-3 kinase.

Tyrosine kinases, G-proteins, PLC and PKC

In the presence of genistein [9, 17-19], pertussis toxin [9, 20, 21] and U73122 [9, 22-24], HICC currents were significantly reduced to -1.08 ± 0.16 pA/pF ($n = 6$, $p < 0.001$), -1.09 ± 0.55 pA/pF ($n = 10$, $p < 0.01$) and -0.48 ± 0.02 pA/pF ($n = 5$, $p < 0.001$), respectively. In contrast, with U73343 [22-24] as an inactive analogue of U73122, currents were virtually identical to control values (i.e. -3.47 ± 0.61 pA/pF, $n = 5$; Figure 3a). These data suggest that tyrosine kinases, G-proteins and PLC are employed in the signalling machinery of HICC activation. Moreover, bis-indolyl-maleimide-I, a rather specific inhibitor of PKC [9, 25-27], was found to reduce HICC currents to -

1.73 ± 0.44 pA/pF ($n = 7$, $p < 0.01$; Figure 3a).

In the Coulter-counter recordings, genistein and bis-indolyl-maleimide-I exhibited effects that were very similar to the inhibition of the HICC, i.e. RVI values that were reduced to $7.9 \pm 0.8\%$ ($n = 10$, $p < 0.001$) and $14.0 \pm 2.1\%$ ($n = 10$, $p < 0.001$), respectively (see Figure 3b). In the presence of pertussis toxin, on the other hand, RVI was no longer detectable; rather, there was a slow further decrease of cell volume following the initial period of shrinkage leading to an RVI value of $-19.5 \pm 2.7\%$ ($n = 10$, $p < 0.001$). Interestingly, with PLC blocked by U73122, a negative RVI as low as $-68.9 \pm 3.3\%$ ($n = 10$, $p < 0.001$) was obtained. Of note, this is close to the data

Fig. 5. HICC currents (A) and RVI (B) under control conditions (CTR), in the presence of SB203580 (1 μ M, $n = 7$ and 10) and SB202190 (2 μ M, $n = 8$ and 10) as blockers of p38 MAP kinase, and with SP600125 (20 μ M, $n = 10$ and 10) and U0126 (5 μ M, $n = 8$ and 10) as inhibitors of JNK 1/2 and ERK 1/2, respectively.

derived from one additional set of reference experiments performed in the presence of 300 μ M flufenamate *plus* 100 μ M amiloride, i.e. under conditions where both HICC and NHE1 are supposed to be blocked, namely $-51.9 \pm 3.0\%$ ($n = 10$, $p < 0.001$; Figure 3b). This may be interpreted in terms of the dual role of PLC in the regulation of HICC as well as NHE1. As expected, U73343 as the inactive analogue of U73122 had no effect on RVI, which was $42.3 \pm 2.9\%$ in the presence of the drug ($n = 10$).

PI-3 kinase

In the next screen, we tested for a possible role of PI-3 kinase in the process of HICC activation. With wortmannin and LY294002 as supposed inhibitors of the enzyme [9, 28-30], HICC currents were -3.32 ± 0.56 pA/pF ($n = 6$) and -2.90 ± 0.82 pA/pF ($n = 6$), both not significantly different from control (Figure 4a). Together

Fig. 6. HICC currents (A) and RVI (B) under control conditions (CTR), and in the presence of Nocodazole (NOC, 30 μ M, $n = 6$ and 10) and Brefeldin A (BREF A, 5 μ M, $n = 7$ and 10) as blockers of exocytosis.

these data render a role of PI-3 kinase in the activation of the HICC rather unlikely.

In sharp contrast, however, RVI was significantly reduced to $20.7 \pm 2.0\%$ ($n = 10$, $p < 0.001$) and $25.4 \pm 3.2\%$ ($n = 10$, $p < 0.001$) in the presence of wortmannin and LY294002, respectively (Figure 4b). This result is in accordance with a recent report from this laboratory showing the activation of Akt/PKB in HeLa cells under hypertonic stress as well as an inhibition of RVI when Akt/PKB is blocked [31]. Since the RVI values with wortmannin and LY294002 match the one obtained in the presence of 100 μ M amiloride (Figure 4b), i.e. under conditions where NHE1 will be blocked, it is supposed here that the PI-3 kinase and Akt/PKB pathway is actually functioning as the mediator for the hypertonic activation of this transporter but with no effect on the HICC of HeLa cells at all.

Compound	Target	References	Concentration	Incubation time	Cell culture / Experiment	HICC Current (pA/pF)	RVI (%)
Control						-3.62	42.7
H-1152	ROCK	[9, 10]	100 nM	20 - 80 min	+ / +	-3.39	40.4
Swinholide A	F-actin (stabilizing)	[11, 12]	2 nM	20 - 60 min	+ / +	-4.02	44.3
Latrunculin B	F-actin (severing)	[10-13]	20 nM	20 - 80 min	+ / +	-1.65**	30.2***
Genistein	tyrosine kinases	[9, 17-19]	50 μ M	80 - 200 min	+ / +	-1.08***	7.9***
Pertussis toxin	G-proteins	[9, 20, 21]	100 ng/ml	15 - 20 h	+ / -	-1.09**	-19.5***
U73122	PLC	[9, 22-24]	5 μ M	30 - 60 min	+ / +	-0.48***	-68.9***
U73343	PLC (negative control)	[22-24]	5 μ M	30 - 60 min	+ / +	-3.47	42.3
Bis-indolyl-maleimide I	PKC	[9, 25-27]	200 nM	100 - 200 min	+ / +	-1.73**	14.0***
Wortmannin	PI-3 kinase	[9, 29-31]	100 nM	40 - 120 min	+ / +	-3.32	20.7***
LY294002	PI-3 kinase	[9, 29-31]	20 μ M	30 - 80 min	+ / +	-2.90	25.4***
U0126	ERK 1/2	[33-35]	5 μ M	30 - 150 min	+ / +	-2.96	46.0
SP600125	JNK1/2	[36-38]	20 μ M	30 - 70 min	+ / +	-2.53	37.0
SB203580	p38 MAP kinase	[39-40]	1 μ M	20 - 80 min	+ / +	-1.43***	29.3**
SB202190	p38 MAP kinase	[37, 41, 42]	2 μ M	20 - 80 min	+ / +	-2.49*	30.4**
Nocodazole	vesicular transport	[9, 49, 50]	30 μ M	5 - 8 h	+ / -	-4.18	46.2
Brefeldin A	vesicular transport	[50-52]	5 μ M	30 - 100 min	+ / +	-3.12	44.2

Table 1. Concentration and incubation time of the compounds used (*, **, ***, significantly different from Control with $p < 0.05, 0.01, 0.001$, respectively).

In an additional series of measurements with wortmannin *plus* amiloride, the RVI of HeLa cells was $25.8 \pm 2.0\%$ of the control value ($n = 10$) which is not significantly different from the experiments where both compounds were applied separately (see above). The lack of additivity of these both effects would support the hypothesis that the hypertonic activation of NHE1 in HeLa cells is actually achieved via PI-3 kinase.

MAP kinases

Next, we tested for the putative contribution of various MAP kinase pathways to HICC activation. In the presence of U0126 [32-34] and SP600125 [35-37], supposed inhibitors of ERK 1/2 and JNK 1/2, however, HICC currents equalled -2.96 ± 0.68 pA/pF ($n = 7$) and -2.53 ± 0.79 pA/pF, respectively, which is both not significantly different from control. In contrast, SB203580 [37-39] and SB202190 [36, 40, 41], reported blockers of p38 MAP kinase, reduced HICC currents to -1.43 ± 0.37 pA/pF ($n = 6$, $p < 0.001$) and -2.49 ± 0.31 pA/pF ($n = 8$, $p < 0.05$; Figure 5a).

In the Coulter-counter experiments, RVI equalled $46.0 \pm 1.6\%$ ($n = 10$) and $37.0 \pm 2.2\%$ ($n = 10$) with

U0126 and SP600125, respectively, supporting the electrophysiological data that ERK 1/2 and JNK 1/2 are probably not part of the machinery for HICC activation. In the presence of SB203580 and SB202190, however, RVI was reduced to $29.3 \pm 1.1\%$ ($n = 10$, $p < 0.01$) and $30.4 \pm 2.4\%$ ($n = 10$, $p < 0.01$; Figure 5b) supporting an actual role of p38 MAP kinase in this process.

Of note in this respect, in the human leukaemia cell line U-937, hypertonic stress led to a sizeable activation of p38 MAP kinase which, however, was found to occur independently from activation of NHE-1 [42]. In rat kidney medullary thick ascending limb of Henle's loop, cell shrinkage activated p38 MAP kinase and SB203580 inhibited RVI [43]. In the isolated perfused heart of *Rana*, hypertonic stress was also found to phosphorylate p38 MAP kinase; but unfortunately, its targets were not defined [44]. Interestingly, in rat hepatocyte HTC cells, p38 MAP kinase appeared to *inhibit* the hypertonic activation of non-selective cation channels [45]. Since the HICC in rat hepatocytes is sensitive to amiloride [46, 47] whereas the one in HeLa cells clearly is not [8], however, the actual molecular target of p38 MAP kinase in both systems may differ significantly (see also [1, 6, 7]).

Exocytosis

Finally, the possible contribution of vesicular transport to HICC current activation was tested. In the presence of nocodazole, a supposed promoter of tubulin depolymerization and inhibitor of microtubule-dependent vesicular transport [9;48;49], and brefeldin A, for that a block of protein transport from the ER to the Golgi has been reported [49-51], HICC currents were -4.18 ± 0.79 pA/pF ($n = 6$) and -3.12 ± 0.75 pA/pF ($n = 7$), respectively, which is not significantly different from control (Figure 6a). Likewise, RVI equalled $46.2 \pm 1.3\%$ ($n = 10$, n.s.) and $44.2 \pm 2.2\%$ ($n = 10$, n.s.) with these compounds (Figure 6b). Together, the results render exocytotic processes as a mechanism of HICC current activation rather unlikely.

Conclusions

From the data presented herein, an overview of the signalling elements employed in HICC activation could be obtained. It was found that the process appears to occur via tyrosine kinases, G-proteins, PLC, PKC and

p38MAP kinase, and that it probably relies on an intact actin cytoskeleton. In contrast, RhoA, ERK 1/2, JNK 1/2, PI 3-kinase as well as channel exocytosis, probably do not contribute to the signalling machinery. One may speculate that the activation of NHE1 (by way of PI-3 kinase) and of HICC occur on separate pathways that possibly may converge on the level of PLC (but not PKC).

This working model is supposed to provide the basis for a future detailed systemic analysis of HeLa cell signalling during RVI. In addition, it may offer useful tools for further defining the interplay of cell volume regulation vs. proliferation and apoptosis.

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