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Heat stress preconditioning does not protect renal epithelial Na^+,K^+,Cl^- and Na^+,P_i cotransporters from their modulation by severe heat stress

France Gagnon, Sergei N. Orlov, Marie Josée Champagne, Johanne Tremblay, Pavel Hamet *

Centre de Recherche du C.H.U.M., Campus Hôtel-Dieu, Université de Montréal, 3850 St. Urbain St., Montréal, Que. H2W 1T8, Canada

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Abstract

This study compares the effects of heat and osmotic stress on heat stress protein (HSP) production while examining the putative protective action of HSPs on modulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters in Madin-Darby canine kidney (MDCK) epithelial cells by severe heat stress (46°C, 15 min). Preconditioning heat stress (43°C, 20 min) followed by 4 h recovery at 37°C led to a 35-fold increase of HSP70 mRNA expression measured by Northern blot analysis. The protein content of HSP70 and HSP27, assessed by Western blots, was augmented by 5- and 2-fold, respectively, after 6 h of recovery. In contrast to preconditioning heat stress, hyperosmotic stress (520 vs. 320 mosm) elevated HSP70 mRNA content only by 7-fold and did not significantly affect the protein content of HSP70 or HSP27. Neither cell survival, assessed as lactate dehydrogenase (LDH) release, nor the basal activities of the ion transporters and their modulation by protein kinase C, P_2-purinoceptor and cell volume were altered by preconditioning heat stress. Severe heat stress increased extracellular LDH content from 3 ± 2 to $23 \pm 5\%$ and enhanced Na^+,K^+,Cl^- and Na^+,P_i cotransport activity by 2–3-fold. The volume- and protein kinase C-dependent regulation of these carriers was abolished by severe heat stress while regulation by P_2-purinoceptors was preserved. Preconditioning heat stress diminished severe heat stress-induced LDH release to $11 \pm 4\%$ but did not protect Na^+,K^+,Cl^- and Na^+,P_i cotransporters from activation by severe heat stress and did not prevent severe heat stress-induced inactivation of protein kinase C- and volume-dependent signaling pathways. These results show that in MDCK cells, preconditioning heat stress-induced HSPs are not involved in the regulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters and do not protect them from modulation by severe heat stress.

Keywords: Heat stress protein; Renal epithelium; Na^+,K^+,Cl^- cotransporter; Na^+,P_i cotransporter

1. Introduction

Heat stress proteins (HSPs) are encoded by a conserved gene family whose expression is increased in response to environmental stressors such as heat, hyperosmolarity, heavy metals, reactive oxygen species and chronically applied excitatory stimuli [1–4]. Functioning as chaperons, HSPs play a role in both assembly and transport of newly synthesized protein within cells as well as in the removal of denatured proteins, thus preventing protein damage under environmental stress [5,6] and providing cellular resistance to subsequent stress that would other-

* Corresponding author. Fax: +1-514-843-2911; E-mail: hamet@ere.umontreal.ca

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wise be lethal [7]. This protection of cellular functions induced by sublethal heat stress against subsequent stress is termed thermotolerance or thermoprotection. It has been shown that transfection with HSP70 cDNA protects fibroblasts from severe heat stress, whereas microinjection of HSP70 antibody sensitizes these cells to heat [8], thus providing direct evidence for the role of HSPs in cell survival.

Special interest in acquired cytoprotection has arisen from experiments on ischemic preconditioning of cardiac tissue. These studies have demonstrated that preconditioning under transient ischemia leads to myocardial protection against subsequent ischemia and reperfusion injury (for recent reviews, see [9–11]). Such research on preconditioning has spread to its putative application in the protection of other tissues including the kidney. Thus, it has been shown that mild heat stress or a brief period of ischemia-reperfusion in the rat kidney is associated with resistance to subsequent ischemic or oxidative stress [12,13]. Moseley and co-workers reported that preconditioning heat stress elevates the threshold temperature required to disrupt transepithelial electrical resistance of Madin-Darby canine kidney (MDCK) cell monolayers [14]. In these studies, both mild preconditioning stress and brief ischemia were accompanied by induction of HSP70 production. However, the role of HSPs in kidney thermoprotection is still a matter of controversy [15,16].

It should be mentioned that thermoprotection of kidney has been demonstrated mainly by estimation of cell survival, whereas data on thermotolerance of renal ion transporters are limited to reports on thermoprotection of transepithelial glucose transport in primary monolayer cultures from the flounder kidney [17] and in monolayers of porcine-derived renal epithelial cell line, LLC-PK1 [18]. To the best of our knowledge, nothing is known about thermotolerance of other transporters in kidney epithelium and their regulation by HSPs. This study examines the effect of preconditioning heat stress and osmotic stress on HSP70 and HSP27 production and compares these results with the action of preconditioning and severe heat stress on the baseline activity of Na^+,K^+,Cl^- and Na^+,P_i cotransport and their regulation by protein kinase C, P_2-purinoceptors and cell volume in epithelial MDCK cells derived from collecting duct of canine kidney. Previously, it was shown that in these cells transcription of HSP70 mRNA is sharply potentiated by mild heat stress and by elevation of osmolality of extracellular fluid (for review see [3]).

2. Methods

2.1. Cell culture

MDCK cells from the American Type Culture Collection (ATCC No. CCl 34) between passages 54 and 62 were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and grown in MEM, supplemented with sodium bicarbonate 2.5 g/l, HEPES 2 g/l, penicillin 100 U/ml, streptomycin 100 μg/ml and 10% fetal bovine serum (Gibco, Burlington, Ont., Canada). The medium was changed every 2–3 days. The cells were passaged at subconfluent density by treatment with 0.05% trypsin (Gibco) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline, scraped from the flasks with a rubber policeman and inoculated at 1.25×10^3 cell/cm^2. Both stock cultures and cultures for experiments were grown in 80 cm^2 culture flasks and 24 well plates, respectively, for 6 –8 days to attain subconfluency.

2.2. Heat and osmotic stress

For preconditioning heat stress, cells were incubated for 20 min in a water bath at 43°C, then transferred to an incubator at 37°C for up to 8 h. To induce severe heat stress, they were incubated for 15 min in a water bath at 46°C. The medium was changed before heat stress was applied and replaced by prewarmed medium without fetal bovine serum. After heat stress, the medium was removed immediately and replaced by new medium prewarmed at 37°C, except in experiments for Western blot analysis where it was not changed after heat stress. Hyperosmotic stress was induced by the addition of NaCl or sucrose (final osmolality 520 mosm) to the MEM containing fetal bovine serum for up to 24 h. The osmolality of solutions was estimated with a Knauer milliosmometer (Berlin, Germany). To control the cytotoxicity of stress, lactate dehydrogenase (LDH) release and the relative content of attached cells were

measured using an enzymatic assay kit (Sigma, St. Louis, MO, USA) and the modified Lowry method [19], respectively. Table 1 shows that these parameters were not changed under preconditioning heat stress, whereas severe heat stress increased LDH release from 3 to 23% and decreased the content of attached cells by 28%. Both parameters were partly normalized when the cells were subjected to preconditioning heat stress. In the ion transport experiments, the effect of heat stress on cell survival was estimated by the measurement of protein content in attached cells.

2.3. Extraction of RNA and Northern blot analysis

Total RNA from cells seeded in 80 cm^2 plates was isolated by the acid guanidinium thiocyanate-phenol-chloroform method [20]. Samples containing 10 μg of total RNA in a volume of 5 μl were denatured by heating at 65°C for 15 min in 20 μl of a buffer containing 20 mM MOPS, 50% formamide and 2.2 M formaldehyde, mixed with 5 μl of a loading buffer containing 20 mM MOPS, 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol. RNA was size-fractionated by electrophoresis on 1% agarose and 1.8% formaldehyde gel in 20 mM MOPS, 5 mM sodium acetate and 0.7 mM EDTA, pH 7.0, at 100 mA for 4 h. The gels were stained with ethidium bromide, and RNA was transferred to Hybond nylon membranes following the manufacturer's instructions. To fix RNA, the membranes were treated for 5 min under UV light and stored at 4°C until prehybridization. The blots were prehybridized at 65°C for 2–3 h in a buffer containing 1 M NaCl, 1% SDS and 10% dextran and hybridized overnight at 65°C in the prehybridization solution with the addition of 100 μg/ml of denatured salmon sperm DNA and 10^6 cpm/ml ^{32}P-labeled cDNA probes of rat HSP27 and human HSP70. The HSP27 probe was radiolabeled by the polymerase chain reaction amplification method. The HSP70 probe was radiolabeled by the random priming technique. After hybridization, the membranes were washed three times, exposed on phosphorus-sensitive cassettes for 24 h, and HSP mRNA content was quantified by PhosphorImager densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and normalized for β-actin mRNA content.

2.4. Protein extracts and Western blot analysis

Cells seeded in 80 cm^2 flasks were washed twice in ice-cold PBS, scraped and centrifuged for 10 min at $650 \times g$ at 4°C. Cell pellets were resuspended in 50 μl of hypotonic buffer (10 mM Tris, pH 7.4; 1 mM EDTA; 1 mM DTT; 1 mM PMSF; 50 μg/ml leupeptin), frozen immediately on dry ice and stored at −80°C. After four cycles of freezing-thawing, extracts were centrifuged at $15\,000 \times g$ for 45 min at 4°C and supernatants were kept for further analysis. The protein concentration of the supernatants was determined by the method of Bradford using bovine serum albumin as standard [21] and 100 μg of cytoplasmic protein were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Mississauga, Ont., Canada) in 25 mM Tris and 192 mM glycine for 4 h at 100 V. Nonspecific sites of the nitrocellulose membrane were blocked overnight at 4°C with phosphate buffered saline containing 5% non-fat dried milk (w/v) and 0.1% Tween 20 (v/v). HSP72, the inducible form of HSP70, and HSP27 were respectively detected with mouse Ab SPA-810 and rabbit polyclonal Ab SPA-801 (both from StressGen, Victoria, B.C., Canada) diluted 1:1000 and 1:2500 with phosphate buffered saline containing 1% non-fat dried milk (w/v). Immune complexes were revealed with specific ^{125}I-labeled second antibody at 0.25 μCi/ml. The membrane was exposed and analyzed with a PhosphorImager.

2.5. ^{86}Rb and ^{32}P uptake studies

MDCK cells were washed twice with 2 ml of medium A containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-Tris buffer (pH 7.4, room temperature), then incubated for 30 min at 37°C in 1 ml of medium B with and without agents indicated in the figures. Medium B contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, 20 mM HEPES-Tris (pH 7.4). For ^{86}Rb studies, the preincubation medium was replaced by 0.25 ml of the same medium B with 1 mM ouabain and with or without 20 μM bumetanide. For ^{32}P uptake studies, the cells were washed once with 1 ml of medium C containing (in mM): choline chloride 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, D-glucose 5, 20 mM

HEPES-Tris (pH 7.4). Then, 0.25 ml of medium B or C, with and without the agents indicated in the figures, was added. The cells were incubated at 37°C for 5 min, and thereafter 0.25 ml of medium B containing 1–2 μCi/ml ^{86}RbCl, or medium B or C containing 1 μCi/ml [^{32}P]orthophosphate and 0.2 mM K_2HPO_4 was added. Radioisotope uptake was terminated by the addition of 2 ml of ice-cold medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-Tris buffer (pH 7.4). The cells were then washed four times with 2 ml of ice-cold medium W and lysed with 1 ml of 1% SDS/4 mM EDTA mixture. The radioactivity of the cell lysate was measured with a liquid scintillation analyzer. ^{86}Rb (K^+) and ^{32}P uptake were calculated as $V = A/amt$ where A is the radioactivity in the sample (cpm), a is the specific radioactivity of ^{86}Rb (K^+) or ^{32}P (cpm/nmol) in the incubation medium, m is the protein content (mg) and t is the incubation time (min). Fig. 1 shows that kinetics of ^{86}Rb and ^{32}P uptake were linear up to 20 min. The absolute values of Na^+,K^+,Cl^- cotransport (ouabain-resistant bumetanide-sensitive ^{86}Rb influx, 49.1 ± 5.4 nmol/mg protein/15 min, $n = 20$) and Na^+,P_i cotransport (Na_o^+-dependent component of ^{32}P influx, 1.80 ± 0.01 nmol/mg protein/15 min, $n = 3$) were in accordance with previously reported data [22,23].

2.6. Chemicals

4β-Phorbol 12-myristate 13-acetate (PMA), ATP, ouabain, bumetanide, sucrose were from Sigma (St. Louis, MO, USA); ^{86}RbCl, [^{32}P]orthophosphate were from Dupont (Boston, MA, USA); ^{125}I was from Amersham (Arlington, IL, USA); salts, D-glucose and buffers were from Sigma and Anachemia (Montreal, Ont., Canada).

3. Results

3.1. Effect of preconditioning heat stress and osmotic stress on HSP production

Fig. 2 shows that preconditioning heat stress (43°C, 20 min) rapidly increased HSP70 mRNA content for a maximum 35-fold elevation after 4 h recovery. In contrast to heat treatment, a maximal 8-fold increment of HSP70 mRNA was observed at 8 h of osmotic stress. Prolongation of stress up to 24 h did not significantly modify the HSP70 mRNA level (Fig. 2C,D). Therefore, the absolute values of the osmotic stress-induced increment were 4–5-fold less than with heat stress (Fig. 2E).

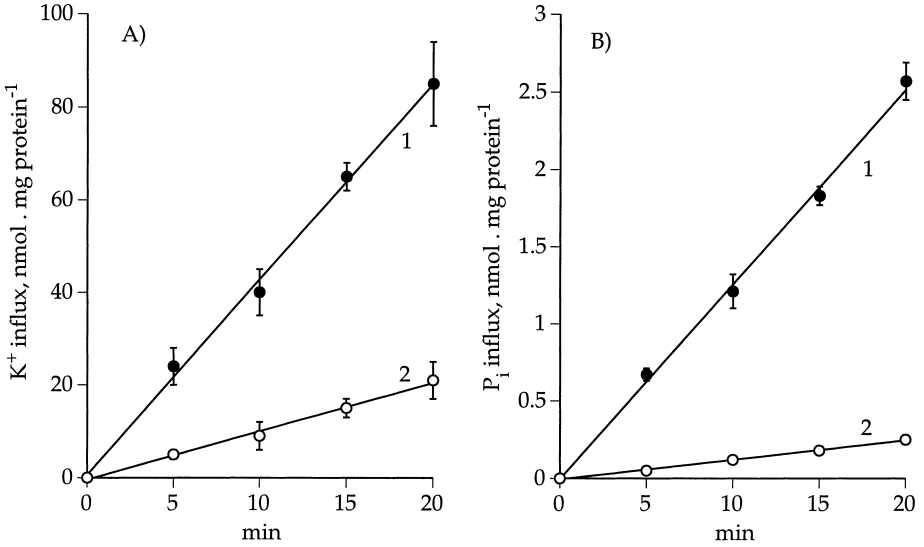

Fig. 1. Kinetics of K^+ (^{86}Rb) (A) and P_i (B) influx in MDCK cells. (A) K^+ influx was measured under control conditions (1, medium B+1 mM ouabain) and in the presence of 20 μM bumetanide (2). (B) P_i influx was measured in medium B containing 0.1 mM K_2HPO_4 (1) or under equimolar substitution of Na^+ with choline chloride (2). Means ± S.E.M. obtained in experiments performed in quadruplicate are given.

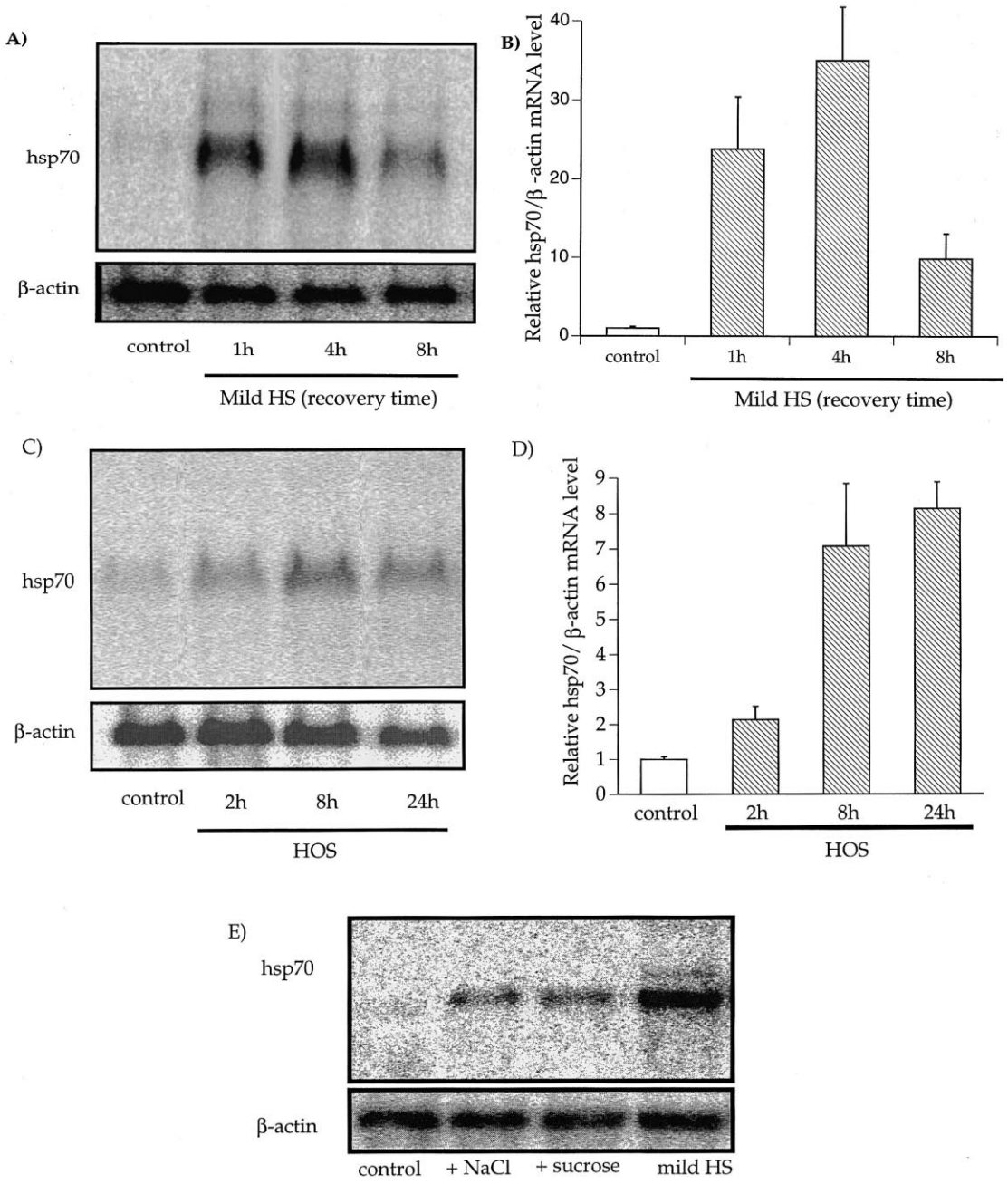

Fig. 2. Northern blot (A,C) and HSP70/β-actin mRNA ratio (B,D) in MDCK cells 1, 4 and 8 h after preconditioning heat stress (HS) (43°C for 20 min) (A,B) and 2, 8 and 24 h after hyperosmotic stress (HOS) induced by the addition of NaCl to MEM (final osmolality 520 mosm vs. 320 mosm in control medium) (C,D). (E) Northern blot comparing the effects of preconditioning heat stress (43°C, 4 h recovery) and hyperosmotic stress induced by increased osmolality from 320 to 520 mosm by the addition of 100 mM of NaCl or 200 mM of sucrose to MEM for of 8 h. The HSP70/β-actin mRNA ratio under control conditions was taken as 1.00. Means ± S.E.M. obtained in experiments performed in triplicate and duplicate are given.

Fig. 3 shows the effect of preconditioning heat stress (43°C for 20 min followed by 6 h recovery at 37°C) and 8 h osmotic stress on HSP70 and HSP27 protein content. Preconditioning heat stress induced 5- and 2-fold increases of HSP70 and HSP27 protein production, respectively. We did not observe any significant effect of osmotic stress on HSP70 and HSP27 protein content (Fig. 3). In view of the stron-

Table 1
Effects of preconditioning and severe heat stress on the survival of MDCK cells

Cell treatment	LDH release (%)	Content of attached cells (%)
1. Control	3 ± 2	100 ± 5
2. Preconditioning heat stress (43°C, 20 min; 37°C, 4 h)	7 ± 3	92 ± 8
3. Severe heat stress (46°C, 15 min)	23 ± 5	72 ± 7
4. Preconditioning heat stress+severe heat stress	11 ± 4	86 ± 8
$P_{1,3}$	< 0.05	< 0.01
$P_{1,4}$	NS	NS

MDCK cells grown in 24 well plates were washed twice with medium A and incubated for 4 h in 1 ml of medium B at 37°C. In part of the experiment, cells were subjected to preconditioning and/or severe heat stress at the beginning and at the end of incubation, respectively. LDH release was estimated as the ratio of extracellular and total (extracellular+intracellular) enzyme activity. To determine intracellular LDH activity, the incubation medium was removed and the cells treated with 0.5% Triton X-100. To estimate the content of attached cells, the incubation medium was aspirated and cells washed five times with 2 ml of ice-cold medium W and treated with 0.5 N NaOH for protein measurement. Protein and total LDH content in control cells were taken as 100%. For the composition of medium A, B and W see Section 2.5. Means ± S.E.M. obtained in 3 experiments performed in quadruplicate are given.

ger effect of preconditioning heat stress on HSP production compared to hyperosmotic stress, heat stress was used as HSP inducer for subsequent experiments.

3.2. *Effect of heat stress on the activity of ion transporters*

Fig. 4 shows the effect of the temperature of preincubation medium on the rate of K^+ and P_i influx. The rate of (ouabain+bumetanide)-resistant K^+ influx (curve 2) was not modulated after 15 min of preincubation in the range from 37 to 47°C, whereas the rate of Na^+-insensitive P_i influx (curve 4) was increased after preincubation at 46–47°C by 70–80%. In contrast, the activity of Na^+,K^+,Cl^- and Na^+,P_i cotransport (curves 1 and 3, respectively) was slightly augmented in the range from 42 to 45°C but was activated by 2–3-fold after preincubation of MDCK cells at 46–47°C. Considering this,

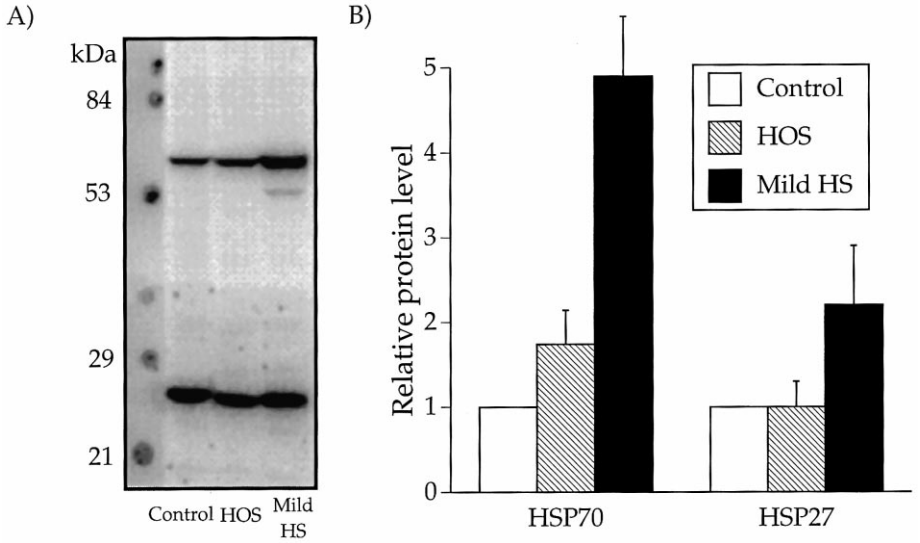

Fig. 3. Western blot (A) and relative content (B) of HSP70 and HSP27 in MDCK cells subjected to hyperosmotic stress (HOS) induced by the addition of sucrose to MEM for 8 h (final osmolality 520 mosm) and by preconditioning heat stress (HS) (43°C, 20 min) followed by 6 h recovery at 37°C. The level of HSPs in control conditions was taken as 1.00. Means ± S.E.M. from two experiments performed in duplicate are given.

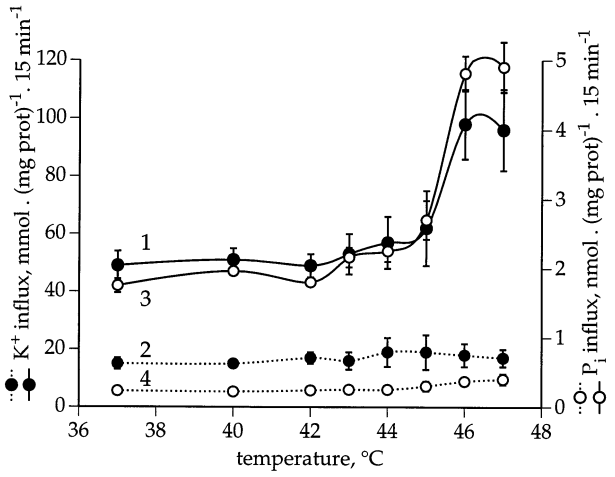

Fig. 4. Dependence of the activity of Na^+,K^+,Cl^- cotransport (1), (ouabain+bumetanide)-resistant K^+ influx (2), Na^+,P_i cotransport (3) and Na^+-independent component of P_i influx (4) in MDCK cells on the temperature of preincubation medium. The medium was changed before heat stress was applied and replaced by prewarmed medium. After 15 min heat stress, the medium was removed and replaced by new medium prewarmed at 37°C. All measurement of ion fluxes was done immediately after heat stress as indicated in Section 2. Means ± S.E.M. obtained in experiments performed in quadruplicate are given.

15 min preincubation at 46°C was used in the further experiments as a severe heat stress. Fig. 5 shows that preconditioning mild heat stress did not protect Na^+,K^+,Cl^- and Na^+,P_i cotransport from their modulation by severe heat stress.

3.3. Effect of heat stress on the regulation of ion transporters by PMA and ATP

We demonstrated previously that an activator of protein kinase C, PMA, completely blocked Na^+,K^+,Cl^- cotransport in MDCK cells and decreased the activity of Na^+,P_i cotransport by ~30%. In contrast to PMA, activation of P_2-purinoceptors by ATP inhibited both carriers by 40–60% [24,25]. Therefore, we also studied the effect of mild and severe heat stress on the regulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters by PMA and ATP.

Preconditioning heat stress did not affect the regulation of Na^+,K^+,Cl^- cotransport and Na^+,P_i cotransport by PMA (Fig. 6) and by ATP (Fig. 7). Severe heat stress completely blocked the regulation of both cotransporters by PMA (Fig. 6) but did not significantly modify their sensitivity to regulation by ATP (Fig. 7). Preconditioning heat stress did not protect the PMA-sensitive signaling pathway from its inactivation by severe heat stress (Fig. 6).

3.4. Effect of heat stress on volume-dependent regulation of Na^+,K^+,Cl^- cotransport

It is known that Na^+,K^+,Cl^- cotransport is activated by hyperosmotic shrinkage in all types of cells studied so far [26] with the exception of human

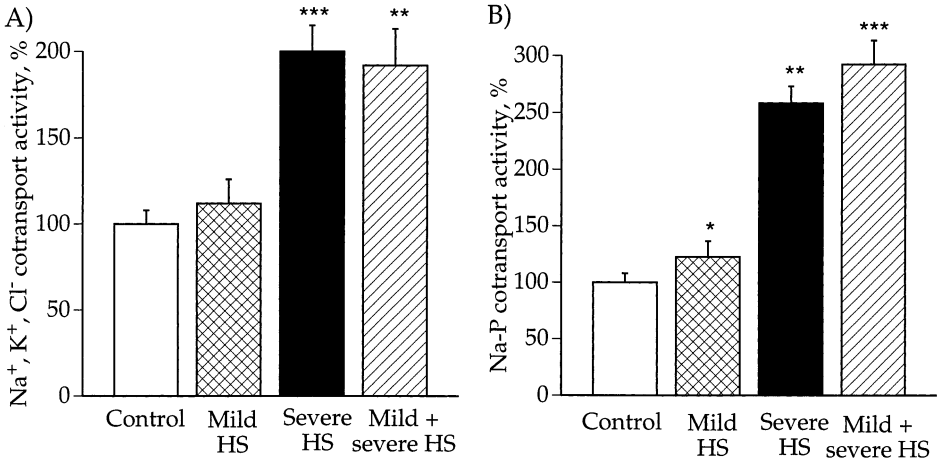

Fig. 5. Effects of preconditioning mild heat stress (HS) (43°C for 20 min, 4 h recovery at 37°C) and severe heat stress (46°C for 15 min) on the activity of Na^+,K^+,Cl^- (A) and Na^+,P_i (B) cotransport. Values of the activity of these transporters under control conditions were taken as 100%. Means ± S.E.M. obtained in seven (A) and two (B) experiments performed in quadruplicate or triplicate are given. *, **, *** for $P < 0.02$, 0.002 and 0.0001, respectively, as compared to the controls.

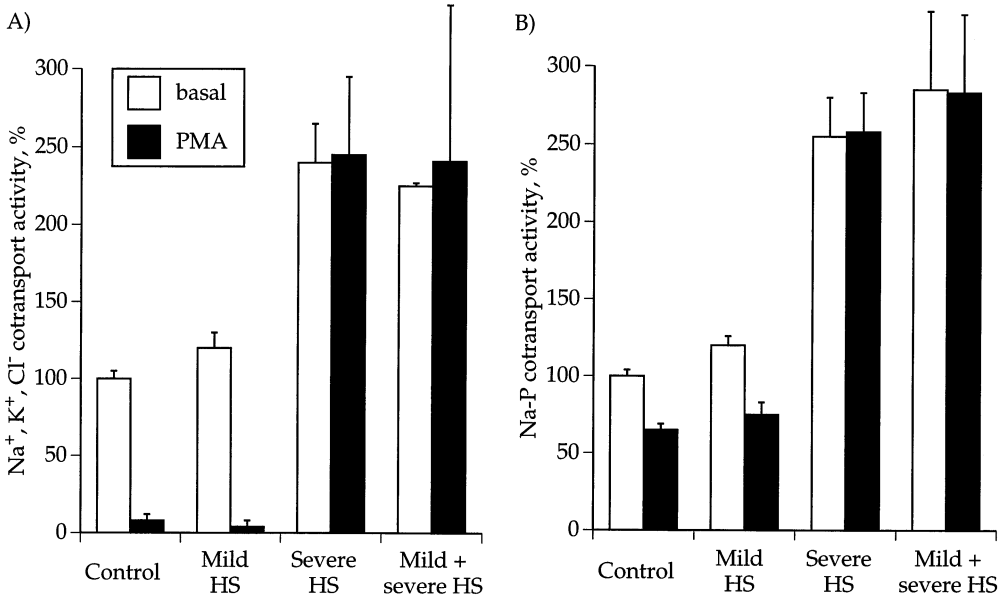

Fig. 6. Effects of preconditioning mild heat stress (43°C for 20 min, 4 h recovery at 37°C) and severe heat stress (46°C for 15 min) on the regulation of Na^+,K^+Cl^- cotransport (A) and Na^+,P_i cotransport (B) by PMA. Following the heat stress protocol, cells were pre-incubated for 30 min with or without 0.1 μM PMA in medium B or C before the measurement of ^{86}Rb or ^{32}P influx, respectively. The value of cotransporter activities in control PMA-untreated cells was taken as 100%. Means ± S.E.M. obtained in three (A) and two (B) experiments performed in quadruplicate and triplicate are given.

erythrocytes [27,28]. Fig. 8 shows that cell shrinkage caused by increment of osmolality from 160 mosm to 470 mosm led to 10-fold activation of Na^+,K^+,Cl^- cotransport. Preconditioning heat stress did not significantly affect volume-dependent regulation of the activity of this carrier, whereas severe heat stress drastically diminished its efficiency. Preconditioning heat stress did not protect the volume-dependent reg-

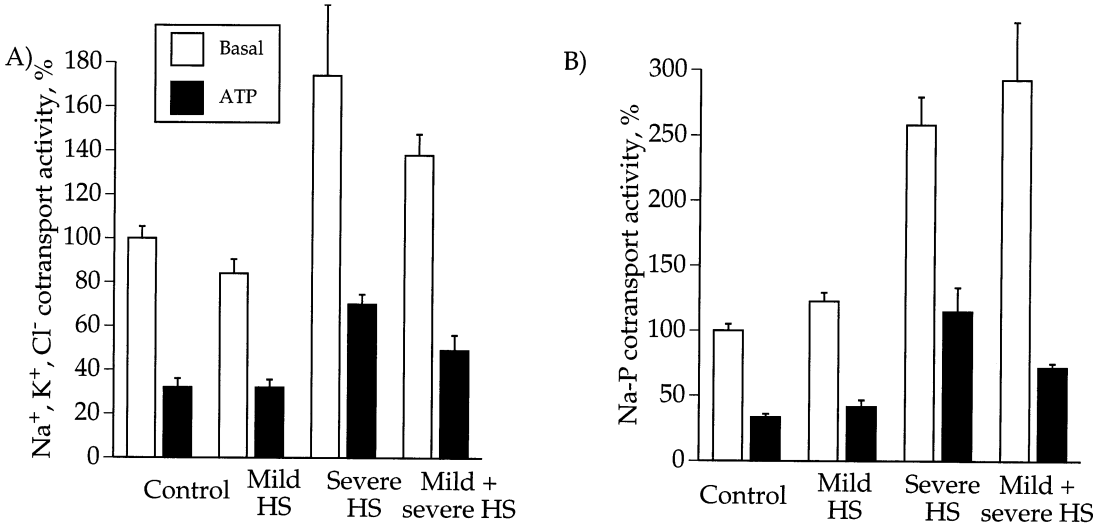

Fig. 7. Effects of preconditioning mild heat stress (43°C for 20 min, 4 h recovery at 37°C) and severe heat stress (46°C for 15 min) on the regulation of Na^+,K^+,Cl^- cotransport (A) and Na^+,P_i cotransport (B) by ATP. Following the heat stress protocol, cells were pre-incubated for 30 min with or without 100 μM ATP in medium B or C before the measurement of ^{86}Rb or ^{32}P influx, respectively. The value of cotransporter activities in control ATP-untreated cells was taken as 100%. Means ± S.E.M. obtained in three (A) and two (B) experiments performed in quadruplicate and triplicate are given.

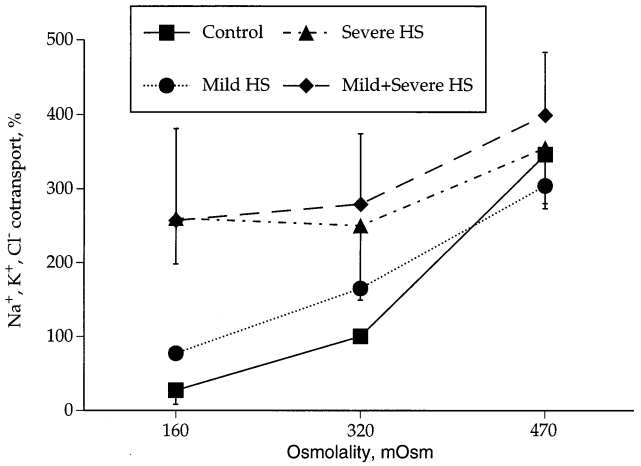

Fig. 8. Effects of preconditioning mild heat stress (42°C for 20 min, 4 h recovery at 37°C) and severe heat stress (46°C for 15 min) on the volume-dependent regulation of Na^+,K^+,Cl^- cotransport. Following the heat stress protocol, cells were preincubated for 15 min in 0.25 ml of isosmotic medium B with 1 mM ouabain and with or without 20 μM bumetanide. Then, 0.25 ml of hyposmotic medium B containing 20 mM NaCl, isosmotic medium B and hyperosmotic medium B containing sucrose was added to adjust the final osmolality of the media at 160, 320 and 470 mosm, respectively. These media also contained 1–2 μCi/ml $^{86}RbCl$. The value of Na^+,K^+,Cl^- cotransport activity in control cells in isosmotic medium without heat stress was taken as 100%. Means ± S.E.M. obtained in two experiments performed in quadruplicate are given.

ulation pathway from its inactivation by severe heat stress.

4. Discussion

The present study demonstrates that in MDCK cells severe heat stress drastically activates Na^+,K^+,Cl^- and Na^+,P_i cotransport, and abolishes their regulation by protein kinase C and cell volume. Mild preconditioning heat stress led to the increased production of HSP70 and HSP27 (Fig. 3) and protected MDCK cells from destruction by severe heat stress (Table 1). However, we did not observe a protective effect of preconditioning heat stress on the modulation of the activity of ion transporters by severe heat stress (Fig. 5). This finding demonstrates that in this model of the mammalian renal epithelium, neither Na^+,K^+,Cl^- nor Na^+,P_i is subjected to thermoprotection and regulation by mild heat stress-inducible HSPs.

To study the influence of HSPs on ion transport in MDCK cells, we compared the effects of preconditioning heat stress (43°C for 20 min, 4 h recovery) and hyperosmotic stress on HSP70 and HSP27 mRNA and protein production. We observed a 35-fold increase of HSP70 mRNA content following heat stress (Fig. 2) that is in accordance with previously reported data [29]. It was demonstrated previously that after 6–10 h of induction of hyperosmotic stress, HSP70 mRNA content in MDCK cells is elevated by 6–8-fold [29,30]. These results concur with data obtained in our study (Fig. 2D). Using the rat DNA probe, we did not detect HSP27 mRNA in MDCK cells probably because of the lower homology of this probe with canine HSP27 compared to the human HSP70 probe used for the detection of HSP70 in MDCK cells. To further examine the effect of preconditioning heat stress and osmotic stress on HSP production, we used Western blot analysis. This study shows that preconditioning heat stress increased HSP70 and HSP27 protein content by 5- and 2-fold, respectively (Fig. 3). These results demonstrate that the mild heat stress-induced increment of HSP70 protein content is much lower than the elevation of its mRNA level, which is consistent with data obtained for a neuroblastoma-derived cell line [31]. In contrast to heat stress, neither HSP70 nor HSP27 protein content in MDCK cells was significantly affected by hyperosmotic stress (Fig. 3). The lack of effect of osmotic stress on HSP70 protein content compared with the moderate induction of HSP70 mRNA is probably due to nonspecific inhibition of protein synthesis under hyperosmotic conditions [32]. Thus, based on data obtained by Western blot analysis, the involvement of HSP27 and HSP70 in the protection of kidney epithelial cells under long-term increment of the osmolality of tubular fluid seems unlikely. This conclusion is in accordance with the negligible effect of dehydration of mice on HSP70 expression in the inner medulla as compared with a recently discovered member of the HSP70/BiP superfamily, osmotic stress protein Osp94 [33,34]. In view of the stronger effect of heat stress on HSP production in comparison to hyperosmotic stress, preconditioning heat stress (43°C for 20 min followed by 4 h recovery) was used as HSP inducer in subsequent experiments.

We did not see any effect of preconditioning heat

stress on the basal activity of Na^+,K^+,Cl^- and Na^+,P_i cotransporters and their regulation by the P_2-purinergic agonist ATP, protein kinase C and cell volume (Figs. 5–8). These results suggest that the biochemical pathways involved in the above-listed cellular functions of mammalian kidney epithelial cells are resistant to modulation of the content of preconditioning heat stress-sensitive HSPs, including HSP70 and HSP27. In contrast to mild preconditioning heat stress, severe heat shock (46°C, 15 min) led to 2–3-fold activation of Na^+,K^+,Cl^- and Na^+,P_i cotransporters (Fig. 4). Under analysis of the possible mechanisms of this phenomenon it is important to underline that the activity of these carriers was sharply increased after elevation of the temperature of the preincubation medium from 45°C to 46°C (Fig. 4). It seems unlikely that severe heat stress activates Na^+,K^+,Cl^- and Na^+,P_i cotransporters via a direct effect on these two non-homologous proteins. The most likely explanation is that this action is mediated by a common thermosensitive element that is involved in the regulation of activity of these carriers. The role of protein kinase C in the activation of the carriers by severe heat stress should be probably excluded because of the slight (20–25%) inhibition of these enzyme observed after 1 h exposure of cells at 45°C [35]. Under analysis of these data it should be underlined that a complete inhibition of protein kinase C with calphostin C increased Na^+,K^+,Cl^- cotransport by 30–35% only [36] and did not affect Na^+,P_i cotransporter in MDCK cells (data prepared for publication), whereas 15 min of exposure at 46°C increased the activity of Na^+,K^+,Cl^- and Na^+,P_i cotransporters by $\sim$2- and 2.5-fold, respectively (Fig. 4). Considering this, we speculate that a thermosensitive regulator of these ion carriers is related to the cytoskeleton network. Several lines of evidence support our assumption.

1. In several types of cells, including aortic smooth muscle cells and renal epithelial cells, modification of the cytoskeleton network drastically affects the activity of Na^+,K^+,Cl^- [37,38] and Na^+,P_i [39,40] cotransporters.
2. Treatment of aortic smooth muscle cells and renal epithelial cells with a disintegrator of microfilament bundles, cytochalasin B, or with a stabilizer of F-actin, phalloidin, prevents the hormonal regulation of Na^+,K^+,Cl^- cotransporter [37,38].
3. The interaction of protein kinase C with anchoring proteins of the cytoskeleton network plays a crucial role in the regulation of activity of the membrane-bound enzyme and transporters [41]. Fig. 6 shows that severe heat stress completely inhibited the regulation of both cotransporters in MDCK cells by the protein kinase C activator PMA.
4. In rat erythrocytes, disruption of the cytoskeleton network by annealing of the spectrin carcass under 10 min preincubation at 49°C completely abolishes the shrinkage-induced activation of Na^+,K^+,Cl^- cotransporter [42] which is in accordance with data on the inhibition of volume-dependent regulation of Na^+,K^+,Cl^- cotransporter in MDCK cells subjected to severe heat stress (Fig. 8).

In contrast to the complete inhibition of volume- and PMA-induced regulation, severe heat stress did not significantly modify the regulation of Na^+,K^+,Cl^- and Na^+,P_i cotransporter by ATP. These results suggest that the mechanisms of inhibition of these carriers by PMA and ATP are different. This conclusion is supported by data on the lack of effect of protein kinase C inhibitors and down-regulation of protein kinase C under long-term treatment with PMA on the regulation of Na^+,K^+,Cl^- cotransporter by ATP [24,36].

In conclusion, in contrast to protection of the viability of severe heat stress-treated MDCK cells by mild preconditioning heat stress, we did not discern any thermoprotection of two major renal ion pathways, i.e. Na^+,K^+,Cl^- and Na^+,P_i cotransporters, by preconditioning heat stress. We also did not observe any involvement of preconditioning heat stress-inducible HSPs in the modulation of activity of these carriers. These data suggest that in contrast to protection by mild heat stress of Na^+-coupled glucose transporter from their modulation by severe heat stress revealed in monolayers of flounder kidney epithelial cells [17] and in LLC-PK_1 cells [18] which are highly abundant in proximal tubules, mild heat stress-inducible HSPs are not involved in the protection of Na^+,K^+,Cl^- and Na^+,P_i cotransporters in renal epithelial cells from collecting duct of mamma-

lian kidney. However, to draw a final conclusion, additional experiments with monolayers of epithelial cells from collecting duct possessing transcellular movement of salt and osmotically obliged water should be performed.

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