Regulation of stanniocalcin in MDCK cells by hypertonicity and extracellular calcium

DAVID SHEIKH-HAMAD, DIANE ROUSE, AND YU YANG
Renal Section, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

Sheikh-Hamad, David, Diane Rouse, and Yu Yang. Regulation of stanniocalcin in MDCK cells by hypertonicity and extracellular calcium. Am. J. Physiol. Renal Physiol. 278: F417–F424, 2000.—Differential display RT-PCR cloning method was applied to poly(A)⁺ RNA isolated from Madin-Darby canine kidney (MDCK) cells in isotonic or hypertonic medium. A differentially expressed 360-bp PCR fragment was isolated, subcloned, sequenced, and used to screen an MDCK cDNA library constructed in λZapII. A composite sequence of two overlapping cDNA clones provided 1,053 bp of sequence that was 93% identical to human stanniocalcin and corresponded to the 3'-end of the mRNA. Although the fish homolog of this hormone inhibits calcium uptake by the gill and intestine, the function of mammalian stanniocalcin remains unknown. Stanniocalcin cDNA probe hybridizes to a 4.4-kb mRNA that is induced eightfold by hypertonicity, in a manner that is dependent on medium organic osmolytes. The mRNA induction correlates with increased total cellular content of the protein and its concomitant release to the medium, consistent with secretion for autocrine or paracrine activity. Furthermore, induction of the mRNA by hypertonicity is dependent on extracellular calcium and displays a threshold phenomenon. The data suggest that kidney stanniocalcin may have a role in the adaptation of kidney cells to osmotic stress, in a manner that is extracellular calcium dependent.

Kidney; osmotic stress; calcium; Madin-Darby canine kidney cells; thick ascending limb

In our pursuit of identifying mRNAs that are induced by hyperosmotic stress in Madin-Darby canine kidney (MDCK) cells, we used differential display RT-PCR cloning method to display and clone partial cDNA products corresponding to down- or upregulated mRNA (9, 15, 23, 38, 39). To date, despite evidence to suggest that the hormone interacts with a receptor on the serosal membrane of the gill cells and, through an unknown second messenger system, blocks calcium entry through the apical membrane (surface exposed to ambient water) (37).

In mammals, stanniocalcin is expressed in multiple organs including heart, kidney, prostate, placenta, lung, skeletal muscle, pancreas, thymus, small intestine, colon, thyroid, spleen, and ovary (3, 4). Immunohistochemical studies in rat, mouse, and human kidney localize stanniocalcin to distal nephron segments including thick ascending limb, distal convoluted tubules, and collecting duct cells (principal and type A intercalated) (9, 15, 23, 38, 39). To date, despite evidence to indicate this fact.

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suggest the presence of stanniocalcin in mammalian blood and its expression in multiple organs (23), the function of this hormone remains unknown. Although the expression of stanniocalcin in distal nephron segments, where calcium reabsorption occurs, suggests a regulatory role in renal calcium handling, the following data suggest that stanniocalcin may play a role in the adaptation of kidney cells to osmotic stress.
METHODS

Cell culture. MDCK cells (American Type Culture Collection, Rockville, MD) were used in passages 62–68. Cells were grown in a serum-free, defined medium, containing 1.55 mM Ca\(^{2+}\) (315 mosmol/kgH\(_2\)O) (31). This medium contains 120 µM myo-inositol (inositol) and no betaine or choline. In some experiments, the media were made as above but with one of the following modifications: 1) no inositol, to deplete medium osmolytes; 2) addition of 5 mM inositol and 5 mM betaine, to supplement medium osmolytes; and 3) addition of 200 mosmol/kgH\(_2\)O of one of the following, for osmotic stress: NaCl, raffinose, or urea. These osmotically active constituents were added to the basic medium, making it hyperosmotic to varying extents (515–525 mosmol/kgH\(_2\)O). For another portion of the experiments, EGTA (0.0–1.5 mM) was added to the experimental medium for chelation of calcium. Alternatively, measured amounts of calcium were added to calcium-deficient medium to control for EGTA effects. All cultures were maintained in 5% CO\(_2\)-95% air at 37°C.

Differential mRNA display. Confluent MDCK cells were exposed to isotonic medium (315 mosmol/kgH\(_2\)O) lacking betaine and inositol, or the same medium made hypertonic (515 mosmol/kgH\(_2\)O) by addition of NaCl. Total RNA was purified using RNazol (Tel-Test, Friendswood, TX) (7). Poly(A)^+ RNA was isolated as previously described (14). Differential mRNA display (19, 20) was performed using RNAmap Kit A (GeneHunter, Brookline, MA) according to manufacturer's instructions. Briefly, poly(A)^+ RNA was subjected to DNase I treatment, reverse-transcribed, and amplified by PCR using a modified 14-mer oligo(dT) and a 10-mer arbitrary primer, in the presence of [\(\alpha\)-\(^{35}\)S]dATP (Amersham, Arlington Heights, IL). Radiolabeled PCR products were resolved on denaturing 6% polyacrylamide DNA sequencing gels and analyzed for differentially expressed products (19, 20). Each gel piece, corresponding on the autoradiographs to hypertonically induced cDNA, was cut, and the DNA was extracted. The cDNA was then reamplified by PCR using the same set of primers, cloned in pCR-II vector (TA Cloning Kit, Invitrogen, San Diego, CA), and sequenced.

Northern blot analyses. Total and poly(A)^+ RNA were isolated as described above. Electrophoresis was performed, loading equal amounts of poly(A)^+ RNA per lane in a 1% agarose/2.2 M formaldehyde gel, followed by transfer to a GeneScreen membrane (New England Nuclear) (14). Human (2 kb) full-length \(\beta\)-actin cDNA (Clontech) and mRNA-display PCR products were labeled with [\(\alpha\)-\(^{32}\)P]dCTP (Random Primed DNA Labeling Kit, Boehringer-Mannheim, Indianapolis, IN) for use as probes. The probes were hybridized to the blots overnight at 42°C in a solution containing 40% formamide, 5× SSC (750 mM NaCl, 75 mM trisodium citrate), 0.5× SSC, 0.5% SDS, 30 min twice in 0.1× SSC, 0.5% SDS.

Fig. 2B—Continued.
AUTORADIOGRAHS were prepared using X-Omat AR film (Kodak; Rochester, NY) with an intensifying screen. Bands on Northern blots were scanned using UMAX Astra 1200S scanner (Fremont, CA) and quantitated using Adobe Photoshop 4 and UTHSCSA Image Tool software. Band intensities were determined relative to the corresponding β-actin bands.

RESULTS

Clone HT9 corresponds to dog kidney stanniocalcin. HT9, a 360-bp PCR fragment amplified with primers AP-3 and T12MG (RNAmap Kit A) using MDCK cells poly(A)⁺ RNA, appeared to be differentially induced by extracellular hyperosmolality. When used as a probe on Northern blots, the cDNA hybridized to a 4.4-kb mRNA that is induced sixfold (after 24 h) by the addition of NaCl, raffinose, or urea, and the abundance of stanniocalcin mRNA was determined using Northern hybridization. Addition to the medium of equiosmolar amounts (200 mosmol/kg H₂O) of either NaCl or raffinose, but not the membrane permeable solute urea, induces stanniocalcin mRNA sixfold at 24 h (Fig. 3, A and B). We conclude that stanniocalcin is induced by hypertonic stress imposed by impermeable solutes and may have a role in the adaptation of kidney cells to osmotic stress.

Induction of stanniocalcin mRNA correlates with increased abundance of the protein and its release to the medium. To determine the kinetics of stanniocalcin mRNA induction by hypertonicity, we used Northern hybridization to measure stanniocalcin mRNA abundance. Poly(A)⁺ RNA (3 µg) was loaded per lane on RNA gels, and Northern blots were probed with dog stanniocalcin cDNA. Representative Northern blots are shown. Data are based on at least 3 independent determinations.
Stanniocalcin in MDCK cells at various time points after the addition of NaCl (200 mosmol/kg \(H_2O\)) to the medium, or at a fixed time point (16 h) after the addition of various amounts of NaCl to the medium. Induction of the mRNA occurs as early as 6 h after exposure of the cells to hypertonicity, peaks (8-fold) after 16 h (Fig. 4A), and shows maximal induction with 100 mosmol/kg \(H_2O\) increment in medium osmolality (Fig. 4B). This pattern of induction is similar to that of other hypertonicity-induced mRNAs in its timing, but not in its dose response. For example, peak induction of mRNAs corresponding to genes involved in the accumulation of organic osmolytes occurs after 16–24 h of exposure to hypertonicity (8, 33). In addition, they demonstrate further increase in abundance as medium tonicity increases beyond 100 mosmol/kg \(H_2O\) (8, 33). We then used immunoprecipitation and Western blotting to determine the effect of hypertonicity on the total cellular content of stanniocalcin protein and examined for its presence in the medium. As shown in Fig. 5, stanniocalcin protein (band broad spanning 29–34 kDa) is detected simultaneously in cells and medium after 16 h of exposure to hypertonicity. Stanniocalcin was not detected in immunoprecipitates of an irrelevant antibody (anti-\(\beta_1\)-integrin, K20). Of note, although the mRNA is detectable in unstressed cells and at all time points after exposure to hypertonicity, the protein is measurable only after prolonged exposure to hypertonicity (16 h). The significance of this discrepancy remains to be determined. Collectively, these data are consistent with induction and release of stanniocalcin protein to the medium upon exposure of the cells to hypertonicity and suggest that stanniocalcin acts as a paracrine or juxtacrine hormone in mammalian kidney in a hypertonicity-dependent manner.

Accumulation of intracellular organic osmolytes attenuates the induction of stanniocalcin mRNA by hypertonicity. As mentioned above, hypertonicity induces genes responsible for the accumulation of organic osmolytes by the cell. Accumulation of organic osmolytes in turn attenuates the induction of these genes, presumably by decreasing intracellular ionic strength (25, 27). To test whether stanniocalcin mRNA induction by hypertonicity is modulated by medium organic osmolytes, MDCK cells were exposed to hypertonic medium under conditions that would exaggerate or minimize organic osmolytes accumulation (24). Under hypertonic conditions, MDCK cells accumulate four major organic solutes: betaine, inositol, taurine, and glycerophosphocholine (GPC) (13). Betaine, taurine, and inositol are accumulated only when they are present in the medium, and the magnitude of their transport is dependent on their concentration in the medium and the extent of extracellular hyperosmolality (13, 22). Although GPC can be synthesized from intracellular stores of choline, it does not accumulate in the cell in vitro to appreciable amounts in the absence of medium choline (12). Hence, stanniocalcin mRNA abundance was measured in cells exposed to medium containing high NaCl (final osmolality of 515 mosmol/kg \(H_2O\)) and either no betaine and inositol in the medium (to prevent their accumulation) or with 5 mM of each added (to enhance their accumulation) (25). Under hypertonic conditions, and in the absence of medium betaine and inositol, stanniocalcin mRNA is induced sixfold at 24 h. However, under hypertonic conditions, and in the pres-
ence of medium betaine and inositol, stanniocalcin mRNA induction is attenuated to a level equal to that seen in cells under isotonic conditions (Fig. 3A). The modulation of hypertonic induction of gene expression by cellular accumulation of organic osmolytes was originally considered unique to genes that are directly involved in the accumulation of organic solutes, such as transporters for betaine, taurine, and inositol (25). However, recent data from this laboratory suggest that a number of genes that are induced by hypertonicity and are not directly involved in organic solute transport display such behavior. Among these genes are the 70-kDa heat shock protein and the adhesion molecules CD9 and β1-integrin (24–26). This suggests a common regulatory pathway for the plethora of genes that are induced by hypertonicity, irrespective of their direct involvement in organic osmolytes transport.

Induction of stanniocalcin by hypertonicity is extracellular calcium dependent. Stanniocalcin is considered a major regulator of body calcium in fish, and the release of stanniocalcin from corpuscles of Stannius in vivo and in tissue culture is regulated by variations in extracellular calcium. For this reason, we asked whether stanniocalcin expression in MDCK cells is regulated by extracellular calcium. Confluent MDCK cells were exposed to hypertonic medium for 24 h in the presence of increasing medium EGTA concentrations (0–1.55 mM), and stanniocalcin mRNA abundance was determined using Northern hybridization. As shown in Fig. 6, stanniocalcin mRNA induction by hypertonicity is blunted only when EGTA concentration reaches 1.5 mM, suggesting a threshold response. With the use of equations derived from Bulos and Sactor (2), this EGTA concentration corresponds to calculated medium calcium of 0.05 mM.

The above observations are based on the assumption that the effects attributed to EGTA are related solely to decreasing calcium concentration in the medium. To control for nonspecific EGTA effects, MDCK cells were placed in calcium-deficient hypertonic medium, to which varying concentrations of calcium were added. cells were then analyzed for stanniocalcin mRNA abundance. Poly(A) RNA (3 µg) was loaded per lane on RNA gels, and Northern blots were probed with stanniocalcin cDNA. Representative Northern blot is shown (duplicate experiments). Data are based on at least 3 independent determinations.

Fig. 6. Hypertonic induction of stanniocalcin is EGTA dependent. MDCK cells were grown to confluence in isotonic medium (315 mosmol/kgH2O) containing 1.55 mM calcium. Cells were then placed in same medium containing no betaine and inositol, to which NaCl (HT) was added (final osmolality of 515 mosmol/kgH2O), in the presence or absence of increasing EGTA concentrations. Control cells were maintained in isotonic medium without betaine and inositol, which contained 1.55 mM calcium. After 24 h, cells were analyzed for stanniocalcin mRNA abundance. Poly(A) RNA (3 µg) was loaded per lane on RNA gels, and Northern blots were probed with stanniocalcin cDNA. Representative Northern blot is shown (duplicate experiments). Data are based on at least 3 independent determinations.

Fig. 7. Hypertonic induction of stanniocalcin is calcium dependent. MDCK cells were grown to confluence in isotonic medium (315 mosmol/kgH2O) containing 1.55 mM calcium. A: at time 0, cells were placed in calcium-deficient medium, containing no betaine and inositol, to which NaCl ([HT]), final osmolality of 515 mosmol/kgH2O) and increasing amounts of calcium were added. B: control cells were maintained in isotonic medium, containing no betaine and inositol, to which increasing amounts of calcium were added. Cells were then analyzed for stanniocalcin mRNA abundance, using stanniocalcin cDNA as a probe. Poly(A) RNA (3 µg) was loaded per lane. Representative Northern blots are shown (duplicate experiments). Data are based on at least 3 independent determinations.

Fig. 8. Stanniocalcin mRNA expression in rat tissues. Twenty micrograms of total RNA were loaded per lane, and Northern blot was probed with stanniocalcin cDNA.
lar calcium threshold phenomenon under isotonic conditions, MDCK cells were exposed to isotonic medium containing various calcium concentrations for 24 h, and the abundance of stanniocalcin mRNA was measured. As shown in Fig. 7B, under isotonic conditions, stanniocalcin mRNA expression is not affected by medium calcium concentrations shown earlier to blunt its induction by hypertonicity. From these data, it can be concluded that the induction of stanniocalcin by hypertonicity requires an extracellular calcium concentration greater than 0.1 mM. Whether this finding is pertinent to the regulation of the adaptive response to high osmolality or to other physiological functions of kidney stanniocalcin remains to be determined.

Stanniocalcin mRNA distribution in rat tissue. To better understand the role of stanniocalcin in mammals, we examined the expression and distribution of its mRNA in rat tissue. As shown in Fig. 8, stanniocalcin mRNA (3.8 kb) is detected in kidney cortex and medulla, adrenals, colon, and lungs, whereas it is weakly expressed in heart, parathyroid, and cerebrum. This pattern of expression is notably different from previous reports (3, 4) in its expression in brain, adrenals, and parathyroid glands and in its absence in spleen, testes, and skeletal muscle. The expression in parathyroid, colon, and kidney suggests involvement in calcium homeostasis, whereas the significance of its expression in other organs remains to be determined.

DISCUSSION

Our data suggest the involvement of stanniocalcin in the adaptation of kidney cells to osmotic stress. In normal calcium media (1.55 mM), stanniocalcin mRNA expression is increased by hypertonicity. However, when medium calcium is decreased to 0.1 mM range, the induction by hypertonicity is blunted. The data suggest dual regulation of stanniocalcin mRNA in these cells by hypertonicity and extracellular calcium. MDCK cells are considered to be of distal nephron origin and display characteristics of thick ascending limb cells (17). Thus the expression of stanniocalcin mRNA in these cells is consistent with literature reports of stanniocalcin expression in distal nephron. In addition, it may be deduced from our data that MDCK cells are capable of sensing extracellular calcium and modulate gene expression in response to it. These cells have recently been reported to express calcium receptors (1). However, it remains to be determined whether calcium receptors are involved in the response of stanniocalcin to hypertonicity or extracellular calcium.

The extracellular calcium threshold to which stanniocalcin responds in MDCK cells is likely between 0.1 and 0.5 mM (the narrowest calcium concentration range tested, through which induction of stanniocalcin by hypertonicity is altered). This is still significantly lower than that reported in fish, being 1–4 mM in the cells of Stannius corpuscles (16, 34). It is suggested that, although the function of stanniocalcin across the evolutionary tree from fish to mammals was maintained, possibly to inhibit calcium entry into cells, the calcium levels to which it responds vary. Furthermore, although stanniocalcin functions as a true hormone in fish, it may be operating in the kidney as an autocrine or paracrine substance.

Finally, we can only speculate about the physiological role of stanniocalcin in the kidney. The calcium concentrations in the lumen of the thick ascending limbs and in nephron segments beyond remain largely unknown. Thus extrapolation about the physiological relevance of the calcium concentration threshold at which stanniocalcin is regulated remains speculative. However, assuming that the function of stanniocalcin is preserved across the evolutionary tree, that is, to inhibit calcium entry or uptake, we speculate that stanniocalcin may function to inhibit calcium entry into cells under hypertonic conditions, for cytoprotection. Alternatively, it may function to regulate calcium uptake in the distal nephron. In the latter case, we speculate that stanniocalcin either directly inhibits calcium channels or that it binds to a putative receptor and sets off a signaling cascade that eventually leads to inhibition of calcium channels.

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Address for reprint requests and other correspondence: D. Sheikh-Hamad, Renal Section, Dept. of Medicine, Baylor College of Medicine, 6535 Fannin St., MS F505, Houston TX 77030 (E-mail: sheikh@bcm.tmc.edu).

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REFERENCES


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