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Topical Review

Intracellular Monovalent Ions as Second Messengers

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Abstract. It is generally accepted that electrochemical gradients of monovalent ions across the plasma membrane, created by the coupled function of pumps, carriers and channels, are involved in the maintenance of resting and action membrane potential, cell volume adjustment, intracellular Ca^{2+} handling and accumulation of glucose, amino acids, nucleotides and other precursors of macromolecular synthesis. In the present review, we summarize data showing that side-by-side with these classic functions, modulation of the intracellular concentration of monovalent ions in a physiologically reasonable range is sufficient to trigger numerous cellular responses, including changes in enzyme activity, gene expression, protein synthesis, cell proliferation and death. Importantly, the engagement of monovalent ions in regulation of the above-listed cellular responses occurs at steps upstream of $Ca^{2+}{}_i$ and other key intermediates of intracellular signaling, which allows them to be considered as second messengers. With the exception of HCO_3^--sensitive soluble adenylyl cyclase, the molecular origin of sensors involved in the function of monovalent ions as second messengers remains unknown.

Key words: Sodium — Potassium — Proton — Bicarbonate — Chloride — Intracellular signalling

Introduction

In accordance with Earl Sutherland's signal transduction hypothesis [113], any intracellular molecule can be considered a potential second messenger in the signal transduction pathway if it satisfies 3 major criteria. i) Modulation of intracellular concentration of the potential second messenger triggered by external stimuli precedes cellular responses and normalizes after removal of these stimuli. ii) The transient modulation of intracellular second messenger concentration per se is sufficient to trigger cellular responses in the absence of investigated external stimuli. iii) Cellular responses triggered by external stimuli are mediated by the interaction of second messengers with their intracellular sensors.

In pioneering studies performed more than 40 years ago, it was shown that cAMP satisfies the above-listed criteria and provides coupling between excitation of plasma membrane receptors by catecholamines and peptide hormones with gluconeogenesis and lipolysis in hepatocytes and adipocytes, respectively [105]. Later on, the list of second messengers was broadened in experiments demonstrating a key role for cGMP, Ca^{2+} and lipid molecules, such as diacyl glycerol, inositol 1,4,5-triphosphate etc., in the regulation of other cellular functions, such as myocyte contraction and relaxation, hormone and neurotransmitter release, light sensing, cell proliferation and apoptosis, etc. [10, 18, 63, 119].

Similarly to the above-listed second messengers, the intracellular concentration of monovalent cations is transiently affected by diverse extracellular stimuli and normalized to baseline values via feedback activation of the pumps, carriers and channels shown in Fig 1. Thus, transient activation of Na^+/H^+ exchange and $[Na^+]_i$ elevation appear to be a universal response of quiescent cells to growth-promoting stimuli [15, 39, 44, 70, 90, 101, 125]. In neurons, short periods of synaptic activity produce large increases of $[Na^+]_i$, from ~10 to 30 and 100 mM in apical dendrites and dendritic spines, respectively, mainly due to Na^+ influx via N-methyl-D-aspartate (NMDA) receptor channels [106]. In erythrocytes and other cells with low resting potential, transient activation of

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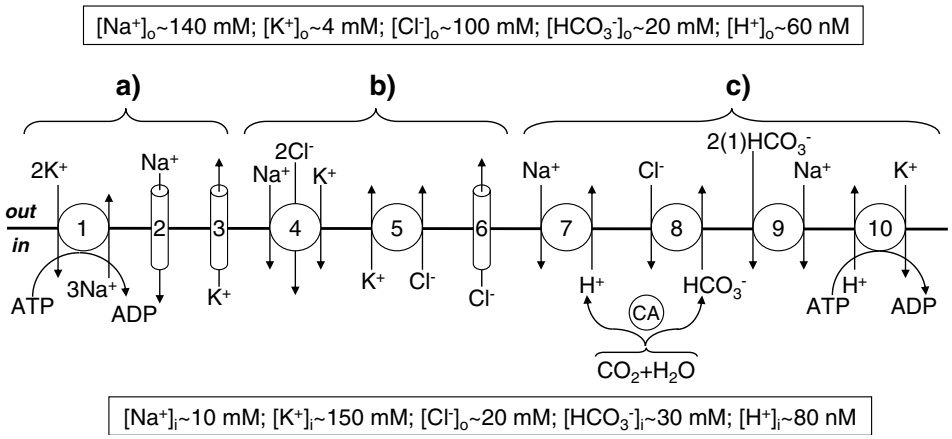

Fig. 1. Major plasma membrane ion transporters involved in intracellular Na^+/K^+ (*a*), Cl^- (*b*) and H^+/HCO_3^- (*c*) handling. *1:* Na^+/K^+-ATPase; *2*: Na^+ channels; *3:* K^+ channels; *4*: Na^+, K^+, $2Cl^-$ cotransport; *5*: K^+, Cl^- cotransport; *6*: Cl^- channels; *7:* Na^+/H^+ exchange; *8:* anion exchanger; *9*: Na^+,$NaHCO_3^-$ cotransport; *10:* H^+/K^+-ATPase; *CA*: carbonic anhydrase. The representative values of extra- and intracellular concentrations of monovalent ions are shown.

K^+ channels results in 5- to 8-fold attenuation of $[K^+]_i$ [9]. The regulation of these and other monovalent ion transporters by extracellular stimuli is considered in detail in several comprehensive reviews [1, 31, 33, 36, 45, 108, 125]. Here, we just summarize data showing that modulation of the intracellular concentration of monovalent ions triggered by external stimuli is sufficient to affect cellular responses by acting upstream or even independently of the signaling pathways evoked by well-defined second messengers. We would like also to underline that the goal of our mini-review is to support the concept of monovalent ions as second messengers, to consider the possible pathophysiological implications of these signaling cascades, and to provoke the search for intracellular monovalent ion sensors rather than to provide a complete list of monovalent ion-dependent cellular functions documented so far.

Sodium

In cells abundant with Na^+/Ca^{2+} exchanger, elevation of $[Na^+]_i$ is sufficient to activate this carrier and to elicit diverse $Ca^{2+}{}_i$-mediated responses, including positive inotropic effect in cardiomyocytes and neurotransmitter release in nerve terminals treated with low doses of Na^+/K^+-ATPase inhibitors, such as ouabain and other cardiotonic steroids (CTS) [11]. Data obtained in these studies should probably be considered as first evidence for the involvement of $Na^+{}_i$ in the regulation of cellular function as a second messenger. This section is focused on data showing that $Na^+{}_i$ can modulate cellular function independently of $[Ca^{2+}]_i$ elevation and of activation of other Na^+-coupled ion carriers.

In the late 1990s, we observed that almost complete Na^+, K^+ pump inhibition with ouabain protects rat vascular smooth muscle cells (VSMC) from apoptosis triggered by growth factor withdrawal, staurosporin or inhibitors of serine-threonine phosphatases and potentiated by transfection with c-myc or its functional analogue E1A adenoviral protein [86]. Suppression of apoptosis in CTS-treated VSMC can be mediated by membrane depolarization, accumulation of Na^+ or loss of K^+. Conformational transition of the Na^+/K^+-ATPase α-subunit triggered by interaction with CTS may be sufficient per se to generate the antiapoptotic signal. In addition, CTS interaction with target(s) distinct from the Na^+/K^+-ATPase α-subunit can not be excluded. We found that apoptosis in VSMC is sharply suppressed by incubation of VSMC in K^+-free medium. Keeping in mind an obligatory role of $K^+{}_o$ in the activation of Na^+/K^+-ATPase, this observation strongly suggests the antiapoptotic action of ouabain is mediated by inhibition of the Na^+/K^+ pump. To further examine the role of monovalent cations, we treated VSMC with ouabain in high-K^+ medium. Sustained depolarization in high-K^+, low-Na^+ medium did not affect apoptosis. In contrast, dissipation of the transmembrane gradient of monovalent cations occurring in this medium sharply diminished the effect of ouabain on $Na^+{}_i$ and $K^+{}_i$ content and completely abolished its antiapoptotic action [86]. These data led us to conclude that Na^+/K^+ pump inhibition protects VSMC against apoptosis via elevation of the $[Na^+]_i/[K^+]_i$ ratio.

Later on, the antiapoptotic action of ouabain and K^+-depleted medium was detected in a cultured renal proximal tubule cell line [131], in freshly-isolated rat cerebellar granule cells [49] and in human umbilicial vein endothelial cells [120]. It should be underlined that relatively low concentrations of ouabain were used in these studies, and its action on the $[Na^+]_i/[K^+]_i$ ratio was not examined. More recently, we

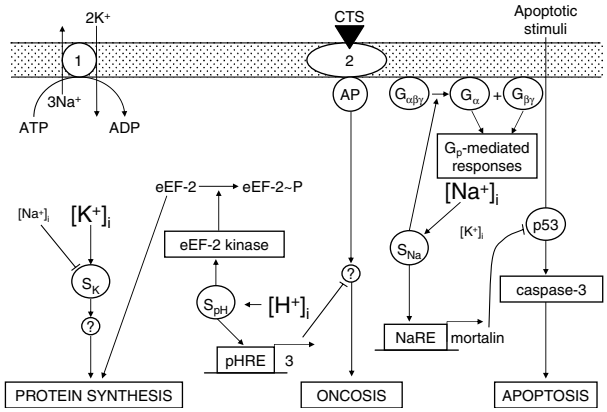

Fig. 2. Intracellular monovalent cations as second messengers. *1* and *2*: active and inactive conformation of the Na^+/K^+-ATPase α-subunit triggered by its interaction with CTS; *3:* H^+_i-sensitive gene(s) involved in the inhibition of oncosis; S_K, S_{Na} and S_{pH} – hypothetical sensors of intracellular K^+, Na^+ and H^+, respectively; *AP:* adapter protein(s) whose interaction with Na^+, K^+-ATPase α-subunit is affected by CTS; *pHRE* and *NaRE*: H^+- and Na^+-sensitive elements implicated in the regulation of gene expression; *eEF-2*: elongation factor-2; *Gαβγ*:: α, β and γ subunits of GTP-binding proteins; *?*: unknown intermediates; - → and --| : activatory and inhibitory signals, respectively. For more details, *see* text.

noted that K^+-free, Na^+-containing medium rescues vascular endothelial cells from apoptosis triggered by [^{3}H]-decay-induced DNA damage. Because this protection was absent in K^+-free, low-Na^+ medium, we concluded that the antiapoptotic signal triggered by Na^+/K^+ pump inhibition is mediated by $[Na^+]_i$ elevation rather than by loss of K^+_i [87].

To further explore the novel Na^+_i-mediated antiapoptotic pathway, we treated cells with actinomycin D or cycloheximide. Both inhibitors of macromolecular synthesis abolished protection against apoptosis documented in VSMC pretreated with ouabain [84]. Deploying a rat multi-probe template set, we failed to detect, in ouabain-treated VSMC, elevation of mRNA species encoding major pro- and antiapoptotic proteins such as Bcl-2, Bcl-xL, Bcl-xS, Bax, and caspases 1-3 [82]. With these negative data in mind, we took a proteomics approach to identify a set of Na^+_i-sensitive genes. Twelve soluble proteins, including mortalin, whose expression is triggered by ouabain, were identified by mass spectrometry [117]. Previous studies demonstrated the pancytosolic and mitochondrial/juxtanuclear localization of mortalin in mortal and immortal cells, respectively [114, 122–124]. Northern and Western blotting confirmed the induction of mortalin expression in ouabain-treated VSMC and documented its mitochondrial localization. We established that, similarly to ouabain, transfection with mortalin delayed the development of apoptosis in serum-deprived VSMC-E1A. We also found that transfection with mortalin inhibits p53 translocation to the nucleus [117]. Viewed collectively, these data suggest that elevated $[Na^+]_i$ suppresses programmed cell death via augmented mortalin expression that, in turn, blocks p53 nuclear translocation triggered by apoptotic stimuli (Fig. 2).

In the last decade, it was found that, in several types of cells, sustained inhibition of the Na^+/K^+ pump triggers the expression of the α1- and β1-subunits of the Na^+/K^+-ATPase, myosin light chain, skeletal muscle actin, atrial natriuretic factor and tumor growth factor-β (for recent review, *see* [116, 128]). These data together with augmented RNA synthesis [85] and the appearance of numerous protein spots, detected in ouabain-treated VSMC by 2-D electrophoresis [82, 117], suggest that this action of Na^+, K^+ pump inhibitors is at least partially mediated by early response genes. Indeed, in VSMC, we observed 10- and 4-fold elevations of immunoreactive c-Fos and c-Jun after 2- and 12-h treatment with ouabain, respectively [115]. A 4-fold increment of c-Fos mRNA content was detected in 30 min of ouabain addition. Importantly, within this time interval, $[Na^+]_i$ was increased by ~5-fold whereas $[K^+]_i$ was decreased by only 10–15%. This result shows that $[Na^+]_i$ augmentation rather than $[K^+]_i$ attenuation generates a signal that leads to c-Fos expression.

In accordance with known signaling pathways triggered by CTS, gene expression, seen under elevated $[Na^+]_i$, can be mediated by cell volume modulation or/and activation of Na^+/H^+ and Na^+/Ca^{2+} exchangers. The latter hypothesis is also consistent with the presence of (Ca^{2+} + cAMP) response element (CRE) within the c-Fos promoter [107]. Indeed, we have demonstrated that K^+_o-induced depolarization leads to c-Fos expression that is completely abolished by the selective L-type Ca^{2+} channel blocker nicardipine [115]. However, the data listed below strongly indicate that c-Fos expression in ouabain-treated VSMC is a Ca^{2+}-independent phenomenon. First, c-Fos expression in ouabain-treated cells is not sensitive to nicardipine. Second, neither

$[Ca^{2+}]_i$ nor total exchangeable Ca content in VSMC is affected by ouabain [115]. This observation is consistent with negligible Na^+/Ca^{2+} exchanger activity detected in VSMC [83]. Third, neither extracellular (EGTA) nor intracellular (BAPTA-AM) Ca^{2+} chelators abolish ouabain-induced c-Fos expression [115]. These data as well as the lack of a significant effect of ouabain on pH_i and cell shrinkage on c-Fos content allow us to hypothesize that gene expression in ouabain-treated VSMC is mediated by a novel *$Na^+{}_i$ -dependent, $Ca^{2+}{}_i$ -insensitive mechanism of excitation-transcription coupling*.

Gene expression is probably not the only cellular function controlled by the $Na^+{}_i$-sensor. Indeed, it has been shown in neuronal cells that elevation of $[Na^+]_i$ is sufficient to activate heterotrimeric G-proteins via the GTP-independent mechanism of dissociation of their α- and βγ-subunits [104]. Moreover, in these cells, $[Na^+]_i$ elevation modulates the activity of NMDA receptors, K^+ and Ca^{2+} channels by G-protein-dependent and -independent mechanisms [12, 104, 130]. Are these cellular responses and $Na^+{}_i$-dependent gene expression, demonstrated in our studies [115], mediated by the same $Na^+{}_i$-sensor? We will address this question in future studies.

Potassium

More than 40 years ago, it was demonstrated that protein synthesis in prokaryotes is sharply inhibited in the absence of K^+ [65]. Later on, the requirement of K^+ for protein synthesis was detected in animal cells of different origins [56, 59, 64, 73, 100]. Using human fibroblasts subjected to sustained inhibition of Na^+/K^+-ATPase with ouabain, it was shown that inversion of the $[Na^+]_i/[K^+]_i$ ratio inhibits protein synthesis without any impact on mRNA function, ATP content and amino acid transport [59], thus suggesting direct influence of $[K^+]_i$ on the protein synthesis machinery.

In reticulocytes, globin contributes to more than 90% of total protein synthesis. In these cells, it was found that $K^+{}_i$ depletion inhibits the elongation step of globin synthesis without any impact on ribosome subunit assembly [16]. The half-maximal activation of globin synthesis by reticulocyte lysate in medium containing 60, 90 and 125 mM Na^+ was observed at $[K^+]$ of 15, 25 and 40 mM, respectively [16]. These data indicate that elevation of $[Na^+]_i$ diminishes the efficacy of protein synthesis regulation by $K^+{}_i$ via attenuation of K^+ interaction with its hypothetical sensor (Fig. 2). Intermediates of the protein synthesis machinery involved in $K^+{}_i$ sensing remain unknown.

It should be underlined that the effect of $K^+{}_i$ loss on protein synthesis is cell type-specific. Thus, in contrast to the above-mentioned cells showing 2- to 4-fold attenuation of protein synthesis after sustained inhibition of Na^+/K^+-ATPase in K^+-free medium or in the presence of CTS, we did not see any significant effect on [^{3}H]-leucine protein labelling after 24-h ouabain treatment of cultured VSMC from the rat aorta [85]. Two hypotheses could explain these data. First, the $K^+{}_i$-sensitive element of the protein synthesis machinery is absent in VSMC. Second, attenuation of protein synthesis in K^+-depleted VSMC is masked by augmented mRNA synthesis. Indeed, we discerned a 6-fold elevation of total RNA synthesis in VSMC treated with ouabain for 10 h [85] that could be attributed to $Na^+{}_i$-mediated expression of c-Fos and other early response genes detected in VSMC after 1–2 h of ouabain addition [115].

Proton

The functioning of numerous proteins is affected by cellular acidification. Thus, for example, acidification inhibits TASK-3 K^+ channels [102] but activates TREK-1 K^+ channels [67] and Ca^{2+}-permeable acid-sensitive ion channels [129]. It should be underlined, however, that modulated activity of these ion transporters was detected in pH ranges from 7.4 to 5.0, which corresponds to ~200-fold elevation of $[H^+]_i$ concentration, thus suggesting the involvement of these proteins in pH sensing under severe hypoxia and/or HCO_3^- depletion. A system with much higher pH sensitivity has been detected in our studies of oncosis in CTS-treated epithelial and endothelial cells.

In contrast to rat VSMC [85, 86], NIH 3T3 mouse fibroblasts, HEK-293, HeLa, human renal carcinoma Caki cells [81], renal epithelial cells from the Rhesus monkey [22, 23], human lymphocytes [32] and rat astrocytes (*unpublished data*), 24-h exposure to ouabain results in massive death of renal epithelial cells from Madin-Darby canine kidney (MDCK) and endothelial cells from the porcine aorta (PAEC). Both types of ouabain-treated cells possess combined markers of necrosis (cell swelling, negligible labelling with nucleotides in the presence of terminal transferase, nuclei staining with cell-impermeable dyes, such as propidium iodide) and apoptosis (nuclear condensation, chromatin cleavage, caspase-3 activation) [23, 87, 96]. We also demonstrated that in contrast to classical cell culture models of apoptosis, death of ouabain-treated MDCK cells was insensitive to the pan-caspase inhibitor z-VAD.fmk [96]. To underline the striking difference in cell volume behavior (swelling *vs* shrinkage detected in cells undergoing apoptosis), revised terminology has been proposed, claiming that necrosis was originally offered as a concept to characterize any post-mortem changes in cell morphology. In accordance with this nomenclature, the label "oncosis", derived from the Greek word for swelling, describes cell death that is distinct from apoptotic shrinkage [68].

Surprisingly, we found that more than 500-fold inhibition of the Na^+/K^+ pump in K^+-free medium does not affect the survival of C7-MDCK cells [96]. As predicted, 6-h incubation of C7-MDCK cells in K^+-free medium led to a sharp $[Na^+]_i$ elevation, and the addition of ouabain only slightly altered this parameter, whereas incubation in high-K^+/low-Na^+ medium did not impact the baseline values of $[Na^+]_i$ and $[K^+]_i$, but completely abolished the K^+_i loss triggered by ouabain. However, similarly to control medium, ouabain killed cells to the same extent in K^+-free and high-K^+/low-Na^+ media [96]. Moreover, the same left-hand shift was noted in comparison to the dose-dependent action of ouabain on Na^+/K^+ pump activity and death of C7-MDCK and PAEC [4, 87, 96]. These results strongly indicate that in both cell types, CTS trigger Na^+_i, K^+_i-independent oncosis via interaction with the Na^+/K^+-ATPase α-subunit rather than with other potential K^+_o-insensitive receptors. Considering these data, we proposed that CTS-induced conformational transition of the Na^+/K^+-ATPase α-subunit is sufficient to trigger its interaction with an unidentified adapter protein(s), resulting in Na^+_i, K^+_i-independent oncosis of renal epithelial and vascular endothelial cells (Fig. 2). This adapter protein or downstream intermediates of the Na^+_i, K^+_i-independent death machinery are absent in CTS-resistant cells, including VSMC.

Under analysis of the role of extracellular ions in CTS-induced oncosis, we observed that decreases of $NaHCO_3$ concentration from 44 to 11 mM sharply attenuated the death of C7-MDCK cells triggered by ouabain. Keeping in mind that total Na^+ concentration in control and $NaHCO_3$-depleted medium was the same, 2 hypotheses can explain this finding. First, medium acidification caused by a decreased HCO_3^-/CO_2 ratio is sufficient to inhibit the cell death machinery. Second, a decreased HCO_3^-/CO_2 ratio suppresses cell death independently of medium acidification. Data obtained in additional experiments do not support the latter hypothesis. Indeed, cell death inhibition was also detected in medium with high $NaHCO_3$ concentration and acidified by HEPES, whereas alkalinization with Tris abolished the protective action of $NaHCO_3$ depletion [5]. Finally, we used $NaHCO_3$-free, HEPES-Tris-buffered medium and observed that the death of ouabain-treated PAEC and C7-MDCK cells is suppressed by acidification of the medium from pH 7.4 to 7.0, i.e., under conditions when pH_i was decreased from ~7.2 to 6.9. The rescue of ouabain-treated C7-MDCK cells was also detected under selective intracellular acidification caused by inhibition of the Na^+/H^+ exchanger with ethylisopropyl amiloride [5].

Neither [^{3}H]-ouabain binding nor ouabain-sensitive ^{86}Rb influx was significantly affected by modest acidification [5], showing that the H^+_i-sensitive element of the cell death machinery is located downstream of Na^+/K^+-ATPase. It should be noted that acidification from 7.2 to 5.0 activates rather than inhibits caspases [71] and nucleases [97], excluding these downstream intermediates as a potential H^+_i-sensor involved in the suppression of death signaling triggered by CTS.

Elongation factor-2 (eEF-2) is the most prominently phosphorylated protein detected in mammalian tissue extracts, and its phosphorylation by eEF-2 kinase leads to inactivation and inhibition of protein synthesis [110]. By comparing liver homogenates from wild-type and eEF-2 kinase knockout mice, it was shown that eEF-2 phosphorylation is completely blocked by pH elevation from 6.6 to 7.4 [30, 109], i.e., in the range where switch off/on regulation of the CTS-induced cell death machinery is detected. Considering this, it may be proposed that acidosis suppresses the death signal via eEF-2 phosphorylation that in turn abolishes eEF-2-mediated activation of protein synthesis. However, the death of ouabain-treated MDCK cells was noted in the presence of RNA and protein synthesis inhibitors, whereas the protective effect of acidification was sharply diminished by these compounds at modest non-toxic concentrations [5]. These results strongly suggest that the rescue by modest intracellular acidification of renal epithelial and vascular endothelial cells from Na^+_i, K^+_i-independent oncosis triggered by CTS is mediated by the de novo expression of gene(s) containing pH_i-response element.

Bicarbonate

Mammalian spermatozoa undergo activation processes induced by bicarbonate and mediated by elevation of intracellular cAMP content. It has been assumed that this action of HCO_3^- is caused by alkalinization of the cytoplasm. However, several laboratories have reported that spermatozoa are highly abundant in soluble adenylyl cyclase (sAC) [19, 37, 79] stimulated by HCO_3^- in a pH_i-independent manner [19]. It was also shown that bicarbonate activates purified and recombinant sAC with an EC_{50} of 20 mM [19] relevant to the physiological range of $[HCO_3^-]_i$ (Fig. 1*c*). Amino acid residues involved in the organization of the HCO_3^--binding site of sAC and the role of these sites in the conformational transition of this enzyme remain unknown.

Side-by-side with spermatozoa, sAC was also detected in the kidney and choroid plexus [19, 95], indicating involvement of HCO_3^- as a second messenger in the regulation of cAMP-dependent functions of these tissues. Based on the identification of sAC within nuclei, it has been proposed that this enzyme contributes to $[H^+]_i/[HCO_3^-]_i$-dependent

modulation of gene expression via activation of CRE-binding protein [132]. It was also suggested that pH_i-coupled modulation of sAC activity is responsible for pH-dependent recycling of vacuolar H^+-ATPase [95].

Chloride

The first data suggesting the role of Cl^-_i in cellular signaling were probably obtained in the study of regulation of the ubiquitous isoform of Na^+, K^+, $2Cl^-$ cotransporter (NKCC1). Several research groups demonstrated that inwardly-directed NKCC1 contributes to the accumulation of Cl^- above the Nernst equilibrium potential. For example, in secretory epithelial, renal epithelial, vascular endothelial, mesangial and neuronal cells, inhibition of NKCC1 with bumetanide decreases $[Cl^-]_i$ by 2- to 6-fold [52, 69, 77]. In resting neuronal cells with high permeability for K^+ ($P_K >> P_{Cl}$), NKCC1-mediated elevation of $[Cl^-]_i$ does not affect membrane potential (E_m) but leads to transient depolarization under activation of GABA-sensitive anion channels [29]. In VSMC, P_K and P_{Cl} values are somewhat similar [21], indicating NKCC1 involvement in the regulation of resting E_m. Indeed, bumetanide decreases $[Cl^-]_i$ [89], hyperpolarizes [28] and abolishes differences in $[Cl^-]_i$ and E_m between VSMC from normotensive and deoxycorticosterone-salt-hypertensive rats [14]. In recent studies, we demonstrated that in HCO_3^--depleted medium, NKCC1 inhibition sharply suppresses smooth muscle cell contraction triggered by modest depolarization or by α-adrenergic stimulation [7, 55].

Since the seminal studies of dialyzed squid axons [13], it was shown that in all types of cells studied so far NKCC1 activity is decreased by 5- to 10-fold under elevation of $[Cl^-]_i$ from 20 to 150 mM (108), thus providing feedback regulation of this carrier. In Cl^--depleted tracheal epithelial cells [42] and shark rectal glands [66], NKCC1 activation is accompanied by phosphorylation of the carrier, indicating the presence of protein kinases whose activity is negatively regulated by $[Cl^-]_i$. The presence of Cl^-_i-sensitive intermediates of signal transduction, including protein kinases and phosphatases, was also proposed in the study of activation of permeabilized neutrophils [38], GABA receptors [58], and phosphorylation of membrane-bound proteins in airway epithelium, including nucleoside diphosphate kinase [75, 118]. In contrast, we failed to detect any impact of Cl^-_i depletion on baseline phosphorylation of proteins in MDCK cells or on protein phosphorylation triggered by activators of protein kinases A, C and mitogen-activated protein kinases (*unpublished data*).

Several laboratories have reported that growth factors and other mitogens transiently activate

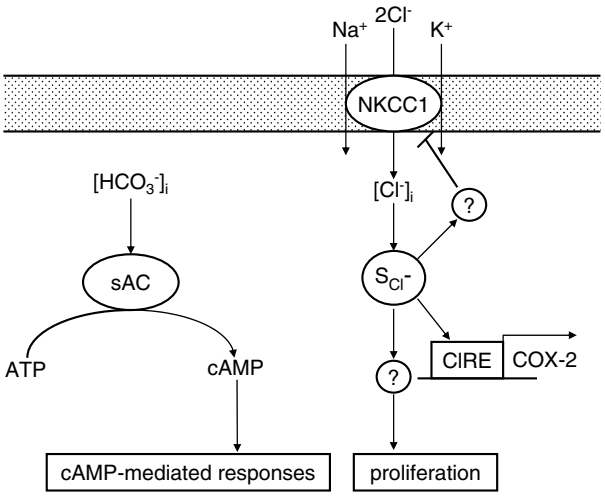

Fig. 3. Intracellular monovalent anions as second messengers. S_{Cl}: hypothetical sensor of intracellular Cl^-; *ClRE*: Cl^--sensitive element implicated in the regulation of gene expression; *?*: unknown intermediates; - → and --| : activatory and inhibitory signals, respectively. For more details, *see* text.

NKCC1 [91, 92], whereas chronic inhibition of the carrier with bumetanide or furosemide suppresses the growth of fibroblasts [91], VSMC [17], lymphocytes [92], vascular endothelial cells [93] and airway smooth muscle cells [51]. More recently, Panet and co-workers reported that NKCC1 overexpression sharply accelerates the proliferation of mouse Balb/c 3T3 cells in serum-free medium [94]. Since there is no conclusive data on the involvement of NKCC in the regulation of intracellular Na^+ and K^+ content under baseline conditions [61, 89], elevation of $[Cl^-]_i$ seen under NKCC activation in the above-listed cells [52, 69, 77] seems to be crucial for proliferative responses (Fig. 3).

Pathophysiological implications

Augmented activity of NKCC1 has been detected in blood cells and VSMC from spontaneously hypertensive rats (SHR) and in some patients with essential hypertension [80]. Two hypotheses explain the possible involvement of abnormal activities of this carrier in the pathogenesis of hypertension. First, NKCC1 activation leads to augmented contractile responses of VSMC, as demonstrated by our group [7, 55] and by O'Neill and co-workers [2, 3]. Second, NKCC1-mediated elevation of $[Cl^-]_i$ causes heightened proliferation of VSMC (Fig. 3), i.e., a well-documented hallmark of vascular remodeling (increased wall-to-lumen ratio) detected in hypertension and implicated in the development of cardiovascular complications of this disease [34, 88]. The latter hypothesis is consistent with data showing shortening of the G_0/G_1 phase in cultured VSMC

from SHR [43, 121] and lengthening of the same cell-cycle phase in bumetanide-treated cells [51]. Importantly, vascular remodeling might be further enhanced by elevation of $[Na^+]_i$ caused by augmented production of endogenous CTS documented in hypertension and several other extracellular fluid volume-expanded disorders [81]. This hypothesis is consistent with data on $Na^+{}_i$-dependent inhibition of apoptosis in VSMC considered in the *Sodium* section.

So-called "kidney resetting", i.e., normal salt and water excretion under elevated blood pressure, is the most powerful servomechanism of the long-term maintenance of severe hypertension independently of the origin of this disease, including hypertension caused by enhanced salt consumption [40, 41]. Several lines of evidence strongly suggest that elevated intake of Cl^- rather than Na^+ triggers the development of NaCl-induced hypertension [53, 111]. It may be assumed that similar to VSMC (*Chloride* section) elevation of $[Cl^-]$ in the macula densa, caused by augmented NaCl intake via renal specific NKCC2 isoform [57, 78] leads to the increased contraction of mesangial cells, thus providing an explanation for the altered tubuloglomerular feedback regulation of kidney function. In addition, luminal $[Cl^-]$ negatively correlates with renin production in juxtaglomerular preparations [46, 54]. In the macula densa, renin secretion is under the control of cyclooxygenase (COX) activity and prostaglandin production [35] (Fig. 3). More recent studies show that COX-2 expression is augmented in Cl^--depleted medium and in the presence of NKCC inhibitors [20]. The mechanism of the involvement of $[Cl^-]$ in the regulation of COX-2 expression remains unknown.

As for the physiological significance of $H^+{}_i$-sensing, it must be noted that acidosis with $pH_i < 6.5$ is considered a hallmark of hypoxia and ischemia [112]. In several tissues, including the heart [126], brain [8] and kidney [50], short ischemic preconditioning protects cells from death caused by a subsequent severe ischemic event. Importantly, both ouabain-treated cells (*Proton* section) and cells subjected to severe ischemia [60] possessed combined markers of apoptosis and necrosis. Moreover, similarly to ouabain-treated cells, the protective action of ischemic preconditioning on severe ischemia-induced tissue damage was transient [62] and diminished sharply in the presence of cycloheximide [8]. The protective action of acidification was also demonstrated in mouse macrophages subjected to UV-irradiation [98], serum-deprived bovine and human umbilical vein endothelial cells [26, 27] and endothelial cells from human pulmonary arteries treated with staurosporin [25]. Viewed collectively, these data suggest that intracellular signaling triggered by $H^+{}_i$ sensor plays a universal role in modulation of efficacy of the cell death machinery.

Search for Intracellular Monovalent Ion Sensors

The ability of proteins to sense the modulation of monovalent ion concentration is strongly supported by cell physiology and molecular biology data obtained in studies of superfamilies of monovalent ion pumps, carriers and channels (Fig. 1). However, with the exception of HCO_3^--sensitive sAC, the molecular origin of monovalent ion sensors distinct from ion transporters and involved in intracellular signaling is still a mystery. This statement is in contrast with rapid progress in the identification of $Ca^{2+}{}_i$ sensors and may be explained by several features of these molecules. First, the high thermostability of several Ca^{2+}-binding proteins, such as calmodulin, and the well-defined molecular origin of their targets, such as phosphodiesterase and plasma membrane Ca^{2+}-ATPase, allowed researchers to purify them and to identify their amino acid sequence, even before the molecular biology era. As shown above, the downstream targets of monovalent cations and Cl^- sensors are still unknown. Second, high-affinity $Ca^{2+}{}_i$ sensors are almost completely saturated at $[Ca^{2+}]_i$ of 1 μM, and their Ca^{2+}-binding sites are slightly affected by the presence of monovalent cations and Mg^{2+}. This feature led to the identification of amino acid residues by ^{45}Ca binding assay. In contrast to Ca^{2+}, Na^+, K^+, HCO_3^- and Cl^- affect cellular function in the millimolar range. If monovalent ions bind with low-affinity sensors, these interactions may be affected by numerous factors, which complicates their identification by screening with radioisotopes. Third, high-affinity binding sites, initially detected in parvalbumins and calmodulin, are formed by a highly conservative linear amino acid sequence consisting of 14 amino acid residues (the so-called "EF-hand" domain). This knowledge led to the rapid identification of more than 30 other $Ca^{2+}{}_i$ sensors by the screening of cDNA libraries [47]. In contrast, monovalent ion sensors are probably formed by 3-D protein structures and recruit space-separated amino acid residues. This hypothesis is consistent with data obtained by the identification of amino acid residues in monovalent ion transporters performed with single-point mutated constructs. Thus, it was shown that Ala^{330}, Glu^{786}, Glu^{796}, Asn^{783} and Asp^{815} contribute to Na^+ binding within one of the 3 Na^+-binding sites of the Na^+/K^+-ATPase α-subunit [103].

Keeping these data in mind, we tried to identify a Na^+ response element (NaRE) involved in c-Fos expression triggered by the sustained inhibition of Na^+/K^+-ATPase (*Sodium* section). In case of positive results, this approach can lead to the identification of an upstream $Na^+{}_i$-sensor in a 2-hybrid yeast system, i.e., by mating yeast transformed with NaRE of the c-Fos promoter as a bait, with yeast expressing an activation domain fusion cDNA library. This approach was supported by previous

data on mouse NIH 3T3 cells transfected with human c-Fos showing the lack of an activatory action of ouabain on human c-Fos mRNA content after deletion of the −222 to −70 promoter region [76]. To achieve our goal, we transfected HeLa cells with the vector encoding the luciferase reporter gene under the control of the human c-Fos −650 to +103 region, which contains all known transcription elements of c-Fos promoter. With this construct, we failed to detect any significant elevation of luciferase expression in HeLa cells subjected to 6-h inhibition of Na^+/K^+-ATPase with ouabain or in K^+-free medium, which contrasted with massive accumulation of endogenous c-Fos mRNA and immunoreactive protein in ouabain-treated HeLa cells (*unpublished data*). We also did not observe any positive impact of Na^+/K^+-ATPase inhibition on luciferase expression driven by Elk-1, SRF, CREB and AP1 transcription factors [115]. At least two hypotheses could explain our negative results. (i) Chromatin architecture involved in the formation of NaRE is different in c-Fos and luciferase driven by c-Fos promoter. Indeed, nuclease digestion assays documented that chromatin transcription-sensitive c-Fos sites are located in a region centered to −350 base pair as well as at a position −1900, i.e., upstream of the classical promoter [48]. (ii) Side-by-side with 5′-untranslated region, introns contribute to organization of c-Fos NaRE. The role of introns in transcriptional regulation has been proved in the study of several genes, including c-Fos (24;72) and WNK kinase [127].

In conclusion, we would like to stress that the proposed models of monovalent ion sensors (Figs. 2 and 3) are based on the assumption that their function occurs in a fixed environment. This is not a case of the cytoplasm containing up to 0.4 g of protein and 0.1 g of other macromolecules, such as carbohydrates, lipids and nucleic acids, per ml of intracellular water [6, 74]. Because of extreme macromolecular crowding, cytoplasm functions as a gel undergoing gel-sol phase transitions in response to diverse stimuli, including modulation of the content of monovalent ions [99]. We speculate that these transitions per se can affect the functions of target proteins, thus contributing to the mechanism sensing monovalent ion concentrations. Further investigations should be performed to confirm or reject this hypothesis and to design new approaches for identification of the molecular nature of monovalent ion sensors.

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