

Review

ATP release via anion channels

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Abstract

ATP serves not only as an energy source for all cell types but as an ‘extracellular messenger’ for autocrine and paracrine signalling. It is released from the cell via several different purinergic signal efflux pathways. ATP and its Mg^{2+} and/or H^+ salts exist in anionic forms at physiological pH and may exit cells via some anion channel if the pore physically permits this. In this review we survey experimental data providing evidence for and against the release of ATP through anion channels. CFTR has long been considered a probable pathway for ATP release in airway epithelium and other types of cells expressing this protein, although non-CFTR ATP currents have also been observed. Volume-sensitive outwardly rectifying (VSOR) chloride channels are found in virtually all cell types and can physically accommodate or even permeate ATP^{4-} in certain experimental conditions. However, pharmacological studies are controversial and argue against the actual involvement of the VSOR channel in significant release of ATP. A large-conductance anion channel whose open probability exhibits a bell-shaped voltage dependence is also ubiquitously expressed and represents a putative pathway for ATP release. This channel, called a maxi-anion channel, has a wide nanoscopic pore suitable for nucleotide transport and possesses an ATP-binding site in the middle of the pore lumen to facilitate the passage of the nucleotide. The maxi-anion channel conducts ATP and displays a pharmacological profile similar to that of ATP release in response to osmotic, ischemic, hypoxic and salt stresses. The relation of some other channels and transporters to the regulated release of ATP is also discussed.

Abbreviations: AAC – ADP/ATP carrier; ABC – ATP-binding cassette; ANT – adenine nucleotide translocase; CF – cystic fibrosis; CFTR – cystic fibrosis transmembrane conductance regulator; Cx – connexin; DPC – diphenylamine-2-carboxylate; JGA – juxtaglomerular apparatus; MDR – multidrug resistance; NBD – nucleotide-binding domain; pl-VDAC – plasmalemmally expressed VDAC; PEG – polyethylene glycol; PKA – protein kinase A; RVD – regulatory volume decrease; TAL – thick ascending limb; TGF – tubuloglomerular feedback; VDAC – Voltage-dependent anion channel; VSOR – volume-sensitive outwardly rectifying

ATP release in the purinergic world

Adenosine-5'-triphosphate (ATP) is a universal energy source constantly produced by purine-generating reactions including mitochondrial oxidative phosphorylation and cytosolic glycolysis. It is utilized by cells at high rates. When cells are stimulated, they release small amounts of signalling molecules which include ATP. Once released, the extracellular ATP binds to P2 purinergic receptors expressed in virtually all cell types [1] to act as an ‘extracellular ligand’ for autocrine and paracrine signalling at cellular and organic levels [2–6]. P2 purinergic receptors consist of seven ionotropic P2X receptor subtypes [7] and 8 G-protein-coupled P2Y receptor subtypes [4, 8]. Between

these purinergic receptors and the purine-generating reactions, there exist purinergic signal efflux pathways and purino-converting enzymes (Figure 1). The latter includes at least nine different ectonucleotidases, which hydrolyse ATP, ADP and AMP to adenosine [9], as well as ecto-adenylate kinase, which converts 2ADP to ATP and AMP, and ectonucleoside diphosphate kinase, which converts ADP to ATP [10].

ATP, a relatively large and hydrophilic molecule, can exit cells using several different purinergic signal efflux pathways (Figure 1). One obvious source of extracellular ATP is cell lysis, which occurs when massive cell death takes place during injury or inflammation. In this case, ATP originates from injured cells, macrophages or lymphocytes at the inflammation site [11]. A non-lytic source of ATP would be the release of secretory granule or vesicle content during stimulated exocytosis, which occurs in some secretory cell types [2, 5, 13–15]. However, it is evident

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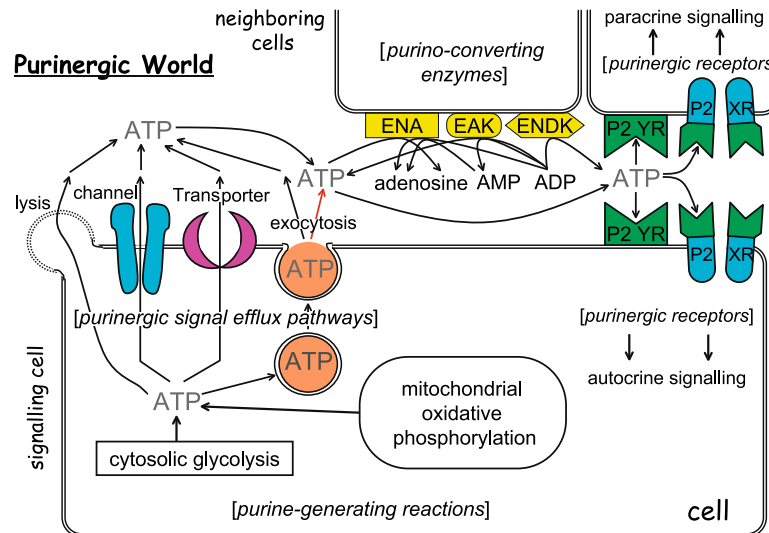


Figure 1. ATP in a purinergic world consisting of purine-generating reactions, purinergic signal efflux pathways, purino-converting enzymes and purinergic receptors. *ENA*, *EAK* and *ENDK* represent ectonucleotidase, ectoadenylate kinase and ectonucleoside diphosphate kinase, respectively. Arrows indicating *ENA*-mediated degradation of ATP to ADP and AMP as well as of ADP to AMP are missing because both ADP and AMP are rapidly degraded to adenosine by the same enzyme. Although the purino-converting enzymes are shown only on a cell different from the cell expressing purinergic signal efflux pathways and purinergic receptors, they may coexist on the same cell.

that some other mechanisms of ATP release are at work, because ATP release takes place even when cell damage or vesicular exocytosis does not occur. A recent study by real-time bioluminescence imaging showed brief point-source bursts of ATP release, which suggest the transient opening of channels [16]. Since the dissociation constants (pK values) of the α , β and γ phosphate groups are <2 , most ATP molecules exist in anionic forms (ATP^{4-} , $HATP^{3-}$, $MgATP^{2-}$ and $MgHATP^{-}$) at physiological pH (Table 1). Therefore, it is possible that an anion channel or transporter can electrogenically translocate anionic ATP, thereby serving as a conductive pathway for ATP release. Between the extracellular nanomolar and intracellular millimolar ATP conditions, there should exist an outwardly directed electrochemical potential gradient of the order of 10^{10} and 10^8 for ATP^{4-} and $MgATP^{2-}$, respectively, when the intracellular potential is around -60 mV. In this review we shall survey experimental arguments *pro* and *con* for the involvement of different types of channels and transporters in the electroconductive release of ATP.

Connexin as an ATP-releasing channel

Connexin (Cx) hemichannels, precursors to gap junction intercellular channels, are nonselective channels that are

Table 1. Anionic forms of ATP in the absence or presence of an equivalent concentration of Mg^{2+} at pH 7.4.

	ATP (%)	Mg^+ATP (%)
ATP^{4-}	89.5	10.7
$HATP^{3-}$	10.5	2.1
$MgATP^{2-}$	0	87.0
$MgHATP^{-}$	0	0.1

All the dissociation constants were taken from Sigel [284].

permeable to molecules of less than Mr 1000. Thus, there is a possibility that the Cx-hemichannel serves as a release pathway for ATP of molecular weight 507.21. In fact, enhanced ATP release was observed in C6 glioma cells and epithelial HeLa cells overexpressing Cx43 [17–20] and in *Xenopus* oocytes transfected with Cx50 or Cx46 [21]. Also, cells endogenously expressing Cx, such as astrocytes, endothelial cells and bronchial epithelial cells were shown to respond with ATP release to hemichannel stimulation by reduction of extracellular Ca^{2+} or mechanostress [16, 17, 19, 20]. However, Cx43, Cx46 and Cx50 are known to form cation-selective, but not anion-selective, channels [21–23] and to be insensitive to SITS [21] or DIDS [21, 23], stilbene-derivative Cl^{-} channel blockers. In contrast, SITS was shown to block Cx43- or Cx32-associated ATP release [17]. Also, it must be noted that Cx overexpression was reported to be associated with altered expression of other genes [24]. Thus, it is possible that Cx expression upregulates the activity of an ATP-releasing anion channel which is distinct from the Cx-hemichannel. The differential sensitivity to various anion transport inhibitors (reported in these studies as well as in other papers discussed below) might be sometimes misleading given the notable sensitivity of the luciferin–luciferase reaction itself to these substances. Therefore, the drugs should be screened for their effect on the ATP-detecting assay (luciferase, PC12-cell biosensor, etc.).

CFTR as an ATP-releasing channel

A hereditary disease, cystic fibrosis (CF), is widespread among Caucasians, and to a lesser extent among people of other races. Intensive investigations over the last several decades have led to the identification of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) [25].

Certain mutations in this gene cause an autosomal recessive disease characterized by severe dysfunction of fluid and electrolyte transport in secretory epithelia. CFTR has been shown to function as a cAMP-activated chloride channel (for reviews see [26–29]) with a small single-channel conductance (8–10 pS) and a nonrectifying I–V relationship. Many groups reported ATP release associated with CFTR expression (reviewed by [30, 31]) in human airway epithelial cells [32], C127 cells [33, 34], red blood cells from normal and CF patients upon mechanical stress [35], cardiac myocytes [36, 37] and retinal pigment epithelium cells [38].

If anionic forms of ATP are released via the CFTR channel, then actual ATP-mediated conductance should be measurable in CFTR-expressing cells under certain experimental conditions. Reisin et al. [39] were the first to detect such a conductance in C127/CFTR cells. When both pipette and bath contained ATP solutions, a 4.8-pS channel could be observed with a reversal potential of around 0 mV which was independent of employed cations (Na^+ , Mg^{2+} or Tris^+), suggesting that the current was anionic. The channels were activated by PKA and inhibited by diphenylamine-2-carboxylate (DPC), indicating that the currents were related to or mediated by CFTR. The permeability ratio of ATP^{4-} to Cl^- was 0.4 from whole-cell and 0.2 from single-channel current measurements. Schwiebert et al. [32] also demonstrated the presence of a glibenclamide-sensitive 6-pS channel in human airway epithelial cells bearing either normal or mutant CFTR, using symmetrical ATP solutions (140 mM TrisATP). The reversal potential shifted by -22 mV when 140 mM TrisCl was present on one side, indicating that the channel was permeable to chloride ions as well. Cantiello and coworkers repeatedly observed PKA-stimulated whole-cell and single-channel ATP-mediated currents from shark rectal gland cells [40] as well as from rat [36] and mouse [37] cardiac myocytes in primary culture. However, it must be pointed out that one cannot completely rule out a possible contamination of Cl^- currents in the currents measured during these patch-clamp experiments, because the shank of the patch pipette was back-filled with Cl^- -containing solution which may have been pushed down into a Cl^- -free, ATP-containing tip region by hydrostatic pressure.

In an attempt to rule out the contribution of non-CFTR proteins in the CFTR-mediated ATP conductance, Cantiello et al. [41] reconstituted purified CFTR proteins into lipid bilayers and observed single-channel ATP currents in a more defined environment. In these experiments, when conditions were similar to those used in single-channel patch-clamp experiments, two types of channels with unitary conductances of 8.6 and 34.3 pS and $P_{\text{ATP}}/P_{\text{Cl}}$ of 0.1 and 0.2 were observed. Surprisingly, when lower (more physiological) ATP concentrations were used, the authors observed single-channel events which had a $P_{\text{ATP}}/P_{\text{Cl}}$ of 9–17 and with unitary conductances varying from 26.6 to 511 pS with multiple sublevels.

Rather small single-channel conductances of about 10 pS at physiological Cl^- concentrations could be indicative of a relatively narrow pore. What is the actual dimension of

the CFTR channel pore and does it fit its ATP releasing function? Linsdell et al. [42] estimated the minimum functional pore diameter to be 0.53 nm ($R_p \sim 0.27$ nm) from the permeability of CFTR to different anions. A similar result was obtained in Calu-3 cells with an endogenous CFTR conductance [43]. These values are clearly smaller than the size of ATP (Table 2).

However, there exist a possibility that binding of a permeant ion may change the conformation and thus the size of the pore. For instance, CFTR displayed a strong ATP-hydrolysis-dependent asymmetry in permeation properties and allowed the passage of large organic anions (like lactobionate) added from the intracellular, but not from the extracellular, side. The functional pore diameter was 1.38 nm ($R_p \sim 0.69$ nm) for kosmotropic organic anions [44]. A recent electron crystallography study of negatively stained two-dimensional crystals of CFTR protein has revealed a 1.5 nm wide shaft which might represent the CFTR channel pore in one of its AMP-PNP-dependent conformations [45]. A 1.4–1.5 nm-wide pore is already large enough to accommodate ATP, providing a plausible structural basis for ATP release.

The attractive hypothesis that CFTR is a channel not only for Cl^- but also for ATP prompted many other researchers to test this idea. However, in a number of studies the results were different from those in the papers cited above. Li et al. [46] examined purified CFTR incorporated into lipid bilayers and failed to detect any ATP currents, while Reddy et al. [47], using human airway Calu-3 cells and sweat duct cells, and Grygorczyk et al. [48], using CFTR-expressing CHO cells, could not observe any consistent ATP conductance by patch-clamp in conditions apparently

Table 2. Effective radii of some physiologically significant inorganic and organic anions and osmolytes that potentially permeate anion channels.

Anion	Effective radius (nm)	Reference
Cl^-	0.181	[44]
NO_3^-	0.212	[44]
HPO_4^{2-}	0.275	Unpublished calculation
Gluconate	0.349	[44]
Aspartate	0.339	Unpublished calculation
Glutamate	0.345	[44]
Taurine	0.263	[285]
Proline	0.28	[285]
Myo-inositol	0.306	[285]
Betaine	0.285	[285]
GPC	0.367	[285]
ATP^{4-}	0.57–0.58	[212]
HATP^{3-}	0.56–0.58	Unpublished calculation
MgATP^{2-}	0.59–0.61	[212]
ADP^{3-}	0.53–0.56	Unpublished calculation
UTP^{4-}	0.53–0.54	Unpublished calculation

The unhydrated radii were calculated as a geometric mean of three dimensions according to the formula: $R_x = (1/2)(l_1 l_2 l_3)^{1/3}$ where l_1 , l_2 and l_3 are ion dimensions estimated from space-filling models. The dimension data were taken either from the indicated references or calculated using Molecular Modeling Pro computer software (Norgwyn Montgomery Software Inc., North Wales, PA).

GPC – Glycerophosphocholine.

similar to those used by the Cantiello and Schwiebert groups alluded to above. These disappointing discrepancies were thoroughly discussed by Abraham et al. [49].

At the same time, Foskett and coworkers did observe ATP-conductive channels related to CFTR expression. First, Pasyk and Foskett [50] provided convincing evidence of the existence of an ATP-conductive pathway in CFTR-expressing CHO and MDCK cells. The channel I–V relationship, which gave a slope conductance of 4.5 pS (in symmetrical 100 Na₂ATP solutions containing 5.2 mM Cl[−] to provide reversibility for Ag/AgCl electrodes), shifted by about −12 mV in the presence of a 10-fold ATP gradient at symmetrical Cl[−] concentrations (close to the Nernst potential for ATP^{4−} of −15 mV). Moreover, the currents were insensitive to cation replacement (Na⁺ to Tris⁺) and to a decrease in the Cl[−] concentration from 5.2 to 0.7 mM. In a subsequent paper, Sugita et al. [51] used 100 mM Na₂ATP (pipette) and 140 mM NMDG-Cl (bath) solutions and observed three kinds of single-channel events: normal CFTR (7.4 pS) with an extremely low ATP permeability ($P_{\text{Cl}}/P_{\text{ATP}} \sim 140$), a CFTR-associated ATP channel (5.2 pS) with $P_{\text{Cl}}/P_{\text{ATP}} = 2.5$ and a CFTR-independent ATP channel (6.3 pS) which was more permeable to Cl[−]. The activity of the CFTR-associated ATP channel, similar to that of CFTR, required PKA-mediated phosphorylation, was affected by non-hydrolysable ATP analogs, and was altered by mutations in the R-domain and NBDs. However, it exhibited pharmacology different from CFTR, and furthermore, was insensitive to mutations in the pore region. The authors thus concluded that the ATP-conductive pathway is associated with but distinct from CFTR.

A similar conclusion was reached by Braunstein et al. [52] based on the finding that ATP channel currents were observed with crude membrane fractions, but not highly purified CFTR proteins or protein preparations immunodepleted of CFTR, in lipid bilayer reconstitution experiments. *Xenopus* oocytes provided another test system in which CFTR could be expressed and ATP release measured from a single cell. In such experiments, Jiang et al. [53] found that only a subset of oocytes exhibited ATP release in association with CFTR expression. The nucleotide release required a special stimulation procedure consisting of Cl[−] depletion and replenishing, was sensitive to the Cl[−] concentration, and was selective for Cl[−] over Br[−]. The ATP release was affected by mutations in the CFTR protein, and it did not need Cl[−] conductance (because a non-conductive mutant did release ATP), but mutations in the pore region which change the ionic selectivity of the CFTR channel also altered the halide sensitivity of ATP release. The authors concluded that CFTR serves as the Cl[−] sensor for a separate ATP-releasing pathway, which is endogenous to oocytes and present in only about half of the cells.

CFTR-independent ATP release was observed by Takahashi et al. [54] in 3T3 fibroblasts, by Grygorczyk and Hanrahan [55] in T84, CHO, Calu-3, NIH3T3 and some other cells, by Mitchell et al. [56] in ocular ciliary epithelial cells, by Watt et al. [57] in human nasal epithelial cells in primary culture, by Hazama et al. [58–61]

in human Intestine 407 and mouse C127 cells, and by Donaldson et al. [62] in nasal airway surface liquid from normal and CF subjects. Kawano et al. [63] studied cardiac sarcoplasmic reticulum (SR) membrane vesicles reconstituted into lipid bilayers and have identified a PKA-activated Cl[−] channel distinct from CFTR. This channel was clearly ATP-conductive with $P_{\text{ATP}}/P_{\text{Cl}}$ of 0.5 indicating that there may exist other types of ATP-permeable pathways different from CFTR in cardiac SR.

Other ABC transporters as ATP-releasing pathway

CFTR belongs to a large family of ATP-binding cassette (ABC) transporter proteins and shares with them great structural similarity. ABC proteins have been found in all organisms from bacteria to humans and represent the largest and most diverse ATPase superfamily [64]. ATP binding to nucleotide-binding domains (NBDs) initiates a cycle of conformational changes that ultimately lead to active transport (either uptake or extrusion) of a wide variety of hydrophobic and hydrophilic solutes including amino acids, sugars, polysaccharides, peptides, lipids, bile salts, metals, toxic drugs and even proteins [65]. A plausible hypothesis would be that ATP could also be one of the substrates or, alternatively, that the transporter could work as a channel in some circumstances to allow conductive transport of anionic forms of ATP.

Indeed, Abraham et al. [66] observed enhanced release of ATP from CHO cells overexpressing a P-glycoprotein, murine MDR1, in a manner dependent on the amount of the expressed protein. Moreover, the authors were able to detect the inward whole-cell currents (corresponding to the efflux of ATP^{4−} or MgATP^{2−}) when the pipettes were filled with Cl[−]-free solutions containing either 100 mM MgATP or 100 mM TrisATP. This current was seen only in transfected cells, and mutations at NBDs significantly suppressed the ATP currents. Single-channel events observed in the presence of Cl[−]-free ATP solutions were rectifying with a unitary conductance of 14.7 pS for outward currents and 43 pS for inward currents. The reversal potential was insensitive to the cation (Mg²⁺ or Tris⁺) used as a counterion, suggesting that the current was carried by ATP^{4−} or MgATP^{2−}. Similar results were obtained by Bosch et al. [67] with MDR49 and MDR65, the two P-glycoprotein genes of *Drosophila melanogaster*, expressed in Sf-9 cells. MDR65-expressing cells had a measurable basal whole-cell ATP current, which was further increased by large pulses to −150 mV, while MDR49 had no significant basal ATP current, but did develop the current upon electrical stimulation. The authors speculated that MDR65 is a functional analog of CFTR as it can conduct both Cl[−] and ATP, whereas MDR49 is closer to mammalian MDR1 because it does not conduct Cl[−] and needs activation by voltage pulse in order to conduct ATP. Single-channel conductances in symmetrical ATP conditions observed by Bosch et al. [67] varied from 5.6 to 115 pS. However, a judgment on the degree of

relevance of these results awaits a determination that Cl^- added to the shank of the patch pipette was not the cause of a technical problem in which currents were contaminated.

In rat hepatoma HTC cells, hypotonic swelling increased the amplitude of whole-cell currents measured using extracellular and intracellular solutions both containing not only 100 mM MgATP but also 5 mM MgCl_2 [68]. In hepatoma HTC-R cells overexpressing MDR1 protein, an increase in the whole-cell current (carried not only by ATP but also by Cl^-) induced by hypotonicity was greater than that in parental HTC cells [69]. The same group later reported that MDR1-overexpressing cells (HTC-R and NIH3T3/MDR1) exhibited an approx. three-fold higher level of ATP release which was sensitive to the MDR inhibitors cyclosporin and verapamil [70]. The whole-cell currents were inhibited by anti-MDR1 antibodies, but the ATP release was insensitive to the mutation of G185V, which alters the substrate selectivity of MDR1. Thus, the authors suggested that MDR1 is not an ATP channel itself, but rather a regulator of a separate ATP channel [70].

Studying mice with disrupted ABC transporter genes, Abraham et al. [71, 72] found a decreased basal blood ATP level in MDR1- and MRP1-deficient mice, and a slower rate of ATP release from erythrocytes isolated from these animals. Interestingly, CFTR knockout animals, in which MRP1 expression was found to be augmented, had an increased rate of ATP release from unstimulated erythrocytes, suggesting that ATP release was mediated by MRP1 but not by CFTR. The electrical mobility of doxorubicin was dependent on the ATP concentration, indicating that a complex formed between the MDR substrate and ATP, and that ATP was co-released with the substrate [71]. Recent crystallography studies, consistent with the hypothesis of ABC transporter-mediated ATP release, showed that MDR possesses a cavity large enough to accommodate and translocate molecules as large as ATP [73]. Also, Darby et al. [74] recently suggested that MRP-mediated ATP release from rat astrocytes is present based on the observation that hypotonicity-induced ATP release was inhibited by an MRP inhibitor (MK571). However, these lines of evidence are still circumstantial. Direct electrophysiological evidence is required before making a definite conclusion that conductive ATP release occurs via MDR1 or MRP1.

VSOR channel as an ATP-releasing pathway

Cellular swelling in response to a hypo-osmotic challenge activates anion channels in most cell types [75–78]. At both microscopic single-channel and macroscopic whole-cell levels, this Cl^- current exhibits moderate outward rectification and inactivation kinetics at large positive potentials [77, 79]. The Volume-Sensitive Outwardly Rectifying (VSOR) chloride channels feature a low-field anion selectivity with a permeability sequence of $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$ in human epithelial cells and most other cell types [77, 80, 81]. Single VSOR Cl^- channels exhibit an intermediate unitary conductance of 50–70 pS [77, 79,

82], and voltage-dependent sensitivity to extracellular ATP [77, 83, 84] and intracellular protons [85]. The VSOR Cl^- channel activity requires intracellular ATP; a prerequisite for channel activation is not the hydrolysis of ATP, but the direct binding of ATP molecules to the channel protein or its accessory protein [77, 86–89].

Although cell volume regulation was first recognized as the primary function of this type of channel [75–77, 80], numerous studies have evolved around several other physiological processes in which VSOR Cl^- channels are key players. These include maintenance of intracellular acid-base balance through permeability to lactate and bicarbonate ions [90], regulation of cell proliferation [91, 92], and regulation of the cell cycle [93, 94]. Recent studies indicated that the VSOR channel also plays a cell-rescuing role by counteracting necrotic cell swelling [95–97]. Other studies demonstrated a cell-killing role by inducing apoptotic cell shrinkage [98–101].

Substantial evidence has been provided for VSOR Cl^- channel-mediated release of anionic amino acids such as glutamate and aspartate [75, 102, 103], but not the zwitterionic amino acid, taurine [104–107]. Thus, there exists a possibility that the VSOR Cl^- channel mediates conductive release of large anions such as ATP as well. Swelling-activated whole-cell currents are substantial enough (5–10 nA for a medium-sized cell at 50–100 mV) that even a subtle ATP permeation, if it existed, would generate a physiologically significant flux of the nucleotide. The first question to be addressed, however, is whether the VSOR channel pore is wide enough to permit the passage of ATP. Certainly, the pore size is not the only determinant of channel permeability. However, knowing the approximate dimensions of the pore is helpful in getting a picture of the overall transport process. Calculations using the permeability of the VSOR channel to anions of different size yielded a pore diameter of 0.73 or 1.15 nm (radius of 0.37–0.58 nm) depending on whether or not frictional forces were taken into account [108]. Droogmans et al. [109, 110] studied the voltage-dependent block of VSOR currents by basket-shaped compounds, calixarenes, and demonstrated that this block can be released at high positive voltages, suggesting that calixarenes act as ‘permeant blockers,’ which cannot only block the pore but also pass through it. Varying the size of the calixarenes, these authors estimated the lower and upper limits for the cross-sectional dimensions of the channel pore to be 1.1×1.2 nm and 1.7×1.2 nm, respectively. The minimum and maximum of the pore radii calculated as a geometric mean of these dimensions are 0.57 and 0.71 nm, respectively. Ternovsky et al. [111] measured the partitioning of ethylene glycol and its polymeric forms into the pore of the VSOR channel and found the cut-off radius of the VSOR channel lumen to be 0.63 nm. Thus, three unrelated and independent approaches (anion permeation, voltage-dependent block and polymer partitioning) have yielded a converging estimate of the VSOR pore radius at 0.6–0.7 nm. This value is compatible with the role of the VSOR channel as a mediator of swelling-induced efflux of intracellular osmolytes, including Cl^- ions with an effec-

tive radius of 0.18 nm and most amino acids with effective radii of app. 0.3–0.4 nm (Table 2).

Surprisingly, the estimates of VSOR pore size practically coincide with the radius of ATP^{4-} or MgATP^{2-} , which is about 0.6–0.7 nm (Table 2). This result agrees with the observation that profound voltage-dependent block of VSOR channel is caused by extracellular ATP [83, 84, 112, 113]. However, can ATP molecules be translocated through the VSOR channel? Hisadome et al. [113] found that the voltage-dependent block by ATP can also be released at large positive potentials (analogous to block by calixarenes), suggesting that the negatively charged nucleotide acts as a 'permeant blocker' and thus can physically pass through the channel. In this study, VSOR inhibitors such as glibenclamide, verapamil, tamoxifen, and fluoxetine suppressed the ATP release, suggesting that the VSOR channel can serve as a conductive pathway for swelling-induced ATP release in aortic endothelial cells [113].

The tantalizing hypothesis that the VSOR channel is a pathway for release of ATP was scrutinized in several detailed studies. However, most of the results obtained were at variance with this prospect, as follows. In human Intestine 407, mouse mammary C127/CFTR and bovine ciliary epithelial cells, swelling-induced ATP release was not inhibited by a number of VSOR anion channel blockers, such as glibenclamide [56, 58–60], DPC [56], DIDS [56], SITS [59] and arachidonic acid [59]. Gd^{3+} , which is an effective blocker of swelling-induced ATP release [59, 61, 114], did not inhibit VSOR anion currents in Intestine 407 [59], and C127/CFTR [60] and C127 [115] cells. Monoclonal antibodies raised against membrane proteins from swollen cells could block swelling-induced ATP release from Intestine 407 cells [59], but failed to affect swelling-induced activation of anion currents in Intestine 407 [59] and C127/CFTR [61] cells. Heterologous expression of CFTR was shown to downregulate VSOR anion channel activity in CPAE and COS cells [116] as well as in HEK293T cells [117], but upregulate swelling-induced ATP release from C127/CFTR cells [58, 60]. The expression of CFTR was shown to be required for swelling-induced ATP release from airway epithelial cells [114].

Thus, on one hand, the available biophysical data suggest that the VSOR channel lumen can accommodate a bulky ATP^{4-} anion added from the extracellular side and even translocate it to the cytoplasm at high positive voltages. On the other hand, however, in most cells studied to date, the bulk of pharmacological results argues against an actual involvement of VSOR anion channel in electrogenic release of ATP. Further studies are necessary to clarify this important issue.

Maxi-anion channel as an ATP-releasing pathway

Anionic current fluctuations of very high amplitude were first described by Blatz and Magleby [118] in plasma membrane patches excised from rat skeletal muscles in primary culture. These events had relatively slow voltage-

dependent gating with a mean open time of 0.48 s at +30 mV and 1.19 s at –30 mV. The current-to-voltage relationship was linear with a slope conductance of 430 ± 15 pS in symmetrical 143 mM KCl conditions. When the bath KCl concentration was varied, the reversal potential approached the calculated Nernst potential for Cl^- indicating high anion selectivity of this channel. Calcium ions were not required for the channel activity. Later, very similar channels were observed by Schwarze and Kolb [119] in myotubes obtained from chick embryos and in mouse peritoneal macrophages. These channels spontaneously appeared in only 5% of patches, and could be activated by the calcium ionophore A23187 in 30% of silent patches. Patch excision significantly increased the channel incidence, and once opened, the channels were insensitive to Ca^{2+} ions. The channels had a unitary conductance of 340 pS with a substate at 208 pS and a Q_{10} of 1.3 in the temperature range of 4.5 to 38 °C. The permeability ratio $P_{\text{Cl}}/P_{\text{Na}}$ was estimated to be 4–6 from the reversal potential shifts induced by NaCl concentration changes. The authors studied the gating kinetics in detail and found at least three non-conducting states with very steep voltage dependence. The burst-like gating pattern had bell-shaped voltage dependency. At about the same time, large-conductance channels with anion selectivity and bell-shaped voltage dependency were described in Schwann cells from neonatal rats in primary culture [120] and in A6 *Xenopus* kidney epithelial cells [121].

In the following two decades the patch-clamp technique has been applied to a broad range of cell types and tissues, and the activity of a maxi-anion channel with a unitary conductance of 300–400 pS has been reported in almost every part of the whole organism. For instance, the maxi-anion channel activity has been found in freshly isolated frog skeletal muscle [122, 123] and smooth muscle from uterus [124] and colon [125, 126], as well as in cultured vascular smooth muscle cells of rat aorta [127–129], neonatal cardiac myocytes in primary culture [130–132], cultured L6 rat muscle cells [133–136] and BC3H1 myoblasts [137]. Maxi-anion channels were detected in neuronal [138–147] and glial [120, 148–154] cells. Epithelial cells from bladder [155], stomach [156], pancreas [157–159], colon [160–162], trachea [163–165], choroid plexus [166], bile duct [167, 168], ciliary body [169–171], kidney [121, 172–181], inner ear vestibule [182] and placenta [183–188] were also found to express maxi-anion channels with properties similar to those in muscle and neuronal cells. Similar maxi-anion channels were also discovered in fibroblasts [189–192] and endothelial [193–197] cells. In the immune system, maxi-anion channel activity has been confirmed in B-lymphocytes [198–201], T-lymphocytes [202–204] and in peritoneal macrophages [119, 205]. Mast cells [206], keratinocytes [207], osteogenic cells [208], cultured glomus cells of the carotid body [209], PC12 pheochromocytoma cells [210], pavement cells from the gills of the trout [211] and mammary gland C127 cells [115, 212, 213] have also been shown to possess this channel. Patch-clamping intracellular organelles revealed maxi-anion channel activity in sarco-

plasmic reticulum 'sarcoballs' [214], while the presence of the channel in the endoplasmic reticulum [215] and the Golgi complex [216] was demonstrated by reconstituting these membranes into liposomes and lipid bilayers, respectively.

Role of maxi-anion channels in swelling-induced ATP release from mammary gland cells

Osmotic cell swelling induces the release of intracellular ATP in a large variety of cell types [217]. Swelling-induced ATP release was shown to facilitate the process of volume regulation of osmotically swollen cells, called regulatory volume decrease (RVD), by the purinergic receptor-mediated stimulation of the volume-regulatory K^+ efflux [128–220] or Cl^- efflux [52, 68, 74, 221] pathways.

Mammary gland C127 cells also responded to the hypotonic stimulation by massive release of ATP. However neither CFTR nor VSOR contributed significantly to this flux of nucleotide [58, 60, 61]. Like in most other cell types studied so far, the conventional VSOR chloride channel is the main component of the swelling-induced macroscopic whole-cell anion conductance. However, when VSOR chloride currents were suppressed by omitting ATP from the intracellular (pipette) solution and by supplementing the hypotonic bath solution with phloretin, a relatively selective blocker of VSOR Cl^- channels [222], another type of anion current could be observed in C127 cells [115]. This current was not outwardly rectifying and exhibited profound time-dependent inactivation at positive and negative voltages greater than around ± 25 mV. Importantly, the current was sensitive to the most powerful inhibitor of ATP release, Gd^{3+} . The single-channel fluctuations underlying this swelling-induced macroscopic conductance were observed in cell-attached patches after hypotonic stimulation and in excised inside-out patches. They had a large unitary conductance (approx. 400 pS) and displayed a voltage-dependent inactivation very similar to that observed for whole-cell current. ATP^{4-} added from both the extracellular and intracellular sides caused a profound voltage-dependent blockage revealing a weak ATP-binding site with a K_d of about 12 mM. This was located in the middle of the pore and was accessible to the nucleotide from either side, satisfying one criterion for being a translocator of ATP. Indeed, when all anions were replaced with ATP^{4-} , inward ATP-mediated currents were observed [115], as shown in Figure 2A, and a permeability ratio P_{ATP}/P_{Cl} of about 0.1 was obtained. Macroscopic whole-cell currents, single maxi-anion channel currents and swelling-induced ATP release shared the same pharmacological profile, i.e., sensitivity to Gd^{3+} , SITS and NPPB, but not to phloretin, niflumic acid or glibenclamide. Moreover, swelling-induced activation of this channel was facilitated in CFTR-expressing C127 cells, a fact which is in agreement with the upregulation of swelling-induced ATP release by CFTR in these cells [58, 60]. Based on these observations, Sabirov et al. [115] proposed that maxi-anion channels serve as a conductive pathway for the swelling-induced release of ATP in mammary C127 cells.

For its newly proposed function as a conductive pathway for ATP release, it would be favorable that the maxi-anion channel has a pore sufficiently large to permit the passage of bulky ATP anions. Recently, we [212] attempted to estimate the maxi-anion channel pore size using two different approaches. First, we used a conventional method and measured the permeability of organic anions of different size. However, we found a linear relationship between the relative permeability of these anions and their relative ionic mobility (measured as the ratio of ionic conductances) with a slope close to 1. This result suggests that the organic anions tested, with radii up to 0.49 nm (from formate to lactobionate), move inside the channel by free diffusion. In the second approach, we succeeded, for the first time, in pore-sizing by the nonelectrolyte exclusion method in single-channel patch-clamp experiments. In this method, electroneutral hydrophilic polymers, polyethylene glycols (PEGs), are added to the pipette, to the bath or to both the pipette and bath, and only molecules that can freely access the pore lumen can suppress the channel conductance. The cut-off radii of PEG molecules that could access the channel from the intracellular (1.16 nm) and extracellular (1.42 nm) sides indicated an asymmetry of the two entrances to the channel pore. Measurements by symmetrical two-sided application of PEG molecules yielded an average functional pore radius of ~ 1.3 nm. These three estimates are considerably larger than the radii of ATP^{4-} and $MgATP^{2-}$ (Table 2). Therefore, it was concluded that the nanoscopic maxi-anion channel pore provides sufficient room to accommodate ATP and is well suited to its function as a conductive pathway for ATP release [212, 217].

Since the radii of ADP^{3-} and UTP^{4-} are smaller than those of ATP^{4-} and $MgATP^{2-}$ (Table 2), it is very possible that maxi-anion channels conduct ADP^{3-} and UTP^{4-} , which are agonists for many P2 receptors subtypes and the P2U subtype, respectively. In fact, as shown in Figure 2 (B, C), sizable inward currents carried by ADP^{3-} and UTP^{4-} were detected in the inside-out patches when all the anions in the intracellular (bath) solution were replaced with ADP^{3-} or UTP^{4-} . The P_{ADP}/P_{Cl} and P_{UTP}/P_{Cl} values evaluated from the reversal potentials are approximately 0.1 (see legends for Figure 2).

The relation between intracellular signalling pathways and ATP release mediated by the maxi-anion channel is poorly understood at present. One of the regulatory signals, arachidonic acid, has been shown to inhibit both maxi-anion channels and swelling-induced ATP release at a physiological concentration range with a K_d of 4–6 μM [213]. The arachidonate effects were insensitive to inhibitors of arachidonate-metabolizing oxygenases. They were mimicked by *cis*-unsaturated fatty acids (which are not substrates for oxygenases), suggesting a direct action on the channel. The maxi-anion channel activity was inhibited by arachidonic acid in two different ways: channel shutdown (K_d of 4–5 μM) and reduced unitary conductance (K_d of 13–14 μM), both of which did not affect the voltage dependence of open probability. ATP^{4-} -conducting inward currents measured in the presence of 100 mM ATP in the bath were also reversibly inhibited by arachidonic acid.

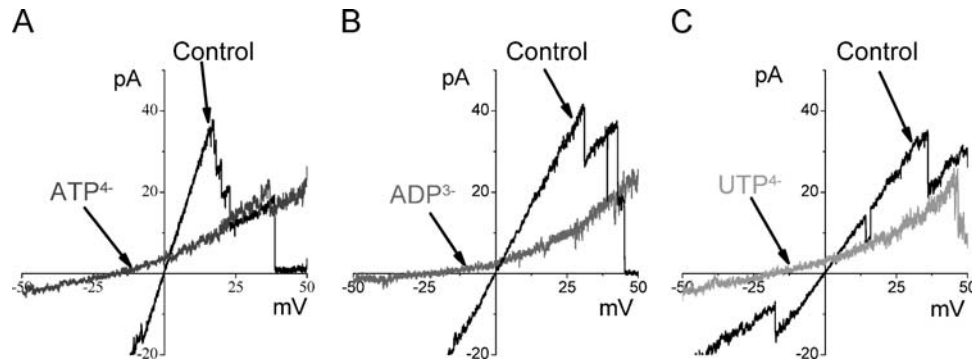


Figure 2. Representative current–voltage (*I*–*V*) curves composed of outward Cl^- currents and inward currents carried by ATP^{4-} (A), ADP^{3-} (B) and UTP^{4-} (C). The currents were recorded by ramp-clamp in macro-patches excised from C127 cells. The extracellular (pipette) solution was Cl^- -rich Ringer solution, and the intracellular (bath) solution was Cl^- -free solution containing 100 mM Na4ATP (A), Na3ADP (B) or Na4UTP (C). The reversal potentials are -16.2 ± 1.8 mV ($n = 5$; A), -20.3 ± 1.7 mV ($n = 10$; B) and -17.1 ± 0.8 mV ($n = 10$; C). The calculated values of $P_{\text{ATP}}/P_{\text{Cl}}$, $P_{\text{ADP}}/P_{\text{Cl}}$ and $P_{\text{UTP}}/P_{\text{Cl}}$ are 0.10 ± 0.014 , 0.12 ± 0.010 and 0.09 ± 0.005 , respectively.

Sensitivity of maxi-anion channels to arachidonate has previously been observed in an L6 rat muscle cell line [136] and in human term placental membranes reconstituted into giant liposomes [185]. On the other hand, gastric endothelin-activated maxi-anion channels were insensitive to arachidonic acid added from the outside [156].

Role of maxi-anion channels in salt stress-induced ATP release from macula densa cells

Glomerular filtration rate in the kidney is tightly regulated via a highly elaborate feedback mechanism. The juxtaglomerular apparatus (JGA), a morphologically complex junction between the thick ascending limb (TAL), glomerulus and afferent and efferent arterioles, is the site where tubuloglomerular feedback (TGF) takes place. Macula densa cells are located within the cortical TAL and have their basolateral membrane in contact with glomerular mesangial cells, which, in turn, are contiguous with smooth muscle cells and renin-secreting granular cells of the afferent arteriole. Macula densa cells sense a rise in the TAL luminal NaCl concentration induced by salt load in body fluids. As a consequence, they transmit signals that cause alterations both in vascular tone of the afferent arteriole and in renin secretion from granular cells of the juxtaglomerular apparatus [223–226].

There are no gap junctions between macula densa cells and mesangial cells (see citations in [226]). Therefore, it has been suggested that the macula densa cells release a humoral factor at their basolateral membrane. Interstitial ATP levels correlate with the TGF response, and afferent arterioles express P2X receptors, whose activation by ATP causes sustained vasoconstriction (see citations in [181, 226]). Thus, ATP was and is currently considered to be one candidate for such a humoral factor.

In this light, Bell et al. [181] performed a patch-clamp study on the lateral membrane of macula densa cells. Together with a Ca-activated 20-pS non-selective cation channel [227], a maxi-anion channel with a unitary conductance of 380 pS was identified in cell-attached patches when 135 mM NaCl was present in the bathing

solution [181]. Interestingly, when extracellular NaCl was removed from the bathing solution, the channel became quiescent. Channel activity was restored within some 10 s after readdition of NaCl to the bathing solution. In excised inside-out patches this channel exhibited an ATP conductance with the permeability ratio $P_{\text{ATP}}/P_{\text{Cl}}$ of 0.14 and was sensitive to the most effective blocker of ATP release, Gd^{3+} . Therefore, this ATP-conducting anion channel was thought to function as an ATP release pathway in macula densa cells. To verify this, the authors used rat pheochromocytoma PC12 cells that are rich in plasmalemmal P2X receptors as biosensors [14, 228] to detect ATP release. When a PC12 cell was brought into close proximity to a macula densa plaque, clear ATP-induced responses to an increase in the tubular NaCl concentration were detected as P2X receptor-mediated cation currents measured by patch-clamp or as cytosolic Ca^{2+} concentration increases in fura-2-loaded cells [181]. Importantly, very similar Ca^{2+} responses to an increase in the NaCl concentration of the TAL luminal perfusate were also observed in fura-2-loaded glomerular mesangial cells when they were placed close to the basolateral membrane of macula densa cells [181]. These results led to the conclusion that the maxi-anion channel served as a basolateral ATP-conductive pathway opened in response to changes in luminal content in the cortical TAL in which the macula densa resides. Cell-to-cell communication between macula densa cells and mesangial cells, which express P2Y₂ receptors [181, 226], involves the release of ATP from macula densa cells via maxi-anion channels at the basolateral membrane in response to salt stress, which is associated with drastic changes in the volume of macula densa cells [229]. This mechanism may represent a new paradigm in cell-to-cell paracrine signal transduction mediated by ATP in TGF [181, 217].

Role of maxi-anion channel in ischemia-induced ATP release from cardiomyocytes

The normal basal levels of plasma and interstitial ATP are very low and do not exceed 20–40 nM in human venous

plasma [230] and in the cardiac interstitial space [231]. However, the local concentration of extracellular ATP is known to often exceed micromolar levels due to ATP release associated with local trauma, vascular injury and platelet aggregation [232, 233]. It is also well known that ATP is released into the interstitial space during electrical stimulation [234], application of cardiotoxic agents [235–239], mechanical stretch [240] and increased blood flow [235–241]. Hypoxia [236, 237, 242–244] and ischemia or ischemic preconditioning [231, 237, 240, 245] can also induce the release of ATP into the cardiac interstitial space. Purinergic nerves innervating the heart [12], cardiac vascular endothelial cells [246] and/or cardiomyocytes themselves [243] may release ATP. In the heart, many ionotropic P2X receptor and metabotropic P2Y receptor subtypes are expressed [247], and a variety of effects on the heart mediated by extracellular ATP have been reported [248, 249]. However, the mechanism by which ATP is transported across the cell membrane is not well understood in these tissues either.

Neonatal rat cardiomyocytes grow well in primary culture and have long been studied as a model for cellular ischemia/hypoxia. Application of osmotic stress, ischemia and hypoxia induces a quick and reversible increase in the level of ATP in superfusates, as estimated by luciferin–luciferase assay [132]. Using a single-cell based biosensor technique [14, 228], the local concentration of the released ATP was found to reach a level of over 10 μM [132]. The swelling-induced release of ATP from neonatal rat ventricular myocytes was sensitive to the anion channel blockers SITS and NPPB, suggesting an electrogenic mechanism of ATP release via an anion channel. Several types of anion channels, including CFTR, VSOR and maxi-anion channels, have been observed in cardiomyocytes [131, 132, 250, 251]. Lader et al. [36] reported that neonatal rat cardiomyocytes possess a cAMP-activated, glibenclamide-sensitive ATP-conductive pathway associated with CFTR. However, swelling-induced ATP release from neonatal rat ventricular myocytes was found to be insensitive to glibenclamide, which is a potent blocker of cardiac CFTR [252] and VSOR Cl^- channels [253]. Our patch-clamp experiments have confirmed the robust expression of maxi-anion channels in cardiomyocytes and demonstrated that the cardiac maxi-anion channels can be activated readily not only by hypotonicity [131, 132] but also by ischemic or hypoxic stress [132]. Cardiac ATP release and maxi-anion channel activity shared the same pharmacological profile and were sensitive to Gd^{3+} and arachidonic acid, which are the most effective blockers of maxi-anion channels in C127 cells [115, 213]. These data, together with the actual conductivity of ATP^{4-} and MgATP^{2-} through the cardiac maxi-anion channel, indicate that the channel constitutes a major electrogenic pathway for the exit of ATP during purinergic paracrine and autocrine signalling in the heart [132].

Considering intracellular ATP and Cl^- concentrations are approximately 2 and 20 mM, respectively, and using the measured permeability to ATP^{4-} and MgATP^{2-} and the measured rate of ATP release, Dutta et al. [132]

estimated that a brief activation of only a few maxi-anion channels is sufficient to produce the observed physiologically meaningful ATP signal. In contrast, the total number of maxi-anion channels expressed in a single cardiomyocyte (around 60, as estimated from the whole-cell current) seems to be much larger, indicating that the cells keep many channels in the inactive state or ‘on standby’ for extracellular purinergic signalling. Since cardiac cell swelling is known to be induced during ischemia or hypoxia [254–256], it seems likely that cell swelling underlies the mechanism by which maxi-anion channels are activated in response to not only hypotonic but also hypoxic or ischemic stress.

Molecular identity of the maxi-anion channel

Maxi-anion channels have very large single-channel conductance and bell-shaped voltage-dependent inactivation with maximal open probability at near 0 mV. These biophysical properties are similar to those of the Voltage-Dependent Anion Channel (VDAC) expressed in the outer membrane of mitochondria [257–259]. Therefore, it has been hypothesized that maxi-anion channels observed in patch-clamp experiments in different cells represent a plasmalemmally expressed VDAC (pl-VDAC) protein [147, 153, 192, 210]. A large number of research groups have indeed reported the presence of VDAC protein in the plasma membrane of various cells [147, 153, 192, 210, 260–267]. However, how can the same protein be targeted to such different locations? One possible mechanism was suggested by Buettner et al. [210], who identified an alternative first exon in the murine *vdac-1* gene that leads to the expression of a form of porin with a leader peptide at its N-terminus, a signal that targets the protein to the plasma membrane through the Golgi apparatus. This signal peptide is eventually cleaved away to produce a plasmalemmal VDAC protein identical to the mitochondrial one. Other mechanisms involving untranslated regions of the mRNA have also been considered to explain the extra-mitochondrial localization of porin [263].

Probing the pore of mitochondrial porin by the nonelectrolyte partitioning method yielded a value for the pore radius of 1.5 nm for the fully open state of the channel in lipid bilayers [268]. This figure is very close to the cut-off size of around 1.3 nm obtained in our experiments with the maxi-anion channel [212]. Later, using the asymmetric PEG application method, Carneiro et al. [269, 270] described an asymmetrical pore for mitochondrial VDAC with radii of ~ 1 and ~ 2 nm for its *cis* and *trans* entrances, respectively (*cis* designates the side of the bilayer from which the protein was added). This asymmetry parallels the asymmetry of the maxi-anion channel revealed in our experiments using one-sided application of PEG: 1.16 nm and 1.42 nm for radii of the intracellular and extracellular vestibules, respectively [212]. Electron microscopic images demonstrated that mitochondrial porin has an inner radius of ~ 1.4 nm [271], which is very close to the value obtained by polymer size exclusion for VDAC in lipid bilayers [268–270] and for the maxi-anion channel in our patch-

clamp study [212]. Thus, the structural features of mitochondrial porin and the ATP-conductive maxi-anion channel do converge. Maxi-anion channels also resemble mitochondrial porins with respect to its open-channel block by ATP [272] and ATP conductivity [272–274]. Moreover, consistent with our hypothesis of maxi-anion channel-mediated ATP release, ATP release was diminished in VDAC-1 knock-out mice and augmented in cells over-expressing pl-VDAC-1 [275]. Thus, it is tempting to conclude that the above hypothesis that the maxi-anion channel and pl-VDAC are identical is valid. However, such similarities could be circumstantial. Closer inspection of channel properties reveals some crucial differences. For instance, the single-channel conductance of the maxi-anion channel saturates at 617 pS ($K_d = 77$ mM) and 640 pS ($K_d = 112$ mM) with increases in the chloride concentration in skeletal muscle ‘sarcoballs’ [214] and L6 myoblasts [134], respectively. In contrast, the VDAC single-channel conductance may reach levels of over 10 nS at high salt concentrations without any saturation [257], suggesting a fundamentally different mechanism of ionic transport in these two pores.

Plasmalemmal VDAC is not the only candidate for the maxi-anion channel. Suzuki and Mizuno [276] suggested that maxi-anion channels might be related to the human analog of the *tweety* gene found in the *flightless* locus of *Drosophila*. We believe that more thorough testing of the candidates is needed. It will be necessary to reproduce the maxi-anion channel phenotype by heterologous expression of the genes encoding the channel in cells lacking maxi-

anion channels and to demonstrate that the permeation properties of the expressed channels are sensitive to site-directed mutagenesis.

The hemichannel protein Cx can be excluded from being a candidate for the ATP-conductive maxi-anion channel, because of the following four lines of evidence: (1) Cx43-hemichannels are not selective to anions but rather, are cation-selective [23, 277]; (2) Cx-hemichannels were sensitive to extracellular Ca^{2+} , but maxi-anion channel activity was consistently observed in C127 cells in the presence of extracellular Ca^{2+} (2 mM) [115]; (3) the voltage dependence of open probability which is typical of Cx45-hemichannels [278] is distinct from the bell-shaped voltage dependence of open probability of maxi-anion channels; (4) octanol, a known blocker of gap junction hemichannels, had no effect on maxi-anion channels in cardiomyocytes [132] or C127 cells at a concentration of 1 mM (Figure 3).

The mitochondrial adenine nucleotide translocase (ANT), or ADP/ATP carrier (AAC), which mediates ATP/ADP exchange at the inner mitochondrial membrane, was suggested to reside in the plasma membrane in neurons [279] and was shown to form a large-conductance (300–600 pS) channel when reconstituted in giant liposomes [280, 281]. However, this mitochondrial permeability transition pore-like large-conductance channel was cation selective ($P_K/P_{Cl} = 4.3$) [280]. Also, as shown in Figure 3, both atractyloside and bongkreikic acid, potent AAC blockers, failed to affect the activity of the maxi-anion channel in C127 cells either from intracellular or from extracellular side. These results may suggest that

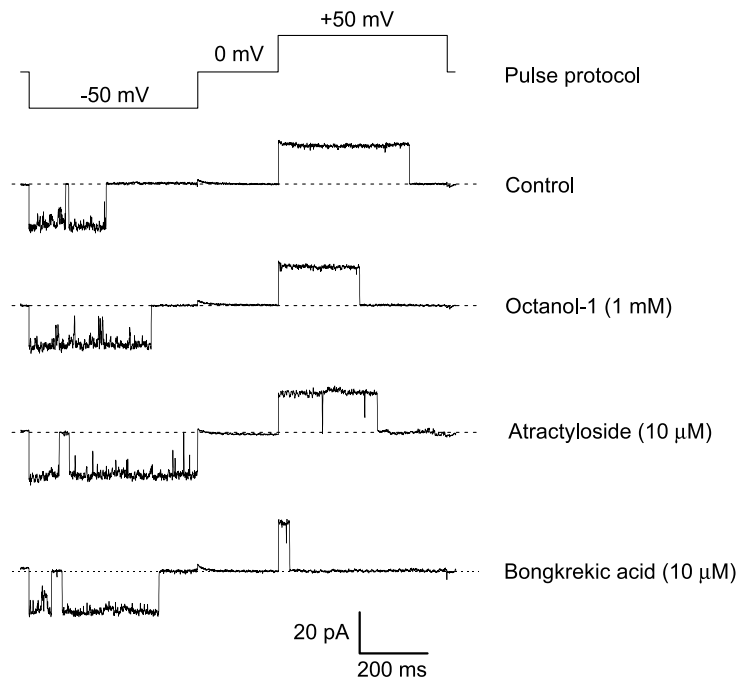


Figure 3. Lack of effect on maxi-anion channel activity of octanol, atractyloside and bongkreikic acid added to the intracellular solution in inside-out patches excised from C127 cells. Dashed lines indicate the zero current level. The test pulse protocol applied is shown on the top. Traces are representative of 4, 3 and 3 experiments with octanol-1 (1 mM), atractyloside (10 μM) and bongkreikic acid (10 μM), respectively. Experimental conditions were same as in Sabirov et al. [115]. Maxi-anion channel activity was also not affected by the addition of these drugs to the extracellular (pipette) solution (data not shown, $n = 2-4$).

ANT/AAC is not the molecule corresponding to the maxi-anion channel. Detail heterologous expression and/or reconstitution studies supplemented with site-directed mutagenesis are necessary in order to rule out this possibility with more confidence.

Concluding remarks and perspectives

In a wide variety of cell types, it has been shown that ATP release is induced in response to several kinds of stress, including hypoxia, ischemia, osmotic swelling and mechanical stimulation (Figure 4). The regulated release of ATP plays an essential role in autocrine and/or paracrine cell-to-cell signalling (Figure 1). There has been an upsurge of interest in non-lytic and non-exocytic mechanisms of regulated ATP release. Since ATP cannot be transported across lipid bilayers by simple diffusion, some channel or transporter (carrier or pump) may be involved in its transmembrane transport. The main candidates suggested up until now include ABC transporters, the ADP/ATP exchange carrier, gap junction hemichannels, and anion channels such as CFTR, VSOR and maxi-anion channels (4). Recently, much evidence has been accumulated for ATP release via maxi-anion channels. Taken together, existing evidence points to the maxi-anion channels as prime candidates for ATP release channels in mammalian cell plasma membranes.

Ion channels are traditionally considered to be selective permeation pathways for small inorganic ions. This view is perhaps true for Na^+ , K^+ and Ca^{2+} -selective channels of excitable cells where even a small leak of an unwanted species would be detrimental to cell function. Chloride channels are more promiscuous, allowing passage of not only chloride ions, but also of some other negatively-charged substances including intracellular metabolites. The experimental studies surveyed in the present review indicate that even species as bulky as ATP^{4-} and MgATP^{2-} can be

transported through anion channels, suggesting that they serve as signalling gates for cell-to-cell purinergic signalling. The structural basis for channel-mediated ATP release remains obscure. Recent advances in crystallographic methods may provide us with a biophysically solid framework for understanding channel-mediated ATP release. We are close to such a level of understanding with the CFTR protein [45], but not with other putative ATP-releasing channels such as VSOR and the maxi-anion channel.

Could other types of chloride channels be involved in purinergic signalling? To date, a number of different chloride channels have been identified in a variety of cell types (see for review [282]). For instance, Ca^{2+} -dependent Cl^- channels (CaCC) are activated in pancreatic cells and colonic epithelial cells whenever the intracellular Ca^{2+} concentration rises, and the resulting Cl^- transport drives fluid movement. The outwardly rectifying Cl^- channels (ORCC), in concert with CFTR, perform a similar function in airway epithelia.

Voltage-dependent Cl^- channels of the CIC family have different physiological roles depending on their type and localization. CIC-1 is known to be responsible for the Cl^- conductance which sets the resting potential of skeletal muscle and regulates muscle contractility; CIC-2 is activated upon hyperpolarization and cell swelling. Most of the other members of this group are intracellular channels that provide anionic conductances coupled to the operation of vesicular H^+ -ATPases. Transport of ATP as yet another function of these channels has not been considered so far. The three-dimensional structure of a bacterial CIC channel was established recently by X-ray crystallography [283]. It revealed a rather narrow path for Cl^- without much room for the possible transport of large organic anions such as ATP^{4-} . However, given the striking increase of studies suggesting capability of CFTR to conduct large anions when they are present on the intracellular side and where ATP hydrolysis can occur [44], one may suppose that, in certain physiological

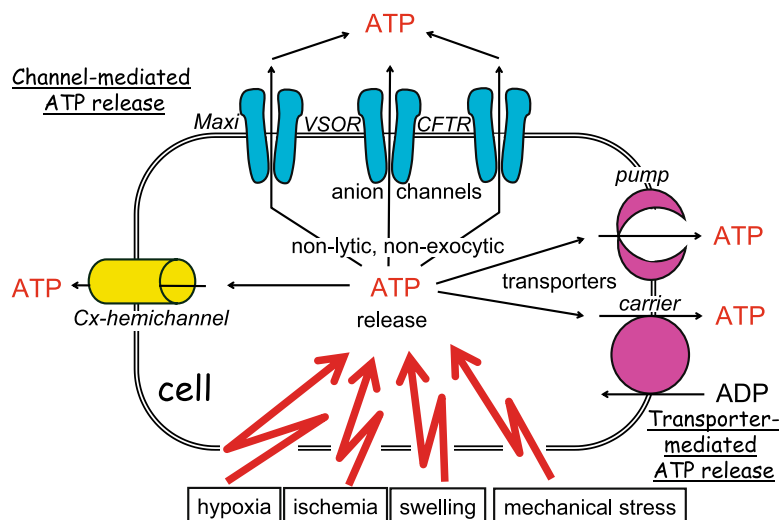


Figure 4. Putative non-exocytic pathways of regulated ATP release in response to hypoxia, ischemia, osmotic cell swelling or mechanical stimulation (see text for details).

conditions, even normally ATP-impermeable Cl⁻ channels may translocate the nucleotide at physiologically relevant rates. Further detailed investigations may reveal new participants in the fundamental process of regulated adenosine-5'-triphosphate release.

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