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COMMENTARY

ION CHANNELS AS SENSORS OF CELLULAR ENERGY

MECHANISMS FOR MODULATION BY MAGNESIUM AND NUCLEOTIDES

BRIAN O'ROURKE*
Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A.

The profound influence of nucleotides on cell physiology has been studied intensely since the elucidation of the central role of ATP as the intracellular currency of energy exchange more than half a century ago [1]. Even before this discovery, magnesium was recognized as an important activator of alkaline phosphatase and has since been implicated in nearly all cellular functions including growth, energy metabolism, membrane excitability and muscle contraction [Refs. 2–6]. Magnesium and nucleotide effects are inextricably linked as a result of the high concentrations of each in the cytoplasm of the cell and the predilection for complex formation between the two species. Assessment of the role of each molecular species in a given biological process is complicated not only by the innumerable systems affected, but also by the multiple modes of action of free magnesium (Mg^{2+}), free nucleotide, and the magnesium–nucleotide complex. The overriding physiological effect is thus the result of the interplay between each individual action in an additive, cooperative or antagonistic manner. The degree of complexity in addressing the role of magnesium and nucleotides on cell function precludes a detailed review in a single article. Nonetheless, a general overview of the various mechanisms by which Mg^{2+} and Mg–nucleotides act serves as a guideline for experimental design and interpretation of results. This is one objective of the present article. The primary and more specific objective of this article, however, is to bring to light an emerging literature on the direct effects of magnesium and nucleotides on ion channel activity, with special emphasis on recent interesting and novel mechanisms suggesting that ion channels act as sensors of intracellular energy levels. For some important topics not discussed in detail, the reader is referred to several other articles for related information on the effects of Mg^{2+} on ion channels [7, 8], the regulation of cellular magnesium transport [9–11], and the activation of G-proteins by Mg^{2+} and MgGTP [12, 13].

* Correspondence: Brian O'Rourke, Ph.D., 844 Ross Building, The Johns Hopkins University School of Medicine, 720 Rutland Ave., Baltimore, MD 21205. Tel. (410) 614-0027; FAX (410) 955-7953.

How is magnesium unique?

Despite a longstanding recognition of the requirement for magnesium in almost all cellular processes, the abundance of magnesium and its relative insensitivity to perturbation on a rapid time scale have previously relegated magnesium to a minor role in considerations of intracellular signalling. This view has changed in recent years, as the techniques for measuring Mg^{2+} have improved and pathological and hormonally induced changes in intracellular Mg^{2+} have been detected [14, 15]. The total concentration of magnesium inside the cell (approximately 17 mM [9]) makes it the most abundant intracellular divalent cation, but it is largely bound to intracellular buffers (including ATP), leaving only 0.5 to 3 mM free Mg^{2+} in the cytoplasm [7, 10]. The avid binding of magnesium to other molecules within the cell is governed by its large charge-to-size ratio (Mg^{2+} has a crystal radius of 0.65 Å) compared with the other physiologically relevant cations (Na^+ 0.95 Å; K^+ 1.33 Å; Ca^{2+} 0.99 Å; Pauling radii from [16]). This property confers upon Mg^{2+} the ability to polarize surrounding ligands without becoming polarized itself, leading to the formation of stable complexes with highly electronegative groups such as phosphates, carboxylates, and amines. In many chemical reactions, this interaction is strong enough to enable magnesium to decrease the unfavorable entropic cost of forming intermediate complexes, resulting in a lower free energy of activation for the reaction [17]. It is also important to note that the interaction is not so strong that it interferes with charge transfer, e.g. between the γ phosphate of ATP and a substrate (magnesium binds to ATP with an apparent affinity on the orde of 30–100 μM [18, 19]). The polarizing ability of Mg^{2+} also contributes to a large hydration energy for Mg^{2+} compared with other ions, accounting for the impermeability of Mg^{2+} through most ion channels [16].

Mechanistic models

The multitude of mechanisms by which magnesium exerts an influence on cellular processes can be broadly divided into two classes: (1) those depending

upon Mg^{2+} and (2) those depending on Mg–nucleotide complexes. Effects of free Mg^{2+} can then be further subdivided into the following schemes:

(1a) Mg^{2+} screening the negative surface charge on membranes,
(1b) Mg^{2+} as a blocking particle in ion channels,
(1c) Mg^{2+} stabilization of the structure of intracellular polymers (e.g. DNA) or multimeric enzyme complexes,
(1d) Mg^{2+} as an allosteric regulator of proteins via metal–protein binding sites,
(1e) Mg^{2+} interaction with the substrate or product of an enzyme-catalyzed reaction at the active site.

Effects involving Mg–nucleotide complexes can also be separated into two mechanistic models:

(2a) Mg–nucleotides in a hydrolytic role participating in chemical reactions either acting as a substrate for the reaction or providing the energy required to induce a conformational change in the enzyme (this category includes many different catalytic paradigms),
(2b) Mg–nucleotides altering protein function by binding to allosteric sites on the protein without a requirement for hydrolysis of the nucleotide.

It is also important to note that the effects of Mg^{2+} and Mg–nucleotides may differ depending on whether the site of action is extracellular or intracellular.

Finally, one must consider the effects of free nucleotides on protein activity. In certain instances, allosteric control is achieved without a strict requirement for magnesium. Because there are several examples of ion channels regulated in this manner, this distinction is quite important; however, due to the predominant concentrations of Mg–nucleotides in the physiological milieu and a lack of knowledge about the actual regulator *in vivo*, these cases will be discussed along with the non-hydrolytic Mg–nucleotide effects (mechanism 2b) described above.

Effects of Mg^{2+} *on protein activity*

Perhaps the most general influence of Mg^{2+} and other divalents on the activity of proteins in membranes is their ability to screen fixed negative charges on the inner and outer aspects of the bilayer (mechanism 1a; see Refs. 16 and 20 for discussion). The functional effects on voltage-dependent processes will differ depending on the site of interaction. Charge screening results in perturbation of the electrical field surrounding the voltage-sensing gates of ion channels causing a shift in activation and inactivation processes in the hyperpolarizing direction when intracellular Mg^{2+} increases and in the depolarizing direction when extracellular Mg^{2+} increases. In the latter case, this means that for a given depolarizing stimulus in the range of channel activation, fewer Na^+ and Ca^{2+} channels will open, thus raising the threshold for regenerative excitation of the cell. Surface charge screening by divalents is relatively non-selective (Ca^{2+} is only slightly more effective than Mg^{2+} [21]) and requires a large change in concentration (a 10-fold change in Mg^{2+} produces a 10–15 mV shift in voltage-dependent properties).

A second influence of Mg^{2+} on both extracellular and intracellular sites is the ability of Mg^{2+} to enter the pore of an ion channel (mechanism 1b). Mg^{2+}-dependent block of ion flux can occur by interaction with high affinity sites (with a K_D in the range of 10^{-6} to 10^{-5} M) on the extracellular side of the pore, as in the case of the *N*-methyl D-aspartate (NMDA)* receptor/channel [22], or on the cytoplasmic side of a channel, as for the inwardly-rectifying potassium channels I_{K1} [23, 24] and $I_{K,Ach}$ [25]. Mg^{2+}-dependent block can also occur through interaction with low affinity sites (K_D values on the order of 10^{-3} M) as exemplified by the blocking sites in voltage-dependent Na^+ [26, 27], Ca^{2+} channels [28, 29], and ATP-sensitive K^+ channels [30]. Mechanisms 1a and 1b are possible explanations for the observed effects of extracellular Mg^{2+} to reduced neurotransmitter release ("membrane stabilizing" effect) [6, 31, 32], decrease vascular tone in pathological states [33], and decrease the incidence of arrhythmia subsequent to myocardial infarction [34, 35].

Mg^{2+} plays an important role in stabilizing nucleic acid polymers and protein complexes (mechanism 1c). Specific examples include a direct effect of Mg^{2+} to increase the helix-coil transition temperature (melting temperature) of DNA up to a Mg^{2+}:DNA ratio of one, the stabilization of tRNAs and ribosomal particles by Mg^{2+}, and the structural stabilization of enolase [see Ref. 2]. The possible effects of Mg^{2+} on the structure of ion channels have not been investigated. Channels are commonly multimeric complexes that may be associated with other cytosolic proteins and cytoskeletal components. With regard to the latter, recent evidence indicates that compounds which stabilize cytoskeletal components reduce the rate of loss of activity of L-type Ca^{2+} channels in excised patches in a MgATP-dependent manner [36]. Conversely, cytoskeletal destabilizers accelerated the loss of channel activity. These results suggest that Ca^{2+} channel function depends not only on the assembly of channel subunits in the membrane, but also on the maintenance of a higher order structural complex in the intact cell.

The effect of Mg^{2+} on structure and function may also overlap with and extend to allosteric sites for Mg^{2+} on proteins (mechanism 1d). In addition to the many enzymes involved in the control of energy metabolism (e.g. hexokinase and pyruvate kinase) and growth (e.g. DNA and RNA polymerases) that have a demonstrable requirement for Mg^{2+} in the physiological range, key proteins involved in the regulation of ion channels have been shown to be allosterically regulated by intracellular Mg^{2+} [7]. One such multicomponent system is the β-adrenergic receptor/G-protein/adenylate cyclase cascade. Mg^{2+} interacts with low affinity metal binding sites on both the G-protein and adenylate cyclase to enhance the basal activity of the system. This requirement for Mg^{2+} is reduced when the receptor is occupied by an agonist, apparently by lowering

* Abbreviations: NMDA, *N*-methyl D-aspartate; SR, sarcoplasmic reticulum; and CFTR, cystic fibrosis transmembrane conductance regulator.

the K_m for Mg^{2+} activation [13]. Ion channels may also be allosterically regulated by Mg^{2+}, although there is little specific information about the nature or location of the Mg^{2+} binding site. In addition to the open channel blocking mechanism already discussed for inwardly rectifying potassium channels, Mg^{2+} influences channel gating. For I_{K1}, Mg^{2+} induces the appearance of subconductances approximately one-third of the full conductance amplitude and prolongs the open time of the channel [23], suggesting that Mg^{2+} may alter the conformation of the channel in addition to blocking the pore.

The open channel blocking effect of Mg^{2+} on L-type Ca^{2+} currents is relatively weak (the K_D for extracellular block is ~10 mM [28]); however, effects of Mg^{2+} on channel inactivation have been observed. In frog cardiomyocytes internally perfused with various concentrations of Mg^{2+} (with MgATP held constant), Hartzell and White [37] observed an inverse relationship between Ca^{2+} current amplitude and internal Mg^{2+}. Furthermore, when the L-type currents were carried by Ba^{2+} rather than Ca^{2+}, so that inactivation of the current was primarily voltage dependent, raising internal Mg^{2+} greatly enhanced the rate of decay of Ba^{2+} current, implying that Mg^{2+} was interacting with the inactivation gate of the channels. The effects of Mg^{2+} on current amplitude were larger when the channels were previously phosphorylated, suggesting that the affinity of the Mg^{2+} binding site was altered by phosphorylation [38]. With regard to this possibility, phosphorylation may alter the efficacy of Mg^{2+} block of NMDA channels [39], although the phosphorylation site is likely to be on the side of the channel opposite the blocking site. In guinea pig cardiomyocytes, millimolar concentrations of Mg^{2+} reportedly reduce the amplitude of L-type Ca^{2+} or Ba^{2+} currents; however, no increase in the rate of current inactivation was observed [40].

An inverse relationship between intracellular Mg^{2+} and the delayed rectifier potassium current amplitude has been noted by Duchatelle-Gourdon *et al.* [41]. This effect was suggested to result from stimulation of intracellular phosphatases. Most protein phosphatases (types 1, 2A, 2B, and 2C) require a divalent cation for activation (nomenclature from Ref. 42). For phosphatases 1, 2A, and 2B, Mn^{2+} is usually more effective than Mg^{2+} in activating the enzyme; in contrast, the activation of type 2C is highly specific for Mg^{2+}. Maximal activation of type 2C occurs at a Mg^{2+} concentration of 5 mM [43, 44].

The last mechanistic scheme involving Mg^{2+} (mechanism 1e) is a role for Mg^{2+} at catalytic sites. Consideration of the mechanism also encompasses reactions utilizing MgATP as a substrate (mechanism 2a). The numerous ways in which Mg^{2+} participates in the catalysis of a chemical reaction can be broadly divided into three molecular arrangements, as described by Mildvan [45]: (i) a metal bridge between substrate and enzyme occurring in either a simple (S-Mg-E) or cyclic ($E < \begin{matrix} Mg \\ | \\ S \end{matrix}$) conformation, (ii) a substrate bridge between the enzyme and magnesium (E-S-Mg), or (iii) magnesium binding to the enzyme without interacting with the substrate (M-E-S). A specific and detailed role for Mg^{2+} at the active site has been worked out for a number of enzymes [45]. A reduction in the entropic cost of substrate–enzyme association, the ability to act as an acid catalyst, and a role in charge transfer may all contribute to the general functionality of Mg^{2+} at the active site. Magnesium often catalyzes a reaction in a manner similar to that of a permanent functional group of an enzyme, as in the case of the ribozyme reaction of *Tetrahymena* [46]. It remains to be determined whether these types of mechanistic models will provide a useful framework for interpreting the unusual effects of Mg^{2+} on ion channel function that have been mentioned above.

Mg*ATP as a substrate for phosphoryl transfer reactions*

To date, all known phosphoryl transfer reactions using ATP as a substrate require the MgATP complex at the active site (mechanism 2a). In the past twenty years, modulation of ion channel function by phosphorylation has emerged as a predominant regulatory mechanism. Examples of channels subject to phosphorylation-dependent modulation include L-type Ca^{2+} channels, Na^+ channels, several types of K^+ channel, Cl^- channels, and NMDA channels among others. The effect of phosphorylation on the cardiac L-type Ca^{2+} channel has been the subject of numerous studies [47]. Activation of the cyclic AMP-dependent phosphorylation cascade by β-adrenergic agonists (the primary pathway for positive inotropy in the heart) leads to an increase in availability of channel opening during a depolarizing stimulus [48]. In addition, phosphorylation increases the appearance of long lasting channel openings [49]. The overall effect of β-adrenergic receptor stimulation on cardiac muscle is a large increase in whole-cell Ca^{2+} current, leading to an enhanced intracellular Ca^{2+} release from the sarcoplasmic reticulum, and an increase in developed force. The requirement for MgATP in this process in the intact cell is unknown; however, *in vitro* estimates of the K_m for MgATP activation of both adenylate cyclase and the cyclic AMP-dependent protein kinase are between 3 and 20 μM [50–52]. Thus, under all conditions other than severe metabolic inhibition there should be ample MgATP available for phosphorylation. This conclusion is contradictory to the notion that phosphorylation is facilitated when intracellular MgATP is increased from low to high millimolar concentrations. One example is the widely observed effect of MgATP on Ca^{2+} current rundown, a phenomenon in which currents decline with time after gaining access to the cytoplasmic compartment of the cell. While other factors mediating this process have been implicated (e.g. Ca^{2+}-dependent protease activity [53, 54] and voltage-dependent processes [55]), a decrease in channel phosphorylation has been proposed to explain the loss of channel activity with time [56]. Similarly, a phosphorylation hypothesis has been postulated to explain the effects of ATP on Ca^{2+} currents in internally perfused guinea pig cardiomyocytes, even though the K_m for the ATP effect was several millimolar [57]. These findings are also at odds with the observation that in the absence of hormonal stimulation, only 20%

of the basal Ca^{2+} current amplitude can be attributed to phosphorylated channels [58]. Several possible explanations for these discrepancies can be considered. First, the actual concentration of MgATP available for kinase activation at the plasma membrane may be lower than in the bulk intracellular pipet solution as a result of consumption by other energy-requiring reactions or diffusion limitations within the cell. This would result in overestimation of the K_m for phosphorylation *in vivo*. This first mechanism seems unlikely, since β-adrenergic responses are still robust when Mg^{2+} is highly buffered and MgATP levels are presumed to be quite low [40, 59]. Second, other factors like the ADP, P_i or Mg^{2+} concentrations may alter the apparent K_m for MgATP activation of phosphorylation. In this regard, changes in the energy charge [60] (equal to $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$), or phosphorylation potential [61] (equal to $[ATP]/[ADP][P_i]$) of the cell have been proposed as control mechanisms for many enzymes. Furthermore, the Mg^{2+} buffering capacity of the cell may change when ATP is added internally (a factor not taken into account in Ref. 57), leading to direct effects of Mg^{2+} on the channels in addition to indirect effects on the phosphorylation cascade. Finally, the Ca^{2+} channels could be modulated in a novel way through a pathway with a high K_m for MgATP. This possibility will be explored in a later section.

Nucleotide effects on G-protein-dependent activation of ion channels

Modulation of ion channels by G-proteins through a direct membrane delimited pathway has been described for acetylcholine-gated K^+ channels [62], neuronal Ca^{2+} [63] and K^+ channels [64], and cardiac L-type Ca^{2+} channels [65, 66]. Mg^{2+} and MgGTP are critically required for this mechanism and the details of this process have been reviewed elsewhere [7, 12]. Flash photolysis of intracellular caged guanine nucleotides (including non-hydrolyzable analogs) in the presence of Mg^{2+} and ATP increases the amplitude of L-type Ca^{2+} currents in guinea pig cardiomyocytes [67] and decreases N-type Ca^{2+} currents in neurones [68], providing a clear example of specific G-protein activation through occupation of a nucleotide-binding site not requiring hydrolysis. Recent evidence indicates that MgATP may also be an important cofactor in the activation of G-proteins. In addition to the ability of ATP to activate G-proteins indirectly by being converted to GTP via nucleoside diphosphate kinase [69], adenine nucleotides (including non-hydrolyzable analogs) can modulate cooperative interactions among G-proteins [70]. Furthermore, MgATP, acting by a non-hydrolytic mechanism, may be required to preserve G-protein-mediated modulation of N-type Ca^{2+} channels by norepinephrine in sympathetic neurons [71].

Direct effects of ATP and MgATP on proteins

Specific binding sites for nucleotides exist on the extracellular surface of cells as well as on intracellular proteins (mechanism 2b). Purinergic receptors for nucleotides released from nerve terminals or nucleosides generated by cellular metabolism have been classified according to their relative selectivity for adenosine over ATP and by the functional effects initiated by receptor activation. P_1 receptors, further divided into A_1 and A_2 subtypes, have a higher affinity for adenosine. P_2 receptors, which prefer ATP, have been subclassified into P_{2X}, P_{2Y}, P_{2Z} and P_{2T} subtypes [72]. While the likely physiological mediator for P_2 receptors is MgATP, a third category of purinergic receptor (P_3) has been proposed recently based on its requirement for MgATP. Functionally distinct responses to extracellular ATP and MgATP were observed in cardiac cells [73].

Similarly, distinctions can be made when considering the effects of nucleotides on intracellular nucleotide-binding sites. All known hydrolytic sites for nucleotides require the Mg–nucleotide complex. However, the oft-cited converse reasoning, that a magnesium requirement for nucleotide action implies a hydrolytic role, is not true. One clear counter example is the extensively studied ATP synthase of the mitochondrial membrane. It possesses at least six MgATP binding sites, three of which are catalytic and three of which are allosteric regulatory sites crucial for activity. MgATP induces a protein conformation different from that of ATP alone [74] and MgAMP-PNP stabilizes the hexameric form of the thermophilic F1 synthase [75]. Other examples of enzymes regulated by the non-hydrolytic binding of MgATP include phosphoprotein phosphatase type 1 [42], the Rep Helicase of *Escherichia coli* [76], AMP-nucleosidase [77], and phosphofructokinase [78].

For both extracellular and intracellular nucleotide binding sites, non-hydrolytic effects of ATP are usually demonstrated by replacement with analogs having the oxygen of the phosphoanhydride bonds of ATP substituted with a nitrogen or carbon atom (e.g. AMP-PNP, AMP-PCP, AMP-CPP) [79]. Using this approach, several types of ion channel have been shown to be modulated by ATP via non-hydrolytic binding of the nucleotide, as summarized in Table 1. The first was the ATP-sensitive potassium channel initially described in cardiac myocytes [80–82]. Early experiments showed that ATP or non-hydrolyzable ATP analogs could inhibit channel opening in excised patches [83]. The inhibitory effect of ATP is observed in the absence of magnesium, although a secondary requirement for MgATP in the preservation of channel activity by phosphorylation has been suggested [84]. Subsequent studies have shown that nucleotide regulation of the channel depends not only on the number of phosphates on the nucleotide (ATP, ADP, AMP), but also on whether magnesium is present [85]. MgADP and other nucleoside diphosphates are capable of opening K_{ATP} channels in excised patches after channel run-down [86]. In addition, MgADP can shift the ATP sensitivity of the channel in excised patches. This effect may be important *in vivo* when ADP levels rise as a consequence of metabolic inhibition [87].

The Ca^{2+} release channel (ryanodine receptor) of cardiac or skeletal muscle sarcoplasmic reticulum (SR) has been shown to be modulated by ATP via a non-hydrolytic mechanism. The channel is activated

Table 1. Summary of the direct effects of Mg^{2+} and MgATP on ion channels that may be involved in sensing cellular energy levels

Channel	Mg_i^{2+} effect	$MgATP_i$ effect
K_{ATP}	Mg^{2+} blocks outward current	ATP or AMP-PxP (non-hydrolyzable) inhibits channel opening Mg–nucleotide not required for ATP inhibition, but required for MgADP activation
SR Ca^{2+} release	Mg^{2+} inhibits opening	ATP or AMP-PxP increases channel opening by Ca^{2+} Mg–nucleotide not required
IP_3-gated Ca^{2+} release	Mg^{2+} decreases IP_3 binding	MgATP or MgAMP-PxP increases channel opening by IP_3, but inhibits IP_3 binding at very high concentrations Mg–Nucleotide required for photoaffinity labeling
Cl^- (CFTR)	Unknown	MgATP directly activates channel after prior phosphorylation MgAMP-PxP activates in an intact preparation, but not in excised patches
L-type Ca^{2+}	Mg^{2+} inactivates channel	MgATP or MgAMP-PxP increases channel activity

by Ca^{2+}, caffeine, or adenine nucleotides and inhibited by Mg^{2+}, H^+, calmodulin, and ruthenium red. In canine SR vesicles, optimal Ca^{2+} efflux was observed in the presence of 5 mM AMP-PCP [88]. In this case, Mg^{2+} antagonized the positive effects of the free nucleotide by shifting the Ca^{2+}-dependence of channel opening to a higher K_D. The inositol 1,4,5-trisphosphate (IP_3) receptor, responsible for second messenger-mediated Ca^{2+} release from the endoplasmic reticulum, may also be modulated by cytosolic ATP. Ehrlich and Watras [89] reported that the addition of 0.1 mM AMP-PCP doubled the open probability of the aortic IP_3-gated channel in the presence of IP_3 but not in its absence.

The K_{ATP} and SR Ca^{2+} release channels are examples of ion channels modulated by nucleotide-binding sites which prefer free nucleotide triphosphates over Mg–nucleotides. Recently, a second paradigm for nucleotide-dependent modulation of ion channels has become apparent. The cystic fibrosis transmembrane conductance regulator (CFTR) is a protein structurally homologous to a family of transporter proteins referred to as ATP-binding cassette, or ABC, proteins [90]. Uniquely, the CFTR protein is a chloride channel subject to direct regulation by MgATP in addition to being controlled by phosphorylation. Studies on excised patches containing expressed CFTR channels show that following a brief exposure and subsequent withdrawal of phosphorylating conditions, MgATP facilitates channel opening directly [91]. CFTR channel activation strictly requires the Mg–nucleotide complex, and it can also be observed in the absence of prior phosphorylation when mutant proteins lacking an intracellular regulatory (phosphorylation) domain are expressed. The direct effect of MgATP has a high K_m (approximately 270 μM) in comparison to phosphorylation and is relatively non-specific in that other nucleoside triphosphates can substitute, a result which is also distinct from phosphorylation. Still to be resolved is the question of whether or not nucleotide hydrolysis is required for channel opening. The available evidence thus far indicates that non-hydrolyzable ATP analogs cannot open CFTR Cl^- channels in excised patches [91]. On the other hand, the observation of non-hydrolytic activation of transepithelial Cl^- conductance by MgAMP-PNP in permeabilized microperfused sweat ducts has led to the proposal that MgATP hydrolysis may not be required for the direct effect in intact tissue [92]. One explanation for the differing results may be that hydrolysis of MgATP may be required for the low K_m phosphorylation process, but not for the high K_m direct activation pathway. This points out the fundamental difficulty of examining the role of non-hydrolytic ATP binding in mediating a functional effect—if phosphorylation is required to make an allosteric site available, the inhibition of phosphorylation by a non-hydrolyzable ATP analog will mask the direct effects of the Mg–nucleotide.

Direct effects of Mg–*nucleotides on cardiac L-type* Ca^{2+} *channels*

Results of experiments in our laboratory have revealed that Mg–nucleotides can directly increase the activity of L-type Ca^{2+} channels in whole-cell

patch clamped guinea pig cardiomyocytes [59]. Utilizing DM-nitrophen as photolabile caged-Mg^{2+}, rapid increases in intracellular MgATP were initiated by flash photolysis while minimizing the influence of free Mg^{2+} on channel activity. Raising MgATP from less than 10 μM to several hundred micromolar resulted in a pronounced and specific increase in whole-cell Ca^{2+} currents occurring with a time constant of approximately 30 sec. A similar effect was observed when intracellular MgATP was increased rapidly by flash photolysis of caged-ATP in the presence of a physiological Mg^{2+} concentration. Interestingly, the response was preserved when phosphorylation was blocked by substitution of non-hydrolyzable ATP analogs or by inclusion of specific peptide inhibitors of protein kinases. Recent experiments have shown that enhancement of peak inward Ca^{2+} current can also be observed when MgAMP-PNP is increased by intracellular perfusion (O'Rourke B and Marban E, unpublished observation). On the level of single channels, the Mg–nucleotide-induced up-regulation of whole-cell Ca^{2+} current appears to be due to an increase in the availability of the channel opening during a depolarization. Although further experimentation will be necessary to determine the exact intracellular site of action, the effect of Mg–nucleotides on the L-type Ca^{2+} channel represents a unique mechanism for ion channel regulation. It requires the Mg–nucleotide complex but does not depend upon ATP hydrolysis or phosphorylation.

Ion channels as MgATP sensors

An obvious question that arises in considering the direct effects of MgATP on ion channels or enzymes is what is the significance of this mechanism in the regulation of cellular function. This is particularly important for MgATP effects with K_m values much lower than the normal intracellular MgATP of 5–8 mM. For instance, one would not expect MgATP to be the limiting factor for phosphorylation since the K_m for this process is roughly 500- to 1000-fold lower than the MgATP concentration. With higher K_m effects, like the direct effect of MgATP on CFTR Cl^- channels (~0.3 mM), a regulatory role is more plausible. Unfortunately, there is currently little information about the actual K_m for direct nucleotide-dependent regulation in intact cells. Despite this limitation, there are several important examples of the physiological relevance of nucleotides as regulators of cell function. ATP-sensitive K^+ channels figure prominently in the control of insulin release from pancreatic islet β cells in response to glucose [82]. Exposure to glucose results in depolarization of the β cell membrane leading to the activation of Ca^{2+} influx, an increase in intracellular Ca^{2+} and insulin secretion. Depolarization results from the block of K_{ATP} channels responsible for determining the resting membrane potential. The observation that Ca^{2+} currents increase in response to glucose in the β cell suggests that a direct influence of glycolytic metabolites on Ca^{2+} channels may be significant in this process [93], perhaps providing evidence for a physiological role for the direct effects of ATP on L-type Ca^{2+} channels.

Discrepancies between the K_i for ATP block of ATP-sensitive K^+ channels in excised patches and the estimated ATP levels in intact cells have been rationalized by speculating that other factors like Mg^{2+}, ADP, P_i, lactate and pH may alter the affinity of the channel for ATP in the intact cell, as they do in isolated patches. Similarly, the phosphorylation potential has been proposed to be a determinant of contractile failure in the heart during hypoxia [94], and the PCr:P_i ratio may account for differences in contractility in the presence of glycolytic or mitochondrial energy substrates [95]. A second possibility is that the MgATP concentration at a given allosteric site is determined by the relative proximity of ATP-generating versus ATP-consuming processes. There is evidence for metabolic compartmentation of this sort for the regulation of K_{ATP} channels in cardiac cells [96] and for ion transport in smooth muscle [97].

Finally, it is important to note that, in the intact cell, an alteration in nucleotide levels will be accompanied by an inverse change in intracellular Mg^{2+}. For ion channels, the two species generally mediate opposing effects (e.g. activation by MgATP and inhibition by Mg^{2+} of L-type and SR Ca^{2+} channels), suggesting that a reduction in energy supply would initiate changes in ion channel activity as a result of the additive effects of increased Mg^{2+} and decreased MgATP. This strategy would increase the sensitivity of the cellular response to changing energy levels. It is interesting to note that a drop in energy levels leads to a reduction in Ca^{2+} entry and Ca^{2+} release, and an increase in the activity of ATP-sensitive K^+ channels, all serving to decrease the metabolically demanding task of Ca^{2+} handling. Direct sensing of the metabolic state by ion channels permits this to occur with maximal economy (ATP is not hydrolyzed), further preserving the cellular energy stores.

Molecular structure of nucleotide binding sites of ion channels

The molecular structure of an ATP binding site is highly conserved across a remarkably diverse number of enzymes. Two common nucleotide binding fold motifs have been described by Walker *et al.* [98], the first consisting of a glycine-rich amino acid sequence G-X-X-X-X-G-K(T)-X-X-X-X-X-X-I/V (motif A; X denotes a variable amino acid) preceded by a basic amino acid six positions before the first glycine and the second consisting of the sequence R/K-X-X-X-G-X-X-X-L-H-H-H-H-D (motif B; H denotes a hydrophobic amino acid). Of the ATP-regulated channels that have been discussed thus far, only the nucleotide-binding domains (NBD) of the CFTR Cl^- channel have clearly defined Walker A (GSTGAGKT in NBD1 and GRTGSGKST in NBD2) and Walker B (LYLLD in NBD1 and ILLLD in NBD2) consensus sites [99].

The amino acid sequence of the K_{ATP} channel is currently unknown; however, a novel K^+ channel (ROMK1) containing the Walker A motif (G-S-H-I-Y-G-K-L-L-K-T-T-I) has been cloned and expressed recently [100]. Interestingly, ROMK1 channels were activated rather than inhibited by 2.5 to 5 mM MgATP applied to the intracellular face of excised patches. Whether the MgATP effect involves

phosphorylation or is a direct nucleotide effect was not reported. Similar MgATP-dependent regulation of Ca^{2+}-activated K^+ channels of pulmonary airway smooth muscle has been described [101]. In the latter study, the effect was only poorly supported by MgAMP-PNP, suggesting that hydrolysis of the nucleotide is required. This interpretation is subject to the same pitfalls associated with the CFTR channel studies (see earlier discussion).

The mouse cerebellar IP_3-gated Ca^{2+} release channel contains a sequence similar to the Walker A motif, specifically G-L-G-L-L-G-L-Y [102]. In the same preparation, Maeda *et al.* [103] have photoaffinity labeled this regulatory site using 8-azido-ATP. The binding affinity was determined to be 17 μM and required the presence of Mg^{2+}. In the same study, MgATP was maximally effective in stimulating channel opening at a concentration of 0.6 mM. Ca^{2+}-release channels of the SR also contain at least two poorly matched Walker A-like motifs (G-X-G-X-X-G) [104]. Taken together, the available evidence suggests that the allosteric regulatory sites of channels proven to be modulated by ATP without a requirement for hydrolysis may contain nucleotide binding folds somewhat divergent from the accepted consensus sequence. The lysine of the G-X_4-G-K consensus sequence is believed to participate in chelating the phosphates of MgATP in the active sites of many proteins and is important in determining the rate of ATP hydrolysis in the F_1-ATPase [105], adenylate kinase [106], the yeast RAD3 protein [107], or the recA protein of *E. coli* [108]. However, this does not necessarily mean that it is critical for MgATP binding to a non-hydrolytic site. In fact, mutating this lysine to arginine in the catalytic domain of the recA protein or the RAD3 protein attenuates MgATP hydrolysis but does not alter the capacity of the mutant protein to bind MgATP. Furthermore, mutation of this lysine to a methionine in the second nucleotide binding domain of the CFTR Cl^- channel does not prevent MgATP-dependent opening of the phosphorylated channel. Taking these findings into consideration, it is interesting to note that the β-subunit of the L-type Ca^{2+} channel contains the conserved sequence G-P-S-L-K-G-Y, with an arginine in the correct position 6 amino acids upstream [109]. Furthermore, photoaffinity labeling of an ATP-binding site in an isolated dihydropyridine receptor fraction similar in size to the β-subunit has been reported [110]. The significance of these findings will require further experimentation but may be important in light of our evidence in support of allosteric regulation of Ca^{2+} channel activity by MgATP.

Conclusion

The accumulating evidence that both Mg^{2+} and Mg–nucleotides are important in the control of ion channels is not surprising in light of the wealth of evidence available for the regulation of intracellular enzymes. Indeed, ion channels can be thought of as enzymes mediating the translocation of ions across the impermeable phospholipid bilayer. As for other enzyme-catalyzed reactions, the modulation of ion channel activity by magnesium and nucleotides results from interactions among several mechanistic designs, including allosteric binding at metal and metal–nucleotide sites, as well as catalytic interactions at the active site.

The discovery of low affinity metal–nucleotide regulation coupled with evidence for low affinity effects of Mg^{2+} on membrane currents leads to the proposal that ion channels can detect alterations in the energy state of the cell by a direct sensing mechanism. In the β cells of the pancreas, this model has been put forth to explain the normal physiological coupling between substrate availability and insulin secretion. For other cell types, when the ratio of energy supply to demand is high, these direct effects are likely to be saturated, and regulation would largely be accomplished by the energy-consuming phosphorylation/dephosphorylation cycle. In pathological or substrate-deficient states, however, direct energy sensing by ion channels may be important in limiting cellular activity, thus preventing or delaying cell death.

Further investigation will be required to demonstrate that direct energy sensing by ion channels is a physiological control mechanism. In particular, the role of allosteric regulation of ion channels under normal or pathological conditions needs to be assessed, with the K_m for the effect determined in intact cells or tissues. On the molecular level, the structure of the nucleotide-binding sites of known nucleotide-sensitive channels must be defined more precisely in order to clarify the role of non-hydrolytic versus hydrolytic effects of Mg–nucleotides. In a similar vein, apart from some recent advances for the NMDA channel [111, 112], little is known about the structural requirements for a Mg^{2+} binding site. More information of this type may lead to the discovery of additional examples of energy-sensing ion channels.

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