Antagonistic Regulation of Native Ca\(^{2+}\)-and ATP-sensitive Cation Channels in Brain Capillaries by Nucleotides and Decavanadate

LÁSZLÓ CSANÁDY and VERA ADAM-VIZI

Department of Medical Biochemistry, Semmelweis University, and Neurochemical Group of the Hungarian Academy of Sciences, Budapest H-1444, Hungary

ABSTRACT Regulation by cytosolic nucleotides of Ca\(^{2+}\)- and ATP-sensitive nonselective cation channels (CA-NSCs) in rat brain capillary endothelial cells was studied in excised inside-out patches. Open probability (P\(_o\)) was suppressed by cytosolic nucleotides with apparent K\(_i\) values of 17, 9, and 2 \(\mu\)M for ATP, ADP, and AMP, as a consequence of high-affinity inhibition of channel opening rate and low-affinity stimulation of closing rate. Cytosolic [Ca\(^{2+}\)] and voltage affected inhibition of P\(_o\), but not of opening rate, by ATP, suggesting that the conformation of the nucleotide binding site is influenced only by the state of the channel gate, not by that of the Ca\(^{2+}\) and voltage sensors. ATP inhibition was unaltered by channel rundown. Nucleotide structure affected inhibitory potency that was little sensitive to base substitutions, but was greatly diminished by 3'-5' cyclization, removal of all phosphates, or complete omission of the base. In contrast, decavanadate potently (K\(_i\) = 90 nM) and robustly stimulated P\(_o\), and functionally competed with inhibitory nucleotides. From kinetic analyses we conclude that (a) ATP, ADP, and AMP bind to a common site; (b) inhibition by nucleotides occurs through simple reversible binding, as a consequence of tighter binding to the closed-channel relative to the open-channel conformation; (c) the conformation of the nucleotide binding site is not directly modulated by Ca\(^{2+}\) and voltage; (d) the differences in inhibitory potency of ATP, ADP, and AMP reflect their different affinities for the closed channel; and (e) though decavanadate is the only example found to date of a compound that stimulates P\(_o\) with high affinity even in the presence of millimolar nucleotides, apparently by competing for the nucleotide binding site, a comparable mechanism might allow CA-NSC channels to open in living cells despite physiological levels of nucleotides. Decavanadate now provides a valuable tool for studying native CA-NSC channels and for screening cloned channels.

KEY WORDS: microscopic reversibility • competitive binding • kinetic model • Trp channels • blood-brain-barrier

I N T R O D U C T I O N

Ca\(^{2+}\)- and ATP-sensitive nonselective cation channels (CA-NSCs) are present in various tissues, such as acinar (Maruyama and Petersen, 1982; Suzuki and Petersen, 1988) and ductal (Gray and Argent, 1990) cells of the exocrine pancreas, kidney (Paulais and Teulon, 1989), brain endothelium (Popp and Gögelein, 1992), brown adipose tissue (Koivisto et al., 2000), cultured rat cardiac cells (Colquhoun et al., 1981; Guinamard et al., 2002), vomeronasal sensory neurons (Liman, 2003), and astrocytes (Chen and Simard, 2001). For many other nonselective channel that have been found in native tissues (Marty et al., 1984; Gögelein and Capek, 1990), as well as in some endothelial (Kamouchi et al., 1999; Suh et al., 2002) and nonendothelial (Yellen, 1982; Sturgess et al., 1986; Jung et al., 1992) cell lines, inhibition by direct application of nucleotides in inside-out patches has not yet been studied. Although suggested to belong to the Trp family of ion channels (Petersen, 2002), the molecular identity of CA-NSC channels is not yet certain. These channels can be activated by Ca\(^{2+}\) from the cytosolic side (Ca\(^{2+}\)) in excised patches (Popp and Gögelein, 1992; Csanády and Adam-Vizi, 2003) or through stimulation of various cell-surface receptors that increase Ca\(^{2+}\) in intact cells (Maruyama and Petersen, 1982; Kamouchi et al., 1999; Koivisto et al., 2000). Differing sensitivity profiles for inhibition by micromolar cystolic nucleotides have been described for CA-NSC channels in astrocytes (Chen and Simard, 2001), in brown fat cells (Halonen and Nedergaard, 2002), and in the kidney (Paulais and Teulon, 1989), as well as in an insulinoma cell line (Sturgess et al., 1986), but the mechanism of this inhibition has not yet been elucidated.

In a previous study we have examined the regulation by Ca\(^{2+}\) and voltage of the CA-NSC channels present in rat brain capillary endothelial cells (Csanády and Adam-Vizi, 2003), and the present work was aimed at clarifying the mechanism of regulation by nucleotides. If micromolar concentrations of cytosolic nucleotides do indeed inhibit CA-NSC channels, it remains unclear.

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how they could become activated, as reported, in intact cells, in which nucleotide concentrations are generally in the millimolar range. ATP-sensitive K⁺ channels are also inhibited by binding of micromolar cytosolic ATP to the pore-forming subunit (Tucker and Ashcroft, 1998). In contrast, most CA-NSC channels are inhibited not only by cytosolic ATP, but also by ADP and AMP (Sturgess et al., 1986; Paulais and Teulon, 1989; Halonen and Nedergaard, 2002), raising the question of how the reported strong inhibition by nucleotides is counteracted in living cells to allow the channels to open.

Using inside-out patches excised from rat brain capillary endothelial cells we have examined in detail the effect on CA-NSC gating of cytosolic ATP, ADP, AMP, as well as a number of other nucleotides. Our results suggest that all of these nucleotides share a common binding site on the CA-NSC channel, and that inhibition occurs through simple reversible binding that leads to a stabilized closed state in which the nucleotide is more tightly bound. Furthermore, we find that decavanadate (DV) can counteract nucleotide inhibition, apparently by binding competitively to the same site. Robust stimulation of CA-NSC channels by decavanadate, even in the presence of millimolar nucleotide, is a consequence of its extremely high (nanomolar) affinity for the open-channel conformation. An as yet unknown putative activator molecule might act through a similar mechanism to permit CA-NSC function in intact cells.

**Materials and Methods**

**Preparation and Culture of Rat Brain Capillary Endothelial Cells**

Primary cultures of rat brain capillary endothelial cells were prepared as described (Domötör et al., 1998) by isolating microvessels from rat brain by gradient centrifugation. Collagenase/disparag-digested capillaries were seeded on glass coverslips pre-coated with a biological matrix, and nonendothelial cells were selectively killed by complement-mediated lysis. Recordings were made after 5–6 d of culture.

**Calibration of Decavanadate Concentrations**

DV stock solution was prepared by adjusting the pH of a 50 mM Na₂VO₄ solution to 2.0, at which pH DV is the major vanadium species (Gersmely et al., 1985). This stock was stored at +4°C and used within 24 h for dilution into the bath freshly (<90 min) before each experiment. The rate of decay of DV in our bath solution (pH = 7.1, T = 25°C) was measured by photometry (Varága et al., 1985) as peak absorption at ~390 nm (against a blank with identical composition, but boiled; see Fig. 4), and actual concentrations were calculated accordingly for each experiment.

**Excised-patch Recording**

Excised, inside-out patch recordings were performed at 25°C using an Axopatch 200B amplifier and Pclamp 8 software for data acquisition (Csányád and Adam-Vizi, 2005). Currents were filtered at 1 kHz with a 4-pole Bessel filter and digitally sampled at 5 kHz. Pipette (extracellular) solution contained (in mM) 140 NaCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, and 30 μM benzamil (pH = 7.4). The continuously flowing bath (intracellular) solution contained (in mM) 140 KCl, 2 MgCl₂, 10 HEPES (pH = 7.1). Because CA-NSC channels rapidly lose their sensitivity for Ca²⁺ after patch excision (Csányád and Adam-Vizi, 2003), channels were reversibly activated by adding 1 mM CaCl₂ to the bath solution (bars in Figs. 1 and 4–6), except in Fig. 2, A and C, where ATP-inhibition was tested in the presence of 10 mM and 100 μM Ca²⁺, respectively. Nucleotides (Sigma-Aldrich) were diluted into the bath from pH-adjusted aqueous stock solutions, and free Ca²⁺ and Mg²⁺ was kept constant by adding appropriate amounts of CaCl₂ and MgCl₂ (calculated with WinMaxx). Except for Fig. 2 B, pipette holding potential was +40 mV (Vₘ = −40 mV). CA-NSC channels, identified by their obligatory dependence on cytosolic Ca²⁺ for opening, as well as by their nucleotide sensitivity, were responsible for all unitary channel openings we could observe under the above conditions, since the other two possible sources of inward unitary Na⁺ current in our cells, stretch-activated channels and amiloride-sensitive cation channels, were eliminated by the absence of stretch and by pipette benzamil, respectively (Csányád and Adam-Vizi, 2003).

**Kinetic Analysis**

Kinetic analysis was done using custom-written software. Current records were digitally filtered with a 200-Hz Gaussian filter, slow baseline-drifts removed using a baseline-update algorithm sensitive to single-channel current transitions (Csányád et al., 2000). Baseline-subtracted currents were idealized using half-amplitude threshold crossing with a fixed dead time of 1 ms (Csányád, 2000).

Dwell-time histograms of single CA-NSC channels are complex even in the absence of nucleotides, due to complex regulation by Ca²⁺ and voltage (Csányád and Adam-Vizi, 2003). In the absence of a reliable scheme to describe Ca²⁺- and voltage-dependent gating on its own, mechanistic interpretation of nucleotide-mediated changes in the dwell-time distributions is not feasible at present. On the other hand, most of the data presented here were recorded from patches with multiple (1–10) active channels to obtain sufficient numbers of events even under strong inhibition by nucleotides. Therefore, kinetic parameters were not extracted from traditional fitting of dwell-time histograms. Instead, Pₜₜ opening and closing rates were obtained using the cycle-time method (Csányád and Adam-Vizi, 2003). Open probability (Pₒ) was calculated from the events lists as Σₜₜ(nₜₜ)/(NT), where nₜₜ and tₜₜ denote the number of open channels and the duration, respectively, of the kth event. N is the number of channels in the patch, and T = Σₜₜ is the total duration of the record. (The number of channels (N) was taken as the maximum number of simultaneously open channels observed immediately upon excision into 1 mM Ca²⁺ when Pₒ was close to unity.) Mean open time (m.o.t.) and mean closed time (m.c.t.), i.e., the arithmetic average of the open and closed dwell times of a single channel, were calculated as m.o.t. = Σₜₜ(nₜₜ)/(number of upward transitions), and m.c.t. = (1/m.o.t.)/(1/Pₒ) − 1. Average opening rate was then defined as 1/m.c.t., and closing rate as 1/m.o.t.

Experiments were performed by applying test concentrations of nucleotides or DV bracketed by control segments recorded in the absence of ligand. Normalized Pₒ, opening and closing rates (in Figs. 1, 2, 5, 6, and 8) were obtained by normalizing the kinetic parameter in the presence of ligand to the average of that parameter in the bracketing control segments.
Individual normalized dose response curves in Figs. 1, 2, and 5–7 were fit, using a nonlinear least-squares fitter (SigmaPlot 8.0), by Hill-functions of the form

\[ Y([S]) = \frac{Y(\infty)}{[S]^n + K_{1/2}^n} + Y(0) \frac{K_{1/2}^n}{[S]^n + K_{1/2}^n}, \]  

where \([S]\) denotes the concentration of ligand, \(K_{1/2}\) is the half-maximally effective ligand concentration, and \(n\) is the Hill-coefficient (and \(Y(0)\) was fixed to 1). Fitted \(K_{1/2}\) values are printed in the figure panels, and \(n\) values are given in the figure legends.

Ensemble fitting of all dose response curves (Fig. 8) by various models was done by custom-written software. To calculate the error function, the differences between the predicted parameters (see equations for each model in online supplemental material, sections 2.1–2.6) and the measured values of normalized \(P_o\) opening and closing rate were squared and summed for all conditions (18 dose response curves, several ligand concentrations each). (Errors for closing rates in the presence of nucleotides were weight by 1/2 to compensate for the fact that these curves were normalized to their minimum values; see Fig. 1 D.) Because at steady-state the ligand binding/unbinding steps influence channel opening rates, closing rates, and \(P_o\), only through their equilibrium constants (\(K_8\), not through the absolute values of on- and off-rate, only \(K_8\) were fitted for these transitions (e.g., “horizontal” transitions in the schemes shown in Fig. 1 E and Fig. 8). The error function was then minimized with respect to the kinetic constants involved, using a downhill simplex algorithm (Caceci and Cacheris, 1984).

**Single-channel Conductances**

All-points histograms of segments of current recordings, obtained at membrane potentials between −80 and +80 mV, were fitted with sums of Gaussians, and distances between adjacent peaks were plotted against voltage, to obtain current-voltage (i/V) plots. For the \(Na^+\)/K⁺ condition (\(Na^+\) in the pipette, K⁺ in the bath), with or without DV, channel conductances and reversal potentials were obtained from straight-line fits to each plot. For the \(Na^+\)/NMDG condition, with or without DV, i/V plots were fitted to the Goldmann-Hodgkin-Katz current equation written for two permeant ions,

\[ i_{\text{p}}(V) = \frac{\gamma \cdot V}{\left( \frac{p_{\text{NMDG}}}{p_{\text{Na}}} \cdot \frac{[\text{NMDG}^+]_o - [\text{NMDG}^-]}{[\text{Na}^+]_o - [\text{Na}^-]} \cdot e^{-\frac{F(V + RT)}{RT}} \right) + \frac{[\text{Na}^+]_o - [\text{Na}^-]}{1 - e^{-\frac{F(V + RT)}{RT}}}}. \]  

\[ \text{(2)} \]

to obtain parameters (\(p_{\text{NMDG}}/p_{\text{Na}}\)) and \(\gamma\) [pS/nM] (\(p_x\), permeability and valence of ion \(X^+\)). Reversal potentials were obtained by solving \(i_{\text{p}}(V) = 0\) and conductances were defined as the asymptotic slopes of the fits at very negative potentials (where, from the above equation, \(g_{\text{p}} = \gamma \cdot [Na^+]_o\)).

**Statistics**

Unless otherwise indicated, all data points in the figures represent the averages of at least five measurements, and error bars are SEM.

**Online Supplemental Material**

Mathematical details of some of the analyses used in this paper are available at http://www.jgp.org/cgi/content/full/jgp.200309008/DC1. Section 1 of this supplemental material provides the mathematical proof that the loop-criterion for microscopic reversibility can be applied to average rates among compound states. Section 2 contains the derivations of the equations that describe open probabilities (and opening and closing rates for the scheme in Fig. 1 E) as a function of various ligand concentrations for all of the kinetic schemes considered. Subsection 2.1 deals with the basic scheme in Fig. 1 E, subsections 2.2–2.6 discuss five different possible ways of extending that model to include two different ligands. Section 3 discusses the results of fitting the five extended models to the ensemble of the data, as illustrated in Fig. S1. All sections are referenced by the appropriate locations in the main text.

**RESULTS**

**Micromolar Cytosolic Nucleotides Inhibit CA-NSC Channels by Slowing Opening Rate and Speeding Closing Rate**

CA-NSC channels activated by cytosolic \(Ca^{2+}\) in inside-out patches from brain endothelial cells promptly and reversibly close upon superfusion with millimolar cytosolic ATP (Popp and Gögelein, 1992; Csanády and Adam-Vizi, 2003). We found a similar inhibition of open probability (\(P_o\)) when we superfused our patches with ATP, ADP, or AMP (Fig. 1 A); fits to the Hill-equation (Eq. 1, MATERIALS AND METHODS) yielded apparent \(K_{1/2}\) values of 17 ± 1, 9.4 ± 1, and 2.4 ± 0.2 \(\mu\)M, respectively (Fig. 1 B, solid lines). This suggests that inhibition is not dependent on ATP hydrolysis. Kinetic analysis showed that all three nucleotides inhibited \(P_o\) by slowing channel opening (Fig. 1 C) and speeding closing (Fig. 1 D). Interestingly, \(K_{1/2}\) (from fits to the Hill-equation; Fig. 1 C, solid lines) for inhibition of opening rate was ~2-fold lower in each case than for inhibition of \(P_o\) (11 ± 2, 4.3 ± 0.5, and 1.1 ± 0.2 \(\mu\)M for ATP, ADP, and AMP), whereas closing rates were stimulated only at higher cytosolic nucleotide concentrations, and failed to saturate even in the 100-micromolar range. (The plots in Fig. 1 D could not be reliably fitted with the Hill equation since closing rates failed to saturate even in the presence of millimolar nucleotide, while slow opening rates precluded collection of events at even higher concentrations.)

These findings can be rationalized by a simple model (Fig. 1 E) in which nucleotides bind both to the closed and the open channel. According to the thermodynamic principle of microscopic reversibility, for a closed kinetic scheme the product of the rates around a loop is identical in both directions. Because nucleotide binding stabilizes the closed state of the CA-NSC channel, to maintain reversibility, the nucleotide must be bound more tightly in the closed than in the open state. For the scheme in Fig. 1 E, \(K_{1/2}\) for opening rate (Fig. 1 C) equals the dissociation constant (\(K_d\)) of the nucleotide from the closed channel, while \(K_{1/2}\) for closing rate (compare, Fig. 1 D) reflects the \(K_d\) of the open channel (see online supplemental material, section 2.1, Eqs. 9 and 10). Therefore, the large separation between
the efficiencies of nucleotides for affecting opening vs. closing rate is consistent with our simple scheme.

**Inhibition by Nucleotides Is Not Affected by Channel Rundown**

CA-NSC channels from brain capillaries are opened by micromolar cytosolic \([\text{Ca}^{2+}]_i\) ([Ca\(^{2+}\)]_i), but after patch excision they rapidly (within 30–60 s) deactivate (“run down”); ~1 min after excision millimolar \([\text{Ca}^{2+}]_i\) is required for channel opening (Csanády and Adam-Vizi, 2003). To detect any parallel changes in nucleotide sensitivity, we performed experiments in which test concentrations of ATP were briefly applied immediately after patch excision into a bath containing 100 \(\mu\)M free \([\text{Ca}^{2+}]_i\). CA-NSC channels were initially opened to a \(P_o\) of close to unity in 100 \(\mu\)M \([\text{Ca}^{2+}]_i\) (Fig. 2 A, patch with 6 channels); superfusion with ATP, only ~10 s after excision, resulted in prompt inhibition of channel activity. Quantitation of such experiments was hampered by rapid rundown (note, only two of the six channels were still recovered with high \([\text{Ca}^{2+}]_i\) affinity after the brief, ~12-s exposure to ATP, Fig. 2 A). Nevertheless, a tentative analysis of gating parameters in ATP, normalized to the bracketing segments, yielded dose response curves of \(P_o\), opening and closing rates (Fig. 2, D–F, open circles) qualitatively similar to those obtained for deactivated channels at steady-state in 1 mM \([\text{Ca}^{2+}]_i\) (open triangles, replotted from Fig. 1, B–D, for comparison). In particular, inhibition of \(P_o\) resulted from high-affinity inhibition of opening rate and low-affinity stimulation of closing rate, with fits to the Hill equation (dotted lines) yielding \(K_{1/2}\) values of 34 ± 3, and 15 ± 2 \(\mu\)M, respectively, for \(P_o\) and opening rate. Because rundown had no great influence on nucleotide inhibition (especially considering opening rate, see also below), and because the time window before deactivation was too narrow, we restricted the present study on nucleotide inhibition to partially deactivated CA-NSC channels gating steadily in 1 mM \([\text{Ca}^{2+}]_i\) (Figs. 1–8, except for Fig. 2, A and B, below).

**Figure 1.** Inhibition of CA-NSC channel gating by ATP, ADP, and AMP. (A) Current traces from patches superfused with 1 mM cytosolic nucleotide during activation by 1 mM \([\text{Ca}^{2+}]_i\) (bars). (B–D) \(P_o\) (B), opening (C), and closing rates (D) as a function of nucleotide concentrations, normalized to control values in the absence of nucleotide (see **Materials and Methods**). Solid lines in B and C are fits to the Hill equation (Eq. 1, **Materials and Methods**), midpoints \((K_{1/2})\) are printed in each panel. Hill constants \((n)\) were 1.1 ± 0.03, 1.2 ± 0.1, and 1.2 ± 0.2 for \(P_o\) and 1.1 ± 0.2, 1.2 ± 0.1, and 1.1 ± 0.1 for opening rate in ATP, ADP, and AMP. The leftmost data point in B–D (and Figs. 2 and 5–8) represents control. (E) Simple model of reversible nucleotide binding.
Inhibition of Open Probability, but Not of Opening Rate, by Nucleotides Is Sensitive to [Ca\(^{2+}\)] and Voltage

Because the equilibrium between open and closed states of the CA-NSC channel (i.e., vertical transitions in Fig. 1 E) is regulated by [Ca\(^{2+}\)] and voltage (Csánády and Adam-Vizi, 2003), we tested how these parameters affect nucleotide inhibition.

Raising [Ca\(^{2+}\)] from 1 to 10 mM stimulated partially deactivated CA-NSC channels from a typical P\(_o\) of 0.4–0.5 to a P\(_o\) of close to unity (Fig. 2 C; Csánády and Adam-Vizi, 2003). Under such conditions cytosolic ATP still inhibited (Fig. 2 B), but the dose response curve for inhibition of P\(_o\) was shifted to the right (Fig. 2 D, solid triangles); the K\(_{1/2}\) value obtained from a Hill-fit (solid line) was 52 ± 6 μM. Conversely, when the membrane potential (V\(_m\)) was hyperpolarized from −40 mV (used throughout this study) to −80 mV (all in 1 mM Ca\(^{2+}\)), P\(_o\) typically decreased to ∼0.2 (Fig. 2 C; Csánády and Adam-Vizi, 2003). ATP inhibited P\(_o\) at −80 mV (Fig. 2 C) slightly more potently than at −40 mV; a Hill-fit to the dose response curve (Fig. 2 D, solid diamonds) yielded a K\(_{1/2}\) of 7.3 ± 1 μM (solid fit line).

Under both of these altered conditions, ATP inhibition of P\(_o\) resulted from inhibition of opening rate (Fig. 2 E) together with stimulation of closing rate (Fig. 2 F). In both cases inhibition of opening rate occurred with high affinity while stimulation of closing rate required high concentrations of ATP with no saturation apparent at 1 mM. These results further corroborate the scheme put forward in Fig. 1 E. Interestingly, while [Ca\(^{2+}\)] and voltage shifted the apparent affinity for ATP as reflected by P\(_o\) (Fig. 2 D), no such effect was apparent in the dose response curves for inhibition of opening rate (Fig. 2 E). Hill-fits (solid lines) yielded K\(_{1/2}\) values for opening rate of 14 ± 5 and 8.4 ± 2 μM, respectively, in 10 mM Ca\(^{2+}\) (−40 mV) and at −80 mV (1 mM Ca\(^{2+}\)), none of which differed significantly from the control value obtained at −40 mV in 1 mM Ca\(^{2+}\) (11 ± 2 μM, Fig. 2 E, open triangles and solid line).

ATP Inhibition Is a Function of Total [ATP], Rather Than of Free [ATP]

Because altering free [Ca\(^{2+}\)] alters the fractional distribution of ATP among its free (ATP\(^{4+}\), free ATP) and divalent-bound (Ca-ATP, Mg-ATP) forms, we tested whether the ATP-binding site on the CA-NSC channel selectively binds any one of these ATP species. As inhibition of P\(_o\) is a complicated function of several parameters, including control P\(_o\) in the absence of ATP (online supplemental material, section 2.1., Eq. 12, available at http://www.jgp.org/cgi/content/full/jgp.200309008/DC1; see also Discussion below), a
more reliable parameter for assessing nucleotide binding affinity is inhibition of opening rate (or closing rate; however, we could not obtain complete dose response curves for the latter); from the scheme in Fig. 1 E, \( K_{1/2} \) for opening rate is equal to the \( K_{C} \) of ATP from the closed channel (online supplemental material, section 2.1., Eq. 9).

We therefore measured opening rates under various free \([Ca^{2+}]\) and \([Mg^{2+}]\) and either 10 or 100 \( \mu M \) total [ATP], and normalized these rates to those obtained in bracketing control segments under identical ionic conditions (same free \([Ca^{2+}]\) and \([Mg^{2+}]\)) but no ATP. Table I summarizes normalized opening rates, and lists total [ATP], as well as [Mg-ATP], [Ca-ATP], and [free-ATP] calculated using the freeware program Winmaxc. Inhibition of opening rate was similar in all three cases where total [ATP] was 10 \( \mu M \) (conditions a–c in Table I), while [Mg-ATP] varied over an \(~5\)-fold, [Ca-ATP] over an \(~20\)-fold, and [free-ATP] over an \(~5\)-fold range. Likewise, 100 \( \mu M \) total [ATP] caused similar inhibition of opening rate (conditions d–f in Table I), while [Mg-ATP] varied by \(~6\)-fold, [Ca-ATP] by \(~10\)-fold, and [free-ATP] by \(~10\)-fold. These results cannot be explained by selective binding of any of the three ATP species; rather, they indicate that the ATP binding site of the CA-NSC channel does not greatly discriminate, at least between Ca-ATP and Mg-ATP. We cannot rule out that free ATP does not bind at all, since this species represents a negligible fraction of total [ATP] (\(<0\%) under all conditions tested, and CA-NSC channels cannot be studied in divalent-free solutions where free ATP would be the predominant species.

**Distinct Profile of Nucleotide Inhibitory Potency for Brain Endothelial CA-NSC Channels**

We examined how inhibition depends on the structural elements of the nucleotide (Fig. 3, A and C). The poorly hydrolyzable ATP analogue AMPPNP effectively inhibited, further supporting the above conclusion that simple binding of ATP, not hydrolysis of its \( \gamma \)-phosphate, was required. Substitutions of the adenine ring by guanine (GTP) or uracil (UTP) were compatible with inhibition, in contrast to complete removal of the base (ribose-5P) or its replacement with a negatively charged phosphate group (phospho-riboyl-PP). Although AMP was the most potent inhibitor (Fig. 1), its 3’-5’ cyclic form cAMP failed to affect gating, as did cGMP. High-affinity inhibition required the presence

![Figure 3](image-url)
of at least one phosphate, as adenosine (Ado) inhibited only at high concentrations. Inorganic phosphate (P_i) alone had little effect (but 2 mM pyrophosphate caused 92±2% inhibition (n = 4)). While high concentrations of dinucleotides like NAD^+, NADP^+, NADH, NADPH, NAAADP, and ADP-ribose (but not cADP-ribose) inhibited to a variable extent, we cannot rule out the possibility of a slight contamination of our dinucleotide stocks by AMP. Importantly, none of the above compounds failed to inhibit channel activity (e.g., P_i, cAMP, cGMP, cADP-ribose, Ado, ribose-5P, PRPP) could prevent inhibition by concomitantly added ATP (Fig. 3 B), suggesting that these compounds failed to inhibit gating because they did not bind to the channels. Thus, as summarized in the sketch in Fig. 3 C, inhibitory potency requires a purine or pyrimidine base and one or more phosphate groups (with one phosphate being optimal) without 3’-5’ cyclization.

Although a lipid, not a nucleotide, phosphatidyl inositol 4,5-bisphosphate (PI(4,5)P_2) is known to modulate a variety of cation channels, including several Trp channels (Liu and Liman, 2003; Prescott and Julius, 2003), as well as ATP-sensitive K^+ channels (Loussouarn et al., 2001; Rohacs et al., 2003), where it competes off inhibitory ATP (MacGregor et al., 2002). We therefore tested a short-chain (dioctanoyl), water-soluble, form of PI(4,5)P_2, which, however, affected neither CA-NSC gating (Fig. 3 A) nor inhibition by 100 μM ATP (Fig. 3 B).

**Decavanadate Is a Potent Activator of CA-NSC Channels That Stimulates P_o with Nanomolar Affinity**

While P_i was without effect, when we perfused our channels with a 1-mM solution of the P_i-analogue orthovanadate, we saw, to our surprise, robust stimulation of channel gating to a P_o close to unity, together with a clear (~30%) increase in single-channel current size (Fig. 4 A); however, activity remained Ca^{2+} dependent as witnessed by prompt closure of all channels when Ca^{2+} was washed off in the continued presence of vanadate. When millimolar crystalline orthovanadate is dissolved at neutral pH, trace amounts of its decamer (DV) inevitably form, causing the appearance of a faint yellow color. DV-free, colorless, vanadate solution can be prepared, e.g., by boiling (Csermely et al., 1985). Such DV-free vanadate solutions were without effect on CA-NSC gating, while, in the same patches, the control (yellow) vanadate solution repeatedly produced maximal stimulation (Fig. 4 B), suggesting that DV, and not orthovanadate, was responsible for the effect. To verify this idea, and to quantify the amount of DV in our bath solution, we prepared a stable DV stock solution (pH = 2, see MATERIALS AND METHODS), from which we diluted fresh DV into our bath solution immediately before each experiment. [DV] of our bath solution was monitored with a spectrophotometer as absorption at ~390 nm (Fig. 4 C). Repeated photometry showed that DV decayed with a time constant of ~4 h in this solution (pH = 7.1, T = 25°C) (Fig. 4 D). Actual [DV] was therefore calculated for each experiment by correcting for the decay expected from the time lag between the dilution of DV and the experiment itself.

Recordings in a range of cytosolic [DV] showed stimulation of channel P_o already at nanomolar [DV], at which concentrations no effect on channel conductance was apparent (Fig. 5 A). P_o was stimulated with a K_1/2 of 90 ± 16 nM (Fig. 5 B), mainly due to slowed channel closure (Fig. 5 C), together with a slight increase in average opening rate (Fig. 5 D).

**Stimulation of Gating by Decavanadate and Inhibition of Gating by Nucleotides are Functionally Competitive Effects**

Interestingly, DV not only robustly stimulated gating, but could also prevent inhibition by concomitantly added ATP, ADP, or AMP. Thus, in the presence of ~8 μM DV (~90× its own K_1/2, see Fig. 5 B) channel gating was maximally stimulated, and remained insensitive...
to superfusion with 1 or 10 μM AMP; even in 1 mM AMP, Po remained ~0.25, 10 mM AMP being needed for near-complete inhibition (Fig. 6 A). K1/2 for inhibition by AMP was increased ~90× (to 232 ± 51 μM, Fig. 6 B), as would be expected for a simple competitive mechanism, i.e., DV and AMP competing for a single binding site (see online supplemental material, section 2.2, Eq. 14b). Similarly, ~1.6 μM DV (~20× its own K1/2) increased K1/2 for inhibition by ADP and ATP ~20× (to 215 ± 33 μM, Fig. 6 C) and ~40× (to 677 ± 159 μM, Fig. 6 D), respectively. Conversely, AMP could prevent stimulation by DV. When the effect on gating of increasing [DV] was assessed in the presence of constant 1 mM AMP (~400× its own K1/2), 24 ± 5 μM DV was required for half-maximal activation (Csanády and Adam-Vizi, 2003), similar to that reported in excised patches for large conductance Ca2+-activated K+ (Pérez et al., 2001), TrpM4b (Nilius et al., 2003), and TrpM5 (Hofmann et al., 2003) channels (although higher Ca2+ affinities were concluded for the latter two in some whole-cell studies; Launay et al., 2002; Prawitt et al., 2003). Within ~1 min of excision, Ca2+ affinity of our CA-NSC channels declines such that channel activation requires millimolar Ca2+ (Csanády and Adam-Vizi, 2003). The cause of this loss of sensitivity toward activating Ca2+ is incompletely understood. Because Ca2+ dependence is dramatically altered by this rundown phenomenon, and because our detailed steady-state studies on nucleotide inhibition were done minutes after excision, we verified whether CA-NSC channels could be inhibited by ATP freshly after excision, in their presumably more “native-like” state. Our experiments confirmed ATP inhibition of freshly excised channels with characteristics similar to those of deacti-

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**DISCUSSION**

**Inhibition by Nucleotides Is an Intrinsic Property of CA-NSC Channels**

CA-NSC channels freshly excised from brain endothelial cells are activated by micromolar Ca2+ (compare Fig. 2 A), with a [Ca2+]i of ~20 μM required for half-maximal activation (Csanády and Adam-Vizi, 2003), similar to that reported in excised patches for large conductance Ca2+-activated K+ (Pérez et al., 2001), TrpM4b (Nilius et al., 2003), and TrpM5 (Hofmann et al., 2003) channels (although higher Ca2+ affinities were concluded for the latter two in some whole-cell studies; Launay et al., 2002; Prawitt et al., 2003). Within ~1 min of excision, Ca2+ affinity of our CA-NSC channels declines such that channel activation requires millimolar Ca2+ (Csanády and Adam-Vizi, 2003). The cause of this loss of sensitivity toward activating Ca2+ is incompletely understood. Because Ca2+ dependence is dramatically altered by this rundown phenomenon, and because our detailed steady-state studies on nucleotide inhibition were done minutes after excision, we verified whether CA-NSC channels could be inhibited by ATP freshly after excision, in their presumably more “native-like” state. Our experiments confirmed ATP inhibition of freshly excised channels with characteristics similar to those of deacti-
vated channels including high-affinity inhibition of opening rate and low-affinity stimulation of closing rate (Fig. 2, A and D–F). In particular, $K_{1/2}$ for inhibition of opening rate, a direct measure of ATP affinity of the closed channel (see below), was comparable for freshly excised channels (15 ± 2 μM) and deactivated ones (11 ± 2 μM). Thus, nucleotide inhibition is an intrinsic property of CA-NSC channels that is not altered during post-excision rundown.

Inhibition by Cytosolic Nucleotides Is Consistent with Simple Reversible Binding

We found that micromolar cytosolic nucleotides inhibited gating of CA-NSC channels by slowing opening rate and speeding closure. The higher apparent affinity for affecting opening rate than closing rate was consistent with a microscopically reversible four-state scheme (Fig. 1 E) in which nucleotides bind more tightly to the closed channel and therefore, in return, stabilize the closed state. We have previously reported that Ca²⁺ and voltage regulate channel gating in a complex fashion consistent with multiple open and closed states (Csanády and Adam-Vizi, 2003). How can our analysis based on the simple four-state model in Fig. 1 E be reconciled with those findings? The four states of the simple scheme have to be understood as compound states, e.g., the bottom-left state comprises all elementary states of the channel in which the pore is closed and the nucleotide binding site unliganded, regardless of the status of the Ca²⁺ binding site(s) and the voltage sensor(s). It is easy to show that the principle of microscopic reversibility holds up when applied to the average rates of transition among such compound states (see online supplemental material, section 1, Eq. 8, available at http://www.jgp.org/cgi/content/full/jgp.200309008/DC1). Although our average opening and closing rates, defined simply as the inverse of the arithmetic means of single-channel open and closed dwell-times and extracted from patches with multiple channels (see MATERIALS AND METHODS), are relatively simple measures of channel gating, their use in this study is justified by two arguments. First, extraction of these average parameters by the cycle-time method is robust and model independent. Second, the equations that predict opening and closing rates and $P_o$ as a function of nucleotide concentration for the model in Fig. 1 E (online supplemental material, section 2.1, Eqs. 9, 10, and 12) are valid regardless of whether the 4 states in that scheme are simple (“elementary”) states, or “compound” states composed themselves of sets of in-
parameters could also affect the equilibrium of the “horizontal” transitions in that scheme, i.e., nucleotide binding affinity of the closed, and/or open, channel, thereby altering apparent sensitivity to nucleotide inhibition. Indeed, a 10-fold elevation of $\text{Ca}^{2+}$ (to 10 mM, Fig. 2 B) resulted in an ~3-fold increase of $K_{i/2}$ for inhibition of $P_{o}$ ($K_{i}$) by ATP (Fig. 2 D), while hyperpolarization by ~40 mV (to ~80 mV) decreased that value by ~2-fold relative to control (Fig. 2, C and D), consistent with reports on CA-NSC channels in other tissues (Gray and Argent, 1990; Halonen and Nedergaard, 2002; Liman, 2003). However, while $K_{i/2}$ for inhibition of opening rate has a simple physical meaning, in that it is equal to the dissociation constant of the ligand from the closed channel ($K_{i}$, online supplemental material, section 2.1, Eq. 9), $K_{i}$ is expected, as found for ATP, ADP, and AMP (compare Fig. 1 B and C). At ~80 mV in 1 mM $\text{Ca}^{2+}$, $P_{o}$ was small (~0.2), therefore, $K_{i}$ is expected to be similar to $K_{i}$ ($K_{i} = 1.3 K_{i}$), not inconsistent with that found for ATP (Fig. 2, D and E). Finally, at ~40 mV in 10 mM $\text{Ca}^{2+}$, $P_{o}$ was ~0.8 (and $P_{i} = 0.08$), therefore, $K_{i}$ = 4.6 $K_{i}$ is expected, in good agreement with our ATP data (Fig. 2, D and E). (Indeed, the comparison holds up even for our tentative analysis on freshly excised channels in 100 mM $\text{Ca}^{2+}$ [Fig. 2 A], for which average $P_{i}$ in bracketing control segments was ~0.6, and thus, $K_{i} = 2.5 K_{i}$ is expected.) Importantly, the measured $K_{i}$ values for ATP were not significantly changed by a 10-fold increase in $\text{Ca}^{2+}$ or a ~40-mV change in voltage (Fig. 2 E). Thus, the apparent $\text{Ca}^{2+}$ and voltage dependence of nucleotide inhibition of $P_{o}$ (Fig. 2 D) simply reflects different $P_{o}$ in the absence of nucleotide under those conditions. That is, $\text{Ca}^{2+}$ and voltage alter the fraction of time the channel spends in the high-affinity closed state vs. the low-affinity open state, while ATP-affinity itself of (at least) the closed channel is not sensitive to $\text{Ca}^{2+}$ and voltage. The conformation of the ATP binding site senses only whether the channel is closed or open, not the actual state of the $\text{Ca}^{2+}$ and voltage sensors.

Nucleotide Sensitivity Profile Outlines a Subfamily of CA-NSC Channels

$\text{Ca}^{2+}$-activated monovalent cation-selective channels are present in several native tissues and differing nucleotide sensitivity profiles have been found for the ones

The Conformation of the Nucleotide Binding Site Senses the Conformation of the Gate, but Not of the $\text{Ca}^{2+}$ and Voltage Sensors

The equilibrium between open and closed states of the CA-NSC channel (i.e., “vertical” transitions in Fig. 1 E) is regulated by $\text{Ca}^{2+}$, and voltage. In principle these

terconnected “elementary” states. In the latter case, of course, the transition rates between states have to be taken as average rates of transition between compound states; for the “vertical” transitions in the scheme, these are exactly the average opening and closing rates we extract from our data using the cycle-time method.) Our study clearly shows that, using these simple parameters, the mechanism of the nucleotide and DV effects can be deciphered in the absence of a detailed understanding of the underlying $\text{Ca}^{2+}$- and voltage-dependent gating.

![Figure 7](http://example.com/figure7.png)

**Figure 7.** Stimulation of single-channel conductance by micromolar concentrations of DV. (A) Single-channel $i/V$ plots in the absence of (empty symbols), or in saturating (70 μM) DV (solid symbols). Pipette solution was 140 mM NaCl, bath solution was 140 mM of either KCl (circles and straight-line fits) or NMDG-Cl (triangles and fits to the Goldman-Hodgkin-Katz current equation). (B) Single-channel current sizes at −40 mV holding potential in the absence and presence of increasing [DV], or DV (Control, black), or in the absence of (empty symbols), or in saturating (70 μM) DV (solid line is a fit to the Hill equation (Eq. 1, supplemental material, section 2.1, Eq. 9)). (C) Normalized single-channel conductance as a function of cytosolic [DV] in the absence of nucleotides (control, black), or in the presence of 1 mM ATP, ADP, or AMP (empty symbols). Solid line is a fit to the Hill equation (Eq. 1, MATERIALS and METHODS) for the control condition, $K_{i/2}$ is printed in the panel, $n = 1.7 ± 0.4$. (D) Fractional change in single-channel conductance upon addition of 1 mM ATP, ADP, or AMP, in the presence of various [DV].
that were examined in detail. The CA-NSC channels present in astrocytes, for example, are clearly different from the brain endothelial channel, in that they are inhibited by ATP but are insensitive to ADP and AMP and have been suggested to be coupled to the type 1 sulphonylurea receptor (Chen et al., 2003). The order of inhibitory potency AMP > ADP > ATP >> Ado we found for the brain endothelial channel (Figs. 1 and 3) is similar to that reported for CA-NSC channels in the endocrine pancreas (Sturgess et al., 1986), in the kidney (Paulais and Teulon, 1989), and in brown fat cells (Halonen and Nedergaard, 2002), suggesting that these might form a unique subfamily of CA-NSC channels, even though subtle differences exist among them; e.g., cyclic nucleotides, which inhibit the CA-NSC of brown fat cells (Halonen and Nedergaard, 2002), failed to affect gating of the brain endothelial channel (Fig. 3). Interestingly, Ca2+ sensitivity (micromolar) of this latter subfamily of channels is also similar, while the astrocyte channel is activated by nanomolar Ca2+.

At present it is not known which gene(s) encodes the native CA-NSC channels, and whether both their Ca2+ and nucleotide sensitivities are accounted for by a single polypeptide. Possible candidates include some members of the Trp ion channel family that form cation channels and require intracellular Ca2+ for gating. The Ca2+-permeable TrpM2 is also nucleotide sensitive in that it is activated (unlike the brain endothelial CA-NSC, see Fig. 3 A) by cytosolic ADPribose and NAD+, which bind to a COOH-terminal domain of the TrpM2 protein (Perraud et al., 2001; Sano et al., 2001). The closest functional matches to native CA-NSC channels are TrpM4b (Launay et al., 2002; Nilius et al., 2003) and TrpM5 (Hofmann et al., 2003; Liu and Liman, 2003; Prawitt et al., 2003), both ~25-pS, monovalent cation selective, Ca2+- and voltage-activated channels. While nucleotide sensitivity has so far not been reported for TrpM5, in a recent report (Nilius et al., 2004) TrpM4b, expressed in HEK-293 cells, was shown to be inhibited by micromolar cytosolic adenine nucleotides, although some of the details were distinct from those described here for the brain endothelial CA-NSC channel; e.g., ~20% of TrpM4b current was insensitive to even millimolar [ATP], ADP was the most potent inhibitor, GTP and UTP were poor inhibitors, and it was concluded that free ATP is the species responsible for ATP inhibition.

Mutually Exclusive Binding Explains Antagonistic Regulation of Gating of CA-NSC Channels by Nucleotides and Decavanadate

DV stimulated gating with extremely high (nanomolar) affinity (Fig. 5). This tight binding of the highly charged DV molecule (6 negative charges) implies strong electrostatic interactions, which in turn suggests that positively charged arginine sidechains may contribute to its binding site (Soman et al., 1983; Toyoshima et al., 2000). The fact that DV, when added concomitantly, could similarly prevent inhibition by either ATP, ADP, or AMP, implicitly suggests that the latter three nucleotides all share a common binding site; unlike ATP-sensitive K+ channels which possess distinct binding sites for inhibitory ATP (on the pore-forming Kir6.x channel subunit) and stimulatory MgADP (on the regulatory SUR subunit), but are insensitive to AMP, the most potent inhibitor of the CA-NSC channel.

DV also increased single-channel conductance by ~30% without grossly altering selectivity properties (Popp and Gögelein, 1992; Csanády and Adam-Vizi, 2003). This effect on the pore required micromolar [DV] (Figs. 5 A and 7 B) and was not influenced by the presence of nucleotides. We therefore conclude that DV affects the permeation properties of CA-NSC channels by binding with lower affinity to a site, presumably close to the pore, separate from the one at which it exerts its gating effect. Possibly, DV bound in the pore alters the local electrostatic potential so as to increase the local concentration of permeating cations, and hence the unitary conductance, as has been reported for both cation (MacKinnon et al., 1989; Nimigean et al., 2003) and anion channels (Middleton et al., 1996; Chen and Chen, 2003). A detailed study should be undertaken to address this issue for the CA-NSC channel.

Setting aside this effect on permeation, all our observations on channel gating in the presence of ATP, ADP, AMP, and DV, including changes in Popen as well as gating kinetics, are easily explained by extending the model in Fig. 1 E (cartoon in Fig. 8). A single site on the cytosolic face of the CA-NSC channel which binds either ATP, ADP, or AMP undergoes an obligate conformational change upon channel opening (and closure). Nucleotides bind tightly (micromolar Kd) in the closed, but only loosely (millimolar Kd) in the open, channel conformation, thereby stabilizing the closed state (Fig. 1, C–E). DV binds competitively to the same site, but is bound more tightly in the open (nanomolar Kd) than in the closed state (micromolar Kd), thereby stabilizing the open-channel conformation. The graphs in Fig. 8 replot the 18 dose response curves of steady-state Popen, opening, and closing rates from Figs. 1, 5, and 6. Whereas in the above figures these plots were individually fitted with the Hill equation, the bold lines in Fig. 8 (and predicted K1/2 values in each graph; derived in online supplemental material, section 2.1, Eqs. 9, 10, and 12, and section 2.2, Eq. 14b) result from a simultaneous fit of the cartooned gating model to the ensemble of all 18 curves. With only nine adjustable parameters (gray boxes), the good overall fit to all 18 curves supports this simple model. (Opening rate of the unliganded channel was fixed to its average value, white
Nucleotide Regulation of Ca\(^{2+}\)-activated Cation Channels

We have also considered the alternative à priori possibility, namely that ATP and DV bind to separate sites (noncompetitive model; the kinetic treatment of this model can be found in section 2.3 of the online supplemental material). Such a model with independent binding sites for nucleotides and DV did not fit our data. If binding of DV was allowed to allosterically destabilize the nucleotide binding site (in which case, by microscopic reversibility, nucleotide binding must destabilize the DV binding site), our data could be fitted, but predicted huge decreases in nucleotide affinity brought upon by DV binding (\(\sim 200\), \(10^5\), and \(10^3\)-fold for ATP, ADP, and AMP). This would mean that simultaneous binding of nucleotide and DV would almost never occur, i.e., from a kinetic point of view, the model reduces to the mutually exclusive model (competitive binding), which is a subset of the more general model (allowing simultaneous binding). E.g., the fits for ADP suggest that even when both [ADP] and [DV] are 1,000× their own \(K_{1/2}\), the channel spends 99.5% of its time without both ligands simultaneously bound.

While we could reasonably fit our data assuming one ATP binding site (Fig. 8), most cation channels are multimeric; Trp channels, for example, are believed to function as tetramers (Harteneck et al., 2000). Therefore, even if the CA-NSC channel is a heteromultimer, it is plausible to expect more than one ATP binding site (possibly 2, 3, or 4) per channel molecule. Our data did not imply any significant cooperativity (Hill coefficients of most dose response curves were close to 1, the random scatter in the slopes of some of the curves, e.g., in Fig. 6, most likely reflect the stochastic nature of our single-channel data), but could be equally well fitted assuming 1–4 independent subunits with nucleotides and DV competing at each site (thin lines in the \(P_o\)-plots of Fig. 8 show the fit for four subunits; see kinetic treat-
ment in section 2.4. of the online supplemental material). Fitting of models with 2–4 independent subunits each containing separate binding sites for nucleotides and DV (compare online supplemental material, section 2.5) still required strong negative allosteric interaction, although the extent of mutual destabilization required for a good fit decreased with the number of assumed subunits (∼50–70-fold for n = 4). A scheme assuming four binding sites for ATP and a single separate allosteric binding site for DV (modeled on the binding of 2,3-bisphosphoglycerate and O2 to hemoglobin; Benesch and Benesch, 1969; see online supplemental material, section 2.6) could not be adequately fitted to our data. (See fits to various models in section 3 [Fig. S1] of the online supplemental material.)

Among the proteins known to interact with DV, several contain nucleotide binding sites, including the sulfonylurea-receptor subunits of ATP-sensitive K+ channels (Proks et al., 1999). For most of these enzymes the exact site of binding of DV relative to that of the nucleotides was not addressed (Boyd et al., 1985; Proks et al., 1999) or was inferred simply from the competitive (Menon and Goldberg, 1987; Krivanek, 1994) or non-competitive (Choate and Mansour, 1979; Soman et al., 1983; Pezza et al., 2002) kinetic patterns. However, x-ray crystallographic data for adenylate kinase (Pai et al., 1977) and combined evidence from biochemical (Csermely et al., 1985; Coan et al., 1986; Ross and McIntosh, 1987; Hua et al., 2000) and crystallographic (Toyoshima et al., 2000) data for the sarcoplasmic Ca2+-ATPase have unambiguously confirmed that DV binds at the nucleotide binding site in these two proteins.

In summary, we conclude that ATP, ADP, and AMP inhibit by binding to a common site. Our data are consistent with 1–4 independent channel subunits, and indicate mutually exclusive binding of nucleotides and DV. While a strong negative allosteric interaction between separate nucleotide and DV sites is formally consistent with our data per se, the comparatively good fit with the simpler competitive model (Fig. 8) and the known binding of DV to nucleotide binding sites (Pai et al., 1977; Toyoshima et al., 2000) argue in favor of the latter explanation. A similar strategy of antagonistic regulation by two ligands binding competitively at the same site is employed by the enzyme ribonucleotide reductase, which is activated when ATP, but inhibited when dATP, binds to its primary regulatory site (Reichard et al., 2000).

Significance of Our Findings

The significance of our decavanadate results is twofold. First, decavanadate is the only high-affinity activator for CA-NSC channels known so far, and it provides a powerful new tool for future studies on these channels. In excised patch recordings, for example, maximal stimulation by micromolar decavanadate can be used to determine the number of active channels. Furthermore, as the exact molecular identity of the CA-NSC channels is still unknown, decavanadate stimulation provides a new functional fingerprint that can be used to screen heterologously expressed cloned channels. Second, the fact that decavanadate allows channels to gate in the presence of millimolar cytosolic nucleotides suggests a possible explanation for how cytosolic Ca2+ signals (Maruyama and Petersen, 1982; Kamouchi et al., 1999; Koivisto et al., 2000) can activate CA-NSC channels in living cells. Binding of some negatively charged metabolite to the decavanadate binding site (regardless of its location) could provide a simple way for the cell to regulate the readiness of CA-NSC channels to respond to rises in cytosolic Ca2+ in the presence of millimolar nucleotides. Future studies will have to identify any such natural activating ligand of the CA-NSC channel.

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