

Trinitrophenyl-ATP Blocks Colonic Cl⁻ Channels in Planar Phospholipid Bilayers

Evidence for Two Nucleotide Binding Sites

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ABSTRACT Outwardly rectifying 30–50-pS Cl⁻ channels mediate cell volume regulation and transepithelial transport. Several recent reports indicate that rectifying Cl⁻ channels are blocked after addition of ATP to the extracellular bath (Alton, E. W. F. W., S. D. Manning, P. J. Schlatter, D. M. Geddes, and A. J. Williams. 1991. *Journal of Physiology*. 443:137–159; Paulmichl, M., Y. Li, K. Wickman, M. Ackerman, E. Peralta, and D. Clapham. 1992. *Nature*. 356:238–241). Therefore, we decided to conduct a more detailed study of the ATP binding site using a higher affinity probe. We tested the ATP derivative, 2',3',O-(2,4,6-trinitrocyclohexadienylidene) adenosine 5'-triphosphate (TNP-ATP), which has a high affinity for certain nucleotide binding sites. Here we report that TNP-ATP blocked colonic Cl⁻ channels when added to either bath and that blockade was consistent with the closed-open-blocked kinetic model. The TNP-ATP concentration required for a 50% decrease in open probability was 0.27 μM from the extracellular (*cis*) side and 20 μM from the cytoplasmic (*trans*) side. Comparison of the off rate constants revealed that TNP-ATP remained bound 28 times longer when added to the extracellular side compared with the cytoplasmic side. We performed competition studies to determine if TNP-ATP binds to the same sites as ATP. Addition of ATP to the same bath containing TNP-ATP reduced channel amplitude and increased the time the channel spent in the open and fast-blocked states (i.e., burst duration). This is the result expected if TNP-ATP and ATP compete for block, presumably by binding to common sites. In contrast, addition of ATP to the bath opposite to the side containing TNP-ATP reduced amplitude but did not alter burst duration. This is the result expected if opposite-sided TNP-ATP and ATP bind to different sites. In summary, we have identified an ATP derivative that has a nearly 10-fold higher affinity for reconstituted rectifying colonic Cl⁻ channels than any previously reported blocker (Singh, A. K., G. B. Afink, C. J. Venglarik, R. Wang, and R. J. Bridges. 1991. *American Journal of Physiology*. 260 [*Cell Physiology*. 30]:C51–C63).

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Thus, TNP-ATP should be useful in future studies of ion channel nucleotide binding sites and possibly in preliminary steps of ion channel protein purification. In addition, we have obtained good evidence that there are at least two nucleotide binding sites located on opposite sides of the colonic Cl^- channel and that occupancy of either site produces a blocked state.

INTRODUCTION

Rectifying Cl^- channels are widely distributed in nature and are thought to mediate volume regulation, signal transduction, and transepithelial transport (Welsh, 1987; Lukács and Moczydlowski, 1990; Solc and Wine, 1991). In addition to their medium conductance in symmetric solutions containing 140 mM Cl^- (i.e., 30–50 pS) and characteristic outwardly rectifying current–voltage relation, these channels can be distinguished from other anion-selective channels based on their sensitivity to disulfonic stilbenes, anthranilic acids, and indanyloxyacetic acids (Lukács and Moczydlowski, 1990; Alton, Manning, Schlatter, Geddes, and Williams, 1991; Singh, Afink, Venglarik, Wang, and Bridges, 1991; Solc and Wine, 1991; Paulmichl, Li, Wickman, Ackerman, Peralta, and Clapham, 1992). Patch-clamp studies have also revealed that rectifying Cl^- channels can be activated by protein kinase (PK) and ATP in excised apical membrane patches from normal but not cystic fibrosis (CF) airway epithelial cells (Schoumacher, Shoemaker, Halm, Tallant, Wallace, and Frizzell, 1987; Li, McCann, Leidtke, Nairn, Greengard, and Welsh, 1988). However, the relationship between CF and rectifying Cl^- channels is complicated. Transfection of the normal gene into CF-defective cells causes another regulated Cl^- channel with different properties to appear and restores the PK- and ATP-mediated activation of rectifying Cl^- channels (Egan, Flotte, Afione, Solow, Zeitlin, Carter, and Guggino, 1992).

Several recent reports indicate that extracellular ATP can alter the activity of rectifying Cl^- channels. Addition of ATP to the extracellular side caused a dose-dependent, flickery-type blockade of reconstituted rectifying Cl^- channels obtained from airway epithelial cells and platelets with an estimated IC_{50} of 40 μM (Manning and Williams, 1989; Alton et al., 1991). Blockade by extracellular ATP is not limited to reconstituted channels since Stutts, Chinet, Mason, Fullton, Clarke, and Boucher (1992) have presented data showing that external ATP increased burst duration of rectifying Cl^- channels in excised apical membrane patches from airway epithelial cells, which is the result expected if ATP is acting as a fast or flickery-type blocker. More recently, an outwardly rectifying Cl^- channel has been cloned from the Madin Darby canine kidney (MDCK) cell line (Paulmichl et al., 1992). The Cl^- conductance resulting from expression of this channel mRNA in *Xenopus* oocytes was also sensitive to extracellular nucleosides and nucleotides. Thus it may be possible to identify higher affinity ligands for the nucleotide binding site of rectifying Cl^- channels from the numerous nucleotide derivatives used in previous studies of ATP binding proteins. These ligands could then be used as probes to extend our understanding of the pharmacology, physiology, and biophysics of rectifying Cl^- channels. Additionally, these derivatives would permit future study of the structure–activity relation of the binding site and possibly aid in the purification of ion channel protein.

Based on the notion that rectifying Cl^- channels possess an extracellular nucleo-

tide binding site, we conducted experiments to identify a higher affinity probe. We tested the ATP derivative 2',3'-O-(2,4,6-trinitrocyclohexadienyldene) adenosine 5'-triphosphate (TNP-ATP), which was first synthesized by Hiratsuka and Uchida (1973) and has a relatively high affinity for certain nucleotide binding sites. As shown in Fig. 1, the trinitrophenol group is attached to the ribose of ATP via a dioxolane ring. This ATP derivative was used to characterize the nucleotide binding sites of myosin ATPase (Hiratsuka, 1982; Hiratsuka and Uchida, 1973), (Na,K)-ATPase (Moczydlowski and Fortes, 1981a, b), *Escherichia coli* F₁ ATPase (Rao, Al-Shawi, and Senior, 1988), beef heart mitochondrial ATPase (Grubmeyer and Penfensky, 1981a,b), arsenate anion pump (Rosen, Weigel, Karkaria, and Gangola, 1988; Karkaria and Rosen, 1991), and synthetic peptides of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel (Thomas, Shenbagamurthi, Ysern, and Pedersen, 1991). TNP-ATP binding is competitive with ATP (Grubmeyer and Penfensky, 1981a; Moczydlowski and Fortes, 1981b; Rosen et al., 1988; Karkaria and Rosen,

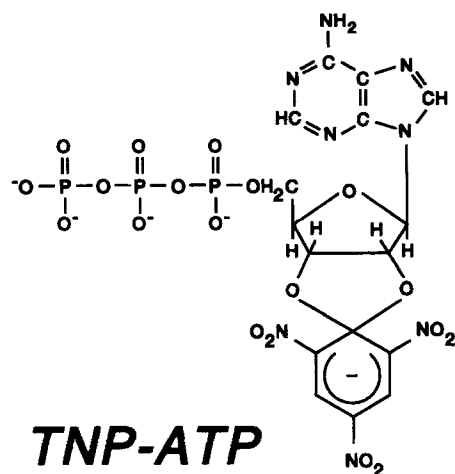


FIGURE 1. Chemical structure of TNP-ATP at neutral and basic pH. Acidic pH causes the protonation of the 2'-oxygen (pK_a 5.2), resulting in the opening of the dioxolane ring (Hiratsuka and Uchida, 1973). Since the pH of all solutions used in this study was 6.9, >98% of the TNP-ATP was in the form shown.

1991) and TNP-ATP can be hydrolyzed slowly by certain ATPases (Hiratsuka and Uchida, 1973; Watanabe and Inesi, 1982).

The purpose of this report is to show that TNP-ATP acts as a potent reversible blocker of rat colonic Cl⁻ channels reconstituted into planar phospholipid bilayers. Addition of TNP-ATP to either the *cis* or *trans* bath produced a new population of nonconducting events. However, there was a marked difference in the kinetic constants depending on the side of TNP-ATP addition. Blockade of the reconstituted rectifying Cl channels by either *cis* or *trans* TNP-ATP conformed to the simple linear closed-open-blocked kinetic scheme. We also conducted experiments to test for possible competition between TNP-ATP and ATP and obtained direct evidence that there are at least two nucleotide binding sites located on opposite sides of the channel with different affinities for TNP-ATP.

Portions of this study have been published in abstract form (Venglarik, Wang, and Bridges, 1991; Venglarik and Bridges, 1992).

MATERIALS AND METHODS

Bilayer Technique

Single channel current records of rectifying colonic Cl^- channels in planar phospholipid bilayers were obtained as previously described (Reinhardt, Bridges, Rummel, and Lindemann, 1987; Bridges, Worrell, Frizzell, and Benos, 1989; Bridges and Benos, 1990; Singh et al., 1991). We used conventional polystyrene chambers for painted bilayers that were modified so the volume of each bath was 0.4 ml. The *cis* bath was connected to a voltage divider that supplied the command potential via a 4% agar 1 M KCl bridge, a small reservoir containing a 1 M KCl solution, and a Ag-AgCl electrode (In Vivo Metric, Healdsburg, CA). The *trans* bath was connected to the inverting input of a current to voltage converter using an identical circuit arrangement. The current to voltage converter was configured to hold the *trans* bath at virtual ground. Initially both baths contained identical 50 mM KCl solutions (see below). 200–300-pF membranes were formed by dissolving 14 mg/ml phosphatidylethanolamine (PE) and 6 mg/ml phosphatidylserine (PS) in *n*-decane and then painting the phospholipid-decane mixture over a 300–400- μm -diam aperture. Membrane capacitance was monitored continuously during bilayer formation and briefly during experiments by applying a small triangle wave potential. After bilayer formation, the KCl in the *cis* solution was increased to 300 mM by adding a small volume of 3 M KCl. All experiments were performed at room temperature ($\sim 20^\circ\text{C}$).

Vesicles were prepared from isolated rat colonic crypts and stored at -20°C in a sucrose buffer (250 mM sucrose, 1 mM MOPS, and 1 mM EGTA, pH 7.0) for up to 6 mo before use (Bridges, Garty, Benos, and Rummel, 1988; Bridges et al., 1989). 2–5 μl of membrane vesicles containing 1 mg/ml of protein was added to the *cis* bath. Single channel currents were monitored with a digital storage oscilloscope (model 310; Nicolet Instrument Corp., Madison, WI) and recorded to VHS videotape using a modified digital audio PCM (model 2000T; A. R. Vetter Co., Inc., Rebersburg, PA). Reconstituted rat colonic Cl^- channels have the same conductance, halide selectivity ($\text{I} > \text{Br} > \text{Cl} > \text{F}$), and kinetics as voltage- or kinase-activated channels recorded from the apical membranes of airway and intestinal epithelial cells using the patch-clamp technique (Reinhardt et al., 1987; Halm, Reckemmer, Schoumacher and Frizzell, 1988; Halm and Frizzell, 1992).

Data Analysis

Analysis was performed only on records that contained a single channel. Records from experiments where the addition of blocker produced discrete events were played back, filtered at 400 Hz (model 3200; Khron-Hite, Avon, MA) and acquired to an IBM-compatible computer using a TL-1 DMA A/D interface and pClamp software (Axon Instruments, Inc., Foster City, CA) at a sample rate of 2 kHz. Modified pClamp software was used to construct idealized records, calculate open probability, and construct and fit event duration histograms. Open and closed events were measured from idealized records obtained using a threshold setting of one-half the single channel current amplitude. Events < 2.5 ms were not properly resolved and were excluded from histograms. Approximately 2,000–8,000 events representing 5–60 min of channel activity were used for each event duration histogram.

Records from experiments where the addition of blocker caused an apparent reduction in current amplitude were analyzed twice: once to estimate the mean burst duration (\bar{T}_{burst}) and once to determine the change in amplitude. In this study, a burst is defined as the time that the channel spends in the open and fast-blocked states. Thus, during a burst the channel appears to be open but has a reduced amplitude. Data for calculation of mean burst duration were played back and filtered at 400 Hz. A TL-1 DMA interface and modified pClamp software were used to construct an idealized record and calculate \bar{T}_{burst} from the events list using a critical

closed time of 2.5 ms (Colquhoun and Hawks, 1982, 1983). Data for amplitude measurements were obtained by filtering representative portions of the record at 100–400 Hz, digitizing the record with the oscilloscope, and transferring the data to the computer. We used a BASIC program to group the data into two sets corresponding to the two current levels (i.e., open and closed) and to calculate the mean and standard deviation for each level. Estimates of amplitude obtained in this way were nearly identical to values obtained by binning the data and fitting the resulting histograms to two Gaussian distributions using pClamp software.

Materials

The solutions contained (mM): 50 (or 300) KCl, 1 MgCl₂, 0.6 CaCl₂, 1 EGTA, and 1 MOPS-KOH (pH 6.9).¹ PE and PS were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). TNP-ATP was obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO). TNP-ATP and MgATP were added as small volumes (1–20 μ l) of concentrated stock solutions containing 50 mM KCl buffer (pH 6.9). Equivalent volumes were removed after any addition to maintain bath volume.

RESULTS

TNP-ATP Blockade

Fig. 2 shows representative current records that illustrate the effect of TNP-ATP when added to the *cis* (A) or *trans* (B) baths. We can deduce that the external domain of the channel faces the *cis* bath and the cytosolic domain faces the *trans* bath based on previous reports of the rectifying current–voltage relation (Reinhardt et al., 1987; Halm et al., 1988; Li et al., 1988) and current amplitude measurements (i.e., 2.4–3.2 pA). All channels used in this study incorporated into the bilayer with the extracellular side facing the *cis* bath ($n = 35$). To be consistent with previous reports, current records are oriented to show *trans* to *cis* or “outward” current as positive. Note that addition of TNP-ATP to either bath caused the appearance of a new closed (blocked) state and reduced the open probability of the channel. This effect was rapidly reversed by replacing the bath with a solution containing no TNP-ATP (not shown). Blockade by *trans* TNP-ATP was unexpected since earlier studies report no change in channel currents after addition of intracellular ATP (Manning and Williams, 1989; Alton et al., 1991; Stutts et al., 1992).

The current records shown in Fig. 2 suggest that TNP-ATP blocked with higher affinity from the *cis* (extracellular) bath compared with the *trans* (cytosolic) bath. The difference in affinity was quantified by plotting the open probability of the channel (P_o) as a function of *cis* or *trans* TNP-ATP concentration and fitting the data to a single site Michaelis-Menten function. This is shown in Fig. 3A. The TNP-ATP concentration required for a 50% decrease in P_o (K_i) was 0.27 μ M for *cis* or external TNP-ATP and 19.8 μ M for *trans* or cytosolic TNP-ATP. Table I shows that similar K_i 's were obtained in eight additional experiments. The nearly 75-fold difference in

¹ Hanrahan and Tabcharani (1990) have shown that addition of 10 mM MOPS to rectifying Cl channels in excised membrane patches caused a 25% reduction in current amplitude. MOPS has a similar effect on reconstituted rectifying Cl channels, but the reduction in current amplitude produced with 1 mM MOPS is small (i.e., <5%; Venglarik, C. J., and R. J. Bridges, unpublished observations).

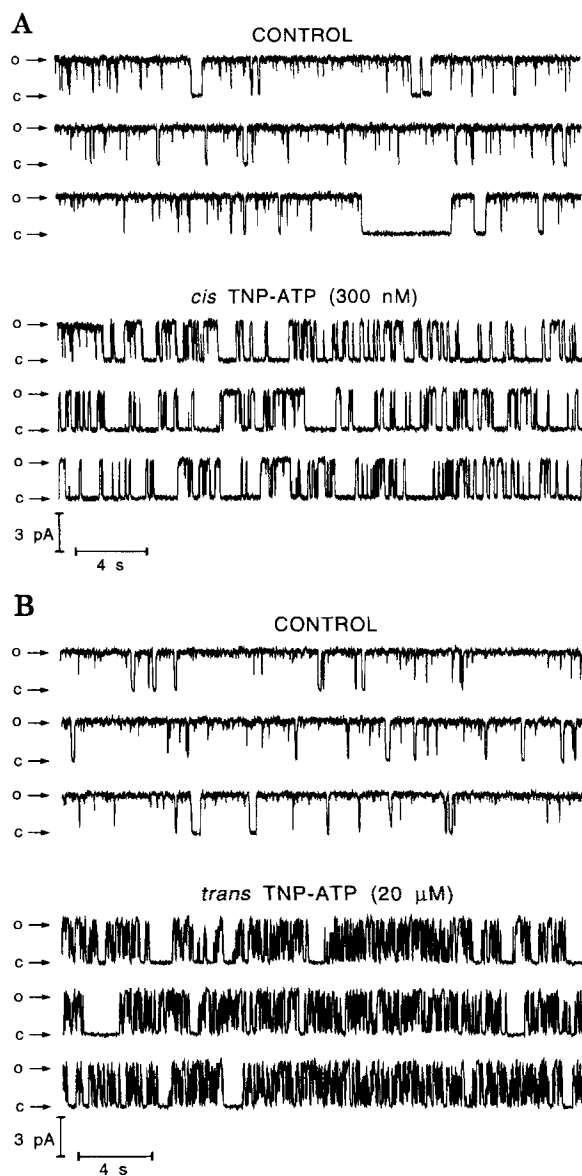


FIGURE 2. Current traces of Cl⁻ channel activity before (*control*) and after addition of (A) 0.30 μM TNP-ATP to the *cis* bath or (B) 20 μM TNP-ATP to the *trans* bath. Conditions: 300 mM KCl *cis*, 50 mM KCl *trans*, -30 mV *cis* with respect to *trans*. Unless noted, all experiments were conducted under similar conditions. Arrows indicate closed (C) and open (O) current levels.

cis versus *trans* affinity for TNP-ATP as estimated by the K_i 's suggests that there are at least two different sites that are accessible from opposite sides. The ability to describe these data with simple Michaelis-Menten functions suggests that TNP-ATP interacts with a single site on either side. To further test this hypothesis, we transformed these data and constructed Hill plots (Fig. 3 B). The lines in Fig. 3 B show the best fit of the transformed data obtained using linear regression. The Hill coefficients (n) were not different from 1. The Michaelis-Menten kinetics of blockade (Fig. 3 A), the lack of cooperativity (Fig. 3 B), and the 75-fold difference in affinity (Table I) provide

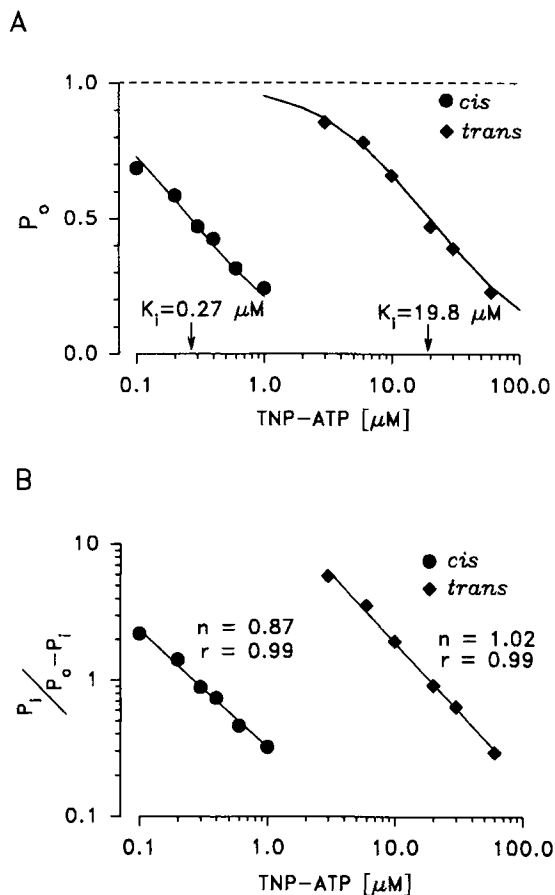


FIGURE 3. Concentration dependence of TNP-ATP inhibition of channel P_o (A) and the corresponding Hill plots (B). TNP-ATP was added to the *cis* (●) or *trans* (◆) bath at the indicated concentrations. P_o of control channel activity (>95%) was used to normalize the effect of TNP-ATP on P_o . The lines in A are the theoretical fits predicted for a single-site Michaelis-Menten type function obtained using nonlinear regression for the data shown. The lines in B were obtained by linear regression of the transformed data. n is the Hill coefficient and r is the regression coefficient.

suggestive evidence that there is an interaction between a single TNP-ATP molecule and one of two sites located on opposite sides of the membrane. This hypothesis is tested more rigorously in later sections. However, first we determined if TNP-ATP is an open channel blocker.

TABLE I
Kinetic Constants of TNP-ATP Block of Colonic Chloride Channels

	K_i	k_{on}	k_{off}	K_D
	μM	$\mu\text{M}^{-1}\text{s}^{-1}$	s^{-1}	μM
<i>cis</i> TNP-ATP	0.26 ± 0.009	8.2 ± 1.03	1.9 ± 0.25	0.23 ± 0.041
<i>trans</i> TNP-ATP	20.1 ± 0.39	1.8 ± 0.07	54.5 ± 5.24	30.5 ± 3.14

Values are the means \pm SE for $n = 5-8$ channels. Each channel was tested for three to five concentrations of TNP-ATP from either the *cis* or *trans* side of the bilayer. K_i 's were obtained by nonlinear regression of the concentration-dependent decrease in P_o . Rate constants were determined as described in Materials and Methods.

TNP-ATP Is an Open Channel Blocker

Having shown that TNP-ATP blocked the rectifying colonic Cl^- channel from either side, we performed event duration analysis to determine if TNP-ATP block conformed to the simple linear closed-open-blocked kinetic model. Control open and

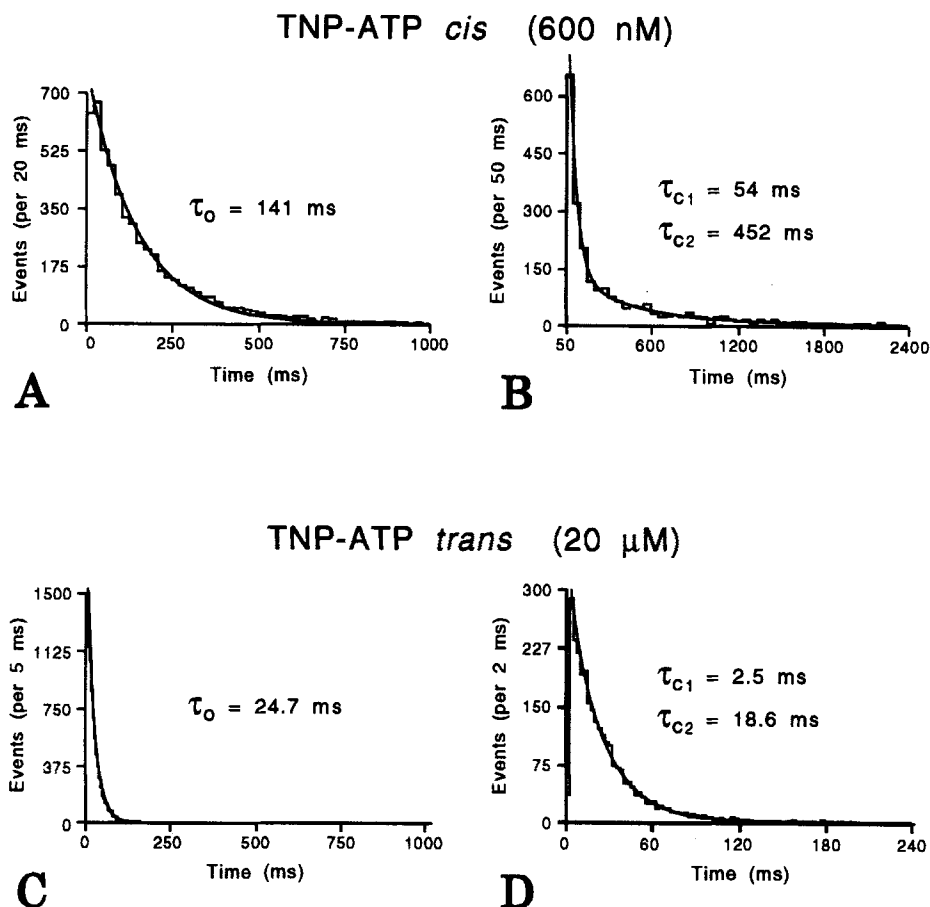


FIGURE 4. Representative open and closed time histograms of Cl^- channel activity after the addition of TNP-ATP to the *cis* (A and B) or *trans* (C and D) baths. Sample durations were 55 min for *cis* TNP-ATP and 5.7 min for *trans* TNP-ATP. Records were filtered at 400 Hz and sampled at 2 kHz. Total events in the idealized records were: *cis* TNP-ATP, 5,655; *trans* TNP-ATP, 5,191. Bin width: (A) 20 ms; (B) 50 ms; (C) 5 ms; and (D); 2 ms. Fit range: (A) 20–1,000 ms; (B) 50–2,400 ms; (C) 5–200 ms; (D) 4–200 ms. Note that the time scale in B is 10-fold less than in D.

closed time histograms obtained from current records before blocker addition could be described by single exponentials with an open time constant (τ_o) of 270 ± 20 ms and a closed time constant (τ_c) of ~ 3.0 ms (Reinhardt et al., 1987; Bridges et al., 1989; Singh et al., 1991). These control values indicate that there is a single open

state and one dominant closed state.² In order for block to be consistent with the closed-open-blocked model, kinetic analysis must satisfy four additional criteria. First, we should still find a single open time constant (τ_o) in the presence of blocker. Second, τ_o^{-1} should vary as a linear function of the blocker concentration with a slope corresponding to the on rate constant (k_{on}). Third, the blocker should produce an additional exponential distribution in the closed time histograms corresponding to the blocked time constant (τ_B). Fourth, τ_B^{-1} should be independent of blocker concentration.

Analysis of the open time histograms after TNP-ATP addition revealed a single time constant. For example, addition of 600 nM TNP-ATP to the *cis* bath decreased τ_o to 141 ms from a control value of 285 ms (Fig. 4 A) and addition of 20 μ M TNP-ATP to the *trans* bath decreased τ_o to 24.7 ms from the control value of 265 ms (Fig. 4 C). Fig. 5 plots τ_o^{-1} as a function of *cis* (A) and *trans* (B) TNP-ATP concentration. In both *cis* and *trans* experiments τ_o^{-1} varied as a linear function of TNP-ATP concentration with a slope corresponding to a k_{on} of $8.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for *cis* (extracellular) TNP-ATP and a k_{on} of $1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for *trans* (cytosolic) TNP-ATP. Table I shows that similar concentration-dependent k_{on} 's were obtained in four additional experiments. Analysis of the closed time histograms after addition of TNP-ATP revealed three time constants for *cis* additions and two time constants for *trans* additions. For example, after addition of 600 nM TNP-ATP to the *cis* bath we found a time constant of 3.6 ms that corresponded to τ_c (not shown), a time constant of 452 ms that dominated the distribution (i.e., τ_B) and an intermediate time constant of 54 ms (Fig. 4 B). Subsequent experiments revealed that the intermediate time constant of 54 ms was due to the presence of contaminating TNP-ADP in the TNP-ATP.³ Similar results were obtained in four additional experiments (Table I). In the presence of 20 μ M TNP-ATP in the *trans* bath (Fig. 4 D) we found a time constant of 2.5 ms corresponding to τ_c and a time constant of 18.6 ms that dominated the distribution (i.e., τ_B). Table I shows that nearly identical values were

² Inspection of the current records shown in Fig. 2 reveals that in addition to the relatively frequent short-lived closed state, long-lived closed events occasionally appear in the record. Thus, at least one additional closed state is required to describe the control channel kinetics. However, due to the relatively low frequency of these long-lived closures we have excluded this state in our analysis. After blocker addition the control short-lived closed state ($\tau_c = 2\text{--}3$ ms) and the less frequent long-lived closed states are expected to make a relatively minor contribution to the event duration histogram (see Fig. 4).

³ The simplest explanation for two block time constants after *cis* additions is that the TNP-ATP contained an impurity. We determined the composition of TNP-ATP using thin layer chromatography as described by Hiratsuka (1982). We found that the TNP-ATP contained $\sim 20\text{--}30\%$ picric acid and $10\text{--}20\%$ TNP-ADP. Addition of picric acid had no apparent effect on channel activity but *cis* TNP-ADP produced a τ_B that was similar to the 54-ms time constant observed with *cis* TNP-ATP (not shown). We also found that the τ_B 's for *trans* TNP-ATP and TNP-ADP were similar, explaining why we did not resolve two blocked time constants for *trans* additions (Fig. 4 D). The presence of picric acid and TNP-ADP in the "TNP-ATP" obtained from Molecular Probes, Inc. raises two caveats. First, TNP-ATP is a more potent blocker than reported herein since we are overestimating the concentration of TNP-ATP by $20\text{--}40\%$ when determining the K_i , k_{on} , and K_D . Second, to simplify the presentation of the analysis in the following section we will ignore the contribution of TNP-ADP in the kinetic models. The reasons why this omission does not affect the outcome of the analysis are presented in Appendix I.

obtained in four additional experiments. Fig. 5 plots the reciprocal of the block time constant (τ_B^{-1}) as a function of *cis* (*A*) and *trans* (*B*) TNP-ATP concentration. In both cases, τ_B^{-1} (k_{off}) appeared to be independent of TNP-ATP concentration. The TNP-ATP concentration dependence of k_{on} and the TNP-ATP concentration invariant k_{off} indicates that TNP-ATP blockade conformed to the linear closed-open-blocked kinetic model.

Equilibrium dissociation constants (K_D 's) were calculated from the ratio of the off and on rate constants and are presented in Table I. The general agreement between the K_i 's calculated from the decrease in P_o and the K_D 's provides independent

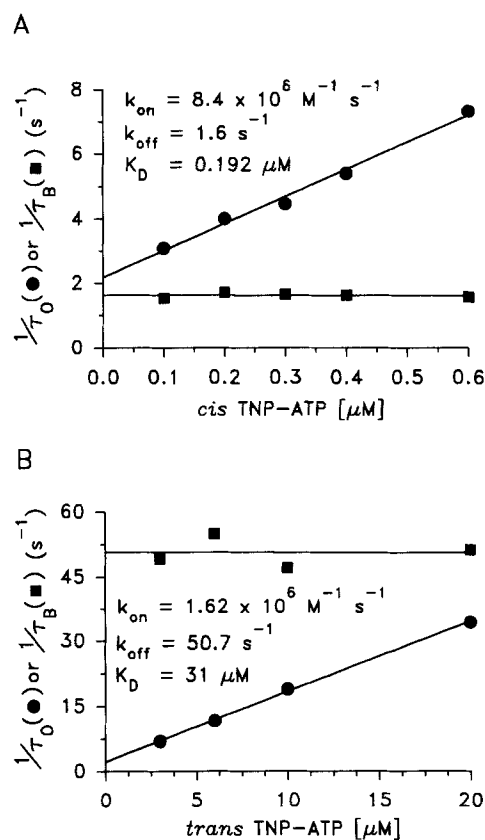


FIGURE 5. TNP-ATP concentration dependence of the reciprocal of the open (τ_o^{-1}) and blocked (τ_B^{-1}) time constants. TNP-ATP was added to either the *cis* (*A*) or *trans* (*B*) bath at the indicated concentrations. Time constants were estimated from event duration histograms similar to those shown in Fig. 4. Records at each concentration were selected to insure that a minimum of 2,000 events were present in each histogram (range 2,133–8,800 events).

confirmation of the estimates of k_{on} and k_{off} . The nearly 130-fold difference in the K_D for *cis* versus *trans* TNP-ATP (Table I) was due to a 4.5-fold difference in the k_{on} 's and a 28-fold difference in the k_{off} 's. The marked difference in kinetic constants provides further suggestive evidence that there are two TNP-ATP binding sites located on opposite sides of the membrane.⁴ However, these kinetic data do not exclude a more

⁴ Preliminary experiments revealed that the K_D 's were weakly voltage dependent (Venclarik, C. J., and R. J. Bridges, unpublished observations). However, the voltage dependence can only account for a three- to fivefold difference in *cis* versus *trans* K_D at -30 mV .

complicated hypothesis with a single binding site that is capable of discriminating between TNP-ATP in the *cis* or *trans* bath and additional experiments were performed to eliminate this possibility.

ATP and TNP-ATP Compete for Block When Added to the Same Side of the Channel

The equilibrium binding constants for TNP-ATP blockade of the rectifying colonic Cl⁻ channel presented in the previous sections fall within the range of the binding constants previously reported for other nucleotide binding proteins (Hiratsuka and Uchida, 1973; Grubmeyer and Penfensky, 1981a; Moczydlowski and Fortes, 1981b; Watanabe and Inesi, 1982; Rao et al., 1988; Rosen et al., 1988; Thomas et al., 1991). Thus, our results suggest that TNP-ATP binds to a nucleotide binding site on the Cl⁻ channel. The purpose of this section is to provide additional support for this notion by showing that TNP-ATP and ATP compete for block when added to the same side of the channel. Previous studies have shown that extracellular ATP blocks rectifying Cl⁻ channels (Manning and Williams, 1989; Alton et al., 1991). We recently obtained preliminary evidence that ATP caused a fast-type block of the rectifying rat colonic Cl⁻ channel and that ATP block also conformed to the linear closed-open-blocked kinetic model (Venglarik et al., 1991; Venglarik and Bridges, 1992). Since ATP and TNP-ATP have markedly different kinetic constants, it is possible to construct two simple models for competition and independence and predict the kinetic behavior of channels in the presence of a fixed amount of TNP-ATP and increasing concentrations of ATP. Comparing the predicted results with experimental data should reveal if ATP and TNP-ATP compete for block (Miller, 1988; Wang, 1988).

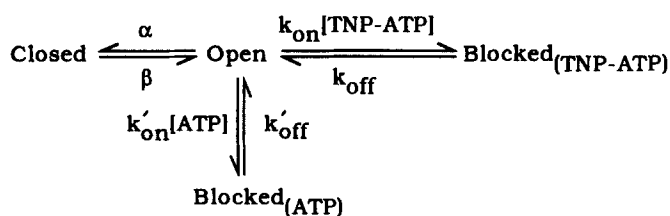
Combining the closed-open-blocked kinetic schemes for ATP and TNP-ATP blockade yields the two solutions shown in Fig. 6. The first model (Fig. 6 A) illustrates the kinetic scheme expected if ATP and TNP-ATP share a single site, which is the simplest model for competition between TNP-ATP and ATP. Note that the channel cannot be blocked simultaneously by TNP-ATP and ATP. The second model (Fig. 6 B) shows the kinetic scheme expected if ATP and TNP-ATP do not share a binding site. Note that this scheme contains an additional state representing simultaneous block by TNP-ATP and ATP.

Since ATP is a fast-type blocker and TNP-ATP produces discrete events, it is possible to distinguish between these two kinetic models by adding TNP-ATP and then comparing the effect of increasing ATP concentration on the ratio of the current amplitude obtained before (i_0) and after ATP addition (i) with \bar{T}_{burst} (Miller, 1988; Wang, 1988). \bar{T}_{burst} is defined as the mean time that the channel spends in the open and ATP-blocked states. Since the dwell times of the channel in the fully open and ATP-blocked states are too brief to be resolved by our recording system, the open and ATP-blocked states result in a current level with reduced amplitude that may be accompanied by an increased noise (Yellen, 1984; Venglarik et al., 1991). The advantage of comparing i_0/i and \bar{T}_{burst} is that both can be obtained directly from current records. Miller (1988) and Wang (1988) used a similar approach to study competition based on a two-state model of open channel block. Our analysis based on the closed-open-blocked three-state model yields similar results since the presence of additional transitions from the open state to closed or blocked states is not expected to affect the general outcome (see Appendix I).

The relationship between the concentration of fast blocker [ATP] and the ratio of the current amplitude before and after blocker addition (i_o/i) has been derived previously (Colquhoun and Hawks, 1982, 1983; Coronado and Miller, 1982; Miller, 1988):

$$i_o/i = 1 + [\text{ATP}]/K'_D \quad (1)$$

A



B

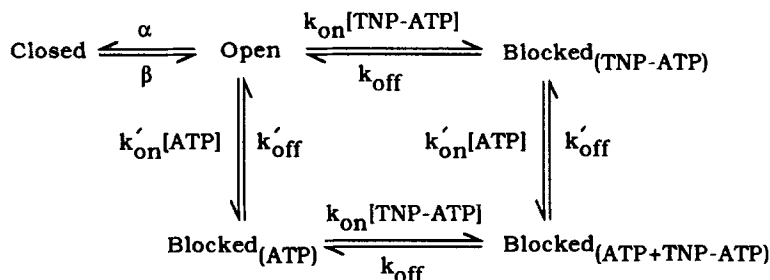


FIGURE 6. Two kinetic schemes that result from combining the linear closed-open-blocked models for ATP and TNP-ATP. Both models contain a single open state, a single closed state, an ATP blocked state and a TNP-ATP blocked state. Based on the closed-open-blocked models we expect that the channel can undergo reversible transitions from the open state to the closed state, the ATP blocked state, and the TNP-ATP blocked state. We also expect that the closed state cannot communicate with the various blocked states. *A* illustrates the kinetic scheme expected if ATP and TNP-ATP share the same binding site. *B* shows the kinetic scheme expected if ATP and TNP-ATP do not bind to the same site. *B* contains an additional state representing simultaneous block by TNP-ATP and ATP and assumes that the presence of nucleotide at one site does not influence the rate constants for the second site.

where K'_D represents the equilibrium dissociation constant for the fast-type blocker (ATP). This equation is a variation of the single-site Michaelis-Menten type saturation function.

The relationship between the concentration of fast blocker [ATP] and \bar{T}_{burst} was derived for the two models shown in Fig. 6 using approaches described by Colquhoun

and Hawks (1982, 1983). For the kinetic scheme shown in Fig. 6A, the simplest approach to obtain \bar{T}_{burst} is to sum the mean open time before the addition of ATP ($\tau_o = (\alpha + [\text{TNP-ATP}]k_{\text{on}})^{-1}$) and the total amount of time that the channel spends in the ATP blocked state ($([\text{ATP}/K'_D] (\alpha + [\text{TNP-ATP}]k_{\text{on}})^{-1})$). Thus it follows that

$$\bar{T}_{\text{burst}} = (\alpha + [\text{TNP-ATP}]k_{\text{on}})^{-1} (1 + [\text{ATP}/K'_D]) \quad (2)$$

Combining Eqs. 1 and 2 and substituting τ_o for $(\alpha + [\text{TNP-ATP}]k_{\text{on}})^{-1}$ yields:

$$\bar{T}_{\text{burst}} = \tau_o(i_o/i) \quad (3)$$

Therefore, plotting \bar{T}_{burst} as a function of i_o/i should produce a linear relationship with a slope equal to τ_o , which corresponds to the mean open time before ATP addition. Since the smallest value of i_o/i is 1, the corresponding y-intercept will also be τ_o .

For the kinetic scheme shown in Fig. 6B, one approach is to consider the open and fast blocked states within the burst as a single state. Therefore, the time that a channel spends in a burst state is the reciprocal of the sum of the three exit rates (Colquhoun and Hawks, 1983). These rates are: (a) $\alpha(1 + [\text{ATP}/K'_D])^{-1}$ for entering the closed state, (b) $([\text{TNP-ATP}]k_{\text{on}})(1 + [\text{ATP}/K'_D])^{-1}$ for going to the TNP-ATP blocked state, and (c) $([\text{TNP-ATP}]k_{\text{on}})(1 + K'_D/\text{ATP})^{-1}$ for entering the TNP-ATP and ATP blocked state. Adding the three rates, combining the terms containing $[\text{TNP-ATP}]k_{\text{on}}$, and taking the reciprocal yields:

$$\bar{T}_{\text{burst}} = (\alpha(1 + [\text{ATP}/K'_D])^{-1} + [\text{TNP-ATP}]k_{\text{on}})^{-1} \quad (4)$$

This equation shows that if ATP and TNP-ATP bind to separate sites \bar{T}_{burst} will vary as a nonlinear function of $(1 + [\text{ATP}/K'_D])$ or i_o/i . There are two ways to deal with this nonlinearity. First, when $\alpha < [\text{TNP-ATP}]k_{\text{on}}$, plotting \bar{T}_{burst} as a function of i_o/i will yield a plot with an intercept of τ_o , which corresponds to the mean open time before ATP addition and a slope of approximately zero. Second, it is possible to linearize Eq. 4 with respect to $(1 + [\text{ATP}/K'_D])^{-1}$, which corresponds to i/i_o from Eq. 1 by taking the reciprocal of both sides. Since we can ensure that $\alpha < [\text{TNP-ATP}]k_{\text{on}}$ by choosing appropriate concentrations of TNP-ATP, we will use the first method to avoid the nonlinearity.

Having derived the two predicted relationships between \bar{T}_{burst} and i_o/i , we conducted experiments to test the hypothesis that TNP-ATP and ATP compete for block when present in the same bath (Fig. 7A). We added a fixed concentration of TNP-ATP (0.60 μM *cis* or 20 μM *trans*) and then added increasing amounts of ATP to the same bath. Fig. 7A shows a control single channel current trace, current traces after addition of 0.6 μM TNP-ATP to the *cis* bath, and channel activity in the presence of 0.6 μM TNP-ATP and 1 mM ATP in the *cis* bath. Fig. 7B shows the single channel current records for a similar experiment where TNP-ATP (20 μM) and ATP (20 mM) were added to the *trans* bath. These records show that addition of ATP to the same bath containing TNP-ATP decreased the current amplitude and increased the time the channel spent bursting.

Fig. 8 plots \bar{T}_{burst} as a function of (i_o/i) for the *cis* (A) and *trans* (B) additions of TNP-ATP and ATP for the experiments shown in Fig. 7 and includes results obtained at several additional ATP concentrations. The lines illustrate the predicted relation-

ship from Eq. 3 if TNP-ATP and ATP compete for block. Similar results were obtained in six additional experiments (data not shown). It is clear from Fig. 8 that ATP and TNP-ATP compete for block since the addition of ATP increased \bar{T}_{burst} . In fact, the deviation of the data above the theoretical relationship suggests that ATP is

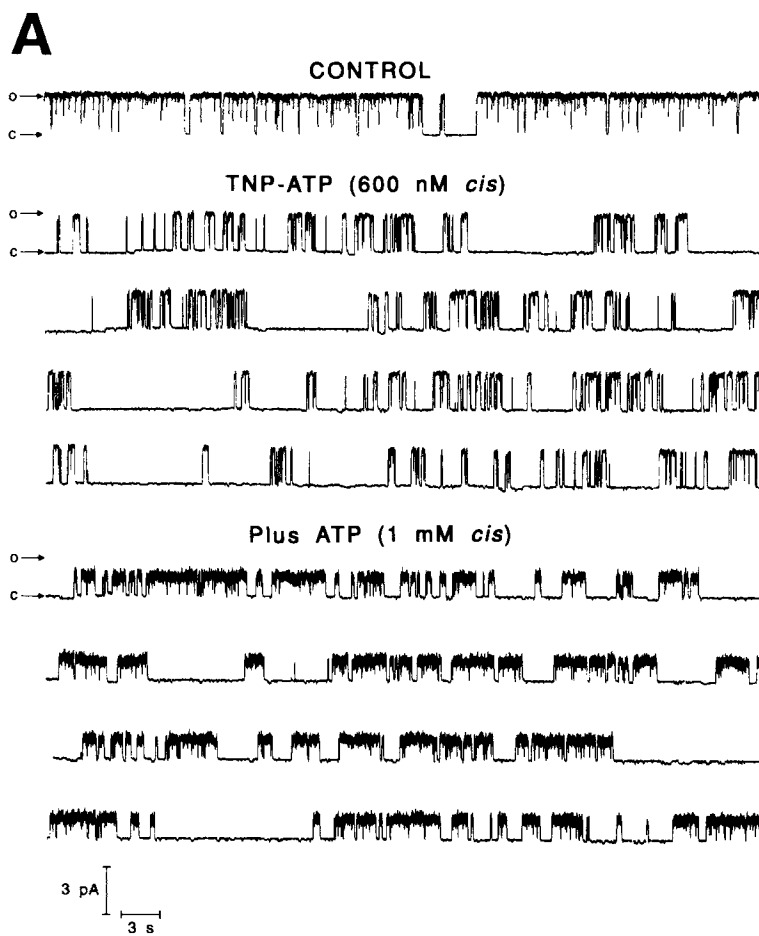


FIGURE 7. Current traces of Cl^- channel activity that demonstrate the competitive interaction of TNP-ATP and ATP when added to the same bath. The upper records show control channel activity. The middle records were obtained after addition of $0.6 \mu M$ TNP-ATP to the *cis* bath (A) or $20 \mu M$ TNP-ATP to the *trans* bath (B). The bottom records are channel activity after addition of $0.6 \mu M$ TNP-ATP and $1 mM$ ATP to the *cis* bath (A) or $20 \mu M$ TNP-ATP and $20 mM$ ATP to the *trans* bath (B). Note that the time scale is 10-fold less in B. Conditions are the same as in Fig. 2.

better at displacing TNP-ATP than it is at blocking the channel. This unexpected result indicates that the kinetic model (Fig. 6A) could be revised to include an ATP-occupied open state. However, it is unclear if this additional state is best described by a simple modification of the single-site model shown in Fig. 6 to include

an intermediate ATP-occupied open state between the open and blocked states (Castillo and Katz, 1957), which can account for the deviation as described in Appendix II, or if the model should be more extensively modified to include additional ATP binding sites with no cooperativity (Colquhoun and Ogden, 1988; Bean, 1990). These hypotheses could be tested in future studies.

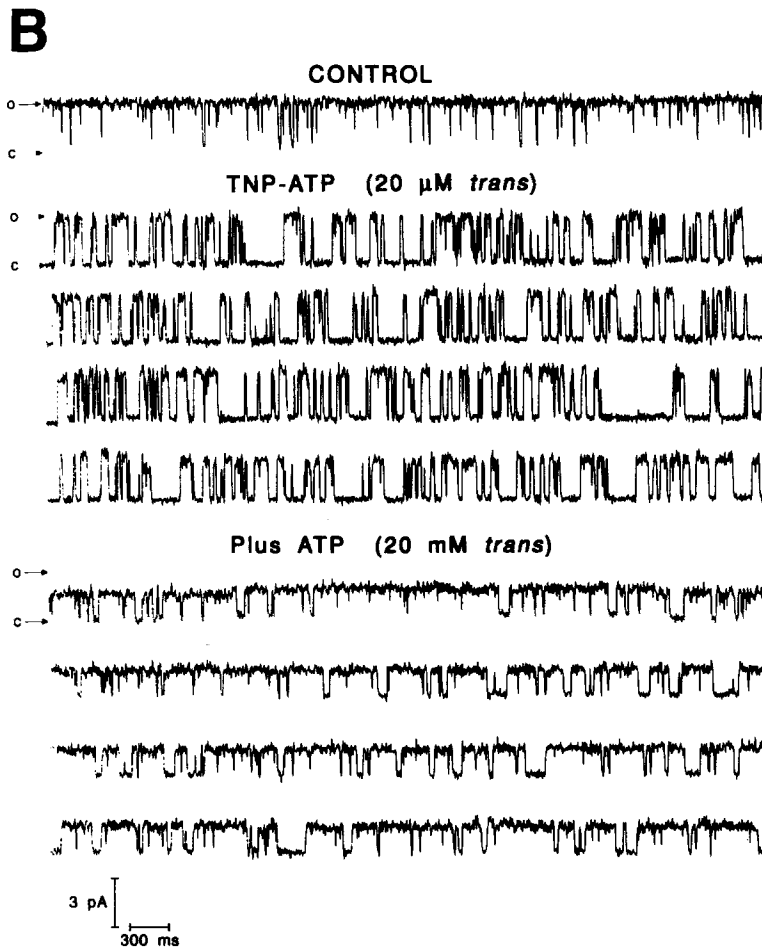


FIGURE 7.

ATP and TNP-ATP Bind to Separate Sites When Added to Opposite Sides of the Colonic Cl⁻ Channel

The analysis derived in the previous section to show that TNP-ATP competes with ATP for block when both were added to the same side of the channel can also be used to test the hypothesis that there are different nucleotide binding sites located on opposite sides of the channel. The markedly different *cis* versus *trans* kinetic constants

in Table I provide indirect support for this hypothesis. Since we can ensure that $\alpha < [\text{TNP-ATP}]k_{\text{on}}$ by choosing an appropriate concentration of TNP-ATP, we expect that \bar{T}_{burst} will remain fairly constant when plotted as a function of i_o/i if there are two different sites. Fig. 9 plots \bar{T}_{burst} as a function of (i_o/i) for two experiments with opposite-sided addition of TNP-ATP and increasing concentrations of ATP. The data presented in Fig. 9A were obtained with 0.60 μM TNP-ATP in the *cis* bath and varying concentrations of *trans* ATP. The data shown in Fig. 9B were obtained with 20 μM TNP-ATP in the *trans* bath and varying concentrations of *cis* ATP. The current traces from these experiments (not shown) clearly indicated that ATP caused a

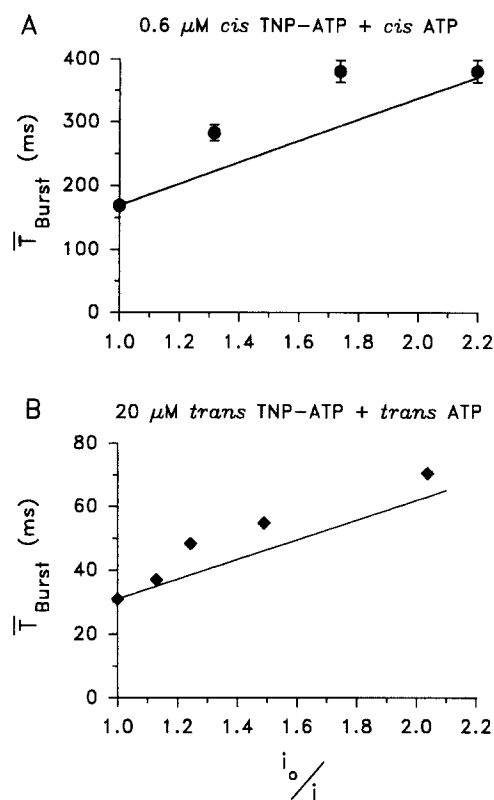


FIGURE 8. Effect of same-side addition of ATP on the mean burst duration (\bar{T}_{burst}) and change in apparent current amplitude (i_o/i) of TNP-ATP blocked channels. Experiments were conducted as described in Fig. 7. TNP-ATP was added to the *cis* bath (A; 0.6 μM) or the *trans* bath (B; 20 μM) followed by ATP addition to the same side. Each data point denotes the \bar{T}_{burst} calculated for 500–5,000 bursts as a function of the corresponding i_o/i obtained for a single ATP concentration. A critical closed time of 2.5 ms was used to estimate the burst duration from the idealized records (Colquhoun and Hawks, 1982). Error bars are shown except when smaller than the symbols. The lines illustrate the theoretical relation predicted for a competitive-type interaction given by Eq. 3.

decrease in current amplitude but did not alter burst duration. Thus, as shown in Fig. 9, \bar{T}_{burst} did not vary with i_o/i when ATP was added to the side opposite TNP-ATP. These data provide strong evidence that there are two nucleotide binding sites located on opposite sides of the channel, and that both sites can be simultaneously and independently occupied by nucleotides.

DISCUSSION

Our results show that TNP-ATP blocked reconstituted rectifying colonic Cl channels when added to either the extracellular or the cytoplasmic side. In both cases blockade

conformed to the closed-open-blocked kinetic model. Reconstituting the channels in the absence of cytoplasmic second messengers and the agreement of our data with this simple kinetic model provides strong evidence that the binding sites are part of the channel. Based on the concentration of TNP-ATP causing a 50% reduction in P_o (0.27 μM), we have identified a blocker that has a nearly 10-fold higher affinity for reconstituted rectifying colonic Cl⁻ channels than any previously reported blocker. Thus, TNP-ATP should be useful in future studies of ion channel nucleotide binding sites and possibly in preliminary steps of channel protein purification. In addition, we have obtained good evidence that there are at least two nucleotide binding sites

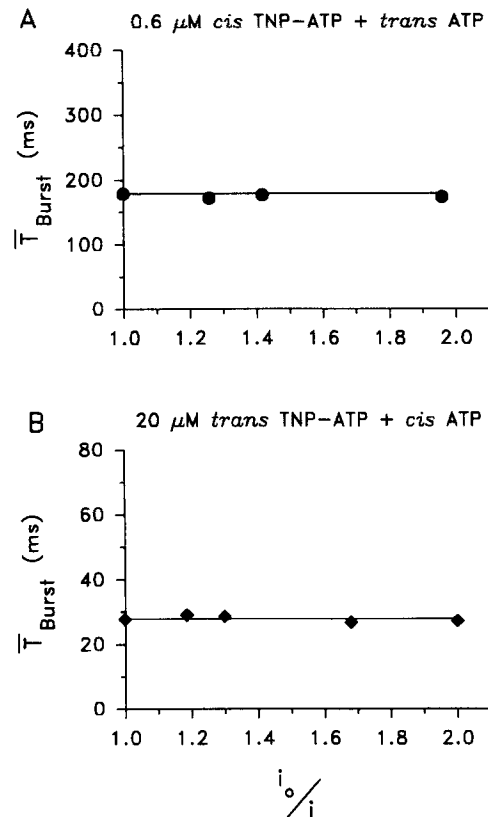


FIGURE 9. Effect of opposite-side addition of ATP on the mean burst duration (\bar{T}_{burst}) and change in apparent current amplitude (i_o/i) of TNP-ATP blocked channels. Experiments were conducted as described in Fig. 7, except that ATP was added to the side containing no TNP-ATP. TNP-ATP was added to the *cis* bath (A; 0.6 μM) or to the *trans* bath (B; 20 μM) followed by ATP addition to the opposite bath. Each data point plots the \bar{T}_{burst} calculated for 500–5,000 bursts as a function of the corresponding i_o/i obtained for a single ATP concentration. Error bars are shown except where they were smaller than the symbols. The lines illustrate the theoretical relationship predicted if TNP-ATP and ATP block independently (Eq. 4) and if $\alpha < k_{\text{on}}[\text{TNP-ATP}]$.

located on opposite sides of the colonic Cl⁻ channel and that occupancy of either site leads to a blocked state. We have also extended a method of burst analysis (Colquhoun and Hawks, 1982; Coronado and Miller, 1982; Miller, 1988; Wang, 1988) to test for competition between open channel blockers for channels that undergo additional transitions to nonconducting states from the open state. Analysis of the bursting behavior of a channel can be used to test for a competitive interaction between open channel blockers as well as for the independence of blocker binding sites.

Comparison of TNP-ATP with Other Cl⁻ Channel Blockers

Comparing the kinetic constants of TNP-ATP (Table I) with results presented in an earlier study of three other classes of open channel blockers (i.e., disulfonic stilbenes, anthranilic acids, and indanyl alkanolic acids) on reconstituted colonic Cl⁻ channels (Singh et al., 1991) yields several interesting observations. Based on the concentration needed for half-maximal reduction of P_o (0.27 μ M), TNP-ATP is at least 10 times more potent from the *cis* (extracellular) side than any of the blockers previously tested and is approximately equipotent to the best blocker from the *trans* (cytoplasmic) side. Thus, TNP-ATP is an excellent probe for future studies of rectifying Cl⁻ channels.

The side-dependent difference in k_{off} 's for TNP-ATP represents a significant departure from our earlier study where the blockers had similar k_{off} 's regardless of the side of addition (Singh et al., 1991). The similarity in k_{off} 's for the disulfonic stilbene, 4,4'-dinitro-stilbene-2,2'-sulfonic acid (DNDS) and the indanyl alkanolic acid, 2-[(cyclopentyl-6,7-dichloro-2,3-di-hydro-2-methyl-1-oxo-1H-inden-5-yl)oxy] acetic acid (IAA-94), from the *cis* and *trans* sides was used to argue for the presence of a single blocker binding site that was accessible from either side of the open channel (DNDS, 52 s⁻¹ *cis* versus 63 s⁻¹ *trans*; IAA-94, 107 s⁻¹ *cis* versus 99 s⁻¹ *trans*). In contrast, the nearly 29-fold difference in the *cis* k_{off} (1.9 s⁻¹) versus *trans* k_{off} (55 s⁻¹) for TNP-ATP suggests that there are two TNP-ATP binding sites and we provide additional evidence for separate binding sites in subsequent experiments (i.e., Fig. 9). Consequently, it is unclear how the single binding site proposed for the three other classes of blockers might be related to the two nucleotide binding sites reported herein. However, future experiments designed to test for interaction between ATP, TNP-ATP, and these other blockers should reveal whether the other classes of blockers bind to the nucleotide binding sites identified in this study.

Comparison of Ion Channel ATP Binding Sites

ATP binds to at least three other types of ion channels in addition to rectifying Cl⁻ channels. The presence of extracellular ATP activates cation-selective channels in a variety of tissues (Krishtal, Marchenko, and Pidoplichko, 1983; for review see Bean, 1992). Conversely, intracellular ATP blocks channels that are K⁺ selective (Noma, 1983; for review see Ashcroft, 1988). Recent evidence indicates that the Cl⁻ channel that is defective in CF can be activated by intracellular ATP (Riordan, Rommens, Kerem, Alon, Rozmahel, Grelczak, Zielenski, Lok, Pavsic, Chou et al., 1989; Anderson, Berger, Rich, Gregory, Smith, and Welsh, 1991). Finally, several reports, including this study, document that rectifying Cl⁻ channels can be blocked by extracellular ATP (Manning and Williams, 1989; Alton et al., 1991). Thus there are both cation and anion channels with nucleotide binding sites on either the extracellular or intracellular side and ATP produces either blockade or activation.

The presence of more than one binding site is a conserved feature of many ATP-gated channels (Ashcroft, 1988; Riordan et al., 1989; Bean, 1992). For example, Hill coefficients of 2–3 have been reported for blockade of K channels by cytosolic ATP and for activation of cation channels by extracellular ATP (Ashcroft, 1988; Bean, 1992). Thus, both families of channels possess members with multiple cooperative

binding sites. In addition, deviations in the low concentration range of the concentration-effect curve of extracellular ATP-activated cation channels provide evidence for multiple noncooperative binding sites (Colquhoun and Ogden, 1988; Bean, 1990). We have also obtained good evidence that the rat colonic Cl⁻ channels possess at least two nucleotide binding sites. An important difference between the other ATP-gated channels and the rectifying rat colonic Cl⁻ channel is the topology of the binding sites. For each of the other ATP-gated channels the multiple nucleotide binding sites are restricted to the same side of the channel. In contrast, the rat colonic Cl⁻ channel possesses at least two nucleotide binding sites on opposite sides of the membrane. Hence, the opposite-sided topology of the nucleotide binding sites in the reconstituted rectifying Cl⁻ channels is a novel observation. Further investigation should reveal if other ATP-gated channels have multiple binding sites on opposite sides of the membrane. However, our results suggest that care should be taken in deducing the location of nucleotide binding sites for other ion channel proteins from the primary structure and resulting hydropathy plots.

A detailed comparison of ion channel nucleotide binding site pharmacology should reveal additional similarities and differences. Many nucleotide derivatives and several other unrelated compounds have previously been shown to affect ATP-gated cation channels (Ashcroft, 1988; Bean, 1992). These agents could also be tested for possible effect on rectifying Cl⁻ channels. Likewise, TNP-ATP could be a useful probe in future studies of the other types of ATP-gated ion channels.

Significance of the Nucleotide Binding Sites

One question that arises from this study concerns the physiological roles of the nucleotide binding sites. Although extracellular ATP activates cation-selective channels in a variety of cell types (Bean, 1992) and blocks rectifying Cl⁻ channels (Manning and Williams, 1989; Alton et al., 1991) as shown in Fig. 8, the role of these extracellular ATP binding sites has not been clearly identified. One possible function of the external ATP binding site is to report cell injury. Additionally, since ATP is copackaged with neurotransmitters in synaptic vesicles (Sneddon, Westfall, and Fedan, 1983), it may have a transmitter-like role. Based on this observation, we can also speculate that the presence of ATP within secretory vesicles regulates channels before exocytosis. The role of intracellular ATP on ion channels is less ambiguous since there is good evidence that intracellular ATP binding sites couple cation channel gating to the metabolic status of the cell (Ashcroft, Harrison, and Ashcroft, 1984). Presumably the extracellular and intracellular nucleotide binding sites associated with rectifying Cl⁻ channels have similar functions. However, additional experiments are clearly needed to address these issues and TNP-ATP may be a useful probe in these studies.

Testing for Interaction between Blockers

We extended an approach used by Miller (1988) and Wang (1988) to test for interaction between open channel blockers. We were specifically interested in answering two questions: (a) Are the TNP-ATP binding sites nucleotide binding sites? and (b) Are there two binding sites located on opposite sides of the channel? The criterion used in previous studies to test whether TNP-ATP was binding to a

nucleotide binding site was to provide evidence showing that ATP and TNP-ATP compete for binding. In general, inhibition of ATP hydrolysis by TNP-ATP (Moczydlowski and Fortes, 1981*b*; Grubmeyer and Penfensky, 1981*a*) or a quench of bound TNP-ATP fluorescence by ATP (Watanabe and Inesi, 1982; Rosen et al., 1988; Karkaria and Rosen, 1991) was used to assay for competition. In our study we took advantage of the marked difference in the TNP-ATP and ATP blocked states to test for competition as well as the presence of two binding sites located on opposite sides of the channel. Comparison of the outcome predicted from the derived kinetic equations (Eqs. 3 and 4) with the experimental results clearly demonstrates that TNP-ATP and ATP compete for block when added to the same side of the bilayer (Figs. 6 and 7). However, these data do not exclude the possibility that there are separate ATP and TNP-ATP binding sites with an allosteric mechanism of mutual exclusion (Miller, 1988). The structural similarity of TNP-ATP and ATP (Fig. 1), the relatively high affinity of TNP-ATP for rectifying Cl⁻ channels (Fig. 3 and Table I), and the notion that ATP is better at displacing TNP-ATP than it is at blocking the channel (Fig. 8) suggest that an allosteric mechanism is unlikely. The simplest interpretation is that TNP-ATP blocks the channel by binding to a nucleotide binding site. Moreover, we demonstrate that ATP and TNP-ATP do not bind to the same nucleotide binding site when present on opposite sides of the channel (Fig. 9). Based on these results, we can conclude that there are at least two different nucleotide binding sites located on opposite sides of the reconstituted colonic Cl⁻ channel.

The extended method of burst analysis we present provides a general approach that can be used to test for the competitive interaction between open channel blockers as well as the independence of blocker binding sites. This approach can be applied to any channel and set of blockers provided that: (a) there is a single open state, (b) the off rates of the blockers are different enough from each other and from the closing rate constant(s) to permit bursting periods to be defined and measured, and (c) the channel can only enter the closed or blocked states from the open state. For example, it is now possible to determine whether the various different classes of Cl⁻ channel blockers (Singh et al., 1991) competitively interact with ATP. Similarly, this approach can be applied to Ca²⁺ channels (Winegar and Lansman, 1990) and NMDA receptor-mediated channels (Huettnner and Bean, 1988; Christine and Choi, 1990) to test for blocker interaction or independence. Therefore, this extended analysis can provide important new insights regarding ion channel blocker binding sites.

APPENDIX

I. Possible Effect of Contaminant TNP-ADP in Tests for Interaction between ATP and TNP-ATP

It is of interest to note that the contaminant TNP-ADP did not affect the outcome of experiments testing for competition between TNP-ATP and ATP. This is because the kinetic schemes in Fig. 6 can be modified to include additional closed or blocked states and will produce the same general relationships presented in Eqs. 3 and 4. Since TNP-ADP is an open channel blocker (Venglarik, C. J., and R. J. Bridges, unpublished observations), it is possible to construct the two additional kinetic

models (not shown) and derive two equations that can be used to predict channel behavior in the presence of a fixed amount of two open channel blockers ([TNP-ATP] and [TNP-ADP]) and varying concentrations of a third ([ATP]). Assuming that TNP-ADP, TNP-ATP, and ATP all compete for the same site yields:

$$\bar{T}_{\text{burst}} = (\alpha + [\text{TNP-ATP}]k_{\text{on}} + [\text{TNP-ADP}]k''_{\text{on}})^{-1} (1 + [\text{ATP}]/K'_D) \quad (\text{A1})$$

This equation can be obtained in the same way as Eq. 2. $[\text{TNP-ADP}]k''_{\text{on}}$ is the product of the on rate for TNP-ADP and the TNP-ADP concentration. Combining Eqs. 1 and A1 and substituting τ_o for $(\alpha + [\text{TNP-ATP}]k_{\text{on}} + [\text{TNP-ADP}]k''_{\text{on}})^{-1}$ yields Eq. 3.

Assuming that TNP-ATP and TNP-ADP share the same sites and that ATP binds to separate sites yields:

$$\bar{T}_{\text{burst}} = \{\alpha(1 + [\text{ATP}]/K'_D)^{-1} + [\text{TNP-ATP}]k_{\text{on}} + [\text{TNP-ADP}]k''_{\text{on}}\}^{-1} \quad (\text{A2})$$

This equation can be obtained in the same way as Eq. 4. When $\alpha < [\text{TNP-ATP}]k_{\text{on}}$, plotting \bar{T}_{burst} as a function of i_o/i will yield a plot with an intercept of τ_o , which corresponds to the mean open time before ATP addition, and a slope of approximately zero, which is also the result expected for Eq. 4. Therefore, the presence of contaminant TNP-ADP will alter the value of τ_o but will not affect the general outcome of the analysis. Furthermore, this analysis of TNP-ADP block underscores a general conclusion that additional or fewer open-closed transitions will yield identical results. Thus, Eqs. 3 and 4 are directly related to those derived by Miller (1988) and Wang (1988) using less complicated two-state models for open channel block.

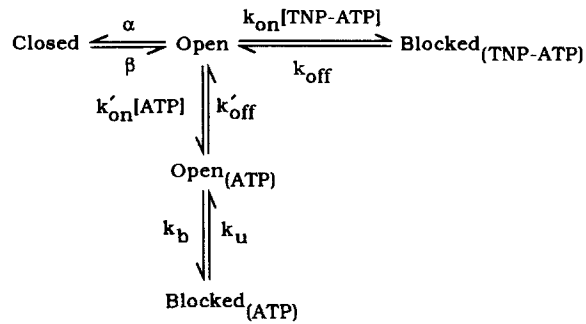
II. A Simple Modification of the Model for Competition Can Account for the Observation That ATP Is Better at Displacing TNP-ATP Than It Is at Blocking the Channel

In Fig. 8 the data consistently deviate above the theoretical relationship, which indicates that ATP is better at preventing TNP-ATP binding than it is at blocking the channel. This result suggests that the kinetic model (Fig. 6A) should be revised to include an ATP-occupied open state. A simple revision of the model which may account for this difference is shown in Fig. 10 and includes an intermediate ATP-occupied open state. This intermediate state is equivalent to the bound inactive state, which has been used to describe enzyme kinetics and ligand-activated channels (Castillo and Katz, 1957). We derived the expected relationship between \bar{T}_{burst} and i_o/i for this simple model, fitted the data in Fig. 8 to the expected function, and found good agreement. Thus we can provide a simple explanation for the difference between the data and the predicted function in Fig. 8.

The equation describing the relationship between the mean time that the channel spends bursting (\bar{T}_{burst}) and the ratio of the current amplitude before and after ATP addition (i_o/i) can be derived in the same way as outlined for Eqs. 1–3:

$$\bar{T}_{\text{burst}} = \tau_o(i_o/i) (1 + k_u/k_b) \quad (\text{A3})$$

where τ_o corresponds to the mean open time before ATP addition and k_u/k_b represents the ratio of the unblocking and blocking rate constants. Note that the plot of \bar{T}_{burst} as a function of i_o/i is a linear relationship with a slope of $\tau_o (1 + k_u/k_b)$ and a y-intercept (i.e., $i_o/i = 1$) of τ_o . As shown in Fig. 11, A and B, the data shown in Fig.



for the presence of the intermediate ATP-occupied open state and the additional set of transitions consisting of blocking (k_b) and unblocking (k_u) rate constants.

FIGURE 10. Modified kinetic scheme that includes an intermediate ATP-occupied open state ($Open_{(\text{ATP})}$). This kinetic model contains a single closed state, an unoccupied open state, a TNP-ATP blocked state, an ATP-occupied open state, and an ATP blocked state. The transitions are identical to those shown in Fig. 6A except

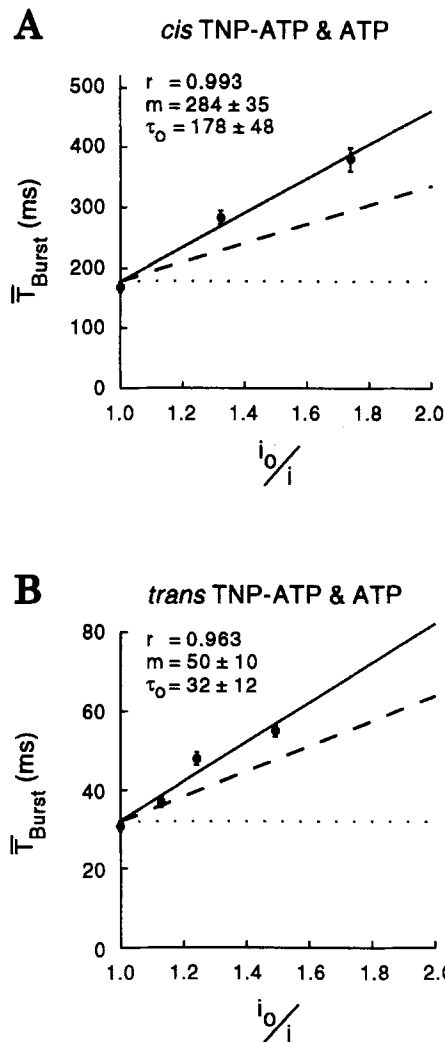


FIGURE 11. Comparison of the data shown in Fig. 8 with the predicted relationship derived for the modified kinetic model illustrated in Fig. 10. From Eq. A3 we expect the data in Fig. 8 to conform to linear functions with intercepts of τ_o and slopes (m) of $\tau_o (1 + k_u/k_b)$. Therefore, we fit these data using linear regression and the solid lines illustrate the best fits. The estimated y-axis intercept (τ_o), slope (m), and Pearson r (r) are indicated in the figures. The estimates of \bar{T}_{burst} when $i_o/i > 2$ were excluded since the signal to noise ratio was $\sim 2.5:1$ at $i_o/i = 2$ and these data are not reliable. The dashed lines represent the predicted results for a competitive-type interaction as given by Eq. 3 based on the estimate of τ_o from the fit. Additionally, the figures include dotted lines which illustrate the expected results for an independent-type interaction (Eq. 4).

8 are well described by this equation, excluding the estimates of \bar{T}_{burst} when $i_o/i > 2$. Since the signal to noise ratio is $\sim 2.5:1$ at $i_o/i = 2$, these data are not reliable. The intercept (τ_o) is 178 ± 48 ms for *cis* addition of ATP (Fig. 11 A) and 32 ± 12 ms for *trans* additions of ATP (Fig. 11 B) and the slope (m) is 284 ± 35 ms for *cis* addition of ATP and 50 ± 12 ms for *trans* additions. The ratio of the unblocking and blocking rate constants (k_u/k_b) can be calculated from τ_o and m and is 0.59 for *cis* addition of ATP and 0.56 for *trans* additions of ATP. However, despite the good agreement between our data and the theoretical functions (Pearson $r = 0.993$ in Fig. 11 A and 0.963 in Fig. 11 B) we cannot exclude more complicated models, and this possibility could be tested in future studies.

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