ATP-Dependent Activation of the Atrial Acetylcholine-Induced K⁺ Channel Does Not Require Nucleoside Diphosphate Kinase Activity

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Abstract—Prior reports by others have shown that cytoplasmically applied ATP can activate the acetylcholine-induced K⁺ channel in inside-out atrial membrane patches when no guanine nucleotides are present in the solution bathing the cytosolic face of the membrane. A nucleoside diphosphate kinase mechanism was proposed to explain the activation by ATP. We show in the present study that cytoplasmic adenylylimidodiphosphate mimics the activation by ATP. Unlike ATP, the activation by adenylylimidodiphosphate does not subside on washout. Although commercially available adenylylimidodiphosphate is contaminated by guanylylimidodiphosphate, the activation by adenylylimidodiphosphate still occurs after HPLC purification to remove guanine nucleotide contamination. Adenylylimidodiphosphate does not support phosphotransferase activity by nucleoside diphosphate kinase. Therefore, nucleoside diphosphate kinase activity cannot explain the activation of atrial acetylcholine-induced K⁺ current by ATP and adenylylimidodiphosphate. We hypothesize that the activation by millimolar concentrations of ATP is due to binding of adenosine nucleotide to the guanine nucleotide binding site of the G protein(s) responsible for stimulating the acetylcholine-induced K⁺ current. (Circ Res. 1998;82:971-979.)

Key Words: acetylcholine-induced K⁺ channel ■ adenylylimidodiphosphate ■ ATP ■ atrial myocyte ■ nucleoside diphosphate kinase

The acetylcholine-induced K⁺ current, I_{K,ACH}, plays a major role in vagal control of the heart. Activation of I_{K,ACH} contributes to vagally mediated slowing of spontaneous rate, slowing of atrioventricular nodal conduction, and decreasing the force of contraction of the atria. The mechanism of activation is well studied but still incompletely understood. Muscarinic agonists stimulate I_{K,ACH} via a membrane-delimited pathway.¹ A pertussis toxin–sensitive G protein couples the cardiac m2 muscarinic receptor to the channel.² There is evidence to suggest that both the α-subunit from the Gi family of G proteins or G-protein βγ-subunits can activate I_{K,ACH}³–⁸ Physiological activation of I_{K,ACH} requires an extracellular agonist (either acetylcholine or adenosine), a membrane-bound receptor, and intracellular GTP. The channel can also be activated by hydrolysis-resistant analogues of GTP at the intracellular side of the membrane.⁹,¹⁰

G proteins are a family of heterotrimeric proteins that serve as transducing elements for a large number of cell surface receptors. In the absence of agonist, G proteins exist predominantly in the inactive heterotrimeric (αβγ) GDP-ligated state. This is because the rate of hydrolysis of GTP by the G-protein α-subunit is much faster than the rate of dissociation of GDP (reviewed in Reference 11). The binding of an agonist to a G-protein–coupled receptor increases the dissociation rate for GDP, allowing a significant fraction of the subset of G proteins that are coupled to that receptor to bind GTP and become activated. The activated G protein is thought to dissociate into α- and βγ-subunits.¹¹ Both portions of the G protein can then interact with downstream effector molecules. In the case of I_{K,ACH}, the channel protein is the effector molecule. The G protein deactivates by hydrolyzing GTP to GDP. The GDP-ligated state favors the reassociation of the α- and βγ-subunits and terminates the response if agonist is no longer present.¹² In frog atrial cells, indirect measures of the basal GDP dissociation rate from G_{i} resulted in an off rate of 0.3 min⁻¹ in the absence of agonist.¹³ The rate of GTP hydrolysis by G_{i} was estimated to be 135 min⁻¹.¹⁰

In the absence of intracellular guanine nucleotides, I_{K,ACH} can be activated by millimolar concentrations of ATP on the cytoplasmic face of inside-out membrane patches.¹²,¹³ The stimulation by ATP can occur in the absence of agonists¹²,¹³ but is not inhibited by the absence of agonists.¹⁴ The effects of ATP can be inhibited by nanomolar concentrations of GTP or GDP.¹²,¹³ Two groups concluded independently that an NDP kinase was responsible for the effect of ATP.¹²,¹³ This interpretation was based on the observations that (1) the effect of ATP required Mg²⁺, (2) the hydrolysis-resistant ATP analogue AppNHp did not activate I_{K,ACH} in their hands, (3) ATPγS mimicked the effect of ATP, and (4) the effect of ATP was inhibited by cytoplasmic guanine nucleotides.¹²,¹³
Since these studies were conducted on inside-out membrane patches in the absence of cytoplasmic guanine nucleotide, the hypothetical NDP kinase mechanism required the kinase to phosphorylate GDP while it was bound to the G-protein α-subunit. A whole-cell patch-clamp study using intracellular dialysis with ATPγS also supported the notion that NDP kinase activity could affect $I_{\text{K,ACH}}$. The data were consistent with the proposal that ATPγS served as a phosphate donor for the conversion of cellular GDP to GTPγS. A subsequent biochemical study demonstrated that NDP kinase was present in atrial membranes. One difficulty with the NDP kinase hypothesis is the requirement for phosphorylation of GDP while it is bound to the G-protein α-subunit. Attempts to demonstrate phosphorylation of G-protein–bound GDP were not successful. Experiments with two anti-NDP kinase antibodies, one that inhibited the phosphotransferase activity and one that did not, supported the idea that NDP kinase was involved in agonist-dependent activation of $I_{\text{K,ACH}}$. Notably, it was concluded that the action of NDP kinase on agonist-dependent activation of $I_{\text{K,ACH}}$ did not involve the conversion of GDP to GTP. However, antibody experiments suggested that NDP kinase activity was not required for agonist-independent activation of $I_{\text{K,ACH}}$ by 4 mmol/L ATP.

The present study resulted from a serendipitous observation. We were investigating $I_{\text{K,ACH}}$ activity in inside-out membrane patches. AppNHp was included in the bath solution to prevent the activation of $I_{\text{K,ATP}}$ after patch excision. Surprisingly, we observed an activation of $I_{\text{K,ACH}}$ that was similar to the ATP-dependent activation described by others. This initiated a re-investigation of the effect of AppNHp on $I_{\text{K,ACH}}$.

Materials and Methods

Primary cultures of canine atrial cells were prepared as previously described. Cells were used between 1 and 4 days in culture. The inside-out configuration of the patch-clamp technique was used to record single-channel activity. Dow Corning Corporation) had resistances of 5 to 20 MΩ when filled with the pipette solution. An Axoclamp 1D amplifier was used at a gain of 100 mV/pA and a low-pass filter setting of 2 or 5 kHz. The amplifier voltage offset was adjusted to zero before forming a high-resistance seal.

Vesicles or restricted access patches were frequently formed after patch excision. We could successfully convert some of the restricted access patches to an inside-out membrane patch by briefly moving the pipette tip out of the bath solution into air. Patches that did not respond to bath application of 2 mmol/L ATP with an increase in $I_{\text{K,ACH}}$ channel activity that reached a peak by 3 minutes were rejected.

Current and voltage were recorded using a modified videocassette recorder (model 420 CR, A.R. Vetter) and analyzed off-line using PClamp 6.02 software (Axon Instruments). A Labmaster TL-1 board (Techmar) was used to digitize the data at 8, 10, or 20 kHz. Sampling frequency was chosen to be at least four times the corner frequency of the low-pass filter used before digitization.

NP was determined either by dividing the time integral for channel activity by the product of the single-channel current amplitude and the time interval being analyzed or by event lists generated using the Fetchan module of PClamp 6.02. NP was determined from event lists using the PStat module of PClamp 6.02. All patches used for this study contained multiple channels. Estimates of $\tau_n$ were made from portions of traces with infrequent overlapping channel events. Open-time histograms with a minimum of 750 openings were fit with a single exponential using a maximum likelihood estimator to determine $\tau_n$.

Positive ions flowing from the extracellular to the cytoplasmic side of the membrane are represented as downward deflections (i.e., the normal whole-cell current convention is used). The voltages reported correspond to the voltage on the cytoplasmic side of the membrane relative to the solution in the patch electrode on the extracellular side of the membrane. Patches used for this study either had no ATP-sensitive K+ channels or were used after the activity of ATP-sensitive channels had run down.

Symmetrical bath and pipette filling solutions were used. The solutions contained (mmol/L) potassium aspartate 150, MgCl2 0.3, or 10, HEPES 10 (titrated to pH 7.25 with KOH), dextrose 5.5, and EGTA 1. In experiments with 0 or 3 mmol/L MgCl2, 10 mmol/L KC1 was added to the solutions. When used, the disodium or magnesium salt of ATP, AppNHp, or GDP was added directly to the bath solution. Calculated free Mg2+ concentrations with 3 mmol/L total Mg2+ were 9.64 and 7.73 mmol/L in the absence and presence, respectively, of 2 mmol/L disodium ATP. Calculated free Mg2+ concentrations with 3 mmol/L total Mg2+ were 2.86 and 1.15 mmol/L in the absence and presence, respectively, of 2 mmol/L disodium ATP. When ATP was added, the pH was adjusted back to 7.25 with KOH. A conventional gravity-fed flow system was used for these studies. The flow rate was ~4 mL/min. The bath volume was 0.8 mL. All studies were performed at room temperature (21°C to 23°C).

For one set of experiments (Figure 8), a fast-flow system was used to change the solution superfusing the cytoplasmic face of inside-out membrane patches within 2 seconds. We have described this fast-flow system previously. A six-inlet single-outlet polytetrafluoroethylene manifold was used for the fast-flow system. The membrane patch was placed in the opening of the polyethylene outlet tube (1.14-mm internal diameter). Whole-cell currents were measured as previously described. Holding potential was −10 mV in whole-cell experiments. Currents were measured in response to slow hyperpolarizing voltage ramps (~7 mV/s). The bath solution for this study contained (mmol/L) NaCl 144, KCl 25, HEPES-NaOH 10 (pH 7.4), CaCl2 1.8, MgCl2 1, glucose 5.5, and glibenclamide 0.01. When present, the concentration of carbacol was 10 μmol/L. The electrode filling solution contained (mmol/L) potassium aspartate 125, KCl 15, HEPES-KOH 10 (pH 7.2), EGTA 4, disodium phosphocreatine 5, MgATP 3, MgCl2 1, and GTP 0.2.

Commercially available AppNHp (Sigma Chemical Co) was used for analysis and further purification. AppNHp was stored at −20°C or colder and used within 3 months of purchase or purification. Analytical HPLC was performed using an HP 1050 Liquid Chromatograph (Hewlett Packard) supplied with diode array detector and Primosphere 5 C18-HC column (0.2×15 cm, Phenomenex). The mobile phase was 5% MeOH in 0.1 mol/L triethylammonium acetate (pH 7.0). The flow rate was 0.2 mL/min.

Purification of commercial AppNHp was carried out by preparative HPLC using a Dynamax system (Rainin Instrument Co Inc) supplied with a UV absorbance detector at 254 nm and using a Primosphere 10 C18-HC column (2.1×25 cm, Phenomenex). The mobile phase was the same as in analytical HPLC at a flow rate of 20 mL/min.

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**Selected Abbreviations and Acronyms**

- $\tau_n$ = open time constant
- AppNHp = adenylylimidodiphosphate
- ATPγS = adenosine 5′-O-thiotriphosphate
- Gx = G-protein coupling muscarinic receptors to $I_{\text{K,ACH}}$
- GppNHp = guanylylimidodiphosphate
- $I_{\text{K,ACH}}$ = acetylcholine-induced K+ current
- $I_{\text{K,ATP}}$ = ATP-sensitive K+ current
- NDP = nucleoside diphosphate
- NP = product of the number of channels (N) and single-channel open probability (P_o)
I
access to the cytoplasmic face. ATP-dependent activation of patches is the formation of vesicles or patches with restricted technical difficulty when working with inside-out membrane requires 2 to 3 minutes to reach steady state. A common has been reported previously by Kaibara et al.13 and Heidbüchel and colleagues.12,14 The response typically develops slowly and appeared different from that normally reported for \( I_{\text{K,ACH}} \) by Cytoplasmic ATP: Mg\(^{2+}\) Dependence

The agonist-independent activation of \( I_{\text{K,ACH}} \) channels by ATP has been reported previously by Kaibara et al.13 and Heidbüchel and colleagues.12,14 The response typically develops slowly and requires 2 to 3 minutes to reach steady state. A common technical difficulty when working with inside-out membrane patches is the formation of vesicles or patches with restricted access to the cytoplasmic face. ATP-dependent activation of \( I_{\text{K,ACH}} \) was used as a positive control to ensure that bath-applied agents could reach the cytoplasmic surface of the membrane. All of the patches included in this study responded to bath-applied ATP (2 mmol/L) with a steady-state activation of \( I_{\text{K,ACH}} \) within 3 minutes.

Activation of \( I_{\text{K,ACH}} \) by Cytoplasmic ATP: Mg\(^{2+}\) Dependence

The agonist-independent activation of \( I_{\text{K,ACH}} \) channels by ATP has been reported previously by Kaibara et al.13 and Heidbüchel and colleagues.12,14 The response typically develops slowly and requires 2 to 3 minutes to reach steady state. A common technical difficulty when working with inside-out membrane patches is the formation of vesicles or patches with restricted access to the cytoplasmic face. ATP-dependent activation of \( I_{\text{K,ACH}} \) was used as a positive control to ensure that bath-applied agents could reach the cytoplasmic surface of the membrane. All of the patches included in this study responded to bath-applied ATP (2 mmol/L) with a steady-state activation of \( I_{\text{K,ACH}} \) within 3 minutes.

An example of the ATP-dependent activation and the Mg\(^{2+}\) dependence of this effect is shown in Figure 1. Superfusion with 2 mmol/L ATP in the presence of 3 mmol/L Mg\(^{2+}\) induced an inward current at \(-80\) mV. When 3 mmol/L Mg\(^{2+}\) was removed from the bath in the continued presence of 2 mmol/L ATP, channel activity decreased back to baseline levels. Channel activity resumed when Mg\(^{2+}\) was reintroduced. Similar Mg\(^{2+}\) dependence was seen in nine of nine patches.

Current-Voltage Relationship for the ATP-Induced Current

The current-voltage relationship for the ATP-activated single channel is shown in Figure 2. The continuous trace at the top of Figure 2 shows that the current reverses near 0 mV. The inwardly rectifying current-voltage relationship and slope conductance between \(-40\) and \(-120\) mV of 44 pS are similar to what have been previously reported for \( I_{\text{K,ACH}} \). Mean open time for the ATP-activated channels was also consistent with the properties of \( I_{\text{K,ACH}} \), \((0.60±0.03\) milliseconds at \(-80\) mV).

If one extrapolates a linear fit to the data between \(-120\) and \(-40\) mV in Figure 2 to estimate the reversal potential, the value is negative to 0. This is probably due to the inward rectification of the channel, since large uncompensated liquid junction potentials would be unlikely when working with symmetrical bath and pipette solutions. We have observed a similar phenomenon with whole-cell \( I_{\text{K,ACH}} \) measured in 25 mmol/L extracellular K\(^{+}\) (Figure 3). Theoretically, the nonlinearity in the whole-cell current-voltage relationship could be due to voltage-dependent changes in open probability. However, we did not detect a decrease in NP, for ATP-activated channels when the voltage was shifted positive from \(-120\) to \(-40\) mV. This suggests that the nonlinearity of the whole-cell current as the reversal potential is approached is due to rectification of the single-channel current amplitude.

At first glance, the nonlinear current-voltage relationship appears different from that normally reported for \( I_{\text{K,ACH}} \) in freshly isolated myocytes. The apparent discrepancy could

![Figure 1. Cytoplasmic ATP activates an inward current at \(-80\) mV in a Mg\(^{2+}\)-sensitive manner. When present, the Mg\(^{2+}\) concentration in the bath solution was 3 mmol/L. The pipette solution was nominally Mg\(^{2+}\) free. Top, Continuous record low pass–filtered at 400 Hz. Superfusion with 2 mmol/L disodium ATP in the presence of 3 mmol/L total Mg\(^{2+}\) (estimated free Mg\(^{2+}\), 1.15 mmol/L) resulted in the activation of an inward current at a holding potential of \(-80\) mV. Channel activity subsided when Mg\(^{2+}\) was returned to the bath. Calibration bars represent 20 seconds and 5 pA. Bottom, Selected segments of the record on an expanded time base. These traces were low pass–filtered at 2 kHz. Scale bars represent 20 milliseconds and 5 pA.](http://circres.ahajournals.org/)

![Figure 2. Current-voltage relationship for the ATP-induced current. Top, Continuous record in the presence of 2 mmol/L ATP and 3 mmol/L total Mg\(^{2+}\). The holding potential was changed in 20-mV increments as shown above the current record. The inward rectification of the channel activity at positive holding potentials is apparent. Noise level was lowest and no channel activity was present at 0 mV. Calibration bars are 10 seconds and 10 pA. Bottom right, Average single-channel current (i)–voltage relationship from five patches. Mean and standard errors are plotted. Errors are within the symbol size at \(\pm -80\) mV. The continuous line is a least-squares fit to a second-order polynomial. The nonlinear current-voltage relationship was observed in each of the individual patches examined.](http://circres.ahajournals.org/)
Activation of $I_{K, ACh}$ by Adenylylimidodiphosphate

Figure 3. Rectification properties of whole-cell $I_{K, ACh}$ in 25 mmol/L extracellular K$^+$. Note that linear extrapolation of the current-voltage relationship from data points negative to $-60$ mV estimates a reversal potential that is negative to the actual reversal potential. Currents were measured in response to a slow hyperpolarizing voltage ramp. Carbachol (10 μmol/L)–induced currents were normalized to cell membrane capacitance and then averaged ($n=6$). A similar shape was seen for the current-voltage relationship from all individual cells. Error bars represent standard error. For clarity, every 10th data point is plotted.

Note: Rectification properties are important in understanding the behavior of ion channels under different conditions.

Inhibition of ATP-Activated $I_{K, ACh}$ by GDP

Figure 4 shows an example of $I_{K, ACh}$ activation by ATP in the presence of 10 mmol/L total Mg$^{2+}$. Early in the activation, discrete single-channel events could be resolved (Figure 4, trace b). By 3 minutes, the effect of ATP had reached a steady state, and many overlapping channel openings were observed (Figure 4, top and trace c). A similar activation by ATP was observed in 11 patches in the presence of 10 mmol/L total Mg$^{2+}$. The inward rectification of the channel was documented during voltage-clamp steps to $+80$ mV (Figure 4, trace g). In agreement with other reports, ATP was inhibited by the application of 10 μmol/L GDP (Figure 4, top and trace d). The inhibitory effect of GDP subsided after several minutes of washout, and channels gradually reactivated in the continued presence of ATP. The effect of ATP subsided on washout. The inhibitory effect of GDP was observed in two of two patches.

Although we normally used the disodium salt of ATP to activate the channels, the activation was not Na$^+$ dependent. The magnesium salt of ATP was as effective as disodium ATP (not shown). This observation differs from results obtained in cultured embryonic chick atrial cells, where ATP alone had little effect, but a priming by ATP was reportedly required to observe a Na$^+$-dependent activation.

Activation by Commercially Available AppNHp

Figure 5 is a continuation of the experiment shown in Figure 4. After ATP was washed out, the patch was exposed to 2 mmol/L AppNHp and 10 μmol/L GDP. Channel activity did not increase during simultaneous exposure to AppNHp and GDP (Figure 5, top and trace b). The guanine nucleotide was then washed out. After several minutes of superfusion with AppNHp alone, the inhibitory effect of GDP subsided, and $I_{K, ACh}$ channels were activated by AppNHp (Figure 5, top and traces c and d). Unlike the effect of ATP, the effect of AppNHp did not subside on washout. Although the activation by AppNHp conflicts with published reports, the persistent activation by commercially available AppNHp was observed in six of six patches when the patches were first screened to determine if they responded to ATP. The inhibitory effect of 10 μmol/L GDP was observed in two of two patches. The inhibitory effect of GDP on activation by AppNHp could only be observed before activation. Application of 10 μmol/L GDP after persistent activation by AppNHp was ineffective ($n=2$, not shown). The results presented in the present study were obtained with AppNHp purchased from Sigma, but we have also seen activation of neonatal rat atrial $I_{K, ACh}$ by AppNHp purchased from Boehringer Mannheim (S. Sorota, unpublished data, 1990).

Figure 4. The effect of cytoplasmic ATP is inhibited by GDP. Top, Ten minutes of continuous recording from an inside-out membrane patch. Channel openings are represented as downward deflections. ATP (5 mmol/L) and GDP (10 μmol/L) were superfused into the recording chamber at the times indicated by the bars above the record. Holding potential was $-80$ mV during most of this recording. During the gap in the continuous record, the holding potential was stepped to $+80$ mV. The continuous record was low pass–filtered at 400 Hz. The scale bars represent 10 pA and 50 seconds. Bottom, Individual traces on an expanded time scale. Traces are from times that correspond to the letters above the continuous record. Traces a through d were recorded at a holding potential of $-80$ mV. Trace g was recorded at $+80$ mV to examine the rectification of the single channels. The expanded traces were low pass–filtered at 2 kHz. The scale bars represent 10 pA and 50 milliseconds.
Contamination of Commercial AppNHp
At first glance, the effect of commercially available AppNHp could have been interpreted to indicate that NDP kinase was not required for the activation of $I_{K_ACh}$ by ATP. Further investigation revealed that interpretation of these experiments was problematic because of guanine nucleotide contamination in commercially available AppNHp. Commercial suppliers synthesize AppNHp from adenosine that has significant guanosine contamination. The specifications of most commercial suppliers of AppNHp indicate that guanine nucleotide contamination is less than or equal to one part in 10,000.

HPLC analysis in ion-pair reversed-phase mode was used to look for contamination of AppNHp by guanine nucleotides. Absorbance at 254 nm was monitored with full spectra being determined at maxima of the corresponding peaks (Figure 6, top). We detected the presence of GppNHp impurity at the level of 0.1% to 0.2% when 10 µg of commercial AppNHp was injected. The peak at 4.8 minutes had a spectrum that was identical to a reference standard of GppNHp (not shown). The adenosine analogues ran as two peaks with retention times of 5.9 and 8.3 minutes in Figure 6, top. The UV spectra of these two peaks were nearly identical, indicating that both peaks represent adenosine analogues (see Figure 6, bottom, inset). The main peak at 8.3 minutes corresponds to AppNHp. The peak at 5.9 minutes is likely to be the diphosphate breakdown product of AppNHp, which is a common contaminant in AppNHp preparations.

Activation by GppNHp
The presence of GppNHp in the AppNHp solutions could be problematic because hydrolysis-resistant GTP analogues are known to activate $I_{K_ACh}$. The patch-clamp studies above were performed using 2 mmol/L of commercially available AppNHp. If GppNHp were present at 1:10,000, this would result in a final GppNHp concentration of 0.2 µmol/L. This concentration of GppNHp was found to effectively and persistently activate $I_{K_ACh}$ in two of two patches (not shown). This result rendered the experiments with commercially available AppNHp uninterpretable, since it could not be determined whether the activity of the preparation was due to AppNHp or the contaminating GppNHp.

Purification of AppNHp
To circumvent the difficulty with commercial AppNHp, preparative HPLC purification was used to separate the GppNHp impurity from the AppNHp sample. AppNHp solution (1 mL) in the mobile phase (25 mg/mL) was applied to the column (10 injections total), and fractions containing pure AppNHp (due to analytical HPLC data) were pooled, evaporated using a rotary evaporator at room temperature, kept in vacuo for ~16 hours to remove the excess of triethylammonium acetate, dissolved in water, and finally were freeze-dried to yield the triethylammonium salt of AppNHp (264 mg). Purity was confirmed by applying 200 µg of AppNHp (20-fold more than in Figure 6, top) to the HPLC system. After purification, there was no detectable GppNHp peak eluting from the column between 3 and 5 minutes (Figure 6, bottom).

Effect of Purified AppNHp
After confirming that triethylammonium did not activate $I_{K_ACh}$ on its own or inhibit the activation by ATP (not shown), we repeated the inside-out patch-clamp experiments using the purified AppNHp. A representative experiment is shown in Figure 7. Superfusion with ATP activated $I_{K_ACh}$ channels in the patch, and channel activity subsided on washout of ATP (Figure 7, top and trace b). Next, 2 mmol/L purified AppNHp was applied in the presence of 10 µmol/L GDP. There was little change in channel activity over the next 3 minutes (Figure 7, top and trace d). At that time, GDP was washed out, but the application of AppNHp was continued. A persistent activation of $I_{K_ACh}$ resulted (Figure 7, top and trace e). As expected, the channel activated by AppNHp exhibited strong inward rectification (Figure 7, trace f). The effect of purified AppNHp was seen in four of four patches. The inhibition of the AppNHp effect by GDP was observed in two of two patches.

At a concentration of 2 mmol/L, ATP increased channel activity more than AppNHp. The ratio of $N_p$ in the presence of AppNHp to the $N_p$ in the presence of ATP was 0.60±0.04. A portion of the smaller $N_p$ could be attributed to a decreased $\tau_e$ value in the presence of AppNHp (ATP, $\tau_e=0.56±0.04$ milliseconds; AppNHp, $\tau_e=0.41±0.02$ milliseconds).
of Kaibara et al.\textsuperscript{13} in that the mean open time for ATP-activated channels (in the absence of GTP) is <1 millisecond.

**Activation Time Course: Effect of Recent GDP Application**

If the NDP kinase hypothesis is correct, ATP-dependent activation will require the presence of GDP on the G protein. However, guanine nucleotides will dissociate from G-protein \( \alpha \)-subunits in the presence of millimolar concentrations of \( \text{Mg}^{2+} \).\textsuperscript{11} The dissociation of guanine nucleotides from G proteins is \( \text{Mg}^{2+} \)-dependent.\textsuperscript{11} If the NDP kinase hypothesis is correct, then recent application of GDP should not inhibit and may in fact enhance the activation of \( I_{\text{K,ACH}} \) by ATP. If, however, GDP dissociation from G-protein \( \alpha \)-subunits is limiting activation by ATP, then recent application of GDP will slow the activation time course.

We examined the effect of recent GDP exposure on the activation time course using a fast-flow system that allowed rapid and complete solution changes. The protocol was to expose to ATP and wash out three different times. GDP was applied for 5 minutes as the first ATP washout was initiated. As GDP was washed out, the second exposure to ATP was initiated. GDP was not present during the second washout of ATP or third exposure to ATP.

The results of one experiment are shown in Figure 8. In this patch, channel activity during the first exposure to ATP reached a steady state within several seconds. The first exposure to ATP occurred 12 minutes after patch excision to allow \( I_{\text{K,ATP}} \) channels to rundown. A 5-minute exposure to 10 \( \mu \text{mol/L} \) GDP was initiated as soon as ATP was washed out. The second exposure to ATP was initiated simultaneously with GDP washout. During the second application of ATP, channel activity was initiated with a lag and took much longer to achieve a steady state. The estimated half-time for activation during the second exposure to ATP was 100 seconds. A third exposure to ATP was initiated after a 5-minute washout period. Activation was rapid during the third exposure to ATP. This result is inconsistent with the NDP kinase hypothesis but is consistent with a model in which GDP dissociation limits the activation time course.

Similar results were observed for a second patch (not shown), with half times for activation of 54 and 35 seconds during the first and third exposures to ATP, respectively, and 148 seconds during the second exposure to ATP just after exposure to GDP.

**Discussion**

We have reproduced several aspects of the findings of Heidbüchel and colleagues\textsuperscript{12,14} and Kaibara et al.\textsuperscript{13} with regard to the activation of \( I_{\text{K,ACH}} \) by millimolar concentrations of cytoplasmically applied ATP. The time course for activation by ATP, \( \text{Mg}^{2+} \) dependence, current-voltage relationship, channel \( \tau \) values, and the inhibition of the response by 10 \( \mu \text{mol/L} \) GDP indicate that we are studying the same phenomenon. The prior studies had further determined that the response to ATP was not blocked by pertussis toxin and could be mimicked by ATP\( \gamma \text{S}. \) These data in combination with their failure to activate \( I_{\text{K,ACH}} \) with AppNHp were consistent with the hypothesis that a phosphorylation event was involved. Both Heidbüchel and
colleagues and Kaibara et al proposed that the phosphorylation of GDP that was bound to G-protein \( \alpha \)-subunit by NDP kinase was responsible for the stimulatory effect of ATP. This action would convert GDP to GTP and could promote activation of the G protein. Heidbuchel et al\(^{16} \) subsequently used biochemical methods to demonstrate that NDP kinase activity is detectable in atrial membranes from frogs, guinea pigs, and humans.

Before the present study was undertaken, there were several aspects of the NDP kinase hypothesis that were problematic. Whole-cell \( I_{K,AC} \) is very low in the absence of agonist despite the presence of cytoplasmic GTP. Low basal activity is attributed to the ratio of the GTP hydrolysis rate and the rate for GDP dissociation (reviewed in Reference 11). Since GDP dissociates from the G protein very slowly relative to the rate of GTP hydrolysis, the majority of the G protein is GDP-liganded. How then could NDP kinase activity result in sustained activation of \( I_{K,AC} \) in inside-out membrane patches during exposure to ATP? The phosphorylation of GDP that is bound to the G protein would have to be fast relative to the rate of GTP hydrolysis to cause most of the G protein to be GTP-liganded. Furthermore, the requirement that NDP kinase acts on bound GDP places significant constraints in terms of the spatial orientation of G protein, NDP kinase, GDP, and ATP that would allow for this proposed rapid rephosphorylation. A biochemical study to detect the phosphorylation of GDP while bound to G proteins has concluded that this does not occur.\(^{17} \)

It is expected that GDP will dissociate from the G protein in inside-out membrane patches. Indirect estimates of the basal GDP dissociation rate from the G protein(s) that

Figure 7. Purified AppNHp persistently activates \( I_{K,AC} \), and the effect is blocked by GDP. Top. Fifteen minutes of continuous recording from an inside-out membrane patch. ATP (2 mmol/L), AppNHp (2 mmol/L), and GDP (10 \( \mu \)mol/L) were superfused into the recording chamber at the times indicated by the bars below the record. Holding potential was \(-80 \) mV during most of this recording. During the gaps in the continuous record, the holding potential was stepped to \(+80 \) mV. The scale bar represents 10 pA. Middle, NP\(_o\) vs time. The time scales of the continuous record and the NP\(_o\) determination are aligned. Bottom, Individual traces on an expanded time scale. Traces are from times that correspond to the letters above the continuous record. Traces a, b, d, and e were recorded at a holding potential of \(-80 \) mV. Traces c and f were recorded at \(+80 \) mV to examine the rectification of the single channels. The scale bars represent 10 pA and 50 milliseconds. Magnesium is present at 10 mmol/L.

Figure 8. Recent GDP superfusion slows the activation of \( I_{K,AC} \) by ATP. Each panel shows a continuous record of channel activity in the patch and NP\(_o\). All records are from the same patch. The 5-minute gaps between three successive applications of ATP are not shown. Solid bars above the traces indicate times when 2 mmol/L ATP was applied. Open bars indicate the times when 10 \( \mu \)mol/L GDP was applied. A fast-flow local superfusion system was used to allow exchange of solution within 2 seconds. Top, Rapid activation of \( I_{K,AC} \) during the first exposure to ATP is shown. Channel activity reached steady state within several seconds. Middle, Recent exposure to GDP slows channel activation by ATP. The patch was perfused continuously with GDP for 5 minutes before initiating superfusion with ATP. Bottom, Channels activate rapidly again after a 5-minute washout of ATP in the absence of GDP. Activity was estimated for 2.5-second segments after superfusion with ATP was initiated. One estimate of NP\(_o\) was made for the entire interval before ATP application because the number of events was low during this period. No estimates of channel activity were made during the electrical artifacts that correspond to switching solution lines. There were occasional openings of \( I_{K,ATP} \) channels during the times when ATP was not present (eg, the middle trace \( \sim 10 \) seconds after ATP washout). These channels did not open in the presence of ATP and were ignored in the calculation of NP\(_o\). \( I_{K,ATP} \) can readily be distinguished from \( I_{K,AC} \) because \( I_{K,ATP} \) has a higher conductance and opens in bursts. The scale bar on the top right represents 20 seconds and 5 pA. Magnesium is present at 10 mmol/L.
activates \( I_{K,\text{ACh}} \) in native cells suggest that 30% of the GDP dissociates every minute.\(^{10}\) Heidbüchel et al\(^{12}\) have reported that the application of GTP to the cytoplasmic face of the membrane in the absence of extracellular agonist produces a transient activation of \( I_{K,\text{ACh}} \) in the continued presence of cytoplasmic GTP. To account for this transient activation, one would have to propose that GTP had access to unliganded G protein (no guanine nucleotide bound), resulting in a short pulse in which the G protein exists primarily in the GTP-ligated state. This implies that there was no GDP on many of the G-protein molecules. GDP dissociation from the G protein in inside-out membrane patches clearly presents a problem for the NDP kinase hypothesis, since GDP-liganded state. This could occur if either (1) the rate of GDP hydrolysis was much lower that the rate of GTP dissociation would be the limiting factor for activation shortly after patch excision or shortly after exposing the patch to guanine nucleotides. If a patch is exposed to guanine nucleotide–free solutions for long times, the activation rate is shorter. These data are consistent with the experimental observations of Heidbüchel et al.,\(^{12}\) indicating that in the absence of guanine nucleotides the time course for channel activation is shorter during a second exposure to ATP.

The data in Figure 8 suggest that the rate of GDP dissociation would be the limiting factor for activation shortly after patch excision or shortly after exposing the patch to guanine nucleotides. If a patch is exposed to guanine nucleotide–free solutions for long times, the activation rate is shorter. These data are consistent with the experimental observations of Heidbüchel et al.,\(^{12}\) indicating that in the absence of guanine nucleotides the time course for channel activation is shorter during a second exposure to ATP.

In summary, we found that AppNHp can support the activation of \( I_{K,\text{ACh}} \) in the absence of extracellular agonist or intracellular guanine nucleotide. These data are not consistent with the NDP kinase hypothesis for activation of \( I_{K,\text{ACh}} \) by ATP. This observation does not rule out a role for NDP kinase under some yet-to-be-identified conditions. Our results do indicate that the activation of \( I_{K,\text{ACh}} \) by ATP in the absence of guanine nucleotides can be explained without invoking a role for NDP kinase.

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References


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