Agonist-bound muscarinic receptors open atrial $K^+$ channels through a GTP-dependent pathway mediated by the G protein $G_s$. However, nucleotides other than GTP are also able to support channel activity, even in the absence of agonists. This process was proposed to be mediated by nucleoside-diphosphate (NDP) kinase, which would transfer phosphate from nucleotide triphosphates to the GDP bound to $G_s$, producing $G_s$-GTP without the need for receptor-induced GDP-GTP exchange. We examined the effect of antibodies to NDP kinase on the ATP-supported activity of atrial muscarinic $K^+$ channels and the corresponding GIRK1/CIR channels expressed in HEK 293 cells. Inhibitory antibodies reduced ATP-induced channel openings, but this effect displayed an absolute requirement for agonist and was also seen with antibodies that do not inhibit the enzyme. Both types of antibodies also reduced agonist-dependent channel activity in the presence of GTP, ruling out a role for NDP kinase in GTP rephosphorylation. Channel activity was not affected by the antibodies in preparations where ATP-induced muscarinic channels are not under tight receptor control, namely pertussis toxin-treated atrial patches and membranes from cells expressing $K_{ACh}$ channel subunits. Thus, participation of NDP kinase in this pathway requires activated receptors and has a function distinct from phosphate transfer between nucleotides.

The effects of acetylcholine in the heart are mediated by $m_2$ muscarinic receptors, which couple to various effectors through specific G proteins (1). Activated receptors interact with GDP-bound, inactive G proteins and accelerate the basal rate of exchange of GDP for GTP. Binding of GTP promotes activation of G proteins that are then capable of regulating the activity of a number of effectors (2, 3), including the muscarinic $K^+$ channels of atrial myocytes, $K_{ACh}$ (4, 5). Deactivation of G proteins through their intrinsic ability to hydrolyze GTP promotes the return of the system to the basal, inactive state; this cycle is repeated while agonists are present. When GTP is substituted by one of its poorly hydrolyzable analogues, interruption of the hydrolytic step leads to activation of G proteins in a persistent manner. This is well illustrated by the gradual appearance of muscarinic K$^+$ currents ($I_{K_{ACh}}$) in the absence of agonists when cells are internally perfused with solutions containing GTP analogues (6).

G proteins display strict selectivity for guanine nucleotides (7, 8). Therefore, enzymatic interconversion of nucleotides is usually found to underlie activation of G protein-coupled pathways by nucleotides other than GTP. In most cases, the enzyme implicated in these effects is NDP kinase (EC 2.7.4.6), which can rephosphorylate GDP using the terminal phosphate of most nucleotide triphosphates (9). For instance, intracellular application of ATP-$S$ into atrial myocytes activates $I_{K_{ACh}}$ indirectly, due to the endogenous NDP kinase activity which transfers the \( \gamma \)-thiophosphate group of this ATP analogue into GTP, forming GTP-$S$ (10, 11). By analogy, transphosphorylation catalyzed by NDP kinase was presented as an explanation for the finding that exposure of excised patches of atrial membranes to high (1-5 mM) concentrations of nucleotides other than GTP could induce $K_{ACh}$ activity, even in the absence of muscarinic agonists (12–15). It was suggested that activation by these nucleotides required membrane-bound NDP kinase, which would utilize their terminal phosphate to phosphorylate directly the GDP bound to $G_s$, thus forming GTP-bound $G_s$ without agonist-induced GDP-GTP exchange.

Such a functional link between NDP kinase and G proteins had been proposed previously (reviewed in Refs. 16–18), but the idea lacks convincing experimental support, in part because of technical difficulties which often give rise to artifacts, and also due to the lack of a specific NDP kinase inhibitor. The recent development of inhibitory antibodies against the amphibian enzyme, which neutralize its activity in vitro as well as in intact cells (11), provided a unique tool to examine whether or not NDP kinase is involved in a given cellular process. In the present study, we examined the characteristics of the process by which ATP activates $K_{ACh}$, utilizing tools such as pertussis toxin and antibodies to NDP kinase, to determine whether this enzyme mediates ATP-induced channel activation in inside-out atrial myocyte membrane patches. Additionally, we determined whether NDP kinase is involved in the stimulatory effects of ATP in $K_{ACh}$ channels from membrane patches from HEK 293 cells expressing the protein components of muscarinic $K^+$ channels, GIRK1 and CIR. Our results show that NDP kinase does perform an essential role in channel gating by agonists, but its function in this system is not in situ GTP regeneration.

**EXPERIMENTAL PROCEDURES**

HEK 293 cells co-expressing the GIRK1/CIR polypeptides were a generous gift from Dr. Gabor Szabo (University of Virginia). Pertussis toxin was a gift from Dr. Erik Hewlett (University of Virginia); antibodies to human erythrocyte NDP kinase A, affinity-purified on immobilized human NDP kinase, were a gift from Drs. Marie-Lise Lacome
Atrial myocytes were dissociated from bullfrog hearts as described (19). Single channel activity was recorded from excised, inside-out atrial membrane patches (20) at 22–24°C using pipettes made from quartz capillaries (1-mm outer diameter, Sutter Instruments). The external (pipette) solution contained 0.1 M KCl, 2.5 mM CaCl₂, 20 mM HEPES, pH 7.2 with KOH and 10 μM carbamylcholine when noted. The resistance of the pipettes when filled with this solution was 6–9 MΩ. The internal (bath) solution contained 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA-KOH, 20 mM HEPES, pH 7.2 with KOH, with the additions described in the text and figure legends. To ensure access of bath solution to the intracellular aspect of the membranes, after formation of a patch, the tip of the pipette was briefly passed through the air-bath solution interface. This procedure helps to disrupt closed vesicles that may form during patch excision (20). Patches showing significant rundown of Kₐch currents during the first 5 min of recording were discarded. Single channel recording from patches excised from HEK 293 cells expressing GSK160543 is performed as described previously (21).

Single channel currents were measured with an Axopatch 200A patch clamp amplifier (Axon Instruments), monitored on a digital storage oscilloscope (Hameg), and stored on video tape using a VR-10B converter (Instrutec). Single channel analysis was performed with the program TRANSIT (Baylor College of Medicine, Houston, TX). For in depth analysis, the data were filtered at 5 kHz with a Bessel filter (8 decibel/octave, Frequency Devices 900) and digitized at 15 kHz. For large files not requiring high time resolution analysis, the data were filtered at 2 kHz and digitized at 6 kHz. Channel activity in patches is reported as the number of channels (N) multiplied by the probability of opening (Pₒ). To determine relative channel activities, the Nₒ during a period of 2 min at the end of the application of control or test solutions to a patch was averaged. The value obtained in test solution was then normalized to the control Nₒ. Results are reported as means ± S.E.; differences between means were assessed by Student’s t test (p > 95%). Pertussis toxin (100 μg/ml) was diluted 1:1 with internal solution supplemented with 3 mM ATP and 20 mM dithiothreitol and activated by incubation at 35°C for 20 min. This solution was then diluted 50-fold with bath solution containing 10 μM GTP and 1 mM NaCl, to a final toxin concentration of 1 μg/ml. The protein A-purified IgG fraction from anti-frog NDP kinase serum fraction (F1) was applied at 10 nM, unless otherwise specified; affinity-purified antibodies to human NDP kinase A (HA) were applied at 1 nM.

For the determination of antibody effects on the NDP kinase activity of frog cardiac sarcolemma, purified membranes (22) were permeabilized with saponin (2 mg/ml) for 20 min and then incubated on ice with either antibody in 50 μl of 75 mM KCl, 5 mM MgCl₂, 1 mg/ml bovine serum albumin, and 50 mM Tris-HCl, pH 7.5. After 1 h, the NDP kinase activity of each mixture was assayed in duplicate or triplicate. Enzyme assays, protein determination, and immunoblots were performed as described elsewhere (11). Dot blots were performed by spotting 0.1–3 μg of sarcoclemmal or 0.1–25 ng of purified frog NDP kinase into nitrocellulose filters. Blots were air-dried, blocked, and probed as described (13).

To control for the possibility of GTP contamination of ATP, solutions of the latter were analyzed by HPLC on a Radial-Pak cartridge type 8PSAX 10 μm (Waters) run at 3 ml/min with 87% ammonium phosphate, pH 5.4, containing 13% methanol (23). This system produced a good separation of GTP from ATP. No GTP was detected in the ATP utilized in these experiments; calculation of the detection limit of this system indicates that if GTP is present at all as a contaminant, its concentration in 4 mM ATP must be lower than 2 μM. To establish whether the concentration of GTP would be high enough to support the level of channel activity observed with ATP, we measured carbamylcholine-induced muscarinic K⁺ channel activity in patches, first with 4 mM ATP in the bath solution and then in the presence of 2 μM GTP (not shown). The open probability of the channels decreased from 0.13 to 0.04 (n = 5) when ATP was replaced by GTP, indicating that any contamination by GTP is too low to account for the effects of ATP described below.

**RESULTS**

As reported by other investigators (12–15), ATP activates inwardly rectifying muscarinic K⁺ channels in excised, inside-out patches of atrial membranes in the absence of GTP (see initial portion of recording in Fig. 1A). The single channel conductance (γ = 36.6 pS), and mean open time (τ = 1.25 ms) of the channels recorded under these conditions are similar to values measured in cell-attached patches with the agonist carbamylcholine in the pipette, 40.3 pS and 1.17 ms, respectively. To examine whether NDP kinase was involved in the effect of ATP, we measured the activity of Kₐch channels activated by ATP before and after 10 min of application of an IgG fraction (F1) purified from antiserum to frog skeletal muscle NDP kinase. These antibodies have been shown to bind to frog NDP kinase with high affinity and inhibit its activity in vitro and in vivo (11). As illustrated in Fig. 1A, when the pipette contained no agonist, application of F1 at 10 nM in the presence of 4 mM ATP
Application of GTP\(_\text{gS}\) at the end of the experiment did not stimulate KA\(_\text{Ch}\) activity further (\(n = 3\)). It is unlikely that the antibodies would not inhibit NDP kinase in cardiac membrane patches, since they neutralize the enzyme in subcellular fractions as well as in intact cells (11). Therefore, the simplest explanation for the lack of effect of the antibodies is that inhibition of NDP kinase activity has no effect on the stimulation of muscarinic K\(_\text{1}\) channels by ATP.

However, these results were obtained in the absence of agonist, and previous reports suggest that agonists can alter the interaction between NDP kinase and elements of signal transduction pathways (24, 25). Therefore, we performed a similar set of experiments, this time with 10 \(\mu M\) carbamylcholine added to the pipette solution. As seen in Fig. 1B, in the presence of agonist the same concentration of F1 reduced markedly the activity of KA\(_\text{Ch}\) channels.

Application of GTP\(_\text{gS}\) after maximal inhibition had been attained led to a rapid reversal of the effect of F1 (Fig. 1B) and restored channel activity to a level equal or superior to the control value. In contrast, attempts to reverse the block by removing the antibodies from the bath were unsuccessful: the reduction in KA\(_\text{Ch}\) activity persisted after washout periods of 10 min (\(n = 4\); not shown).

Inhibition was observed only with native immunoglobulins: in the presence of boiled F1 the activity of muscarinic K\(^+\) channels remained at 74.6% of the control (Fig. 2A, panel b), a value not significantly different from the one measured in bath solution (Fig. 2A, panel a). The specificity of the effect of F1 was checked in experiments where purified frog skeletal muscle NDP kinase (1 nM) was present both before and during the application of F1 (Fig. 2, panel c). The presence of NDP kinase for 7.5–23 min had no significant effect in channel activity. Application of GTP\(_\text{gS}\) at the end of the experiment did not stimulate KA\(_\text{Ch}\) activity further (\(n = 3\)). It is unlikely that the antibodies would not inhibit NDP kinase in cardiac membrane patches, since they neutralize the enzyme in subcellular fractions as well as in intact cells (11). Therefore, the simplest explanation for the lack of effect of the antibodies is that inhibition of NDP kinase activity has no effect on the stimulation of muscarinic K\(^+\) channels by ATP.

However, these results were obtained in the absence of agonist, and previous reports suggest that agonists can alter the interaction between NDP kinase and elements of signal transduction pathways (24, 25). Therefore, we performed a similar set of experiments, this time with 10 \(\mu M\) carbamylcholine added to the pipette solution. As seen in Fig. 1B, in the presence of agonist the same concentration of F1 reduced markedly the activity of KA\(_\text{Ch}\) channels. This effect was usually slow, taking several minutes to reach an apparent steady state, and was not due to spontaneous channel rundown: when F1 was not added, KA\(_\text{Ch}\) activity decreased only slightly after 10–15 min of recording, indicating that the extent of rundown is minimal under these conditions (Fig. 2A, panel a). The single channel amplitude and mean open time were not affected by the antibodies, being, respectively, 2.76 ± 0.07 pA and 1.35 ± 0.14 ms in controls and 2.81 ± 0.11 pA and 1.00 ± 0.34 ms after exposure to F1. Normalization of the NP\(_\text{o}\) in the presence of F1 to the NP\(_\text{o}\) in 4 mM ATP alone showed an average decrease of 74.2% in KA\(_\text{Ch}\) activity after 10 min of exposure to the antibody (Fig. 2B).

Application of GTP\(_\text{gS}\) after maximal inhibition had been attained led to a rapid reversal of the effect of F1 (Fig. 1B) and restored channel activity to a level equal or superior to the control value. In contrast, attempts to reverse the block by removing the antibodies from the bath were unsuccessful: the reduction in KA\(_\text{Ch}\) activity persisted after washout periods of 10 min (\(n = 4\); not shown).

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in the bath had little effect on channel activity, and the mean channel activity in NDP kinase alone amounted to 87.9% of control (±28.6%, n = 4). Application of F1 in the presence of the enzyme prevented the inhibitory effect of the antibodies (Fig. 2B), indicating that the effect of the antibodies was specific and reflected binding of IgG to endogenous NDP kinase. Also, the IgG fraction purified from the preimmune serum had little effect on the ATP-supported, agonist-induced KACh activity (Fig. 2A, panel d). These results are summarized in Fig. 2B.

Since G proteins have high selectivity for GTP, and this nuclease supports high channel activity in the presence of activated receptors, transfer of phosphate from ATP into GDP by NDP kinase should play a negligible role when GTP is also present. Thus, F1 should not affect muscarinic K channels when the bath solution contains ATP and a saturating (26) concentration of GTP. The next set of experiments was designed to test this prediction; K channels were incubated with antibodies HA or F1 at the concentration indicated. Activity was assayed as described under “Experimental Procedures” and plotted as percent of the value measured in the absence of immunoglobulins. Results are from three separate experiments. B, channel activity recorded from excised frog atrial patches, with the bath solution containing 4 mM ATP; HA antibodies were applied to patches at a concentration of 1 nM. The pipette contained no agonist (upper panel) or 10 μM carbamylcholine (lower panel). After a few minutes of perfusion with HA, channel activity in the patch shown in the lower panel consists chiefly of openings of the background inward rectifying K channel, I(K1). C, summary of the data obtained with HA antibodies in ATP alone, as well as in the presence of GTP. Relative channel activities were calculated as described under “Experimental Procedures.”

Fig. 4. Effect of non-neutralizing antibodies to the activity of KACh channels. A, effect of antibodies to frog (F1) or human (HA) NDP kinase on the NDP kinase activity of frog cardiac sarcolemma. Membranes (5 μg) were incubated with antibodies HA or F1 at the concentration indicated. Activity was assayed as described under “Experimental Procedures” and plotted as percent of the value measured in the absence of immunoglobulins. Results are from three separate experiments. B, channel activity recorded from excised frog atrial patches, with the bath solution containing 4 mM ATP; HA antibodies were applied to patches at a concentration of 1 nM. The pipette contained no agonist (upper panel) or 10 μM carbamylcholine (lower panel). After a few minutes of perfusion with HA, channel activity in the patch shown in the lower panel consists chiefly of openings of the background inward rectifying K channel, I(K1). C, summary of the data obtained with HA antibodies in ATP alone, as well as in the presence of GTP. Relative channel activities were calculated as described under “Experimental Procedures.”

that this particular role of NDP kinase is not related to GTP regeneration from GDP and ATP.

The catalytic function of NDP kinase is not always required for its cellular function (27, 28); for instance, a mutant NDP kinase that has no enzymic activity can still function as a transactivator of c-myc (27). Furthermore, although the simplest explanation for an inhibitory effect of a blocking antibody is that it causes enzyme inhibition, immunoglobulins can also affect function by other mechanisms, for example through disruption of macromolecular assemblies (29, 30). To establish whether enzyme inhibition is relevant to the effect of F1 antibodies, we utilized affinity-purified polyclonal antibodies to the A chain of human NDP kinase (HA). Although these antibodies recognize NDP kinase from frog cardiac sarcolemma in immunoblots and native dot blots (not shown), they do not inhibit its enzymatic activity, in contrast to the behavior of the antibodies to the frog enzyme (Fig. 4A). When applied to patches at 1 nM, HA antibodies did not affect the KACh activity induced by ATP, provided that the pipette contained no agonist (Fig. 4B). However, application of HA in the presence of agonist led to a pronounced decrease in the number of KACh openings (Fig. 4B), to an extent (Fig. 4C) comparable with the effect of the neutralizing F1 antibodies (Fig. 2B), and the presence of GTP did not influence the outcome of the experiments (Fig. 4C). Therefore, block of NDP kinase catalytic function is not necessary for the inhibitory effect of antibodies to NDP kinase on KACh channels. Instead, what seems to determine whether the antibodies have or not an effect is the presence of agonist.

Although ATP and GTP activate KACh channels to a similar extent, there is an important difference between these two nucleotides: while treatment with pertussis toxin abolishes GTP-dependent channel activity of muscarinic K channels
with 1 g/ml pertussis toxin and 1 mM NAD (PTX) until muscarinic channel activity was abolished; the residual channel activity observed was due only to ATP-sensitive channels. The bath was then perfused with a solution containing ATP (4 mM) and finally with ATP and HA antibodies (1 nM). Similar results were obtained with F2 antibodies. B, current traces recorded in the inside-out configuration from HEK 293 cells expressing GIRK1/CIR channels. ATP (1 mM) was applied during the period indicated, first in the absence and then in the presence of antibodies to human NDP kinase (HA, 1 nM).

(26, 31), the channels are still activated by ATP (13). Stimulation of K_{ACh} channels by ATP under these conditions was proposed to be mediated by NDP kinase. However, our results imply that NDP kinase only participates in channel opening when it is subject to control by agonist-bound receptors and should not play a role in this process when the coupling between receptor and G protein is disrupted by ADP-ribosylation of G_{i}. Indeed, as illustrated in Fig. 5A, in pertussis toxin-treated patches antibodies to NDP kinase have no effect on the ATP-supported channel activity (n = 10), confirming the view that NDP kinase does not participate in channel activation by ATP unless activated receptors and G proteins are also involved. This conclusion is supported in experiments performed on membranes of HEK 293 cells expressing the two subunits of muscarinic K^+ channels, GIRK1 and CIR (32). Functional expression of GIRK1 and CIR or their homologues produces channels with the characteristics of K_{ACh} in various cell types and does not require joint co-expression of muscarinic receptors or G proteins (32–34). In HEK 293 cells2 (Fig. 5B), as well as in Xenopus oocytes (33–34), expressed GIRK/CIR channels are strongly stimulated by ATP, thus providing a model to study channel activation by ATP without the interference of G proteins or receptors. As expected, application of HA antibodies to patches of membranes from HEK 293 cells had no effect on the GIRK1/CIR channel activity induced by ATP (Fig. 5B; n = 3). These data corroborate the hypothesis that NDP kinase plays a role in channel opening only when this process also involves G protein activation by a receptor.

2 J. Murphy and G. Szabo, unpublished results.

DISCUSSION

We demonstrate here that in atrial membrane patches NDP kinase participates in the gating of muscarinic K^+ channels by agonists. Antibodies to NDP kinase reduce markedly the open probability of muscarinic K^+ channels, and this effect has an absolute requirement for receptor activation by agonist and intact receptor-G_{i} coupling. The similarity between the effects of blocking and null antibodies, as well as their ability to reduce channel openings in the presence of GTP, argue against a role for NDP kinase in GTP formation. Instead, the data imply that, as seen in other systems (27, 28), the role played by NDP kinase in the muscarinic K^+ channel pathway does not involve nucleotide rephosphorylation at the expense of ATP.

Although our experimental approach cannot provide structural or mechanistic information about the connection between NDP kinase and the process that leads to channel opening, some characteristics of this link can be deduced from the data. Thus, the agonist dependence of the antibody effect and the loss of inhibition observed after pertussis toxin treatment suggest that the binding of antibodies to NDP kinase interferes with K_{ACh} gating at the level of the interaction between agonist-bound receptors and G_{i}. Moreover, while the antibodies attenuate agonist-induced channel activity in a manner that cannot be reversed by washout, the subsequent reactivation by GTP does indicate that the interaction between the G protein and the channel is not markedly affected. A direct effect of the antibodies on the channel is unlikely, considering the lack of effect of the antibodies on agonist-independent channel activity in atrial cells or in HEK 293 cells expressing GIRK1/CIR channels. Therefore, the antibody-induced block does not result from nonspecific binding to elements of the pathway or a generalized disruption of interactions between receptor, G protein, and channel. Rather, the antibodies must disturb some process that takes place only when the receptors are active and occurs early in the activation sequence. The precise site of action of the antibodies, and therefore the protein that interacts with NDP kinase, has yet to be defined, although the obvious candidates are the muscarinic receptor and/or G_{i}. Some observations suggest that NDP kinase and G-protein-coupled receptors may interact; for instance, activation of surface cAMP receptors from Dictyostelium stimulates membrane NDP kinase activity (35). Although this functional connection does not necessarily imply that the receptor acts directly on NDP kinase, the hypothesis of a physical association is supported by the observation that NDP kinase activity is also stimulated by receptor-mimetic compounds such as mastoparan (36). On the other hand, there is evidence for reversible complex formation between NDP kinase and the α-subunit of G_{i}, and this interaction is regulated by G protein-coupled receptors and by guanine nucleotides (24, 25). Therefore, it is conceivable that similar interactions occur in atrial membranes, with NDP kinase being associated with muscarinic receptors, G_{i}, or both.

Finally, what is the role of NDP kinase in muscarinic K^+ channel activation? Clearly, the best known cellular function of NDP kinase, nucleotide rephosphorylation, is not involved in this case. However, NDP kinase has other, less well characterized functions that may be key to its role in transmembrane signaling. In particular, the phosphohistidine intermediate of NDP kinase can transfer phosphate onto histidine (37) or serine and threonine residues of several proteins (38, 39) in what appears to be a stoichiometric, rather than catalytic process (39). If NDP kinase phosphorylates some element of the muscarinic K^+ channel pathway, and to do so must associate stoichiometrically with its substrate, the binding of immunoglobulins could hinder complex formation regardless of their ability to inhibit phosphate transfer. On the other hand, it is equally
possible that the role of NDP kinase in this system is totally dissociated from its catalytic activity. These possibilities are currently under investigation.

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Does Not Involve GTP Formation
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