Membrane-Bound Nucleoside Diphosphate Kinase Activity in Atrial Cells of Frog, Guinea Pig, and Human

Hein Heidbüchel, Geert Callewaert, Johan Vereecke, and Edward Carmeliet

Muscarinic \( K^+ \) channels in inside-out patches of atrial cells from guinea pig or rabbit can be activated by Mg\(^{2+}\)-ATP in the absence of acetylcholine and GTP or GDP. The ATP-dependent activation involves a phosphorylation and is postulated to be due to the association of a membrane-bound nucleoside diphosphate kinase (NDPK) with the G protein \( G_K \); direct phosphorylation of the \( G_K \)-bound GDP into GTP, catalyzed by NDPK, would result in activation of the G protein and, hence, activation of the channels. The aim of this study was to identify the presence of NDPK activity in atrial membranes by investigating the phosphate transfer between tritium-labeled nucleotides. We show that frog, guinea pig, and human atrial membranes contain a substantial NDPK activity since they catalyze the conversion from \([3H]GDP+nucleoside triphosphate\) to \([3H]GTP\) (or \([3H]GTP+yS\)), from \([3H]ADP+nTP\) to \([3H]ATP\), and from \([3H]GTP+nucleoside diphosphate\) to \([3H]GDP\). The phosphate transfer rates for the \([3H]GDP+ATP\) to \([3H]GTP\) conversion are 1.8, 0.5, and 2.4 \( \mu \)mol inorganic phosphate formation/mg per 10 minutes at 37°C in frog, guinea pig, and human, respectively. The order of substrate efficiency for different NTPs was ATP > ITP = GTP > UTP > CTP, which parallels the efficiency of these nucleotides in their activation of the muscarinic \( K^+ \) channels. Addition of other nucleotides blocked the transphosphorylation reaction, indicating that the NTP-NDP conversion mechanism is aspecific, as is expected for an NDPK-catalyzed reaction. In conclusion, the data support the concept of NDPK involvement in the atrial muscarinic signal transduction cascade. (Circulation Research 1992;71:808–820)

**Key Words** • acetylcholine • muscarinic \( K^+ \) channels • nucleoside diphosphate kinase • EC 2.7.4.6 • G proteins

**A** ctivation of atrial muscarinic \( K^+ \) channels by acetylcholine requires the presence of intracellular GTP since a GTP-binding protein (denoted as \( G_K \)) is involved in signal transduction. However, we and others have recently shown that in inside-out patches of guinea pig or rabbit atrial cells, muscarinic \( K^+ \) channels can be activated by Mg\(^{2+}\)-ATP in the absence of agonist and GTP or GDP. This activation was explained by assuming the presence of a membrane-bound nucleoside diphosphate kinase (NDPK) associated with the G protein \( G_K \). The NDPK enzyme (or group of enzymes) catalyzes the conversion of nucleoside diphosphates (NDPs) to nucleoside triphosphates (NTPs) by the general reaction sequence N\(_i\)TP+N\(_{N+1}\)DP\(_{N+1}\)=N\(_{N}\)DP+N\(_i\)TP (for an overview, see Parks and Agarwal\(^3\)). \( G_K \)-associated NDPK can activate the G protein by directly transferring a phosphate group from ATP to GDP on the G protein,\(^1\) a mechanism that is different from the agonist-induced GDP to GTP exchange. The presence of NDPK may have major physiological relevance for the regulation of muscarinic \( K^+ \) channel activity and, hence, for the electrical properties of atrial tissue: 1) In the absence of agonists, direct transphosphorylation can contribute to the basal activity of the muscarinic channels (and hence to the basal \( K^+ \) conductance of atrial cells). 2) In the presence of agonist, local GTP formation (from ATP+GDP) can maintain a high local GTP/GDP ratio, which is essential for efficient G protein activation (and hence reliable parasympathetic control of heart function).

Although NDPKs are ubiquitous\(^3\) and their presence in atrial cells was postulated by others,\(^4\) we wanted to ascertain that atrial membranes indeed contain this enzymatic activity. The aim of this study was to identify the presence of NDPK in atrial membranes and characterize its enzymatic activity with different nucleotides as substrates to correlate the data with the efficiency of these nucleotides in the activation of muscarinic \( K^+ \) channels. In most experiments, we used the crude atrial membranes of the frog (Rana esculenta) because they are less contaminated by sarcoplasmic reticulum. Initially, we will show that muscarinic \( K^+ \) channels from frog cells can be activated in an ATP-dependent way, indicating that this activation mechanism is not restricted to mammalian cells. The second part of the present study deals with experiments on crude atrial membranes of the guinea pig and on different membrane fractions of human atrium, showing that an important membrane-bound NDPK enzymatic activity is present in these species also.

---

From the Laboratory of Physiology, University of Leuven, Campus Gasthuisberg, Leuven, Belgium.

H.H. is working as a Research Assistant of the National Fund for Scientific Research (Belgium).

Address for correspondence: Dr. Hein Heidbüchel, Laboratory of Physiology KU Leuven, Gasthuisberg, O&K, Herestraat 49, B-3000 Leuven, Belgium.

Received November 18, 1991; accepted May 13, 1992.
Materials and Methods

Isolation of Single Frog Atrial Myocytes and Patch-Clamp Recordings

Single atrial cells of the frog species *Rana esculenta* were dissociated by enzymatic dispersion by Langendorff perfusion. The aorta was cannulated, and the heart was perfused at room temperature. The following perfusions were performed consecutively: 1) 5 minutes with a Ca²⁺-free solution containing (mM) NaCl 130, KCl 5.4, KH₂PO₄ 1.2, MgSO₄ 1.2, gluconolactone 10, and HEPES 6 at pH 7.2 (NaOH), 2) 5 minutes with a Ca²⁺-free solution containing 76 units/ml collagenase (Worthington Biochemical Corp., Freehold, N.J.) and 0.7 units/ml protease VII (Sigma Chemie GmbH, Deisenhofen, Germany), and 3) 5 minutes with a low (180 μM) Ca²⁺ solution. After the perfusions, the cells were dispersed and stored at room temperature in the same low Ca²⁺ solution.

Cells were transferred to a tissue bath placed on the stage of an inverted microscope. The bath was perfused at a flow rate of 3–4 ml/min with a bathing solution containing (mM) potassium aspartate 130, MgCl₂ 10, K₂HPO₄ 0.3, glucose 5.5, EGTA 1, HEPES 5, and ATP 4 at pH 7.3 (KOH) (total K⁺ concentration, ≈140 mM). No GTP was added unless otherwise mentioned. The resting membrane potential of the cells under these conditions is estimated to be 0 mV. The free Mg²⁺ concentration was kept constant at 5.7 mM. We performed single-channel experiments in the cell-attached and inside-out mode by means of the patch-clamp technique described by Hamill et al. using the EPC-7 amplifier (List Medical Electronic, Darmstadt, Germany). Membrane potentials are expressed with the extracellular side as reference. All experiments were carried out at room temperature. The patch-clamp pipettes were made from hard-glass capillaries (Jencons, Leighton Buzzard, Bedfordshire, UK) by a two-stage vertical puller. The electrode resistance was 5–10 MΩ when filled with the pipette solution containing (mM) KCl 140, CaCl₂ 1.8, MgCl₂ 1, and HEPES 5 at pH 7.3. Pipettes were Sylgard-coated, and their tips were fire-polished.

Voltage and current data were recorded on an FM tape recorder (model 3964A, Hewlett-Packard Co., Palo Alto, Calif.) at a bandwidth of 5 kHz. Later, data were filtered at 2.5 kHz with an eight-pole Bessel filter (Kemo type VBF 8, Kemo, Beckenham, UK), and analog-to-digital conversions were made at a sampling rate of 5 kHz. Amplitude histograms and kinetic analysis were performed after subtraction of leak currents. Exponential fits were obtained by nonlinear regression using the least-squares method. Mean open probabilities during given time intervals were calculated as the mean patch current/single-channel current ratio. Whenever possible, data are expressed as mean ± SEM, unless otherwise mentioned.

Membrane Preparation From Frog, Guinea Pig, and Human Atria

Atrial membranes were prepared with a modified protocol according to procedures reported for canine- or rabbit cardiac membranes. The whole procedure was performed at 4°C. Human, guinea pig, or frog atria were minced using scissors and an Ultraturrax homogenizer in a buffer containing (mM) sodium pyrophos-
mg/ml + 10 mg/ml bovine serum albumin) to unmask latent activity in rightside-out vesicles. The membrane protein concentrations that yielded reliable ATPase measurements ranged from 15 to 500 \( \mu \)g/ml. All incubation media contained membranes within this concentration range; most experiments were performed with 100 \( \mu \)g/ml proteins.

Ca, Mg-ATPase activity was determined in the same assay medium as the Na,K-ATPase activity but in the presence of 1 \( \mu \)g/ml of the calcium ionophore A23187 and in the absence of SDS. CaCl\(_2\) was added to get a free-Ca\(^{2+}\) concentration of 2–15 \( \mu \)M. Previous experiments had shown that these free Ca\(^{2+}\) concentrations resulted in maximal Ca\(^{2+}\)-dependent ATPase stimulation.

5'-Nucleotidase determinations were performed using a commercially available kit making use of the auxiliary enzymes adenosine deaminase and L-glutamate dehydrogenase (Sigma Diagnostics, St. Louis, Mo.). The membranes were not incubated with SDS or with alamethicin, because preliminary experiments had shown that these manipulations always led to a decrease of the measured 5'-nucleotidase activity.

The amount of \( ^{32} \)P-phosphorylated intermediates of the 130-kd (sarcolemmal) and 100-kd (sarcoplasmic reticular) Ca, Mg-ATPases was determined in order to estimate their relative presence in membrane preparations. The method was based on the protocol described by Wuytack et al\(^10\) for smooth muscle Ca, Mg-ATPase. The assay medium contained (mM) Na\(_3\) 5, imidazole HCl 20 at pH 6.9, and either CaCl\(_2\) 0.05 or potassium EGTA 0.5. The reaction was performed on ice, started by the addition of 6 \( \mu \)M \( [\gamma-^{32}P] \)ATP at 13 Ci/mmol, and stopped 10 seconds later by the addition of a mixture containing 3% SDS, 10% sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 40 mM dithiothreitol. SDS-polyacrylamide gel electrophoresis was run on a 10% resolving gel, and the Cerenkov radiation in the 100- and 130-kd bands was counted after their location was determined by autoradiography of the gels.

**Measurement of NDKP Enzymatic Activity in Atrial Membranes**

The NDKP assays were performed in a buffer solution composed of (mM) KCl 150, MgCl\(_2\) 6 (free Mg\(^{2+}\), 3.6 mM), glucose 5.5, EGTA 0.8, HEPES 5, leupeptin 10\(^{-5}\),

---

**Figure 1.** Activation of frog atrial muscarinic K\(^+\) channels in the absence of acetylcholine. NTP, nucleoside triphosphate. Panel A: Muscarinic K\(^+\) channel activity during the cell-attached (CA) and inside-out recording configuration. Channel activity quantified by NPo (where N is the number of channels and Po the open probability of each channel) is plotted as a function of time. NPo was averaged for every recording of 410 msec. The bathing solution contained 4 mM ATP and 5.7 mM free Mg\(^{2+}\) at the beginning of the experiment; removal of ATP and application 0.1 mM GTP are indicated. The pipette did not contain acetylcholine. The holding potential was \(-58\) mV. The time of patch excision from the cell (forming an inside-out patch) is indicated by a big arrow at the top of the graph. Current tracings a–e are shown below the graph. Panel B: Muscarinic K\(^+\) channel activity in an inside-out patch. The changes in the composition of the bathing solution are as indicated. The number of channel openings had progressively been increasing after excision of the patch from the cell: the first recording (a) was started after steady state had been reached (± 5 minutes after excision).
tritiated $[\text{3H}]\text{GDP}$ (88.5 $\mu$M), $[\text{3H}]\text{GTP}$ (92.5 $\mu$M), or $[\text{3H}]\text{ADP}$ (84.5 $\mu$M) was incubated with 1 $\mu$M of another NTP or NDP, and the transphosphorylation reaction was started by adding membranes. The total volume of the assay medium was 25 $\mu$L; the reaction was conducted at 37°C. After different incubation times (from 0 to 120 minutes), 1-$\mu$L samples of the reaction mixture (3.7 kBq) were spotted on polyethyleneimine cellulose thin-layer chromatography sheets containing a fluorescence indicator together with 2 nmol of each of the marker nucleotides GTP$\cdot$P$\cdot$S, GTP, GDP, and GMP or 2 nmol of their adenine equivalents. The plates were developed in 5 M sodium formate buffer, pH 3.4 at room temperature. Mean $R_0$ values were as follows: ATP$\cdot$S 0.18, ATP 0.52, ADP 0.82, AMP 0.83, GTP$\cdot$S 0.05, GTP 0.24, GDP 0.57, GMP 0.73, and inorganic phosphate (P) 0.88. For autoradiography, the plates were exposed for 1–3 days at $-80\degree$C after application of an enhancer spray (En'hance, NEN Research Products, Boston). The resolution was determined in control experiments; spots of less than 37 Bq could be detected, equivalent to a conversion of less than 1% of the incubated tritiated nucleotide. When hot spots were localized, they were cut out and quantified by scintillation counting after several hours of vigorous shaking in the scintillation cocktail (Ready Safe, Beckman). Counts per minute were recalculated as disintegrations per minute for each vial, using quenching parameters determined with a commercial $^3$H standard. Percentage conversion was calculated as the number of counts in a given spot relative to the whole lane, after subtraction of the background radioactivity in control lanes at time 0. The specific activity of the tritiated nucleotide that was applied was used to calculate the absolute amount of transformed substrate. Conversion rates were roughly assessed by taking the 30- or 60-minute data points for calculation; because the reaction deviated from linearity during this observation period, the calculated conversion rates are an underestimation of the real catalytic activity.

**Products**

All nucleotides (except ATP), pyruvate kinase, and lactic acid dehydrogenase were from Boehringer Mannheim, Mannheim, Germany. ATP was from Sigma Chemie Gmbh. All other reagents were from Merck, Darmstadt, Germany. $[\text{3H}]\text{GDP}$, $[\text{3H}]\text{GTP}$, $[\text{3H}]\text{ADP}$ were from Amersham International plc, Amersham, UK; the other labeled nucleotides were from Du Pont de Nemours, Homburg, Germany.

**Results**

$\text{Mg}^{2+}\cdot\text{ATP}$-Dependent Activation of Muscarinic K$^+$ Channels in Atrial Cells of Rana esculenta

Figure 1A demonstrates that, similar to guinea pig$^1$ or rabbit$^2$ atrial cells, muscarinic K$^+$ channels in frog atrial cells can be activated by $\text{Mg}^{2+}\cdot\text{ATP}$, whereas acetylcholine and GTP are absent from the extracellular and intracellular solutions, respectively. Only sporadic openings of muscarinic K$^+$ channels were observed in cell-attached conditions (basal activity) and during the first minute after formation of an inside-out patch (tracings a and b in Figure 1A). Thereafter, channel activity gradually increased, reaching a steady-state level after approximately 5 minutes (tracing c). The activity of the muscarinic K$^+$

---

### Figure 2

**Activity of frog atrial muscarinic K$^+$ channels in the presence of extracellular acetylcholine.** NTP, nucleoside triphosphate. A representative experiment shows openings (panel A) and the evolution of NPo (where N is the number of channels and P is the open probability of each channel) (panel B) of muscarinic K$^+$ channels during cell-attached (CA) and inside-out patch-clamp configurations. Tracings a–e are shown. The initial bathing solution contained 5.7 mM free Mg$^{2+}$ but no nucleotides. The pipette contained 10 $\mu$M acetylcholine. The holding potential was $-58$ mV. NPo in panel B was averaged for every recording of 410 msec. Multiple channel openings were present in the CA mode. Excision of the patch from the cell is indicated by a big arrow above the graph in panel B. Within the first minute after excision, NPo of the muscarinic K$^+$ channels decayed to a basal level. The 3-minute period during which openings of ATP-dependent K$^+$ channels were present was omitted. Application of 100 $\mu$M GTP (+4 mM ATP) resulted in activation of the muscarinic K$^+$ channels, and openings rapidly disappeared after washout (wo) of GTP. The muscarinic K$^+$ channels were, however, reactivated by ATP alone in the course of the following minutes (tracing e).
channels was dependent on ATP, because openings disappeared after removal of the nucleotide (tracing d). The ATP-dependent activation is the result of the direct NDPK-catalyzed transphosphorylation of GDP that is bound to the G protein G\(\kappa\); the \(\gamma\)-phosphate of ATP is transferred to GDP by membrane-bound NDPK, hence transforming the G nucleotide into GTP. The GTP-bound form of G\(\kappa\) is the active conformation that opens the K\(^+\) channels. Application of 100 \(\mu M\) ATP alone transiently activated the channels in some experiments (to \(\approx 6.7\%\) of the activity observed in the presence of 4 mM ATP), but steady-state NPo (where N is the number of channels and Po is the open probability of each channel) always returned to the level of basal activity within less than 20 seconds (tracing e). This is explained by the fact that although GTP can act as a phosphate donor for NDPK (in analogy with ATP), it will also block the direct NDPK-mediated activation of G\(\kappa\), as we have previously reported for muscarinic K\(^+\) channels in inside-out patches of guinea pig cells. The block is due to competition of exogenous GTP (or GDP) with G\(\kappa\)-bound GDP, resulting in uncoupling of NDPK from G\(\kappa\) and cessation of direct phosphate transfer. In frog cells also, addition of 100 \(\mu M\) GTP or GDP to the bathing solution caused a reversible block of the ATP-induced muscarinic K\(^+\) channel activation to approximately 1% of the activity observed in the presence of 4 mM ATP alone (Figure 1B, tracings b and d). ATP-dependent activation and block by G nucleotides were observed in four other experiments.

With \(10^{-5} M\) acetylcholine present in the pipette, a marked cell-attached activity was observed with multiple superimposed openings (Figure 2, tracing a); acetylcholine stimulates GDP to GTP exchange on the G protein, leading to activation of G\(\kappa\) and opening of the channels. The channel activity disappeared on excision of the patch in a bathing solution without nucleotides (tracing b), since no exchange is possible under these conditions. Muscarinic K\(^+\) channel openings resumed within seconds after application of 100 \(\mu M\) GTP alone (not shown) or in combination with 4 mM ATP (tracing c). Washout of GTP, in the continuing presence of ATP, resulted in an initial fast drop of NPo to 1.4% (36% in another experiment) after approximately 15 seconds (tracing d) and a secondary slow reactivation that reached its steady state after several minutes (tracing e); this secondary activation is due to NDPK-mediated transphosphorylation, using ATP as a phosphate donor and only starting after the complete washout of blocking GTP (as described above). In two experiments, the mean open time during cell-attached activity and after
activation with ATP was determined; it slightly increased from approximately 1.0 to approximately 2.1 msec. Again, these results confirm the findings in guinea pig atrial cells, indicating that the ATP-dependent activation is not only seen in mammalian cells.

**NDPK Enzymatic Activity in Atrial Membranes of Rana esculenta**

As described in "Materials and Methods," we investigated the phosphate transfer between NDPs and NTPs (tritium labeled at position 8 of the purine ring) in the presence of atrial membrane vesicles. The crude atrial membrane preparation of frog was used without further purification. The Na,K-ATPase activity was 2.27 μmol P, formation/mg per 10 minutes, and the Ca,Mg-ATPase activity was 0.74 μmol P, formation/mg per 10 minutes; the ratio of the ATPase activities was 3.07.

**γ-Phosphate or γ-Thiophosphate Transfer From Different NTPs to [3H]GDP**

When 88.5 μM [3H]GDP and 1 mM ATP were incubated with atrial membranes at concentrations between 0.15 and 1.5 μM/ml, a time-dependent increase of [3H]GTP in the incubation medium could be observed (Figures 3A and 3B). Incubation led to a net transformation of 1.80±0.34 μmol [3H]GDP/mg atrial membranes within 10 minutes at 37°C (n = 5). No GTP formation was detectable in the absence of membranes or after membranes had been boiled for 15 minutes (Figure 3C). In the absence of ATP, no [3H]GTP formation could be demonstrated. When high membrane concentrations (15 μg/ml) were used, [3H]GTP production was estimated to be less than 0.005 μmol/mg per 10 minutes, i.e., <0.3% of the conversion rate in the presence of ATP. Breakdown of the substrate [3H]GDP to [3H]GMP was negligible (0.04 μmol/mg per 10 minutes, n = 4).

The net transformation of [3H]GDP to [3H]GTP in the presence of ATP could be reduced by adding 0.1 or 1 mM ADP or UDP (Table 1). The reduction in [3H]GTP formation was somewhat more pronounced for ADP than for UDP: 100 μM of both ADP and UDP resulted in only a minor reduction (<20%), but 1 mM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Absolute transfer rate (μmol/mg per 10 minutes)</th>
<th>Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]GDP (n=2)</td>
<td>&lt;0.005</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>+1 mM ATP (n=5)</td>
<td>1.80±0.34</td>
<td>100</td>
</tr>
<tr>
<td>+1 mM ATP+0.1 mM ADP (n=2)</td>
<td>...</td>
<td>80±5</td>
</tr>
<tr>
<td>+1 mM ATP+1 mM ADP (n=2)</td>
<td>...</td>
<td>12±8</td>
</tr>
<tr>
<td>+1 mM ATP+0.1 mM UDP (n=2)</td>
<td>...</td>
<td>100</td>
</tr>
<tr>
<td>+1 mM ATP+1 mM UDP (n=2)</td>
<td>...</td>
<td>35±5</td>
</tr>
<tr>
<td>+1 mM ITP (n=3)</td>
<td>...</td>
<td>70±7</td>
</tr>
<tr>
<td>+1 mM GTP (n=3)</td>
<td>...</td>
<td>70±6</td>
</tr>
<tr>
<td>+1 mM UTP (n=2)</td>
<td>...</td>
<td>42±1</td>
</tr>
<tr>
<td>+1 mM CTP (n=2)</td>
<td>...</td>
<td>20±2</td>
</tr>
<tr>
<td>+1 mM ATP γS (n=3)</td>
<td>0.02±0.002</td>
<td>1.5</td>
</tr>
<tr>
<td>+1 mM GTP γS (n=3)</td>
<td>0.006±0.001</td>
<td>0.6</td>
</tr>
<tr>
<td>+1 mM AMP-PNP (n=2)</td>
<td>...</td>
<td>0</td>
</tr>
<tr>
<td>+1 mM GMP-PNP (n=2)</td>
<td>...</td>
<td>0</td>
</tr>
<tr>
<td>[3H]ADP (n=2)</td>
<td>&lt;0.01</td>
<td>...</td>
</tr>
<tr>
<td>+1 mM ITP (n=2)</td>
<td>0.73±0.13</td>
<td>41</td>
</tr>
<tr>
<td>+1 mM ITP+0.1 mM GDP (n=2)</td>
<td>[39±7%]</td>
<td>...</td>
</tr>
<tr>
<td>+1 mM ITP+1 mM GDP (n=2)</td>
<td>[9±8%]</td>
<td>...</td>
</tr>
<tr>
<td>+1 mM ITP+0.1 mM UDP (n=1)</td>
<td>[59%]</td>
<td>...</td>
</tr>
<tr>
<td>+1 mM ITP+1 mM UDP (n=2)</td>
<td>[32±6%]</td>
<td>...</td>
</tr>
<tr>
<td>[3H]GTP (n=3)</td>
<td>0.05±0.003</td>
<td>...</td>
</tr>
<tr>
<td>+1 mM UDP (n=3)</td>
<td>0.45±0.11</td>
<td>25</td>
</tr>
<tr>
<td>+1 mM ADP (n=3)</td>
<td>0.11±0.02</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

The transformation of tritiated nucleotides was measured at 37°C, and net transfer rates (i.e., after subtraction of conversion rates with the tritiated nucleotide alone) were determined by taking the 30- or 60-minute conversion percentages, as described in "Materials and Methods." The concentrations of [3H]GDP, [3H]GTP, and [3H]ADP were 88.5, 92.5, and 84.5 μM, respectively. The free Mg2+ concentration was kept constant at 3.65 mM. The number of measurements is indicated for each condition. Relative activities are expressed as percentages of the transfer rate between [3H]GDP+ATP. Percentages between square brackets are with respect to the reaction with [3H]ADP+ITP as substrates.
ADP decreased [3H]GTP formation by 88±8%, and 1 mM UDP decreased [3H]GTP formation by 65±5% (n=2 for all determinations). Incubation of [3H]GDP with ADP, in the absence or presence of membranes, did not result in any [3H]GTP formation, indicating that the guanylate kinase activity was negligible.

Not only ATP but also other NTPs could serve as phosphate donors. This is shown in Figures 4A and 4B. The order of substrate efficiency was ATP>ITP=GTP>UTP>CTP (Table 1). When [3H]GDP was incubated with ATPγS or GTPγS, slow formation of [3H]GTPγS was detectable, as is shown in Figure 4C. The rate of triphosphate transfer was 0.02±0.002 and 0.006±0.001 µmol/mg per 10 minutes with ATPγS and GTPγS as donors, respectively, which was 1.5±0.3% and 0.6±0.2% of the transfer rate with ATP. The efficiency of GTPγS was 36±8% relative to that of ATPγS. The degradation of the substrates ATPγS and GTPγS was minute, even when incubated with seven-fold higher membrane concentrations (not shown). Breakdown of [3H]GDP to [3H]GMP was clearly visible in these experiments, in contrast to the experiments with NTPs, because of the much higher membrane concentration (20 versus 1.5 µg/ml); moreover, it was more pronounced in the presence of ATPγS than in the presence of GTPγS. It is also apparent from Figure 4 that, mainly in the case of ATPγS, [3H]GTPγS production was preceded by a faster and more pronounced [3H]GTP production that was due to contaminating ATP or GTP in the respective γ-thiophosphate nucleotide preparations. When the observed [3H]GTP formation was used to assess the magnitude of these contaminations, 5±0.7% ATP and less than 0.2±0.1% GTP were present in the ATPγS and GTPγS preparations, respectively.

Addition of 1 mM of the nonhydrolyzable analogues guanylylimidodiphosphate (GMP-PNP) or adenylylimidodiphosphate (AMP-PNP) did not induce [3H]GMP-PNP formation. The estimated contamination of these analogues with GTP and ATP was less than 10% and less than 1%, respectively.

Other Nucleotide Pairs Tested for γ-Thiophosphate Transfer

The addition of 1 mM ITP to 84.5 µM [3H]ADP resulted in the formation of [3H]ATP (Figures 5A and 5B), in analogy with the foregoing transphosphorylation reactions. The transition rate was 0.73±0.13 µmol/mg

**FIGURE 4.** Formation of [3H]GTP or [3H]GTPγS from [3H]GDP and different nucleoside triphosphates, ATPγS, or GTPγS by frog atrial membranes. Panel A: Autoradiography of thin-layer chromatograms showing the separation of [3H]GDP from [3H]GTP. [3H]GDP (88.5 µM) was incubated with a 1.5-µg/ml frog atrial membrane preparation and 1 mM ATP (twice), CTP, ITP, GTP, or UTP, as indicated. For each condition, 1-µl samples were spotted on polyethyleneimine cellulose at time 0 (before the addition of membranes) and after 10, 30, 60, and 120 minutes. Panel B: The percentage of formed [3H]GTP plotted as a function of time for the different conditions shown in panel A. Panel C: Autoradiography of thin-layer chromatograms showing the separation of [3H]GDP from [3H]GTP, [3H]GMP, and [3H]GTPγS. [3H]GDP (88.5 µM) was incubated with a 20-µg/ml frog atrial membrane preparation and 1 mM ATPγS or GTPγS, as indicated. For each condition, 1-µl samples were spotted on polyethyleneimine cellulose at time 0 (before the addition of membranes) and after 10, 30, 60, and 120 minutes.
Autoradiographs of thin-layer samples estimated was preparation, membrane

\[ \text{M} \text{UDP} \] observed \( (0.05 \pm 0.003 \text{ mmol/mg}) \) and membranes, \( \text{ADP} + \text{ITP} \) from \( \text{NDP} \). The breakdown of \( \text{ADP} \) or \( \text{ATP} \) resulted in negligible or \( \text{GDP} \) formation, as can also be seen in Figures 5A and 5B: 0.1 and 1 mM GDP reduced the net transition to 39±7% and 9±8%, respectively, and 1 mM UDP reduced the formation to 59% in one experiment, and 1 mM UDP blocked the formation to 32±6% in two experiments (Table 1).

When 92.5 \( \mu \text{M} \) \( \text{H} \text{GTP} \) was incubated with atrial membranes, a moderate hydrolysis to \( \text{H} \text{GDP} \) could be observed (0.05±0.003 \( \mu \text{mol/mg} \) per 10 minutes, \( n=3 \) (Figure 5C). \( \text{H} \text{GTP} \) chromatograms always showed a discrete \( \text{H} \text{GDP} \) spot at time 0, even when no membranes had been added. This \( \text{H} \text{GDP} \) contamination was estimated to be 1.5±0.4% \( (n=9) \). Adding 1 mM ADP or 1 mM UDP to \( \text{H} \text{GTP} \) and membranes resulted in a pronounced increase of \( \text{H} \text{GDP} \) formation. The increase in apparent \( \text{H} \text{GTP} \) breakdown to \( \text{H} \text{GDP} \) after incubation with UDP or ADP is the result of the additional \( \text{H} \text{GDP} \) formation via NDK-catalyzed transphosphorylation between \( \text{H} \text{GTP} \) and \( \text{NDP} \). The additionally formed \( \text{H} \text{GDP} \) amounted to 0.11±0.02 \( \mu \text{mol/mg} \) per 10 minutes \( (n=3) \) in the case of 1 mM ADP and 0.45±0.11 \( \mu \text{mol/mg} \) per 10 minutes with 1 mM UDP \( (n=3) \) (Table 1).

**NDPK Enzymatic Activity in Atrial Membranes of Guinea Pigs**

Guinea pig atrial membranes were used without further purification because of the limited amount of available material. The Na,K-ATPase and Ca,Mg-ATPase activity in this crude fraction were 0.47 and 5.06 \( \mu \text{mol/mg} \) per 10 minutes, respectively, with a ratio of 0.09.

Transformation of \( \text{H} \text{GDP} + \text{ATP} \) to \( \text{H} \text{GTP} \) was apparent with a rate of 0.50±0.05 \( \mu \text{mol/mg} \) per 10 minutes \( (n=5) \), and this transition was reduced 60% by addition of 1 mM ADP \( (n=2) \) (Figure 6A). Boiled membranes did not catalyze any conversion. \( \text{H} \text{GMP} \) production was 0.02±0.08 \( \mu \text{mol/mg} \) per 10 minutes in the absence of ATP but could hardly be detected in the presence of ATP. In the presence of 1 mM GTP\( \gamma \text{S} \), a minor conversion to \( \text{H} \text{GTP} \) and a slow formation of \( \text{H} \text{GTP} \gamma \text{S} \), which were not quantified, were detected. In analogy, ATP\( \gamma \text{S} \) resulted in the fast and slow production of \( \text{H} \text{GTP} \) and \( \text{H} \text{GTP} \gamma \text{S} \), respectively, which were absent if the membranes had previously been boiled (not shown). Two other conversion reactions \( \text{H} \text{ADP} + \text{ITP} \) to \( \text{H} \text{ATP} \) and \( \text{H} \text{GTP} + \text{UDP} \) to

**FIGURE 5. Formation of \( \text{H} \text{ATP} \) from \( \text{H} \text{ADP} \) and formation of \( \text{H} \text{GDP} \) from \( \text{H} \text{GTP} \) by frog atrial membranes. Autoradiographs of thin-layer chromatograms show the separation of \( \text{H} \text{ATP} \) from \( \text{H} \text{ADP} \) (panel A) and the separation of \( \text{H} \text{GDP} \) from \( \text{H} \text{GTP} \) (panel C). \( \text{H} \text{ADP} \left(84.5 \mu \text{M}\right) \) or \( \text{H} \text{GTP} \left(92.5 \mu \text{M}\right) \) was incubated with 3-\( \mu \text{g/ml} \) or 6-\( \mu \text{g/ml} \) frog atrial membrane preparation, respectively, alone or in the presence of other unlabeled nucleotides, as indicated. For each condition, 1-\( \mu \)l samples were spotted on polyethyleneimine cellulose at time 0 (before the addition of membranes) and after 30, 60, and 120 minutes. In panel B, the percentage of formed \( \text{H} \text{ATP} \) is plotted as a function of time for the different conditions shown in panel A.
[3H]GDP were present at concentrations of 88.5, 92.5, and 84.5 μM, respectively. The concentration of unlabeled nucleotides was always 1 mM. For each condition, 1-μl samples were spotted on polyethyleneimine cellulose at time 0 (before the addition of membranes) and after 30, 60 (except for the GDP-only condition in panel A), and 120 minutes. H.T. denotes incubation of [3H]GDP and ATP with heat-treated membranes (boiled for 15 minutes); only samples at time 0 and 120 minutes are shown.

**Discussion**

Muscarinic K+ channels in inside-out patches of frog atrial cells can be activated by ATP in the absence of acetylcholine and GDP or GTP, in a similar way as was described for atrial cells of the guinea pig and rabbit. Thus, the ATP-dependent activating mechanism is not restricted to mammalian cells. Moreover, this activation shows the same characteristics in all species: channel openings can be induced by Mg2+-ATP in the presence or in the absence of agonist, and GDP or GTP block the activation in the absence of agonist. It was postulated that the ATP-dependent activation is due to NDPK, which is present in atrial membranes and which can directly activate Gx by phosphorylation of Gx-bound GDP. That direct transphosphorylation of Gx-bound GDP by NDPK is possible has recently been shown in reconstitution experiments with GTP binding proteins Gx, Gz, and Gβ. The block by G nucleotides is explained by competition of exogenously applied GDP or GTP with Gx-bound GDP for NDPK, hence uncoupling...
the activating association between $G_K$ and NDPK. Agonist-mediated exchange can mask the blocking effect of GTP on NDPK (manifested by the transient drop in NPo on washout of GTP before reactivation by ATP and NDPK, as shown in Figure 2).

Kim$^{12,13}$ reported that phosphorylation of rat atrial muscarinic $K^+$ channels (or of a regulatory protein) by protein kinase A$^{12}$ and/or by a hitherto unidentified kinase$^{13}$ results in a significant increase of the mean open time (and hence of the NPo) of the channels. This effect may add to the final activity level of the channels in the presence of Mg$^{2+}$ATP. In accordance with Kim's results, the mean open time of the channels in our preparation was prolonged after ATP-dependent activation compared with cell-attached conditions, as we have also reported for guinea pig cells (from 1.8±0.8 to 2.5±0.7 ms, $n=7$). However, the kinase-mediated effects described by Kim cannot be responsible for the channel activation itself, as we have reported in the present study and in a previous study: 1) Protein kinase A is not membrane associated;$^{12}$ 2) Exogenously added protein kinase A+ATP could not induce opening of the channels by itself;$^{12}$ 3) Good substrates for NDPK such as UTP and ITP could not mimic the effect of ATP on the prolongation of the mean open time as observed by Kim.$^{13}$

Our results show that frog, guinea pig, and human atrial membranes can indeed catalyze the conversion between NDPs and NTPs; i.e., they contain NDPK enzymatic activity. The γ-phosphate transfer was mainly demonstrated by using $[^3H]$GDP as a phosphate acceptor (Table 1). However, there was great variability between the different NTPs regarding their efficiency as phosphate donors: the order was ATP>GTP>CTP>UTP. This order parallels the observed efficiency of these nucleotides in activation of the muscarinic $K^+$ channels in guinea pig atrial membrane patches. It is known that, although NDPK has a relative lack of substrate specificity, different isozymes vary in their Michaelis constants and maximum velocity with different nucleotides. The order of efficiency reported by other authors was variable among different NDPK preparations, but ATP was always the most efficient substrate.$^{14-16}$ The average rate of phosphate transfer between ATP and $[^3H]$GDP was 1.8 μmol/mg per 10 minutes, which is of the same order of magnitude as the Na,K-ATPase activity in the same preparation; i.e., the membranes have a substantial transphosphorylating capacity. Crude rat liver and heart membranes were reported by other authors to have specific activities of 0.17 and 0.8 μmol/mg per 10 minutes under comparable experimental conditions.$^{12,17}$

We also demonstrated the NDPK activity of the membranes by using $[^3H]$ADP as the phosphate acceptor with ITP as the donor and by using $[^3H]$GTP as the
phosphate donor with UDP or ADP as the acceptor (Figure 5). Although these reactions were slower than some of those with \([\text{H}]\text{GDP}\) as the acceptor, they were at least six to 18 times more effective than the thophosphorylations by ATP\(\gamma\)S or GTP\(\gamma\)S (Table 1). It is known that ATP\(\gamma\)S can be used by kinases but that the thophosphoryl transfer has slow kinetics. This observation also correlates with the slow onset of irreversible channel activation seen in atrial membrane patches. \(\text{ATP\gamma}S\) was a better thophosphoryl donor than GTP\(\gamma\)S in our experiments (ratio of 2.8), as ATP was a better thophosphoryl donor than GTP (ratio of 1.4). In HL-60 membranes, however, the transfer with GTP\(\gamma\)S was 1.4 times more efficient than with ATP\(\gamma\)S. Nonhydrolyzable NTP analogues such as GMP-PNP and AMP-PNP did not result in phosphate transfer in analogy with the described inability of AMP-PNP to stimulate the channels.

Some guanylate kinase or GTP–GMP kinase activity may have been present, because limited \([\text{H}]\text{GTP}\) production could be observed when \([\text{H}]\text{GDP}\) alone was incubated with membranes, but the GTP-producing capacity of these pathways is much less than the NDPK-dependent one (0.005 versus 1.8 \(\mu\text{mol/mg per 10 minutes}\)). Moreover, no additional formation of \([\text{H}]\text{GTP}\) could be detected on incubation of 88.5 \(\mu\text{M} [\text{H}]\text{GDP}\) with 1 \(\text{M} \text{ADP or UDP}\). Also, the production of \([\text{H}]\text{GMP}\) from \([\text{H}]\text{GDP}\), which was mainly observed at higher membrane concentrations, can be due to guanylate kinase and GTP–GMP kinase activity or nonspecific \([\text{H}]\text{G-nucleotide breakdown}. Addition of an excess cold nucleotide (e.g., 1 \(\text{M} \text{ATP}\)) could markedly reduce this breakdown (compare Figures 6A and 7A), although further addition of 1 \(\text{M} \text{ADP}\) again increased \([\text{H}]\text{GMP}\) production (possibly via increased guanylate kinase action). Moreover, G nucleotides seem to be better blockers of \([\text{H}]\text{GMP}\) production than A nucleotides (see Figure 4C).

Addition of ADP or UDP reduced the rate of phosphate transfer between ATP and \([\text{H}]\text{GDP}\), and GDP or UDP blocked the conversion of \([\text{H}]\text{ADP}+\text{ITP}\). Block can be the result of mere competition between different NDPs for the NDP-selective acceptor site on NDPK, but it is known that an excess of either NDP or NTP can lead to the formation of a nonfunctional “abortive” NDPK complex when NDPS also bind to the NTP-selective donor site or vice versa: both sites are occupied by the same nucleotide, and no phosphate transfer will occur. Therefore, the inhibition patterns are complex: for each nucleotide they depend on the type and concentration of the partner nucleotides, and they have no relation with the nucleotide’s efficiency as a substrate during transphosphorylation. It was not our aim to make a detailed study of these inhibitory interactions, but the observation that different nucleotides interfere with the phosphate transfer indicates that the NTP–NDP conversion mechanism is relatively aspecific, as is expected for an NDPK-catalyzed reaction. Our results indicate that the order of affinity for the acceptor site is GDP > ADP > UDP because 1) equimolar concentrations (100 \(\mu\text{M}\) of ADP or UDP block less than 50% of the conversion between 88.5 \(\mu\text{M} [\text{H}]\text{GDP}+1 \text{mM ATP}\),

### Table 2. Rate of Net Phosphate Transfer by Different Atrial Membrane Preparations From Frog, Human, and Guinea Pig for Different Combinations of Nucleotides

<table>
<thead>
<tr>
<th>Condition</th>
<th>Absolute transfer rate ((\mu\text{mol/mg per 10 minutes}))</th>
<th>Relative versus ([\text{H}]\text{GDP}+\text{ATP}) in same preparation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frog</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP (n=5)})</td>
<td>1.80±0.34</td>
<td>...</td>
</tr>
<tr>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP}+1 \text{mM ADP (n=2)})</td>
<td>...</td>
<td>12</td>
</tr>
<tr>
<td>([\text{H}]\text{ADP}+1 \text{mM ITP (n=2)})</td>
<td>0.73±0.13</td>
<td>41</td>
</tr>
<tr>
<td>([\text{H}]\text{GTP}+1 \text{mM UDP (n=3)})</td>
<td>0.45±0.11</td>
<td>25</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP (n=2)})</td>
<td>2.40±0.2</td>
</tr>
<tr>
<td>SL</td>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP (n=3)})</td>
<td>2.36±0.18</td>
</tr>
<tr>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP}+1 \text{mM ADP (n=2)})</td>
<td>...</td>
<td>13</td>
</tr>
<tr>
<td>([\text{H}]\text{ADP}+1 \text{mM ITP (n=1)})</td>
<td>0.50</td>
<td>21</td>
</tr>
<tr>
<td>([\text{H}]\text{GTP}+1 \text{mM UDP (n=1)})</td>
<td>0.25</td>
<td>11</td>
</tr>
<tr>
<td><strong>Guinea pig</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP (n=5)})</td>
<td>0.50±0.05</td>
</tr>
<tr>
<td>SL</td>
<td>([\text{H}]\text{GDP}+1 \text{mM ATP}+1 \text{mM ADP (n=2)})</td>
<td>...</td>
</tr>
<tr>
<td>([\text{H}]\text{ADP}+1 \text{mM ITP (n=1)})</td>
<td>0.16</td>
<td>32</td>
</tr>
<tr>
<td>([\text{H}]\text{GTP}+1 \text{mM UDP (n=1)})</td>
<td>0.07</td>
<td>15</td>
</tr>
</tbody>
</table>

Crude, crude membrane fraction; SL, sarcolemma-enriched fraction; SR, sarcoplasmic reticulum–enriched fraction. Values are mean±SEM.

The transformation of tritiated nucleotides was measured at 37°C, and transfer rates were based on the 30- or 60-minute conversion percentages, as described in “Materials and Methods.” The number of measurements is indicated for each condition.
2) block by ADP is more pronounced than block by UDP, and 3) \(^{[3]}\text{H}\)ATP formation (in the presence of 84.5 \(\mu\text{M}\) \(^{[3]}\text{H}\)ADP+1 mM ITP) was more reduced by 100 \(\mu\text{M}\) GDP than by 100 \(\mu\text{M}\) UDP (61% versus 41% block). On the other hand, \(^{[3]}\text{H}\)GTP to NDP phosphate transfer was faster with UDP compared with ADP as the phosphate acceptor, illustrating the unpredictable relation between a nucleotide’s efficiency as substrate or blocker, as mentioned above.

Interestingly, 100 \(\mu\text{M}\) GDP did not completely block the \(\gamma\)-phosphate transfer, in contrast with the 99% block of channel activation in inside-out patches.\(^1\) This may indicate that the apparent very high affinity of the acceptor site that was evident in the electrophysiological experiments was lost in the membrane preparation. The difference can be due to the absence of direct interaction with a G protein, as has also been reported by others: five times more \(^{[\gamma-32\text{P}]\text{ATP}}\) than \(^{[\gamma-32\text{P}]\text{GTP}}\) was formed when the \(^{[3]}\text{P}\)phosphoepoxide of Ehrlich’s ascites tumor cell NDPK was incubated with GDP and ADP, but the amount of formed \(^{[\gamma-32\text{P}]\text{GTP}}\) dose-dependently increased with the addition of G proteins.\(^2\)

Similar results were reported for NDPK preparations of HeLa S3 cells.\(^2\) Block of channel activation by GDP is due to uncoupling of NDPK from \(G_\text{K}\) (and not to inhibition of the catalytic activity), whereas in the experiments of the present study the enzymatic activity of the uncoupled enzyme is studied (i.e., without direct interaction with the G protein).

We have shown that atrial membranes from human and guinea pig atria contain NDPK enzymatic activity. The relative efficiencies for the three sets of phosphate transfer studied were similar in all species, as is summarized in Table 2. The formation of \(^{[3]}\text{H}\)GTP from ATP and \(^{[3]}\text{H}\)GDP was reduced by the addition of 1 mM ADP. The specific NDPK activity in human membranes was nearly identical for crude and partially purified sarclemma-enriched and sarcoplasmic reticulum–enriched fractions, and it was comparable to that in frog atrial membranes. In crude guinea pig atrial membranes, the activity was only slightly lower (0.5 \(\mu\text{mol/mg per minute}\)). However, the Na,K-ATPase and CaMg-ATPase activities, and their ratios, vary greatly between the different membrane preparations. Thus, the NDPK activity does not seem to be linked to the purification of sarclemmaal or sarcoplasmic reticular markers. It is known that NDPK is a mainly cytosolic enzyme, but its presence in the plasma membrane of liver,\(^1,2\) thyroid,\(^2\) HeLa S3 cells,\(^2\) and platelets\(^2\) and in mitochondrial outer membranes\(^2\) has been reported. The liver plasma membrane NDPK was indistinguishable from the cytosolic enzyme by physicochemical methods or by peptide mapping.\(^2\) However, sonic disruption followed by wash of our vesicle preparations did not reduce their transphosphorylating capacity, so that contamination by soluble NDPK seems unlikely. It was reported that liver endoplasmic reticulum did not contain NDPK activity,\(^2\) but the extent to which sarcoplasmic reticulum contains NDPK remains to be determined.

In conclusion, the findings that atrial membranes contain a high NDPK activity and that the NDPK activity measured shows the same order of substrate efficiency as observed in the channel activation experiments support the concept of NDPK involvement in the muscarinic signal transduction cascade.

Acknowledgment

We thank Frank Wuytack for his careful reading of the manuscript and his constructive comments.

References

10. Wuytack F, Raeymaekers L, De Schutter G, Casteels R: Demonstration of the phosphorylated intermediates of the Ca-transport ATPase in a microsomal fraction and in a (Ca+Mg)-ATPase purified from smooth muscle by means of calmodulin affinity chromatography. Biochim Biophys Acta 1982;693:45–52


Membrane-bound nucleoside diphosphate kinase activity in atrial cells of frog, guinea pig, and human.

H Heidbüchel, G Callewaert, J Vereecke and E Carmeliet

Circ Res. 1992;71:808-820
doi: 10.1161/01.RES.71.4.808

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/71/4/808

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/