Korean J Physiol Pharmacol
Vol 2: 743−752, December, 1998

Modulation of ATP-Induced Activation of the Muscarinic K^+ Channel Activity by Protein Kinase C

Yang-Mi Kim[1], Hong-Ki Park, Jae-Hee Han[2], Choon-Ok Park, and Seong-Geun Hong

Department of Physiology, Gyeongsang National University College of Medicine, 92 Chilam-Dong, Chinju 660−280, Korea

The atrial acetylcholine-activated K^+ (K_{ACh}) channel is gated by the pertussis toxin-sensitive inhibitory G (G_K) protein. Earlier studies revealed that ATP alone can activate the K_{ACh} channel via trans-phosphorylation mediated by nucleoside-diphosphate kinase (NDPK) in atrial cells of rabbit and guinea pig. This channel can be activated by various agonists and also modulated its function by phosphorylation. ATP-induced K_{ACh} channel activation (AIKA) was maintained in the presence of the NDPK inhibitor, suggesting the existence of a mechanism other than NDPK-mediated process. Here we hypothesized the phosphorylation process as another mechanism underlying AIKA and was undertaken to examine what kinase is involved in atrial cells isolated from the rat heart. Single application of 1 mM ATP gradually increased the activity of K_{ACh} channels and reached its maximum 40~50 sec later following adding ATP. AIKA was not completely reduced but maintained by half even in the presence of NDPK inhibitor. Neither ADP nor a non-hydrolyzable ATP analogue, AMP-PNP can cause AIKA, while a non-specific phosphatase, alkaline phosphatase blocked completely AIKA. PKC antagonists such as sphingosine or tamoxifen, completely blocked AIKA, whereas PKC catalytic domain increased AIKA. Taken together, it is suggested that the PKC-mediated phosphorylation is partly involved in AIKA.

Key Words: muscarinic K^+ channel, PKC, ATP, Phosphorylation, Atrial myocytes

INTRODUCTION

Binding of acetylcholine (ACh) to atrial muscarinic receptors causes the opening of K^+ (K_{ACh}) channel to increase the K^+ conductance via inhibitory pertussis toxin-sensitive GTP-binding protein (G_K) (Pfaffinger et al, 1985; Clapham & Kim, 1989). Increased K^+ conductance hyperpolarizes the membrane potential and then reduces the cardiac excitability. Recently it was established that interaction of $\beta\gamma$-subunit of G_K ($G\beta\gamma$) with carboxyl-termini of K_{ACh} channel protein initiates the opening of K_{ACh} (Clapham & Neer, 1993; Inanobe et al, 1995; Kobayashi et al, 1995; Wickman et al, 1994). This channel shows the conductance of 30~40 pS and has a property of the inward rectification, so called, G protein-coupled inward rectifying K^+ (GIRK) channel (Hille, 1992).

As well as ACh, several substances such as calcitonin-gene related peptides (CGRP) (Kim, 1991a), adenosine (Oguchi et al, 1991; Stiles, 1991) and endothelin (Kim, 1991b) can also serve as agonists for activating K_{ACh} channels. Since K_{ACh} channels are gated by G_K, these agonists should share a common pathway leading to dissociation of $G\beta\gamma$ from heterotrimeric G_K. Therefore, it is possible to open K_{ACh} channels (1) by ACh or other agonists at the membrane receptor level, (2) by $G\beta\gamma$, for example, by application of GTP or purified $G\beta\gamma$ subunits even without an agonist. This possibility was experi-

Corresponding to: Seong-Geun Hong, Department of Physiology, Gyeongsang National University College of Medicine, 92 Chilam-Dong, Chinju 660-280, Korea. e-mail: hong149@gshp.gsnu.ac.kr
Present address: [1]Department of Physiology and Biophysics, University of Health Science/The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL60064, USA; [2]Department of Physiology, Seonam University College of Medicine, 720 Kwangchi-Dong, Namwon 590-711, Korea

mentally tested and well documented (Zang et al, 1993; Wickman et al, 1994).

Another way to activate K_{ACh} channels is the single application of ATP to the cytoplasmic side of the membrane in the absence of GTP. Several studies showed that ATP activated gradually K_{ACh} channels with time, implying the involvement of an enzyme system. They suggested the membrane-bound nucleoside diphosphate kinase (NDPK) as an enzyme involved in ATP-induced K_{ACh} channel activation (AIKA) (Heidbüchel et al, 1990; Heidbüchel et al, 1991; Kaibara et al, 1991; Heidbüchel et al, 1993). Since NDPK transfers a terminal phosphate of nucleoside triphosphates to nucleoside diphosphates in the presence of ATP, NDPK transphosphorylates GDP-G_K into GTP-G_K. Then GTP-G_K dissociated from NDPK releases $G\beta\gamma$ to activate K_{ACh} channels (Heidbüchel et al, 1993). Cytoplasmic application of ATP without GTP consequently allows NDPK-induced formation of GTP to open K_{ACh} channels via gating of G_K. Interestingly, function of the K_{ACh} channel can also modulated by phosphorylation by way of G_K-independent way (Hong et al, 1996). This modulatory effect could be clearly detected when ATP was added on the K_{ACh} channel function caused normally in the presence of both external ACh and internal GTP. Channel activities maintained constantly by ACh and GTP were dramatically increased up to five fold after adding ATP, while phosphatase reduced the increment induced by ATP to the level before adding ATP, suggesting that this effect of ATP on the channel function might be due to the phosphorylation/dephosphorylation process (Kim, 1991c; Kim, 1993; Hong et al, 1996). To date, an membrane-bound kinase is believed to be involved in phosphorylation contributing ATP-modulation on the K_{ACh} channel function but it remains still unclear what kind of enzyme is (Hong et al, 1996).

Without GTP, AIKA can be thought either by NDPK-mediated transphosphorylation, or by phosphorylation via G_K-independent way on the basis of regulatory mechanisms described above. If AIKA were entirely due to the NDPK-mediated transphosphorylation, it is required that GDP has to be existed in a membrane phase or a localized space inside the small patch so that GDP can be exchanged into GTP in the presence of ATP (Heidbüchel et al, 1993). Without GTP, GDP might be diffused out of membrane phase and finally became depleted. In accordance with depletion of GDP from the membrane phase, the K_{ACh} channel activity should be gradually disappeared. Since GDP or GDPβS, a non-hydrolyzable GDP analogue, has been known as a NDPK antagonist, NDPK-mediated AIKA should be blocked (Heidbüchel et al, 1993). However in the presence of GDPβS, it was observed that AIKA was maintained although the channel activity reduced by half in the preliminary study. These imply that the NDPK-mediated transphosphorylation cannot be secured and requires another mechanism underlying AIKA.

Here we set an assumption that the phosphorylation may contribute to AIKA as well as transphosphorylation by NDPK. To test our idea, we selected the protein kinase C (PKC) as the potent candidate for eliciting AIKA because molecular studies on the K_{ACh} channel suggested many PKC putative sites in GIRK protein (Kubo et al, 1993). This study suggests that a phosphorylation is an important mechanism underlying AIKA and PKC involves in this process.

METHODS

Single cell preparation

Atria from Sprague Dawley rats (supplied by the Yuhan Central Research Institute, Korea) more than 8 weeks old were used in this experiments.

Whole heart was isolated from the chest of rat anesthetized with i.p. injection of ketamin and rompun (5 mg/kg, respectively) before quickly transferred to the Lagendorff column. Normal Tyrode solution containing 1.8 mM Ca^{2+} saturated with gas (95% O_2 and 5% CO_2) was applied to maintain the coronary circulation hydrostatically (100 cmH_2O) through the orifice of coronary arteries at 37°C for 5 mins. Following washing the heart with Tyrode solution for 5 mins, Ca^{2+}-free Tyrode was perfused. Then, the heart was enzymatically digested with 0.4 mg/ml collagenase (Sigma Type II) for 30 mins. Thereafter the enzyme-containing solution was flushed out with Ca^{2+}-free Tyrode solution and two atria were cut into small pieces. Suspension of single cells harvested by gently agitating atrial pieces was divided in a 35 mm plastic Petri dishes with four pieces of 12 mm round cover glasses and kept at 4°C before use.

Table 1. The composition of the experimental solutions (in mM)

Reagents	Bathing solution	Pipette solution
KCl	120	120
$MgCl_2$	2	2
EGTA/KOH	5	5
HEPES/KOH	10	10
Acetylcholine	–	10 μM

Solutions

The compositions of the normal Tyrode solution, Ca^{2+}-free solution, the pipette solution, and the bathing solution were given in Table 1. The pH of all solutions was adjusted to 7.2 with HEPES-KOH.

Acetylcholine (ACh), 12-O-tetra-decanoylphobol-13-acetate (TPA), protein kinase C (PKC) catalytic subunit and tamoxifen, PKC antagonist, were purchased from Sigma Ltd., USA. Adenosine 5'-triphosphate (ATP, sodium salt), adenylyl-imidodiphosphate (AMP-PNP), guanosine 5'-triphosphate (GTP, trilithium salt), guanosine 5'-O-(3-thio-triphosphate) (GTPγS) were purchased from Böhringer Mannheim Ltd., Germany. Sphingosine and 1-(5-isoquinolinyl-sulfonyl)-2-methyl piperazine (H-7) were obtained from Research Biochemicals International (RBI), USA. Alkaline phosphatase was obtained from Amresco Inc (Ohio, USA).

Sphingosine, tamoxifen and TPA were dissolved in dimethyl sulfoxide (DMSO). When chemicals dissolved in DMSO were applied into bath, they were diluted more than a thousand times not to exceed 0.1% of DMSO concentration. Cytoplasmic sides of excised patches were perfused with desired solutions through plastic tubing at a rate of ~2 ml/min. When solutions containing ATP were used, free [Mg^{2+}] was calculated using the computer program given in Fabiato & Fabiato (Fabiato, A. 1988) and was kept relatively constant at 1.85 mM.

Electrophysiology

Gigaseals were formed with Sylgard (Dow Corning Chemical Co.) coated pipette of 5 MΩ resistances, and channel currents were recorded using the method described by Hamill et al (1981). Membrane potential was held at −60 to −80 mV. Channel activities were recorded with a patch clamp amplifier (EPC-7, List, FRG), low-pass filtered at 3 kHz using an eight-pole Bessel filter (AI 2040, Axon Instruments, USA), and stored on magnetic tape for video cassette recorder (Samsung, SV-606) via a pulse code modulator (PCM-2, Medical Systems, USA). Later, digitized data were transferred directly into an IBM-clone personal computer and analyzed to obtain histograms for duration, amplitudes and probabilities as well as channel activity (averaged $N \cdot P_o$) using analysis program (pClamp 6.02, Axon Instrument, USA), where N is the number of channels in a membrane patch and P_o is the probability of the channel opening.

Single-channel openings in expanded scale presented in figures were filtered at 1 kHz and channel tracings obtained from pen recorder were filtered at 50 Hz. All experiments were performed at 22~24°C, and data were presented as means±SD. Because of multiple channel openings in most patches, accurate measurements of mean open times (τ_o) were not possible. However since alterations in open time duration occurred in these experiments, mean open times were determined by selecting patches having one or two channels.

In this study, patches only containing K_{ACh} channels were used. Sometimes when K_{ATP} channels were activated, experiments were undergone because K_{ATP} channels differ from K_{ACh} channels in amplitudes and opening kinetics. In all experiments, the mean single channel amplitude of the K_{ACh} channels ranged from 1.8 to 2.1 pA at −60 mV.

RESULTS

ATP-induced activation of the muscarinic K^+ channel

Acetylcholine of 10 μM present in the pipette elicited single channel currents of 2.66±0.18 pA (n=18) at −80 mV in the cell-attached patch configuration. These channels currents were inwardly rectified and had conductances of 33 pS at −80 mV. They disappeared in the symmetrical solution containing 140 mM KCl without GTP, which is essential to the G protein gating for activating the K_{ACh} channel in inside-out patch mode.

As shown in Fig. 1A, spontaneous openings of the K_{ACh} channel in the cell-attached patch configuration were disappeared upon excising membrane patch

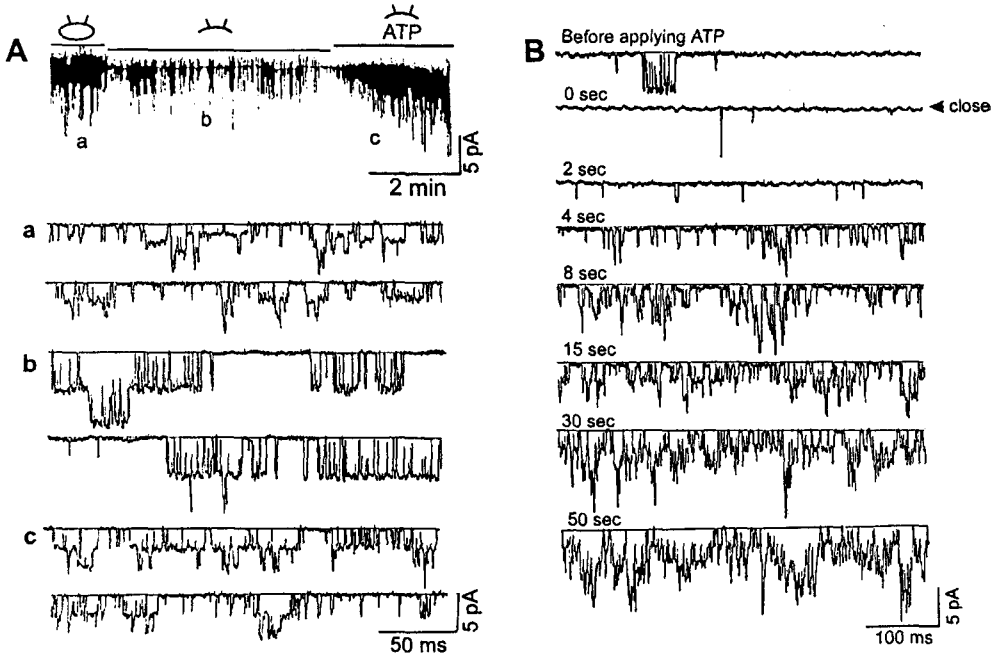

Fig. 1. ATP-induced activation of the K_{ACh} channel. A. Representative activation of the K_{ACh} channel only by 1 mM ATP applied to the cytoplasmic side in the upper panel. Channel currents were recorded at the cell-attached configuration (a), and before (b) and after (c) perfusing ATP at the inside-out (I/O) configuration, respectively. Note the opening of ATP-sensitive K^+ channels in the absence of ATP at I/O configuration (trace b). B. Gradual increase in the ATP-induced activation of K_{ACh} channel (AIKA). Single channel records were obtained at a time represented on the left of each trace. Top tracings in A and B were filtered at 50 Hz. Traces in expanded scale the upper panel were filtered at 1 kHz.

from atrial cell and ATP-sensitive K^+ (K_{ATP}) channels were abruptly activated (trace b) without both GTP and ATP. Subsequent application of 1 mM ATP to the bathing solution ceased the firing of K_{ATP} channel currents and then activated gradually single channel currents of 33 pS again (trace c) even in the absence of GTP. This kind of the K_{ACh} channel activation by ATP was able to observe from more than 90% of patches used in this study. The ATP-induced activation of the K_{ACh} channel (AIKA) was gradually increased with time. Fig. 1B shows a typical AIKA, which activity was gradually increased. Before perfusing ATP to the bath, one could not observe the single K_{ACh} channel current except openings of K_{ATP} channels. Few K_{ACh} channel current appeared as soon as adding ATP, however, its activity became clear 10 s later and reached a maximal activity 50 s later following addition of ATP lowest trace in Fig. 1B). A gradual rise in AIAK could be observed in several trials ($n > 3$), due to the increase in the open probability (P_o), not in the mean open time (τ_o). Half maximum in P_o was 24 s, although not analyzed in all cases. This slow increase in AIKA was thought that ATP did not play directly as an agonist to the K_{ACh} channel.

The ATP-induced activation of K_{ACh} channel by phosphorylation

As shown in Fig. 1B, ATP did not cause the spontaneous activation of K_{ACh} channels, suggesting that its effect on K_{ACh} channels was likely to serve as a regulator responsible for triggering the channel activation. Since it has been reported that AIKA resulted from the transphosphorylation mediated by NDPK in a membrane-delimited manner (Heidbüchel et al, 1993), we confirmed whether ATP might act as a substrate for phosphorylation initiating activation of the K_{ACh} channel. To test this assumption, chemicals that can not be served as substrates for

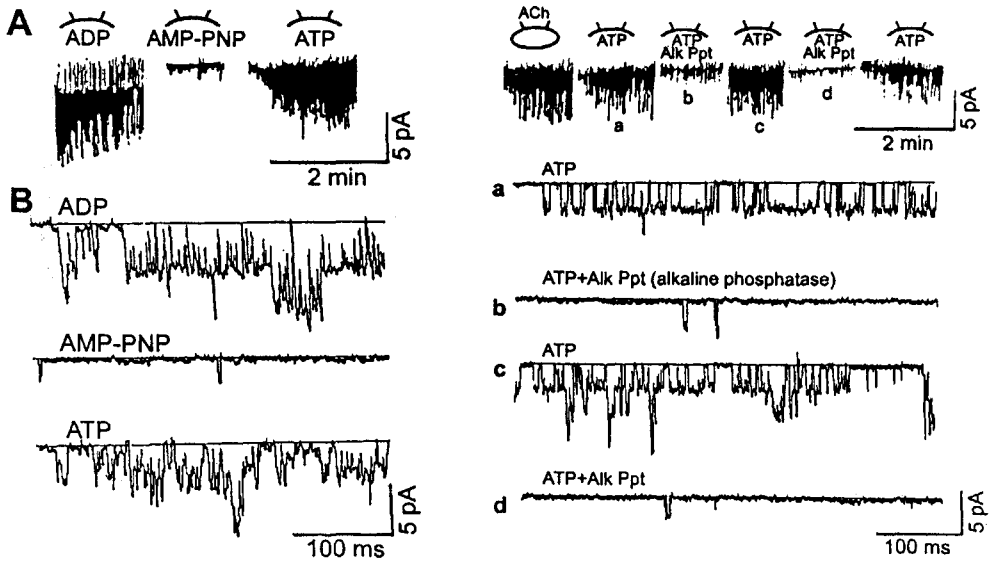

Fig. 2. ATP as a potent substrate for phosphorylation. A, Single channel currents in the presence of ADP, AMP- PNP and ATP. Single channel traces in expanded scale from recordings of top panels. K_{ACh} channel was activated only by application of ATP as shown in the third recordings. These recordings were made in a patch. B. Effect of alkaline phosphatase on the ATP-induced activation of K_{ACh} channels. Six recordings in the top panel were obtained in a same patch and their experimental sequences were in order from left to right. Lower four recording traces in expanded scale were the channel activities represented from the each trace of the same label on the top panel. Addition of 2 units of alkaline phosphatase was repeated two times. Top tracing in each panel was continuously recorded in a same membrane patch and artifacts due to changes of the solution were omitted to leave as blank interruptions.

phosphorylating process, such as ADP or AMP-PNP, a non-hydrolyzable ATP analogue, was applied but failed to initiate the activation of K_{ACh} channels (Fig. 2). When ADP was applied, only K_{ATP} channels were activated (first trace in Fig. 2A). On adding AMP-PNP, neither activation of K_{ATP} channel nor AIKA were observed (middle trace in Fig. 2A).

Since these results implied that AIKA might be brought about by phosphorylation, we applied alkaline phosphatase which acts on phosphorylating sites to cause dephosphorylation. As expected, alkaline phosphatase potently antagonized the effect of ATP (Fig. 2B). After forming the inside-out patch configuration, AIKA was elicited following addition of 1 mM ATP (trace a). Then the further addition of 2 unit alkaline phosphatase blocked completely AIKA as presented in trace b. Again AIKA was recovered by removing the alkaline phosphatase (trace c). The N^*P_o was remarkably reduced from 0.135 ± 0.084 (n=1627) to 0.007 ± 0.004 (n=207) by alkaline phosphatase. Closer single channel analysis revealed that alkaline phosphatase decreased both mean open time (τ_o) and open probability (P_o) shown in Fig. 3. In the presence of 2 unit alkaline phosphatase, P_o was reduced to 16% of that before the perfusion of alkaline phosphatase (0.045 vs. 0.007) and τ_o did from 3.65 ms (n=1226) to 1.50 ms (n=2151). Antagonistic actions between ATP and phosphatase indicated that AIKA resulted from phosphorylation.

AIKA is not entirely dependent on the NDPK-mediated transphosphorylation

There have been evidences that NDPK plays a crucial role in AIKA (Heidbüchel et al, 1990; Heidbüchel et al, 1991; Heidbüchel et al, 1993; Kaibaraet al, 1991). That is, NDPK converts GDP into GTP in the presence of ATP, resulting in enabling the K_{ACh} to open. When GDPβS, a non-hydrolyzable GDP analogue, was bound with G protein, ATP could not bring about the activation of K_{ACh} channels, since GDPβS can not be a substrate of NDPK. Therefore

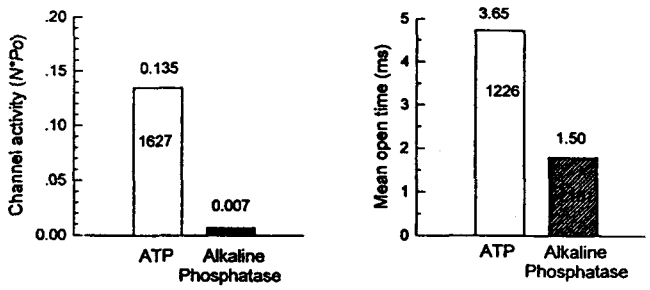

Fig. 3. Effect of the alkaline phosphatase on N^*P_o and τ_o. N^*P_o and τ_o measured before and after adding alkaline phos- phatase were compared in the left and right panel, respectively. Numerics represent data and number of events, respectively (see text).

GDP or GDPβS has been used as NDPK blockers.

In this experiment, we confirmed whether AIKA is entirely dependent on the NDPK-mediated trans-phosphorylation. If AIKA was mediated by NDPK, blockers of NDPK should have inhibited AIKA. As shown in Fig. 4B, either GDP (100 μM) or GDPβS, nonspecific NDPK inhibitor, was added for more than 2 min (trace a) and then washed out (trace b). GDP decreased $N \cdot P_o$ from 0.34 to 0.18 (52.9%), with single channel amplitude and mean open time (τ_o) unchanged. $N \cdot P_o$ was soon recovered to 0.43 after removing GDP, and decreased to 0.21 (48.8%) with subsequent administration of GDPβS (trace c). This result showed that role of NDPK was critical. However in the presence of ATP, AIKA by a NDPK blocker was not totally responsible for AIKA. Actually AIKA was maintained even in the presence of GDPβS as long as ATP existed in the bathing solution, although channel activity was reduced to 50% of maximal activity (Fig. 4).

This result that AIKA remaining by half even in the presence of NDPK blockers such as or GDPβS suggested the presence of another mechanism underlying AIKA. Especially another mechanism might be likely to phosphorylating process involved in protein kinase other than NDPK, since AIKA was lost by dephosphorylating process caused with a nonspecific phosphatase, alkaline phosphatase (Fig. 2 and Fig. 3).

Protein kinases C involved in AIKA

To test which protein kinase(s) is involved in the activation of these K^+ channels, we observed if there would be some changes in AIKA treated with a protein kinase or kinase inhibitor.

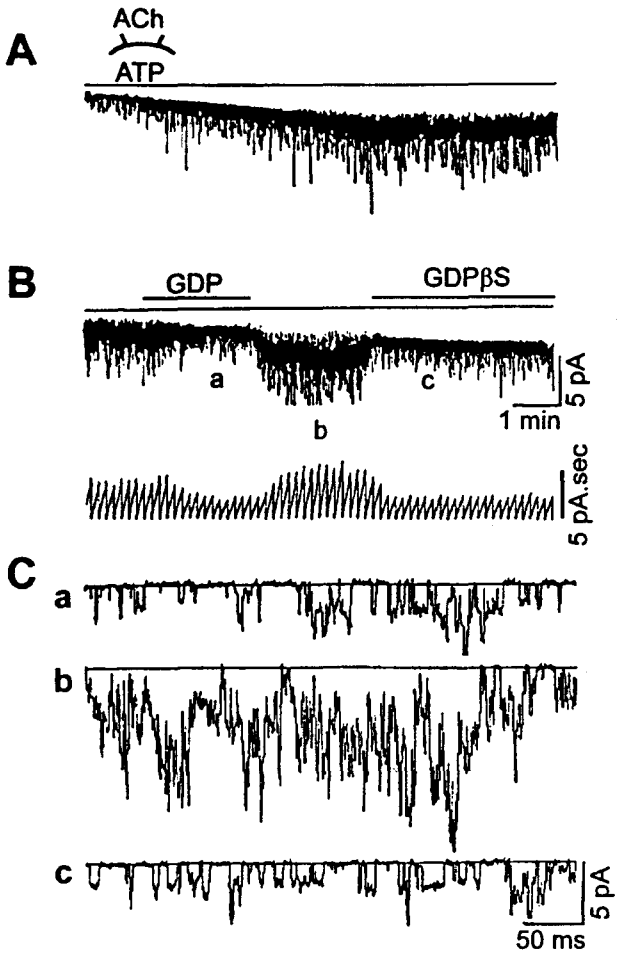

Fig. 4. Effect of GDP and GDPβS on the ATP-induced activation of the K_{ACh} channel. A, The K_{ACh} channel activated by ATP were gradually increased to the steady-state. B, Effect GDP and GDPβS on the K_{ACh} channel activated by ATP. Tracing A and B were in series. Pulses below tracing in B represented the relative channel activity obtained at every 10 sec. Peaks of pulses were calculated from the area occupied by the channel opening multiplied with a given time (10 s) (unit: pA · s). These were used to compare channel activities altered at a given condition. Traces in expanded scale labelled a, b and c were adopted from the channel response to the chemicals denoted above bars.

A potent inhibitor for cAMP-dependent protein kinase (PKA) and Ca^{2+}-dependent protein kinase (PKC), H-7 (50 μM) was added to the cytoplasmic side of the patch pretreated with 1 mM ATP. H-7 decreased τ_o (5.12 ms vs. 3.16 ms) and also open probability (P_o) from 0.079±0.045 (n=502) to 0.050 ±0.023 (n=501), suggesting that the phosphorylation process modulated AIKA by affecting on P_o and τ_o.

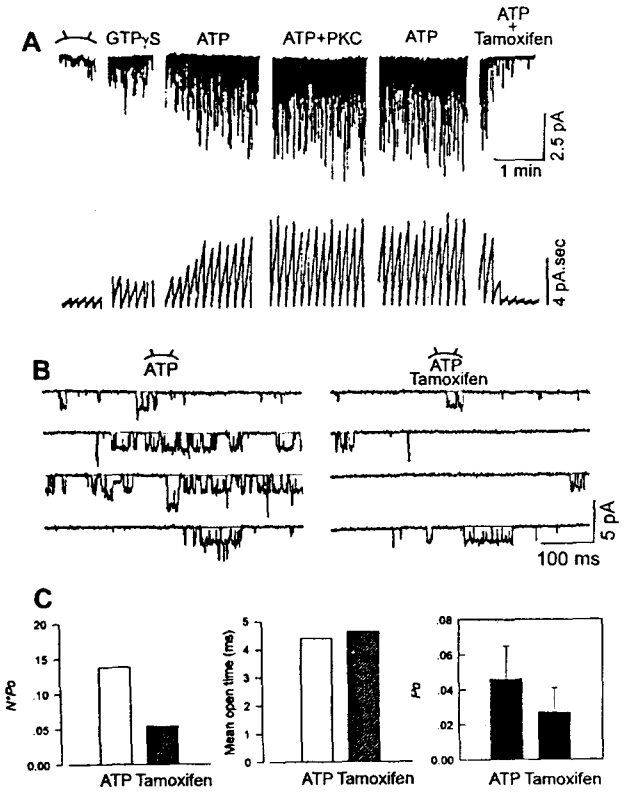

Fig. 5. PKC-induced modulation of AIKA. A, Antagonistic effect between PKC catalytic unit and tamoxifen on the K_{ACh} channel activity induced by ATP. ATP was applied after washout of GTPγS. B, Effect of tamoxifen on the ATP-induced K_{ACh} channel activity in expanded scale. Recordings before and after the addition of tamoxifen were shown in the left and right panel, respectively. Four lines of single channel traces represents recordings for 2 s. C, Changes in N^*P_o and τ_o. and open probability (P_o) by tamoxifen. N^*P_o, τ_o and P_o measured before and after adding tamoxifen were compared in the left, middle and right panel, respectively. Data were represented as mean±S.E.

Recent studies from the amino acid sequence analysis of GIRK1 protein present in the rat atrial muscarinic K^+ channel suggested the presence of the putative binding sites for PKC (Kubo et al, 1993; Kunkel & Peralta, 1995). Therefore, PKC was thought to be potent candidate responsible for AIKA.

Specific PKC antagonists, such as sphingosine and tamoxifen, decreased K_{ACh} channel activities (Fig. 5B & 5C), implying that PKC might be contribute to AIKA. To find concrete evidence that PKC involves in AIKA, PKC catalytic unit was directly applied to membrane patches (Fig. 5A). Upon adding 2.5 μg/ml PKC catalytic domain, the K_{ACh} channel activity was enhanced from 0.204 to 0.24 (17.6%). This difference resulted from the increase in open probability (from 0.068 to 0.08), with the channel amplitude and the open time duration unchanged. The K_{ACh} channel activity remained constant after washing out of PKC catalytic unit, probably due to an absence of the cell cytoplasm, which has been reported to contain a factor acting as phosphatase (Hong et al, 1996). Finally, the K_{ACh} activity was completely inhibited by 30 μM tamoxifen, a PKC antagonist (sixth tracing in Fig. 5A).

In this experiment, when 10 μM sphingosine was applied, K_{ACh} channel activity was rapidly lost but membrane patches were frequently broken out. Thus we failed to record changes in AIKA by sphingosine more than ten minutes. In order to secure recordings, another PKC inhibitor, tamoxifen was used as shown representatively in Fig. 5.

Activities of the K_{ACh} single channel in expanded scale showed clearly the effect of tamoxifen as represented in Fig. 5C. Detailed analysis revealed that addition of tamoxifen reduced N^*P_o to 0.138±0.057 from 0.054±0.028 (left panel in Fig. 13). This reduction in N^*P_o was mainly due to the decrease in the open probability, P_o (from 0.027±0.014 to 0.046 ±0.019), not in τ_o (from 4.65 ms to 4.45 ms). These data indicated that PKC was involved in AIKA.

DISCUSSION

New finding in this study is that the phosphorylating process is an another mechanism underlying ATP-induced K_{ACh} channel activation (AIKA) without GTP, besides NDPK-mediated transphosphorylation which was known as the critical mechanism for AIKA.

As well known as one of the typical G protein (G_K)-gated inwardly rectifying K^+ (GIRK) channel, it has been established that K_{ACh} channel is gated by βγ-subunit of G_K (Gβγ) (Wickman et al, 1994; Kunkel & Peralta, 1995). When ACh bound to the ACh receptor, m_2 subtype of five isoforms, m_2AChR (Haga & Haga, 1990), it activated heterotrimeric inhibitory PTX-sensitive Gαβγ complex (G_K) which dissociates into α-subunit (Gα) and βγ-subunit (Gβγ) in the presence of GTP. Signalling pathways leading to open K_{ACh} channel in atrial cells comprises functional triad structures including m_2AChR-G_K-K_{ACh} channel protein. Therefore GTP or purified Gβγ-

subunit is essential to activate the K_{ACh} channel.

In respect of the established signalling axis, AIKA under the GTP-free condition seems to be quite extraordinary. Since AIKA via NDPK-mediated transphosphorylation is based on transforming GTP, this mechanism can be regarded as same effect as the application of GTP for the activation of K_{ACh} channels. While PKC-mediated phosphorylation is likely to be independent on the G_K-gating. Because this mechanism was still effective on maintaining AIKA in the presence of GDP or GDPβS, which prevents G_K-gating essential to open K_{ACh} channels.

Is the NDPK-mediated transphosphorylation is critical in AIKA?

Several reports already confirmed that ATP could activate K_{ACh} channels with or without ACh stimulation (Heidbüchel et al, 1990; Heidbüchel et al, 1991; Kaibara et al, 1991; Heidbüchel et al, 1993). They suggested that AIKA resulted from the interplay of membrane-bound NDPK which transphophorylates GDP into GTP by transferring the terminal phosphate from ATP. Newly synthesized GTP, in turn, released $G\beta\gamma$ from $G\alpha\beta\gamma$ to turn the K_{ACh} channel on. Consequently, AIKA mediated by NDPK can play only as an alternative route in the physiological signalling pathway.

According to the NDPK hypothesis, NDPK can supply GTP to build up high GTP/GDP ratio enough to activate K_{ACh} channel in a membrane delimited manner, whenever the K_{ACh} channel is normally activated either by the exogenous ACh or by ATP, since the diffusion of the cytosolic GTP to the membrane is too slow to raise the GTP level needed to regulate K_{ACh} channels (Heidbüchel et al, 1993). In addition of this hypothesis, it has been known that the role of NDPK for the activation of the K_{ACh} channel could be switched on or off according to the conditions where stimuli changes. For example, in the absence of agonist such as ACh in the pipette, GTP inhibited the ATP-induced K_{ACh} channel activity because of the competition between GTP and GDP-G_K for the same site on NDPK (Heidbüchel et al, 1993). However when ACh was present, GTP could activate the K_{ACh} channel, resulting from the direct exchange between free GTP and GDP bound to G_K regardless of NDPK. After removing GTP, this process stopped and subsequently the transphosphorylation mechanism took over again. If the K_{ACh} channel activity was elicited by ATP or GTP, it basically resulted from the gating of G_K due to the NDPK-mediated process.

As shown in Fig. 4, the activity in AIKA was reduced by half when NDPK blockers such as GDP or GDPβγ presented. This indicates clearly that NDPK is critical in AIKA. In addition of role of the NDPK blocker, GDP or GDPβS prevents heterotrimeric G_K ($G\alpha\beta\gamma$) not only from dissociating into G βγ subunit but also from G_K-gating. This is, either GDP or GDPβS exerts dual effect on blocking AIKA. Therefore GDP or GDPβS-resistant activities in AIKA implies the presence of a mechanism independent on gating by G_K.

Phosphorylation as well as NDPK-mediated transphodphorylation is responsible for AIKA

Recently a direct phosphorylation has been continuously proposed as the mechanism underlying the regulation of K_{ACh} channel function (Kim, 1991c: Kim, 1993; Zang et al, 1993; Hong et al, 1996). When channel protein was phosphorylated, K_{ACh} channel activities increased 4~5 times up due to the increase in mean open time (Kim, 1993; Hong et al, 1996). This regulatory effect by phosphorylation seemed to be independent on G_K, although not suggested what kind of protein kinase should be involved.

This study revealed several results coincident with phosphorylation-induced modulation of K_{ACh} channel activity. First, ADP or AMP-PNP, which can not be utilized as substrate for protein kinase, failed to elicit the activation of the K_{ACh} channel (Fig. 2A) and alkaline phosphatase completely inhibited AIKA (Fig. 2B & 3), strongly suggesting that AIKA could be caused by phosphorylation. Second, H-7, an inhibitor for both PKC and PKA, decreased τ_o rather than P_o. Third, ATP increased the channel activity more than that induced by GTP alone as shown in Fig. 5 (see compare the first tracing with second tracing shown in top panel). Actually we frequently observed that the ATP-induced channel open time increased. Since an increase in τ_o is characteristic of phosphorylation occurring on addition of ATP (Kim, 1991c: Kim, 1993; Hong et al, 1996), the increased τ_o observed during AIKA, comparing to those recorded at cell-attached configuration, may be served as a valid index for phosphorylation. Whereas τ_o was hardly increased on the GTP-induced activation of the K_{ACh}

channel. If AIKA is caused only by NDPK-mediated transphosphorylation yielding GTP, τ_o should be unchanged. Therefore AIKA might be caused by phosphorylation and transphodphorylation mediated by NDPK.

Protein kinase C may be involved in AIKA

As suggested from the molecular structure of the K_{ACh} channel (exactly GIRK1), PKC has been considered as a potent modulator for the K_{ACh} channel function (Kubo et al, 1993; Krapivinsky et al, 1995; Mileo et al, 1995). The idea that PKC might regulate AIKA could be supported by several results from the present study. First either sphingosine or tamoxifen, a potent and selective PKC inhibitor, reduced or blocked K_{ACh} channel activities induced by ATP. Second direct application of PKC catalytic unit elevated AIKA (Fig. 5). These results indicated that the PKC-mediated phosphorylation can be ascribed to one of mechanisms underlying AIKA.

On the contrary, earlier studies failed to find a role of PKC in K_{ACh} channel function (Kim, 1991c; Lo & Breitwieser, 1994), except in exogenous K_{ACh} current via nicotinic receptor (Mileo et al, 1995). These experiments were performed coincidentally either in the presence of GTP plus ATP or on a intact cell, whereas our experiments were without GTP. In other words, it is hard to observe an effect of PKC on AIKA in the presence of GTP. Then, one may suppose that PKC cannot play a role in AIKA when G_K gated the K_{ACh} channel to activate unless a third molecule is related. If PKC and dissociated $G\beta\gamma$ share a common site in the channel protein, they might compete on the K_{ACh} channel protein to inhibit AIKA. Actually C-terminal has been known to provide site (s) for $G\beta\gamma$ and for the PKC phosphorylation (Huang et al, 1995; Inanobe et al, 1995; Kunkel & Peralta, 1995). We can suppose that the K_{ACh} channel can be modulated in various ways. For instance, when the channel was activated via G_K, a conformational change might cause the channel to switch off the response to PKC. When the signalling via G_K was absent, a change might drive to switch on the response to PKC. Whatever it may happen, it became clear that the K_{ACh} channel function might be flexibly modulated according to the intracellular situation.

Interestingly, we frequently observed that PKC inhibitors used in this study completely blocked AIKA. If AIKA resulted from the NDPK-mediated transphosphorylation combined with the channel phosphorylation, it could be assumed that PKC phosphorylated both NDPK and the channel protein. A further study is required to test this assumption because the regulatory mechanism underlying the K_{ACh} channel activity relates directly to the cardiac function.

In summary, the G_K-gated muscarinic K^+ channel can be activated when ATP was applied to the cytoplasmic side of the I/O membrane patch. The present study provided an evidence that PKC-mediated phosphorylation as well as the transphosphorylation mediated by a nucleoside diphosphate kinase (NDPK) may be responsible for the ATP-induced activation of the K_{ACh} channel without GTP in rat atrial cell. Especially this mechanism underlying the K_{ACh} channel activity induced by ATP may be accomplished in a GTP-binding protein independent way. Precise mechanism linked to ACh-mediated signal transduction processes needs to be further elucidated to understand the physiology of the K_{ACh} channel activation and the regulation of cardiac rate and rhythm.

ACKNOWLEDGEMENT

This research was supported by grant from KOSEF (95-0403-08-0203). We wish to thank Yuhan Central Research Institute, Korea for supplying Sprague Dawley rats.

REFERENCES

Clapham DE, Kim D. G-protein activation mechanisms of the cardiac K^+ channel, $i_{K,ACh}$. *In Secretion and its control.* The Rockefeller University Press, New York: 55–68, 1989

Clapham DE, Neer EJ. New roles for G-protein $\beta\gamma$-dimers in transmembrane signalling. *Nature* 365: 403–406, 1993

Fabiato A. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol* 157: 378–417, 1988

Haga T, Haga K. Interactions of muscarinic acetylcholine receptors (mAChRs) with G-proteins. In Receptor-Effector coupling; A Practical Approach, (Hulme, E. C., editor), IRL press, Oxford: 83–84, 1990

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391: 85−100, 1981

Heidbüchel H, Callewaert G, Vereecke J, Carmeliet E. ATP-dependent activation of atrial muscarinic K^+ channels in the absence of agonist and G-nucleotides. *Pflügers Arch* 416: 213−215, 1990

Heidbüchel H, Callewaert G, Vereecke J, Carmeliet E. Membrane-bound nucleoside diphosphate kinase activity in atrial cells of frog, guinea pig, and human. *Circ Res* 71: 808−820, 1991

Heidbüchel H, Callewaert G, Vereecke J, Carmeliet E. Acetylcholine-mediated K^+ channel activity in guinea-pig atrial cells is supported by nucleoside diphosphate kinase. *Pflügers Arch* 422: 316−324, 1993

Hille B. Ionic channels of excitable membranes, 2nd ed., Sinauer, Sunderland: 170−201, 1992

Hong SG, Pleumsamran A, Kim D. Regulation of atrial muscarinic K^+ channel activity by a cytosolic protein via G protein-independent pathway. *Am J Physiol* 270: H526−H537, 1996

Huang C-L, Slesinger PA, Casey PJ, Jan YN, Jan NY. Evidence that direct binding of $G\beta\gamma$ to the GIRK1 G protein-gated inwardly rectifying K^+ channel is important for channel activation. *Cell* 15: 1133−1143, 1995

Inanobe A, Morishige K, Takahashi N, Ito H, Yamada M, Takumi T, Nishina H, Takahashi K, Kanaho Y, Katada T, Kurachi Y. $G\beta\gamma$ directly binds to the carboxyl terminus of the G protein-gated muscarinic K^+ hannel, GIRK1. *Biochem Biophys Res Comm* 212 (3): 1022−1028, 1995

Kaibara M, Nakajima T, Irisawa H, Giles W. Regulation of spontaneous opening of muscarinic K^+ channels in rabbit atrium. *J Physiol.* 433: 589−613, 1991

Kim D. Calcitonin-gene-related peptide activates the muscarinic-gated K^+ current in atrial cells. *Pflügers Arch* 418: 338−345, 1991a

Kim D. Endothelin activation of an inwarddly rectifying K^+ current in atrial cells. *Circ Res* 69: 250−255, 1991b

Kim D. Modulation of acetylcholine-activated K^+ channel function in rat atrial cells by phosphorylation. *J Physiol* 437: 133−155, 1991c

Kim D. Mechanism of rapid desensitization of muscarinic K^+ current in adult rat and guinea pig atrial cells. *Circ Res* 73: 89−97, 1993

Kobayashi T, Ikeda K, Ichikawa T, Abe S, Togashi S, Kumanishi T. Molecular cloning of a mouse G-protein-activated K^+ channel (mGIRK1) and distinct distributions of three GIRK (GIRK1, 2 and 3) mRNAs in mouse brain. *Biochem Biophys Res Comm* 208: 1166−1173, 1995

Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE. The G-protein-gated artial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+ channel proteins. *Nature* 374: 135−141, 1995

Kubo Y, Reuveny E, Slesinger PA, Jan YN, Jan LY. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature* 364: 802−806, 1993

Kunkel MT, Peralta EG. Identification of domains conferring G protein regulation on inward rectifier potassium channels. *Cell* 83: 443−449, 1995

Lo FC, Breitwieser GE. Protein kinase-independent inhibition of muscarinic K^+ channels by staurosporine. *Am J Physiol* 266: C1128−C1132, 1994

Mileo AM, Palma E, Polenzani L, Limatola C, Grassi F, Eusebi F. Protein kinase C modulates exogenous acetylcholine current in Xenopus Oocytes. *J Neurosci Res* 41: 443−451, 1995

Oguchi T, Furukawa Y, Sawaki S, Kasama M, Chiba S. Are negative chronotropic and inotropic responses to adenosine differentiated at the receptor or postceptor levels in isolated dog heart? *J Pharmacol Exp Ther* 272 (2): 838−844, 1995

Pfaffinger PJ, Martin J M, Hunter DD, Nathanson NM, Hille B. GTP-binding proteins couple cardiac muscarinic receptors to a K^+ channel. *Nature* 317: 536−538, 1985

Stiles GL. Adenosine receptors: Physiological regulation and biochermical mechanisms. *News Physiol Sci* 6: 161−165, 1991

Wickman KD, Iniguez-Lluhi JA, Davenport PA, Taussig R, Krapivinsky GB, Linder ME, Gilman AG, Clapham DE. Recombinant G-protein $\beta\gamma$-subunits activate the muscarinic-gated atrial potassium channel. *Nature* 368: 255−257, 1994

Zang WJ, Yu XJ, Honjo H, Kirby MS, Boyett MR. On the role of G protein activation and phosphorylation in desensitization to acetylcholine in guinea-pig atrial cells. *J Physiol (Lond)* 464: 649−679, 1993