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Modulatory Effect of the Tyrosine Kinase and Tyrosine Phosphatase on the ACh-activated K^+ Channel in Adult Rat Atrial Cells

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Acetylcholine (ACh) activates the inwardly rectifying muscarinic K^+ channel in rat atrial cells via pertussis toxin (PTX)-sensitive G-protein (G_K) coupled with the muscarinic receptor (mAChR). Although this K^+ (K_{ACh}) channel function has reported to be modulated by the phosphorylation process, a kinase and phosphatase involved in these processes are still unclear. Since either PKA or PKC was not effective on this ATP-modulation, the present study examined the possible involvement of the protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) in the function of the K_{ACh} channel.

In the inside-out (I/O) patch preparation excised from the adult rat atrial cell, when activated by 10 μM ACh in the pipette and 100 μM GTP in the bath, the mean open time (τ_o) and the channel activity (N^*P_o) was 1.13 ms (n=5) and 0.19 (n=6), respectively. Following the application of 1 mM ATP into the bath, τ_o increased by 34% (1.54 ms, n=5) and N^*P_o by 66% (0.28, n=6). Channel function elevated by ATP was lasted after washout of ATP. However, this ATP-induced increase in the K_{ACh} channel function did not occur in pretreated cells with genistein (50~100 μM), a selective PTK inhibitor, but occurred in pretreated cells with equimolar daidzein, a negative control of the genistein. On the contrary, PTP which acts on tyrosine residue conversely reversed both ATP-induced increased τ_o by 32% (1.20 ms, n=3) and N^*P_o by 41% (0.15, n=3), respectively.

Taken together, these results suggest that K_{ACh} channel may, at least partly, be regulated by the tyrosyl phosphorylation, although it is unclear where this process exerts on the muscarinic signal transduction pathway comprising the mAChR-G_K-the K_{ACh} channel.

Key Words: ACh-activated K^+ (K_{ACh}) channel, phosphorylation, tyrosine kinase, tyrosyl phosphatase, single channel study, Rat atrial cell

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INTRODUCTION

In atrial cells, acetylcholine (ACh) binds to the muscarinic receptors and activates an inwardly rectifying K^+ (K_{ACh}) current via a guanosine triphosphate (GTP)-binding protein named GK (Breitwieser et al., 1985; Kurachi, 1989; Pfaffinger et al., 1985; Pfaffinger & Siegelbaum, 1990; Sternweis & Pang, 1990). Continuous activation of ACh to the muscarinic receptor causes the K_{ACh} current to decrease, referred as the desensitization. Recently, the mechanism of the desensitization has been known due to the decrease in the channel activity (N^*P_o). This reduction resulted primarily from a time-dependent decrease in the channel activity, which was modulated by the phosphorylating process (Kim, 1991; Kim 1993). The desensitization could be observed only in the cell- attached patch of a intact cell, not in the inside-out membrane patch excised from the cell (Kim, 1991; Kim, 1993; Hong et al., 1996). At inside-out patches, administration of ATP drives the mean open time (τ_o) to elevate. This elevated τ_o can be reverted to a level before applying ATP, simply by adding a phophatase such as alkaline phosphatase, which causes dephosphorylation, to the bathing medium (Kim, 1991). These results suggested that a phosphorylation and dephosphorylation process might be involved in the modulation of the K_{ACh} channel function. If a phosphorylation process indeed modulates the K_{ACh} channel function, what kinase or phosphatase might be involved in the channel regulation?

However it is still unclear which kinase or phosphatase were involved in the regulation of the K_{ACh} channel function. Recent molecular studies revealed that nine sites for Ca^{2+}-dependent protein kinase (PKC) phosphorylation were implicated to exist on the G-protein coupled inward rectifier K^+ (GIRK1) channel protein which is regarded as one of subunits constructing the K_{ACh} channel. Therefore PKC is considered as the potent candidate for the phosphorylation (Kubo et al., 1993; Lo & Breitwieser, 1994). But it has not been reported that PKC was likely to be involved in the regulation of the K_{ACh} channel function. Although there were reports that okadaic acid, a specific protein phosphatase-1 (PP-1) and PP-2A antagonist acting on the serine/threonine residue, were effective on the channel function (Kim, 1991; Zang et al., 1993), their results showed a contradiction for the involvement of PKC in the K_{ACh} channel activity. Therefore the question about enzymes responsible for eliciting phosphorylation on the K_{ACh} channel protein remains to be elucidate.

Several lines of reports suggest that a protein kinase acting on the tyrosine residue rather than kinases on serine/threonine residues could be effective on the K_{ACh} channel function. At the nicotinic receptors in the central nervous system, a receptor protein was phosphorylated by the tyrosine kinase, PTK (Hugarnir et al., 1984; Hopfield et al., 1988). A tyrosine antagonist, methyl-2,5-dihydroxynnamate (MDC) suppressed the K_{ACh} current in frog atrial cells (Otero & Sweitzer, 1993). At present, we do not have clear evidence that PTK plays a role in phosphorylating atrial K_{ACh} channel protein and is considered as a potent candidate involved in the regulation of the K_{ACh} channel function.

This study was performed to confirm whether PTK might be involved in the phosphorylation process that could bring about the alteration in the K_{ACh} channel function. To elucidate this, tyrosine kinase inhibitors and cytosolic tyrosine phosphatase were directly applied to inside-out patches of the membrane in atrial cells isolated from the adult rats.

MATERIALS AND METHODS

Single cell preparation

Atria from adult rats (Sprague Dauley, supplied from Yuhan Pharmaceutical Central Laboratory) more than 12 weeks old were used in this study. Whole heart were quickly excised from the rat anesthetize with ketamin HCl (5 mg/kg) and

xylazine (5 mg/kg) and perfused via a Langendorff column. Initially, the isolated heart was perfused with the bicarbonate/phosphate-buffered Krebs solution containing 1.8 mM Ca^{2+} hydrostatically (100 cmH_2O), ensuring beats to recover and be kept steadily. After 5 min, the Ca^{2+}-free Krebs solution was followed, which cause the heart to dilate and to stop its beating. Finally, perfusion was switched to a Ca^{2+}-free solution containing 0.4 mg/ml collagenase (Type I, Sigma, USA) for 30 min. All solutions were kept at 37°C during perfusion in a column.

Following the enzyme perfusion, both atria were cut out and into small pieces (2×5 mm). The smaller atrial tissues were gently agitated to make a cell suspension of the experimental 140 mM KCl solution. This cell suspension was mounted on 12 mm round cover slips placed in plastic 35 mm Petri dishes. Cells were kept at 4°C before use.

Preparation of the chicken protein tyrosine phosphatase (cPTP)

Tyrosine phosphatase used in this study was donated from the laboratory in the Department of Biochemistry, Gyeongsang National University College of Medicine, Chinju, Korea. The complement DNA (cDNA) of cPTP was cloned from chicken intestine cDAN library, and a glutathione S-transferase (GST)-fusion protein of this gene was expressed in *E.coli* strain BL21 (DE3), isolated by S-hexylglutathione agarose affinity column chromatography. The tyrosine phosphatase activity of this isolated fusion protein was determined by confirming the dephosphorylation of p-nitrophenylphosphate (Jung, 1993)

Chemicals and Solutions

Acetylcholine and collagenase and were purchase from Sigma Chemical (USA). ATP and GTP were purchased from Böhringer Mannheim (Germany). Genistein, daidzein, tyrphostin A1 and tyrphostin 25 were purchased from Biomol (USA). Glibenclamide was obtained from Research Biochemicals International (RBI, USA)

Experimental solution contained 118.5 mM KCl; 2.0 mM $MgCl_2$, 5 mM KOH/EGTA. 10 mM KOH/HEPES and adjusted to pH 7.2 with HCl.

Genistein, tyrphostin and glibenclamide 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 and was kept relatively constant at 1.85 mM.

Single channel recording and data analysis

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 $N*P_o$) 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±SE (standard error). 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 only up to two open channels for general comparison purposes.

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

Properties of the single acetylcholine-activated K^+ (K_{ACh}) channel

Upon touching the atrial cell membrane with the tip of the pipette containing 10 μM ACh, single channel currents were elicited. The conductance of the single channel current was ranged about 35 pS and its mean open time was about 1.0 ms. These are similar to those of the muscarinic K^+ channel (K_{ACh}) current reported by others (Clapham & Kim, 1989; Kurachi et al., 1986; Kim, 1993). It is well known that K_{ACh} current is one of the inwardly rectified K^+ channel currents observed in cardiac cells. As shown in Fig. 1, the current amplitude recorded at inside-out (I/O) patches stimulated with 10 μM ACh in the pipette was −2.99 pA at −80 mV and 1.07 pA at +80 mV, which was only 36% of that recorded at −80 mV, revealing the inward rectification in the current-voltage (I-V) relation.

After forming I/O configuration, K_{ATP} channels were often activated due to the loss of intracellular ATP. Sometimes inward rectifier channels (i_{K1}) were activated in the absence of GTP in the bath. K_{ACh} channels could be distinguished these two channel types from conductances and characteristic opening behaviors. In this symmetrical K^+-rich condition, the

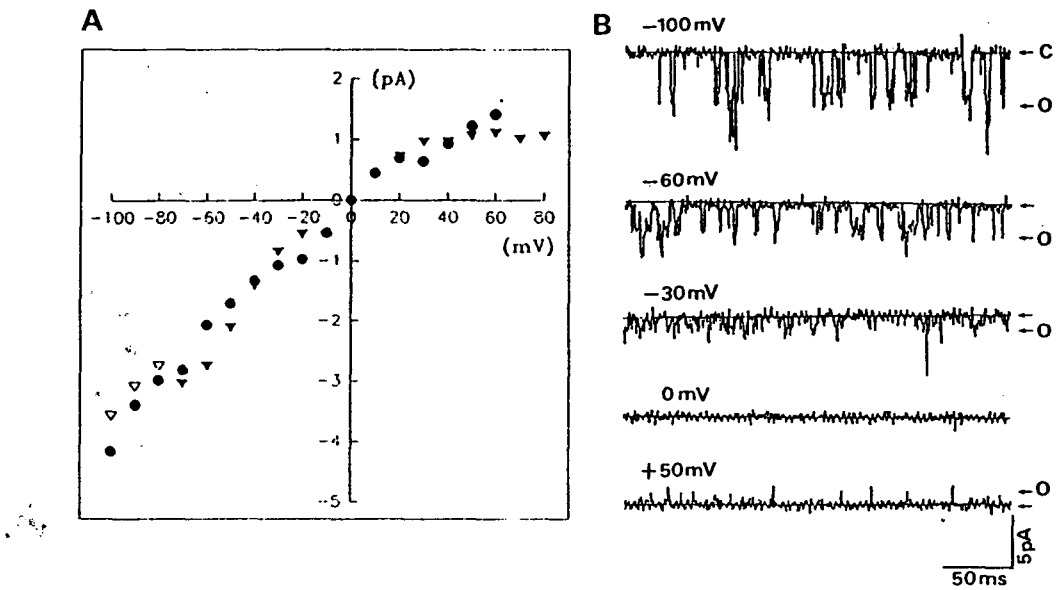

Fig. 1. Current-voltage relation of K_{ACh} channel. A, Inwardly rectified current-voltage (I-V) relation of K_{ACh} channel. Data from three patches are plotted against membrane potential ranged from -100 mV to 80 mV. B, Represented traces are shown with the holding potentials indicated on the left in each trace. These single channel current were recorded from the inside-out patch configuration. Close (c) and open level (o) were indicated with arrows in the left of the trace.

reversal potential was 0 mV, and further addition of 100 μM GTP to the bathing solution resumed channel activities with amplitudes same as those in the cell-attached (C/A) configuration, These results indicated that it was the single channel current flowing through ACh-activated G protein coupled inwardly rectifying K^+ (GIRK) channels.

Regulatory effect of ATP on the K_{ACh} channel current

It has been well established that the K_{ACh} channel is activated via a GTP binding protein (G_K) connected with a muscarinic ACh receptor in the atrial cell. On excising the membrane patch to form I/O configuration, channel activities should be lost due to the absence of the intracellular GTP. Therefore, addition of the GTP to the cytoplasmic side of the membrane patch enables the channel to open via dissociation of the tetrameric G protein. As shown in Fig. 2, single channel currents elicited by the 10 μM ACh present in the pipette were disappeared after forming the inside-out patch and reactivated by application of 100 μM GTP to the bathing solution (trace b in Fig. 2A & B). Further addition of 1 mM ATP to the bath increased the channel function occurred by GTP. The ATP-induced increase in the channel function was maintained after washout of ATP (trace c in Fig. 2B). During the presence of ATP, the open time duration of the K_{ACh} channel was significantly increased from 1.04 ± 0.30 ms to 1.66 ± 0.34 ms ($n=5$, $p<0.05$) and the channel activity (N^*P_o), a product of the number of channel

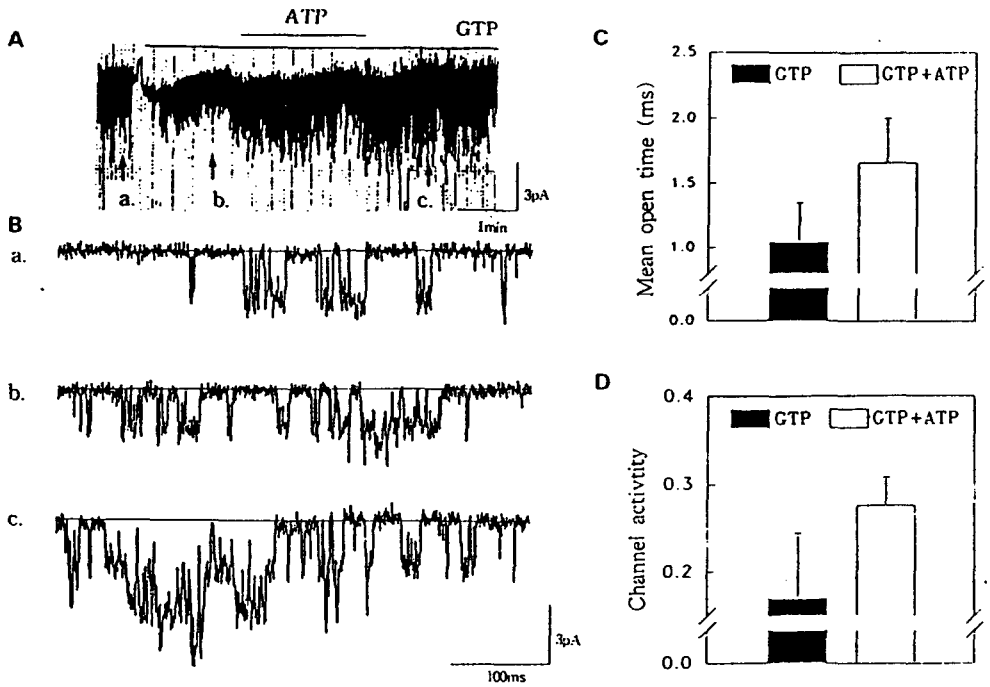

Fig. 2. ATP-induced effect on the K_{ACh} channel activity. A, Representivie traces of the K_{ACh} channel responding to GTP and ATP. B, Tracings in expanded scale selected from the panel A. Channel activities in the cell-attached (a), in the presence of the GTP after forming inside-out patch configuration (b), and following washing 1 mM ATP (c). C, Mean open time (τ_o) obtained before (filled bar) and after ATP administration (open bar). D, Channel activity (N^*P_o) obtained before (filled bar) and after application of ATP (open bar). They were significantly elevated by ATP application ($P<0.05$). Membrane patch was held at -70 mV. Tracing in A were filtered at 50 Hz and in B at 1.0 kHz.

(N) and the open probability (P_o), was also increased to 0.28±0.08 (n=6) from 0.19±0.06 in six patches ($p<0.05$, Fig. 2D). However P_o was indeed insignificantly changed (0.074±0.017 vs. 0.097±0.017, n=6), suggesting that changes in the number of channels might contribute to the effect of ATP on the channel function.

Inhibition of the ATP-induced channel function by the tyrosine kinase inhibitor

As shown in Fig. 2, ATP elevated the K_{ACh} channel function, preliminary due to the increase in the open time (τ_o). This result was similar to those in neonatal rat atrial cells (Kim, 1991). If the ATP effect on the K_{ACh} channel function came from the phosphorylation in the channel protein, a kinase inhibitor should have blocked the effect induced by ATP. In the earlier experiment performed in neonatal atrial cells, either cAMP-dependent protein kinase (PKA) or PKC, both of which exert their effect on serine/threonine residue, did not contribute to the effect of ATP (Kim, 1991). Therefore we tested tyrosine kinase inhibitors (TKI) which act on the tyrosine residue as shown in Fig. 3.

Since a protein kinase inhibitor was not effective on the protein which was already phosphorylated, we compared results recorded in different cells harvested from a same preparation of atrial tissue; one was treated with ATP plus TKI and the other only with ATP as control. As shown in the right panel of Fig. 3, addition of 1 mM ATP elevated the K_{ACh} channel function as expected. While 100 μM genistein, a specific TKI (GN, IC_{50}=1~40 μM), blocked the increase in the channel function even in the presence of a equimolar ATP (right panel of Fig. 3). Following washout of genistein, the channel

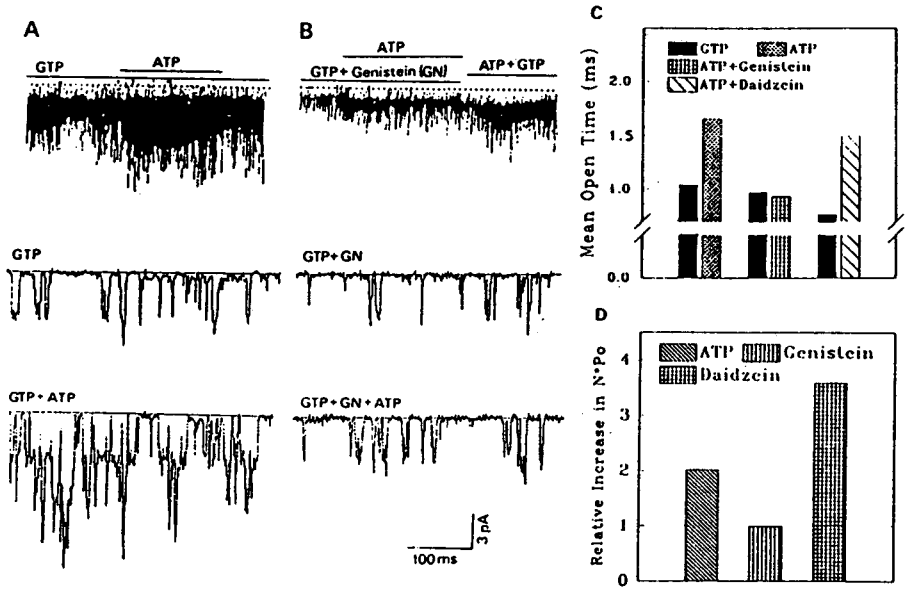

Fig. 3. Inhibition of ATP effect on K_{ACh} channel by the tyrosine kinase inhibitor. A, Example tracings of the channel response to ATP in the absence of the tyrosine kinase inhibitor. Channel activities before (middle trace) and after adding ATP (lower trace) are shown in expanded scale. B, Representitive traces recorded in the presence of (100 μM) genistein, a specific tyrosine kinase inhibitor. Channel activities in panel A and B were obtained in cells isolated from the same rat heart atria. C, Reduction in mean open time by adding genistein in the presence of ATP. D, Genistein-induced effect on the channel activity in the presence of ATP. Data were normalized to the channel activity before adding ATP.

function was slightly increased.

At membrane patches treated with genistein, ATP had no effect on τ_o. When genistein presented in the bath, τ_o was 0.94±0.32 ms (n=4) before adding ATP and 0.94±0.37 ms (n=4) after the addition of ATP as shown in Fig. 3C. Also N^*P_o was not altered from 0.072±0.055 before adding ATP to 0.071±0.056 after adding ATP (n=4, Fig. 3D). However, daidzein, a negative control of genistein (DZ, IC_{50}= ~mM), did not resist to the ATP effect. In the presence of 100 μM daidzein, τ_o and N^*P_o were increased to 1.95 times (0.77 ms vs. 1.50 ms) and 3.6 times (0.065 vs. 0.234) after adding 1 mM ATP.

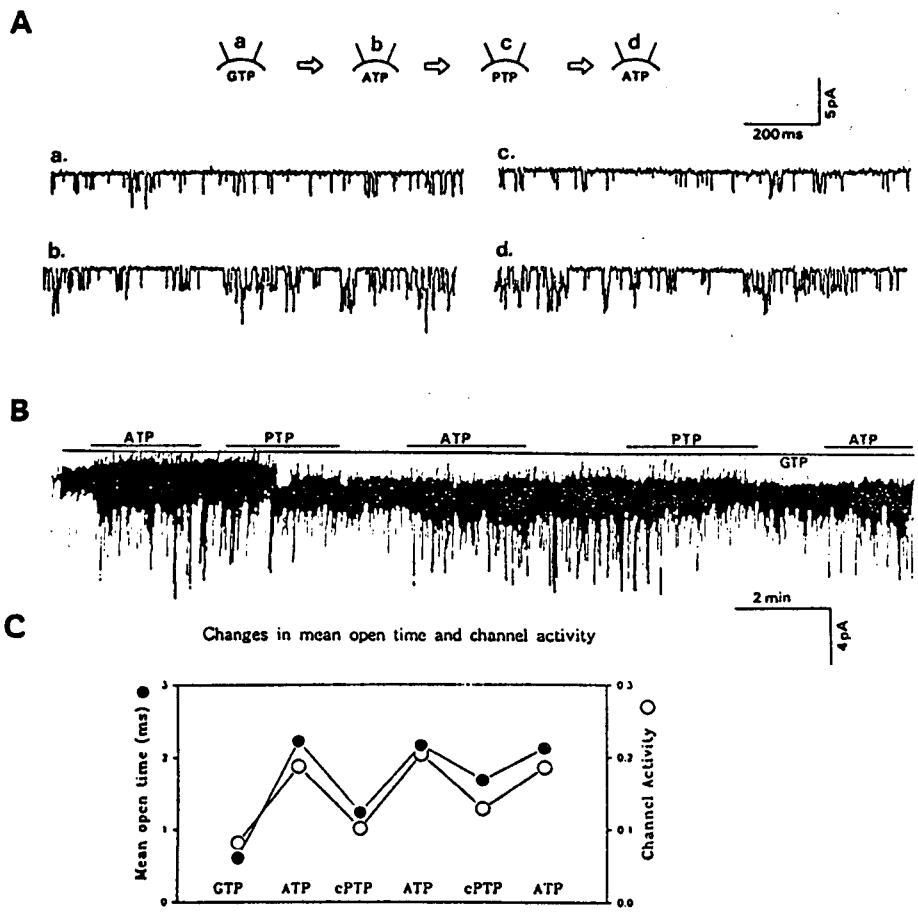

Fig. 4. Modulation effect of the tyrosine phosphatase (PTP) on the ATP-induced K_{ACh} channel activity. A, Effect of the PTP. Upper schematic diagram indicated the sequence of the drug application. Channel activity in expanded scale are shown before adding ATP (a), after adding ATP (b), in the presence of PTP (c). Channel activity recovered by the reapplication of ATP after washing PTP is shown in (d). B, whole trace showing reverse actions between ATP and PTP on the K_{ACh} channel activity. Note channel response by adding ATP and PTP alternatively. C, Calculated τ_o and N^*P_o from the upper panel. Open and closed circles represents values obtained during perfusing solutions containing the chemical corresponding to the data point in the lower inset. Data were analyzed 2 min later following the application of the each drug, respectively.

Antagonistic action between ATP and protein tyrosine phosphatase (PTP)

Results shown in Fig. 2 and 3 suggest that the tyrosine kinase is likely to regulate the K_{ACh} channel function in the presence of ATP. If the K_{ACh} channel function was modulated by the protein tyrosine kinase (PTK), PTP could reverse the channel function increased by ATP. As mentioned earlier, at I/O patches the ATP-induced K_{ACh} channel function was kept constantly after removing ATP while it goes down at the cell-attached configuration, referred as desensitization. This result means that a cytosolic component serving as the phosphatase might be involved. To test this assumption, we selected the cytosolic PTP and applied it to the cytoplasmic side of the I/O membrane patch.

Fig. 4 showed the effect of PTP on the ATP-induced K_{ACh} channel function. After forming I/O configuration, 100 μM GTP elicited the K_{ACh} channel activity with τ_o of 1.05 ms (trace a in Fig. 4). Then the addition of ATP made this channel openings longer (τ_o=2.23 ms, trace b) and these long-lasted opening was steadily maintained after washing ATP (τ_o=2.44 ms, not shown). Subsequent addition of 2 μg/ml PTP reversed channel openings to be short (1.24 ms, trace c), similar to those before adding ATP (trace a). Reversed channel openings by PTP were increased again by reperfusing ATP. Similar result was shown in Fig. 5. Antagonistic action between ATP and PTP was repeated two times in the same patch. Administration of GTP to the cytoplasmic side of the I/O patch caused K_{ACh} channels to activate (τ_o=0.61 ms, N^*P_o=0.082) again nearly same to the basal level recorded at the cell-attached configuration. The function of K_{ACh} channels was almost four times increased by the addition of ATP (τ_o=2.23 ms, N^*P_o=0.188). After washing ATP from the bathing solution, the channel function was kept constant, however it went down to a level of which added only GTP 3 min after applying 2 μg/ml PTP (τ_o=1.24 ms, N^*P_o=0.034). This decreased function was recovered (τ_o=2.16 ms, N^*P_o=0.204) as swithching the perfusion of PTP into ATP, indicating that PTP played the role of the antagonistic action to the effect of ATP on the K_{ACh} channel function.

DISCUSSION

A rapid desensitization of the K_{ACh} channel current occurred upon exposing an atrial cell to ACh. Since earlier studies suggested that this process was depends on the phosphorylation/dephosphorylation cycle (Kim, 1991; Kim, 1993), the present study to find out the kinase involved in this cycle was designed on the basis of the assumption that a phosphorylation could be modulated the K_{ACh} channel function.

Here this study showed clearly that the K_{ACh} channel function can be regulated by the tyrosyl phosphorylation. A line of evidence supported this idea. First, selective TKIs, either genistein or tyrphostin blocked the ATP-induced effect on the K_{ACh} channel function, suggesting that the protein tyrosine kinase (PTK) was involved (Fig. 3). Also although not shown as figure, either ADP or AMP-PNP, a synthetic non-hydrolyzable ATP analogue, did not mimic the effect of ATP, indicating that ATP is used as a substrate of an enzyme and serves as a phosphate donor. Second, the protein tyrosine phosphatase (PTP) reverted the K_{ACh} channel function increased by ATP down to the activity before adding ATP (Fig. 4 & 5), indicating that PTP antagonizes with a role of ATP. Interestingly, the portion reduced by PTP in N^*P_o corresponded nearly to that elevated by ATP. Accordingly it is possible to explain that PTP antagonized to ATP, maybe on the same site where resided on the muscarinic signal transduction triad consisted of m2AChR-G_K-K_{ACh} channel. Manifestation of the exact site for ATP and these enzymes requires further study.

Our finding that PTK and PTP turns on/off the ATP effect on K_{ACh} channel activity was an unexpected result, comparing to suggestions from

other studies. Because Kim (1991) proposed that Ca^{2+}/calmodulin (CaM) and Ca^{2+}/CaM-dependent phosphatase (PP-2B) was a potent candidate for underlying the ATP effect on K_{ACh} channel function. This assumption was based on the facts that, (1) tens of μM Ca^{2+} inhibited the ATP effect. (2) PKA and/or PKC inhibitor (such as H-7, or calphostin C) did not affect the effect of ATP. (3) Okadaic acid, PP-1 (protein phosphatase-1) and PP-2A inhibitor which anatgonizes to a phosphatase competitive with PKA and PKC, hand no effect, but PP-2B reversed partially the ATP-induced channel activity to that unsensitized with ATP. Unlikely to the possible role of Ca^{2+}/CaM, a molecular study revealed that PKC was an another candidate due to the presence of nine PKC phosphorylating sites on the GIRK1 subunit of the K_{ACh} channel protein (Kubo et al., 1993). The effect of PKC on the K_{ACh} channel function was tested but failed (Lo & Brietwieser, 1994).

Actually, several lines of experiments suggested the PTK as an possible modulator acting on the K_{ACh} current in frog atrial cells (Otero & Sweitzer, 1993) and the nicotinic ACh receptor in central nervous system (Hugarnir et al., 1984; Hopfield et al., 1988), although they were indirectly implicated. In addition of these results, recent evidences from a siries of the molecular study on the cardiac K_{ACh} channel support our evidence. That is, cardiac K_{ACh} channel is believed to be consisted of four subunits which comprises GIRK1s and cardiac inward rectifiers (CIR, or referred as GIRK4). On CIR, putative four tyrosine phosphorylating sites were found (Krapivinsky et al., 1995). Although the function of CIR has not still known, the tyrosyl phosphorylation might contribute to the regulation of the K_{ACh} channel function if CIR plays any role as one of K_{ACh} channel constituents. But our idea for the involvement of PTK/PTP in the modulatory effect of ATP on the K_{ACh} channel function requires an evidence that ATP should exert directly its effect at the level of the K_{ACh} channel protein. Further examination needs to be elucidate the exact site for the phosphorylation process related with the channel function.

In this study, high concentration of PTP more than 1 μg/ml was applied to inhibit the channel function (Fig. 5). At the level of hundreds nanogram of PTP, ATP-induced increase in the K_{ACh} channel function was insignificantly reduced. It could be explained that the PTP used in this study is less specific to the rat K_{ACh} channel, because this PTP was isolated from the chicken intestinal cells (cPTP) and its character might be close to the PTP-1 isotype. Nevertheless, it is more probable that the tyrosyl dephosphorylation may be involved in the regulation of the K_{ACh} channel because of several lines of evidences performed in the preliminary experiment. First, tyrosine kinase inhibitors was blocked the channel activity effectively, comparing to other results regarding that kinase inhibitors acting on the serine/threonine residue did not affect on the modulatory effect of ATP (Kim, 1991; Kim et al., 1993; Zang et al., 1993). Second, orthovanadate, known as a PTP inhibitor, blocked the PTP-like cytosolic activity (Hong et al., 1996; Hong & Kim, 1994). Finally, we examined that either heat-inactivated PTP or albumin did not effective on the regulation of the K_{ACh} channel function.

The present study revealed that the tyrosine kinase and the tyrosine phosphatase are partly involved in the regulation of the K_{ACh} channel function. From this result, a tyrosyl phosphorylation can be a important way of the intracellular mechanisms that regulate the ACh-activated muscarinic K^+ channel as well as the classical pathway coupling with G protein and ACh in rat atrial cells.

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