J. Membrane Biol. 147, 241–253 (1995)

The Journal of
Membrane
Biology

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Modulation of the Serotonin-activated K^+ Channel by G Protein Subunits and Nucleotides in Rat Hippocampal Neurons

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Received: 13 March 1995/Revised: 8 June 1995

Abstract. In hippocampal neurons, 5-hydroxytryptamine (5-HT) activates an inwardly rectifying K^+ current via G protein. We identified the K^+ channel activated by 5-HT ($K_{5\text{-HT}}$ channel) and studied the effects of G protein subunits and nucleotides on the K^+ channel kinetics in adult rat hippocampal neurons. In inside-out patches with 10 μM 5-HT in the pipette, application of GTP (100 μM) to the cytoplasmic side of the membrane activated an inwardly rectifying K^+ channel with a slope conductance of 36 ± 1 pS (symmetrical 140 mM K^+) at −60 mV and a mean open time of 1.1 ± 0.1 msec (n = 5). Transducin βγ activated the $K_{5\text{-HT}}$ channels and this was reversed by α-GDP. Whether the $K_{5\text{-HT}}$ channel was activated endogenously (GTP, GTPγS) or exogenously (βγ), the presence of 1 mM ATP resulted in a ~4-fold increase in channel activity due in large part to the prolongation of the open time duration. These effects of ATP were irreversible and not mimicked by AMPPMP, suggesting that phosphorylation might be involved. However, inhibitors of protein kinases A and C (H-7, staurosporine) and tyrosine kinase (tyrphostin 25) failed to block the effect of ATP. These results show that Gβγ activates the G protein-gated K^+ channel in hippocampal neurons, and that ATP modifies the gating kinetics of the channel, resulting in increased open probability via as yet unknown pathways.

Key words: Serotonin — G-protein — Hippocampus — βγ subunit — ATP

Introduction

Agonist-evoked activation of an ion channel via G protein is a widely utilized signal transduction pathway [2, 5, 8, 20]. A well-studied system for such a signal transduction pathway is the atrial muscarinic K^+ channel which is activated by acetylcholine and adenosine via a pertussis toxin-sensitive G protein (G_K). Single-channel properties of the muscarinic K^+ channel and its regulation by G_K and other molecules have been studied extensively during the past ten years [9, 32, 35, 50, 64]. In the brain, it has been shown that several agonists can activate or inhibit a K^+ current via specific membrane receptors coupled to G proteins. These agonists include acetylcholine [42, 51], 5-HT [1, 10], GABA [1, 16], dopamine [51], somatostatin [37, 39], substance P [41], norepinephrine [42], and opioids [38, 43, 44]. Most of these studies have been done by intracellular recording in tissue preparations, and have measured net changes in membrane potential or current in a cell in response to an agonist. Therefore, the populations of single K^+ channels activated by these agonists and the underlying mechanisms of activation have not yet been identified. Although one may expect the neuronal and atrial G protein-coupled K^+ channels to have similar properties, as they probably belong to the same subfamily of G protein-coupled inwardly rectifying K^+ channels [11, 27, 31, 34], very little information is available on the neuronal K^+ channels to support such a view [25]. Recent studies showing evidence that there are at least three members of G protein-coupled K^+ channels in the brain further necessitates that single-channel properties of all such K^+ channels be investigated for each signal transduction pathway [34].

The G protein-gated, muscarinic K^+ channel in atrial cells (K_{ACh} channel) has been studied in detail with respect to both activation by G protein subunits and modulation by cytosolic molecules such as GTP and ATP. Which G protein subunit (α or βγ) activates the K_{ACh} channel has been debated for several years [9, 23, 35,

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64]. However, recent reconstitution studies using recombinant α and βγ subunits, and cloned K_{ACh} channel expressed in *Xenopus* oocytes have demonstrated that the βγ subunit activated the K_{ACh} channel whereas the α subunit reversed the activation produced by the βγ subunit [49, 61]. The activated Giα subunit has been shown to cause apparent activation of the phosphorylated ATP-sensitive K^+ channel in cardiac cells by antagonizing ATP-dependent gating [24, 56]. In cultured neonatal rat hippocampal neurons, however, several K^+ channels including an inwardly rectifying K^+ channel have been reported to be activated by Gαo subunits [59]. In light of the recent evidence that the βγ subunit activates the G protein-gated K_{ACh} channel, it is important to reevaluate the effects of G protein subunits and examine whether βγ subunit can activate the neuronal K^+ channel in the native cell membrane and identify its single-channel properties.

In atrial inside-out patches with ACh in the pipette, addition of GTP to the bath solution has been shown to result in rapid activation of the K_{ACh} channel as expected of a process involving a G protein [32]. In the same patch, further addition of ATP (1 mM) resulted in a ~5-fold increase in channel open probability largely by prolongation of the open time duration [28, 29]. This stimulatory effect of ATP persisted even after its washout, and could not be reproduced with AMPPNP, a hydrolysis-resistant analogue of ATP. Thus, the ATP-induced increase in K_{ACh} channel activity may be mediated via phosphorylation by a membrane-associated, second messenger-independent protein kinase. In hippocampal neurons, whether ATP also modulates the gating kinetics of the G protein-gated K^+ channel via such a membrane-associated kinase or a regulatory protein to prolong the open time duration and increase the open probability of the K^+ channel is not known.

Since very little information is available on the properties of the 5-HT-activated K^+ channel in hippocampal neurons, we asked the following questions. (i) What are the single-channel properties of the K^+ channel opened by 5-HT? (ii) Does Gβγ subunit activate a K^+ channel in excised patches? If so, what are the single-channel characteristics and is the effect reversed by the Gα subunit? (iii) Does the Gα-GDP reverse the K^+ channel activation produced by endogenous G protein (GTP, GTPγS)? (iv) Once the $K_{5\text{-}HT}$ channel is opened via the G protein, can it be further modulated by intracellular ATP in a manner similar to the atrial K_{ACh} channel? That is, does ATP increase the open time duration and channel activity in a manner consistent with phosphorylation? (v) Do protein kinase inhibitors block the effect of ATP on the $K_{5\text{-}HT}$ channel kinetics? These studies represent the first attempt to determine the properties of neuronal, G protein-gated K^+ channels at the single-channel level. The results of these studies obtained in hippocampal neurons were compared with those in atrial cells.

Materials and Methods

Preparation of Hippocampal Neurons

Isolated neurons from the hippocampus were obtained using a procedure described earlier [60]. Adult Sprague-Dawley rats (200–250 g) were anesthetized with ether. After decapitation, the brain was quickly removed and placed in cold (~4°C) solution that consisted of the following (in mM): 120 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 HEPES and 25 *D*-glucose (pH 7.0). The CA3 region of the hippocampus was carefully separated and dissected out from the rest of the brain. The isolated brain region was then cut into ~400–600 μm thick slices and placed in same ice-cold oxygenated solution. The slices were then placed in a spinner flask containing 10 ml of the same solution with 0.33 mg/ml trypsin and kept at 30°C (Worthington). The enzyme solution was oxygenated continuously with 95% O_2/5% CO_2 gas mixture and stirred gently at low speed. After 1 hr, the enzyme solution was discarded and replaced with fresh solution without trypsin. To obtain dispersed neurons, slices were placed in 1 ml of solution containing 0.1 mg DNase I (Behringer Mannheim) and triturated gently with a fire-polished Pasteur pipette. Five to ten μl of the solution containing dissociated cells were placed in the recording chamber and cells were allowed to settle down on glass bottom. Neurons such as that shown in the photomicrograph of Fig. 1 were chosen for electrophysiological recording.

Electrophysiology

Gigaseals were formed with Sylgard-coated pipette of 1–2 (whole cell) or 4–6 (patches) megaohm resistances and channel currents were recorded using the method described by Hamill et al. [18]. For whole-cell recording, capactive transients were canceled before recording. Channel currents were recorded with a patch clamp amplifier (Axopatch 1-D, Axon Instruments, Foster City, CA), low-pass filtered at 5 kHz (−3 dB frequency) using an eight-pole Bessel filter (902-LPF, Frequency Devices, Haverhill, MA) and stored on magnetic tape via a digital data recorder (Instrutech, Great Neck, NY). Later, digitized data were transferred directly into an Atari computer and analyzed to obtain histograms for durations and amplitudes, and channel activity (averaged Np_o) using the analysis protocol described by Sigworth and Sine [52], where N is the number of channels, and P_o is the probability of channel opening. Current tracings lasting several minutes shown in figures were filtered at 100 Hz. Single channel currents shown in expanded scale presented in figures were filtered at 1 kHz. All experiments were performed at 23–24°C and data presented as the mean ± SD. Student's t test was used to determine significance at P level of 0.05.

Drugs and Solutions

Acetylcholine and staurosporine were purchased from Sigma Chemical. Trypsin, ATP, AMPPNP, GTPγS, GTP, CTP, UTP, ITP and GDP were purchased from Boehringer Manheim. Tyrphostin 25 (LC services, Woburn, MA) was dissolved to make 10 mM in a solution containing 50 mM HEPES, 125 mM KCl and 50% ethanol (v/v). H-7 HCl (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) and staurosporine (Research Biochemicals, Natick, MA) were dissolved in DMSO to make a 100 mM stock solution. The stock solutions were diluted with appropriate experimental solutions to desired concentrations just before the experiment. PKI (5–24) was from Research Biochemicals. Sodium orthovanadate was purchased from Fisher (Pitts-

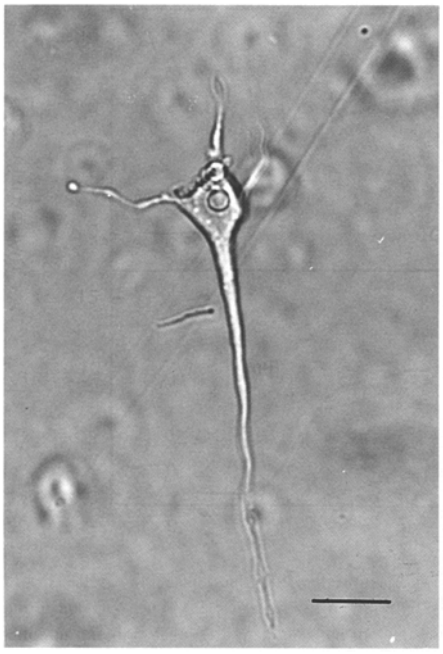

Fig. 1. A photomicrograph of a pyramidal neuron dissociated from the CA3 region of adult rat hippocampus. Scale bar is 20 μM. The average capacitance of pyramidal neurons used in this study was 11 pF.

burgh, PA), and genistein and okadaic acid from LC Services (Woburn, MA). Bovine transducin α and βγ subunits were prepared from rod outer segment membranes as described previously [57]. Pipette and bath solutions contained (in mM): 140 KCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2). When using nucleotide-containing solutions, free Mg^{2+} concentration was maintained at 1.85 mM using the program EQCAL (Biosoft, Milltown, NJ). pH was always adjusted to 7.2 in all solutions. Cytoplasmic side of excised patches were perfused with desired solutions through a plastic tubing at a rate of ~2 ml/min.

Results

Whole-Cell Currents Activated by 5-HT

Neurons from the CA3 region of the adult rat hippocampus were used in this study. Figure 1 shows a photomicrograph of a typical pyramidal neuron dissociated from the CA3 region of the hippocampus. The average capacitance of the neurons was 11 ± 3 pF (n = 6). Whole-cell currents were recorded from such cells with 100 μM GTP and 4 mM ATP in the pipette. In pipette and bath solutions containing 140 mM K^+, cell membrane potential was held at 0 mV and stepped to various potentials ranging from −100 to +60 mV for 500 msec. Under control conditions, the resting (basal) currents exhibited either a small inward rectification in the cell shown in Fig. 2*A* (n = 3) or very little rectification (n = 6). Extracellular perfusion of the cells with 10 μM 5-HT caused a greater increase in the inward current than the outward current (Fig. 2*B*). Thus, the subtracted current representing the current activated by 5-HT was also inwardly rectifying (Fig. 2*C*). Nearly all neurons studied showed an increase in inward current in response to 5-HT (18/22 cells). Although not shown, both the resting and 5-HT-elicited currents were blocked by 2 mM extracellular Ba^{2+}, indicating that they were K^+ currents.

5-HT elicited a rapid inward current when the cell membrane potential was held at −50 mV negative to reversal potential (Fig. 2*D*; E_{rev} is ~0 mV). Activation of an inward current was followed by a small and rapid desensitization to a new steady-state level. The magnitude of the 5-HT-induced increase in current varied markedly among neurons. The average increase in current at −50 mV determined at steady state was 310 ± 80 pA (n = 6). Washout of 5-HT resulted in return of the current level to the control value. When GTP in the pipette was replaced with GDP, only a very small current was activated by 5-HT (Fig. 2*E*). These results confirm earlier studies in brain slices which showed that 5-HT activated a K^+ current in hippocampal neurons via G protein [1]. In the presence of 1 mM Ba^{2+} in the perfusion solution, 5-HT failed to activate an inward current under identical conditions (Fig. 2*F*). As single-channels that give rise to the whole-cell current activated by 5-HT have not been identified or characterized, we studied the ion channels activated by 5-HT in cell-attached and inside-out patches.

Single Channel Properties

Cell-attached patches were formed on the soma of pyramidal cells with or without 10 μM 5-HT in the pipette. Pipette and bath solutions contained 140 mM K^+, and membrane potential was held at −60 mV to record inward channel currents. In the absence of 5-HT in the pipette, opening of five types of ion channels were observed in different cell-attached patches. The open probability of these channels was generally low with the exception of a small conductance (13 pS) channel which opened more frequently (*see* Discussion). When 5-HT was present in the pipette, channels with a conductance of 36 pS were found to be consistently activated in the cell-attached state in 35% of the patches studied (Fig. 3*A*). Upon formation of inside-out patches, the activity of this channel decreased to a low level (NP_o: 0.0005 ± 0.0002; n = 6). Perfusion of the cytoplasmic side of the membrane with 100 μM GTP resulted in a 42-fold increase in channel activity (NP_o: 0.021 ± 0.05; n = 6), showing that this 36 pS channel was the G protein-gated channel opened by stimulation of the 5-HT receptor. The channel openings in an inside-out patch at different membrane potentials are shown in Fig. 3*B*. Amplitude histograms were obtained from channel openings at each membrane potential. An example of an ampli-

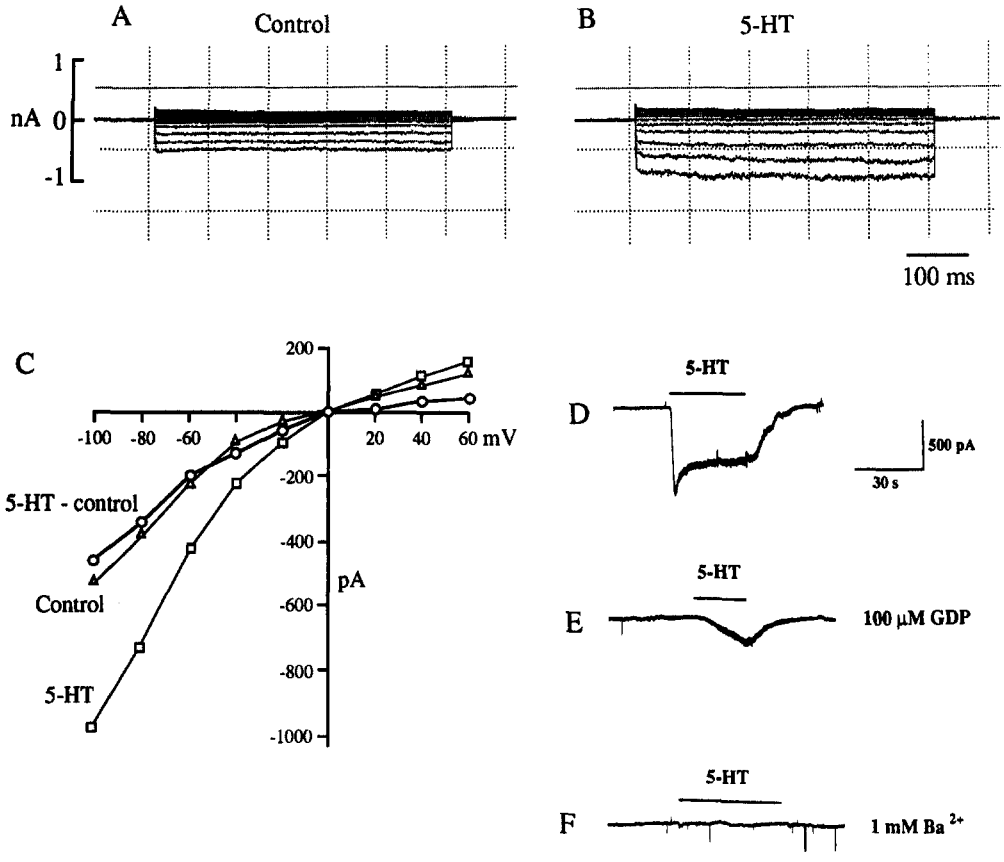

Fig. 2. 5-HT activates an inwardly rectifying K^+ current in hippocampal neurons. Cells were held 0 mV in the whole-cell configuration with 100 μM GTP and 4 mM ATP in the pipette, and currents recorded during 500-msec-voltage steps to voltages ranging from −100 to +60 mV in 20 mV steps before (*A*) and ~15 sec after 5-HT aplication (*B*). Current levels at the end of 500 msec pulses were plotted as a function of membrane potential. The substracted current (5-HT-control) shown in thicker line represents the current activated by 5-HT (*C*). in *D–F*, cells were held at −50 mV and current changes in response to 5-HT were recorded. In *D*, pipette solution contained 100 μM GTP and 4 mM ATP. In *E*, pipette solution contained 100 μM GDP and 4 mM ATP. In *F*, pipette solution contained 100 μM GTP and 4 mM ATP but with 1 mM Ba^{2+} in the bath solution. Holding potential was −50 mV. Bath and pipette solutions contained in (mM): 140 KCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES buffer (pH 7.2).

tude histogram of channel openings at −60 mV is shown in Fig. 3*C*. The mean amplitudes were used to plot the current-voltage relationship shown in Fig. 3*D*, showing the inwardly rectifying property of the current.

The 36 pS channel opened by 5-HT was a K^+ channel as judged by the following criteria. When [KCl] in the pipette solution in inside-out patches was 140, 35 or 280 mM, and the bath [KCl] was kept at 140 mM, the reversal potentials were 0 ± 1, -37 ± 3 or $+15 \pm 3$ mV (n = 3), respectively. These values are close to the expected Nernst potentials for a K^+-selective, but not Cl^--selective, ion channel. In the presence of 1 mM Ba^{2+} in the pipette solution, no inward current was observed. No detectable inward current was present when the K^+ in the pipette solution was replaced with an equal concentration of N-methylglucamine, Na^+, Cs^+ or Li. Thus, 5-HT selectively activated the inwardly rectifying, 36 pS K^+ channel via the GTP binding protein in hippocampal neurons.

Activation of the K^+ Channel by Transducin βγ

The G protein-gated K^+ channel in atrial cells has been shown to be activated by the βγ subunit of the G protein [23, 49, 61]. To test whether the G protein-gated K^+ channel (the $K_{5\text{-HT}}$ channel) in the hippocampal neuron can be activated by βγ, we directly applied the purified bovine transducin βγ to the cytoplasmic side of the membrane of inside-out patches. Figure 4*A* shows an inside-out patch with 10 μM 5-HT in the pipette showing low basal activity of K^+ channels in the absence of GTP. When 100 μM GTP was applied to the bath solution, the $K_{5\text{-HT}}$ channel became active. When GTP was washed off, the $K_{5\text{-HT}}$ channel closed and remained closed as long as GTP was absent. To such patches, βγ (200 nM) was applied to the cytoplasmic surface of the membrane. Within a few seconds, βγ started to activate a channel with kinetics and *I-V* relationship identical to that activated by GTP. Channel activity (NP_o) increased 52-fold

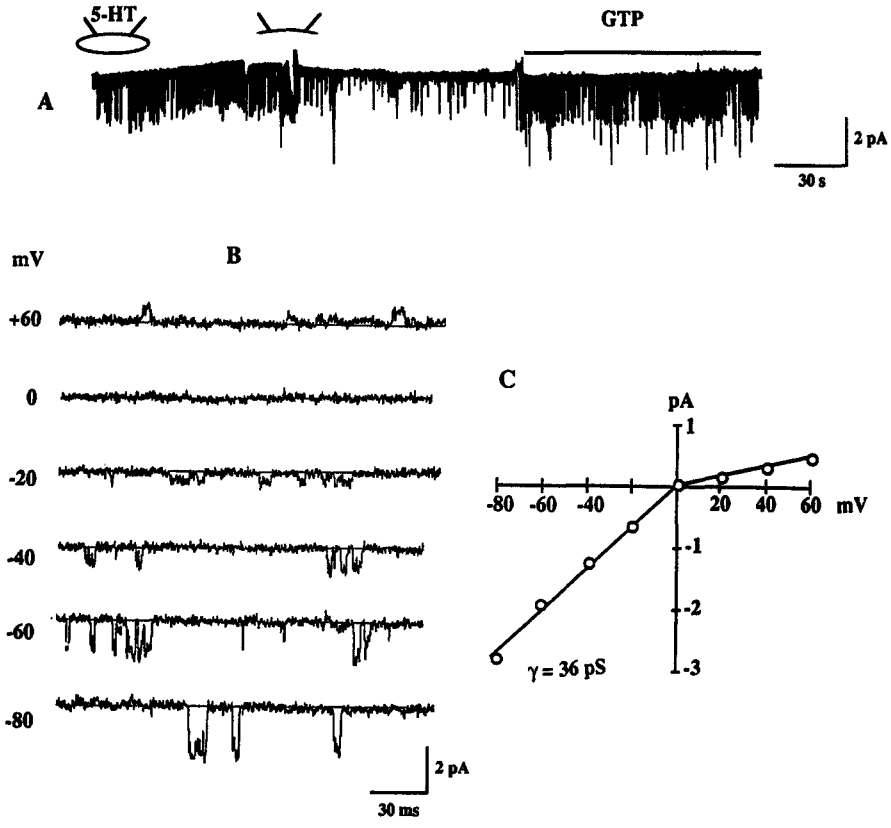

Fig. 3. 5-HT activation of an inwardly rectifying channel via GTP-dependent pathway. (*A*) A cell-attached patch was formed with 10 μM 5-HT in the pipette. Cell membrane potential was held at –60 mV. Subsequently, inside-out patch was formed and 100 μM GTP applied to the cytoplasmic side of the membrane. (*B*) While in the inside-out form, membrane potential was changed from –80 to +60 mV in 20 mV steps, and channel openings were recorded at each potential. (*C*) Amplitude histogram of channel openings recorded at –60 mV is shown. (*D*) From the amplitude histograms obtained at each potential, a current-voltage relationship was obtained. Each point was the mean of three determinations. Single-channel conductance in the inward direction was 36.2 ± 1.2 pS. Conductance in the outward direction was 6.8 ± 0.5 pS.

by βγ application (n = 6). The single-channel conductance was 36 ± 1 pS (n = 4) and the mean open time was 1.1 ± 0.1 msec (n = 4; Fig. 4*C*). Since stock βγ (5.3 mg/ml = 120 μM) was dissolved in 40% glycerol solution, we also tested the effect of the solvent up to a concentration of 0.5%. In all six patches, solution containing 0.5% glycerol failed to activate the $K_{5\text{-HT}}$ channel, but solution containing the βγ subunit activated the $K_{5\text{-HT}}$ channel in the same patch. Boiled βγ subunit did not activate the K^+ channel.

After 1–2 min of channel activation by βγ, transducin α-GDP (0.5 μM) was applied to the bath solution. α-GDP caused a decrease of channel activity, presumably due to removal of the βγ subunit near the channel protein. Boiled α-GDP failed to reduce the activity of βγ-evoked K^+ channel in all six patches tested. In Fig. 4*B*, an inside-out patch was formed without 5-HT in the pipette, and GTPγS (50 μM) applied to the cytoplasmic side of the membrane. In six cells, there was an average of a 65-fold increase in channel activity (NP_o), comparable to that produced by βγ. The K^+ channel activated by GTPγS was inwardly rectifying, and showed single-channel conductance of 36 ± 1 pS (n = 4) and a mean open time of 1.0 ± 0.1 msec (n = 4; Fig. 4*C*), similar to those activated by GTP or βγ. α-GDP (0.5 μM) also reduced the activity of the K^+ channel opened by GTPγS (n = 6). These results showed that βγ subunit of transducin could activate the $K_{5\text{-HT}}$ channel and that α-GDP was able to reverse the effect of βγ as well as that of the endogenous Gβγ in hippocampal neurons.

Effect of ATP on the $K_{5\text{-HT}}$ Channel Activated by GTP and βγ

It has been shown that the activity of the G protein-gated, muscarinic K^+ channel in atrial cells could be further stimulated by intracellular ATP via a mechanism that is not well understood [28, 29]. We studied whether the $K_{5\text{-HT}}$ channel in hippocampal neurons was also sensitive to intracellular ATP. Inside-out patches were formed with 10 μM 5-HT in the pipette and 100 μM GTP applied

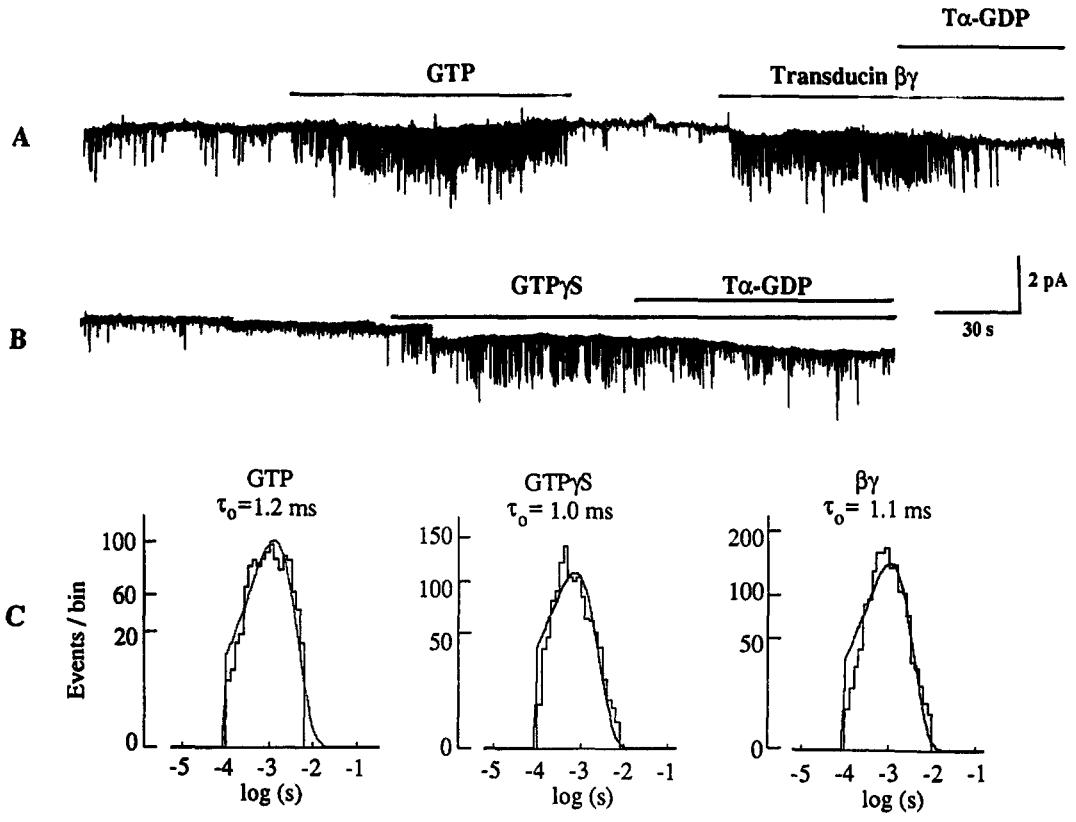

Fig. 4. Transducin βγ activates the $K_{5\text{-}HT}$ channel and α-GDP reverses the effect of βγ. (*A*) An inside-out patch was formed with 10 μM 5-HT in the pipette. GTP (100 μM) was applied to the bath solution and then washed off. After the channel activity decreased, 200 nM βγ was applied to the membrane. After ~1 min, the patch was perfused with both βγ and α-GDP (0.5 μM). (*B*) GTPγS (50 μM) was applied to an inside-out patch formed without 5-HT in the pipette. When the channel was active, α-GDP (0.5 μM) was applied in the presence of GTPγS. (*C*) Open time duration histograms obtained from channels activated by GTP, GTPγS or βγ. Numbers on top of each histogram represent the mean open times. Cell membrane potential was −60 mV.

to the bath solution to activate the $K_{5\text{-}HT}$ channel. In the patch shown in Fig. 5*A*, the mean open time of the $K_{5\text{-}HT}$ channel activated by GTP was 1.1 msec. When the cytoplasmic side of the membrane was exposed to 100 μM GTP and 1 mM ATP, the $K_{5\text{-}HT}$ channel activity increased quickly to a new level of steady state. In five patches, ATP increased the channel activity from 0.012 ± 0.004 to 0.051 ± 0.012 ($P < 0.05$) and increased the open time duration from 1.0 ± 0.1 to 4.2 ± 0.6 msec ($P < 0.05$; n = 5); *see* expanded tracings and the open time duration histogram in Fig. 5). Thus, the ATP-induced increase in $K_{5\text{-}HT}$ channel activity could be explained in large part by the prolongation of channel open time duration. The K^+ channel activity and open time duration remained increased even after washout of ATP for as long as the patches were maintained (~20 min). This indicated that it was not the energy of ATP hydrolysis that modulated the channel kinetics. Increasing the concentration of ATP to 4 mM produced no further significant changes in open time duration or the channel activity (−4 ± 5% change; n = 6). These effects of ATP on the kinetics of the $K_{5\text{-}HT}$ channel were not due to a difference in pH or free $[Mg^{2+}]$, as they were adjusted to be equal in control and ATP-containing solutions. The changes in K^+ channel kinetics produced by ATP were specific to this adenosine nucleotide, as 1 mM ITP, 1 mM CTP or 1 mM UTP did not significantly affect the K^+ channel activity or the open time duration under identical experimental conditions (<5% change in NP_o).

To examine whether ATP itself could alter the gating kinetics of the $K_{5\text{-}HT}$ channel in the absence of GTP, inside-out patches were perfused with 1 mM ATP for several minutes. Under these conditions, G protein would be in the inactive GDP-bound state. ATP (1 mM) itself did not increase the $K_{5\text{-}HT}$ channel above the basal level (Fig. 5*B*). When ATP was washed off and then GTP applied, the $K_{5\text{-}HT}$ channels became active. The mean open time was 4.1 ± 0.3 msec (n = 5; *see* the expanded tracing), showing that ATP had modified the gating of the channel even in the absence of GTP. When patches were preexposed to 100 μM GDP and 1 mM ATP (rather than ATP alone), the mean open time of the K^+ channel subsequently activated by GTP was 3.8 ± 0.4 msec (n = 3) compared to the mean open time of 1.1 ±

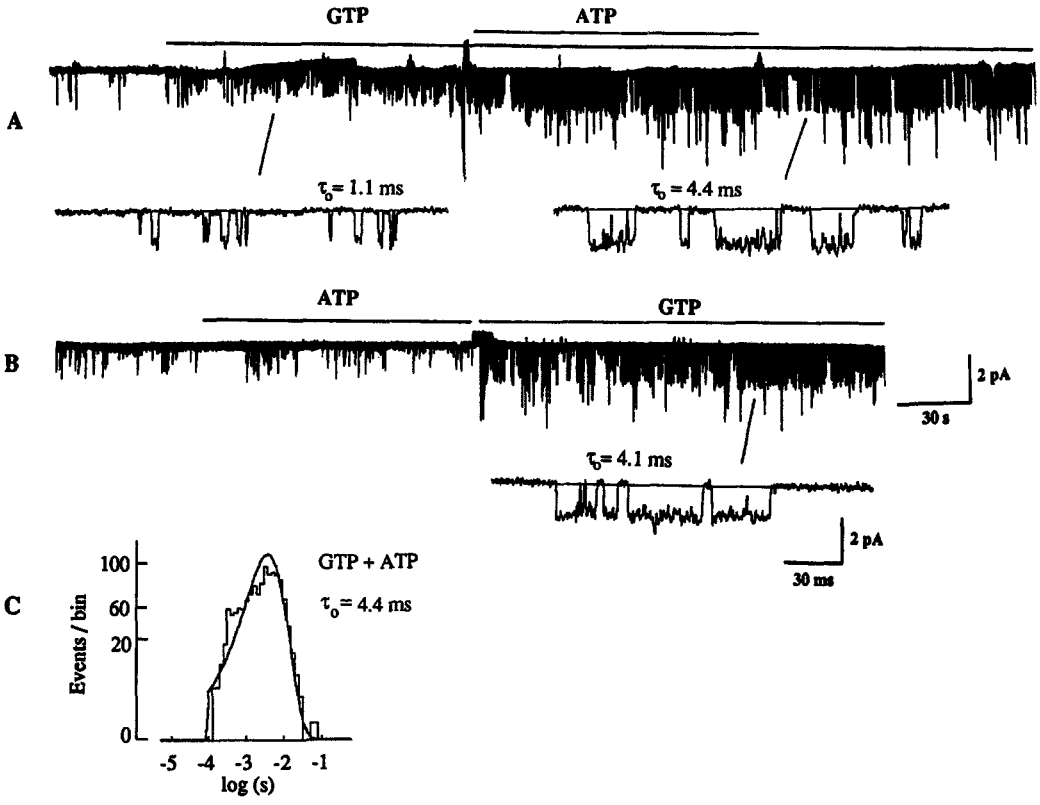

Fig. 5. ATP increases the open time duration and activity of the $K_{5\text{-}HT}$ channel. (*A*) An inside-out patch was formed with 10 μM 5-HT in the pipette. GTP (100 μM) was applied to activate the K^+ channel. When the channel activity reached near steady state, ATP (1 mM) was further applied to the bath solution for ~2 min and then washed off. Channel openings shown at expanded time scale were filtered at 1 kHz. (*B*) ATP (1 mM) was applied to the inside-out patch for ~2 min and then washed off. GTP was then applied to the patch to activate the K^+ channel. (*C*) Open time duration histogram of channel openings recorded in the presence of GTP and ATP (shown in tracing in *A*). Only single openings were analyzed.

0.1 msec (n = 5) observed without ATP pretreatment. Thus, ATP could alter the gating mode of the K^+ channel even when it was in the inactive state.

We also examined whether ATP could alter the kinetics of the $K_{5\text{-}HT}$ channel activated by βγ subunit or GTPγS. In inside-out patches formed without 5-HT in the pipette, applying 200 nM transducin βγ to the cytoplasmic side of the membrane activated the $K_{5\text{-}HT}$ channel as before. The mean open time of the βγ-activated $K_{5\text{-}HT}$ channels was 1.0 msec (Fig. 6*A*). Further addition of 1 mM ATP resulted in a 4.5-fold increase in mean open time duration and a 4.2-fold increase in channel activity. These changes in the kinetics of the K^+ channel produced by ATP can easily be seen in the expanded tracings in Fig. 6*A*. When patches were first exposed to 1 mM ATP, $K_{5\text{-}HT}$ channels remained at basal levels of opening (Figs. 6*B* and *C*). Applying either the βγ subunit (200 nM) or GTPγS (50 μM) to the bath solution resulted in activation of the $K_{5\text{-}HT}$ channel whose mean open times were 3.6 and 3.8 msec, respectively. In both cases, applying α-GDP (0.5 μM) together with βγ or GTPγS resulted in a decrease in K^+ channel activity. The results obtained with βγ and GTPγS from five patches are summarized in Fig. 7 which shows changes in both the relative channel activity and mean open time before and after exposure to ATP. These results provide evidence that the endogenous G protein produced by application of GTP or GTPγS and exogenous βγ subunit activate the same K^+ channel that is also sensitive to intracellular ATP. The results also indicate that ATP can modulate the conformational state of the $K_{5\text{-}HT}$ channel even when it is not active.

Effect of AMPPNP and Protein Kinase Inhibitors

The observation shown in Fig. 5*A* that the effect of ATP on the $K_{5\text{-}HT}$ channel was persistent even after washout of ATP suggests that the underlying mechanism may be via phosphorylation. To further test this possibility, we studied the effect of AMPPNP, a hydrolysis-resistant analogue of ATP, on the $K_{5\text{-}HT}$ channel kinetics. As shown in Fig. 8*A*, 4 mM AMPPNP failed to increase the channel activity and open time duration in every patch tested (n = 5). In the same patch, however, subsequent application of 4 mM ATP caused ~4-fold increases in channel activ-

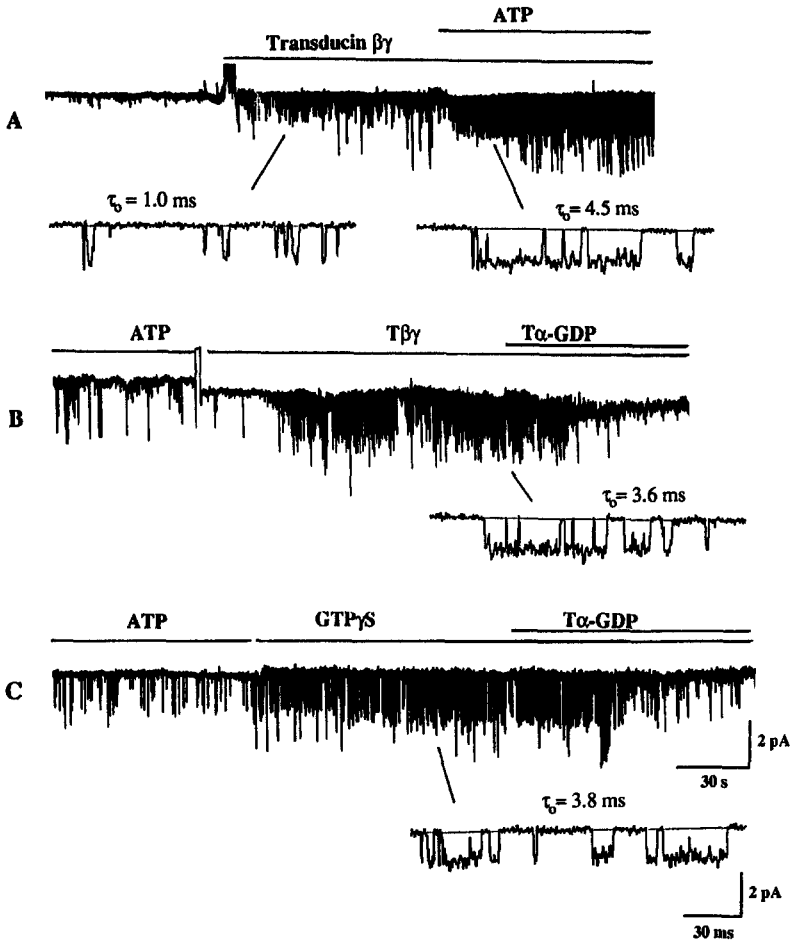

Fig. 6. ATP increases the open time duration and activity of the K^+ channel activated by βγ and GTPγS. (*A*) An inside-out patch was formed without 5-HT in the pipette, and βγ (200 nM) applied directly to the membrane to activate the K^+ channel. ATP (1 mM) was further added to the patch in the presence of βγ. (*B*) ATP (1 mM) was applied first to the inside-out patch for ~2 min and then washed off. Subsequently, βγ (200 nM) was applied. When the channel became active, α-GDP (0.5 μM) was further applied to the patch in the presence of βγ. (*C*) Same experiment as in *B* except that GTPγS was used instead of βγ. Note the prolonged open time duration after treatment with ATP.

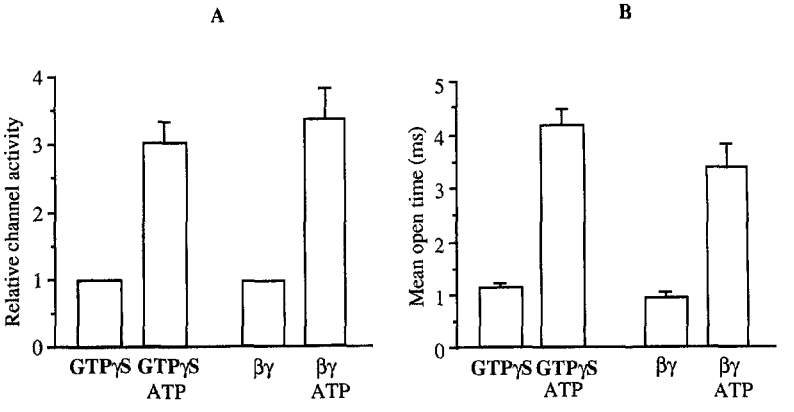

Fig. 7. Summary of the effects of ATP on the relative channel activity and open time duration when the $K_{5\text{-}HT}$ channel is activated by GTPγS or βγ. In each patch, the channels were first activated with 50 μM GTPγS or 200 nM βγ (channel activity observed at this time is taken as 1.0). ATP (1 mM) further applied to the patch, causing significantly increased channel activity and open time duration. Each bar represents the mean ±SD of 6 determinations.

ity (*see* Fig. 9) and open time duration (1.1 ± 0.1 *vs.* 4.2 ± 0.4 msec). These results are consistent with the view that the ATP-induced effect was mediated via a membrane-associated protein kinase.

To further examine the involvement of a protein kinase, we tested the effect of several kinase inhibitors: H-7 (an inhibitor of protein kinases A and C); staurosporine (a nonselective protein kinase inhibitor); and tyrphostin 25 (a tyrosine kinase inhibitor; 17). Inside-out patches with 5-HT in the pipette were perfused with solution containing one of the inhibitors for at least 3 min (3–5 min), and then ATP applied to the membrane. For example, Fig. 8*B* shows an inside-out patch exposed to 50 μM H-7 in the absence of GTP for 4 min. Subsequent application of 100 μM GTP and 4 mM ATP with H-7 caused activation of the $K_{5\text{-}HT}$ channel with a mean open time of 3.7 ± 0.4 msec. Similarly, in patches treated with staurosporine (1 μM) or tyrphostin 25 (50 μM). ATP still

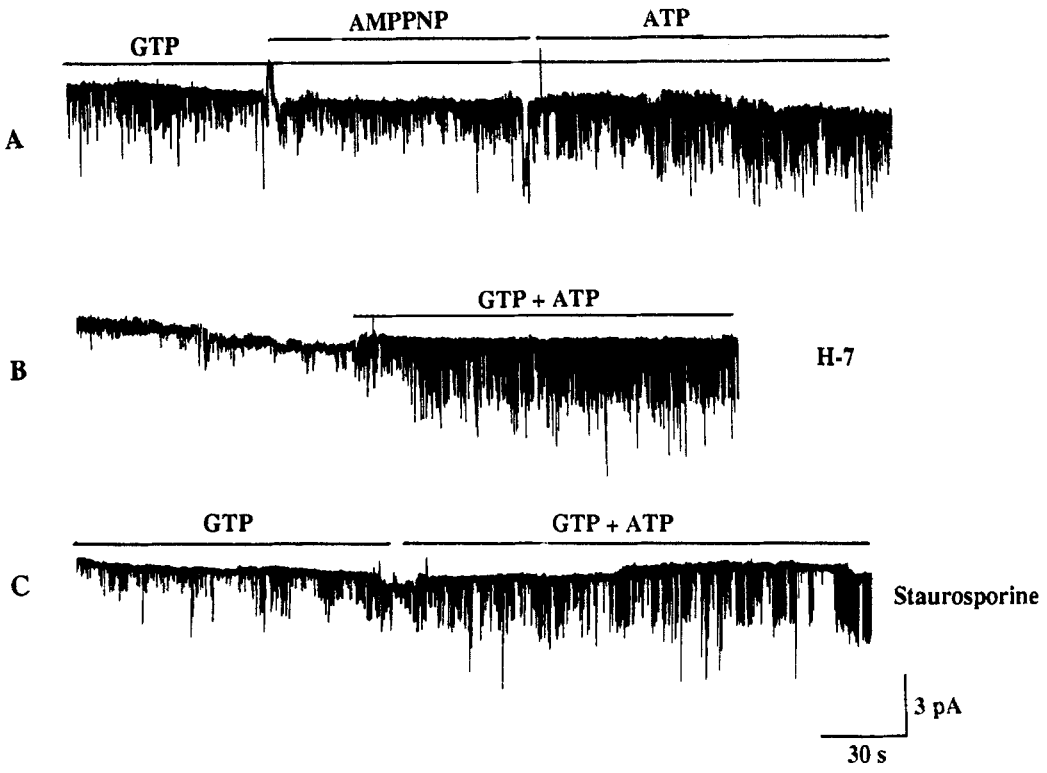

Fig. 8. Lack of effect of AMPPNP and protein kinase inhibitors on the ATP-induced change in K^+ channel activity. (*A*) GTP was applied to the inside-out patch (10 μM 5-HT in the pipette) to activate the K^+ channel. AMPPNP (4 mM) was applied in the presence of GTP for ~2 min. After washing off AMPPNP, ATP (4 mM) was applied to the patch. (*B*) In inside-out patches, H-7 (50 μM) was applied first with (n = 5) or without (n = 3) GTP (100 μM) for ~4 min. A patch without GTP in the bath is shown, and therefore only a basal level of channel activity is present. GTP and ATP (4 mM) were then applied in the presence of the inhibitor. (*C*) Same experiment as in *B* but using staurosporine (0.1 μM) as the inhibitor of protein kinases. GTP was present throughout the experiment.

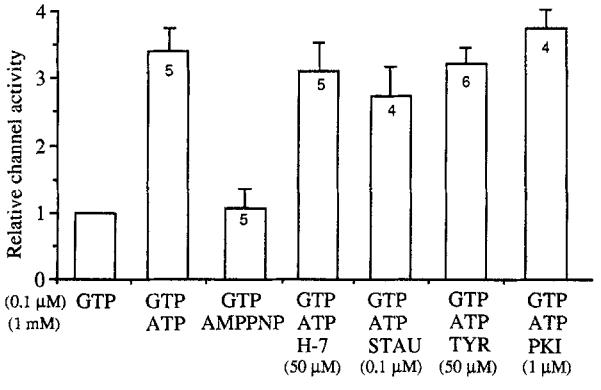

Fig. 9. Effect of ATP, AMPPNP and protein kinase inhibitors on the K^+ channel activity. This is a summary of the experiments described in Fig. 7 plus the effects of two additional kinase inhibitors (50 μM tyrphostin 25 and 1 μM protein kinase inhibitor peptide, PKI 5-24) which block tyrosine kinase and protein kinase A, respectively. Numbers in the bars indicate number of patches tested. No inhibitor was found to significantly block the effect of ATP ($P > 0.05$).

increased the channel activity and open time duration to magnitudes comparable to those in control patches. In four patches, incubation with 1 μM inhibitor peptide (PKI 5–24) specific for protein kinase A failed to block the effect of ATP. Figure 9 shows the lack of effect of kinase inhibitors on ATP-induced increase in K^+ channel activity. Thus, despite the indication that phosphorylation might be involved, the possible role of a kinase in ATP-induced stimulation of the $K_{5\text{-HT}}$ channel was not evident from these studies.

In cell-attached patches, the 5-HT-activated channels have a mean open time of ~1 msec (*see* Fig. 1) despite the presence of ~4 mM ATP in the cell. This suggested that perhaps a phosphatase acts on the $K_{5\text{-HT}}$ channel to maintain it in the dephosphorylated state with an open time duration of ~1 msec. If a phosphatase were indeed involved, inhibitors of this enzyme would allow the $K_{5\text{-HT}}$ channel protein to be phosphorylated. We therefore tested the effect of phosphatase inhibitors, 0.2 mM sodium orthovanadate and 1 μM okadaic acid to determine whether the ATP effect would now be observed in cell-attached patches. Cells were first incubated with one of the inhibitors for 10 min, and then cell-attached patches formed with 5-HT in the pipette. The mean open time durations of the channels in the cell-attached state were 1.2 ± 0.1, 1.1 ± 0.1, and 1.1 ± 0.1 msec, respectively, for each of the inhibitors used (n = 4). Therefore, we obtained no evidence for an involvement of a protein

phosphatase in $K_{5\text{-HT}}$ channel modulation using these inhibitors.

Discussion

Previous studies have shown that several neurotransmitters activate a K^+ current via GTP binding proteins in the brain [1, 38, 42]. However, the individual K^+ channels that give rise to the whole-cell current, and the manner in which G proteins activate the K^+ channel have not yet been identified or characterized. In this study, we examined the single-channel properties of a G protein-gated K^+ channel activated by 5-HT in hippocampal neurons. Our results show that 5-HT activates a population of an inwardly rectifying K^+ channel with single-channel conductance of 36 pS and a mean open time of 1.1 msec whose values are similar to those of the atrial muscarinic K^+ channel. We also show that $\beta\gamma$ subunit of the G protein (transducin) is able to activate the K^+ channel directly and that the α-GDP reverses the $\beta\gamma$ effect. When the K^+ channel was first activated either with GTP or $\beta\gamma$, additional presence of ATP resulted in an increase of the open time duration and thus the channel activity. These findings show that the G protein-coupled K^+ channel in hippocampal neurons have single-channel kinetics and modulatory properties similar to those of the atrial muscarinic K^+ channel.

Properties of G protein-gated K^+ Channels

As many neurotransmitters activate K^+ current via G protein in the brain, it is not known whether the G protein-gated K^+ channels activated by different agonists are the same proteins or belong to a family of structurally similar isoforms. Complementary DNAs for G protein-coupled, inwardly rectifying K^+ channels were first isolated from rat heart (GIRK1/KGA) and rat brain (KGB1), and were found to be identical in primary amino acid sequence displaying two hydrophobic domains (M1, M2) and a pore forming (H5) domain [11, 31]. Study of the GIRK1 channels expressed in oocytes showed that whole-cell and single-channel properties were similar to those in muscarinic K^+ channel in atrial cells. Recently, two additional members of the G protein-coupled K^+ channel cDNAs were isolated from mouse brain (mbGIRK2, mbGIRK3) and showed ~60% identity with GIRK1 [34]. In plant guard cells, the conductance of an inward rectifier K^+ channel activated by GTPγS was reported to be 39 pS in 100 mM KCl [62] compared with the atrial and neuronal 36 pS K^+ channel in 140 KCl. These results show that there are several members in the G protein-coupled K^+ channel family. Therefore, one can not simply assume that all GIRKs will have identical kinetic properties and are regulated the same way by second messenger molecules. It seems necessary to study the signal transduction pathway and single-channel properties and regulation for each agonist for each cell type.

In patches without 5-HT in the pipette, we observed five different types of K^+ channels under basal conditions in cell-attached and inside-out patches. The single-channel slope conductances of these channels at –60 mV were 13, 35, 36, 55 and 88 pS (average of 4 determinations). One or two of these channels were usually observed in a patch. None of the channels were observed with 1 mM Ba^{2+} in the pipette, indicating that they were probably K^+ channels. The 13, 36 and 88 pS channels were inwardly rectifying. The 35 pS channel showed a linear current-voltage relationship and therefore could be clearly distinguished from the 36 pS channel. The 55 pS channel was observed in less than 5% if the patches and opening was infrequent. The 13 pS channel is probably the background inward rectifier K^+ channel in the rat hippocampus, as the human hippocampal inward rectifier (HIR) and mouse brain inward rectifier K^+ channels cloned and expressed in oocytes also have conductances of 10–13 pS [36, 40, 46]. These channels may belong to a large family of inward rectifiers whose conductance ranges from 10 to 40 pS [30]. The 88 pS channel was the ATP-sensitive K^+ channel as it was blocked by intracellular application of 2 mM ATP and activated upon washout. The presence of such ATP-sensitive K^+ channels in hippocampal neurons have been reported earlier [58]. Ca^{2+}-activated K^+ channels were also present in hippocampal neurons but were mostly inactive as $[Ca^{2+}]$ in the experimental solution used in this study was below ~20 nM. Of these K^+ channels, only the inwardly rectifying 36 pS channel was consistently activated by application of $\beta\gamma$ subunit or GTP in the presence of 5-HT.

In an earlier study using cultured neonatal rat hippocampal neurons, K^+ channels with similar conductances (a 12.7 pS channel, two 37.5 pS channels and a 54.5 pS channels) were described [59]. In their study, application of purified and recombinant $G\alpha_o$-GTPγS to the cytoplasmic side of inside-out patches was reported to activate all four types of K^+ channels. Recombinant $G\alpha_i$-1 was found to be ineffective in activating the K^+ channels. Thus, it was concluded that $G\alpha_o$ couples various neurotransmitter receptors to diverse brain K^+ channels. Our results that $\beta\gamma$ subunit activated one of these K^+ channel population and α-GDP reverses the $\beta\gamma$ subunit effect are at variance with their findings. As we used transducin G protein subunits (α or $\beta\gamma$), we can not rule out the possibility that other types of G proteins are also involved in K^+ channel activation. Wickman et al. [61] have shown that transducin $\beta\gamma$ ($\beta1\gamma1$) was the least potent of different $\beta\gamma$ isoforms in the activation of the atrial muscarinic K^+ channel with a $K_{1/2}$ of ~50 nM. This

is consistent with our finding that a relatively high concentration of $\beta 1\gamma 1$ was required to activate the hippocampal $K_{5\text{-HT}}$ channel. Although not tested, other forms of $\beta\gamma$ subunits (such as $\beta 1\gamma 2$) which are nonretinal would probably be more potent in the channel activation. Nevertheless, our data show that $\beta\gamma$ is capable of opening the G protein-gated K^+ channel in hippocampal neurons as it does the muscarinic K^+ channel and GIRK1 expressed in oocytes [49, 63]. This is further supported by recent studies which showed that the carboxyl terminal domain of GIRK1 has potential binding sites for the $\beta\gamma$ subunit [22, 53, 55].

Modulation of the K^+ Channel by ATP

It has been shown that intracellular ATP could either increase or decrease the G protein-gated K^+ channel activity depending on the agonist used to activate the K^+ channel [14]. Therefore, it is necessary to test the effect of the nucleotide for each signal transduction system. Our results show that intracellular application of ATP to hippocampal neuronal membrane patches containing the 36 pS K^+ channel produces effects on the channel kinetics nearly identical to those observed in atrial cells with ACh as the agonist [29]. Thus, in inside-out patches in which the K^+ channel was already activated with 5-HT and GTP, ATP markedly prolonged the open time duration and thereby augmented the open probability by ~4-fold. The effect of ATP was similar whether it was 1 or 4 mM, indicating that maximal effect was produced at a concentration of 1 mM or less. In patches in which the K^+ channel was not active, applying ATP alone did not cause activation of the channel. Therefore, transphosphorylation of GDP-Gα to GTP-Gα via nucleoside diphosphate kinase, which has been reported to take place in atrial cells [19, 26, 45], may be weak or absent in hippocampal neurons.

The lack of effect of AMPPNP suggests that the ATP-induced changes in K^+ channel kinetics could be mediated by phosphorylation via a membrane-associated protein kinase. However, we were unable to inhibit the ATP effect with known inhibitors of protein kinases. Although it was possible that the inhibitors were unable to reach the target molecules in the membrane, this seems unlikely since many other studies using similar preparations showed that the inhibitors were able to block the effects on ion channel function produced via phosphorylation [2, 7, 12, 33, 44]. In our studies, we found that H-7 and staurosporine could block the stimulatory effect of isoproterenol on Ca^{2+} current, clearly showing that the inhibitors reached their target kinase in the cell. Therefore, we failed to obtain any evidence for a role of a kinase in ATP-induced changes in K^+ channel kinetics. Similarly, we found no evidence of phosphatase involvement in $K_{5\text{-HT}}$ channel modulation, as inhibitors of phosphatases showed no effect on the channel kinetics.

Although many ion channels in membrane patches or incorporated in bipid bilayer have been shown to be modulated by ATP via phosphorylation [4, 6, 7, 12, 13, 47, 47], not all of the ATP-modulated ion channels are sensitive to kinase inhibitors, possibly suggesting that a novel class of protein kinases insensitive to currently used inhibitors could be involved [4, 15, 28, 47, 54]. The effects of ATP on the G protein-gated K^+ channels may involve mechanisms biochemically similar to that observed with the ATP-sensitive K^+ channel which require previous exposure to ATP (but not AMPPNP) in order to be converted to the "operative" state and open when ATP is subsequently removed. The protein kinase inhibitors were ineffective in blocking the ATP-induced reactivation of the ATP-sensitive K^+ channel [15]. The mechanism of such ATP effect is therefore different from that observed with the CFTR Cl^- channel [3] or the IRK1 channel [13] which appears to use the energy generated by ATP hydrolysis to alter the channel gating when ATP is present. Since the ATP effect on the $K_{5\text{-HT}}$ channel cannot be washed off, the energy derived from ATP hydrolysis is unlikely to play a role in $K_{5\text{-HT}}$ channel modulation. Therefore, the biochemical nature of the ATP effect on the G protein-gated K^+ channel in both the atrial and hippocampal neurons remains elusive.

The hyperpolarizing effects of K^+ channel activation by 5-HT in hippocampal neurons will be enhanced greatly if intracellular ATP is allowed to increase the K^+ channel activity. However, in cell-attached patches with 5-HT in the pipette and ~4 mM ATP in the cell, the mean open time of the K^+ channel was always ~1.0 msec, similar to that observed in inside-out patches with GTP alone in the bath solution. Thus, despite the presence of a high concentration of ATP in the cell, the ATP effect was not observed in intact cells. This is probably due to the presence of certain cytosolic factors that antagonize the effect produced by ATP. In support of a role of such factors, we found that addition of brain cytosolic extract to ATP-modified K^+ channel resulted in reversal of the ATP effect even in the presence of 4 mM ATP (*preliminary results*). The same effect of atrial cytosol was also observed in the ATP-modified muscarinic K^+ channel in atrial cells [21]. Whether the cytosolic factor is a phosphatase is not clear, as phosphatase inhibitors had no effect on channel gating. As in atrial cells, the G protein-independent pathways (via ATP and certain cytosolic factors) may be involved in the early desensitization of the 5-HT-activated K^+ current in which the open time duration and therefore the channel open probability are modulated in a time-dependent manner [21, 28]. The mechanisms underlying such modulation of the K^+ channel by ATP and cytosolic factors are not known at present and need to be elucidated in future studies. It would be important to identify the modulators of this K^+

channel, as any potential changes in the activity of these factors, possibly via separate receptor-mediated pathways, would result in significantly altered neuronal behavior by increasing or decreasing the G protein-gated K^+ channel activity.

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