DIRECT G PROTEIN GATING OF ACH-SENSITIVE K^+ CHANNEL IN GUINEA-PIG ATRIAL CARDIOMYOCYTES: ANALYSIS BY INSIDE-OUT PATCH CLAMP MODE

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Abstract: Direct actions of guanosine 5'-triphosphate (GTP)-binding protein (G protein) on the K^+ channel in isolated single atrial muscle cells from guinea-pig hearts were investigated. In cell-attached patch-clamp mode, ACh-sensitive K^+ (K^+ [ACh]) channels were stimulated by application of carbachol (10 μM). A unitary channel current identified by carbachol responsiveness was activated inwardly at the resting membrane potential, and was decreased with an increase in membrane potential. At 0 mV, the single-current was zero mV (the reversal potential). The extracellular and pipette solutions (140 mM KCl) were similar. It was a slope conductance of 44.7±3.5 pS (n=8) and an average open time at −80 mV of 1.4±0.3 msec (n=7). It had a strong inwardly-rectifier property at positive potentials. The response was blocked by the addition of atropine (1 μM) to the pipette solution. On switching from a cell-attached to an inside-out patch-clamp experiment (to avoid the influences of intracellular signal transductions), the activation of K^+ [ACh] channels was gradually attenuated. Holding potential was −80 mV. But since other channels (i. e. ATP-sensitive K^+ channel and inward-rectifying K^+ channel) still remained, the cell was transiently taken out from the bath solution (exposed to the air) to inactivate the channels. GTP (100 μM), which was applied by a concentration-clamp technique, activated the K^+ [ACh] channels with the same characteristics within a moment of its application. The responses were reversible. These results indicate that G protein directly activates the K^+ [ACh] channels on the atrial muscle cell membrane, independent of second messengers.

Index Terms

ACh-sensitive K^+ channel, guanosine 5'-triphosphate (GTP), inside-out patch-clamp, single atrial muscle cell

INTRODUCTION

Agonists bind to their own receptors on surface membrane, and produce second messengers [i. e. cAMP, cGMP, IP_3, diacylglycerol (DG), and Ca^{2+}] through cellular signal transductions which cause many physiological functions. Protein kinases [i. e. cAMP-dependent protein kinase (PK-A), cGMP-dependent protein kinase (PK-G), and Ca^{2+}- and phospholipid-dependent protein kinase (PK-C)] appear to be an important component of membrane signal

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transduction for a variety of biological activators of substances [1)-10)]. Acetylcholins (ACh) are released on vagal stimulation to bind the cardiac muscarinic ACh receptor in pacemaker and atrial cells, causing heart rate to decrease. The mechanism for the negative chronotropic effect is due to an increase in K^+ conductance of the cardiac muscles[11),12)]. Furthermore, ACh activates a specific K^+ channel, an ACh-dependent K^+ (K^+ [ACh]) channel, thereby resulting in bradycardia[13),14)].

The signal transductions are mediated through activation of stimulatory and inhibitory G proteins[15),16),17)]. It is unknown whether G proteins have direct actions, or indirect actions on all the ionic channels like the second messengers. For instance, G proteins stimulate phospholipase A_2 to produce arachidonic acid from the membrane, and the form of a 5' lipoxygenase metabolite may then act on ion channels[18),19)]. Hence, G proteins might act upon ion channels or other membrane effectors not directly within the membrane but via intermediary membrane products.

It is not known how the muscarinic ACh is coupled to the K^+ channel, but indirect evidence suggests that G protein is involved[15)-19)]. In the present experiments, thus, I sought to examine whether guanosine 5'-triphosphate (GTP) from inside stimulates the K^+ [ACh] channel (activated by carbachol from outside) in single atrial muscle cells. A major aim was to confirm whether or not the K^+ [ACh] activation induced by GTP was due to a direct action. So, a switch from a cell-attached to an inside-out patch clamp experiment was exerted to avoid the influences of intracellular signal transductions.

MATERIALS AND METHODS

Cell preparation

Guinea-pigs of either sex, weighing 250-400 g, were anesthetized with intraperiotoneal injection of sodium pentobarbital (30 mg/kg), using methods similar to those described previously[8),9),20)]. Under artificial ventilation, the chest was opened and the aorta was cannulated, *in situ*. The heart was then dissected out and perfused with normal Tyrode's solution on the Langendorff apparatus. After the blood was completely washed out, the heart was perfused with Ca^{2+}-free Tyrode's solution, and the spontaneously beating heart ceased. Then, the perfusate was switched to low-Ca^{2+} (30-60 μM) Tryode's solution containing 0.4 mg/ml collagenase (Type I, Sigma Chemical Co., St Louis, MO) for about 20 min. After the collagenase in the heart was washed out by high-K^+ and low-Cl^- solution (KB solution), the atria were dissected with scissors. The final cell suspension was preincubated in the KB solution for at least 60 min at 4°C prior to the experiments.

Cell-attached and inside-out patch clamp recordings

Cell-attached and inside-out patch clamp experiments were performed using glass patch pipettes[9),21)]. The resistance of the patch electrode was 2-3 MΩ, and the tip of the electrode was coated with Sylgard (KE106, Shin-etsu Chemical Co.). Unitary current traces were stored on

a video recorder (BR6400, Victor) using a PCM converter system (RP-880, NF Electronic Circuit Design, Tokyo). The data were analysed on an IBM computer (PCLAMP). Current traces were filtered with a cut-off frequency of 1 kHz for plotting (FV-625, NF).

The compositions of the modified Tyrode's solution was as follows (mM) : NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.0, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Wako Pure Chemical Industries, Ltd., Osaka, Japan) 5.0 (pH 7.4). The pipette solution contained (mM) : KCl 140, $MgCl_2$ 2, EGTA 5, and HEPES 5. In inside-out patch clamp experiments, the same solution as the pipette solution was used as the extracellular solution. The pH in the pipette solution was adjusted to 7.2. The values are represented as mean ± S. E. M. All experiments were performed at room temperature (20 to 22℃).

Concentration-clamp technique

Concentration-clamp technique developed by Akaike's group[22] was used. The cell was aspirated off by a suction pipette, and the pipette was then inserted into the small tube through a hole. Electrical measurements were performed between the agar bridges, using the single-electrode voltage clamp technique. The solution in the tube was rapidly changed to a new solution with suction (−15 cmHg), which was controlled by an electromagnetic valve.

Drugs

Drugs used in this study were guanosine 5'-triphosphate (GTP, Sigma Chemical Co., St Louis, MO, U. S. A.), carbamylcholine chloride(carbachol, Wako Pure Chemical, Osaka, Japan), and atropine sulfate (Nacalai Tesque Inc., Kyoto, Japan).

RESULTS

Modulation of ACh-dependent K^+ (K^+ [ACh]) channels in isolated guinea-pig atrial myocytes were examined by cell-attached patch-clamp technique (Fig. 1). Carbachol (10 μM) was added to the pipette solution, which is a stimulant through muscarinic ACh receptors from surface membrane. The normal Tyrode's solution was used to make a giga seal (high resistance) with the cell membrane, and to perform cell-attached patch clamp experiments. Then, the extracellular solution was changed to the same solution as the pipette solution to take the reversal potential to be zero mV. The solution change was performed by using a concentration-clamp method. Holding potential was changed from −120 to +40 mV to measure the slope conductance of this channel (Fig. 1A). The single-current amplitude at different negative potentials was an inward direction, and had a strong inwardly rectifier property at positive potentials. The values are plotted in Fig. 1B. The average slope conductance was 44.7±3.5 pS (n=8). The average open time at −80 mV was 1.4±0.3 msec (n=7). Addition of atropine (1 μM) to the pipette solution blocked the responses.

From the cell-attached patch clamp mode, the experiment was switched to an inside-out patch clamp mode to avoid the influences of intracellular signal transductions, as shown in Fig.

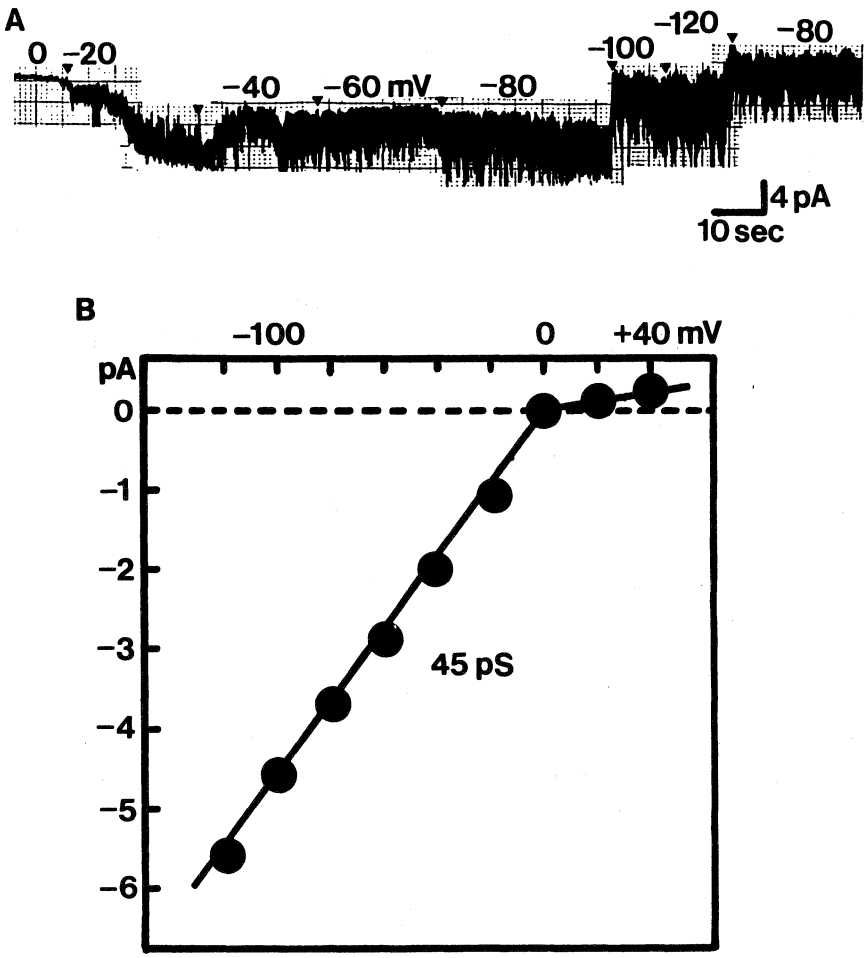

Fig. 1. ACh-sensitive K^+ channels in a guinea-pig atrial myocyte by cell-attached patch clamp mode. Carbachol (10 μM) was added in the pipette solution. The extracellular and intracellular K^+ concentrations were 140 mM. **A** : Unitary current traces at different holding potentials (0 to -120 mV). **B** : Slope conductance of the K^+channels.

2A. The holding potential was−80 mV. The pipette solution still included carbachol (10 μM). Switching to the inside-out patch clamp mode attenuated the conduction of K^+ [ACh] channels, But since the inward current remained, the cell was transiently exposed to the air (removed from the solution) to abolish an ATP-sensitive K^+ channel and a inwardly-rectifying K^+ channel as well as the K^+ [ACh] channel. Then, no activation of channels occurred. Under that condition, the bath solution was changed to a solution containing GTP (100 μM) by a concentration-clamp technique (Fig. 2B). At the same time after the application of GTP, the unitary channel was activated inwardly at −80 mV (which is a property of the concentration -clamp method). Discontinuation of GTP reduced the activation of the channel and recovered to the control level. Similar phenomena were observed in all 15 cells.

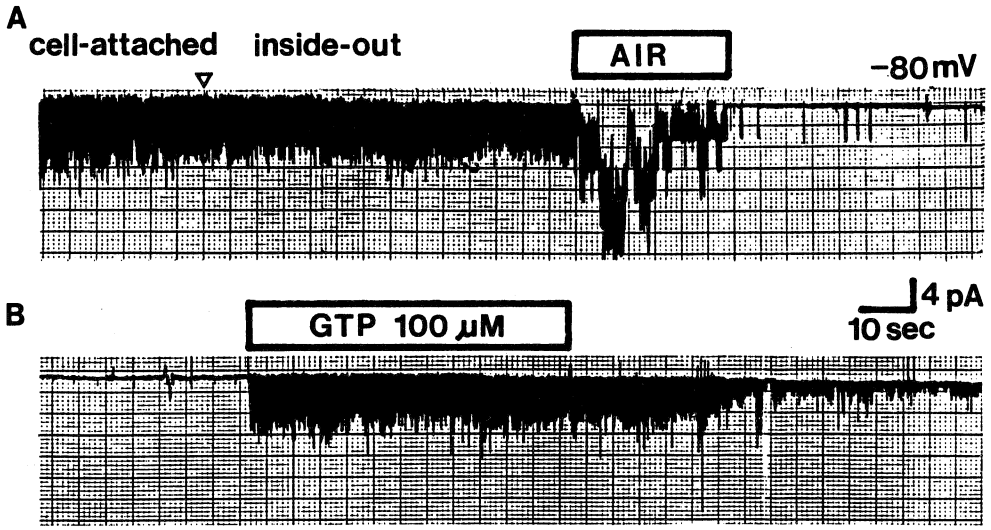

Fig. 2. Direct gating of ACh-sensitive K^+ channels by guanosin 5'-triphosphate in a single atrial muscle cell of guinea-pig. Carbachol (10 μM) was added in the pipette solution. The extracellular and intracellular K^+ concentrations were 140 mM. Holding potential was -80 mV. **A** : Unitary current trace by a switch from cell-attached to inside-out patch clamp mode. **B** : Stimulation of single ACh-sensitive K^+ channel by application of 100 μM guanosine 5'-triphosphate (GTP). The application of GTP and the wash out were performed by concentration-clamp technique.

DISCUSSION

Regulation of G protein on ion channel gating has already been identified for K^+ channels[23),24),25)], Ca^{2+} channels[26),27)], and hyperpolarization-activated inward current (a pacemaker current)[20),28)]. Direct G protein gating of ion channels is distinct from indirect effects of G proteins on ion channels. Phosphorylation of Ca^{2+} channels by PK-A is an example of an indirect effect[29),30)]. The present experiments showed the direct gating of K^+ [ACh] channel by G protein.

Cell-attached patch clamp

Cell-attached patch clamp mode was made by complete seal between a tip of pipette and cell membrane by suction (its resistance was an order of giga ohm), although it is dependent on adhesive activity of the cell membrane. In the present experiments (the extracellular and pipette solutions were similar), the single-currents were activated inwardly at negative potentials, and the reversal potential was zero mV. The current amplitude was increased at more negative potentials. The slope conductance was about 45 pS and mean open time was about 1.4 msec at -80 mV, consistent with other reports ; the former is ranging from approximately 40 to 55 pS and the latter is approximately 1 to 2 msec[19),31),32),33)]. Carbachol included in the pipette solution stimulated the K^+ channels. Atropine inhibited the channel activity. Carbachol would bind to muscarinic ACh receptors on the surface of the membrane, and activate the K^+ channel mediated through G proteins. Therefore, these results indicate that the inwardly activated K^+

channel is an ACh-sensitive K^+ channel.

Inside-out patch clamp

In cell-attached patch clamp mode, stimulation from the receptors transfers to G proteins and produces second messengers (i. e. cyclic nucleotides and Ca^{2+}). To examine direct action of G protein, the experiments were switched to inside-out patch clamp mode. The inside-out mode of the patch-clamp technique is an assay system for determination of the molecular steps leading to channel activation. On formation of the inside-out patch with loss of all intracellular soluble contents (including GTP), K^+ [ACh] channel activity markedly decreases even with ACh (carbachol in this study) in the pipette. Under that condition, GTP application similarly activated the K^+ [ACh] channel in the quiescent patch membrane. These findings indicate that the activation of the channel is not dependent on second messengers, but is due to direct action of G protein. It is also shown that if the intracellular contents are absent, agonists themselves cannot activate K^+ [ACh] channel.

G proteins are heterotrimers consisting of α, β and γ subusits[1),15),34)]. For most ion channels that are stimulated in the membrane-delimited manner, the α subunit is thought to be active. The α subunit is a substrate for ADP ribosylation by bacterial toxins, such as cholera or pertussis (IAP) toxins[35)]. Claims of an effector role for $\beta\gamma$ subunits may be related to recent reports[34),36),37)], in which $\beta\gamma$ may be stimulatory for one type of adenylate cyclase in the presence of α, while it may be inhibitory for another type of adenylate cyclase. In the case of K^+ [ACh] channels, most of the evidence for $\beta\gamma$ favors as the stimulatory subunits. In addition, α is divided to some subunits (α_s, α_i and α_o)[38)]. α_s is related to adenylate cyclase catalytic unit for Ca^{2+} channel. α_i and α_o are related to inhibition of adenylate cyclase activity and muscarinic cholinergic receptor for K^+ channel. In this study, therefore, GTP application would stimulate α_o and $\beta\gamma$ subunits of G protein, and activated K^+ [ACh] channel directly.

CONCLUSION

In the present experiments, carbachol stimulated the K^+ [ACh] channel on atrial muscle cell membrane. The activation of the channel occurred under the conditions designed to avoid the influences of cellular signal transductions (inside-out patch clamp mode). Therefore, these results indicate that the gating of K^+ [ACh] channel is regulated by the direct action of protein, independent of cellular second messengers. The mechanism of activation of K^+ [ACh] channel is shown in Fig. 3.

REFERENCES

1) **Katada, T.** and **Ui, M.** : Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc. Natl. Acad. Sci. U. S. A. **79** : 3129-3133, 1982.

2) **Ui, M.** : Islet-activating protein, pertussis toxin : a probe for functions of the inhibitory guanine nucleotide

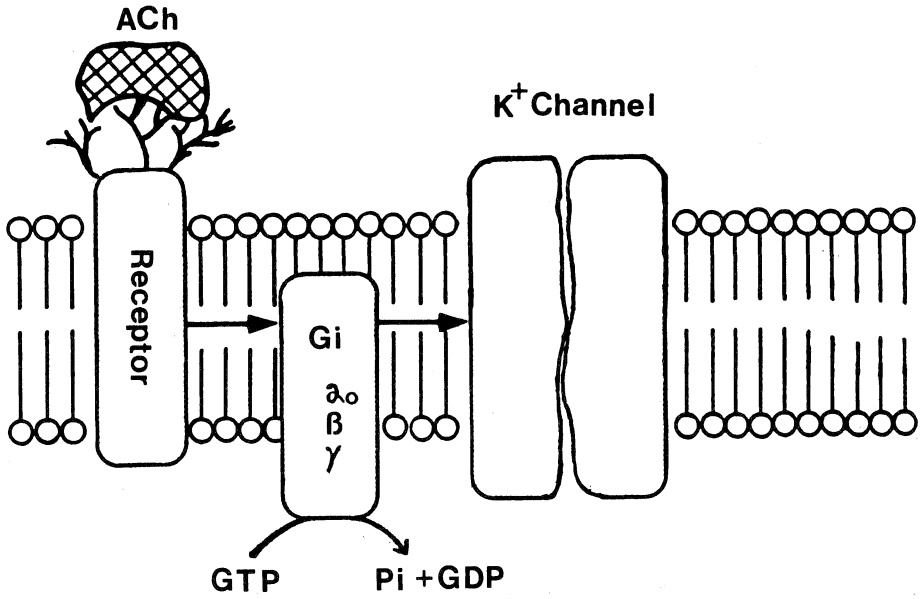

Fig. 3. Schematic representation of the relation between ACh-sensitive K^+ channel and the receptor on the cell membrane of heart muscle. The openning gating of the K^+ channel is activated by G protein mediated through stimulation of muscarinic ACh receptor. α_0, β and γ are subunits of G protein. ACh : acetylcholin. GTP : guanosine 5'-triphosphate. GDP : guanosine 5'-diphosphate.

regulatory component of adenylate cyclase. Trends. Pharmacol. Sci. **5** : 277-279, 1984.

3) **Nishizuka, Y.** : The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature **308** : 693-698, 1984.

4) **Schramm, M.** and **Selinger, Z.** : Message transmission : receptor controlled adenylate cyclase system. Science **225** : 1350-1356, 1984,

5) **Levitzki, A.** : Beta-adrenergic receptors and their mode of coupling to adenylate cyclase. Physiol. Rev. **66** : 819-854, 1986.

6) **Stryer, L.** : Cyclic GMP cascade of vision. Annu. Rev. Neurosci. **9** : 87-119, 1986.

7) **Satoh, H.** and **Hashimoto, K.** : On electrophysiological responses to phorbol esters which stimulate protein kinase C in rabbit sino-atrial node cells. Naunyn-Schmiedeberg's Arch. Pharmacol. **337** : 308-315, 1989.

8) **Satoh, H.** and **Sperelakis, N.** : Calcium and potassium currents in cultured rat aortic vascular smooth muscle cell lines. In : Ion channels of Vascular Smooth Muscle Cells and Endothetial Cells (Sperelakis, N. and Kuriyama, H., eds.). Elsevier, New York, p55-63, 1991.

9) **Satoh, H.** : Inhibition of L-type Ca^{2+} channel by stimulation of protein kinase C in isolated guinea-pig ventricular cardiomyocytes. Gen. Pharmacol. **23** : 1097-1102, 1992.

10) **Satoh, H., Nakashima, T.** and **Tsuchida, K.** : The responses to phorbol esters which stimulated protein kinase C in canine Purkinje fibers. Gen. Pharmacol. **23** : 847-852, 1992.

11) **Trautwein, W.** and **Dudel, J.** : Zum Mechanisms der Membranwirkung des Acetylcholin an der Herzmuskelfaser. Pflügers Arch. **266** : 324-334, 1958.

12) **Satoh, H.** : Electrophysiological actions of adenosine and aminophylline in spontaneously beating and voltage-clamped rabbit sino-atrial node preparations. Naunyn-Schmiedeberg's. Arch. Pharmacol. **347** : 197-204, 1993.

13) **Noma, A.** and **Trautwein, W.** : Relaxation of the ACh-induced potassium current in the rabbit sinoatrial node cell. Pflügers Arch. **377** : 193-200, 1978.

14) **Surawicz, B.** : Role of potassium channels in cycle length dependent regulation of action potential duration in mammalian cardiac Purkinje and ventricular muscle fibers. Cardiovasc. Res. **26** : 1021-1029, 1992.

15) **Katada, T.** and **Ui, M.** : ADP ribosylation of the specific membrane protein of C6 cells by islet-activation protein associated with modification of adenylate cyclase activity. J. Biol. Chem. **257** : 7210-7216, 1982.

16) **Florio, U. A.** and **Sternweis, P. C** : Reconstitution of resolved muscarinic cholinergic receptors with purified GTP-binding proteins. J. Biol. Chem. **260** : 3477-3483, 1985.

17) **Gilman, A. G.** : G proteins and dual control of adenylate cyclase. Cell **36** : 577-579, 1984.

18) **Kim, D., Lewis, D. L., Graziadei, L., Neer, E. J., Bar-Sagi, D.** and **Clapham, D. E.** : G-protein beta gamma-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. Nature **337** : 557-560, 1989.

19) **Kurachi, Y., Ito, H., Sugimoto, T., Shimizu T., Miki, I.** and **Ui, M.** : Arachidonic acid metabolites as intracellular modulators of the G protein-gated cardiac K^+ channel. Nature **337** : 555-557, 1989.

20) **Satoh, H.** and **Sperelakis, N.** : Hyperpolarization-activated inward current in embryonic chick cardiac myocytes : developmental change and modulation by isoproterenol and carbachol. Eur. J. Pharmacol. **240** : 283-290, 1993.

21) **Hamill, O. P., Marty, A., Neher, E., Sakmann, B.** and **Sigworth, F. J.** : Improved patch-clamp techniques for high-resolution current recording from the cells and cell-free membrane patches. Pflügers Arch. **391** : 85-100, 1981.

22) **Akaike N., Inoue, M.** and **Krishtal, O. A.** : 'Concentration-clamp' study of γ-aminobutyric acid-induced chloride current kinetics in frog sensory neurons. J. Physiol. (Lond.) **379** : 171-185, 1986.

23) **Codina, J., Hildebrandt, J. D., Birnbaumer, L.** and **Sekura, R. D.** : Effects of guanine nucleotides and Mg on human erythrocyte N_i and N_s, the regulatory components of adenylyl cyclase. J. Biol. Chem. **259** : 11408-11418, 1984.

24) **Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. M.** and **Hille, B.** : GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature **317** : 536-538, 1985.

25) **Yatani, A., Codina, J., Brown, A. M.** and **Birnbaumer, L.** : Direct activation of mammalian erythrocyte pertussis toxin-sensitive G protein, G_K. Science **235** : 207-211, 1987.

26) **Gomperts, B. D.** : Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. Nature **306** : 64-66, 1983.

27) **Yatani, A., Codina, J., Imoto, Y., Reeves, J. P., Birnbaumer, L.** and **Brown, A. M.** : Direct regulation of mammalian cardiac calcium channels by a G protein. Science **238** : 1288-1292, 1987.

28) **Brown, A. M.** : Regulation of heartbeat by G protein-coupled ion channels. Am. J. Physiol. **259** : H1621-H1628, 1990.

29) **Kameyama, M., Hofmann, F.** and **Trautwein, W.** : On the mechanism of beta-adrenergic regulation of the Ca channel in the guinea-pig heart. Pflügers Arch. **405** : 285-293, 1985.

30) **Flockerzi, V., Oeken, H. J., Hofmann, F., Pelzer, A., Cavalie, A.** and **Trautwein, W.** : Purified dihydropyridine-binding site from rabbit skeletal muscle t-tubles is a functional calcium channel. Nature **323** : 66-68, 1987.

31) **Sakmann, B., Noma, A.** and **Trautwein, W.** : Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. Nature **303** : 250-253, 1983.

32) **Soejima, M.** and **Noma, A.** : Mode of regulation of the ACh-sensitive K channel by the muscarinic receptor in rabbit atrial cells. Pflügers Arch. **400** : 424-431, 1984.

33) **Brown, A. M.** and **Birnbaumer, L.** : Direct G protein gating of ion channels. Am. J. Physiol. **254** :

H401-H410, 1988.

34) **Gliman, A. G.** : G proteins : transducers of receptor-generated signals, Annu. Rev. Biochem. **56** : 615-649, 1987.

35) **Smigel, M., Katada, T., Northup, J. K., Bokoch, G. M., Ui, M.** and **Gilman, A. G.** : Mechanisms of guanine nucleotide-mediated regulation of adenylate cyclase activity. Adv. Cyclic Nucleotide Res. **17** : 1-18, 1984.

36) **Tang, W-J.** and **Gilman, A. G.** : Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Nature **254** : 1500-1503, 1991.

37) **Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J.** and **Clapham, D. E.** : The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. Nature **325** : 321-326, 1987.

38) **Neer, E. J.** and **Clapham, D. E.** : Role of G protein subunits in transmembrane signalling. Nature **333** : 129-134, 1988.