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*Topical Review*

# Membrane-Delimited Cell Signaling Complexes: Direct Ion Channel Regulation by G Proteins

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## Introduction

The demonstration of cytoplasmic second messengers (reviewed in Sutherland, 1972) was one of the most important experimental results in cell signaling. In establishing that receptor activation by the first messenger, the receptor agonist, was coupled to cellular metabolism via a specific molecule, adenosine 3′,5′-cyclic monophosphate (cAMP) produced from a membrane enzyme, adenylyl cyclase (AC), the concept of intracellular second messengers was verified. Ever since, a clear line of experiments identifying various cytoplasmic molecular messengers has dominated the field of cellular signaling. Besides cAMP, other membrane-generated messengers used in signaling are now known to include guanosine 3′,5′-cyclic monophosphate (cGMP), diacylglycerol (DAG), and inositol trisphosphate ($IP_3$). Because cAMP activated protein kinases in turn transphosphorylated cellular effectors with the $\gamma$-phosphate of ATP (reviewed in Krebs, 1986), the exception more often than the rule was the simple sequence of receptor → membrane enzyme → second messenger → cellular effector. Furthermore, ATP was shown not to be the only nucleotide involved in these molecular relays when a requirement for GTP was established (reviewed in Rodbell, 1980). The involvement of GTP culminated in the demonstration that membrane-bound, guanine nucleotide binding, G proteins were the link between receptors and the membrane enzymes which produced second messengers (reviewed in Gilman, 1987; Birnbaumer, Abramowitz & Brown, 1990).

G proteins are a family of heterotrimeric ($\alpha$, $\beta$, $\gamma$) proteins, having as their targets membrane enzymes such as adenylyl cyclase (AC) and cGMP phosphodiesterase (PDE) and having as their activators a large family of receptors which, based upon hydrophobicity plots, share the common structural feature of seven transmembrane segments. G protein receptors include $\beta$- and $\alpha$-adenoreceptors, rhodopsin, muscarinic, dopaminergic and serotonergic receptors, etc. The G$\alpha$ subunit is a GTP-ase and is the usual activator of effectors; dimeric $\beta\gamma$ facilitates the exchange of GTP on $\alpha$ but may have an activator role as well (*see below*). The stimulation by G proteins of membrane enzymes is direct because specific, purified G$\alpha$ subunits such as $\alpha_S$ and $\alpha_T$ when activated, stimulate specific purified enzymes such as AC and cGMP PDE, respectively, following reconstitution in lipid vesicles (Cerione et al., 1984; May et al., 1985). The direct activation of G proteins by G protein receptors has also been shown in reconstitution experiments (Gilman, 1987; Birnbaumer et al., 1990). Thus, G proteins are membrane messengers and are the true second messengers for signaling pathways beginning with G protein receptors and including cytoplasmic third, fourth, etc., messengers.

Recently, a large class of membrane protein targets for G proteins has been identified that do not manifest the substrate-product reactions of enzymes. These are ion channels which have no enzymatic activity but have a central role in signaling due to the large amounts of ionic charge which they transport across the plasma membrane with great rapidity while in the open state. The change in state alters membrane conductance which in turn changes either the membrane potential or the ability to change the membrane potential. A particular class of ion channels, voltage-dependent ion channels, are the fundamental molecular units of excitability and are present in all nerve and muscle cells. Other cells frequently not considered as excitable, but in which

**Key Words** ion channels · G proteins · direct regulation · membrane-delimited pathways · second messengers · cytoplasmic pathways

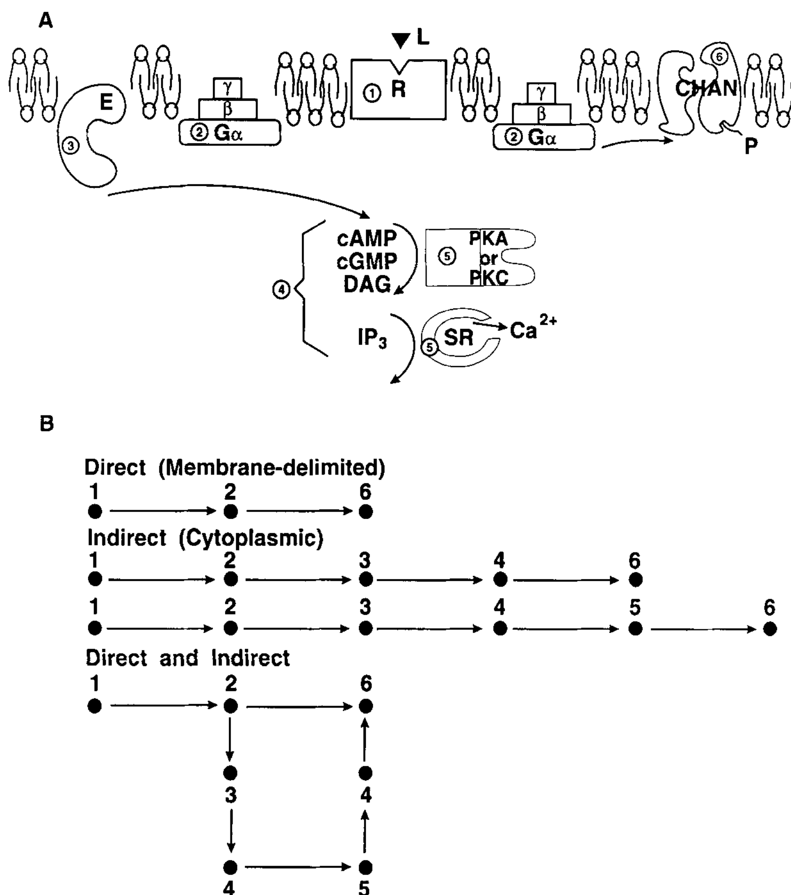



**Fig. 1.** Components of receptor-G protein-ionic channel pathways (*A*) and examples of the pathways (*B*). *1* = R, receptor; *2* = G protein; *3* and *4* = effectors such as adenylyl cyclase, cGMP phosphodiesterase, phospholipase C and phospholipase $A_2$ and their products such as cAMP, cGMP, DAG, and $IP_3$; *5* = PKA, protein kinase A, or PKC, protein kinase C, or SR, sarcoplasmic reticulum.

regulation of membrane potential is crucial to function such as endocrine and epithelial cells, also contain voltage-dependent ion channels. Another class of ion channels, ligand-gated ion channels, exemplified by ATP-sensitive $K^+$ channels are also regulated by G proteins. As we shall see later, an important class of ligand-gated channels are the G protein-gated ion channels. Given their importance, it is no surprise that ion channels are highly regulated and the regulation is frequently provided by G protein receptors. Until recently, it was considered that G protein-receptor regulation of ion channels was mediated mainly by cytoplasmic messengers but it is now clear that G proteins may interact with ion channels within the plane of the membrane in what is referred to as a membrane-delimited manner (Fig. 1). Moreover, reconstitution experiments have established that voltage-dependent $Ca^{2+}$ channels can, just like AC and cGMP PDE, be directly regulated by G proteins (Hamilton et al., 1991). By extension it seems likely that other ion channels will be G protein effectors and, given their diversity, ion channels may be the largest class of G protein effectors. Therefore, a simple sequential system of receptor-second messenger-cellular effector does in fact exist but not as originally envisaged for intracellular effectors. Rather, it exists for G protein receptors with G proteins as the second messenger and ion channels as the cellular effectors. These systems appear simple but like the original cytoplasmic second messenger pathways, may in certain cases be more complicated. It has already been suggested that activated G proteins may stimulate phospholipase A2 (PLA2) to produce arachidonic acid (AA) from membrane lipids and AA may then be a third membrane messenger acting on certain ion channels (Piomelli et al., 1987; Kurachi et al., 1989).

With the demonstration that ion channels are G protein effectors it was also established that a single type of G protein may have several effectors (Mattera et al., 1988). Since one type of receptor may activate several different types of G proteins and different types of receptor may project to a common type of G protein, a great variety of patterns of response is possible and is in fact observed. Consequently, it may be useful to think of G protein receptor complexes consisting of receptor, G protein, and membrane effectors including enzymes and ion channels. Such systems would imply a modular organization within the plasma membrane.

In this review, I will examine the extent of membrane-delimited ion channel regulation by G pro-

teins, the proposition that G protein effects on ion channels are direct and the functional implications for ion channel regulation uniquely determined by membrane-delimited G protein regulation. Inevitably for a new field, some controversies have arisen and these will be referred to. Finally, large G proteins are not the only G proteins coupling receptors to ion channels. Small G proteins, such as ras p21, clearly regulate ion channels although the functional significance of this regulation is unknown. Evidence for this new type of control will be discussed because ion channels are at present the only in vitro readout for this important class of growth regulatory proteins.

## G Protein Regulation of Ion Channels by Membrane-Delimited Pathways

The first channel established as a membrane target for G proteins was the atrial muscarinic or $K^+$[ACh] channel. This channel has been of interest to electrophysiologists ever since it was first shown that acetylcholine (ACh) acting via a muscarinic cholinergic receptor (mAChR), hyperpolarized the atrial membrane by increasing $K^+$ permeability (Trautwein & Dudel, 1958). Subsequently, a latency of 50–150 msec following topical application of ACh or vagal nerve stimulation was established (Glitsch & Pott, 1978; Hill-Smith & Purves, 1978; Hartzell, 1980; Osterrieder et al., 1981; Nargeot et al., 1982, 1983). At the neuromuscular junction, where the nicotinic AChR (nAChR) and its channel are one and the same protein, the latency is about 1 msec. Thus, it seemed likely that there was an intermediary coupling process between the atrial mAChR and atrial $K^+$ channel. Alterations in levels of cGMP and cAMP were found to have no effect on atrial membrane potential (Trautwein, Taniguchi & Noma, 1982; Nargeot et al., 1983). However, all of the experiments were done on cardiac syncytial preparations where the complexity of this tissue made interpretation of voltage-clamped currents difficult (Johnson & Lieberman, 1971). The introduction of methods for dispersing single cardiac cells (Powell & Twist, 1976) and studying the isolated cells electrophysiologically with a suction pipette (Lee et al., 1978, 1979) led ultimately to patch-clamp (Hamill et al., 1981; Sakmann et al., 1983) and single channel studies. Inwardly rectifying single channel $K^+$ currents of atria that were activated by ACh were clearly identified (Sakmann, Noma & Trautwein, 1983). By perfusing the patch pipette, it was shown that ACh activated this $K^+$ channel independently of cytoplasmic mediators (Soejima & Noma, 1984), but a G protein mechanism was not implicated. This result was followed by reports demonstrating that PTX blocked ACh inhibition of atrial pacemaking (Endoh, Manyama & Tajima, 1985), atrial hyperpolarization (Sorota et al., 1985), and the ACh-activated $K^+$ current. In addition, GTP was required for the ACh effect (Pfaffinger et al., 1985) and the ACh-activated $K^+$ current became irreversible in the presence of the nonhydrolyzable guanosine triphosphate (GTP) analog guanyl-5′-yl imidodiphosphate (BMP-P(NH)P) (Breitwieser & Szabo, 1985). The experiments established the involvement of a G protein termed $G_K$[1] (Breitwieser & Szabo, 1985), but a PTX-insensitive nonselective cation current related to phosphoinositide hydrolysis (Sorota et al., 1985; Tajima et al., 1987) complicated the whole-cell current measurements. Nevertheless, the strongest statement on mechanism that could be made was that muscarinic activation was independent of changes in cyclic nucleotides (Pfaffinger et al., 1985). A membrane-delimited or direct pathway between G proteins and the inwardly rectifying $K^+$ channel activated by ACh became clearer when another nonhydrolyzable congener, guanosine 5′-0-(3-thiotriphosphate) GTP$\gamma$S was shown to activate single atrial $K^+$ channel currents in excised, inside-out membrane patches (Kurachi, Nakajima & Sujimoto, 1986*a*, *b*) in a $Mg^{2+}$-dependent manner. The possibility that the G protein was acting indirectly through, for example, a membrane-associated enzyme such as protein kinase C (PKC) had not been specifically excluded although it was noted that adenosine triphosphate (ATP) was not required for the GTP$\gamma$S effect. In the next advance, G proteins purified from human erythrocytes (Codina et al., 1983, 1984*a*, *b*) were applied to ACh-sensitive $K^+$ channels in excised, inside-out, membrane patches from mammalian atrial muscle (Codina et al., 1987; Yatani et al., 1987*a*), and an exogenous PTX-sensitive G protein, now identified as $G_i$-3, preactivated with GTP$\gamma$S and then denoted as $G_k^*$, mimicked the mACH effect on $K^+$[ACh] channels. $G_k^*$ was effective at pM concentrations even in the absence of $Mg^{2+}$, whereas

[1] Abbreviations used are: ATP$\gamma$S, adenosine 5′-0-(3-thiotriphosphate); ARF, ADP-ribosylation factor; CTX, cholera toxin; DHP, dihydropyridine; G protein, guanine nucleotide-binding regulatory protein of hormone-receptor-effector functions; $G_s$, G protein stimulatory to adenylyl cyclase; $G_k$, G protein stimulatory to receptor regulated $K^+$ channels; GDP$\beta$S, guanosine 5′-0-(2-thiodiphosphate); GMP-P(NH)P, guanyl-5′-yl imidodiphosphate; GTP$\gamma$S, guanosine 5′-0-(3-thiothriphosphate); protein kinase A, cAMP-dependent protein kinase; PTX, pertussis toxin (also called IAP or islet activating protein); T-tubule membranes, membrane vesicles of the skeletal muscle traverse tubular system. Cyclic AMP, adenosine 3′,5′-cyclic monophosphate; Cyclic GMP, guanosine 3′,5′-cyclic monophosphate; $NAD^+$, nicotinamide adenine dinucleotide.

GTPγS only had effects at 10 nM or greater and required $Mg^{2+}$ (Kurachi et al., 1986*c*). The other principal G protein purified from human red blood cells (RBC), the cholera toxin (CTX)-sensitive G protein $G_s$, which activates AC when preactivated with GTPγS, had no effect (Codina et al., 1987; Yatani et al., 1987*a*), nor did the preactivated G protein transducin ($G_T$), prepared from bovine retina. Activated $G_0$ from bovine brain had weak effects that may have been due to contamination with an activated G protein with $G_K$ activity (Yatani et al., 1987*a*), but a direct effect of $G_0$ could not be excluded. The preactivated α subunit of $G_K$, $\alpha_i^*$-3 was equipotent with $G_i^*$-3, or the mixture of $\alpha_i^*$-3 plus βγ of $G_i$-3 indicating that the α subunit mediated the effect. Endogenous $G_K$ was not thought to be tightly coupled to the muscarinic receptor since ADP-ribosylation with PTX did not block the effects of exogenous unactivated $G_i$-3. If tight coupling were present, there should have been a permanent loss of receptor-G protein-$K^+$ channel coupling. Nor can the endogenous $G_K$ be tightly coupled to the $K^+$[ACh] channel since exogenous $G_i^*$-3 or $\alpha_i^*$-3 activated the channel. However, the complex of receptor, G protein and $K^+$[ACh] channel was solidly anchored in the membrane since activation could be repeated numerous times in an excised patch of membrane.

The single-channel currents identified by ACh (in this case Carbachol or Carb) responsiveness had a slope conductance of about 40 pS and an average open time at −80 mV of about 1.5 msec. These results were identical for single-channel currents activated by ACh in cell-attached patches, ACh and GTP in the bath solution in excised inside-out patches, GTPγS, $G_i$-3, $\alpha_i^*$-3, inactivated $G_i$-3 plus GTP in the presence of ACh, and a recombinant $\alpha_i^*$-3 expressed in *Escherichia coli* (Brown et al., 1988; Yatani et al., 1988; Mattera et al., 1989). The $G_i^*$-3 and $\alpha_i^*$-3 effects persisted even after washing for as long as 30 min, whereas the ACh effects ceased after GTP was removed. In excised patches opening probability, $P_o$, in the absence of GTP, was zero (Okabe, Yatani & Brown, 1991) and activation by any of the measures described above occurred because of an increase in $P_o$; neither conductance nor open times were affected. This same mechanism was subsequently found in all other cases of membrane-delimited G protein effects. Considerable efforts were made to rule out activation of PKC, and absence of ATP or addition of the nonhydrolyzable congener AMP-PNP, which cannot be used in transphosphorylation, was without effect.

Reconstitution studies established that the specific G protein was either Giα2 or Giα3 but true specificity probably resides in the pathway and cannot be established by reconstitution experiments. Purinergic receptors project to the same $K^+$ channels via a PTX-sensitive G protein (Kurachi et al., 1986*c*). It is possible that the different receptors utilize different $G\alpha_i$s. This has been clearly established in GH3 cells where it has been shown using specific and nonspecific anti-sense cRNAs, that Goα1 couples muscarinic receptors to $Ca^{2+}$ channels and Goα2 couples somatostatin receptors to the same $Ca^{2+}$ channels (Kleuss et al., 1991).

Approaches similar to those described above for the atrial $K^+$[ACh] channel were extended to other channels. Basically, the evidence for membrane-delimited regulation depends on the use of excised membrane patches from intact cells or membrane vesicle preparations from broken cells (Fig. 2). In both situations the single channel currents can be recorded under conditions where there is complete control of substrate and hence the production of second messengers such as cGMP, cAMP, DAG, $IP_3$, etc. These types of experiments established that there were many types of ion channels that could be activated by G proteins or GTPγS in the absence of substrate utilized by membrane enzyme effectors in the production of second messengers. The only other possibility would be a membrane intermediary generated, for example, by a membrane phospholipase. A partial list of channels which are membrane-delimited targets for activated G proteins is given in the Table.

### The Membrane-Delimited Interaction Between G Proteins and Ion Channels is Direct

It has been claimed that G proteins stimulate PLA2 to produce arachidonic acid (AA) from the membrane which in the form of a 5′ lipoxygenase metabolite may then act on ion channels (Kim et al., 1989; Kurachi et al., 1989). Hence, G proteins might act upon ion channels or other membrane effectors not directly within the membrane but via intermediary membrane products. The only way to test this possibility would be to reconstitute ion channels in a defined lipid bilayer system in the absence of any phospholipases. The approach of reconstitution with purified components has been used to justify the claim of a direct action of $G_s$ and $G_T$ on AC and cGMP, PDE, respectively. Only one candidate ion channel has been purified in sufficient quantities to attempt reconstitution in a defined lipid system as has been done for AC and cGMP PDE and that is the $Ca^{2+}$ channel from the T-tubules of skeletal muscle. When this system was reconstituted it was found that preactivated $G\alpha_s$ or $\alpha_s$ could stimulate

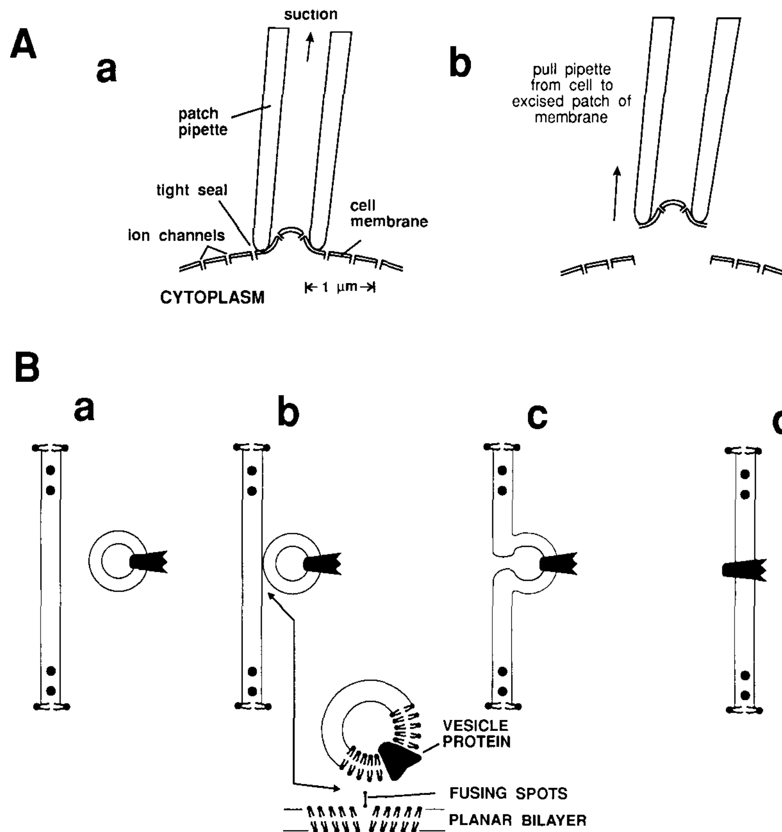



**Fig. 2.** (*A*) Patch clamp method. Current enters or leaves the pipette by passing through the channels in the patch of membrane. Recordings of the current through these channels can be made with the patch still attached to the rest of the cell, as in (*a*), or excised, as in (*b*). *b* was the method we usually employed. (*B*) Method of incorporating membrane vesicles into planar lipid bilayers. The vesicles added to the *cis* chamber are carried to the bilayer by an osmotic gradient, and fusion begins at the fusing spots. The orientation of the $Ca^{2+}$ channels in the vesicle is usually right-side-out for cardiac sarcolemma and inside-out for skeletal muscle T tubules. The conventions for current recording are the same as those used with patch clamp: positive current is outward.

purified $Ca^{2+}$ channel protein in a manner analogous to its effects on membrane preparations of $Ca^{2+}$ channels (Fig. 3). Furthermore, the effect was asymmetrical; application to the cytoplasmic face only was effective. Other preactivated G proteins on their $\alpha$ subunits such as $G_{i3}$ or $\alpha_i$-3, and $G_T$ had no effect. In other experiments it was found that small amounts of labeled G proteins copurified with the $Ca^{2+}$ channels and were coprecipitated when the channels were immunoprecipitated with a specific $Ca^{2+}$ channel antibody. In addition, purified $Ca^{2+}$ channels increased the sedimentation rate of $\alpha_s$ but not $\alpha_i$-3 and both the $\alpha_s$ and $\beta$ subunits of $G_s$ were detected in Western blots of the purified $Ca^{2+}$ channel. Thus, $G_s$ and $Ca^{2+}$ channels are closely associated in the T-tubular membrane (Hamilton et al., 1991).

An experimental shortcoming in these particular reconstitution studies arose from the rundown of purified $Ca^{2+}$ channels. This is a well-known but poorly understood phenomenon that occurs for intact $Ca^{2+}$ channels in the cellular environment. In addition, membrane potential had variable effects, whereas normally membrane potential is the principal activator of $Ca^{2+}$ channels. A better set of test channels might be $K^+$ channels, particularly the $K^+$[ACh] channel. Unfortunately, purified preparations of these channels are presently not available.

## The Subunit Mediator of G Protein Stimulation of Ion Channels

The question regarding which subunit mediates the effect has been raised for $K^+$[ACh] channels where both $\alpha$ and $\beta\gamma$ subunits may be active. For most other ion channels that are stimulated in the membrane-delimited manner, the $\alpha$-subunit is thought to be active. Claims of an effector role for $\beta\gamma$ may be related to a recent report that $\beta\gamma$ may be stimulatory for one type of AC in the presence of $\alpha$ while it is inhibitory for another type of AC (Gilman, 1987). Even in the case of $K^+$[ACh] channels, most of the evidence favors $\alpha$ as the stimulatory subunit. Moreover, stimulatory effects of $\beta\gamma$ could not be dissociated from detergent effects due to the zwitterionic detergent CHAPs used to suspend the hydrophobic $\beta\gamma$. When the hydrophilic transducin $\beta\gamma$ was used, an inhibitory effect was observed. The $\beta\gamma$ activator results are difficult to reproduce because the zwitterionic detergent CHAPS can by itself activate atrial $K^+$ channels (Cerbai et al, 1988; Kirsch et al., 1988) as well as other ionic channels (Kirsch

**Table.** Ion channels directly gated by G proteins

| Gα Protein | Channel | Receptor | Tissue |
|---|---|---|---|
| $\alpha_k$, $\alpha_i$-3, r$\alpha_i$-3 | $K^+$, 40pS, IR | $M_2$, (ACh) | atrium |
| $\alpha_k$, $\alpha_i$-3 | $K^+$, 55pS, ?R | $M_2$, (ACh), SS | $GH_3$ |
| $\alpha_o$, r$\alpha_o$ | $K^+$, 55pS, NR | unknown | hippocampus |
| | $K^+$, 38pS, NR | unknown | hippocampus |
| | $K^+$, 38pS, IR | 5-HT1A | hippocampus |
| | $K^+$, 13pS, NR | unknown | hippocampus |
| $\alpha_i$-1. r$\alpha_i$-1 | $K^+$, 40pS, IR | $M_2$ (ACh) | atrium |
| $\alpha_i$-2, r$\alpha_i$-2 | $K^+$, 40pS, IR | $M_2$ (ACh) | atrium |
| $\alpha_s$ | $Ca^{2+}$, DHP-sens., 25pS | β-AR | atrium, ventricle |
| $\alpha_s$, r$\alpha_s$, splice variants | $Ca^{2+}$, DHP-sens., 10pS | β-AR | skeletal muscle |
| $\alpha_s$ | $Na^+$, TTX-sens. | β-AR | atrium, ventricle |
| $\alpha_i$-3 | Epithelial $Na^+$ | unknown | kidney |
| $\alpha_i$-3 | $K^+_{ATP}$ | | RIN |
| $\alpha_o$ | $K^+_{ATP}$ | | skeletal muscle |
| unknown | $K^+_{Ca}$, 260pS | β-AR | myometrium |
| unknown | $Ca^{2+}$, T-type | unknown | dorsal root ganglion |

From Brown & Birnbaumer, *Annu. Rev. Physiol.* **52:**197–213, 1990.

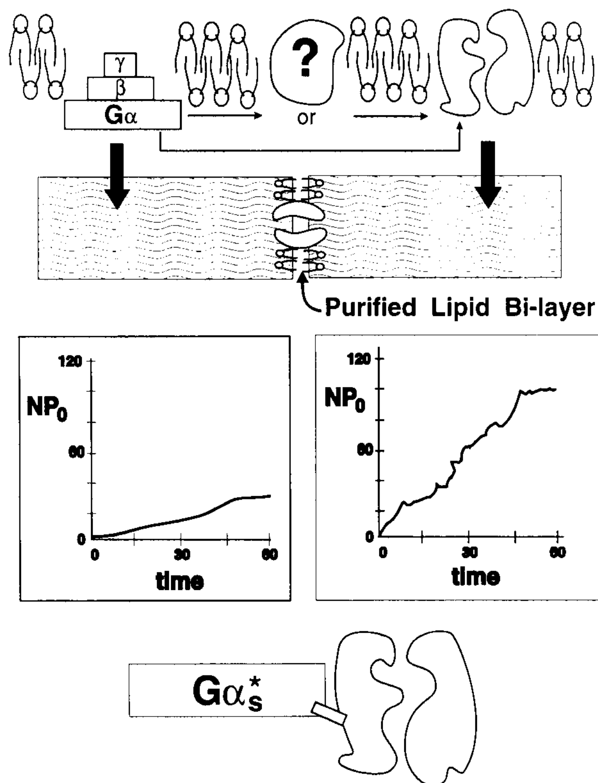



**Fig. 3.** The calcium channel as a G protein effector.

et al., 1988). It is well known that ionic detergents can act upon $Na^+$ channels and other membrane proteins including $PLA_2$ (Pind & Kuksis, 1988). While GTP plus the inactivated trimeric $\alpha_i$-3 stimulated $K^+$[ACh] channels in the presence of agonist and PTX, GTP plus $\beta\gamma$ could not reconstitute the response. Adenosine diphosphate (ADP)-ribosylation with PTX blocked muscarinic activation, and the response was reconstituted by inactivated $\alpha_i$-3 in the presence of GTP. These findings plus the fact that $\alpha_i^*$-3 and holo $G_i^*$-3 were equipotent led us to the conclusion that $\alpha$ subunits and not $\beta\gamma$ subunits mediate the $G_k$ effect.

Most of the experiments on this point have dealt mainly with reconstitution of the $K^+$[ACh] response. To probe functional aspects, a monoclonal antibody mAb 4A (Deretic & Hamm, 1987; Hamm et al., 1988) that binds to $\alpha$ but not $\beta\gamma$ subunits was used. In the reaction, $\alpha$ dissociates from $\beta\gamma$. The results showed that mAb 4A blocked the mAChR effect and, since $\beta\gamma$ was liberated at the same time, the $\alpha$-subunit must be the channel activator. It is now agreed that $\alpha$ subunits are responsible (Logothetis et al., 1988; Bourne, 1989; Kim et al., 1989), although it has been proposed that $\beta\gamma$ subunits activate phospholipase $A_2$ (Bourne, 1989; Kim et al., 1989) and that arachidonic acid (AA) and certain of its metabolites activate these same atrial $K^+$ channels. The latter follows from the observation (Kurachi et al., 1989) that certain eicosanoids activate muscarinic atrial $K^+$ channels. However, this pathway is not operative in muscarinic or purinergic stimulation since eicosanoid pathway blockers are said to have no effect on the agonist-induced responses (Kim et al., 1989; Kurachi et al., 1989). In general, the role of $\beta\gamma$ subunits remains uncertain; it may not be restricted to allosteric regulation of the $\alpha$ subunit and dimeric $\beta\gamma$ may also have the ability to activate effectors.

## A Possible Mechanism by which Gα Subunits and Ion Channels Interact

For obligatory G protein-gated ion channels such as the $K^+$[ACh] channel, several lines of evidence suggested the involvement of an inactivating particle. For example, the $K^+$[ACh] channel is inwardly rectifying due to open channel block by $Mg^{2+}$ and, therefore, has a receptor site for ionic inactivation. Voltage-dependent $K^+$ channels have a peptide moiety at the N-terminus, which has been shown to be the inactivating particle (Zagotta, Hoshi & Aldrich, 1990). Also, in the $G_T$-cGMP PDE interaction, the diesterase is normally inactivated by its $\gamma$-subunit. Trypsin cleaves the $\gamma$-subunit and thereby activates the PDE. The same argument was extended to the $K^+$[ACh] channel. Trypsin produced $K^+$[ACh] currents indistinguishable from those produced by muscarinic acitvation (Kirsch & Brown, 1989). For its effect, trypsin did not require prior muscarinic activation and the trypsin-activated channel was no longer responsive to stimulation by activated G proteins. Trypsin cleaves at arginines and lysines but arginine-specific reagents such as glyoxal and phenylglyoxal were ineffective. Thus, it appears that the site on the $K^+$[ACh] channel with which $G\alpha_K$ interacts contains lysines. It was proposed that the channel protein has a domain which is normally inhibitory to channel opening (Kirsch & Brown, 1989). The domain might resemble an N-terminus blocking particle (Zagotta et al., 1990).

## The Logical Consequences of G Protein-Ion Channel Pathways

One expectation of direct G protein-ion channel pathways is for response times that are slower than those of single-element systems such as the nicotinic cholinergic receptor yet faster than those of five-element cytoplasmic pathways. The time constant for activation of $K^+$[ACh] channels is about 300 msec (rate about 3/sec), and this provides a reasonable estimate for other direct pathways. On the other hand, cytoplasmic pathways have much longer time constants (Powell & Twist, 1976; Buxton & Brunton, 1985). Heart rate is also regulated at comparably fast rates (short time constants). As noted earlier, a prediction from our work on atrial $K^+$[ACh] channels and ventricular $Ca^{2+}$ channels was for the existence of direct pathways in addition (Fig. 1) to the already known indirect pathways (DiFrancesco & Tromba, 1988) from autonomic receptors to the cardiac pacemaker $I_f$ channels (Yatani & Brown, 1990*a*; Yatani et al., 1990*b*). This expectation was fully confirmed in experiments which blocked the cAMP-PKA pathways to cardiac $Ca^{2+}$ channels following stimulation of cardiac $\beta$-adrenoreceptors ($\beta$-ARs) by isoproterenol (ISO) (Pelzer, Pelzer & McDonald, 1990). These experiments established in intact myocytes, a direct link between $\beta$-ARs, $G_s$ and $Ca^{2+}$ channels. Studies with fast solution changes showed a biphasic time course to ISO stimulation, a fast response with the time constant of the $K^+$[ACh] channel response to ACh, and a slow response due to the cAMP-PKA channel phosphorylation pathway. The fast response was attributed to direct coupling, although changes in second messengers such as cAMP could not be excluded.

Recently, the existence of the fast response has been denied (Hartzell et al., 1991). These experiments are to be doubted because other laboratories have confirmed the fast response in guinea pig (Pelzer et al., 1990) and in rat (Bean, *personal communication*). Moreover, a completely different experimental method using photoactivation of caged GTPγS diffused into cardiac myocytes has also demonstrated a fast response (Kozlowski et al., 1991). A fast, direct response has also been observed for G protein-mediated inhibition of neuronal $Ca^{2+}$ channels (Beech et al., 1992).

$Ca^{2+}$ channels are not the only cardiac channels regulated by direct and indirect G-protein pathways. Similar regulation has been shown for $Na^+$ channels and for the cardiac pacemaker channel, $I_f$. These results show that a consideration of the cell's requirements can direct experiments on the regulatory signaling pathways. The fact that in heart cells the coupling of $\beta$-ARs to $Ca^{2+}$, $I_f$, and $Na^+$ channel is indirect as well as direct may be related to long-lasting responses to the signaling agonist (Fig. 4). Even $K^+$[ACh] channels can be targets for indirect, slower G protein-coupled pathways (Kim et al., 1989).

Another expectation of more complex G protein-ion channel pathways is the possibility of coordinated responses. A simple membrane response to an agonist stimulus is not relied on; rather, we have an orchestrated response mediated by a network of ion channels linked by coupling G proteins. Thus, muscarinic agonists in the heart activate $K^+$[ACh] channels and inhibit $Ca^{2+}$ and $I_f$ channels. The effect is to sharply reduce the pacemaker current and the rate at which the pacemaker action potentials depolarize neighboring nonpacemaker cells. $\beta$-adrenergic agonists activate $Ca^{2+}$ currents (Reuter, 1987; Trautwein & Hescheler, 1990; Yatani & Brown, 1991) and have a local anesthetic effect on $Na^+$ channels in depolarized membranes (Yatani et al., 1987*b*; Schubert et al., 1990). The latter would tend to limit the rate at which the cardiac impulse might propagate during sympathetic stimulation in ischemic myocardium and could contribute to the prorhyth-

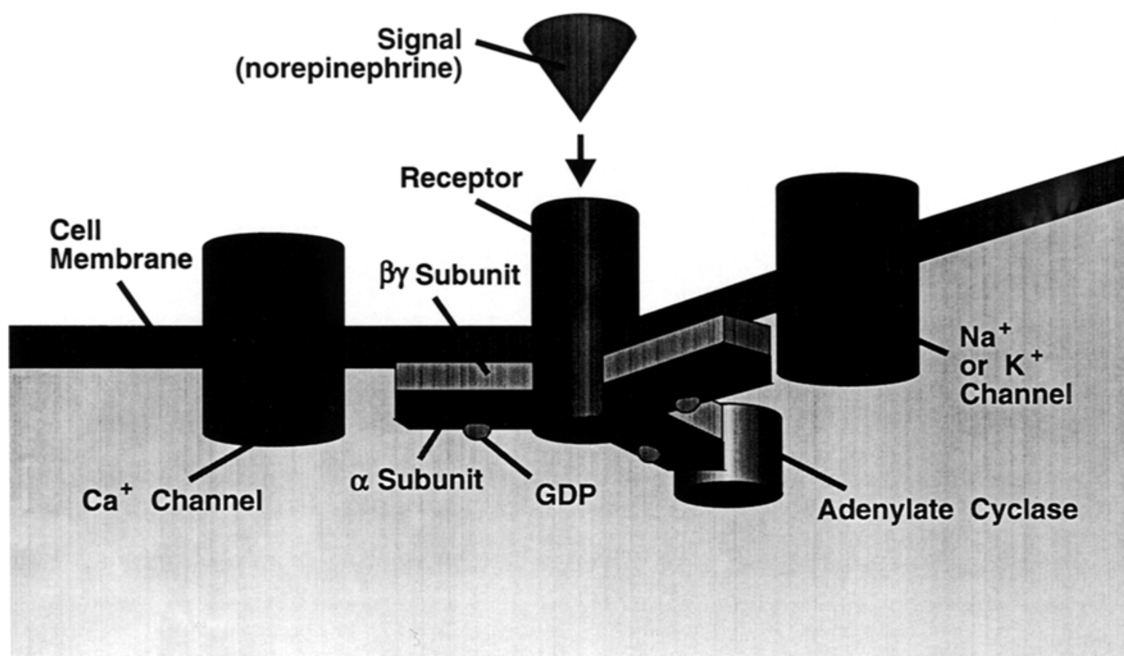



**Fig. 4.** G protein gating of ion channels.

mic effects of local anesthetics (Task Force of the Working Group on Arrhythmias of the European Society of Cardiology CAST and Beyond, 1990). Coordinated responses are also present in neurons and secretory cells. Many G protein receptors activate $K^+$ currents and at the same time inhibit $Ca^{2+}$ currents (Dunlap, Holtz & Rane, 1987; Ewald et al., 1989). This has the effect of using two mechanisms to make it less likely that the cell will secrete neurotransmitters or hormones: first, by hyperpolarizing the cell membrane and reducing any $Ca^{2+}$ influx that is membrane-potential dependent and, second, by inhibiting directly voltage-dependent $Ca^{2+}$ channel pathways. On the other hand, some G protein receptors such as the serotonin receptor inhibit $K^+$ currents, enhance $Ca^{2+}$ currents, and thereby facilitate synaptic transmission (Edmonds et al., 1990). The logic of coordinated receptor-G protein-ion channel pathways might therefore lie in the networks they form. These networks should be thought of as modules that produce coordinated cellular responses to specific stimuli.

As noted for receptor-G protein-effector systems such as $\beta$-AR $\rightarrow$ $G_s$ $\rightarrow$ AC, significant amplification occurs because the $\beta$-Ar can act catalytically on $G_s$ and AC acts catalytically on ATP. Structural organization for such systems must be somewhat loose. In networks that seem to operate as modules such as receptor-G protein-ion channels, for the G protein to encounter its target structural organization is required. If we assume a diffusion co-efficient for $\alpha_s$ along the membrane of $10^{-10}$ $cm^2/sec$ (Edidin, Kud & Sheetz, 1991), a channel density of $1/\mu^2$ and a G protein to channel ratio of one, the average time required for an encounter would be about 3 sec, too slow for the approximately 100-msec delay that has been observed (Yatani & Brown, 1990*a*). A strong prediction, therefore, is that receptors, G proteins, and ion channels will occur as discrete units in the cell membrane. This argument implies that a cellular logic of protein-coupled ion channel networks may be present and that the logic may be understood by considering the specific responses of different cells to a given signaling molecule. The justification is that this approach may be useful in directing our thinking about the complicated signal transducing pathways that appear to be present in all cells. A prediction is that modular networks of similar design (receptor-protein-ion channel or channels) will be identified in many cells and will produce effects that are peculiar to specific cell types. The functional requirements of different cell types may help in understanding the response patterns produced by a particular signal and the modules it activates. In this regard two generalizations seem possible: the cellular logic could be understood in terms of (i) the time constants of the response to a given signal; and (ii) the pattern of response of specific cell types to a given signaling molecule.

## The Spontaneous G Protein Activation of Ion Channels

An interesting phenomenon is the observation that $K^+$[ACh] channels may be active even in the absence of agonist. This was apparent in the report of Soejima and Noma (1984) and can in fact occur for other G protein effectors systems such as Gs and AC (Cerione, 1984). The amount of activity depended upon the concentration of GTP and was inhibited competitively with GDP in the same way as the agonist-activated process. Not surprisingly, muscarinic antagonists had no effect. An endogenous agonist could not be excluded but the most likely explanation is that the spontaneous process is due to thermal interaction between unliganded receptor, G protein and ion channel.

The spontaneous activity has important implications. The first is that the G protein receptor-ion channel effector system is primed in the absence of agonist so that the GDP turnover rate is increased above the basal rate of 0.15 $sec^{-1}$ of the isolated G protein. In this way arriving agonist can produce its effects more quickly than the basal state would allow. This is probably important for regulation that occurs in seconds or less as is the case for the heart rate and synaptic events in the CNS.

A second implication is that the set point for these G protein-ion channel responses will not be zero due to the spontaneous noise. As a result, the dynamic range will be considerably extended. It will also be influenced by the metabolic state of the cell insofar as this state infringes on the concentration ratio of GTP and GDP.

A third implication is that spontaneous activity in opioid G protein-receptor-ion channel coupled systems could be the basis for inverse agonism (Costa & Herz, 1989) observed in these systems.

## G Proteins, $Ca^{2+}$ Channels and Signaling in the CNS

Numerous agonists inhibit the high threshold $Ca^{2+}$ current of sensory neurons (Dunlap & Fishbach, 1978; Dolphin & Scott, 1986; Forscher, Oxford & Schulz, 1986; Marchetti, Carbone & Lux, 1986; Ewald et al., 1988*a*; Bean, 1989; Grassi & Lux, 1989; Kasai & Aosaki, 1989) via a pertussis toxin-sensitive G protein (Holz, Rane & Dunlap, 1986; Hescheler et al., 1987; Ewald, Sternweis & Miller, 1988*b*; Harris-Warrick et al., 1988; Gross et al., 1990). This phenomenon has implications for presynaptic neuronal regulation but knowledge of the mechanism is fragmentary. Suggestions include a multistep mechanism involving protein kinase C (PKC) (Doerner, Pitler & Alger, 1988; Ewald et al., 1988*a*; Rane et al., 1989), an unspecified, messenger-free mechanism (Forscher et al., 1986), and a direct G protein effect (Bean, 1989). Recent reports of voltage-dependent deinhibition of $Ca^{2+}$ channels previously inhibited by either agonists or the nonhydrolyzable guanine nucleotide GTPγS (Marchetti et al., 1986; Bean, 1989; Grassi & Lux, 1989; Kasai & Aosaki, 1989; Elmslie, Zhou & Jones, 1990; Pollo, Taglialatela & Carbone, 1991) offer clues, but how the deinhibition may occur is as yet unclear. Possibilities include the following: a direct effect of depolarization on the G protein-modified channels, voltage-dependent release of guanine nucleotides from the Gα subunit, or voltage-dependent block produced perhaps by the activated G protein itself or a modulating molecule activated by the G protein. These possibilities were tested experimentally by examining the dependence of the processes of deinhibition (unblock) and reinhibition (reblock) on the extent of G-protein activation with intracellular GTPγS. Our results favor the third possibility. We (Lopez & Brown, 1991) propose a hypothesis in which a G protein-dependent blocking molecule is coupled to $Ca^{2+}$ channels to produce inhibition. In our hypothesis unblocking is voltage dependent, and reblocking occurs in a concentration-dependent manner by recombination with the blocking molecule.

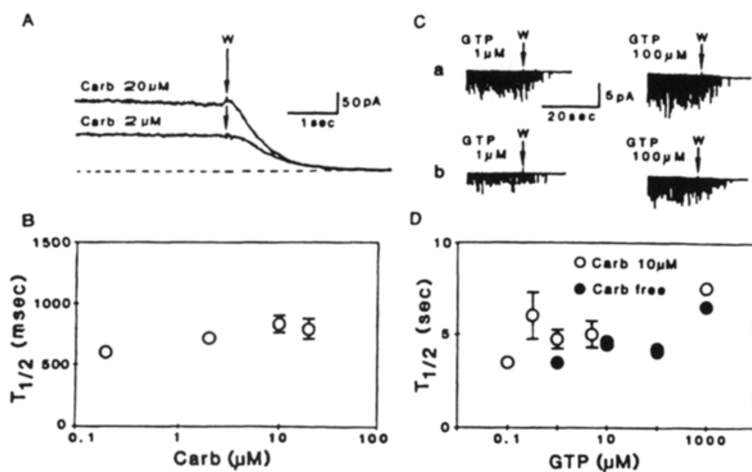
A minimal model of block, unblock, and reblock in the presence of GTPγS which takes into account the dependence on the extent of G protein activation was presented.

The model resembles the scheme recently proposed by Elmslie et al. (1990) in bullfrog sympathetic neurons which in turn was similar to the model of Bean (1989). Reblocking of the current primarily reflects net movement from O to $X_1$ and $X_2$ (Lopez & Brown, 1991). Some previous models did not include a dependence on the concentration of activated G proteins (Kasai & Aosaki, 1989; Marchetti & Robello, 1989; Elmslie et al., 1990), while others explicitly (Grassi & Lux, 1989) or implicitly (Bean, 1989) contemplated that possibility. The present work provides supporting data for such dependence and proposes a model to rationalize the G protein-mediated inhibition of neuronal $Ca^{2+}$ channels.

## Small G Proteins as Second Membrane Messengers

In our experiments on $K^+$[ACh] currents, we observed that deactivation of $K^+$[ACh] currents was about tenfold faster in whole atrial cells compared with deactivation in membrane patches excised from these cells (Fig. 5*A*). The deactivation time in ex-

A




B

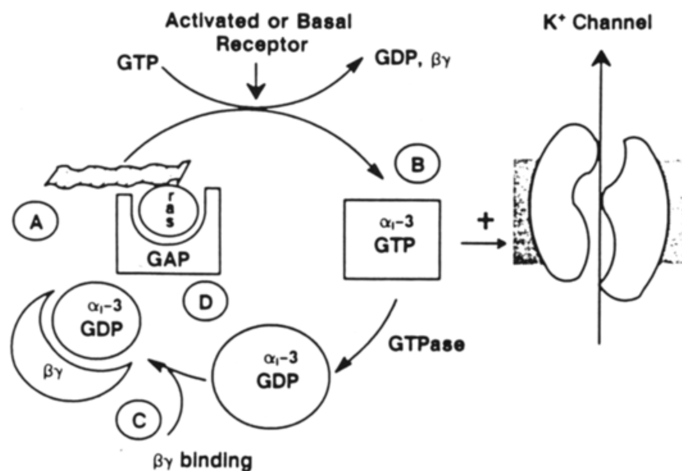



**Fig. 5.** (*A*) Deactivation of whole-cell and cell-free patch $K^+$[ACh] currents. (*B*) Ras/Gap inhibition of $K^+$[ACh] currents.

cised patches corresponded to the rate at which the $\alpha$ subunit hydrolyzes GTP (Gilman, 1987), ruling out a GTP-ase effect of the target $K^+$ channel, and so we wondered if there was a cytoplasmic GTPase activating protein (GAP) for heterotrimeric $G_k$ similar to the one that binds to the small ras p21 guanine nucleotide binding (G) protein (Trahey & McCormick, 1987). We found that ras-GAP did indeed block $K^+$[ACh] channels. The effect, however, was not due to an action on $G_k$ as anticipated, but instead involved the ras p21 protein (Yatani et al., 1990*c*). Thus, ras-GAP did not bind to $G_K$, and $G_K$ had no effect on ras-GAP activity. The inhibitory effect required both ras and GAP since an antibody to ras blocked the GAP effect and vice-versa. Since ras-GAP had no effect after activation of $K^+$[ACh] channels with GTP$\gamma$S, we proposed interruption of coupling between muscarinic receptor and G protein as shown in Fig. 5*B*.

The physiological significance of this observation remains uncertain. Ras p21 is a proto-oncogene product and mutated forms of ras correlate strongly with certain types of cancer and with cellular growth regulation. How this fits in with an effect on coupling between muscarinic receptor and $K^+$[ACh] channel is, therefore, puzzling. It is of interest that some G protein receptors have an oncogenic potential. Whatever the physiology, this large G protein-

coupled system remains the only in vitro readout for the effects of ras p21 and GAP. We took advantage of this to study the mechanism whereby ras and GAP interact. Our results showed that the effects of ras and GAP could be uninhibited by the $SH_2$ (src homology) domains of GAP alone and a model for how ras promotes this activity has been proposed (Martin et al., 1991). The $SH_2$ domains of GAP are known to interact with the PDGF receptor and coupling of this sort to receptor tyrosine kinases may be crucial links in pathways regulating cell growth. It would be of interest to determine if phosphorylated G protein receptors couple to such pathways via this ras-GAP mechanism.

## Summary

Ion channels are signaling molecules and by themselves perform no work. In this regard they are unlike the usual membrane enzyme effectors for G proteins. The pathways of G protein receptor, G protein and ion channels are, therefore, purely informational in function. Because a single G protein may have several ion channels as effectors, the effects should be coordinated and this seems to be the case. Inhibition of $Ca^{2+}$ current and stimulation of $K^+$ currents would have a greater impact than either alone. Additional flexibility is provided by spontaneous noise in the complexes of G protein receptor, G protein, and ion channel. By having a non-zero setpoint, the range of control is extended and the responses become bi-directional.

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