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Specificity of action of guanine nucleotide-binding regulatory protein subunits on the cardiac muscarinic K^+ channel

(muscarinic receptors/patch-clamp/inward-rectifying K channel)

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ABSTRACT **The cardiac muscarinic receptor stimulates a potassium-selective ionic current ($I_{K.ACh}$) through activation of a guanine nucleotide-binding regulatory protein. Purified α and $\beta\gamma$ subunits of the guanine nucleotide-binding regulatory protein have each been reported to open the K^+ channel. We have reported that nanomolar concentrations of purified brain $\beta\gamma$ subunits activated $I_{K.ACh}$ in chicken embryonic atrial patches. In contrast, J. Codina, A. Yatani, D. Grenet, A. M. Brown, and L. Birnbaumer [(1987) *Science* 236, 442–445] subsequently reported that picomolar concentrations of activated erythrocyte α subunits (i.e., the 40-kDa α subunit that the authors call α_K) opened K^+ channels in guinea pig atrial patches. In this paper, we further explore the specificity of various $\beta\gamma$ and α subunits in embryonic chicken and neonatal rat atrial patches. $\beta\gamma$ subunits from either human placenta ($\beta_{35}\gamma$) or bovine brain ($\beta_{35,36}\gamma$) activated $I_{K.ACh}$ whereas transducin $\beta\gamma$ ($\beta_{36}\gamma$) did not. The $\beta\gamma$ activation was consistent in rat and chicken patches [118 of 123 patches (97%)]. $\beta\gamma$ subunits opened K^+ channels at concentrations ≥200 pM and maximally activated the channel at 10 nM. $\beta\gamma$ or guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) channel activation could be reversed by α_{41}–GDP. The purified brain $\beta\gamma$ preparation was contaminated with <0.01% unactivated α. The detergent (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPS), used to suspend the hydrophobic $\beta\gamma$, did not activate $I_{K.ACh}$ alone, with buffer, with heat-inactivated $\beta\gamma$, or with transducin $\beta\gamma$. Unactivated α subunits did not open K^+ channels. Activated, α subunits purified from human erythrocytes (α_{40}–GTP[γ-S]) or bovine brain (α_{39}–GTP[γ-S]) at concentrations of 10 pM or higher (up to 1 nM) opened K^+ channels less frequently in chicken atrial patches [5 of 27 patches (19%) and 9 of 35 patches (26%), respectively] than in rat atrial patches [5 of 11 patches (45%) and 11 of 19 patches (58%), respectively]. Negative results were not due to patch vesicle formation. Other experiments indicated that α and $\beta\gamma$ activated the same population of channels. Activation of the channel by both $\beta\gamma$ and α subunits implies a more complicated scheme for guanine nucleotide-binding regulatory protein action than previously proposed.**

Acetylcholine slows heart rate by hyperpolarizing pacemaker and atrial cells. This hyperpolarization is caused in part by acetylcholine binding to muscarinic receptors that open potassium-selective ion channels ($I_{K.ACh}$) by way of a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein (G protein). Small patches of intact membrane can be isolated by using the inside-out mode of the patch-clamp technique (1) and the intracellular surface can be exposed to GTP or purified G proteins. Muscarinic-gated K^+ currents are induced in the presence of intracellular GTP (2–6). Upon treatment of the cells (4) or cell membrane patches (6) with pertussis toxin (which causes ADP-ribosylation of specific G proteins and uncouples them from the receptor), K^+ channel activity does not increase despite the presence of extracellular acetylcholine and intracellular GTP. Intracellularly applied nonhydrolyzable GTP analogs (5) cause an acetylcholine-independent (2, 6) activation of the muscarinic K^+ channel despite prior cell treatment with pertussis toxin (2, 7).

G proteins are heterotrimers consisting of α, β, and γ subunits. When hormone is bound to receptor in the presence of intracellular Mg^{2+} and GTP, the α subunit binds GTP, is activated, and may dissociate from the $\beta\gamma$ dimer. The $\beta\gamma$ dimer was shown to activate K^+ channels upon perfusion of inside-out patches from chicken embryonic atrial cells (2). Other data have appeared demonstrating activation of muscarinic K^+ channels by the α subunit of an erythrocyte-derived G protein (3). Here, we further examine the specificity of action of the $\beta\gamma$ and α subunits on acetylcholine-activated K^+ channels obtained from embryonic chicken and neonatal rat atrial membrane patches. We have found that $\beta\gamma$ from two sources (bovine brain and human placenta) consistently and specifically activated $I_{K.ACh}$ whereas bovine retinal $\beta\gamma$ did not. Two α subunits (α_{39} and α_{40}) with bound guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) also activated $I_{K.ACh}$.

MATERIALS AND METHODS

Atria from 14-day embryonic chickens and 1-day-old rats were dissociated into single cells and used in short-term tissue culture (<24 hr) (2). Standard patch-clamp methods (1) were used to record single-channel currents from cell-attached and inside-out membrane patches. The solutions in the pipette and bath contained 118.5 mM KCl, 21.5 mM KOH, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes (pH = 7.2, titrated with HCl). Exceptions are noted in the text. The pipette tip was moved into the mouths of a series of tubes where it was perfused by the test solution (2). All experiments were carried out at 20–22°C. Currents were recorded with a List model EPC7 patch-clamp amplifier and recorded on a digital tape recorder. Figures containing original records were played back from digital tape onto a Gould thermal array or pen recorder. Records analyzed quantitatively for

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; $I_{K.ACh}$, potassium-selective ionic current stimulated by the cardiac muscarinic receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate.

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activity were obtained as described (2) by using an INDEC 11/73 computer. The current integral was divided by single-channel amplitude (i) to obtain Np_o, the product of the number of channels (N) and probability of opening (p_o) (2). Averaged Np_o ($\overline{Np_o}$) is given as a percent of $\overline{Np_o}$ over the time noted at the top of Figs. 1 *b* and *d* and 3.

A major problem in inside-out patch recording is the spontaneous sealing over of patches into vesicles. A vesicle no longer has an accessible inner membrane and might give false-negative results. Persistence of unattenuated single channel openings indicates that the patch has not resealed into a vesicle. Fortunately, chicken and rat membrane patches have basal activity (usually >1 opening per 10 sec), thus providing a continuous monitor of the state of the inside-out patch. Vesicle formation is detected by rounding and attenuation of the current steps. GTP (when acetylcholine was in the pipette), GTP[γ-S], or $\beta\gamma$ were often applied to patches at the end of negative experiments to ensure that the patch had not resealed into a vesicle. Data from patches that had sealed over were rejected.

Purification of $\beta\gamma$ subunits from brain, placenta, and retina has been described (8–10). Lubrol 12A9 (Lubrol PX) was removed from $\beta\gamma$ preparations and exchanged for CHAPS [(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] (Sigma and Calbiochem) on a DEAE-Sephacel column. In control experiments, Lubrol PX and cholate caused patch breakdown at levels of more than 100 μM (0.006%). Lubrol PX (Pierce and Sigma) also blocked GTP[γ-S]-induced activity at levels >10 μM (0.0006%) and thus could not be used as a detergent. CHAPS had no effect on recordings at concentrations equal to or less than 200 μM (0.013%); at concentrations more than 200 μM, patch breakdown was frequent. The final CHAPS concentration used in these experiments was equal to or less than 184 μM (as specified). Concentrations are indicated in the figure legends. Dithiothreitol was also included and used at 40 μM. Transducin $\beta\gamma$ from bovine retina did not contain detergent. In some experiments CHAPS was added to the transducin $\beta\gamma$ preparation (as indicated).

The pure α_{41} protein was prepared as described by Neer *et al.* (8) by sequential chromatography over DEAE-Sephacel, Sepharose 6B, DEAE-Sephacel, heptylamine-Sepharose (all in cholate-containing buffer), and DEAE-Sephacel in Lubrol 12A9 buffer containing 50 mM Tris Cl, 75 mM sucrose, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0.6% Lubrol 12A9, 10 mM NaF, and 10 μM $AlCl_3$. A sample was applied to DEAE-Sephacel equilibrated with 50 mM Tris Cl (pH 8), 75 mM sucrose, 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 0.3% CHAPS. The Lubrol 12A9 was washed off the column with equilibrating buffer, and the protein was eluted with equilibrating buffer containing 0.3 M NaCl. Note that this buffer contained no NaF or $AlCl_3$. Other preparations of pure α_{41} were made by a modified procedure in which a larger column was used for the second DEAE-Sephacel step and the heptylamine-Sepharose step was omitted. Lubrol was removed by a final chromatography step over DEAE-Sephacel with a linear gradient of 0–0.3 M NaCl in the CHAPS buffer described above.

The α_{39} protein was purified through the heptylamine-Sepharose step in buffer containing cholate. The final purification was over an Ultrogel AcA 44 column in 50 mM Tris Cl (pH 8) in 75 mM sucrose, 6 mM $MgCl_2$, and 1 mM dithiothreitol with no detergent, NaF, or $AlCl_3$.

Two methods were used to monitor the activation of the α subunit. The first was to measure the amount of GTP[γ-^{35}S] bound to the sample used for reconstitution of the channel. The α_{39} or α_{41} protein was incubated with 2 μM GTP[γ-^{35}S] (900 cpm/pmol) for 15 min at 30°C. GTP[γ-S] was in 2- to 5-fold excess over protein. To separate bound from free GTP[γ-S], each sample was chromatographed on a Sephadex G-50 column equilibrated with a solution of 50 mM Tris Cl (pH 7.6), 75 mM sucrose, 6 mM $MgCl_2$, and 1 mM dithiothreitol for α_{39} or the same buffer with 0.3% CHAPS for α_{41}. The eluted protein contained 0.9 mol of GTP[γ-S] per mol of α subunit.

When α_{39} or α_{41} is activated by GTP[γ-S] or fluoride, trypsin cleaves it into a stable fragment that is 2 kDa smaller than the original. Without activating ligands, the α subunit is rapidly degraded into peptides. We used this property to assess GTP[γ-S] binding and removal of NaF. Samples of α_{41} or α_{39} from which fluoride had been removed were incubated with trypsin as described (11) with or without incubation with GTP[γ-S] (2 μM, 15 min, 30°C). With GTP[γ-S] the entire sample was cleaved to the stable 39-kDa or 37-kDa form, respectively. Without the nucleotide, the protein was entirely degraded, indicating that NaF had been removed and that the protein had reverted to an unactivated conformation.

RESULTS

As we have reported (2), the purified $\beta\gamma$ subunits activate the inward-rectifying, muscarinic-gated K^+ channel in embryonic chicken atria $I_{K.ACh}$ (Fig. 1 *A* and *B*) when diluted to 0.2–10 nM concentrations and applied to inside-out patches. The channel had a conductance of 35–40 pS and a mean open time of ≈1 ms. Within a few seconds of exposure to $\beta\gamma$, $I_{K.ACh}$ channels opened and the channels remained active as long as the recording lasted, albeit with some gradual rundown of activity over time. $\beta\gamma$ from bovine brain (a mixture of $\beta_{35}\gamma$ and $\beta_{36}\gamma$ components) also activated $I_{K.ACh}$ in rat neonatal atria (Fig. 1*A*) and in guinea pig atria (data not shown). $\beta\gamma$ preparations from bovine brain and human placenta applied to patches first activated $I_{K.ACh}$ at a concentration of 200 pM (Fig. 1*B*) and induced maximal activity by 10 nM concentrations. $\beta\gamma$ activation by nanomolar concentration was consistent and reproducible (118 of 123 patches with ≥1 nM $\beta\gamma$).

$\beta\gamma$ activation can be prevented by preincubation with an excess of α–GDP subunits (2). To test the reversal of $\beta\gamma$ activation in the membrane, we applied α–GDP subunits to neonatal rat atrial patches activated by $\beta\gamma$ or by GTP[γ-S] (Fig. 1*C*). Excess α_{41}–GDP (20–40 nM) reversed activation in both cases whereas boiled α_{41}–GDP did not (Fig. 1*C*). The reversal of GTP[γ-S]-activated channels by α–GDP implies that α–GDP may act through two mechanisms: binding of the endogenous activator $\beta\gamma$ and competition with endogenous α–GTP[γ-S] for the regulatory site on the channel. Furthermore, α–GTP[γ-S] and $\beta\gamma$ subunits activate the same population of channels. When α_{39}–GTP[γ-S] activated channels in Fig. 1*D*, $\beta\gamma$ and GTP[γ-S] applied subsequently did not increase activity further. Similarly, when $\beta\gamma$ was applied first, α_{39}–GTP[γ-S] and GTP[γ-S] did not result in higher average channel activity (data not shown).

α Subunit Activation of $I_{K.ACh}$. As shown in Fig. 1, α subunits may also activate $I_{K.ACh}$, but at lower concentrations than required by $\beta\gamma$. α_{40}–GTP[γ-S] obtained from human erythrocytes and generously supplied by J. Codina and L. Birnbaumer (Baylor University, see ref. 3) and α_{39}–GTP[γ-S] from bovine brain could activate $I_{K.ACh}$ in neonatal rat atria at 10 pM in our experiments. α_{40}–GTP[γ-S] and α_{39}–GTP[γ-S] often failed to activate $I_{K.ACh}$ in chicken atria (α subunits could be tested only up to 1 nM since 1 nM GTP[γ-S] alone activates $I_{K.ACh}$). In fact, we were able to see stimulation of $I_{K.ACh}$ in 5 of 27 chicken atrial patches with α_{40}–GTP[γ-S] only at levels equal to or greater than 10 pM. In 5 patches unresponsive to α_{40}–GTP[γ-S], we tested the accessibility of the patch by exposure to GTP (acetylcholine in the pipette) or $\beta\gamma$. In all cases, application of GTP (3 of 3 patches) or $\beta\gamma$ (2 of 2 patches) to the same patch induced channel activity after α_{40}–GTP[γ-S] had failed to activate $I_{K.ACh}$. In neonatal rat cells, α_{40}–GTP[γ-S] activated $I_{K.ACh}$

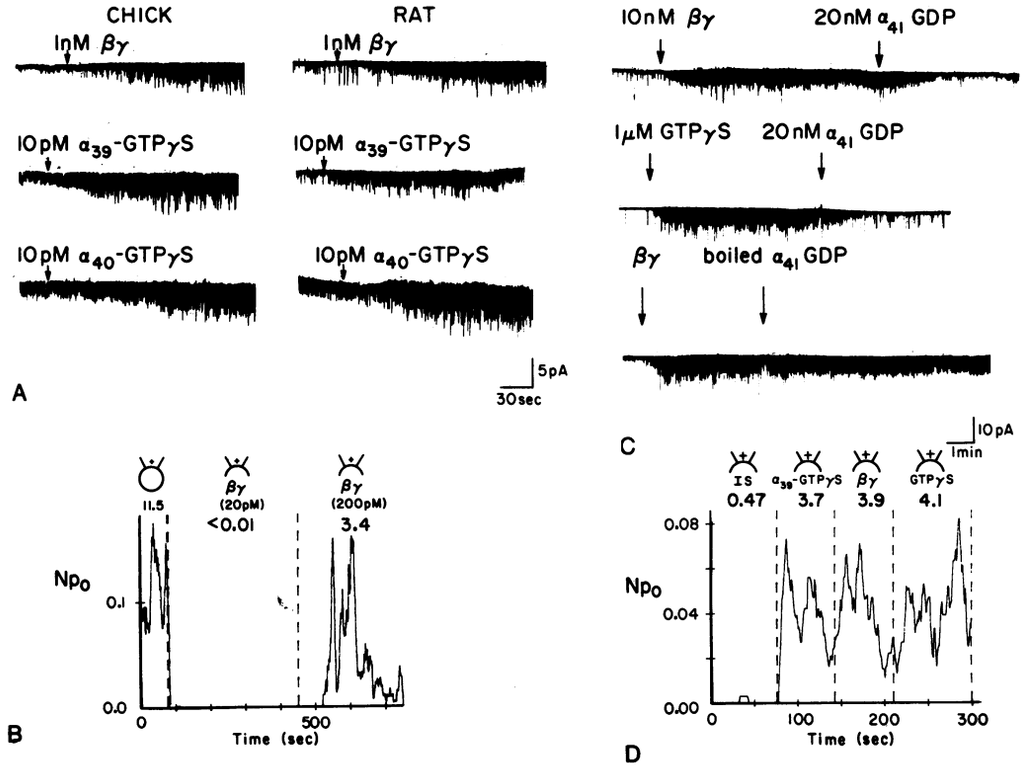

FIG. 1. (*A*) βγ, α_{39}–GTP[γ-S], and α_{40}–GTP[γ-S] activate $I_{K.ACh}$ in embryonic chicken and neonatal rat atria. Each trace shows background single-channel activity (no GTP or acetylcholine) before addition of the specified subunit (arrow). Perfusate before addition of subunit was with normal bath solution; basal activity varies from cell to cell. Holding potential −80 mV. The conductance (35–40 pS) and kinetics (mean open time, 1.0 ms at −80 mV) of rat and chicken single-channel $I_{K.ACh}$ currents were indistinguishable. (*B*) Picomolar concentrations of the βγ subunits activate the muscarinic-gated K^+ channel. βγ (20 pM) failed to activate the K^+ channel but 200 pM βγ activated the channel after ≈1 min. Numbers shown at the top of the figure represent percent average activity (Np_o) over the period shown. (*C*) Reversal of βγ or GTP[γ-S]-activated $I_{K.ACh}$ by α_{41}–GDP in neonatal rat atria. α_{41}–GDP (10 nM) was applied prior to 20 nM α_{41}–GDP in the βγ-activated patch. Boiled α–GDP (20 nM) did not reverse βγ activation of $I_{K.ACh}$. (*D*) α_{39}–GTP[γ-S]-activated channel activity was not increased further by βγ (10 nM) or GTP[γ-S] (100 μM). Likewise α_{39}–GTP[γ-S] (10 pM) did not increase βγ-activated activity (data not shown; $\overline{Np}_o$ = 1.7 vs. 1.5).

more consistently than chicken (5 of 11 patches; 10 pM). α_{39}–GTP[γ-S] (bovine brain) was also effective at low concentrations (10 pM; 11 of 19 patches) in neonatal rat atria but less so in chicken (9 of 35 patches). α_{41}–GTP[γ-S] (from both rabbit liver and bovine brain) has been ineffective to date in chicken (0 of 8 patches) and rat (0 of 14 patches). α_s–GTP[γ-S] (liver, where α_s is the stimulatory α subunit) has also not activated $I_{K.ACh}$ (0 of 4 patches). Again, the negative results in each case were only counted as negative if basal activity was not attenuated or if application of the subunit was followed by activation by GTP (acetylcholine in the pipette). The α subunit was usually applied at high flow rates and with vigorous perfusion by placing the patch in the mouth of a tube through which the subunit-containing solution flowed, making it unlikely that there were diffusion limitations or problems in α–GTP[γ-S] reaching the site of activation. We have reported (2) that α_{39}–GTP[γ-S] did not activate $I_{K.ACh}$. This failure to cause activation may have been due to suboptimal conditions for the binding of GTP[γ-S] to α subunits. Other cell-dependent factors may also be involved since we noted that α_{39}–GTP[γ-S] and α_{40}–GTP[γ-S] activations were not randomly distributed in time, but occurred in clusters of successive patches. In summary, α_{39}–GTP[γ-S] (bovine brain) and α_{40}–GTP[γ-S] (human erythrocyte) activated $I_{K.ACh}$ at concentrations of 10 pM, although not in all patches containing the channel.

βγ Activation of $I_{K.ACh}$. Three factors bear on the specificity of βγ activation of $I_{K.ACh}$: (*i*) α subunits contaminate the βγ preparation from conventional protein purification regimens and thus might cause $I_{K.ACh}$ activation. (*ii*) βγ must be suspended in a buffer containing detergent and this buffer or detergent may have effects on channel activity. (*iii*) α and βγ activated $I_{K.ACh}$, but α activated the channel at a lower apparent concentration than did βγ. We address each of these points with the following experimental evidence.

Purity of βγ. A sensitive test for α contamination of βγ is ADP-ribosylation of the βγ preparation. Various conditions for ADP-ribosylation were investigated to maximize the sensitivity of the assay. In the purest βγ preparation, α (ribosylation substrate) made up 0.01% of the total protein (Fig. 2). Thus, 0.1 pM of unactivated α subunit would be present in 1 nM βγ (a concentration of βγ that consistently activated $I_{K.ACh}$). We never obtained activation of $I_{K.ACh}$ channels from chicken or rat neonatal atria at concentrations <1 pM with activated α–GTP[γ-S]. It is important to stress that any α present in the βγ preparation would be unactivated α for the following reasons. (*i*) The preparation was never exposed to GTP[γ-S] (α–GDP and unbound α subunits did not activate $I_{K.ACh}$). (*ii*) Although an aluminum fluoride step is used in purification of bovine brain βγ (8) and fluoride alone can activate α subunits (12), fluoride was removed from the preparation by gel filtration, ion-exchange chromatography, or by both procedures used sequentially. Furthermore, fluoride activation of α subunits is rapidly reversible (13). Unactivated α subunits from the same preparation from which fluoride had been removed did not activate $I_{K.ACh}$. (*iii*) βγ prepared from human placenta without fluoride (9) activated the $I_{K.ACh}$ channel as well as did bovine brain βγ (Fig. 3*B*). Finally, βγ was present in a 10,000:1 ratio to α. Excess βγ by mass action would have deactivated α even in the presence of GTP[γ-S] or fluoride (14, 15). βγ even activated $I_{K.ACh}$ in the absence of Mg (1); Mg is needed for the dissociation of α from βγ.

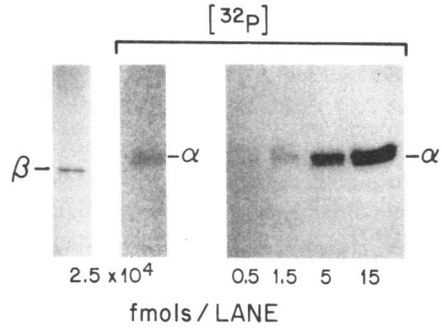

FIG. 2. Pertussis toxin-catalyzed ADP-ribosylation of purified $\beta\gamma$ (lane 2) and of samples containing known amounts of α subunits (lane 3–6) was carried out as described by Yatani *et al.* (24). The reactions were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Similar results were obtained when the samples were ADP-ribosylated as described by Neer *et al.* (8). Lanes 1 and 2 contain 2.5×10^4 fmol of $\beta\gamma$. Lanes: 1, Coomassie blue stain; 2, ADP-ribosylated $\beta\gamma$; 3–6, samples of ADP-ribosylated $\alpha\beta\gamma$ heterotrimers containing the indicated amounts of α subunits. As estimated from the intensity of the ribosylated bands, the $\beta\gamma$ sample contains $\approx$3 fmol of α.

Buffers and Detergents. A second question concerning $\beta\gamma$ activation of $I_{K.ACh}$ relates to the possibility of nonspecific activation by nonprotein components of the $\beta\gamma$ solution. The $\beta\gamma$ buffer alone never activated $I_{K.ACh}$ and was often tested immediately prior to $\beta\gamma$ application. In initial experiments, Lubrol disrupted patches. Furthermore, Lubrol ($\geq$10 μM) blocked channel activity, necessitating exchange of $\beta\gamma$ into CHAPS, a zwitterionic detergent. CHAPS alone (10 μM–200 μM) never activated $I_{K.ACh}$ in embryonic chicken (n = 26) or neonatal rat atrial (n = 6) controls (Fig. 3*A*). Thus, $\beta\gamma$ activation was not an artifact of the detergent in which it was dissolved. The question then arises as to whether $\beta\gamma$ in combination with detergent formed a nonphysiological activator of $I_{K.ACh}$. This seems unlikely since (*i*) $\beta\gamma$ boiled in buffer containing CHAPS failed to activate $I_{K.ACh}$, (*ii*) preincubation of $\beta\gamma$ with α subunits blocked activation (2), and (*iii*) transducin $\beta\gamma$ in CHAPS (see below) did not stimulate $I_{K.ACh}$. Y. Kurachi (personal communication) has also not observed activation in guinea pig atria by CHAPS.

Specificity of $\beta\gamma$. $\beta\gamma$ obtained from bovine brain is a mixture of $\beta_{35}\gamma$ and $\beta_{36}\gamma$. Two other $\beta\gamma$ proteins were perfused onto chicken atrial patches. Placental $\beta_{35}\gamma$ (9) activated $I_{K.ACh}$ whereas transducin $\beta_{36}\gamma$ did not. Placental $\beta\gamma$ was roughly equipotent to bovine brain $\beta\gamma$. Transducin $\beta_{36}\gamma$ did not activate $I_{K.ACh}$ up to 400 nM in the presence or absence of CHAPS (Fig. 3*B*).

α_{39}–GTP[γ-S] and α_{40}–GTP[γ-S] activated $I_{K.ACh}$ at concentrations $\geq$10 pM whereas $\geq$200 pM $\beta\gamma$ was required to stimulate $I_{K.ACh}$ activity. α subunits are more hydrophilic and thus may reach their binding site in the membrane through the aqueous phase whereas $\beta\gamma$ is hydrophobic and must be mixed with detergents. We had to reduce the concentration of detergent used (<200 μM CHAPS) since higher concentrations caused patch breakdown. To determine whether $\beta\gamma$ was aggregated at the dilution and detergent concentrations as close as possible to concentrations used in the reconstitution, the protein was diluted to 100 nM into intracellular solution containing 180 μM CHAPS. A sample was passed over an Ultrogel AcA 44 column (LKB) equilibrated with the same buffer. The protein was diluted by a factor of $\approx$5 during passage over the column. Because the concentration of $\beta\gamma$ was very low, its elution position was determined by immunoblotting. Twenty microliters from each fraction was spotted onto nitrocellulose, probed with rabbit anti-β antibody, and developed with ^{125}I-labeled protein A (16). Eighty percent of the $\beta\gamma$ was eluted at the void volume whereas 20% was eluted at the position expected for unaggregated $\beta\gamma$. Thus, the total $\beta\gamma$ concentrations overestimate the amount of unaggregated $\beta\gamma$. Although increasing dilution would favor the monodispersed form of $\beta\gamma$, the free $\beta\gamma$ in the 200 pM $\beta\gamma$ preparation may actually be only 40 pM. The relative effects of subunit aggregation are not known. The observation that α subunits activate in the pico molar range whereas $\beta\gamma$ activate in the nanomolar range may be due to their different abilities to reach the activation site. Y. Kurachi, T. Katada, and M. Ui (personal communication) have now activated guinea pig atrial $I_{K.ACh}$ channels with 10 pM rat brain $\beta\gamma$.

DISCUSSION

In embryonic chicken and rat neonatal atrial cells, purified $\beta\gamma$ subunits from bovine brain ($\beta_{35}\gamma$ and $\beta_{36}\gamma$) and human placenta ($\beta_{35}\gamma$) consistently activated $I_{K.ACh}$ when applied to the intracellular surface of the patch. In our experiments, α subunits from bovine brain (α_{39}–GTP[γ-S]) and human erythrocyte (α_{40}–GTP[γ-S]) were capable of activating $I_{K.ACh}$ at low concentrations (10 pM) but were not as consistent in stimulating $I_{K.ACh}$ activity as $\beta\gamma$. There was an apparent

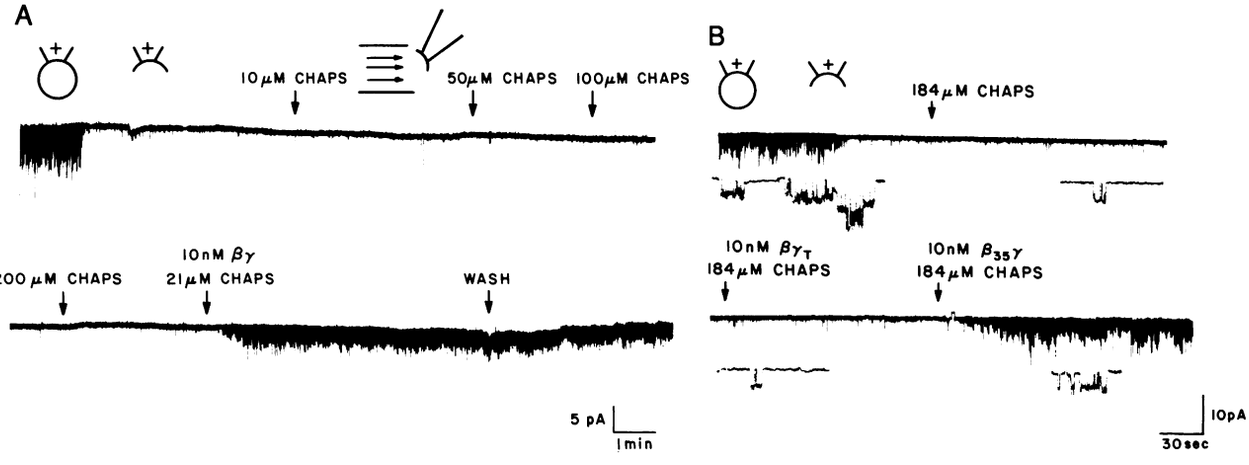

FIG. 3. Detergent (CHAPS) does not activate $I_{K.ACh}$ alone (*A*) or in the presence of transducin (*B*). (*A*) Inside-out patch was exposed to various concentrations of CHAPS with no increase in activity. Application of $\beta\gamma$ (10 nM) with 21 μM CHAPS rapidly activated the channel. (*B*) Transducin $\beta\gamma$ ($\beta\gamma_T$) does not activate $I_{K.ACh}$ even in the presence of CHAPS although $\beta_{35}\gamma$ does. Three other experiments performed by using exactly the same sequence of steps gave the same results.

species difference in that α subunits activated $I_{K.ACh}$ more frequently in rat atria than chicken.

The specificity of $\beta\gamma$ activation is confirmed by the following lines of evidence. (*i*) Boiled $\beta\gamma$ did not activate $I_{K.ACh}$ (2). Buffer alone had no effect. Boiling would not affect the hydrophobicity of $\beta\gamma$ nor would it affect the buffer components. (*ii*) Detergent alone (CHAPS) or detergent in combination with transducin $\beta\gamma$ did not activate $I_{K.ACh}$. (*iii*) $\beta\gamma$ preincubated with α presumably forming an $\alpha\beta\gamma$ heterotrimer did not activate $I_{K.ACh}$. Boiling the α subunit (α_{39} and α_{41}) prior to preincubation with $\beta\gamma$ removed this inhibition by α (2). Most significantly, addition of α_{41}–GDP reversed channel activation in a patch pretreated with $\beta\gamma$ or GTP[γ-S]. (*iv*) $\beta\gamma$ activation of $I_{K.ACh}$ cannot be explained by α contamination since the α was not activated. Furthermore, there was too little α (<0.01%) in the bovine brain $\beta\gamma$ preparation to stimulate the channel even if the α were entirely active.

Our failure to obtain consistent activation with the α subunits, α_{39}–GTP[γ-S] from bovine brain and α_{40}–GTP[γ-S] from human erythrocytes, is surprising (3). α activation was variable in chicken and neonatal rat. Negative experiments were not due to vesicle formation. We cannot rule out the possibility that the active binding site of the channel was otherwise restricted or that an intermediate step may be required. Bovine brain α_{39}–GTP[γ-S] and erythrocyte α_{40}–GTP[γ-S] activated $I_{K.ACh}$ at 10 pM in our experiments. Codina *et al.* (3) reported consistent activation at <1 pM that may be due to a different method of application of α–GTP[γ-S] or larger patch sizes containing more channel sites. Without a detailed dose–response relation, we cannot compare the potency of the two α preparations. Further studies using resolved isoforms of α subunits are needed to establish the relative potencies of α subunits in activating $I_{K.ACh}$.

It is clear that α_{39}–GTP[γ-S], α_{40}–GTP[γ-S], and $\beta\gamma$ activate the same channel in chicken and mammal. One might be tempted to speculate that since Codina *et al.* (3) report stimulation by α_{40} at 1 pM whereas $\beta\gamma$ activated $I_{K.ACh}$ at 200 pM, α_{40} is more likely the mechanism *in vivo* (17, 18). However, we have found that α_{39} activates $I_{K.ACh}$ at $\approx$10 pM concentration and $\beta\gamma$ at a somewhat higher concentration (>200 pM). Since $\beta\gamma$ is hydrophobic, it is likely that $\beta\gamma$ must first be incorporated into the membrane before it can diffuse within the membrane to its site of action. By comparison, α is hydrophilic and may simply bind the channel (or an intermediate) from the cytoplasmic aqueous phase. The uncertainties of $\beta\gamma$ incorporation and binding make it difficult to use concentration differences as a test of specificity. The failure of Codina *et al.* (3) to activate $I_{K.ACh}$ with their $\beta\gamma$ may have been due to the presence of Lubrol (which alone blocks $I_{K.ACh}$ at levels >10 μM).

$\beta\gamma$ activation of $I_{K.ACh}$ is noteworthy since it has been assumed by many that the model of activation of adenylyl cyclase by the stimulatory α subunit and activation of cGMP phosphodiesterase by the transducin α subunit should hold for all other effector systems. There is now evidence for a role of $\beta\gamma$ in activation of $I_{K.ACh}$ (2) phospholipase A_2 (19) and cyclase inhibition (for review, see refs. 20 and 21). Activation of $I_{K.ACh}$ by both α–GTP and $\beta\gamma$ may be physiologically relevant. Release of two signal transduction elements increases the regulatory possibilities. For example, desensitization of the muscarinic-induced current decays in two exponential phases (22, 23), suggesting two separate processes controlling the net current. This could reflect two binding sites, one for α–GTP and one for $\beta\gamma$. Comparison of $\beta\gamma$- or α–GTP-stimulated single-channel kinetics, particularly closed times, would be useful. Alternatively, α–GTP or $\beta\gamma$ may act through an unknown membrane-bound intermediate to provide another mechanism for modulation. Finally, $\beta\gamma$ may act indirectly by removal of an α–GDP that tonically inhibits $I_{K.ACh}$ (2).

Note Added in Proof. L. Birnbaumer has informed us that since α_{40} (α_K) denatures easily and may stick to plastic, the actual concentrations of α_{40} may be different from those we originally provided.

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1. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
2. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. (1987) *Nature (London)* **325,** 321–326.
3. Codina, J., Yatani, A., Grenet, D., Brown, A. M. & Birnbaumer, L. (1987) *Science* **236,** 442–445.
4. Pfaffinger, P., Martin, J., Hunter, D., Nathanson, N. & Hille, B. (1985) *Nature (London)* **317,** 536–538.
5. Breitwieser, G. & Szabo, G. (1985) *Nature (London)* **317,** 538–540.
6. Kurachi, Y., Nakajima, T. & Sugimoto, T. (1986) *Pflügers Arch.* **407,** 264–274.
7. Logothetis, D. E. (1987) Dissertation (Harvard Univ., Cambridge, MA).
8. Neer, E. J., Lok, J. M. & Wolf, L. (1984) *J. Biol. Chem.* **259,** 14222–14229.
9. Evans, T., Fawzi, A., Frazer, E. D., Brown, M. L. & Northup, J. K. (1987) *J. Biol. Chem.* **262,** 176–181.
10. Fung, B. K.-K., Hurley, J. B. & Stryer, L. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 152–156.
11. Winslow, J. W., Van Amsterdam, J. & Neer, E. J. (1986) *J. Biol. Chem.* **261,** 7571–7579.
12. Ross, E. & Gilman, A. (1977) *J. Biol. Chem.* **252,** 6966–6969.
13. Higashijima, T., Ferguson, K., Smigel, M. & Gilman, A. G. (1987) *J. Biol. Chem.* **262,** 757–761.
14. Northup, J. K., Sternweis, P. C. & Gilman, A. G. (1983) *J. Biol. Chem.* **258,** 11361–11368.
15. Neer, E. J., Wolf, L. G. & Gill, D. M. (1987) *Biochem. J.* **261,** 325–336.
16. Huff, R., Axton, J. M. & Neer, E. J. (1985) *J. Biol. Chem.* **260,** 10864–10871.
17. Birnbaumer, L. & Brown, A. M. (1987) *Nature (London)* **327,** 21.
18. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J. & Clapham, D. E. (1987) *Nature (London)* **327,** 22.
19. Jelsema, C. & Axelrod, J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3623–3627.
20. Stryer, L. & Bourne, H. R. (1986) *Annu. Rev. Cell Biol.* **2,** 391–419.
21. Gilman, A. (1987) *Annu. Rev. Biochem.* **56,** 615–649.
22. Carmeliet, E. & Mubagwa, K. (1986) *J. Physiol. (London)* **371,** 239–255.
23. Kurachi, Y., Nakajima, T. & Sugimoto, T. (1987) *Pflügers Arch.* **410,** 227–233.
24. Yatani, A., Codina, J., Brown, A. M. & Birnbaumer, L. (1987) *Science* **235,** 207–210.