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Direct Control of a Large Conductance K^+-selective Channel by G-Proteins in Adrenal Chromaffin Granule Membranes

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Abstract. We report here the presence of a Ca^{2+}-independent K^+-channel of large conductance in adrenal chromaffin cell secretory vesicle membranes which is controlled by inhibitory as well as stimulatory heterotrimeric GTP-binding proteins. Using antibodies against specific α subunits for immunoblot analysis, we were able to identify the presence of the inhibitory G_{i2} and G_{i3} subtypes, as well as the stimulatory G_o and G_s subtypes, but not G_{i1} in adrenal chromaffin granules. Furthermore, functional analysis of the K^+-channel incorporated into planar lipid bilayers showed that GDPβS and GTPγS have opposite effects on channel activity inducing interconversions between a low and a high open-probability state. Consistent with these findings, the same antibodies antagonized the effects of the nonhydrolyzable analogues on the open probability of the K^+-channel.

Key words: K^+-channel — G-protein — Secretory vesicles — Antibodies — GTPγS — GDPβS

Introduction

By allowing intact secretory granules to fuse with phospholipid bilayer membranes, we have previously detected the presence of a Ca^{2+}-independent K^+-selective channel of large conductance (150–300 pS) in the vesicle membranes from adrenal chromaffin cells (Arispe, Pollard & Rojas, 1992). Since in the majority of the K^+-channel incorporations the open probability (p_o) was found to be rather low, we concluded that in our chromaffin granule preparation the K^+-channel was under tonic inhibition by an undetermined mechanism.

G-proteins are responsible for the coupling of cell membrane receptors to membrane resident ion channels or enzymes (Cerione et al., 1986; Yatani et al., 1988; Brown & Birnbaumer, 1990; Spiegel, Shenker & Weinstein, 1992; Brown, 1993), and there is now growing evidence that the distribution of G-protein is not limited to plasma membranes (Carlson et al., 1986; Barr et al., 1991; Donaldson et al., 1991; Carrasco, Sierralta & De Mazancourt, 1994; De Mazancourt, Goldsmith & Weinstein, 1994). In addition, G_o immunoreactivity has been detected in granule membranes from adrenal chromaffin cells (Toutant et al., 1987). Therefore, in the present work we asked whether the receptor-channel mechanism, present in the plasma membrane, might also be operative in the chromaffin granule membrane.

We found and report here that different antibodies, each one raised against a specific α subunit of a trimeric G-protein, identified the presence of G_o, G_s, G_{i2} and G_{i3} but not in G_{i1} in our highly purified preparation of adrenal chromaffin granules (McKenzie et al., 1988; Simonds et al., 1989*b*; Spiegel et al., 1990*a*; Spiegel, 1991; Spiegel, Shenker & Weinstein, 1992). Furthermore, functional analysis of the K^+-channel incorporated into a planar-lipid bilayer membrane showed that the nonhydrolyzable analogues guanosine 5′-O-(2-thiodiphosphate) (GDPβS) and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) had opposite effects on the open probability of the channel. Finally, we found that the same antibodies used in the immunoblot analysis could either activate or inhibit the channel in much the same way as GDP and GTP analogues. We conclude that the activity of the chromaffin granule large conductance K^+-channel is kept under control by direct action of both inhibitory and stimulatory G-proteins on the channel.

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Materials and Methods

Biochemical Procedures

The methods which were used here have been described elsewhere (Arispe et al., 1992). In brief, chromaffin granules were prepared from bovine adrenal medulla tissue by homogenization in 0.3 M sucrose and purified in a metrizamide step gradient, between densities 1.120 and 1.104 g/cm^3 as previously described (Pollard et al., 1979*a,b;* Brocklehurst & Pollard, 1989). The chromaffin granules were found to be substantially purified from contaminating mitochondria, plasma membranes, and lysosomes.

The C-terminal decapeptides of α_o, α_{i3}, α_s, yeast $GP1_\alpha$ (the product of GPA1), the internal decapeptides of α_{i1}, α_{i2} were synthesized (Doherty et al., 1991), conjugated to keyhole limpet hemocyanin with glutaraldehyde, and injected into rabbits. Some antisera were affinity purified on Affi-Gel 15 columns (Bio-Rad) containing the corresponding immobilized peptide (McKenzie et al., 1988; Simonds et al., 1989*a,b;* Spiegel et al., 1990*a,b;* Spiegel, 1991; Spiegel et al., 1992). The protein concentration was determined by the Bradford method.

For immunoblot, P2 membranes and chromaffin granules (150–300 μg protein) were diluted 1:1 in 2× Laemmli buffer and resolved using a SDS-polyacrylamide gel (10% acrylamide, 0.13% bisacrylamide), transferred to PDVF membranes (Millipore) and immunoblotted (20 hr at room temperature) with anti sera. The antibody-antigen complex was detected by ^{125}I-labeled protein A (Amersham).

Bilayer Membranes and Channel Recording

The experimental chamber (made of plexiglass) consisted of two compartments separated by a thin teflon film. Antibodies and reagents were added directly to either the *cis* or *trans* side. After each addition, solutions on both sides were simultaneously mixed by two teflon-coated magnetic stirrers placed in a restricted space at the bottom of each compartment. Ag/AgCl pellet electrodes were immersed in a small pool containing 0.5 M KCl and were electrically connected to the solutions in each compartment via agar bridges (2% agar in 0.5 M KCl). Single-channel currents were recorded using a patch clamp amplifier (Axopatch-1D, equipped with a CV-4B bilayer headstage, Axon Instruments) and were stored on magnetic tape using a PCM/VCR digital system (Digital-4, Toshiba) with a frequency response ranging from DC to 25,000 Hz. Records were made from playbacks through a low-pass filter (8-pole Bessel 902 LPF, Frequency devices) set in the range from 200 to 500 Hz (Arispe et al., 1992).

To fuse intact granules with a lipid bilayer we prepared a suspension of the intact vesicles in a KHepes solution (in mM: 200 KHepes, pH 7.4). Ion channels present in secretory granules from adrenal chromaffin cells were incorporated into a bilayer by adding a small volume (5–10 μl) of this suspension of intact vesicles to a different KHepes solution (either 200 or 400 mM). To facilitate membrane fusion, $CaCl_2$ was added ($[Ca^{2+}] \leq 200$ μM measured with Ca^{2+}-sensitive electrode). Granules were added to the solution in the *cis* side of the chamber, and incorporation occurred directly from the experimental solutions.

Presentation and Statistical Analysis

Every experimental paradigm included in this paper was repeated at least three times. The channel activity records shown in all the figures are representative examples of the phenomena studied. Open probability P_o values were estimated from digitized data (TL-1, 125 kHz module and DMA interface, Axon Instruments, Foster City, CA) using the pClamp 5.5 software. The data base used to construct the histograms ranged from 10^3 to 3×10^3 well-defined events for each condition.

Results

The Large Conductance K^+-channel Present in Chromaffin Granule Membranes Exhibits Two Functional States

The large conductance K^+-channel incorporated into acidic phospholipid planar lipid bilayers exhibits two distinct patterns of activity. As described previously (Arispe, Pollard & Rojas, 1992), in a large proportion of the experiments (162 out of 277 incorporations), K^+-channel openings were characterized by **l**ow **o**pen-**p**robability **(lop)** values at −10 mV ($P < 0.3$). Fig. 1*A* depicts the K^+-channel activity in its **lop** modality. Channel openings occurred in brief bursts (upward deflections of the trace) and the single channel conductance γ_{lop} was 190 ± 6 pS in symmetrical 200 mM KHepes. Long lasting inter-burst intervals (closed conformation of the channel) were often observed. The other type of channel activity (detected in 115 out of 277 incorporations), was characterized by **h**igh **o**pen-**p**robability **(hop)** values ($P_o > 0.7$) at −10 mV and a single channel conductance γ_{hop} of 195 ± 7 pS (symmetrical 200 mM KHepes). As illustrated in Fig. 1*B*, the channel in the **hop** modality remained in the open conformation during long lasting periods with frequent and brief transitions to the closed state (downward deflections of the trace at −10 mV; $P_o = 0.78$). Records with two levels of conductance (Fig. 1*C*) occasionally showed both the **hop** pattern (first level; $\gamma_{hop} = 202 \pm 4$ pS) and the **lop** pattern (second level; $\gamma_{lop} = 198 \pm 8$ pS).

The set of records in Fig. 1 clearly shows that spontaneous transitions between the two patterns of channel activity, and in particular from **lop** to **hop,** did not occur. For example, open probability P_o values remained almost unchanged at *ca.* 0.041 along the four consecutive 10-min intervals of channel activity in the **lop** modality. Similarly, P_o values along the **hop** pattern of channel activity also remained fairly constant for at least 20 min (Fig. 1*B,C*). Thus, if spontaneous conversions from **lop** to **hop** are allowed to occur in our reconstituted system, they were not observed in the time frame of the experiments reported here.

K^+-channel openings in the **lop** modality occurred in bursts (Fig. 2*A:* upward deflections of the trace at −20 and −10 mV; downwards deflections at 10 and 20 mV). Long lasting inter-burst intervals (closed conformation of the channel) were often observed (Fig. 2*A*, record at

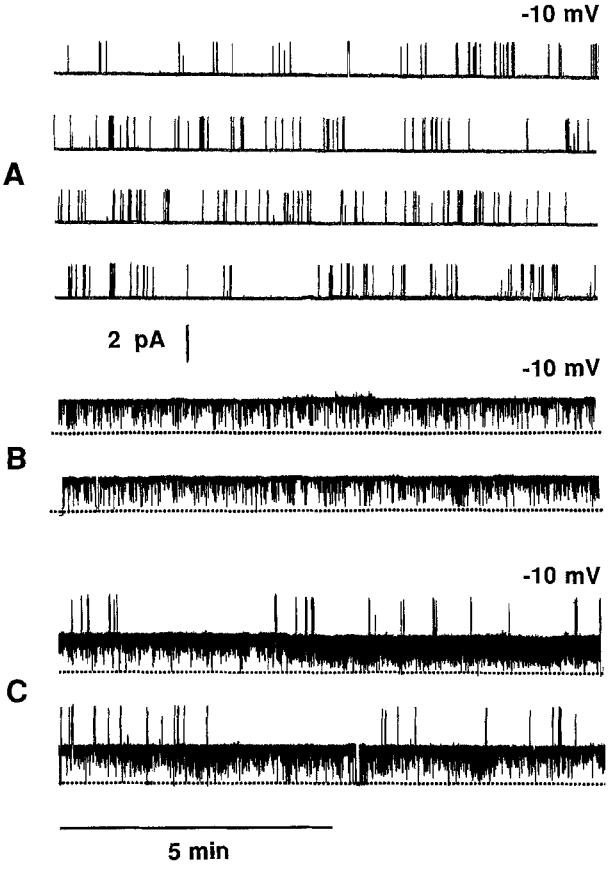

Fig. 1. The chromaffin granule membrane K^+-channel exhibits two modes of activity. Symmetrical KHepes (200 mM) throughout. (*A*) Channel in the low open probability mode. Single-channel conductance at −10 mV estimated from the amplitude histogram was 190 ± 6 pS; P_o = 0.041 ± 0.008 (*B*) Channel in the high open probability mode. Conductance at −10 mV was 195 ± 7 pS; P_o = 0.78 (*C*) Conductances were estimated as 202 ± 4 pS and 198 ± 8 pS for first and second level, respectively.

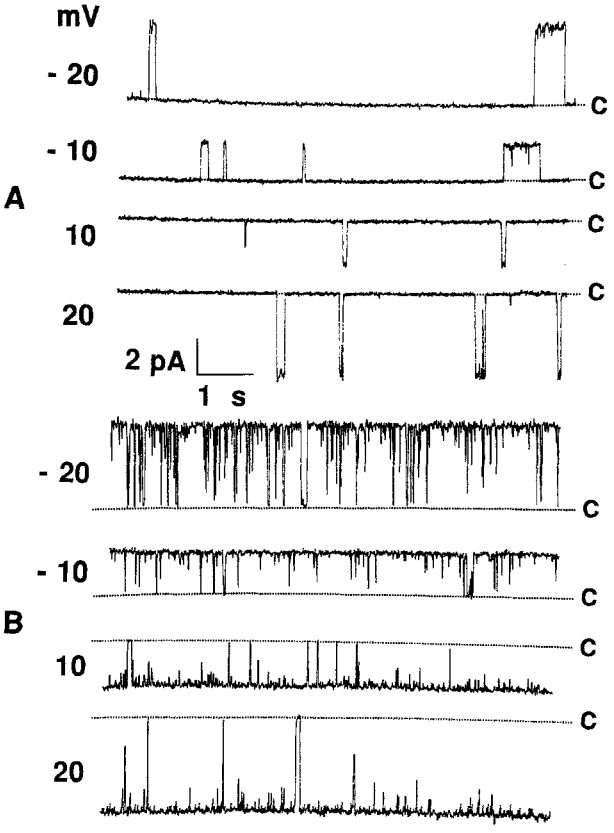

Fig. 2. The chromaffin granule membrane K^+-channel in the **hop** modality exhibits voltage-dependent gating. Symmetrical KHepes (200 mM) throughout. Transmembrane potential is given in mV on the left side of each record, and closed state of the K^+-channel is indicated by the dotted line in each trace. (*A*) Pattern of channel activity defined as **lop.** At −20 mV, K^+-channel openings correspond to the upward deflections of the trace (*B*) Pattern of channel activity defined as **hop.** Transitions to the closed state are less frequent at more positive transmembrane potentials.

−20 mV). In contrast, during the **h**igh **o**pen-**p**robability **(hop)** modality (P_o > 0.7), the channel remained in the open conformation for long periods, with frequent, rapid transitions to the closed state (Fig. 2*B;* brief downward deflections of the trace at −20 and −10 mV; brief upwards deflections at 10 and 20 mV). As anticipated from these data, the current-voltage relationships (*I-V* curves) calculated from both **lop** and **hop** modalities of K^+-channel activity were found to be linear, the single channel conductance γ being equal to *ca.* 195 pS (symmetrical 200 mM kHepes) for both patterns of activity.

In all the experiments in which the channel events occurred in the **lop** modality (P_o < 0.3), the frequency of the K^+-channel openings seemed insensitive to the transmembrane potential (Fig. 2*A*). By contrast, in the **hop** modality (P_o > 0.7), the frequency of closures depended on the transmembrane potential. Indeed, comparing the frequency of closures at −20 and 20 mV (Fig. 2*B*) revealed that P_o increased as the transmembrane potential was made more positive.

Effects of NaF and Nonhydrolyzable Guanosine Nucleotide Analogue on K^+-Channel Activity

G_o immunoreactivity has been detected previously in chromaffin granules (Toutant et al., 1987). Since no specific function of this protein was described, we examined the possibility of G_o involvement in K^+-channel regulation. We resorted to the classical protocol of testing the effects of NaF, GTPγS and GDPβS on chromaffin granule K^+-channel activity. Figure 3 depicts typical effects of 20 mM NaF, 50 μM GTPγS and 500 μM GDPβS. Prior to the application of either NaF (Fig. 3*A*, upper record) or GTPγS (Fig. 3*B*, upper record) to both sides, the channels selected exhibited the **hop** pattern of activity. NaF (20

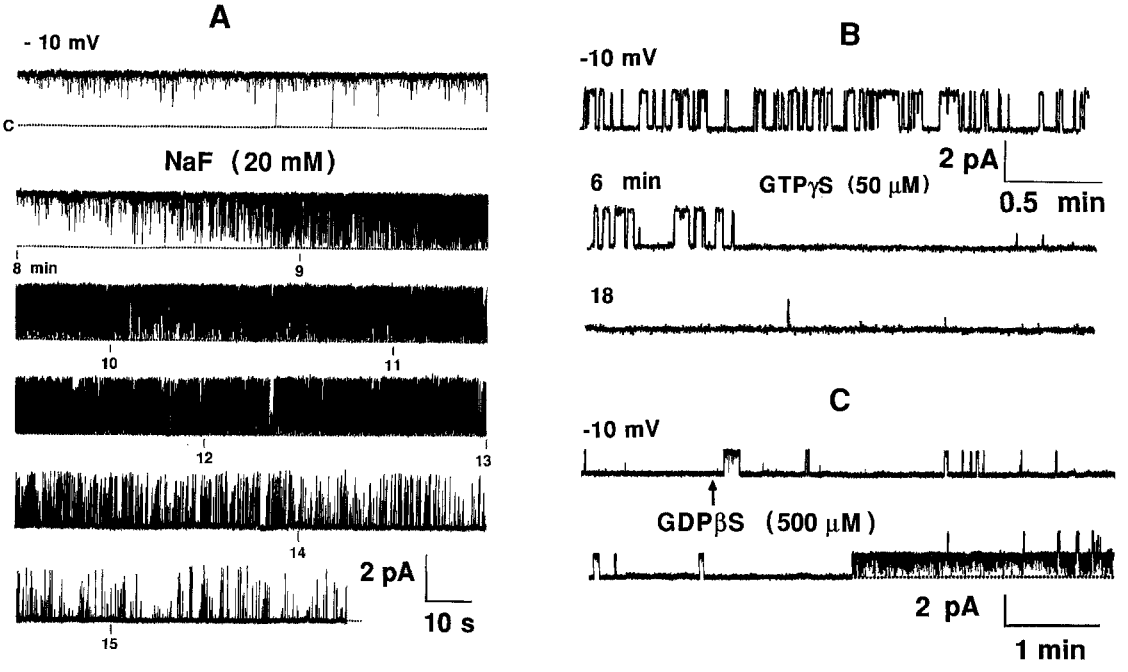

Fig. 3. NaF and GTPγS convert the K^+-channel from **hop** to **lop** mode and GDPβS from **lop** to **hop.** Symmetrical K-Hepes throughout (200 mM; ca. 80 μM free-calcium). (*A*) Time course of the blockade by 20 mM NaF. Upper record: Prior to the application of NaF. (*B*) Prior to the addition of GTPγS channel activity was in the **hop** mode (top record). Lower record was made 6 min after the addition of GTPγS (50 μM) to the *trans* side. (*C*) The control record was made with the channel in the **lop** mode (initial portion of the top trace). GDPβS (500 μM) was added to the *trans* side as indicated by the arrow.

mM) evoked a dramatic reduction in P_o from 0.98 (top record) to 0.08 (bottom record) in *ca.* 16 min. Similar application of NaF (or GDPβS) only to the *cis* side was without effect even after 11 min. As expected further the addition of NaF (20 mM) to the *trans* side induced a transition in the pattern of K^+-channel activity from **hop** to **lop** in less than 5 min. Finally, application of 20 mM NaF to channels in the **lop** modality were without measurable effects on P_o (n = 5; *not shown*). In contrast, a low dose of GTPγS applied to the *trans* side (K^+-channel activity in the **hop** modality) was sufficient to evoke a profound decrease in P_o from 0.68 to less than 0.01 in *ca.* 8 min. Application of higher doses of GTPγS (250 μM) to both sides of the bilayer reduced the time required to observe a discernable decrease in channel activity from 8 min at 50 μM (Fig. 3*B*) to 0.1 min at 250 μM (*not shown*). Similar results were obtained in five additional experiments with GTPγS using different preparations of chromaffin granules. As anticipated, application of the nonhydrolyzable GDP analogue GDPβS (500 μM) to the *trans* side activated a K^+-selective channel which at the start of the experiment depicted in Fig. 3*C* was found to be in the **lop** modality. Thus, GDPβS switched the pattern of activity from the **lop** to the **hop** modality. In contrast, GDPβS (500 μM) added to the *cis* side was without effect even at the end of a 8-min period of exposure to GDPβS (*not shown*). A substantial fraction of the observed increase in P_o evoked by GDPβS could result from the activation of several K^+-channels. However, statistical analysis of records which exhibited only one or two K^+-channels (Fig. 3*C*) revealed that application of GDPβS increases the mean-open time of the K^+-channel. Although totally unexpected, in a few instances (3 out of 8 experiments) we also observed that GTPγS (50 μM) can activate the K^+-channel from the *trans* side, inducing a **lop** to **hop** transition (*not shown*).

One distinctive property of the **hop** mode of K^+-channel activity is the voltage sensitivity of the open probability P_o. Figure 4 shows that GDPβS can induce a switch in the mode of K^+-channel activity from **lop** (Fig. 4; left side, upper record) to **hop** (Fig. 4; left side, records at −40 through 30 mV). Furthermore, while P_o during the control period remained constant (*ca.* 0.1) at different transmembrane potentials, after activation by GDPβS the K^+-channel acquired voltage sensitivity (Fig. 4; right side, lower graph). Current-voltage relationships from measurements obtained prior to (□) and after the application of GDPβS (■) are linear with similar slopes of *ca.* 185 pS (Fig. 4, upper panel). From these experiments with GTP and GDP nonhydrolyzable analogues we concluded that both GTPγS and GDPβS acted on sites accessible from the *trans* side. Since the GTPγS molecules can switch the K^+-channel activity from the **hop** (P_o >

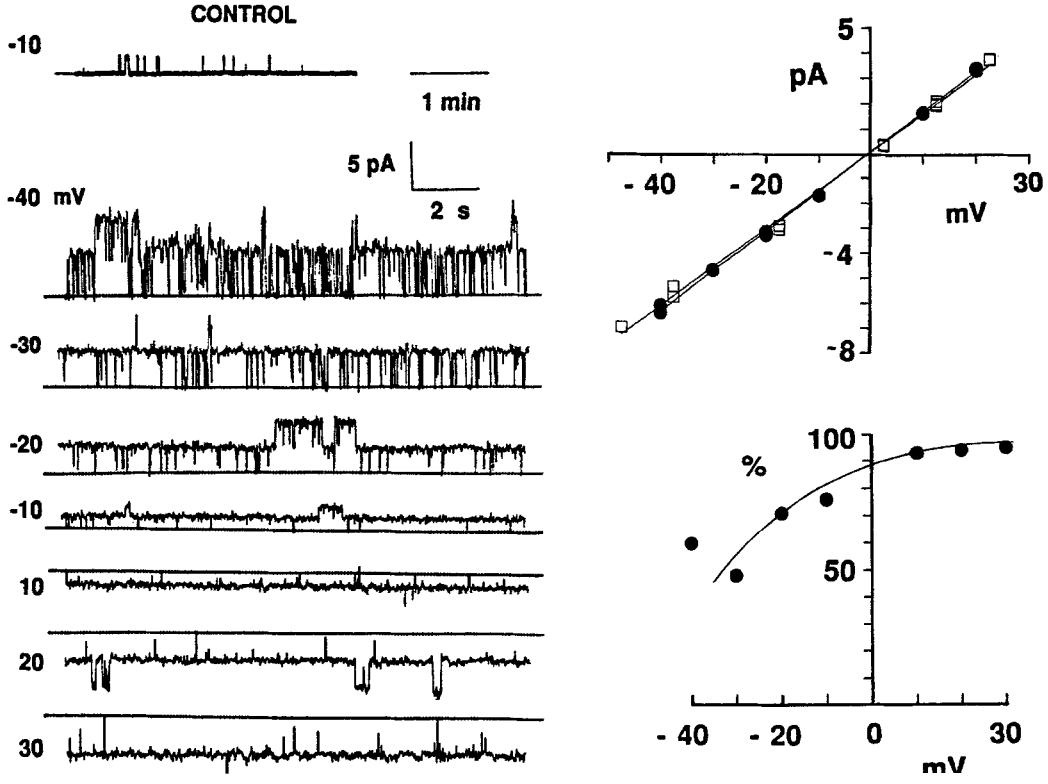

Fig. 4. Current-voltage relationship and fractional-open time after activation of the K^+-channel by ADPβS. Left side: Representative segment of a continuous record of the K^+-channel activity prior to the application of GDPβS (control). Records below were made at different transmembrane potentials (indicated in mV next to each record) after the activation by GDPβS. Right side: Upper panel represents the *I-V* curve, prior to the application of GDPβS (□) and after the activation by GDPβS (■). Lower panel: Fractional open-time as a function of membrane potential after the activation by GDPβS (●).

0.7) to the **lop** ($P_o < 0.3$) mode, and GDPβS can induce a change in the opposite direction, we further hypothesized that the K^+-channel may be under direct control by more than one G-protein.

To determine the molecular components accounting for the observed inhibitory and stimulatory effects evoked by the nonhydrolyzable GTP and GDP analogues, we screened our highly purified preparation of adrenal chromaffin granules for different types of α subunits of trimeric G-proteins using highly selective antibodies against specific subunits.

Identification of G_o, G_{i2}, G_{i3} and G_s but not G_{i1} in Adrenal Chromaffin Granules

Figure 5 depicts seven representative autoradiograms of proteins from highly purified granule membranes (CG, lanes a) and P2 crude membrane fractions (P2MF, lanes b). The antibody AS7, against α_t α_{i1} and α_{i2} (with weak α_{i3} cross reactivity), was used in autoradiogram labeled AS7. Only one thick band (39–41 kDa) is apparent in lane a corresponding to CG and two bands in lane b corresponding to P2MF. The upper band in lane b is a 67 kDa protein only present in P2MF, a result that confirms the purity of the CG fraction tested. The α_{i2} protein is in excess compared to α_{i1} in lane b and, for this reason, the α_{i1} band is absent from lane b of autoradiogram AS7. In another experiment, the lower band labeled α_{i2} in lane a and the lower band in lane b comigrated with the band in brain cholate extract identified as α_{i2} (not shown). Furthermore, both CG and P2MF reacted with another antibody (given name LE) raised against an internal domain of α_{i2} (not shown). Taken together, these data indicate that α_{i2} is expressed in roughly similar amounts in both CG and P2MF.

The antibody LD (autoradiogram labeled LD) raised against an internal domain of the protein α_{i1} revealed a faint band only in the P2MF lane, suggesting that α_{i1} is not present in CG. Autoradiograms labeled GO and GC2 are antibodies raised against the C- and N-terminal domains of α_o, respectively. Both antibodies gave consistent labeling, i.e., strong in CG (lane a) and faint in P2MF (lane b). These results indicate a high content of α_o in CG. Also shown in autoradiogram EC is the labeling with EC, an antibody raised against the C-terminal

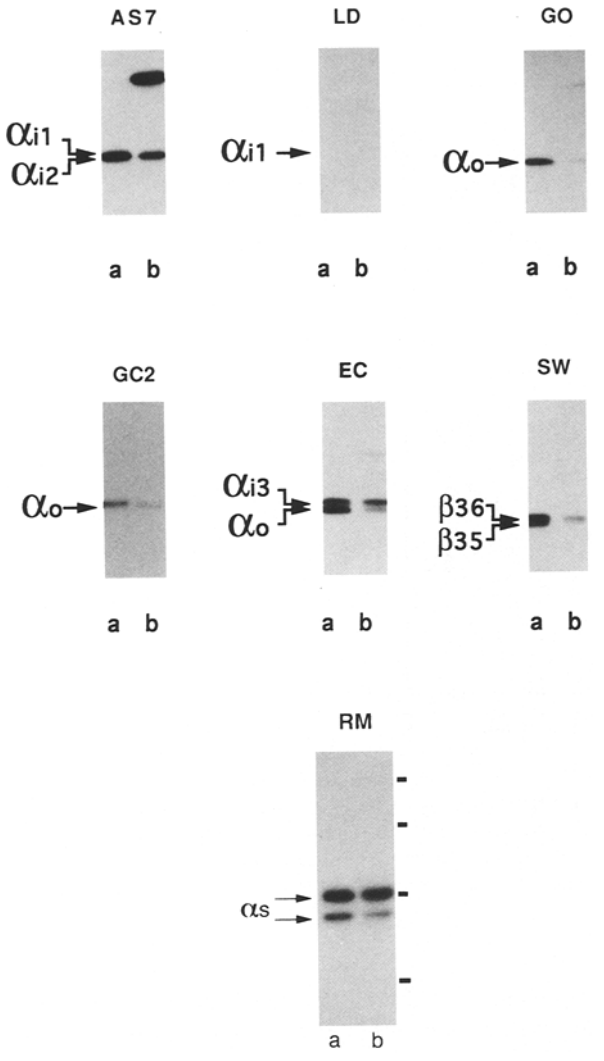

Fig. 5. Immunoblot identification of G_o, G_{i2}, G_{i3} and G_s but not G_{i1} in a highly purified preparation of adrenal chromaffin granules. Each autoradiogram can be identified by the letter code given on the top. Lane a corresponds to the highly purified adrenal chromaffin granule preparation. Lane b corresponds to a P2 crude membrane fraction prepared from bovine adrenal chromaffin cells.

domain of α_{i3} which cross reacts with α_o. As shown on autoradiogram EC, there is a clear doublet at 39–41 kDa in each lane, the lower band being presumably α_o. Consistently with the data displayed on the other autoradiograms GO and GC2, the lower band corresponding to α_o was faint in the P2MF (lane b) (McKenzie et al., 1988; Spiegel et al., 1990; Spiegel, 1991; Spiegel et al., 1992).

Autoradiogram SW shows the labeling with an antibody raised against the C-terminal undecapeptide common to $\beta_{1\text{-}4}$. It is apparent that although SW detected a doublet in both fractions, the labeling in lane a is stronger. This result demonstrates that the β subunit and, probably the γ subunit responsible for membrane attachment of the G-protein (Simonds et al., 1991), are also present in adrenal chromaffin granules. Finally, autoradiogram RM depicts the labeling obtained with antibody against α_s. Autoradiogram RM shows clear labeling of a doublet in the P2MF lane b, corresponding to the short and the long α_s isoforms. The antibody RM also indicated the presence of two α_s isoforms (doublet in lane a) in adrenal chromaffin granules.

In summary, α_o and α_s are by far the predominant G-protein subunits in adrenal chromaffin granules. Of the other subunit types we detected the presence of equivalent amounts of α_{i2} and α_{i3} in both CG and P2MF.

Effects of Antibodies Against Specific α Subunits of Trimeric G-Proteins on K^+-Channel Activity

The same specific antibodies used in the immunoblot analysis were examined for identification of the G protein(s) regulating the large conductance K^+-channel activity. In perfect agreement with the results from the immunoblot analysis, we also found that each one of the antibodies that gave positive labeling had a profound effect on the activity of the K^+-channel. The records shown in Fig. 6 were made during experiments in which the K^+-channel exhibited a $P_o < 0.3$ (top records in *A-C*). As illustrated in Fig. 6*A* (second record from the top), EC at a concentration of 3 μg/cm^3 evoked a dramatic conversion of the pattern of K^+-channel activity from **lop** to **hop** (P_o *ca.* 0.9). EC is not specific for $G\alpha_{i3}$ and cross reacts with $G\alpha_o$ (*see* Fig. 5). However, it is safe to assume that prior to the application of EC the K^+-channel was under tonic inhibition by activated $G\alpha_{i3}$ molecules and the antibody EC removed this inhibition by complex formation with the α_{i3} subunits.

Similar effects were also observed with more specific antibodies raised against other G-proteins. This was the case for AS7 (Fig. 6*B;* 5.4 μg/cm^3) which exhibits a reasonable specificity for $G\alpha_{i2}$ although it cross reacts with $G\alpha_{i3}$. Like the antibody EC, AS7 also switched the K^+ activity from **lop** to **hop.** Taken together, these data suggest that the large conductance K^+-channel is under inhibitory control by G_i-proteins. However, GO raised against α_o, a classical activator of K^+-channels in other cell types (Madison & Nicoll, 1986), also induced the transition from the **lop** to the **hop** activity of the K^+-channel. As shown in Fig. 6*C*, GO (8.3 μg/cm^3) added to the *trans* side activated the channel and, as expected, a further addition of GTPγS on the *trans* side was unable to inhibit the activity (*not shown*). Immunoblots using GO exhibited two clear bands corresponding to α_o and α_{i3}, so cross reaction of GO with α_{i3} provides an alternative explanation to the

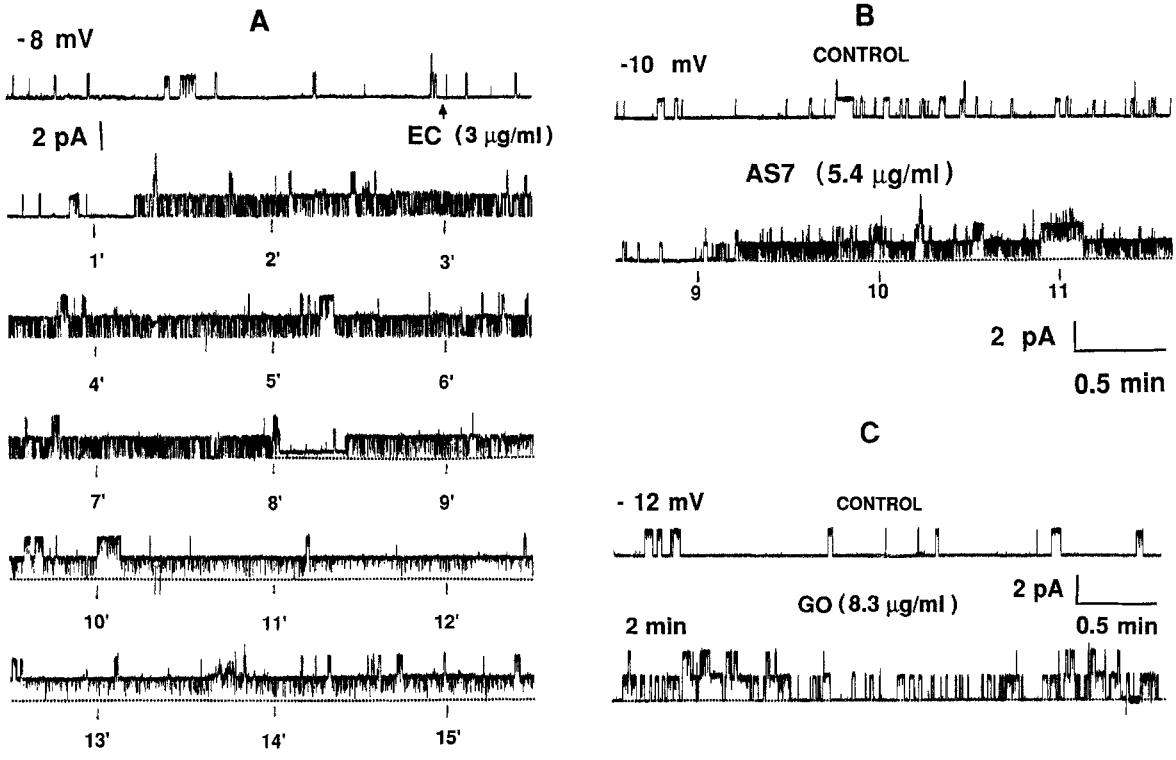

Fig. 6. The antibodies EC to α_{i3}, AS7 to α_{i2} and GO to α_o convert the activity from **lop** to **hop** mode. (*A*) Symmetrical KHepes and −8 mV throughout. EC was added to the *trans* side as indicated by arrow. The numbers underneath the records indicate the time in min. (*B*) AS7 was added to the *trans* side and the second record from the top was made ca. 8 min after its addition. (*C*) GO was applied from the *trans* side. Activation was observed 1–2 min after the addition of GO. After 4 min, GTPγS (40 μM) was also applied without noticeable effects within 10 min.

result. In this case, complex formation of the α_{i3} subunit by GO prevented either the inhibitory interaction between the GTPγS-activated α_{i3} subunit and the K^+-channel or prevented the activation of α_{i3} subunit by GTPγS or both. Consistent with this alternative mechanism we found that GO (8.3 μg/cm^3) applied to the *trans* side can revert the inhibition of the K^+-channel activity induced by NaF (*not shown*). The unavoidable conclusion is that fluoride set the K^+-channel under inhibitory control by a G-protein and, the antibody GO, by binding to the fluoride activated G-protein subunit, released the blockade.

The immunoblot analysis using the RM antibody against α_s also revealed the presence of this subunit in our preparation of adrenal chromaffin granules. The G_s system is a classical example of a plasma membrane trimeric G-protein that couples receptor activation with specific ion channels in different cell types (Scamps et al., 1992). We found that RM is a potent blocker of the K^+-channel activity and is only effective from the *trans* side (Fig. 7). From the functional analysis of the effects of specific antibodies used here we conclude that both inhibitory as well as stimulatory G-proteins are acting on the K^+-channel.

Discussion

We report here the existence of a large conductance K^+-channel in adrenal chromaffin granule membranes that is directly controlled by inhibitory as well as stimulatory G-protein coupled mechanisms. This conclusion is based on three lines of evidence. First, functional analysis of the K^+-channel incorporated into planar lipid bilayer membranes showed two preferred patterns of activity which define the **lop** and **hop** states of the channel. We found that nonhydrolyzable analogues GDPβS and GTPγS have opposite effects on K^+-channel activity. While GDPβS, thought to compete with endogenous GDP for a binding site on the α subunit, stimulated the K^+-channel in the **lop** mode of activity, GTPγS, mimicking the activation of the α subunit by endogenous GTP, inhibited the K^+-channel in the **hop** mode of activity. Second, NaF blocked the large conductance K^+-channel in much the same way as GTPγS. Third, using antibodies against C-terminal decapeptides or internal decapeptides of the α subunit of specific trimeric G-proteins (McKenzie et al., 1988; Simonds et al., 1989*a,b;* Spiegel et al., 1990; Spiegel, 1991; Spiegel et al., 1992), we were able to identify the presence of G_o, G_s, G_{i2}, and G_{i3} but

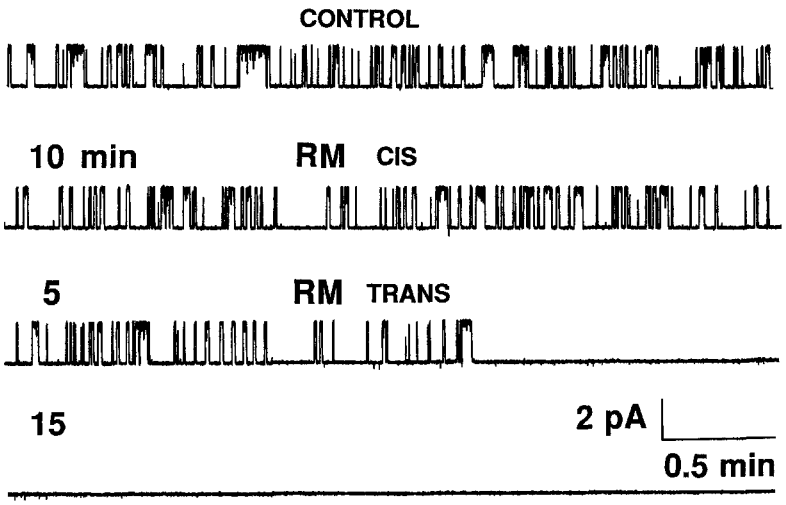

Fig. 7. The antibody RM to α_s converts the activity from **hop** to **lop** mode. Upper record was made prior to the addition of RM. Nearly 10 min after the addition of RM to the *cis* side no noticeable effects were detected. Nearly 5–6 min after the addition of RM to the *trans* side the K^+-channel was blocked (lower record).

not G_{i1} in a highly purified preparation of adrenal chromaffin granules. Since the application of the same specific antibodies used in the immunoblot analysis reverted the effects of the nonhydrolyzable analogues on the activity of K^+-channel, we concluded that the activity of the chromaffin granule large conductance K^+-channel is kept under tight control by a mechanism involving G-proteins.

Hop and Lop Modalities of K^+-Channel Activity Reflect Two Defined Functional States of the Channel

The K^+-channel studied here exhibits two distinct patterns of activity termed **hop** and **lop** modalities. The **lop** pattern of activity, found in a larger proportion of the incorporations of freshly prepared chromaffin granules, was characterized by infrequent bursts of channel openings with long lasting inter-burst intervals and voltage-insensitive P_o (Arispe et al., 1992), while the **hop** pattern was characterized by frequent, rapid transitions to the closed state. A channel trait differing between these two modes of activity was the open-probability P_o. While in the **lop** mode of activity P_o remained constant at *ca.* 0.3 in the transmembrane potential range from −50 to 50 mV, in the **hop** state P_o was substantially larger, varying from *ca.* 0.4 to 0.95 at −40 and 30 mV, respectively (Fig. 4; right side, lower panel). Similar results were always obtained with K^+-channels incorporated in the **hop** modality (115 K^+-channel incorporations). Since all other functional fingerprints, i.e., conductance and selectivity, remained unaltered, we conclude that these two modalities of K^+-channel activity indeed represent two preferred functional conformations of the channel. We now know that adrenergic agonists from the cytosolic side of the vesicles can induce a switch from the **lop** to the **hop** mode of channel activity (*unpublished data*).

Nonhydrolyzable GTP and GDP Analogues Modulate K^+-Channel Activity

Nonhydrolyzable analogues of GTP such as GTPγS have been used to activate GTP-binding proteins and GDP analogue GDPβS to lock GTP-binding proteins in the inactive conformation. We observed and report here that the nonhydrolyzable nucleotide GDPβS activated the channel in much the same way as the adrenergic agonists (*unpublished data*). Furthermore, while GTPγS switched the activity of K^+-channel from **hop** to **lop** mode, GDPβS induced the reverse transition from **lop** to **hop** state. These results provide support for the idea that the control of the CG-type K^+-channel by adrenergic agonists (*unpublished data*) involves GTP binding proteins. The underlying mechanism might include activation of G-proteins resident in the membrane of the secretory vesicles.

Identification of $G\alpha_o$, $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_{i3}$ but not $G\alpha_{i1}$ in our Highly Purified Preparation of Adrenal Chromaffin Granules

Previous works have identified the presence of G_o immunoreactivity in chromaffin cell secretory granule membranes (Toutant et al., 1987). Furthermore, in the hippocampus G_o couples β-adrenergic receptors with adenylate cyclase molecules and thereby regulates cyclic adenosine monophosphate (cAMP) production. This messenger is a crucial component of the mechanism of modulation of the $[Ca^{2+}]_i$ activated K^+-channel present in the plasma membrane of pyramidal cells (Madison & Nicoll, 1986). Although it is well established that antibodies against specific G-proteins disrupt receptor/G-protein coupling, there is growing evidence that G-proteins interact directly with ion channel. Indeed, a recent

report provides some evidence for direct interactions between $G\alpha_s$ and a Ca^{2+}-channel incorporated in a lipid bilayer (Hamilton et al., 1991). Using antibodies against specific C-terminal decapeptides of α_o, α_{i3}, α_s, the internal decapeptides of α_{i1}, α_{i2} and α_{i3} and the common C-terminal decapeptide of α_{i1} and α_{i2}, and the N-terminal domain of α_o (antibody GC2), our immunoblot analysis not only revealed the presence of α_o, but also the presence of α_s, α_{i2} and α_{i3} in the purified granules. Consistent with these data, β subunits were also found in our preparation of chromaffin granules. Thus, we report here for the first time the presence of three additional G-proteins subtypes in adrenal secretory granule membranes. Originally two of these G-proteins were found to couple adrenergic receptors to adenylate cyclase (stimulatory G_s and inhibitory G_{i3}) with either a Ca^{2+}-channel (G_s or a K^+-channel (α_{i2}) at the end of the chain of events, all components within the plasma membrane (Brown, 1993).

The immunoblot analysis used here also identified the dimer $\beta\gamma$ in our preparation of chromaffin granule membranes (Simonds et al., 1991). This structural component, common to many trimeric GTP-binding proteins, plays a crucial role in the functional cycle allowing the release of the activated α subunit after the exchange of GDP for GTP which occurs while the α subunit and the $\beta\gamma$ dimer are tightly associated. This finding lends strong support to the idea the trimeric form of GTP binding proteins also operate within the secretory vesicle membranes. Of the other trimeric G-proteins found in the secretory vesicles from chromaffin cells, α_o couples an adrenergic receptor to adenylate cyclase, providing a mechanism to regulate a *ca.* 55 pS K^+-channel (Madison & Nicoll, 1986); α_{i2} couples a M_2 subtype muscarinic receptor to a *ca.* 40 pS K^+-channel in the plasma membrane (Brown, 1993). Although we cannot completely rule out the possibility of contamination of our secretory granules with membranes from other intracellular organelles or even plasma membrane, we have compelling reasons to believe that these G-proteins labelled by our set of highly specific antibodies are part of the granule membrane (i) The method used to prepare the adrenal chromaffin granules (Pollard et al., 1979*a*; Pollard et al., 1979*b*) yielded highly purified intact vesicles. (ii) For the present experiments, we included additional purification steps to exclude possible contaminating membranes. (iii) The α_{i1} G-protein subunit commonly found in the plasma membrane was completely absent from all the autoradiograms examined with the specific antibody against its internal decapeptide (Spiegel et al., 1990). (iv) The large conductance K^+-channel activated by $[Ca^{2+}]_i$ and voltage, abundant in the plasma membrane of chromaffin cells (Fenwick, Marty & Neher, 1982; Glavinovic & Trifaro, 1988) was never coreconstituted into our bilayer membranes. These data serve to ascertain the purity of the preparation, and thus, we conclude that the set of G-proteins detected are most likely to be part of the granule membrane.

Role of the Channel in Vesicle Function

Regulation of the transport of vesicles from the endoplasmic reticulum to the Golgi apparatus involves heterotrimeric guanidine nucleotide binding proteins (Stow et al., 1991; Colombo et al., 1992; Aridor et al., 1993). Thus, it is possible that G-proteins remain attached to the chromaffin granule membrane after the vesicles are assembled by the Golgi system. On the other hand, modulation of the K^+-channel activity by adrenergic agonists (*unpublished data*) strongly suggests a mechanism whereby only the stimulatory G_s serves to couple adrenergic receptors facing the cytosolic side of the chromaffin granule membrane to the channel. The vectorial character of this model arose from the striking polarity found in terms of sites of action for both nonhydrolyzable GTP and GDP analogues as well as the antibodies. One last argument in support of the present model is the observation that adrenergic agonists activate the channel only from the *cis* side of the bilayer (*unpublished data*).

Functional Adrenergic Receptors are Present in the Membrane of Chromaffin Granules

Early studies of adenylate cyclase activity in chromaffin cell secretory vesicle membranes showed that the enzyme is controlled by β-adrenergic agents. Furthermore, granule adenylate cyclase is activated by fluoride and nonhydrolyzable GTP analogues such as GMP-PNP, indicating the functional existence of heterotrimeric G-proteins in this membrane system (Nikodejevic et al., 1976; Hoffman et al., 1976; Zinder et al., 1977; Winkler & Westhead, 1980). Although the specific receptors which might be present in the chromaffin granule remain to be elucidated, we do not rule out the possibility that, in addition to the adenylate cyclase described by Nikodejevic et al., (1976), the chain of events leading to the expression of the diffusion potential may also be initiated by the binding of epinephrine to β-adrenergic receptors on the cytosolic aspect of the granules. If this were the case, α_s or α_o subunits might couple the receptor to the channel (Spiegel et al., 1990; Brown, 1993).

Dual Stimulatory and Inhibitory Control of the K^+-channel by G-Proteins

As summarized in the Table, we have shown here that $GTP\gamma S$ can induce the transition from **hop** to **lop** or **lop** to **hop.** In addition, antibody GO raised against α_o

Table 1. Summary of the effects of NaF, nonhydrolyzable guanosine analogues (GTPγS and GDPβS), and antibodies on P_o

Treatment	Channel state	
	hop	lop
NaF (α_{all})	$P_o \downarrow$ (Fig. 3*A*)	none*
GTPγS	$P_o \downarrow$ (Fig. 3*B*)	$P_o \uparrow$*
GDPβS	none*	$P_o \uparrow$ (Fig. 3*C*)
EC (α_{i3}, α_o)	none*	$P_o \uparrow$ (Fig. 6*A*)
AS7 (α_{i2})	none*	$P_o \uparrow$ (Fig. 6*B*)
GO (α_o)	none*	$P_o \uparrow$ (Fig. 6*C*)
RM (α_s)	$P_o \downarrow$ (Fig. 7)	none*

* Tested; data not shown

(Morris et al., 1990) is able to revert the effects of F^- and neither NaF nor GTPγS can revert the stimulation of the K^+-channel by each of the antibodies tested, including GO. Furthermore, we have observed, although less frequently, that the antibody RM against α_s can also block the K^+-channel, suggesting that both stimulatory as well as inhibitory G-proteins can interact with the channel. Dual stimulatory and inhibitory regulation has been described before for a $[Ca^{2+}]_i$-activated K^+-channel present in the airway smooth muscle plasma membrane (Kume et al., 1992). However, unlike the situation found in smooth muscle, where the stimulatory control occurs via adenylate cyclase, in chromaffin granules regulation of the K^+-channel is mediated directly by both stimulatory and inhibitory α subunits. Thus, K^+-channel activation (or inactivation) by either GTPγS or NaF may indeed be due to activation of either $G\alpha_o$ or $G\alpha_{i2}$ and/or $G\alpha_{i3}$ subunits by GTPγS or NaF, respectively. Appropriately both events can be prevented if the α subunit is removed by complex formation with the corresponding antibody.

The requirements for a minimal model to explain the interaction of G-proteins and the chromaffin granule K^+-channel can be summarized as follows (*see* Table). GTPγS activates both α_s and α_i. Interaction of GTPγS-activated α_s or α_i with the K^+-channel will increase or decrease P_o, respectively. GDPβS will bind to both α_s and α_i. GDPβS binding will deactivate both types of subunits and release the K^+-channel from the corresponding effect. The highly specific antibodies EC, AS7, GO and RM used to identify the presence of specific Gα proteins (Fig. 5) were also tested in the functional analysis of the K^+-channel. In all instances we obtained results consistent with the inhibition of the specific Gα protein owing to complex formation with the antibody (Table).

In conclusion, the -Ca^{2+}-insensitive K^+-channel of large conductance present in adrenal chromaffin granule membranes is directly gated by inhibitory as well as stimulatory G-proteins. Although the physiological role of the K^+-channel remains to be elucidated, we do not rule out the possibility that this regulation is directed towards catecholamine trafficking across the granule membrane during the secretion cycle and membrane fusion at the site of exocytosis.

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