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Characterization of the G Protein Coupling of SRIF and β-adrenergic Receptors to the Maxi K_{Ca} Channel in Insulin-Secreting Cells

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Abstract. Modulation of the Ca- and voltage-dependent K channel—K_{Ca}—by receptors coupled to the G proteins G_i/G_o and G_s has been studied in insulin-secreting cells using the patch clamp technique. In excised outside-out patches somatostatin (somatotropin-releasing inhibitory factor; SRIF) caused concentration-dependent inhibition of the K_{Ca} channel, an effect that was prevented by pertussis toxin (PTX). In inside-out patches, exogenous α subunits of either G_i- or G_o-type G proteins also inhibited the K_{Ca} channel (IC_{50} 5.9 and 5.7 pM, respectively). These data indicate that SRIF suppresses K_{Ca} channel activity via a membrane-delimited pathway that involves the α subunits of PTX-sensitive G proteins G_i and/or G_o. In outside-out patches, activation of G_s either by β-agonists or with cholera toxin (CTX) increased K_{Ca} channel activity, consistent with a membrane-delimited stimulatory pathway linking the β-adrenergic receptor to the K_{Ca} channel via G_s. In outside-out patches, channel inhibition by SRIF suppressed the stimulatory effect of β-agonists but not that of CTX, while in inside-out patches CTX reversed channel inhibition induced by exogenous α_i or α_o. Taken together these data suggest that K_{Ca} channel activity is enhanced by activation of G_s and blocked by activated G_i and/or G_o. Further, K_{Ca} channel stimulation by activated G_s may be "direct," while inhibition by G_i/G_o may involve deactivation of G_s. In inside-out patches K_{Ca} channel activity was reduced by an activator of protein kinase C (PKC) and enhanced by inhibitors of PKC, indicating that PKC also acts to inhibit the K_{Ca} channel via a membrane delimited pathway. In outside-out patches, chelerythrine, a membrane permeant inhibitor of PKC prevented the inhibitory effect of SRIF, and in inside-out patches PKC inhibitors prevented the inhibitory effect of exogenous α_i or α_o. These data indicate that PKC facilitates the inhibitory effect of the PTX-sensitive G proteins which are activated by coupling to SRIF receptors. To account for these results a mechanism is proposed whereby PKC may be involved in G_i/G_o-induced deactivation of G_s.

Key words: K_{Ca} channel — G proteins — SRIF — β agonists — Protein kinase C

Introduction

The plasma membrane of insulin-secreting cells from the pancreatic islet as well as from tumor cell lines express large calcium-activated potassium channels (K_{Ca} channels). Early studies ascribed a major role to these channels in nutrient-induced electrical activity in the pancreatic β cell (Ribalet & Beigelman, 1981), but subsequent investigations challenged this hypothesis (Kukuljan, Goncalves & Atwater, 1991). These previous studies focussed primarily on K_{Ca} channel modulation by metabolic secretagogues, and it remains to be shown whether nonmetabolic factors that are known to have a modulatory role in other tissues may affect the β cell K_{Ca} channel activity and thus the electrical behavior.

The activity of the K_{Ca} channel is regulated by activation of receptors coupled to the G proteins G_s and G_i/G_o, which have been shown to act either via membrane-delimited pathways or via second messengers and kinases. In hippocampal pyramidal cells, K_{Ca} channel inhibition due to activation of G_s-coupled β-adrenergic receptors has been ascribed to indirect, second messenger-mediated regulation involving increased cAMP production and activation of protein kinase A (PKA) (Madison & Nicholl, 1986; Knöpfel et al., 1990). In consonance with this hypothesis, stimulation of K_{Ca} channels by G_i/G_o-coupled somatostatin (SRIF) receptors in intact GH_4 cells, has been attributed to dephosphorylation of a cAMP-dependent phosphorylation "site" on the channel or a protein associated with it (White, Schonbrunn &

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Armstrong, 1991). By contrast with the indirect inhibitory effect of activated G_s, direct stimulation has been proposed to account for K_{Ca} channel activation by purified α subunits of G_s in lipid bilayers (Scornik et al., 1994). Consistent with this observation, it has been postulated that in smooth muscle direct stimulation by G_s mediates increased K_{Ca} channel activity in response to β-adrenergic stimulation (Toro, Ramos-Franco & Stefani, 1990). Thus activation of G_s may have opposite effects, causing channel stimulation via a direct effect of α_s, as well as channel inhibition via PKA-mediated phosphorylation.

In the case of G_i/G_o-type G proteins, direct regulation of K_{Ca} channel activity by the G protein has not yet been described, however, a second messenger-mediated regulation has been proposed in hippocampal pyramidal neurons. In these studies it was hypothesized that inhibition of the K_{Ca} channel by muscarinic agonists is mediated by activation of protein kinase C (PKC) (Malenka et al., 1986; Knöpfel et al., 1990). It is plausible that PKC-induced inhibition is due to phosphorylation of the channel itself, or alternatively, it may result from phosphorylation-induced reduction of the stimulatory effect of G_s. The latter hypothesis stems from the finding that PKC induces phosphorylation of the β-adrenergic receptor that leads to desensitization and uncoupling of the receptor from G_s (Sibley & Lefkowitz, 1985). Within this scheme deactivation of G_s would account for PKC-mediated channel inhibition.

The present study was carried out to determine the mode of action of G_i/G_o and G_s-type G proteins as well as that of PKC on the K_{Ca} channel. The results reflect complex cross-reactivity between pathways that are involved in K_{Ca} channel regulation.

Materials and Methods

Cell Cultures and Experimental Media

The K_{Ca} channel studies were performed using cells from the insulin secreting cell line HIT (passage #70–86), kindly provided by A.E. Boyd III. The cells were incubated at 37°C in RPMI 1640 medium, supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), streptomycin (100 units/ml) and 2 mM glutamine. The cells were divided once a week by treatment with trypsin and the medium was changed twice between divisions. One hour before starting the experiment the culture medium was exchanged with a glucose-free solution containing (in mM): 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$ and 10 Hepes, the pH being adjusted to 7.4 with NaOH. This solution was also used in the experimental chamber when an extracellular type solution was needed, for outside-out patch experiments. For both inside-out and outside-out patch experiments, the composition of the extracellular type solution was (in mM): 140 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$ and 10 Hepes, pH was adjusted to 7.2 with KOH; that of the intracellular type solution was similar, except that the Ca^{2+}, EGTA ratio was adjusted to give a pCa of 5 or 6×10^{-7} at which simultaneous opening of 1 to 3 channels could be typically observed. For both inside-out and outside-out patch clamp experiments, GTP (50–100 μM) (Boehringer Mannheim Diagnostics, Houston, TX) was added to the solution facing the inside of the membrane (internal type solution) to allow for G protein activation. ATP (Sigma) at a concentration of 200 to 250 μM was also present in the "internal" solution to block the activity of ATP-dependent K channels (Ribalet, Ciani & Eddlestone, 1989). The α subunit of the G proteins G_i and G_o, kindly provided by Drs. J. Codina and L. Birnbaumer (UCLA, School of Medicine), were kept at a concentration of 2 μM in a buffered solution containing (in mM): 10 Tris, 1 EDTA, 1 dithiothreitol, 7.5 $MgCl_2$, 20 KCl. This concentrated solution was then diluted in glucose-free extracellular medium to obtain an α subunit concentration of 10 nM prior to the experiment. The purified G protein α subunits were maintained in their active state by treatment with GTPγS, the ratio of the nucleotide to G protein was 0.7 after dialysis. Somatostatin was obtained from Bachem, Torrance, CA, norepinephrine and isoproterenol from Sigma. Stock solutions of pertussis toxin and of the active A protomer (List Biological Laboratories, Campbell, CA.) were prepared with 10 μg protein diluted in 500 μl of sterile water containing 10 mM TRIS, 0.1 mM Na_2EDTA and 0.04% CHAPS at pH 8.0. Aliquots of these stock solutions were added directly to the experimental chamber to obtain a final concentration of 100 ng/ml. Stock solution of cholera toxin and of its A subunit, (List Biological Laboratories, Campbell, CA.) were prepared with 25 μg of the protein diluted in 1 ml of solution containing (in mM): 50 TRIS, 200 NaCl, 1 Na_2EDTA and 3 NaN_3 at pH 7.5. The peptide (19-36) inhibitor of protein kinase C (PKC) was obtained from Peninsula Laboratories, Belmont, CA; SC-9, [N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide], the PKC activator from Seikagaku America, St. Petersburg, FL., and H-7, 1-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride from Molecular Probes, Eugene, OR. H-7 was dissolved directly into the experimental solution, while SC-9 was stored in dimethylsulfoxide (DMSO). After dilution of the SC-9 stock solution in the experimental medium the final concentration of DMSO was less than 0.05%, at which level there was no significant effect on K channel activity. A stock solution of chelerythrine, a membrane permeant inhibitor of PKC obtained from Calbiochem, was prepared in an external type solution at a concentration of 1.3 mM.

Data Recording and Analysis

The techniques to prepare patch electrodes and record single channel events were similar to those described previously (Ribalet, Eddlestone & Ciani, 1988, for details). The data, filtered at 2 kHz with an 8-pole Bessel filter were recorded either with an EPC 7 List (Darmstadt, FRG) or an Axopatch 1A (Axon Instruments, Burlingame, CA) patch amplifier and stored to video cassette at a fixed frequency of 44 kHz after digitization with a digital audio processor. For analysis, the data were acquired on a computer hard disk at rate of 5.5 kHz, the transfer was carried out with a two-buffer interface allowing continuous acquisition (Bezanilla, 1985). Prior to measurement of channel activity, amplitude histograms of the current steps were built to determine the half amplitude threshold, and this threshold was used to form an idealized record of the original data. This schematized record was utilized for the subsequent analysis. To construct the concentration-dependence curves the percent open time (NP_o), which is the parameter used to assess the level of channel activity, was determined from data samples of 30-sec duration obtained at steady state. Under control conditions, NP_o values varied widely from patch to patch and for this reason ratios of experimental NP_os *vs.* control were used for averages. To build the time course graphs two experiments were used unless specified and NP_o values were estimated using 15-sec bins and plotted as a function of time.

Results

K_{Ca} Channel Modulation by Somatostatin (SRIF)

Insulin-secreting β cells possess SRIF receptors coupled to the PTX-sensitive G proteins G_i/G_o (Ribalet et al., 1991) that have been demonstrated to regulate Ca-dependent K channel (K_{Ca}) behavior in other cell types (White et al., 1991). Of the 2 naturally occurring SRIF products, SRIF-28 and SRIF-14, the former has a tenfold higher affinity for the SRIF receptor in insulin-secreting β cells (Cotroneo, Marie & Rosselin, 1988; Maletti et al., 1992). Outside-out patch clamp experiments were carried out on cells of the insulin-secreting cell line HIT to determine whether the K_{Ca} channel may be modulated by SRIF via a membrane-delimited pathway. The responses to both SRIF-28 and SRIF-14 were investigated over a range of concentrations, and it was determined that SRIF-28 was around 20 times more potent than SRIF-14 to inhibit channel activity. In these experiments, the effect of SRIF-28 increased between 2.5 and 25 nM; partial inhibition by 5 nM and almost complete inhibition by 25 nM are demonstrated in *A* of Fig. 1. In *B* of this figure, average responses from 6 experiments are normalized and plotted as a function of SRIF-28 concentration to illustrate the dose-dependent effect of this product (closed circles). The fit to this plot yields an IC_{50} of about 5 nM. This plot also demonstrates that as SRIF-28 was elevated above 25 nM some relief of inhibition occurred; this is tentatively ascribed to SRIF receptor desensitization. The open circles summarize the data from 5 experiments obtained with SRIF-14; the fit to these data yields an IC_{50} of 104 nM.

In *C* of Fig. 1, average NP_os from 2 experiments are plotted as a function of time to illustrate the time course of channel inhibition induced by 50 nM SRIF-28. The dynamics of the response to SRIF was very similar for both SRIF-28 and SRIF-14, the onset of inhibition became more rapid with increasing concentration, occurring in less than 30 sec at the highest SRIF levels. Reversibility of this effect was very limited; in 5 experiments in which this was studied, only about 30% of control activity was recovered 30 to 45 min after agonist withdrawal.

K_{Ca} Channel Modulation by PTX: Role of Endogenous G_i/G_o

Much evidence favors G_i/G_o as mediators of various responses to SRIF (Birnbaumer, Abramowitz & Brown, 1990). Pertussis toxin (PTX), which catalyzes the ADP ribosylation and prevents the activation of G_i or G_o (Katada & Ui, 1982; Katada, Oinuma & Ui, 1986), was used to confirm this as well as to investigate whether agonist-independent (background) activation of these G proteins occurred in the excised patch, causing tonic inhibition of KCa channel activity.

PTX is formed of an A and B protomer; the B protomer permits the insertion of the A chain across the membrane and into the cell where it becomes enzymatically active. As shown in the upper two traces of Fig. 2, addition of 100 ng/ml of nonpreactivated PTX (A plus B protomers) to the outside-out patch caused an increase of channel activity which was estimated to be 4.14 ± 1.2 times control in 5 experiments. The graph in Fig. 2 indicates that this effect was rapid, occurring in less than one minute. Addition of the A protomer alone to the inside-out patch induced similar channel activation but the effect was less consistent occurring only in 4 of 9 patches; in the other patches there was almost no effect.

The data in the lower trace of Fig. 2 were recorded 5 min after addition of SRIF in the presence of PTX. Comparison of these data with those obtained prior to the addition of SRIF (right upper trace) demonstrate that, in outside-out patches, PTX prevents the inhibitory effect of SRIF. Averaged channel activity recorded after addition of 250 to 500 nM SRIF-14 in the presence of PTX was 2.92 ± 1.4 times that of control, and was not significantly different from that recorded with PTX alone.

It is deduced from these results that agonist-independent activation of G_i or G_o occurs in excised patches resulting in tonic K_{Ca} channel inhibition, and further that SRIF-induced K_{Ca} channel inhibition is mediated via enhanced activation of PTX-sensitive G proteins (G_i or G_o).

K_{Ca} Channel Modulation by Purified α Subunits of the G Proteins G_i and G_o

The effect of PTX suggests that endogenous subunits of G_i and/or G_o modulate K_{Ca} channel activity. To further investigate the role of G protein subunits, purified α subunits of G_{i3}/G_o proteins were applied exogenously to inside-out patches. α_{i3} (from erythrocytes) and α_o (from brain) were selected because they modulate the function of other K channels (Yatani et al., 1987; Ribalet & Eddlestone, 1995). Addition of either type of α subunit caused channel inhibition within 1 to 4 min.; the inhibitory effect of 12.5 pM α_{i3} is illustrated in Fig. 3*A* and that of 12.5 pM α_o in Fig. 3*B*. At concentrations greater than 50 pM to 100 pM both α_{i3} and α_o subunits blocked channel activity by almost 90%. Figure 3*C* is a plot of the concentration-dependent inhibition of channel activity by α_i and α_o; the data points are averaged from 6 and 5 experiments for α_i and α_o, respectively. There was essentially no difference between the potency of the two types of α subunits to block channel activity; the half maximum inhibitory values of 5.95 pM for α_i and 5.74 pM for α_o were not statistically different. Fifteen to forty-five minutes after G protein withdrawal the channel activity was still less than ⅓ of control activity (n = 6)

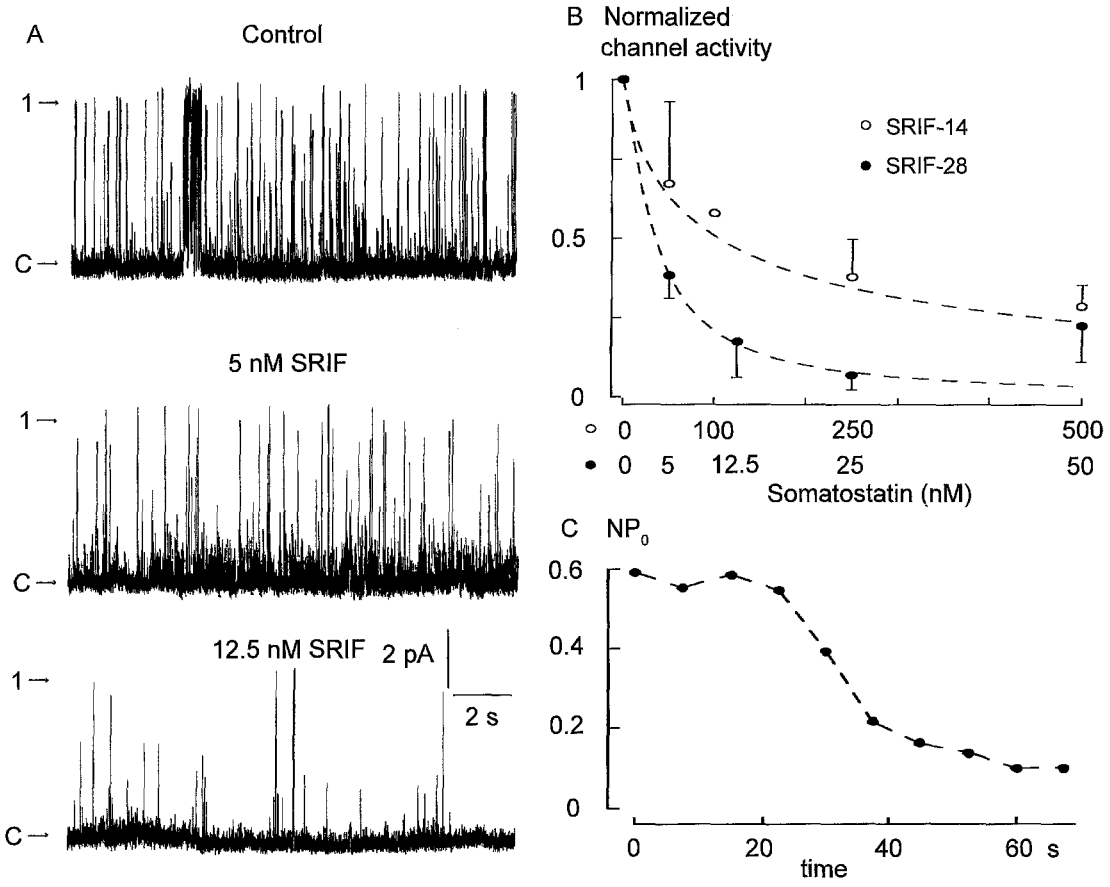


Fig. 1. Concentration-dependent effects of SRIF on K_{Ca} channel activity in the outside-out patch. Recordings in *A* are from the same patch and depict the inhibitory effect of bath applied SRIF-28. The upward current steps represent outward current in symmetrical 140 mM KCl for a pipette holding potential of 25 mV. The pipette solution contained 50 μM GTP and 250 μM ATP. In *B*, the channel activity normalized to the activity measured in the absence of agonist was plotted as a function of SRIF concentration. The upper scale on the horizontal axis is for SRIF-14, and the lower one for SRIF-28. The open and closed circles which represent data obtained with SRIF-14 and SRIF-28 respectively, were fitted with the following equation:

$$I/I_o = 1/1 + (C/K)^n)$$

Where C is the concentration of SRIF, n is a Hill coefficient and K is the IC_{50}. The fit of the open circles yielded $n = .85$ and $K = 104$ nM, that of the closed circles yielded $n = 1.25$ and $K = 5.09$. In *C*, average NP_o values from 2 experiments are plotted as a function of time. In this case NP_os were estimated using data samples of 7.5 seconds duration. SRIF-28 (50 nM) was added at time $t = 10$ sec.

suggesting that the inhibitory effect of the α subunits is poorly reversible. Channel inhibition by α_i or α_o subunits was observed consistently in 21 patches but as shown in Fig. 4 this effect developed with variable time lapses. For instance, the upper panel demonstrates typical, pronounced channel inhibition 1 min after the addition of 25 pM α_i subunit. By contrast the lower panel shows a different and less common pattern of inhibition where addition of α_i subunit is followed by dramatic oscillations of channel activity for up to 10 min between a stimulated and inhibited state prior to inhibition. The inhibitory effect of purified α subunits of G_i and G_o strengthen the hypothesis that SRIF-induced K_{Ca} channel inhibition is mediated via activation of G_i/G_o.

However, in 5 patches purified α subunits (6 pM) stimulated channel activity 4.15 ± 2.04-fold as compared to control (n = 3 with α_i and n = 2 with α_o). In 3 of these experiments, the stimulation was only transient and lasted from 1 to 10 min. Figure 4*B* illustrates the result of one such experiment during which channel activity increased in bursts reaching a level more than 3 times that of control. In 2 other experiments, the increase in channel activity was sustained in the presence of α subunit concentrations less than 25–50 pM; further augmention caused channel inhibition with almost complete block near 250 pM (*results not shown*). This latter effect cannot be ascribed to GTPγ S used to activate α subunits since the GTP analogue had no effect on chan-

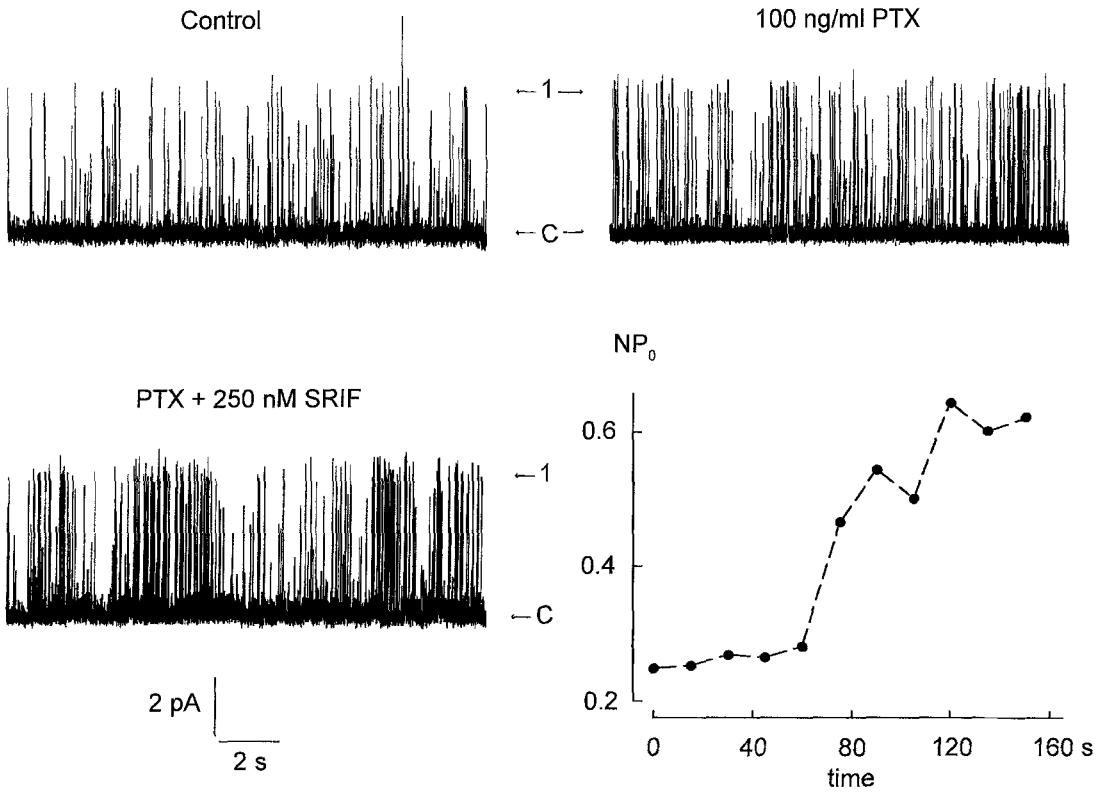


Fig. 2. Effects of Pertussis toxin (PTX) on K_{Ca} channel modulation by SRIF in the excised outside-out patch. The pipette holding potential was 25 mV, and 140 mM KCl was present on both sides of the membrane; the pipette solution contained GTP and ATP. All the data shown in this figure originate from the same experiment. The two upper traces illustrate the effect of 100 ng/ml of PTX (A and B protomers) on channel activity. Control activity shown in the upper left panel was recorded in the presence of NAD^+. The upper right trace showing a stimulatory effect of PTX was taken eight minutes after addition of the toxin. The recording shown in the lower panel was acquired 4 min after addition of 250 nM SRIF-14, and illustrates the lack of inhibitory effect of SRIF in the presence of PTX. In the lower right corner of the figure NP_o values plotted as a function of time are averages from 2 experiments performed with outside-out patches. In this graph 100 ng/ml PTX was applied to the bath at time $t = 20$ sec.

nel activity at these low concentrations, but rather to activation of a secondary, stimulatory pathway. This conclusion is supported by the observation that SRIF had a similar transient stimulatory effect in 3 outside-out patches. In these experiments the activity increased 3.7 ± 0.86 fold and returned to normal after 3 to 5 minutes.

K_{Ca} Channel Modulation by Protein Kinase C (PKC)

As described in the Introduction, modulation of the K_{Ca} channel by α subunits of G_i or G_o may be due to a "direct" effect of the α subunit on the channel or may be mediated by another membrane associated protein. Evidence in favor of protein kinase C (PKC) playing the role of an intermediary protein stems from the observations that α_o/α_i subunits have functional interactions with PKC (Jakobs et al., 1985; Gilman, 1987), and TPA, which acts to stimulate PKC, inhibits K_{Ca} channel activity in cell-attached patches (Ribalet et al., 1988). Based on this latter observation, it may be concluded that PKC activation affects K_{Ca} channel behavior, but this does not show that the kinase is present in the isolated patch. To test this hypothesis the effects of exogenous modulators of PKC on channel activity were investigated in excised membranes patches.

The results from an experiment in which SC-9, a substitute for the PKC activator diacylglycerol (Nishino et al., 1986), was applied to an excised inside-out patch are shown in Fig. 5. The upper panel demonstrates that addition of 150 μM SC-9 caused K_{Ca} channel inhibition after a short delay of 1 to 2 minutes; in 4 experiments channel activity was reduced to 0.37 ± 0.12 of control by SC-9.

Experiments employing inhibitors of PKC yielded complementary data. Peptide 19–36, a synthetic peptide with an amino acid sequence corresponding to a PKC pseudosubstrate (House & Kemp, 1987) caused a marked increase of K_{Ca} channel activity when added to inside-out patch preparations as shown in Fig. 5*B*. In 4 experiments, channel activity in the presence of the PKC inhibitor was 6.8 ± 1.01 times control. Similar results

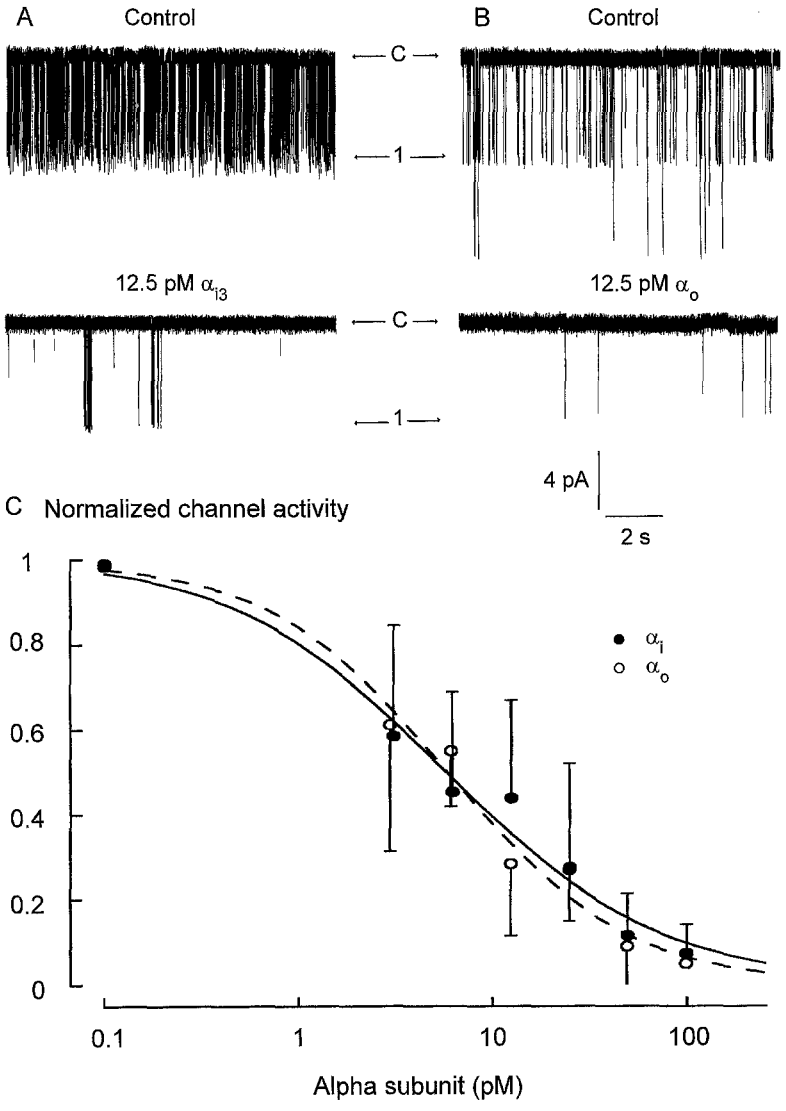


Fig. 3. Effects of G_o/G_i α subunits on K_{Ca} channel activity in excised inside-out patches. The pipette potential was held at + 30 mV, and 140 mM KCl was present on both sides of the membrane; the bath solution contained 200 μM ATP. The upper traces of *A* and *B* illustrate the channel activity prior to addition of α subunit. The lower trace in *A* illustrates the level of activity reached five minutes after addition of 12.5 pM of α_{i3}, that in *B* was recorded six min after addition of 12.5 pM α_o. In these two panels, downward deflections represent inward current. In *C*, the open and closed circles are averaged data points of channel activity measured in the presence of increasing concentrations of α_o and α_i, respectively. The data were normalized to the level of activity measured in the absence of exogenous α subunit and fitted using the equation:

$$I/I_o = A/(1 + (K/C))$$

Where *C* is the concentration of α subunit added to the bath. Fitting of the data yielded an IC_{50} (*K* in the equation) of 5.95 pM for α_i (continuous line) and 5.74 pM for α_o (dotted line).

were obtained in inside-out patches with the Isoquinolinesulfonamide inhibitor of PKC and cyclic nucleotide-dependent protein kinases, H-7 (Hidaka et al., 1984). Channel stimulation commenced rapidly following addition of H-7 (Fig. 5, lower trace) and reached a steady state level within 1 to 2 min. In 9 experiments using 100 μM H-7 channel activity at steady state was 11.02 ± 3.4 times that of control. This effect of 100 μM of H-7 was not sustained but usually decayed after 4 to 5 minutes; the decline proceeded until a level of channel activity comparable to control was restored after 10 to 12 min. At lower inhibitor concentrations the secondary decay of channel activity was not evident; for instance at 25 μM H-7, while channel stimulation was slower to develop, and reached a level typically close to 3 times that of control, this enhanced activity was sustained for at least 15 min, the duration of the longest observation. The difference in the channel response times to the two PKC inhibitors may be due to slower diffusion of the peptide inhibitor, or alternatively, to the competitive type of inhibition that takes place between the synthetic peptide and endogenous PKC substrates (House & Kemp, 1987).

Inhibition of PKC was also investigated in outside-out patches using the membrane permeant PKC inhibitor, chelerythrine (Herbert et al., 1990). In 4 experiments, 13 μM chelerythrine caused K_{Ca} channel activity to increase to 6.2 ± 1.5 times the control value within 1 to 3 minutes of its addition (*see* Fig. 7). As was the case with the other PKC inhibitors as well as the PKC activator, the effect of chelerythrine was fully reversible.

From these experiments, it was concluded that PKC is present in the excised patches of membrane, that it influences K_{Ca} channel activity and that there is background activity of the kinase in the isolated patch.

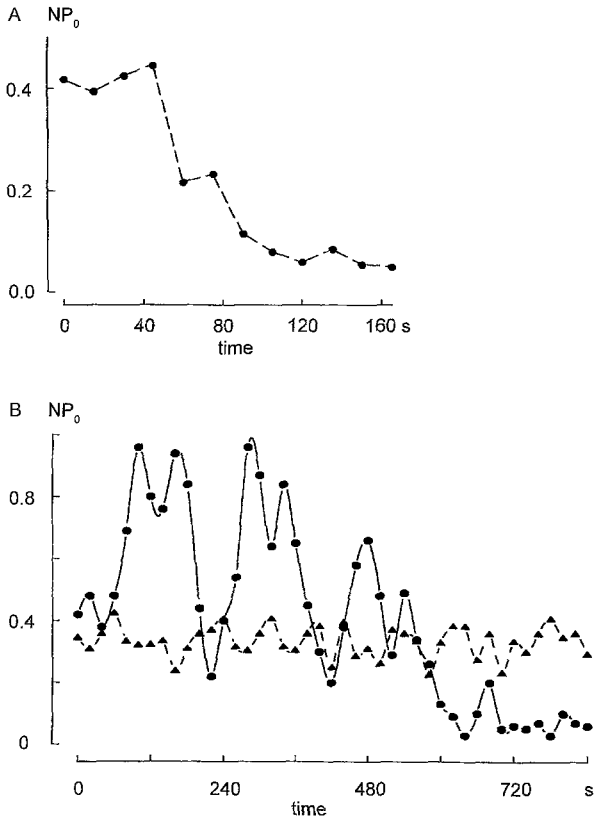


Fig. 4. Time course of K_{Ca} channel inhibition and activation by exogenous G protein α_i/α_o subunits. *A* illustrates the result of one experiment carried out with an inside-out patch. In this case, 12.5 pM α_o was added to the bath at time $t = 15$ sec. The lower panel illustrates channel behavior in a different inside-out patch experiment. In this graph NP_o values shown as triangles were recorded under control conditions prior to the addition of exogenous α subunits. The closed circles correspond to NP_o values recorded after addition of 12.5 pM α_o to the bath.

Role of PKC in K_{Ca} Channel Modulation by G_i/G_o

Having established that activated G_i/G_o proteins and PKC both inhibit the K_{Ca} channel and that there is evidence for interactions between these moieties in other systems (Jakobs et al., 1985; Gilman, 1987), the hypothesis that they act on the K_{Ca} channel in concert was addressed. To determine whether K_{Ca} channel modulation by G protein α_i and α_o subunits requires PKC activity, the effect of α_i and α_o was studied in the presence of the PKC inhibitors, peptide 19–36 and H-7. The data presented in Fig. 6 show that inhibition of PKC prevented K_{Ca} channel inhibition by α_o subunits. The two upper traces of Fig. 6 illustrate the effect of α_o on channel activity in the absence of the protein kinase inhibitor, as already shown in Fig. 3 addition of α_o or α_i caused a substantial decrease in channel activity. In 7 experiments the activity recorded in the presence of 12.5 pM α_i/α_o was equivalent to 0.2 ± 0.05 times control. By contrast, the data shown in the lower panel of Fig. 6 demonstrate that α subunits had no inhibitory effect in the presence of PKC inhibitor. In fact in 2 experiments addition of α_i or α_o caused a 1.7-fold increase in channel activity in the presence of PKC inhibitor. In 8 experiments, the average channel activity measured after addition of α subunits in the presence of PKC inhibitor was 0.98 ± .15 times that recorded under control conditions. These data indicate that PKC mediates a phosphorylating step which favors K_{Ca} channel inhibition by α_i subunits.

Further experiments were carried out to determine whether PKC also facilitates the inhibition of K_{Ca} channel activity by SRIF. The two upper traces of Fig. 7 illustrate the inhibitory effect of SRIF in outside-out patches as already presented in Fig. 1. The lower trace indicates that addition of chelerythrine, the membrane permeant PKC inhibitor, could restore the channel activity previously blocked by SRIF. In 3 experiments, addition of chelerythrine reversed the inhibitory effect of SRIF, causing recovery of channel activity from 0.29 ± 0.1 times control in the presence of SRIF alone to 0.95 ± 0.15 times control when the PKC inhibitor was added. Addition of chelerythrine reversed SRIF-induced channel inhibition only in 3 out of 7 patches. However, when chelerythrine was added to the bath prior to testing for the effect of SRIF, the inhibitor consistently prevented channel inhibition by SRIF. In fact, under these conditions, the activity measured in the presence of SRIF was 1.2 times higher than that recorded with chelerythrine alone (n = 3). Together these observations suggest that SRIF favors a phosphorylation process which is poorly reversible in excised patches, due perhaps to the loss of a dephosphorylating enzyme.

It may be argued that the effect of chelerythrine is independent of PKC regulation. However, the activation of the K_{Ca} channel was accompanied by a concomitant decrease in the activity of occasional ATP-dependent K channels (K_{ATP}) (result not shown). Since we have previously shown that PKC-induced phosphorylation stimulates K_{ATP} channel activity (Ribalet & Eddlestone, 1995), this observation strongly suggests that chelerythrine is indeed inhibiting PKC.

Based on the above data it is postulated that SRIF-activated G_i/G_o proteins only block K_{Ca} channels when concurrent activity of PKC occurs.

K_{Ca} Channel Modulation by Isoproterenol and Norepinephrine

Activation of β-adrenergic receptors has an inhibitory effect on K_{Ca} channel behavior in intact hippocampal pyramidal cells (Knöpfel et al., 1990) and a stimulatory effect in lipid bilayers (Toro et al., 1990). Since β cell function is controlled by β-adrenergic receptors which

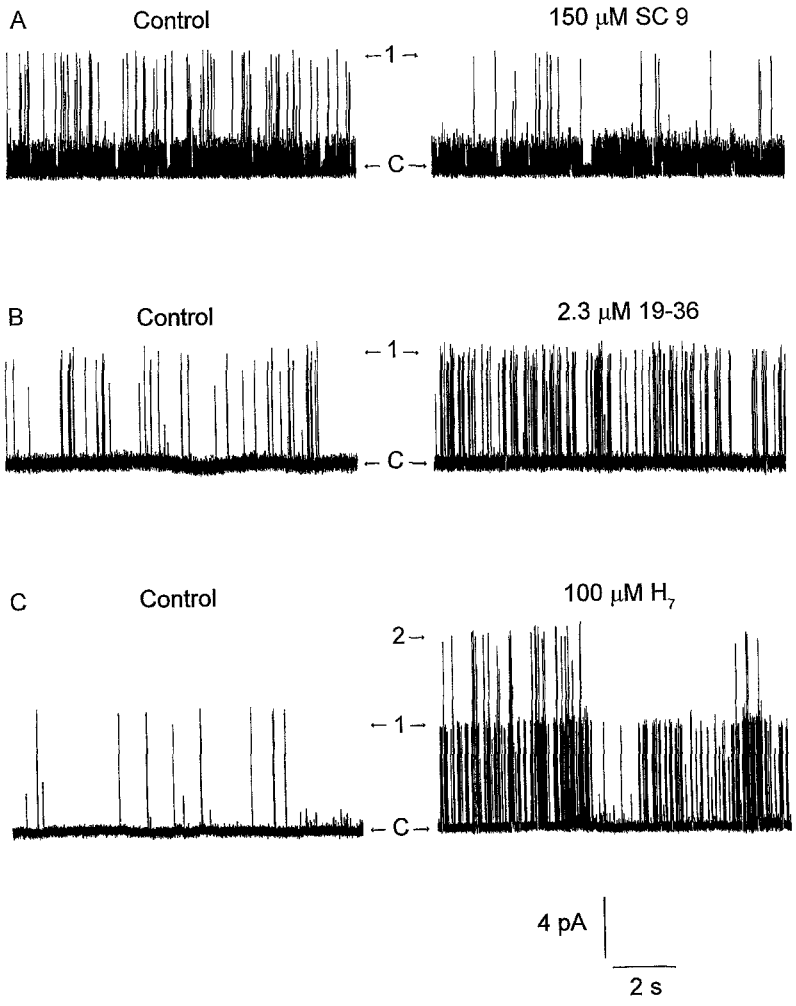


Fig. 5. Effects of protein kinase C (PKC) modulators on K_{Ca} channel activity in the excised inside-out patches. In these experiments there was 140 mM KCl on both side of the membrane and the bath solution contained 200 μM ATP and 50 μM GTP. The data in *A*, *B* and *C* are from 3 different patches, the membrane was held at 35 mV, and upward deflections represent outward currents. *A* shows the effect of the activator SC-9, 4 min after its addition. *B* depicts the effect of the peptide (19–36) inhibitor of PKC, 6 min after its addition (right trace). *C* shows the effect of the PKC inhibitor H-7, the right trace was acquired 1 min after addition of 100 μM H-7.

are coupled to G_s (Ahrén, Taborsky, & Porte, 1986), we investigated whether activation of this receptor type affects K_{Ca} channel activity in insulin secreting cells.

The effects of norepinephrine and isoproterenol, which are specific agonists of β-adrenergic receptors, were tested in outside-out patches. The data presented in the two upper traces and in the lower panel of Fig. 8 illustrate the effect of 100 nM isoproterenol. In 6 experiments the agonist had a stimulatory effect that reached a level 4.98 ± 1.84 times control. Such a stimulation developed rapidly and became maximum within 1 to 2 minutes. Similarly, in 3 other experiments, 10 μM norepinephrine stimulated K_{Ca} channel activity by 6.35± 2.09 fold. Both of these effects were reversible.

Effects of Cholera Toxin on K_{Ca} Channel Activity

β-adrenergic receptors being coupled to the G protein G_s, it may be assumed that K_{Ca} channel stimulation results from activation of G_s. To test whether in insulin-secreting cells, endogenous, membrane-associated G_s provides the functional link between β-adrenergic receptors and K_{Ca} channels, experiments were performed with cholera toxin (CTX) in excised patches.

In outside-out patches, the non-reduced form of CTX (A and B chains), which irreversibly activates G_s, in intact membranes, by ADP-ribosylating α_s, the α subunit of G_s (Birnbaumer et al., 1990), caused rapid and irreversible activation of the K_{Ca} channel (Fig. 8*B*). In 8 out of 11 patches, the effect of CTX occurred within 30 to 90 sec (Fig. 8*C*) and caused a 3.77 ± 1.02-fold increase in channel activity. In inside-out patches, bath application of the reduced toxin (A chain alone) also caused channel stimulation, but stimulation occurred less frequently (3 out of 7 patches). Similarly, a more consistent response to the whole toxin as compared to the active chain alone had also been observed with PTX.

It may be argued that the effect of the toxin is unspecific, and due to formation of Ca^{2+} permeable pores. This is unlikely to be the case since the "leak current" did not change upon application of the toxin to the bath.

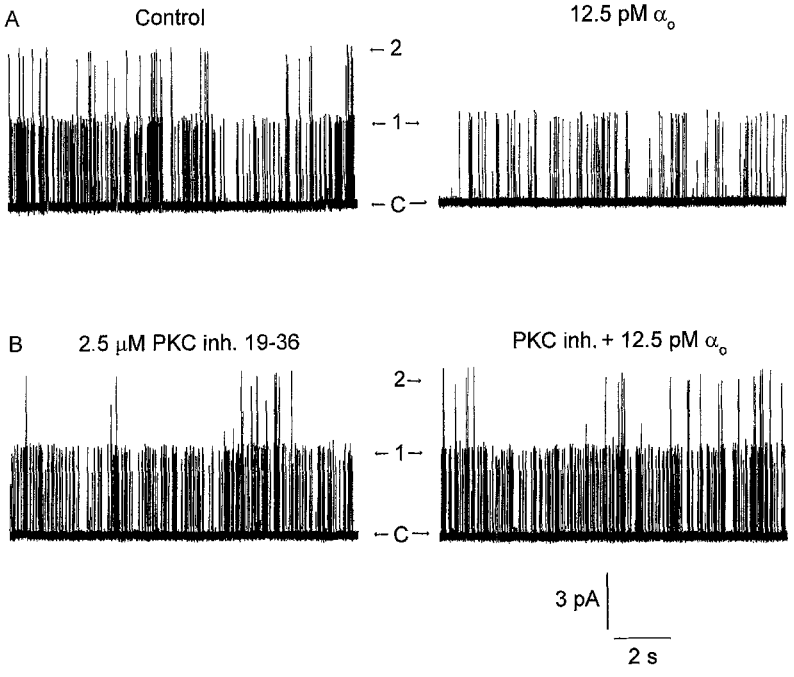


Fig. 6. Effects of PKC modulators on α_o-mediated K_{Ca} channel inhibition in the inside-out patch. The recordings presented in *A* and *B* are outward currents obtained from 2 different patches at 30 mV holding potential. *A* illustrates the inhibitory effect of α_o on K_{Ca} channel activity. Control activity recorded prior to the addition of α subunit is shown in the left trace. The right trace recorded 4 min after addition to the bath of 12.5 pM α_o demonstrates inhibition of K_{Ca} channel activity. By comparison, *B* shows that addition of exogenous α subunits had no effect in the presence of PKC inhibitor. The left trace of panel *B* shows steady state channel activity measured in the presence of peptide 19–36 inhibitor of PKC. The right trace, recorded 7 minutes after addition of 12.5 pM α_o in the presence of inhibitor demonstrates the lack of effect of α_o under these conditions.

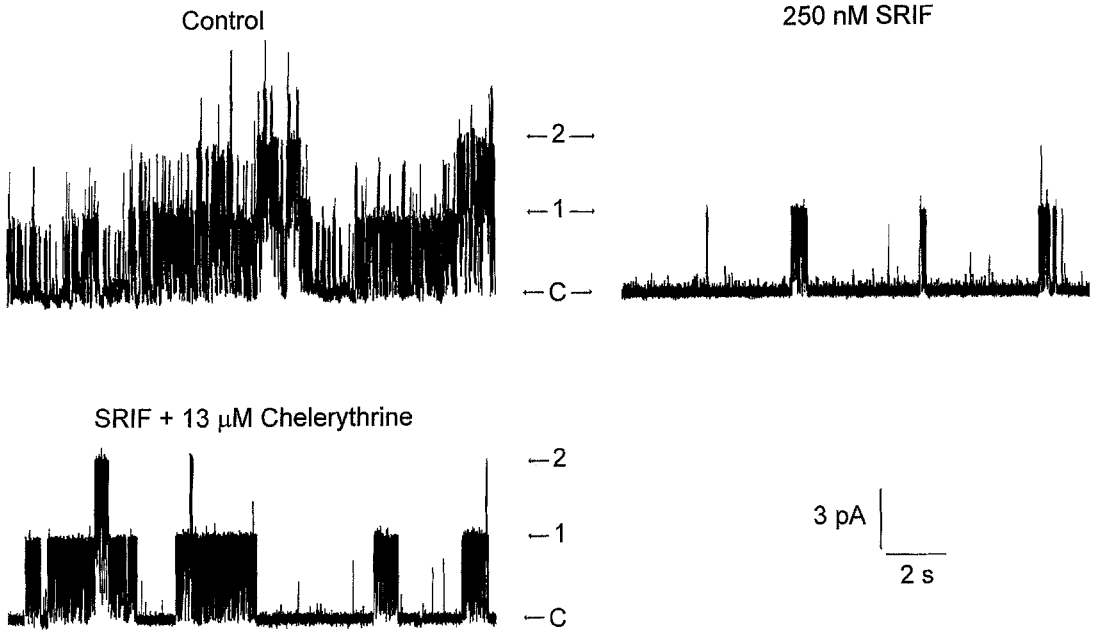


Fig. 7. Effects of PKC modulators on SRIF-induced K_{Ca} channel inhibition in the outside-out patch. The 3 traces in this figure represent outward currents recorded from the same patch at a pipette potential of 30 mV. The two upper traces demonstrate the inhibitory effect of 250 nM SRIF-14. Control activity recorded at steady state, in the absence of agonist, is shown in the left trace, while the right trace shows the inhibitory effect of SRIF, six min after addition of the agonist. The recording shown in the lower panel was acquired 5 min after addition of the PKC inhibitor, chelerythrine, in the presence of SRIF, and illustrates the recovery of channel activity due to PKC inhibition.

In addition, the observation that activation of the K_{Ca} channel was accompanied by a simultaneous decrease in K_{ATP} channel activity (result not shown), suggests that activation of G_s indeed mediates the effect of CTX. We have previously shown that CTX blocks K_{ATP} channel activity in inside-out patches (Ribalet & Ciani, 1994).

The stimulatory effect of CTX indicates that acti-

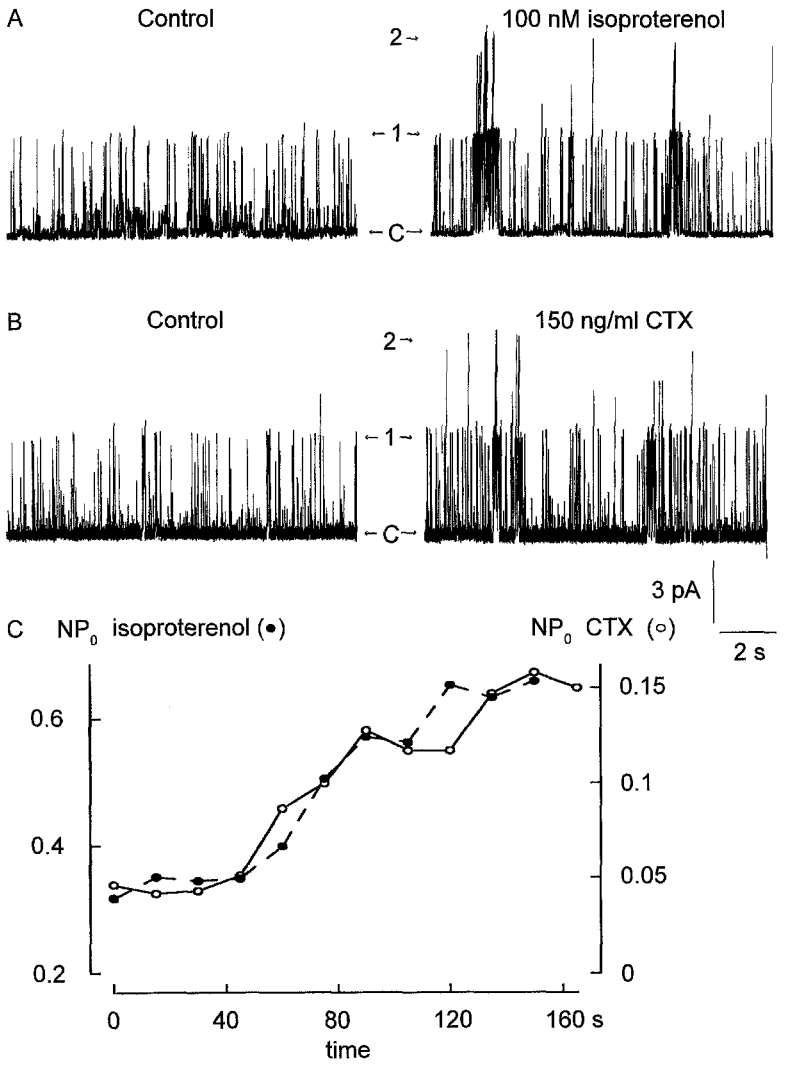


Fig. 8. K_{Ca} channel stimulation by activated G_s in outside-out patches. Data in *A* and *B*, depict single-channel outward currents recorded at a holding potential of 30 mV with symmetrical 140 mM K^+, in two different experiments. In *A*, the left trace was recorded under control conditions with 50 μM GTP and 200 μM ATP in the pipette solution. The right trace recorded 3 min after addition of isoproterenol to the bath, demonstrates K_{Ca} channel stimulation by the β agonist. Data in *B* illustrate the stimulatory effect of cholera toxin (CTX) on K_{Ca} channel activity. Control activity recorded in the presence of NAD^+ and prior to the addition of the toxin is shown in the left trace. The right trace recorded one and a half minute after addition to the bath of CTX demonstrates clear K_{Ca} channel stimulation. In *C* NP_o values recorded from 2 outside-out patch experiments are represented by closed circles. In this case isoproterenol (100 nM) was added to the bath 20 sec after the beginning of the graph. The open circles represent NP_o estimates obtained from 2 outside-out patch experiments using CTX. In this illustration CTX (150 ng/ml) was added to the bath at time t = 20 seconds.

vated G_s mediates K_{Ca} channel stimulation by β-adrenergic receptors.

Cross-reactivity Between G_s and G_i and the Modulation of K_{Ca} Channels

The observation that the K_{Ca} channel activity is enhanced by G_s and inhibited by G_i/G_o leads to the consideration of parallels with the regulation of adenylate cyclase. It may be proposed that the two G proteins act independently on the channel, or alternatively, the channel may interact with only one protein, the activity of which is itself affected by the antagonistic G protein. The results presented below offer evidence that favors the latter interpretation.

Blockage of the Norepinephrine/Isoproterenol Effect by SRIF

Activation of G_i inhibits G_s-stimulated production of cAMP. To determine whether a similar mechanism controls K_{Ca} channel activity we investigated the effect of SRIF on isoproterenol- or norepinephrine-stimulated K_{Ca} channels. The experiment depicted in Fig. 9 was performed in outside-out patches and is representative of 8 other experiments performed with β-adrenergic receptor agonists. The 2 upper traces of Fig. 9 illustrate the stimulatory effect of isoproterenol as shown previously. The data shown in the lower trace demonstrate that addition of 250 nM SRIF completely abolished the stimulatory effect of the β agonist. In 6 experiments, channel activity increased 4.98 ± 1.84-fold in the presence of isoproterenol and returned to a value .97 ± 0.68 times that of control after addition of SRIF. Similarly, SRIF blocked the stimulatory effect of norepinephrine and the activity decreased from 6.35± 0.18 to 0.78 ± 0.37 times the control value after addition of SRIF (n = 3). Other results are consistent with this finding and have shown that neither isoproterenol or norepinephrine could restore the channel activity previously blocked by SRIF.

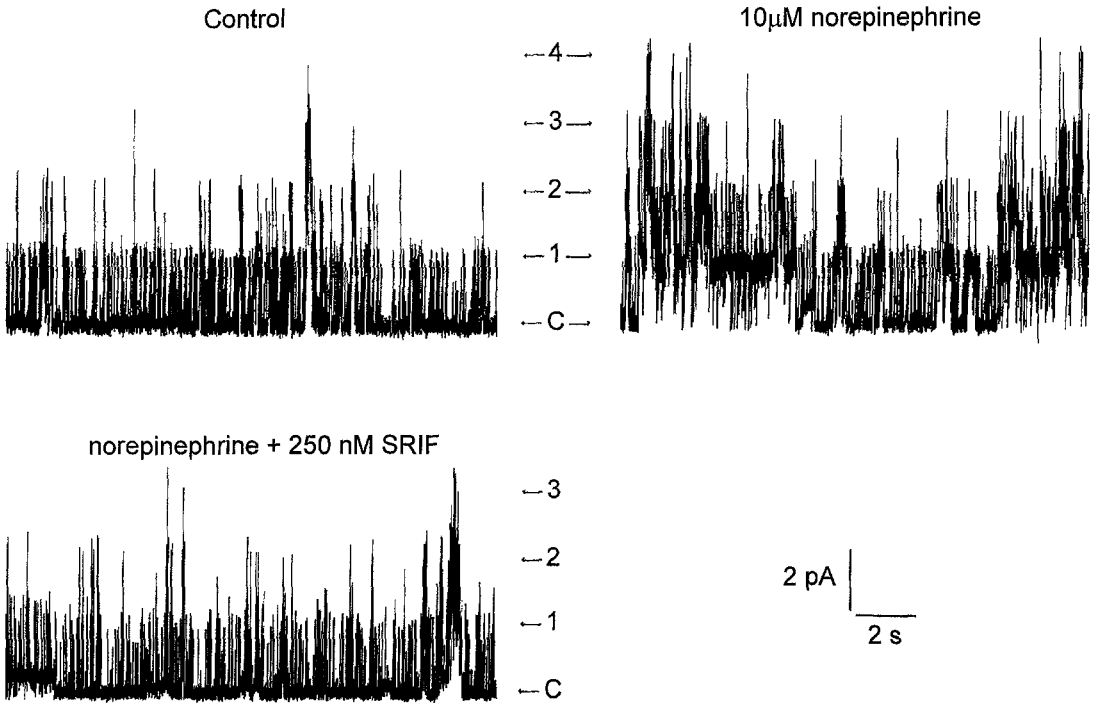


Fig. 9. Inhibition by SRIF of β agonist-induced K_{Ca} channel stimulation in the excised outside-out patch. The pipette holding potential was 20 mV, and 140 mM KCl was present on both sides of the membrane; the pipette solution contained 50 μM GTP and 250 μM ATP. All the data shown in this figure originate from the same experiment. The two upper traces illustrate the effect of norepinephrine on channel activity. Control activity is shown in the upper left panel, and the upper right trace, recorded five minutes after addition of norepinephrine, demonstrates the stimulatory effect of the β agonist. The recording shown in the lower trace was acquired 8 min after addition of 250 nM SRIF-14 to the bath, and illustrates the blockage of norepinephrine-induced channel stimulation by SRIF.

These results suggest that activation of G_i blocks the stimulatory effect of activated G_s.

Blockage of SRIF-induced Channel Inhibition by CTX

By contrast, SRIF did not block the increase in activity induced by CTX and CTX could sometimes reverse the inhibitory effect of SRIF (3 out of 5). The data presented in *A* of Fig. 10 illustrate the latter observation, showing that the inhibitory effect of SRIF (upper traces) is relieved upon addition of CTX (lower trace). This experiment is representative of 3 others in which addition of SRIF caused a 0.35 ± 0.18-fold reduction of channel activity as compared to control, while subsequent addition of CTX reversed this effect, channel behavior becoming equivalent to 1.81 ± 0.74 times control.

These data indicate that irreversible activation of G_s prevents channel inhibition of G_i.

To strengthen this hypothesis experiments were performed to investigate the effect of CTX on α_i/α_o-induced channel inhibition. The results presented in *B* of Fig. 10 show that addition of CTX (lower trace panel *B*) not only reverses the channel inhibition evoked by α_i (upper traces panel *B*) but in fact stimulated channel activity by more than 8.5-fold. On average CTX increased channel activity by 4.9 ± 1.6 fold in the presence of α_i/α_o subunits (n = 4).

This lack of effect of both SRIF and α subunits on CTX-induced K_{Ca} channel activation contrasts with the blockage of the stimulatory effect of norepinephrine and isoproterenol. Based on these data, it will be argued that activated G_i or G_o block K_{Ca} channel activity by preventing activation of G_s rather than blocking "directly" the channel.

Discussion

The focus of this study has been to investigate membrane-delimited interactions between G protein coupled receptors, protein kinase C (PKC) and the K_{Ca} channel. It is clear from the data that a complex relationship exists between them that may involve a number of regulatory steps. We will discuss several of these and propose a mechanism that integrates our observations. While the aim of this work was to provide evidence of channel control in insulin-secreting cells, it is clear that the present data offer insight into channel control in other cell systems.

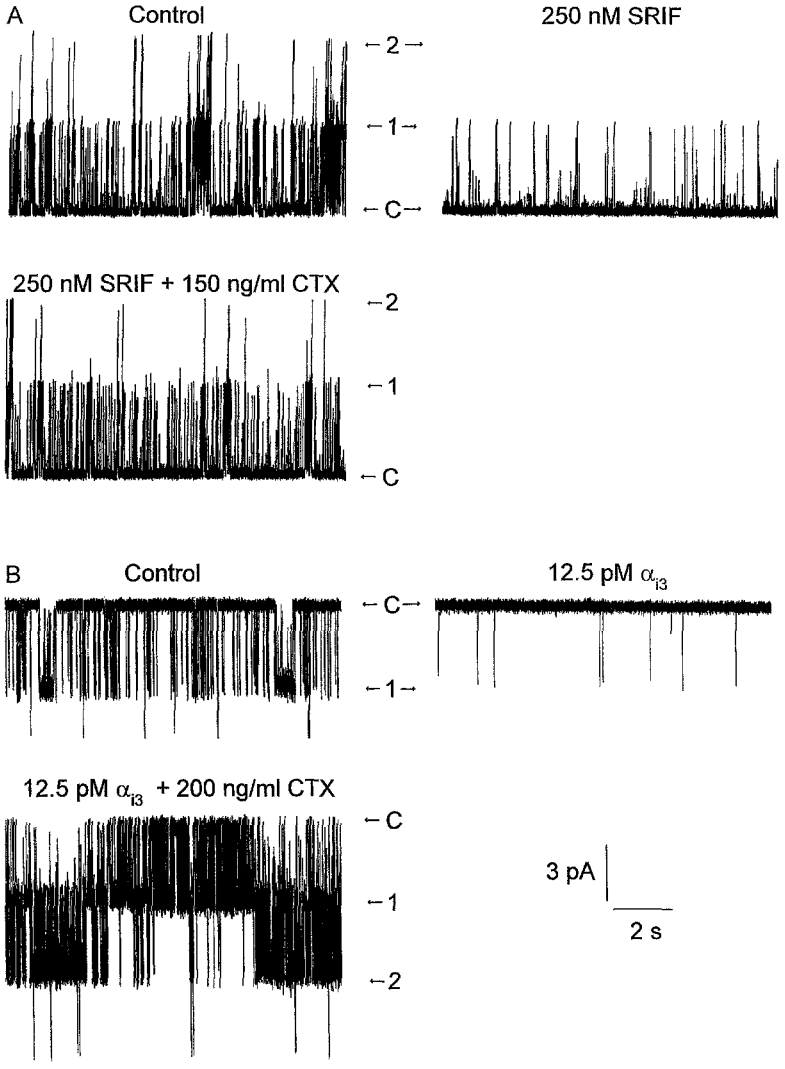


Fig. 10. Reversal of G_o/G_i-induced K_{Ca} channel inhibition by activated G_s in inside-out and outside-out patches. Data in *A* depict single channel outward currents recorded at a holding potential of 25 mV with symmetrical 140 mM K^+ and with 50 μM GTP plus 200 μM ATP in the pipette solution. The upper traces illustrate the inhibitory effect of SRIF-14 on channel activity. Control activity is shown in the upper left panel, and the upper right trace, recorded 6 min after addition of SRIF, demonstrates the inhibitory effect of the agonist. The recording shown in the lower trace was acquired 8 min after addition of the whole cholera toxin (A plus B subunits) to the bath, and illustrates the reversal of SRIF-induced channel inhibition by CTX. The data in *B* illustrate the reversal of α_i-induced channel inhibition by CTX. The three sets of single channel inward current recording were obtained in an inside-out patch of membrane held at −25 mV. The bath solution contained 50 μM GTP and 250 μM ATP. The upper traces illustrate the inhibitory effect of G_i α subunits. Control activity recorded in the presence of NAD^+ and prior to the addition of the α subunit is shown in the left trace. The right trace recorded five minutes after addition of α_{i3} demonstrates clear channel inhibition. The current recording shown in the lower trace was acquired seven minutes after addition of the A subunit of CTX to the bath, and illustrates the dramatic reversal of α_{i3}-induced channel inhibition by CTX.

Concerted effects of PTX-sensitive G proteins and PKC on K_{Ca} Channel Activity

The data presented in Figs. 1–3 identify a membrane-delimited pathway linking the SRIF receptor to the K_{Ca} channel. That SRIF suppresses channel activity in the excised outside-out patch establishes the existence of this pathway, while the observations that the effect of SRIF is blocked by PTX and mimicked by exogenous α_i/α_o in inside-out patches, indicate that α subunits of G_i and/or G_o mediate, at least in part, K_{Ca} channel inhibition by SRIF. The data presented in Figs. 6 and 7 also show that both the inhibitory effect of α subunits and of SRIF requires the presence of active PKC in the patch suggesting that a PKC-catalyzed phosphorylation and a PTX-sensitive G protein α subunit act synergistically to suppress K_{Ca} channel activity. Coordinated control of channel activity by G protein α subunits and PKC has been previously described with respect to the ATP-sensitive K channel in insulin secreting cells (Ribalet & Eddlestone, 1995), and L-type Ca channels in pituitary GH_4 cells (Gollasch et al., 1994).

K_{Ca} Channel Modulation by β-adrenergic Receptors and the Inhibitory Effect of SRIF

To better understand this mechanism of K_{Ca} channel inhibition, we investigated the identity of the target protein of PKC and of the G protein α subunits. One obvious target is the K_{Ca} channel itself as suggested for the L-type Ca channel (Gollasch et al., 1993). However, considering the data presented in Figs. 8–10, it appears that the receptor that activates the G protein G_s is a more likely target than the channel. It is clear from the data illustrated in Fig. 8 and obtained in excised patches using both β-adrenergic agonists and the irreversible activator of G_s, cholera toxin (CTX), that activation of G_s results

in stimulation of K_{Ca} channel activity. That exogenous α subunits of G_s proteins activate K_{Ca} channels in lipid bilayer suggests a direct G protein-K_{Ca} channel interaction (Scornik et al., 1994). Based on this observation it is postulated that the stimulation of K_{Ca} channel activity by β-adrenergic agonists and CTX observed in this study is also due to a direct effect of the α subunit G_s on the channel. Having established that G_s-coupled receptors stimulate K_{Ca} channel activity while G_i/G_o-linked receptors are inhibitory it was instructive to consider whether parallels exist with the mechanism of G protein-mediated control of adenylate cyclase. G_s-mediated activation of adenylate cyclase, whether accomplished by β-adrenergic stimulation or by CTX-induced ADP-ribosylation of α_s, may be suppressed by activation of G_i-coupled receptors. CTX activating irreversibly G_s, it was concluded that the inhibitory effect of G_i is not due to deactivation of G_s and thus to reduction of G_s stimulatory effect but rather to a direct and inhibitory interaction of α_i with the enzyme (*see* for review Birnbaumer et al., 1990). While, as shown in Fig. 9, SRIF was similarly able to suppress K_{Ca} channel stimulation evoked by β-adrenergic agonists, the data presented in Fig. 10 indicate that SRIF failed to suppress the stimulatory effect of CTX. This latter result suggests that, in contrast to the direct association of G_i/G_o with adenylate cyclase, these G proteins do not inhibit K_{Ca} channel activity directly but rather indirectly by reducing the stimulatory effect of G_s.

Role of PKC and G_i/G_o Subunits in the Deactivation of G_s by SRIF

That both PKC and activated G_i/G_o are necessary to mediate K_{Ca} channel inhibition raises the question of how these two elements interact to cause channel inhibition. Assuming that inhibition by PKC results from phosphorylation-mediated deactivation of G_s, two possible events could account for the effect of PKC; it may induce deactivation of G_s via direct phosphorylation of either the α subunit of G_s or of the β-adrenergic receptor. Studies of G protein phosphorylation have shown that the α subunit of G_i but not that of G_s is a target of PKC (Bushfield et al., 1990), suggesting that PKC-induced deactivation of G_s resulting from increased GTPase activity of α_s is unlikely. On the other hand, it is well established that PKC catalyzes phosphorylation of β-adrenergic receptors and that this leads to receptor desensitization and deactivation of G_s (*see* for review Sibley & Lefkowitz, 1985). It is therefore postulated that PKC-mediated K_{Ca} channel inhibition involves β-adrenergic receptor desensitization.

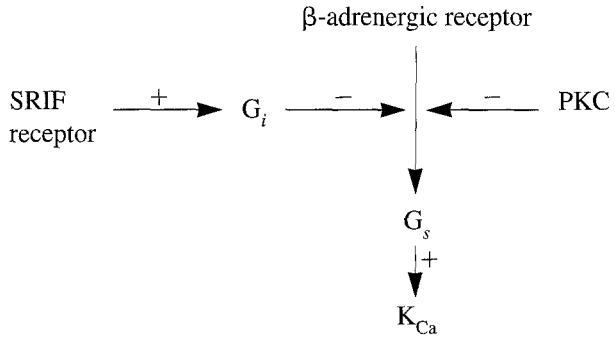
To understand more fully the signal pathway that integrates G_i/G_o and PKC it is necessary to determine how G_i/G_o could interact with PKC-induced β-adrenergic receptor desensitization. It can be easily assumed that activation of G_i/G_o results from the binding of SRIF to its receptor, but it is more difficult to identify the following events that lead to channel inhibition via deactivation of G_s. Consideration of published data suggests two possibilities, the G protein α subunit, via activation of phospholipase C (PLC), increases PKC activity (Gilman, 1987) which in turn causes deactivation of G_s via phosphorylation and desensitization of the β-adrenergic receptor, or alternatively PKC and G_i/G_o α subunits act in parallel to suppress β-adrenergic receptor activity. Evidence suggesting that PTX-sensitive activation of PtdIns-PLC involves most likely the $\beta\gamma$ dimer of G_i/G_o rather than the α subunit (*see* review Sternweis & Smrcka, 1992) mitigates the hypothesis whereby α_i/α_o act via activation of PKC. On the other hand, it has been suggested that PKC-induced β-adrenergic receptor desensitization reduces receptor occupancy by G_s while increasing that by G_i (Okamoto et al., 1991). Based on this observation, it may be hypothesized that G_i/G_o are instrumental in displacing G_s from the desensitized receptor and thus deactivating α_s.

It may be argued that our scheme predicts that SRIF-induced channel inhibition due to deactivation of G_s should only occur when β-agonists are present. However, β-adrenergic receptors are activated (Birnbaumer et al., 1990) and desensitized (Sibley & Lefkowitz, 1985) even in the absence of agonist. Thus, activated G_s proteins may have a tonic stimulatory effect on the K_{Ca} channel and channel inhibition by SRIF may be ascribed to deactivation of G_s even in the absence of β-agonist.

Direct Effect of G_i/G_o α Subunit on K_{Ca} Channels

Having earlier discounted a direct inhibitory role on the K_{Ca} channel for G_i/G_o α subunits, some of our data are suggestive of a direct stimulatory effect. Thus, addition of either SRIF or of purified α subunits often caused a rapidly developing and transient increase of K_{Ca} channel activity, and on some occasions the effect was sustained. Since stimulation was more pronounced when PKC activity was blocked, it is postulated that diffusion of PKC away from the patch of membrane is responsible for the sustained responses to SRIF and α subunits. Such a stimulatory effect may be accounted for assuming direct channel stimulation by α_i/α_o that would act synergistically with α_s. Alternatively, α_i/α_o may act to stimulate serine/threonine phosphatase activity as previously described by White et al., 1991, which may retard phosphorylation and desensitization of the β-adrenergic receptor and allow the stimulatory effect of G_s to be sustained.

In summary: K_{Ca} channel regulation by PKC and the G proteins G_s and G_i/G_o may be schematized as follows



Where β-adrenergic receptors activate the G protein G_s which has a direct stimulatory effect on the channel, PKC acts in concert with G_i/G_o to deactivate G_s and thus induce channel inhibition. In addition, G_i/G_o may also cause channel stimulation either directly or via phosphatase-induced dephosphorylation of the β-adrenergic receptor.

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