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Voltage-dependent calcium channel β-subunits in combination with α_1 subunits, have a GTPase activating effect to promote the hydrolysis of GTP by $G\alpha_o$ in rat frontal cortex

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Abstract The dihydropyridine-sensitive calcium channel agonist (−)-BayK 8644 was found to produce an enhancement of the intrinsic hydrolysis of GTP by G_o in rat frontal cortex membranes. An anti-calcium channel β-subunit antiserum abolished the (−)-BayK 8644-stimulated hydrolysis of GTP by G_o and reduced the dihydropyridine binding capacity of the cortical membranes. A peptide which mimics the β-subunit binding domain of the calcium channel complex, also attenuated (−)-BayK 8644 activation of GTPase. This study suggests that the calcium channel β-subunit is the principal component of the channel complex involved in linking dihydropyridine agonist binding to enhanced hydrolysis of GTP by G_o. This may be a mechanism by which calcium channels can normally act to limit the duration of a G-protein modulatory signal.

Key words: Calcium channel β-subunit; G-protein G_o; GTP hydrolysis

1. Introduction

Neuronal voltage-dependent calcium channels (VDCCs) have been shown to be modulated by a variety of neurotransmitters via an interaction with pertussis toxin (PTX) sensitive GTP-binding proteins (G-proteins) [1]. It has been suggested that the G_o subtype of G-protein may be pivotal in mediating the neurotransmitter inhibition of Ca^{2+} channel currents [2,3,4]. In many neuronal systems, neurotransmitters inhibit N and P/Q type VDCCs [1] with a smaller effect on L-type VDCCs [5,6]. L-type VDCCs are the primary target for modulation in neurosecretory [3] and non-neuronal secretory cells [7].

G-Proteins have also been found to modulate the interaction of dihydropyridine (DHP) agonists with L-type Ca^{2+} channels. The agonist effects of DHPs on dorsal root ganglion neuron Ca^{2+} currents is promoted upon activation of a PTX-sensitive G-protein [8] and GTP analogues have been shown to enhance DHP agonist binding to cortical synaptic membranes [9]. The L-type VDCC has a subunit composition of four non-covalently linked heterologous polypeptides (α_1, α_2-δ, β and γ) [10]. The α_1 subunit forms a functional ion pore which binds DHPs [11] and can be modulated by binding of the VDCC β subunit [12,13,14]. By the use of antisense oligonucleotides complementary to the mRNA of all four cloned VDCC β-subunits we have also revealed the importance of endogenous β-subunits in the amplitude, activation kinetics, DHP modulation [15] and G-protein modulation [6] of Ca^{2+} channel currents in cultured dorsal root ganglion neurones.

G-Protein α-subunits have an intrinsic GTPase activity which hydrolyses GTP, bound to the activated form of the G-protein α-subunit, to GDP, thereby inactivating and recycling the G-protein [16]. Activation of a G-protein linked neurotransmitter receptor results in an increase in GTPase activity due to an increased exchange of GDP for GTP [17,18]. However, GTPase can also be potentiated by an effector protein acting as a GTPase-activating-protein (GAP) to stimulate the intrinsic GTPase activity [19,20,21]. It has been shown previously that the GTPase activity of the G-protein G_o can be stimulated by a number of DHP agonists [22]. Using an anti-peptide anti-VDCC β-subunit antiserum and a peptide, which mimics the β subunit binding site on the VDCC α_1-subunit [23], we now provide evidence that the VDCC β-subunit is the principal component of the L-type calcium channel involved in linking DHP agonist binding with enhanced GTPase activity of the L-type VDCC associated G-protein.

2. Materials and methods

2.1. GTPase assay

The frontal cortex membrane preparation and GTPase assay were performed as described previously [22].

2.2. [^{3}H]PN200 110 binding

200 μl of membrane suspension and 200 μl of either H_2O or 10 μM nicardipine (to assess non-specific binding) were added in duplicate to 5 ml tubes containing 500 μl of binding buffer of the following composition (mM): NaCl 132, KCl 5, $CaCl_2$ 1.2, $MgCl_2$ 1.3, glucose 10, Tris-hydoxymethylaminomethane 25; adjusted to pH 7.4 with HCl. This binding buffer also contained [^{3}H]PN200 110 (Amersham) at a range of concentrations (nM): 0.5, 1.0, 2.0, 5.0, 10.0 in order to determine B_{max} and K_d using Scatchard transformation analysis. Following incubation at 25°C for 60 min the reaction was terminated by filtration through GF/B filters (Whatman). The filters were washed with 3 × 3 ml volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) and then counted for ^{3}H content in a liquid scintillation counter in a 2 ml volume of scintillation fluid (Hionic-Fluor). All experiments using DHPs were performed using sodium lighting.

2.3. Incubation of membranes with antisera

Membranes were incubated at 30°C for 60 min with either VDCC β-subunit antiserum (dilution 1:50), VDCC α2-subunit antiserum (dilution 1:50) or corresponding pre-immune antisera (dilution 1:50). The β-subunit antiserum has been characterised previously [15] and the α2-subunit antiserum characterisation is described in [24]. An antipeptide anti-G protein antibody which recognised the peptide sequence ANNLRGCGLY on the α subunit of G_o was raised, affinity-purified and characterised using the procedures described in [4]. The affinity-

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Abbreviations: DHP, dihydropyridine; PTX, pertussis toxin; VDCC, voltage-dependent calcium channel.

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purified anti-G_i antibody was also raised and characterised as described in [4]. Membranes were incubated at 30°C for 60 min with the anti-G-protein antibodies (50 μg/ml) or control rabbit IgG (50 μg/ml).

2.4. Production of VDCC β-subunit site peptide

The peptide sequences used in this study were synthesized and purified using previously described procedures [25,26]. The VDCC β-site peptide used in this study had the sequence: QQLEEDLKGYLDWI, which is homologous to the VDCC β-α_1 interaction site described in [23]. This peptide sequence corresponds to amino acids 428–441 in the rat brain α_{1C} and α_{1D} calcium channel clones, which are located on the cytoplasmic loop linking transmembrane domains I and II. The control scrambled peptide had the sequence: GYLLEDEKWILDQQ; this peptide has no significant homology with any other peptide sequence in the Genbank/EMBL databases.

The membranes were incubated with the peptides (20 μg/ml; 10 μM) for 1 h at 30°C prior to the experiment.

2.5. Transient calcium channel subunit expression in COS-7 cells

COS-7 cells were grown to confluency in αMEM (Gibco/Life) containing 10% newborn calf serum. Cells were transfected with approximately 20 μg of the appropriate pMT2 construct by electroporation (ElectroporatorII-Invitrogen) in 500 μl of phosphate-buffered saline (PBS), pH 7.4. Three days after transfection the cells were harvested in homogenising buffer (10 mM HEPES, pH 7.4, 10% w/v sucrose, 1 mM EDTA, 1 mM PMSF, 25 trypsin inhibitor units/ml aprotinin, 50 μM leupeptin, 0.1 mg/ml soy bean trypsin inhibitor) at 4°C. Membrane preparation, electrophoresis and immunoblotting were performed essentially as previously described [15].

3. Results and discussion

Binding of the agonist isomer of (−)-BayK 8644 [27] to the L-type calcium channel caused an increase in high affinity GTPase activity in the rat frontal cortex (Fig. 1). The maximally effective concentration was 10–100 nM which produced approximately 20% stimulation of GTPase. In the present study, the weak antagonist isomer, (+)-BayK 8644 [27], was unable to induce any stimulation of GTP hydrolysis (a maximal 3.72 ± 0.63% stimulation of GTPase being observed at 100 nM; Fig. 1). From this we conclude that the GTPase stimulation induced by DHPs occurs when the L-type calcium channel is in the DHP agonist bound conformation. The present study supports the finding of [22,28], which showed an enhancement of GTPase by racemic BayK 8644. It was also found [22] that the stimulation of GTPase by DHP agonists was due to a stimulation of the intrinsic GTPase activity of the G-protein and not due to an increase in the rate of guanine nucleotide exchange, which was the case for $GABA_B$-ergic stimulation of GTP hydrolysis.

We examined the effect of affinity-purified anti-G-protein antibodies, specific to the α subunit of either G_i or G_o, on the DHP agonist and $GABA_B$ receptor mediated enhancement of GTPase. The specificity of the G-protein antibodies used in this study has been confirmed previously [4]. Treatment of the cortical membrane with the anti-G_o antibody caused an 84 ± 5% reduction in the stimulation of GTPase by the DHP agonist (Fig. 2A), the reduction in GTPase activity was prevented by preadsorption of the anti-G_o antibody with its immunising peptide (500 μg/ml, 1 h, 30°C); the stimulation of GTPase by (−)-BayK 8644 was then 15 ± 1.8% (n = 6). The anti-G_i antibody had no effect on DHP agonist-stimulation of GTPase (Fig. 2A). In contrast, $GABA_B$ receptor stimulation of high affinity GTPase was reduced by 89 ± 4% and 41 ± 5% following treatment with the anti-G_i antibody and anti-G_o antibody, respectively (Fig. 2B), indicating that the $GABA_B$ receptor can

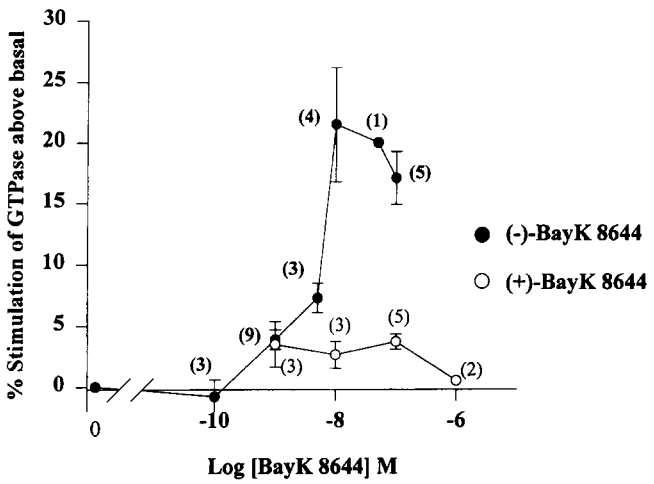

Fig. 1. A concentration–response curve for the stimulation of GTPase by the L-type calcium channel activator (−)-BayK 8644 and L-type calcium channel antagonist (+)-BayK 8644. Basal GTPase activity was 33 ± 6 pmol/mg/min (n = 9) and stimulation by (−)-BayK 8644 (●) or (+)-BayK 8644 (○) is expressed as % stimulation of GTPase above basal activity. Results are presented as mean ± S.E.M. and the number of experiments is given in parentheses.

interact with both G_i and G_o in this preparation. Preadsorption of the anti-G-protein antibodies with their respective immunising peptides (500 μg/ml, 1 h, 30°C) specifically prevented the inhibition of the $GABA_B$ergic-stimulation of GTPase (21 ± 2.1%, n = 6 and 22 ± 3%, n = 7 stimulation of GTPase by (−)-baclofen was observed following pretreatment of the anti-G_i and anti-G_o antibodies, respectively, with their immunising peptides). $GABA_B$ receptors have also been reported to couple to both G_o and G_{i1} in bovine brain [29]. However, it would appear that only the coupling of $GABA_B$ receptors via G_o is involved in the inhibition of calcium channel currents [4,30].

The present study, and that of [22], have shown that the L-type calcium channel, in its DHP agonist bound conformation, promotes the GTPase activity exclusively of G_o. It is possible that in intact polarised systems a significant proportion of L-type VDCCs are normally in this conformation. Since G_o is the principal G-protein involved in mediating the neurotransmitter modulation of the calcium channel current, this enhanced GTPase activity may be the mechanism by which the channel can deactivate such a modulatory signal. A similar mechanism exists for deactivation of the modulatory effects of $G_{q/11}$, induced upon activation of muscarinic receptors [21]. Those authors demonstrated an enhanced GTP hydrolysis of $G_{q/11}$ by its effector protein, phospholipase C. It has also been reported that the GTPase activity of transducin, the photoreceptor G-protein, is promoted by its effector cGMP phosphodiesterase [19,20].

By using an antipeptide antibody raised against the VDCC β-subunit we have found that the VDCC β-subunit has a role in coupling DHP agonist binding to the enhanced GTPase activity of G_o. The anti VDCC β-subunit antiserum used in this study was raised against a VDCC β-subunit peptide as previously described [15]. The anti-β-subunit antiserum recognised β_{1b}, β_3 and β_4 overexpressed in COS cells (Fig. 3A). In the presence of preimmune serum, Eadie-Hofstee analysis showed a 31 ± 6% increase in GTPase V_{max} with no change in K_m for GTP by 10 nM (−)-BayK 8644 (Fig. 3B). However, this stimu-

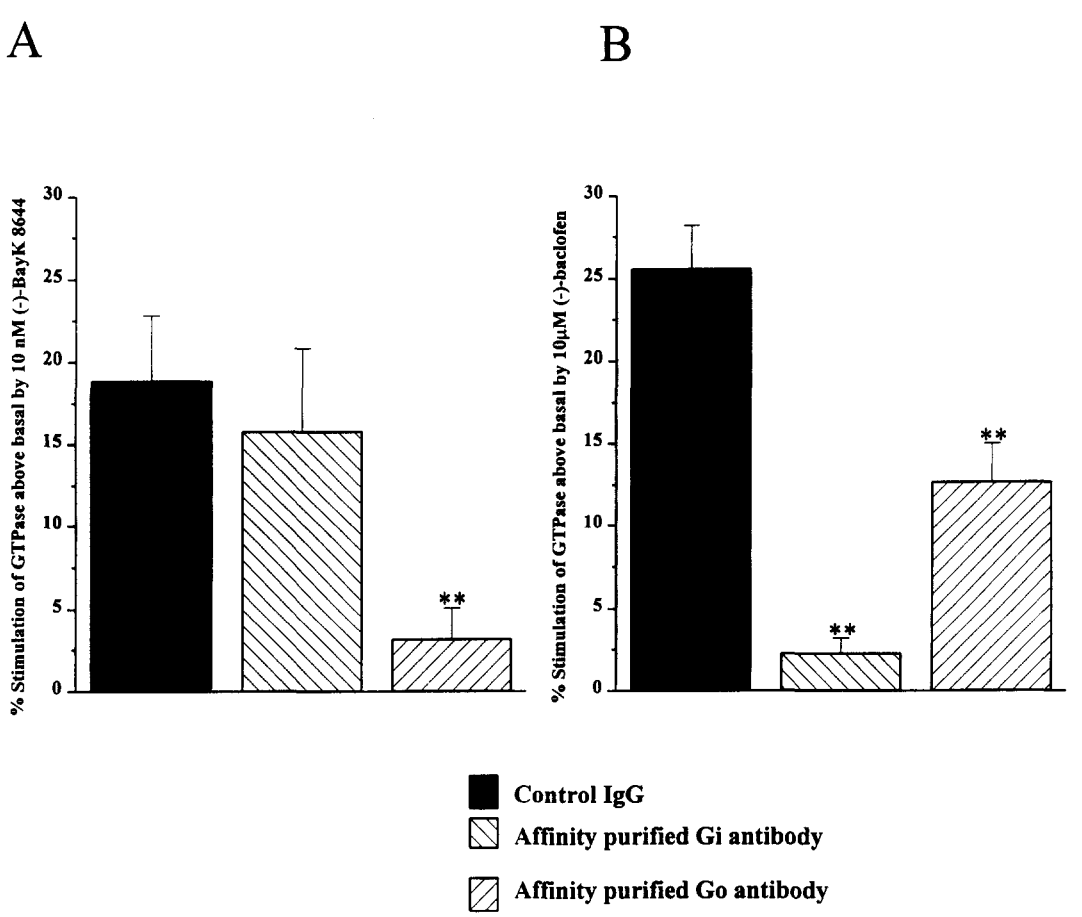

Fig. 2. Effect of anti G-protein antibodies on (−)-BayK 8644 and (−)-baclofen stimulation of GTPase. (A) An affinity purified anti-G_i antibody (50 μg/ml) (▨) had no effect on the ability of (−)-BayK 8644 to stimulate GTPase. Stimulation of GTPase by (−)-BayK 8644 was reduced by 84 ± 5% when the membranes were pretreated with an affinity-purified anti-G_o antibody (50 μg/ml) (▨) ($n = 6$, $^{**}P < 0.01$, paired t-test compared to membranes treated with control rabbit IgG (■). (B) Baclofen-stimulated GTPase was attenuated by 89 ± 4% and 41 ± 5% when the membranes were treated with affinity-purified anti-G_i (▨) and anti-G_o (▨) antibodies respectively ($n = 8$, $^{**}2P < 0.01$, paired t-test compared to membranes treated with control rabbit IgG).

lation of GTP hydrolysis by the DHP agonist was abolished following incubation with the VDCC β-subunit antiserum (Fig. 3B,C). Following preadsorption of the anti-β-subunit antiserum with its immunising peptide, no attenuation in (−)-BayK 8644-stimulation of GTPase was observed (Fig. 3C).

We have reported that the VDCC β-subunit has a role in the G-protein mediated inhibition of the calcium channel current in DRGs [6]. The inhibition of the calcium channel current by the $GABA_B$ agonist (−)-baclofen was potentiated following antisense oligonucleotide-depletion of VDCC β-subunits from these cells. This suggests that in native cells the VDCC β-subunit may act to limit, possibly by competition for a binding site, the extent of the interaction between the VDCC α_1-subunit and G_o [6]. This may be facilitated by the VDCC α_1-β-subunit complex stimulating hydrolysis of GTP-G_o to GDP-G_o, and thus limiting the temporal effectiveness of the G-protein modulating signal.

The primary binding site of the VDCC β-subunit on the α_1-subunit is a conserved motif in the I-II cytoplasmic linker of the α_1-subunit [23], termed the α_1-subunit interaction domain (AID) [Witcher, D.R., De Waard, M., Liu, H.Y. and Campbell, K.P. (1995) Biophys. J. 68, M-AM-H2]. The β-site peptide sequence used in this study is homologous to the AID. The synthetic β-site peptide should thus compete with the AID for interaction with the β-subunit, thereby disrupting the association between the VDCCα_1-subunit and its cytoplasmic β-subunit. The use of this peptide in the GTPase assay system abolished the stimulation of GTP hydrolysis by (−)-BayK 8644 (Fig. 4A) but had no effect on the $GABA_B$-mediated stimulation of GTPase (% stimulation of GTPase by 10 μM (−)-baclofen was 30.8 ± 3.0% and 24.0 ± 2.5% in the presence of the control scrambled peptide and β-site peptide, respectively; Fig. 4B). This result suggests that the VDCC β-subunit must be associated with the L-type VDCC α_1-subunit in order to permit the stimulation of GTPase by DHP agonists. This reinforces the hypothesis that the β-subunit is a prerequisite for coupling DHP agonist binding to increased GTP hydrolysis.

The VDCC β-subunit is involved in modulating DHP binding to the VDCC α_1-subunit. In several studies co-expression of the skeletal muscle VDCC β-subunit with the α_1-subunit enhanced high affinity binding of the DHP antagonist ligand [^{3}H]PN200 110 to the α_1-subunit [31,32,33] although the β-subunit did not appear to increase the expression levels of VDCCα_1-subunit protein [34,35]. In the present study we have observed a 30% reduction in high affinity [^{3}H]PN200 110 binding sites with no change in the affinity for DHPs

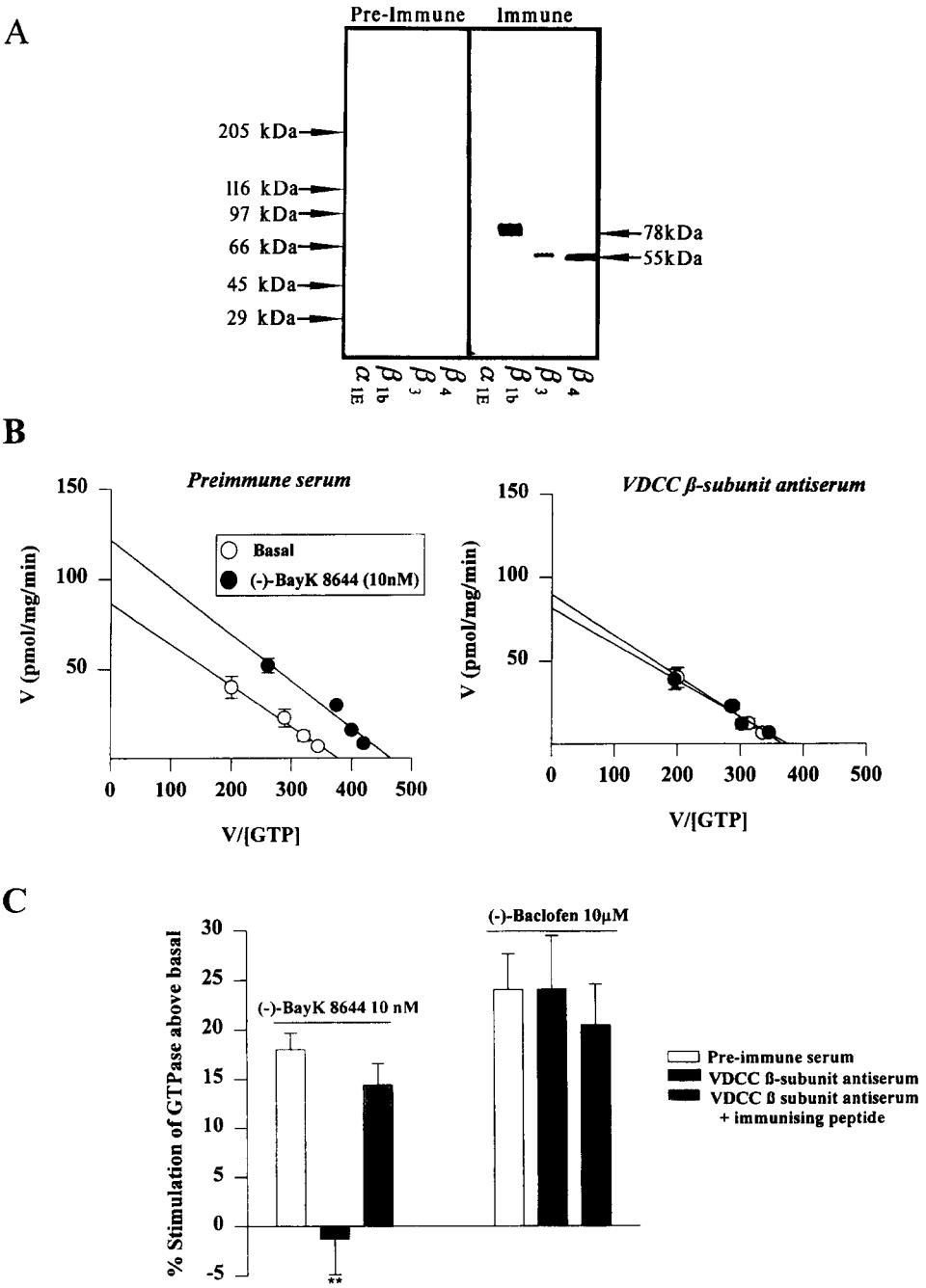

Fig. 3. Effect of VDCC β-subunit antiserum or pre-immune serum on (−)-BayK 8644 and (−)-baclofen-stimulation of GTPase A. Membranes from COS-7 cells transiently expressing either calcium channel α_{1E} as a control [36], β_{1b} [37], β_3 or β_4 [38,39] subunits, as labelled, were immunoblotted with pre-immune serum (left-hand panel) or the anti-β-subunit antiserum β2491 (right-hand panel) at 1:2000 dilution. Positions of molecular mass markers are shown. (B) Following incubation with preimmune serum basal (○) GTPase V_{max} was increased from 91 ± 5 pmol/mg/min to 120 ± 8 pmol/mg/min by 10 nM (−)-BayK 8644 (●) ($P < 0.01$, paired t-test). There was no effect of (−)-BayK 8644 on K_m for GTP (basal K_m = 0.24 ± 0.01 M, K_m in (−)-BayK 8644-stimulated membranes was 0.23 ± 0.02 M). In the presence of anti-VDCC β-subunit antiserum basal GTPase V_{max} was 88 ± 9 pmol/mg/min (basal K_m = 0.24 ± 0.01 M), the subsequent addition of 10 nM (−)-BayK 8644 had no effect on V_{max} (78 ± 10 pmol/mg/min) or on K_m for GTP (0.23 ± 0.007 M) (n = 9). (C) Stimulation of GTPase (at 0.5 M GTP) by 10 nM (−)-BayK 8644 was abolished by pretreatment of the membrane with anti-VDCC β-subunit antiserum (dilution 1:50) (■) (n = 20, **$2P < 0.01$, paired t-test compared to membranes treated with control preimmune serum (□). In contrast, stimulation of GTPase by the $GABA_B$ agonist (−)-baclofen (10 μM) (24.0 ± 3.6%) was unaffected by pretreatment of the membrane with anti-VDCC β-subunit antiserum (24.02 ± 5.42%) (■) (n = 14). Preadsorption of the anti-β-subunit antiserum with its immunising peptide (■), (100 μg/ml, 1 h, 30°C) prevented its effect on (−)-BayK 8644 (10 nM) stimulation of GTPase (14.4 ± 2.15%, n = 5) and had no effect on the stimulation of GTPase by (−)-baclofen (10 μM), (20.4 ± 4.17%, n = 5).

(K_d = 2.00 ± 0.38 nM, n = 8) following treatment of the cortical membranes with anti-VDCC β-subunit antiserum (Fig. 5). A

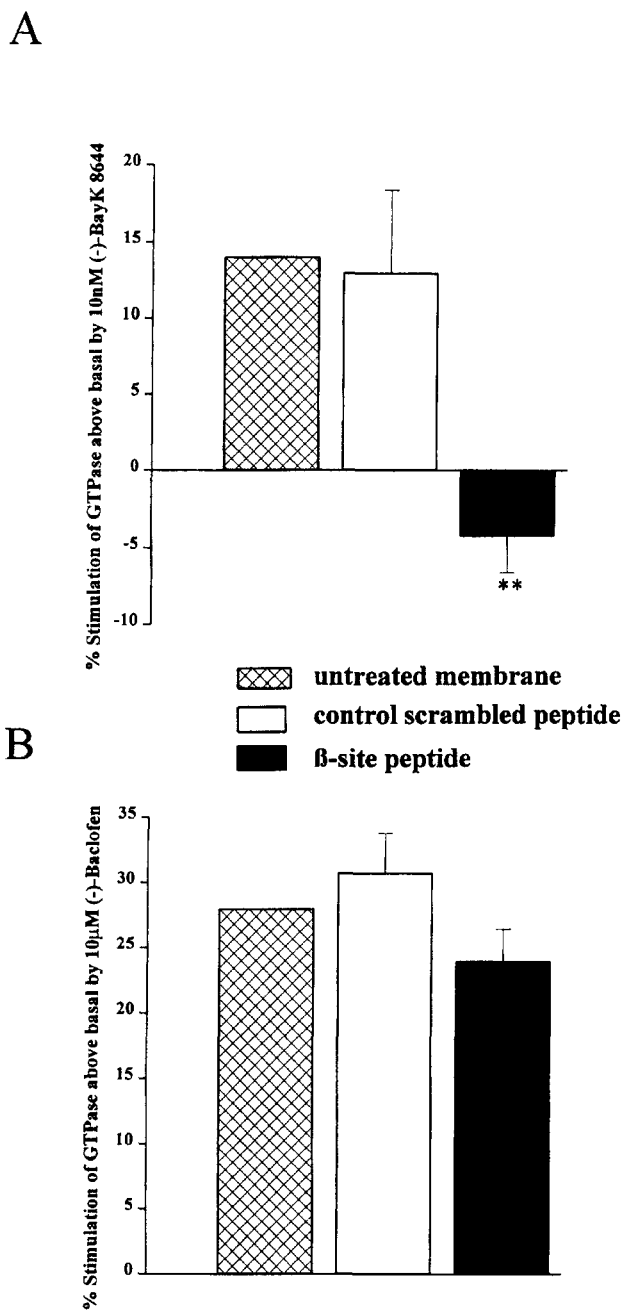

Fig. 4. Effect of a VDCC β-subunit binding site peptide on (−)-BayK 8644 and (−)-baclofen-stimulation of GTPase. (A) Pretreatment of membranes for 1h with the VDCC β-site peptide (■) (20 μg/ml) abolished the ability of (−)-BayK 8644 (10 nM) to stimulate GTPase (at 0.5μM GTP) (n = 10, $^{**}P < 0.01$, paired t-test compared to membranes treated with a control scrambled peptide sequence (□) (20 μg/ml). There was no difference in (−)-BayK 8644 stimulation of GTPase between control non-treated membranes (▩) (11%, 17% stimulation of GTPase, n = 2.) and scrambled peptide treated membranes (12.95 ± 5.4% stimulation of GTPase, n = 10). (B) Stimulation of GTPase by (−)-baclofen (10 μM) is 30.08 ± 3.02% following treatment with the control scrambled peptide and is unaffected by the β-site peptide (24.0 ± 2.5% stimulation of GTPase, n = 10). There was no difference in (−)-baclofen (10 μM) stimulation of GTPase between control non-treated membranes (25%, 33% stimulation of GTPase, n = 2) and scrambled peptide treated membranes.

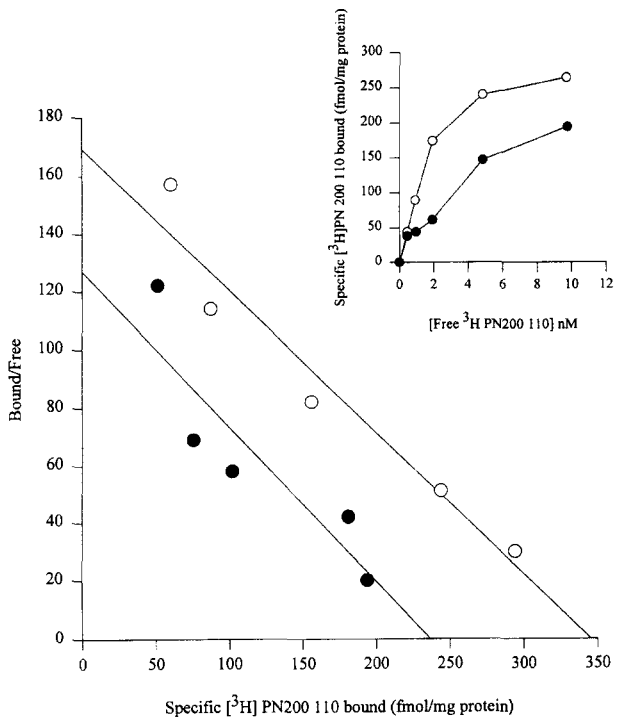

Fig. 5. Effect of VDCC β-subunit antiserum on binding of [^{3}H]PN200 110 to frontal cortex Scatchard transformation of specific [^{3}H]PN200 110 binding to frontal cortex membranes following pretreatment of the membrane with preimmune serum (dilution 1:50) (○) and anti-VDCC β-subunit antiserum (dilution 1:50) (●). In the presence of preimmune serum, B_{max} = 346 ± 29 fmol/mg protein, K_d = 2.4 ± 0.35 nM (n = 8). Incubation with anti-VDCC β-subunit antiserum resulted in a 30 ± 6% reduction in B_{max} (237 ± 47 fmol/mg protein) with no change in the apparent K_d (2.0 ± 0.38 nM) n = 8. Inset: competition binding curve for [^{3}H]PN200 110 binding to cortex membrane in the presence of preimmune serum (○) and anti-VDCC β-subunit antiserum (●).

reduction in the number of high affinity DHP binding sites could contribute to the observed decrease in DHP agonist-stimulated GTPase following incubation with the VDCC β-subunit antiserum. However, since the DHP agonist-mediated inhibition of GTP hydrolysis was completely abolished by this antiserum while the number of available DHP binding sites was only partially reduced, we propose that the decrease in DHP agonist-stimulation of GTPase by VDCC β subunit antiserum is at least in part downstream of agonist binding.

The findings presented in this study indicate that the VDCC β-subunit has a role in modulating the DHP binding site on the VDCC α1-subunit in the rat frontal cortex membranes. The VDCC β-subunit is also the principal subunit involved in the coupling of DHP agonist binding to enhanced GTPase of the G-protein G_o. The proclivity of the VDCC β-subunit to stimulate hydrolysis of G_o may represent a mechanism by which the effector calcium channel deactivates the G-protein modulating signal.

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