

Evidence that a protein–protein interaction ‘hot spot’ on heterotrimeric G protein $\beta\gamma$ subunits is used for recognition of a subclass of effectors

Jamie K.Scott¹, Shan-Fu Huang²,
Beechanahalli P.Gangadhar¹,
Gary M.Samoriski^{2,3}, Peter Clapp⁴,
Robert A.Gross^{2,3}, Ronald Taussig⁴ and
Alan V.Smrcka^{2,5}

¹Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada, V5A 1S6, ²Departments of Pharmacology and Physiology and ³Neurology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642 and ⁴Department of Biochemistry, University of Michigan, Ann Arbor, MI 48109, USA

⁵Corresponding author
e-mail: Alan_Smrcka@urmc.rochester.edu

To understand the requirements for binding to G protein $\beta\gamma$ subunits, phage-displayed random peptide libraries were screened using immobilized biotinylated $\beta\gamma$ as the target. Selected peptides were grouped into four different families based on their sequence characteristics. One group (group I) had a clear conserved motif that has significant homology to peptides derived from phospholipase C β (PLC β) and to a short motif in phosducin that binds to G protein β subunits. The other groups had weaker sequence homologies or no homology to the group I sequences. A synthetic peptide from the strongest consensus group blocked activation of PLC by G protein $\beta\gamma$ subunits. The peptide did not block $\beta\gamma$ -mediated inhibition of voltage-gated calcium channels and had little effect on $\beta\gamma$ -mediated inhibition of Gs-stimulated type I adenylyl cyclase. Competition experiments indicated that peptides from all four families bound to a single site on $\beta\gamma$. These peptides may bind to a protein–protein interaction ‘hot spot’ on the surface of $\beta\gamma$ subunits that is used by a subclass of effectors.

Keywords: G protein $\beta\gamma$ subunits/G protein effectors/peptide/phage display/protein–protein interaction

Introduction

Heterotrimeric G proteins, consisting of multiple isoforms of distinct α , β and γ subunits, mediate the actions of a wide variety of cell surface receptors involved in processes ranging from phototransduction to neurotransmission (Hildebrandt, 1997; Sprang, 1997; Gutkind, 1998; Hamm, 1998). G protein-coupled receptors catalyze exchange of tightly bound GDP for GTP on the α subunit in a process that requires all three subunits. The binding of GTP results in activation of the G protein and dissociation of the α subunit from the $\beta\gamma$ subunits. The α and $\beta\gamma$ subunits both interact with effector molecules, such as phospholipases and ion channels, in a manner that leads to their activation or inhibition (Clapham and Neer, 1997).

A variety of *in vitro* studies have shown that when $\beta\gamma$ subunits are bound to α -GDP they are incapable of activating downstream effectors. Thus, activation and deactivation of $\beta\gamma$ subunit-mediated signal transduction in cells are thought to rely on dissociation and reassociation of GTP- and GDP-bound α subunits, respectively.

G protein $\beta\gamma$ subunit-mediated activation of effectors has diverse roles in the regulation of cell physiology. Some examples of the cellular processes regulated by $\beta\gamma$ subunits are briefly described here. In excitable cells, including neurons and cardiac myocytes, $\beta\gamma$ subunits that are released from G_i regulate inwardly rectifying K⁺ channels, so as to modulate membrane potential or heart rate (Clapham and Neer, 1997). Chemokine receptors, such as the interleukin-8 receptor and the co-receptors for entry of the AIDS virus into leukocytes, are coupled to the release of $\beta\gamma$ subunits from G_i (Kuang *et al.*, 1996; Littman, 1998). Recent evidence indicates that $\beta\gamma$ -responsive phospholipase C (PLC) isoforms β 2 and β 3 play inhibitory roles in cell signaling. Mouse neutrophils, where $\beta\gamma$ -responsive PLC β 2 was eliminated by gene targeting, displayed increased chemotaxis in response to chemotactic peptides and the mice were more resistant to viral infection (Jiang *et al.*, 1997). In transgenic mice lacking $\beta\gamma$ -regulated PLC β 3, morphine acting at G_{i/o}-linked opioid receptors produced pain-killing effects at much lower doses, correlating with greater inhibition of voltage-gated Ca²⁺ channels in dorsal root ganglion neurons (Xie *et al.*, 1999). Activation of multiple G_i- and G_q-coupled receptors, including thrombin, lysophosphatidic acid (LPA) and acetylcholine receptors, results in a mitogenic response in several cell types. MAP kinases are critical components in the growth-promoting pathways regulated by these receptors. $\beta\gamma$ subunits indirectly activate MAP kinase, suggesting that $\beta\gamma$ subunits may mediate the growth-promoting effects of many G protein-coupled receptors (Gutkind, 1998).

The physiological processes regulated by $\beta\gamma$ subunits are mediated by interactions between $\beta\gamma$ subunits and a wide variety of diverse target molecules, ranging from inwardly rectifying K⁺ channels to soluble enzymes such as PLC (for review see Clapham and Neer, 1997). How $\beta\gamma$ subunits regulate such a diverse range of effector molecules is not well understood, and a complete picture of how $\beta\gamma$ subunits interact with various targets has not been forthcoming. Small synthetic peptide-based approaches have been used to dissect $\beta\gamma$ -effector interactions. One such study used a peptide representing amino acids 956–984 of adenylyl cyclase type 2 (QEHA peptide) (Chen *et al.*, 1995). This peptide blocked activation of a number of effectors by $\beta\gamma$ subunits, including adenylyl cyclase 2, PLC β 3, G protein-gated inwardly rectifying potassium channels (GIRK) and β -adrenergic receptor kinase (β ARK) with an IC₅₀ of

Table I. Phage-displayed peptide libraries that were screened against G protein $\beta\gamma$ subunits

Linear libraries	Disulfide-bridged-loop libraries with one-residue flanking regions: LXn	Disulfide-bridged-loop libraries with 4-5-residue flanking regions: Cys	Half-Cys libraries (having only one fixed Cys):	α -conotoxin library
1) X ₆ 2) X ₁₅ 3) X ₃₀	1) LX4 = XCX ₄ CX 2) LX6 = XCX ₆ CX 3) LX8 = XCX ₈ CX 4) LX10 = XCX ₁₀ CX 5) LX12 = XCX ₁₂ CX	1) Cys3 = X ₅ CX ₃ CX ₄ 2) Cys4 = X ₄ CX ₄ CX ₄ 3) Cys5 = X ₄ CX ₅ CX ₄ 4) Cys6 = X ₄ CX ₆ CX ₄	1) X8CX8 2) X15CX 3) X15CX	XCCX ₃ CX ₅ C ₄ GIEG RG

In all cases, the libraries outlined above were fused to the N-terminus of the pVIII coat protein of the phage display vector f88-4. X stands for any of the 20 natural amino acids.

50–100 μ M, and bound at a site overlapping the α subunit binding site on $\beta\gamma$ (Weng *et al.*, 1996). These data suggest that the peptide binds to a site shared by multiple effectors. Based on amino acid substitutions in the peptide and sequence comparison amongst adenylate cyclase, β ARK and GIRK-1, the authors proposed a consensus $\beta\gamma$ -binding sequence, QXXER. However, many proteins are regulated by $\beta\gamma$ subunits but do not contain the QXXER motif [including phosphoinositide 3-kinase γ (PI3K γ) and PLC β 2], so this may not be a universal motif.

As an alternative approach to defining requirements for binding to G protein $\beta\gamma$ subunits, we have screened a panel of phage-displayed random peptide libraries to identify peptides that bind to $\beta\gamma$ subunits. Using this approach we identified multiple peptides that bind specifically to $\beta\gamma$ subunits. This has allowed us to further characterize the sequence requirements for binding to physiologically relevant sites on the surface of $\beta\gamma$ subunits.

Results

Panning

To determine the types of amino acid sequence that can interact with $\beta\gamma$ subunits, phage-displayed random peptide libraries were screened with $\beta_1\gamma_2$ that was bound to immobilized streptavidin via covalently attached biotin. We prepared biotinylated $\beta\gamma$ subunits (b- $\beta\gamma$) using a modification of a previously published protocol (see Materials and methods) (Dingus *et al.*, 1994). A key feature of this method is that $\beta\gamma$ is chemically modified with an amine-specific reagent in the presence of α subunits to protect sites that are critical for protein-protein interactions. b- $\beta\gamma$ is then purified on the basis of its ability to dissociate upon activation of α subunits with aluminum fluoride (Kozasa and Gilman, 1995). This procedure ensures that b- $\beta\gamma$ is functional. The b- $\beta\gamma$ prepared in this way activates PLC β 2, PLC β 3 and PI3K similarly to unmodified $\beta\gamma$ (not shown). Previous work by others indicates that $\beta\gamma$ subunits modified in this way can interact normally with α subunits (Kohnken and Hildebrandt, 1989; Dingus *et al.*, 1994), adenylate cyclase (Kohnken and Hildebrandt, 1989) and phosducin (Satpaev and Slepak, 2000). This indicates that binding sites on $\beta\gamma$ subunits, critical for interactions with α subunits and effectors, were available for interaction with phage-displayed peptides.

We screened 16 different phage-displayed libraries of various lengths, some of which had various constraints

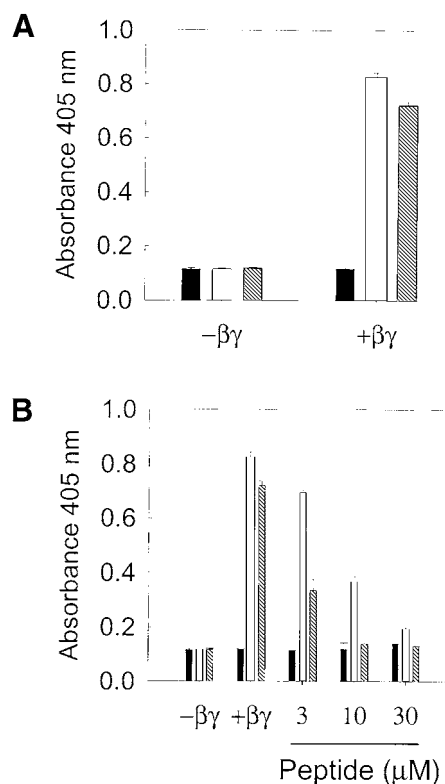


Fig. 1. Phage bearing selected peptides bind specifically to immobilized $\beta\gamma$ subunits and binding is inhibited by a synthetic peptide corresponding to one of the selected sequences. (A) 1×10^{10} f88-4 (black bars), P1 (white) or P21 (dark gray bars) phage were incubated in wells coated with streptavidin with or without prior addition of 50 nM b- $\beta\gamma$. (B) Binding of the same phage to 50 nM b- $\beta\gamma$ was tested at the indicated concentrations of SIKK peptide.

imposed by internal disulfide linkages (Bonnycastle *et al.*, 1996) (Table I). The peptides were displayed as fusions with the pVIII coat protein of the f88-4 phage display vector; $\sim 10\%$ of the coat proteins expressed have peptides fused (~ 250 copies of peptide/phage particle). Each library has a diversity of 1×10^8 – 1×10^9 individual clones. All screening was performed in the presence of detergent, bovine serum albumin (BSA) and salt to minimize non-specific interactions.

Multiple binding clones were obtained from several libraries and were characterized in various ways for binding specificity. For Figure 1A, two selected phage clones were compared with f88-4 in a phage enzyme-

Table II. Binding of phage bearing disulfide-constrained peptides to $\beta\gamma$ in a phage ELISA is inhibited by reduction of disulfides

		A_{405}		
		$-\beta\gamma$	$+\beta\gamma^a$	$+\beta\gamma +10 \text{ mM DTT}^b$
I	ACKRTKAQILLAAPCT	0.129	0.595	0.187
	SIRKALNILGYPDYD	0.118	0.245	0.299
	SCEQTKDRLLGNAC	0.121	0.259	0.143
	ACTLPGKPYSLLGIC	0.121	0.492	0.178
II	TCQKLAWLTGKKEKCL	0.129	0.744	0.330
III	SCEKRYGIEFCT	0.122	0.348	0.159
	SCEKRLGVRSC	0.128	0.717	0.150
IV	PTAVCNFFGQCPMEI	0.116	0.560	0.147
	PSKVCAHFIDICYTLS	0.125	0.275	0.151

^aNinety nanograms of $\beta_1\gamma_2$ were immobilized in each well. Binding of phage was tested in a phage ELISA assay (see Materials and methods). Results are the mean A_{405} of duplicate determinations measured in a microplate reader. Similar experiments were performed three times.

^bDTT (10 mM) was included in the binding solution during incubation with the indicated phage.

linked immunosorbent assay (ELISA) in which binding of phage was detected with a phage-specific antibody and absorbance at 405 nm was monitored after addition of a chromogenic substrate. Absorbance values for wild-type f88-4 phage in the absence of $\beta\gamma$ represent non-specific binding of the phage (and/or the anti-phage antibody) to the plate and can be considered as background. In the presence of $\beta\gamma$ subunits, f88-4 binding was not increased, indicating that the wild-type f88-4 phage did not bind to $\beta\gamma$. Binding of phage bearing the selected peptides in the absence of immobilized $\beta\gamma$ was not significantly different from that for f88-4 (Figure 1A), indicating that the peptides did not interact non-specifically with the plate or the streptavidin. Only in the presence of immobilized $\beta\gamma$ subunits was binding of the selected peptide-bearing phage clones significantly higher than that of wild-type phage (f88-4). These results clearly demonstrate that the peptides expressed as fusions with phage pVIII coat protein bind to $\beta\gamma$ subunits.

Many of the binding clones were derived from disulfide-constrained libraries. To differentiate general non-specific hydrophobic interactions of these peptides with $\beta\gamma$ subunits from interactions dependent on structure, we tested binding of phage to immobilized $\beta\gamma$ after treatment of the phage with 10 mM dithiothreitol (DTT) to reduce intramolecular disulfide bonds. This treatment eliminated binding of all the tested phage derived from the disulfide-constrained libraries (Table II). This indicates that the disulfide constraint within the peptide is critical for binding to the $\beta\gamma$ subunits. DTT treatment had no effect on binding of phage derived from the linear library, indicating that this treatment does not generally inhibit phage binding to $\beta\gamma$ by some other mechanism. These data support the argument that binding of the peptides to $\beta\gamma$ depends on the overall structure of the peptides, and not simply on general hydrophobic or charge characteristics.

Sequence characteristics of the phage

Peptide sequences from specifically binding phage clones were grouped together into four classes based on shared

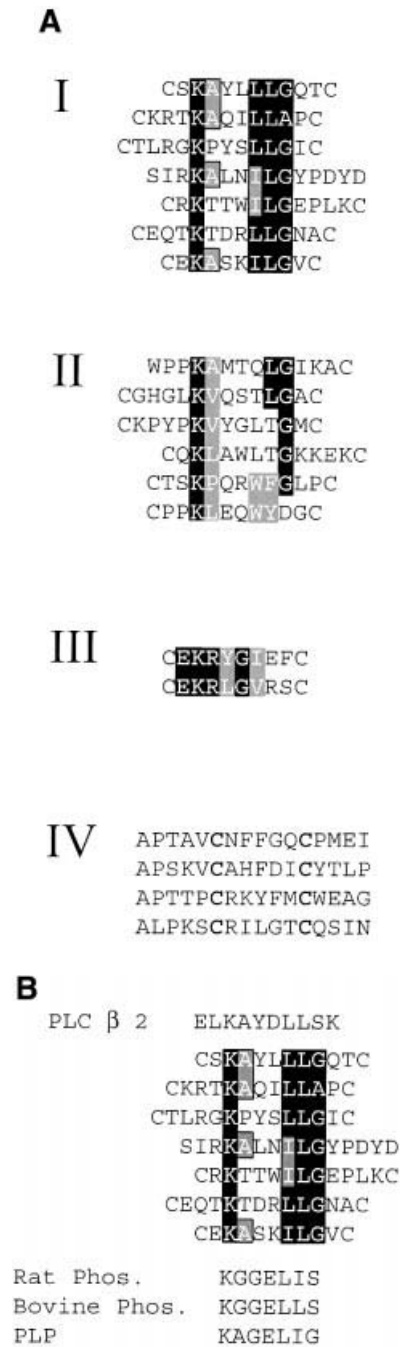


Fig. 2. Alignments of sequences obtained by random peptide phage display screening. (A) Sequences were placed into four groups based on sequence similarity. Black boxes indicate identities and gray boxes conservative substitutions. (B) Comparison of group A sequence with known $\beta\gamma$ -binding sequences from PLC β_2 , rat and bovine phosducins and phosducin-like protein (PLP).

sequence motifs (Figure 2A). The first group of phage had an apparent conserved sequence motif corresponding to KX_3LLG . The second group had a similar motif that was not as strongly conserved, with substitutions at conserved positions possibly representing allowable variations of this motif. Groups III and IV had no obvious homology to these first two consensus groups. For the two clones in group III, there was significant internal sequence similarity, yielding a second consensus motif, $EKRXGX_3$.

Table III. Synthetic peptide SIRK blocks binding to $\beta\gamma$ in a phage ELISA

		A_{405}		
		$-\beta\gamma$	$+\beta\gamma$	$+\beta\gamma + 30 \mu\text{M SIRK}^a$
I	f88-4	0.237 \pm 0.011	0.223 \pm 0.002	0.206 \pm 0.001
	SGRLCSKAYLLLGQTC	0.216 \pm 0.006	0.647 \pm 0.006	0.180 \pm 0.008
	ACKRTKAQILLAAPCT	0.193 \pm 0.009	0.427 \pm 0.010	0.195 \pm 0.002
	ACTLPGKPYSLGIC	0.177 \pm 0.002	0.427 \pm 0.002	0.177 \pm 0.003
II	SIRKALNILGYPDYD	0.206 \pm 0.009	0.663 \pm 0.007	0.260 \pm 0.003
	DCKPYPKVYGLTGMC	0.234 \pm 0.020	0.726 \pm 0.048	0.305 \pm 0.022
	TCTSKPQRWFLPC	0.223 \pm 0.005	0.463 \pm 0.021	0.190 \pm 0.006
	GCPPKLEQWYDGC	0.215 \pm 0.009	0.703 \pm 0.043	0.270 \pm 0.014
III	SCERKYGIEFCT	0.192 \pm 0.002	0.565 \pm 0.065	0.220 \pm 0.013
	SCEKRLGVRST	0.199 \pm 0.011	0.687 \pm 0.002	0.289 \pm 0.072
IV	PSKVCAHFDICYTSL	0.192 \pm 0.002	0.563 \pm 0.017	0.210 \pm 0.02
	PTTPCRKYFMCWEAG	0.192 \pm 0.004	0.672 \pm 0.041	0.259 \pm 0.036

^aPeptides were added to the wells just before addition of the phage. Results are the mean of duplicate determinations \pm SE. Similar experiments were performed at least five times. The phage ELISA assay is the same as in Table I.

Table IV. Synthetic peptide C5 blocks binding of phage to $\beta\gamma$

		A_{405}		
		$-\beta\gamma$	$+\beta\gamma$	$+\beta\gamma + 30 \mu\text{M C5}^a$
f88-4		0.165 \pm 0.010	0.137 \pm 0.002	0.137 \pm 0.006
DCKPYPKVYGLTGMC		0.150 \pm 0.009	0.735 \pm 0.006	0.289 \pm 0.021
TCTSKPQRWFLPC		0.148 \pm 0.003	0.350 \pm 0.020	0.150 \pm 0.004
ACKRTKAQILLAAPCT		0.161 \pm 0.000	0.413 \pm 0.010	0.149 \pm 0.008

^aSynthetic disulfide bridged peptides were dissolved in water at 1 mg/ml and allowed to oxidize overnight by exposure to air. The extent of cyclization was analyzed by reversed-phase HPLC.

The sole apparent unifying characteristic of group IV is the positioning of the cysteine residues, which were fixed before screening.

To determine whether the peptides displayed on the phage bound to $\beta\gamma$ as isolated peptides and whether the displayed peptides had overlapping binding sites, we performed competition ELISA experiments with synthetic peptides corresponding to two of the phage sequences. In these experiments, binding of phage clones displaying various peptide sequences to immobilized $\beta\gamma$ was monitored in the presence and absence of excess synthetic peptide. If the peptide bound to the $\beta\gamma$ at the same site as the phage-borne peptide, it would be predicted to block binding of the phage. The first peptide that we tested in this analysis was the linear peptide that was derived from the screening: SIRKALNILGYPDYD (SIRK peptide). Results are shown in Figure 1B with two selected phage at various concentrations of peptide. The peptide had no effect on the background binding of f88-4, but inhibited binding of both of the selected phage in a concentration-dependent manner, indicating that the peptide binds to the $\beta\gamma$ subunits at the same site as these two selected phage clones. The peptide was more potent at inhibiting binding of phage p21 (IC_{50} 1–3 μM) than P1 (IC_{50} ~10 μM) and may reflect differences in the affinity of these two phage clones for $\beta\gamma$. We predicted that SIRK peptide would block binding of all of the phage displaying the peptides in group I, since this peptide belongs to this consensus group, and that it would not block binding of phage in the other groups. We tested $\beta\gamma$ binding of phage from each group at

a fixed concentration of peptide (30 μM); the results are shown in Table III. The peptide blocked binding of phage in group I and, surprisingly, also inhibited binding of the phage in the other groups. This suggests that all of these disparate sequences bind to a single site or overlapping sites on $\beta\gamma$. To determine whether these effects are specific to peptides from group I, we tested another peptide from group III (SCEKRLGVRST) (C5) and found that it inhibited binding of phage in group I and group II (Table IV); separate experiments showed that this peptide also inhibited binding of selected phage from group IV (not shown). When we initially performed the screening, we predicted that we would obtain sequences that bound to various sites on $\beta\gamma$ subunits that would differ in sequence characteristics. Instead, it seems that there is a single site on $\beta\gamma$ that dominated in the screening, and that it has the capacity to interact with a variety of sequences.

Relevance of the selected binding site to $\beta\gamma$ -effector interactions

The group I consensus sequence is similar to a sequence from PLC β_2 that will also bind to $\beta\gamma$ subunits as an isolated peptide (Sankaran *et al.*, 1998). In fact, the first clone in group I (Table III) displays a high degree of similarity to the PLC β_2 sequence in other positions too (Figure 2B). Based on this, we predicted that peptides from group I would bind to a site on $\beta\gamma$ subunits recognized by PLC and might prevent activation of PLC by $\beta\gamma$ subunits. We examined the ability of the SIRK peptide to inhibit activation of PLC β_2 by $\beta\gamma$ subunits. The peptide

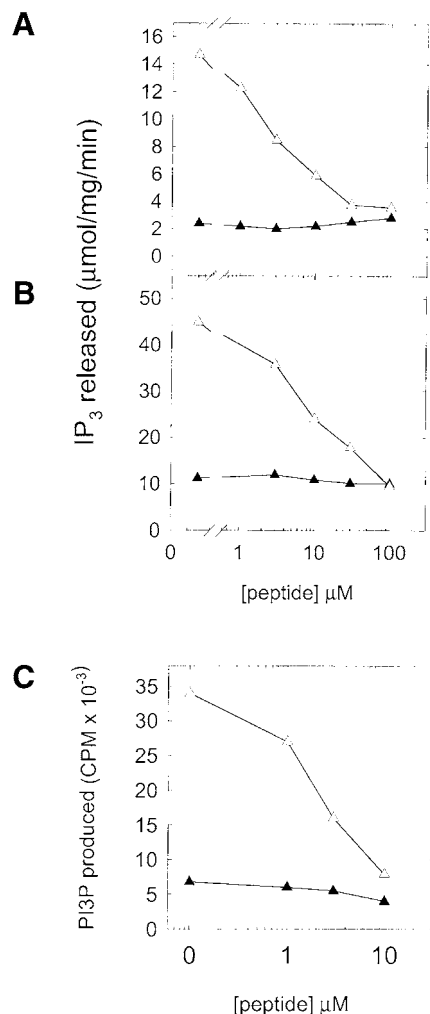


Fig. 3. Phage-displayed peptides inhibit activation of PLC $\beta 2$ and PI3K γ by $\beta\gamma$ subunits. Synthetic peptides with the sequences (A) Sirkalnilgpdyd (linear) and (B) Actlpgkpysllgicd (cyclic) were included in PLC assays at the indicated concentrations. Reactions included either no $\beta_1\gamma_2$ (filled triangles) or 100 nM $\beta_1\gamma_2$ (open triangles) and 1 ng of PLC $\beta 2$, 100 nM free Ca^{2+} ; the reaction time was 5 min. Each data point is the mean of duplicate determinations and the data are representative of experiments repeated at least 10 times (A) and three times (B). (C) Sirk peptide inhibits activation of PI3kinase by $\beta\gamma$ subunits. Sirk peptide was included at the indicated concentrations with 100 nM $\beta_1\gamma_2$ and 10 ng of a heterodimer of p110 γ and EE-tagged p101 PI3K γ subunits purified from Sf9 cells (Stephens *et al.*, 1997).

specifically inhibited PLC $\beta 2$ activation by $\beta\gamma$ subunits (Figure 3A) with an IC_{50} of $\sim 3 \mu\text{M}$, without affecting basal PLC activity. Other peptides from this group, and from the other groups, also inhibited PLC $\beta 2$ activation by $\beta\gamma$ subunits with various potencies (Figure 3B and data not shown).

We also tested whether the Sirk peptide would inhibit interactions between $\beta\gamma$ and other effectors. Activation of PI3K (Figure 3C) and PLC $\beta 3$ (not shown) by $\beta\gamma$ was blocked by peptide concentrations similar to that for PLC $\beta 2$. On the other hand, Sirk peptide did not inhibit $\beta\gamma$ -dependent inhibition of type I adenylate cyclase at concentrations up to 300 μM (Figure 4A). To confirm that PLC activation by $\beta\gamma$ subunits is not inherently more

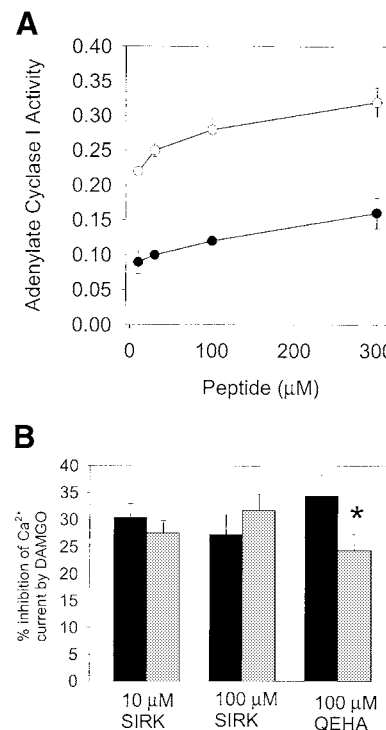


Fig. 4. Sirk peptide is a poor inhibitor of $\beta\gamma$ -mediated inhibition of adenylate cyclase type I and voltage-gated Ca^{2+} channels. (A) Sirk peptide was included at the indicated concentrations with 5 μg of Sf9 membranes expressing type I adenylated cyclase and no $\beta\gamma$ (open circles) or 20 nM $\beta\gamma$ subunits (filled circles). Data points are the mean of duplicate determinations and the data are representative of experiments repeated three times. (B) Sirk peptide is a poor inhibitor of DAMGO-mediated inhibition of N-type Ca^{2+} channels. Black bars are control cells and gray bars are peptide-treated cells. Peptides at the indicated concentrations were included in the patch pipette and allowed to dialyze into the cell for 10 min before stimulation of the cells with 3 μM DAMGO. For 10 μM Sirk, there were 24 cells for control and 37 for peptide treated. For 100 μM Sirk, there were 13 cells for control and eight for peptide treated. For QeHA peptide, there were eight cells for control and 11 for peptide treated. Results are the mean \pm SE. Treatments with Sirk (10 and 100 μM) were not statistically different from control treatments, while the QeHA treatment was significant with $p < 0.04$ in a two-tailed paired t -test.

sensitive to peptide inhibition than $\beta\gamma$ -dependent inhibition of adenylate cyclase I in general, we tested a peptide previously shown to inhibit $\beta\gamma$ interactions with type I adenylate cyclase, QeHA (Chen *et al.*, 1995). This peptide inhibited PLC activation with the same potency as has been reported for its effects on $\beta\gamma$ inhibition of adenylate cyclase ($\text{IC}_{50} \sim 100 \mu\text{M}$) (not shown). This indicates that peptides, in general, are not inherently more potent at disrupting PLC interactions with $\beta\gamma$ than adenylate cyclase- $\beta\gamma$ interactions.

We also examined the effect of this peptide on opioid-mediated inhibition of voltage-gated Ca^{2+} currents in rat dorsal root ganglion neurons. The mechanism of channel inhibition, at least for N-type channels, involves in part direct binding of G protein $\beta\gamma$ subunits to the α subunit of the channel (Herlitz *et al.*, 1996, 1997; Qin *et al.*, 1997; Samoriski and Gross, 2000). In these experiments, whole-cell Ca^{2+} currents were evoked by stepping to +10 mV from a holding potential of -80 mV in the presence and absence of the opioid agonist DAMGO, and in the absence

or presence of selected peptides, dialyzed intracellularly via a patch pipette. Since the pipette contains an infinite reservoir of peptide, the intracellular solution should contain the same concentration of peptide as the patch pipette. Identical methods have been used to introduce $G\alpha_o$ antibodies into these cells to block inhibition of Ca^{2+} channels in this pathway (Wiley *et al.*, 1997). The peptide apparently did not affect the DAMGO-dependent inhibition of the voltage-gated Ca^{2+} current (Figure 4B). There was no significant difference between mean current amplitudes in control and peptide-treated cells (not shown). To confirm that we could indeed inhibit $\beta\gamma$ -mediated inhibition of Ca^{2+} currents with $\beta\gamma$ -binding peptides, we performed the same experiments with the QEHA peptide derived from adenylyl cyclase (Chen *et al.*, 1995). In these experiments, DAMGO-mediated Ca^{2+} channel inhibition was partially inhibited, indicating that $\beta\gamma$ interactions with the Ca^{2+} channel can be inhibited by synthetic peptides. That only a portion of the receptor-mediated effects on Ca^{2+} currents were inhibited by QEHA may be because a submaximal dose of QEHA was used ($IC_{50} \sim 100 \mu M$ for inhibition of other processes) or because pathways other than the $\beta\gamma$ -mediated one may be involved in regulating this Ca^{2+} channel. Based on more extensive experiments (Samoriski and Gross, 2000) we would predict only a partial block, as strong depolarization, which reverses $\beta\gamma$ binding to the channels, only partially reverses DAMGO-induced current reductions.

Taking these data together, we conclude that this peptide-binding site is important for PLC β and PI3K interactions with $\beta\gamma$ subunits, but perhaps not so important for interaction with adenylyl cyclase I and voltage-gated Ca^{2+} channels.

Analysis of K^+ channel interaction sites

To characterize the nature of the effector binding site on $\beta\gamma$ further, we used the competition ELISA assay described earlier to determine whether peptides derived from another effector would prevent binding phage bearing the selected peptides. We tested four peptides derived from inwardly rectifying K^+ channel subunits GIRK1 and GIRK4, which have previously been shown to inhibit interactions between $\beta\gamma$ subunits and solubilized K^+ channels, presumably through direct interactions with $\beta\gamma$ subunits (Krapivinsky *et al.*, 1998). GIRK1 (220–239) and the homologous GIRK4 (226–245) were reported to inhibit $\beta\gamma$ - K^+ interactions with relatively high IC_{50} values (70–100 μM) and we tested them in a competition ELISA at twice the IC_{50} . GIRK1 (364–383) and GIRK4 (209–225) had lower reported IC_{50} values (8 and 0.6 μM , respectively), so we tested GIRK1 (364–383) at 10 times and GIRK4 (209–225) at 100 times their respective IC_{50} values. None of the peptides prevented binding of phage bearing the SIRK sequence (Figure 5). This suggests that these peptides and the regions that they represent on the K^+ channel bind to other determinants on the $\beta\gamma$ subunit.

Analysis of the SIRK peptide

Based on the alignment of the sequences in group I, we predicted that the conserved amino acids would form critical binding determinants. To test this idea, we synthesized a set of 'alanine scan' analogs of the SIRK peptide. Sixteen peptides were synthesized, including

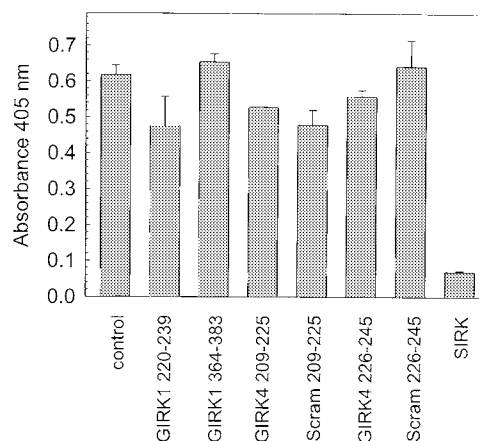


Fig. 5. Peptides derived from inwardly rectifying K^+ channels do not bind to the selected site on $\beta\gamma$ subunits. Peptides derived from GIRK1 and GIRK4 that bind to $\beta\gamma$ were tested for their ability to inhibit binding of phage displaying SCERKYGIEFCT at 1×10^{10} phage particles/well. Peptides were tested at the following concentrations: GIRK1 (220–239), 200 μM ; GIRK1 (364–383), 100 μM ; GIRK4 (209–225), 60 μM ; scrambled GIRK4 (209–225), 60 μM ; GIRK4 (226–245), 200 μM ; scrambled GIRK4 (226–245), 200 μM ; SIRK, 30 μM .

wild-type peptide and peptides with successive alanine substitutions along the sequence. Where an alanine was already present in the sequence, it was substituted by glycine. Each synthetic peptide was purified to $\geq 80\%$ homogeneity and analyzed by mass spectrometry to confirm its identity. Each peptide was tested for its ability to inhibit PLC activation by $\beta\gamma$ subunits at various concentrations and IC_{50} values were estimated for each and are shown in Table V. The data strongly suggest that the predicted amino acids are important for binding. Residues at positions 8, 9 and 10, which correspond to {L/I}-LG in the predicted consensus, are clearly critical for peptide binding to $\beta\gamma$ subunits and inhibition of PLC $\beta 2$ activation. An amino acid that was, unexpectedly, important for binding of this peptide is the alanine at position 5. This amino acid was present in some of the peptide sequences, but not others. While mutation of the lysine at position 4 reduced the binding by ~ 10 -fold, this amino acid is apparently not as critical for binding to $\beta\gamma$ as the LLG sequence. Interestingly, substitution of alanine for asparagine at position 7 increased the apparent binding of the peptide to $\beta\gamma$. Given all the positions that are required for binding of this peptide, why do peptides from group II bind where there are variations at all of the positions except position 4? Perhaps there are allowable substitutions for these amino acids in the peptide (just not alanine) or changes at other positions that are compensating for the loss of binding energy.

Discussion

We have used random peptide phage display screening to identify amino acid motifs involved in binding to G protein $\beta\gamma$ subunits. We predicted that we would obtain sequence groups that bound to different surfaces of the $\beta\gamma$ subunit and that we would be able to identify groups of consensus sequence that would be used by different effectors. Instead, the screen yielded four distinct groups of binding

Table V. Alanine scan analogs of the SIRK peptide sequence and their ability to inhibit activation of PLC by βγ

	Inhibition of βγ-PLC activation: IC ₅₀ (μM) ^a	Position of alanine or glycine substitution
SIRKALNILGYPDYD	5	–
AIRKALNILGYPDYD	5	1
SARKALNILGYPDYD	20	2
SIKALNILGYPDYD	10	3
SIRAALNILGYPDYD	60	4
SIRKGLNILGYPDYD	100	5
SIRKAANILGYPDYD	65	6
SIRKALAILGYPDYD	0.5	7
SIRKALNALGYPDYD	200	8
SIRKALNIAGYPDYD	300	9
SIRKALNILAYPDYD	70	10
SIRKALNILGAPDYD	5	11
SIRKALNILGYADYD	5	12
SIRKALNILGYPAYD	5	13
SIRKALNILGYPDAD	5	14
SIRKALNILGYPDYA	5	15

^aResults are representative data from titrations performed two or three times for each peptide at at least five different concentrations of peptide. Wild-type peptide was included in all titrations to control for experimental variation. Titrations were performed using methods identical to those used for Figure 3 and are described in Materials and methods.

sequences, most of which were derived from disulfide-constrained libraries that appeared to bind to the same site on βγ subunits. Group I sequences had a clear consensus that was definable on the basis of seven distinct sequences, K-X₃-{I,L}-L-G. Based on competition analysis in the phage ELISA assay, all of the peptides share a single binding site. This single binding site appears to be shared by several effectors that bind βγ subunits including PLC β and PI3K γ. Critical binding determinants predicted from the apparent homology of the selected peptides was confirmed by scanning alanine mutagenesis. Most of the critical binding amino acids were non-polar, suggesting that hydrophobic interactions could be a major energetic driving force for binding of βγ subunits to effectors.

Protein–protein interactions are often driven by burial of exposed hydrophobic surfaces. A series of studies has been performed using phage display library screening to study protein–protein interactions. Repeatedly, such screens yield peptides that bind to relevant protein–protein interaction sites on the surfaces of proteins, despite the fact that the libraries screened are entirely random (Wrighton *et al.*, 1996; Fairbrother *et al.*, 1998; Lowman *et al.*, 1998). It has been suggested that phage display methods tend to identify ‘hot spots’ on proteins that form the energetically critical contacts in a protein–protein interaction. A number of studies have analyzed such ‘hot spots’ in detail. For example, for human growth hormone (hGH) >30 contact sites have been identified in the three-dimensional co-crystal (Wells and de Vos, 1996). Alanine scanning mutagenesis of these contacts indicates that only five of these residues contribute to >85% of the binding energy and that these critical interactions are primarily non-polar. This has implications for the specificity of the interaction. Non-polar interactions do not have the strict stereochemical requirements found in electrostatic or hydrogen-

bond-driven interactions and thus may accommodate a number of different sequences. This is different from what has been suggested for other peptides that bind to βγ. In particular, the QEHA peptide derived from adenylate cyclase and a peptide from the C terminus of βARK (Koch *et al.*, 1993; Touhara *et al.*, 1995) are both charged and have been proposed to bind through electrostatic interactions to surfaces on β subunits (Weng *et al.*, 1996). Thus, effector interactions with βγ subunits may occur through hydrophobic interactions. At least one peptide that we have found, SIRK, has a 10- to 100-fold higher affinity for βγ than the βARK and QEHA peptides.

A number of βγ-regulated effectors (PLC β, phosducin and phosducin-like protein) have sequences with similarity to the sequence we derived (Figure 2B). The co-crystal structure of phosducin and βγ has been determined (Gaudet *et al.*, 1996). In this structure, amino acids 193–198 of phosducin (the region that has similarity to the group I consensus) form a loop between two β strands with a lysine and two adjacent leucine residues analogous to the KX₃LLG amino acids in direct contact with β subunit amino acids (Figure 6A and B). It is tempting to speculate that our peptides bind to this same region on βγ. Since many of the peptides we found are disulfide constrained with short inter-cysteine sequences, they are predicted to form a loop. The region that this sequence binds to on the β subunit undergoes major conformational rearrangement when phosducin binds between blades 6 and 7. It is possible that these peptides also cause this conformational change to occur (Loew *et al.*, 1998). We emphasize that the idea that the phage-displayed peptides bind to the site on βγ shown for phosducin binding in Figure 6 is highly speculative, and further experiments are needed to demonstrate more rigorously the structural basis for interactions between these peptides and βγ subunits.

Our results suggest that the peptides we identified bind to a unique site on βγ subunits that is not targeted by the QEHA and βARK peptides. In support of this, the binding site for the peptides we have found seems only to be shared by some effectors. The peptides block activation of PLC and PI3K, but are relatively ineffective at blocking inhibition of adenylate cyclase and Ca²⁺ channels. All the peptides previously found to bind to βγ subunits and block effector activation seem to do so in a general way, in that they block activation of all the βγ-regulated effectors that have been tested. The SIRK peptide is the first peptide that binds to βγ subunits and inhibits effectors with this kind of selectivity. This has important implications for potential pharmacological manipulation of βγ-regulated pathways. Various studies with β subunit mutants (Li *et al.*, 1998) or the β₅-subunit isoform (Yoshikawa *et al.*, 2000) have suggested that effectors can bind to βγ subunits in different ways.

In the analysis presented here, we have begun to define consensus amino acids that could be used by effectors for interactions with βγ subunits. While a consensus did arise from our studies, there was a striking variation in the types of sequence that interacted with the same site on βγ. This suggests that one possible reason for the ability of βγ subunits to interact with a wide diversity of effectors is that there is a binding site on βγ that can accommodate a diverse range of sequences. This is clearly not the whole story, because the peptides we defined only block some

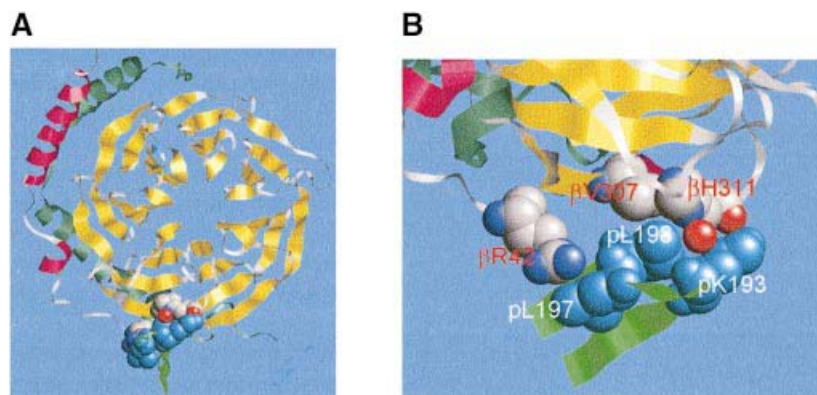


Fig. 6. Model of the interaction between phosducin 193–198 and $\beta_1\gamma_1$. (A) Top view of the β subunit bound to a loop from 189 to 201 between β strands S4 and S5 of phosducin [Sigler nomenclature (Gaudet *et al.*, 1996)]. The β subunit propeller ribbons are in yellow, the β N-terminal helix is in red and the γ subunit is dark green. The β subunit amino acids that contact the K-X₃-LL sequence in phosducin are shown in spacefill representation in CPK colors. The amino acids from phosducin, K193, L197 and L198 are turquoise, while other amino acids in the region from 189 to 201 are displayed only as a ribbon and colored light green. (B) Close-up view of amino acids involved in direct interaction between the K-X₃-LL sequence in phosducin and the β subunit. Amino acids from phosducin are labeled with a 'p' prefix. The models were constructed from coordinates deposited in the SwissProt data bank (Gaudet *et al.*, 1996) using RasTop version 1.3 developed by P.Valadon, A.Meuller and R.Sayle (http://www.bernstein-plus-sons.com/software/RasMol_2.7.1/).

effectors. Thus, there must be other binding sites on $\beta\gamma$ subunits that are used by other effectors. It is interesting that our screen did not identify peptides that bind to these other binding sites nor did it identify sequences with homology to other known $\beta\gamma$ -binding peptides such as the QEHA or the β ARK peptides. We suggest that the binding site on $\beta\gamma$ for the peptides we selected is a dominant binding determinant on $\beta\gamma$ subunits and that the peptides that bound there were specifically enriched in the selection process at the expense of weaker binding peptides that bind at other sites. Further screening in the presence of peptides that block this dominant binding site may reveal peptides that bind at other sites.

Materials and methods

$\beta\gamma$ biotinylation

Biotinylated $\beta_1\gamma_2$ subunits were prepared using a modification of the method developed by Dingus *et al.* (1994). Sf9 insect cells were infected with His₆- α_1 , - β_1 and - γ_2 as described previously (Kozasa and Gilman, 1995). Proteins were extracted from the membrane fraction and bound to a nickel NTA-agarose column. Heterotrimeric $\alpha_1\beta_1\gamma_2$ was eluted from the column in 4 ml of 20 mM HEPES pH 8.0, 100 mM NaCl, 0.1% polyoxyethylene 10 lauryl ether (C₁₂E₁₀), 10 μ M GDP, 150 mM imidazole. The eluted protein was diluted to 1 mg protein/ml with 20 mM HEPES pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 10 μ M GDP; the final detergent concentration was adjusted to 0.05% C₁₂E₁₀. NHS-LC-biotin (Pierce) was added from a 20 mM stock in dimethylsulfoxide to give a final concentration of 1 mM. The reaction was allowed to proceed for 30 min at room temperature, followed by addition of 10 mM ethanolamine pH 8.0 from a 200 mM stock and incubation on ice for 10 min. The sample was diluted to 90 ml with dilution buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 10 μ M GDP, 0.5% C₁₂E₁₀). Washed nickel NTA-agarose (2 ml) was added and incubated at 4°C, with mixing, overnight. The mixture was poured through a column and washed with 20 ml of dilution buffer. The column was warmed to room temperature for 15 min and washed with 5 ml of wash buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 10 μ M GDP, 1% Na-cholate). $\beta\gamma$ subunits were eluted in wash buffer plus 10 mM MgCl₂, 10 mM NaF, 30 μ M AlCl₃. Biotinylation was confirmed by electrophoresis and blotting with streptavidin-horseradish peroxidase and by showing that all of the b- $\beta\gamma$ could be bound to streptavidin-agarose. b- $\beta\gamma$ was capable of

activating PLC β_2 and β_3 with a potency and efficacy similar to those of unmodified $\beta\gamma$ (not shown).

Phage screening

The experiment was designed with several libraries (Bonnycastle *et al.*, 1996); the Cys libraries were kindly donated by G.P.Smith (University of Missouri-Columbia) and combined in single wells of the microtiter plate such that 16 different libraries were screened in seven wells. The libraries that were screened are shown in Table I. The panning process we describe is the standard method for selecting binding phage (Smith and Scott, 1993; Bonnycastle *et al.*, 1996). Briefly, 10¹² virions were incubated with b- $\beta\gamma$ subunits (100 ng) that had been bound to immobilized streptavidin on a microtiter plate. The wells were washed thoroughly to remove unbound phage. Non-specific binding of phage, either to the plate or to the $\beta\gamma$ subunits, was minimized by performing the incubations and washes with buffers containing 150 mM NaCl, 0.5% Tween-20 and 2 mg/ml BSA. Bound phage were eluted at pH 2.2, and the eluates were neutralized and used to infect *Escherichia coli* cells that amplified the phage. Panning of each library mix was repeated three times with each to enrich for $\beta\gamma$ -binding phage.

After panning, the pools of phage were tested for binding to the target using a phage ELISA protocol (Smith and Scott, 1993), in which b- $\beta\gamma$ was bound to immobilized streptavidin and reacted with the amplified phage pools. The purpose of this step was to ensure that the pools contained phage that bound specifically to $\beta\gamma$ subunits. Phage binding was detected with anti-phage antibody conjugated to horseradish peroxidase. From each of the selected pools of phage, individual clones were isolated, purified and tested for binding, and the DNA encoding the peptide was sequenced.

Phage preparation

Isolated colonies of *E.coli* infected with phage were picked from NZY-*tet* (Gibco-BRL) plates and grown overnight in 1.5 ml at 37°C. Phage were purified from the supernatant of the overnight cultures by precipitation with 20% PEG 8000 and heat treatment at 70°C. Phage concentrations were estimated by agarose gel electrophoresis of the purified phage by comparison with a standard curve generated with f88-4 phage that had been grown on a large scale, purified twice by PEG precipitation and quantified spectrophotometrically (Smith and Scott, 1993).

DNA sequencing

Single-stranded DNA was isolated from an aliquot of the purified phage preparation by standard methods (Maniatis *et al.*, 1991). Sequencing reactions were performed according to the protocol supplied with Big Dye Sequencing Kit (Perkin-Elmer) using the primer CTGAGTTCATT-AAGACG; reactions were analyzed by the automated DNA sequencing core facility at the University of Rochester.

Phage ELISA

b-βγ (100 ng) was immobilized in streptavidin-coated wells of a 96-well plate. Phage clones (1 × 10¹⁰) were incubated with immobilized βγ in TBS with 0.1% Tween-20 for 1–4 h. For competition ELISAs, peptides were added just before incubation with the phage. After washing to remove unbound phage, anti-M13 antibody linked to horseradish peroxidase was added for 1 h followed by a chromogenic dye. The extent of color reaction was monitored in a microplate reader. The absorbance values (A₄₀₅) shown are a crude measure of the strength of phage binding to βγ.

PLC and PI3K assays

PLC assays were performed as described (Romoser *et al.*, 1996) with specific details given in the figure legends. Briefly, purified PLC β2 was mixed with sonicated phospholipid vesicles containing 50 μM brain phosphatidylinositol 4,5-bisphosphate (PIP₂), 200 μM liver phosphatidylethanolamine (PE) (Avanti Polar Lipids, Inc.) and [³H]inositol-PIP₂ (New England Nuclear) (6000–8000 c.p.m./assay), with or without 100 nM purified β₁γ₂ and peptides at the indicated concentrations. Reactions were allowed to proceed for 10–30 min at 30°C in the presence of ~100 nM free Ca²⁺. Intact lipids and proteins were precipitated with BSA and 10% trichloroacetic acid, and removed by centrifugation. Supernatant containing soluble [³H]inositol 1,4,5-trisphosphate was removed and analyzed by liquid scintillation counting.

PI3K assays were performed as described (Parish *et al.*, 1995). Briefly, purified p101–p110γ heterodimer was mixed with sonicated phospholipid vesicles containing 300 μM liver phosphatidylinositol (Avanti Polar Lipids, Inc.) and 600 μM PE, with or without 100 nM purified β₁γ₂ and peptides at the indicated concentrations. Reactions were initiated by the addition of 10 μM ATP containing [³²P]ATP at 100 000 c.p.m./assay. Reactions were allowed to proceed for 30 min at 30°C. Lipids were extracted and analyzed by liquid scintillation counting.

Adenylyl cyclase assays

Adenylyl cyclase activity was measured as described (Smigel, 1986). All assays were performed for 10 min at 30°C in a final volume of 100 μl containing 50 mM sodium HEPES pH 8.0, 500 μM ATP, 0.6 mM EDTA, 3 mM K₂-phosphoenolpyruvate, 10 mM MgCl₂, 500 μM 3-isobutyl-1-methylxanthine (IBMX), 0.1 μg/μl BSA, 1 μg/μl pyruvate kinase, 5 μg of membrane protein. Activated G protein α subunits were diluted into 50 mM sodium HEPES pH 8.0, 5 mM MgSO₄, 1 mM EDTA, 1 mM DTT and mixed with membranes before the start of the assay.

Peptide synthesis

The alanine scan mutants of SIRK peptide were synthesized manually using Chiron multipin methodology (Geysen *et al.*, 1984). The 16 peptides shown in Table V were synthesized simultaneously on Fmoc-Rink amide handle-crowns (the substitution level of each crown was 6.9 μmol). Each peptide was synthesized on an individual crown and in a separate well. Each impure peptide was purified by HPLC using a semi-preparative Zorbax C-8 column (9.4 × 250 mm, 5 μm) using a linear gradient from 5 to 70% acetonitrile in 0.05% trifluoroacetic acid over 50 min. The fraction corresponding to the required peptide was checked by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy, concentrated and lyophilized.

Measurement of DAMGO inhibition of N-type Ca²⁺ channels

Electrophysiological measurements of dorsal root ganglion neurons were made as in Samoriski and Gross (2000).

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