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ACCELERATED COMMUNICATION

Dopamine D_2 Receptor-Induced Heterologous Sensitization of Adenylyl Cyclase Requires $G\alpha_s$: Characterization of $G\alpha_s$-Insensitive Mutants of Adenylyl Cyclase V

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ABSTRACT

Whereas acute stimulation of $G\alpha_{i/o}$-coupled receptors inhibits the activity of adenylyl cyclase, a delayed consequence of persistent activation of the receptors is heterologous sensitization, an enhanced responsiveness of adenylyl cyclase to activators such as forskolin or agonists of $G\alpha_s$-coupled receptors. $G\alpha_s$-insensitive mutants of adenylyl cyclase type V were used to test the hypothesis that heterologous sensitization requires $G\alpha_s$-dependent activation of adenylyl cyclase. When adenylyl cyclase was stably expressed in human embryonic kidney (HEK) 293 cells with the D_{2L} dopamine receptor, basal, forskolin-stimulated, and isoproterenol-stimulated cyclic AMP accumulation were all enhanced by 2-h pretreatment with the D_2 receptor agonist quinpirole. Transient expression of wild-type adenylyl cyclase and three $G\alpha_s$-insensitive mutants (F379L, R1021Q, and F1093S) in HEK293 cells stably expressing the D_{2L} receptor demonstrated that all three mutants had little or no responsiveness to β-adrenergic receptor-mediated activation of $G\alpha_s$ but that the mutants retained sensitivity to forskolin and to D_{2L} receptor-mediated inhibition. Transiently expressed adenylyl cyclase V was robustly sensitized by 2-h pretreatment with quinpirole. In contrast, the $G\alpha_s$-insensitive mutants displayed no sensitization of forskolin-stimulated cyclic AMP accumulation, indicating that responsiveness to $G\alpha_s$ is required for the expression of heterologous sensitization.

The dopamine D_2 receptor belongs to the class of G protein-coupled receptors whose intracellular effects, including inhibition of adenylyl cyclase activity, are mediated primarily by activation of the pertussis toxin-sensitive G proteins $G\alpha_i$ and $G\alpha_o$ (Robinson and Caron, 1997). Prolonged stimulation of several $G\alpha_{i/o}$-coupled receptors, including the D_2 and D_4 dopamine receptors, causes heterologous sensitization of adenylyl cyclase, such that the responsiveness of adenylyl cyclase to subsequent activation by forskolin or $G\alpha_s$-coupled receptors is enhanced (Sharma et al., 1975; Jones and Bylund, 1988; Bates et al., 1991; Watts and Neve, 1996). Although heterologous sensitization induced by long-term agonist treatment may be a consequence of changes in the abundance of receptors or G proteins (Hadcock and Malbon, 1993; Van Vliet et al., 1993; Watts et al., 1999), our work has focused on the mechanisms of rapid sensitization induced by short-term (15–120 min) agonist treatment. D_2 receptor-stimulated heterologous sensitization is prevented by pertussis toxin treatment, implicating activation of $G\alpha_{i/o}$ proteins as an early step in heterologous sensitization, and a pertussis toxin-resistant mutant of $G\alpha_o$ can rescue sensitization in NS20Y neuroblastoma cells (Watts et al., 1998). Several lines of evidence have led us to hypothesize that rapid (<2 h) heterologous sensitization is a consequence of an enhanced interaction between $G\alpha_s$ and adenylyl cyclase, whereas the abundance of $G\alpha_s$ is not altered (Watts et al., 1999). For example, heterologous sensitization of adenylyl cyclase is associated with an increase in the number of [^{3}H]forskolin

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ABBREVIATIONS: AC, adenylyl cyclase; $G\alpha_s$, the α subunit of the G protein that stimulates adenylyl cyclase; $G\alpha_i$, the α subunit of a G protein that inhibits adenylyl cyclase; $G\alpha_o$, the α subunit of a pertussis toxin-sensitive G protein that regulates the activity of many enzymes and ion channels; D_{2L}, long alternatively spliced form of the D_2 dopamine receptor; HEK, human embryonic kidney; HSV, herpes simplex virus.

binding sites (Jones and Bylund, 1990) that may represent $G\alpha_s$-adenylyl cyclase complexes. Sensitization also enhances the maximal responsiveness of adenylyl cyclase to the β-adrenergic receptor agonist isoproterenol and increases the potency of forskolin (Watts and Neve, 1996), which is similar to the effect of increased $G\alpha_s$ activity on adenylyl cyclase (Seamon and Daly, 1981; Barovsky et al., 1984). In addition, isoforms of adenylyl cyclase that show synergistic activation by $G\alpha_s$ and subtype selective activators such as Ca^{2+} for ACI or phorbol esters for ACII show marked short-term sensitization (Watts and Neve, 1996). Finally, ACV, which is inhibited by $G\alpha_i$ and intracellular calcium (Yoshimura and Cooper, 1992; Taussig et al., 1993) and synergistically activated by forskolin and $G\alpha_s$, exhibits robust heterologous sensitization of basal and drug-stimulated activity (Watts and Neve, 1996; Cumbay and Watts, 2001). These observations form the basis of the present study, which is designed to examine further the role of $G\alpha_s$ and $G\alpha_s$-adenylyl cyclase interactions in D_2 receptor-mediated heterologous sensitization.

Zimmerman et al. (1998), using a yeast genetic selection system, identified mutants of ACV that are stimulated by forskolin but are insensitive to activation by $G\alpha_s$ when characterized in Sf9 cell membranes. In the present study, we confirmed that three of the mutants, F379L, R1021Q, and F1093S, are insensitive to $G\alpha_s$ but retain sensitivity to forskolin and $G\alpha_{i/o}$ when expressed in mammalian cells. In addition, we assessed the ability of D_2 receptor stimulation to sensitize the $G\alpha_s$-insensitive mutants. Although wild-type ACV is greatly sensitized by activation of the D_{2L} receptor and is the only isoform of adenylyl cyclase that we have tested that exhibits sensitization of basal activity, neither basal- nor forskolin-stimulated activity of $G\alpha_s$-insensitive mutants of ACV was sensitized by activation of D_{2L}, supporting our hypothesis that rapid heterologous sensitization is caused by enhanced activation of adenylyl cyclase by $G\alpha_s$.

Materials and Methods

Expression of the D_{2L} Receptor and Adenylyl Cyclase. Some experiments used a cell line stably expressing ACV and the rat D_{2L} dopamine receptor, designated ACV/D_{2L}. The cell line was created by transfection of pcDNA3-D_{2L} into HEK293 cells already expressing recombinant ACV. Individual colonies were initially screened for expression of D_2-like receptor binding and then tested for maintained expression of the recombinant adenylyl cyclase. Cells stably expressing the receptors and ACV were then maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 5% calf bovine serum with 50 U/ml penicillin and 50 μg/ml streptomycin, 300 μg/ml G418, and 460 U/ml hygromycin. Cells were grown in a humidified incubator in the presence of 10% CO_2. In other experiments, a herpes simplex virus (HSV) vector was used to drive the expression of wild-type and mutant forms of ACV in HEK293 cells stably expressing the D_{2L} dopamine receptor (HEK-D_{2L}). Wild-type canine ACV and three $G\alpha_s$-insensitive mutants described previously, F379L, R1021Q, and F1093S (Zimmermann et al., 1998), were cloned into pHSVPrPUC. Replication-defective HSV vectors were packaged at a titer of approximately 2×10^5 infectious units/μl as described previously (Neve et al., 1997). Cells were infected with HSV-ACV (approximately 1 infectious unit/cell, except where indicated) 18 h before measurement of cyclic AMP accumulation.

Cyclic AMP Accumulation Assay. Cells were plated out at concentrations between 100,000 and 150,000 cells/well in 48-well cluster plates. For sensitization experiments, cells were preincubated for 2 h in the presence of 1 μM quinpirole at 37°C. After pretreatment with quinpirole or vehicle, the cells were washed three times for 3 to 4 min with 200 μl of assay buffer (Earle's balanced salt solution containing 0.02% ascorbic acid and 2% calf bovine serum) and placed on ice, and then drugs (forskolin or isoproterenol) were added. Spiperone (1 μM) was added to the assay buffer to block activation of the D_{2L} receptor by any residual agonist. The cells were then incubated for 15 min at 37°C. The medium was removed and the cells were lysed with 3% trichloroacetic acid. The 48-well plates were stored at 4°C until quantification of cyclic AMP was carried out.

Quantification of Cyclic AMP. Cyclic AMP was quantified using a competitive binding assay (Watts and Neve, 1996) adapted from the assay of Nordstedt and Fredholm (1990). Duplicate samples of the cell lysate (1–15 μl) were added to reaction tubes containing cyclic AMP assay buffer (100 mM Tris/HCl, pH, 7.4, 100 mM NaCl, 5 mM EDTA). [^{3}H]Cyclic AMP (2 nM final concentration) was added to each well. Binding protein (~150 μg of protein in 200 μl of cyclic AMP buffer) was then added to each well. The reaction tubes were incubated on ice for 3 h. The tubes were then harvested by filtration (Unifilter GF/B; Packard Instruments, Meriden, CT) using a 96-well Packard Filtermate Harvester. Filters were allowed to dry and Packard Microscint scintillation fluid was added. The filters were counted on a Packard TopCount scintillation/luminescence detector. Cyclic AMP concentrations from each sample were estimated in duplicate from a standard curve ranging from 0.1 to 100 pmol of cyclic AMP/tube.

Results and Discussion

Heterologous Sensitization of Basal and Drug-Stimulated Activity of ACV. HEK293 cells stably expressing ACV and the D_{2L} receptor (ACV/D_{2L} cells) were pretreated with vehicle or 1 μM quinpirole for 2 h before measurement of cyclic AMP accumulation. As shown previously (Cumbay and Watts, 2001), pretreatment with the D_2 agonist quinpirole increased basal cyclic AMP accumulation in the cells from 11 ± 3.4 to 60 ± 16 pmol of cyclic AMP/well (Fig. 1). Quinpirole pretreatment also caused an increase of more than 10-fold in subsequent stimulation of cyclic AMP accumulation by 1 μM isoproterenol, acting through the endogenous β-adrenergic receptor that is present in low abundance on HEK293 cells (V. J. W., unpublished observations), or 100 nM forskolin in ACV/D_{2L} cells (Fig. 1). These concentrations

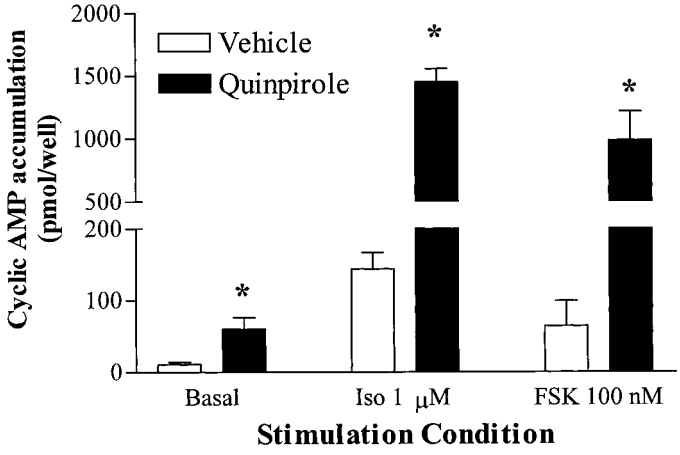

Fig. 1. Sensitization of basal and drug-stimulated ACV activity. HEK-ACV/D_{2L} cells were pretreated for 2 h with vehicle (□) or 1 μM quinpirole (■), then washed extensively before measurement of cyclic AMP accumulation in absence (Basal) or presence of isoproterenol- (ISO 1 μM), or forskolin- (FSK 100 nM) as described under *Materials and Methods*. Data shown are the mean ± S.E.M. of four to five independent experiments. *$P < 0.05$ compared with vehicle-treated cells.

of isoproterenol and forskolin were chosen because they had little effect on the endogenous adenylyl cyclase in HEK293 cells, as can be seen in Fig. 4, B and C; thus, cyclic AMP accumulation under these conditions was almost entirely due to stimulation of the heterologously expressed adenylyl cyclase.

Insensitivity of ACV Mutants to Isoproterenol-Stimulated Activation of $G\alpha_s$. Using a yeast selection system, Zimmermann et al. (1998) identified 25 $G\alpha_s$-insensitive mutants of ACV that were caused by substitutions at 11 highly conserved positions in the cytoplasmic regions C_{1a} and C_2 of ACV. Characterization of the mutants in Sf9 cells demonstrated that responsiveness to forskolin was spared in a subset of the mutants, although they had reduced responsiveness to $G\alpha_s$ and, therefore, a loss of synergism between $G\alpha_s$ and forskolin. Three of the mutants that failed to bind $G\alpha_s$ and showed the least sensitivity to activation by $G\alpha_s$ (Zimmermann et al., 1998) were selected to test our hypothesis concerning the role of $G\alpha_s$ in rapid heterologous sensitization of adenylyl cyclase. We reasoned that if heterologous sensitization involves an enhanced interaction between $G\alpha_s$ and adenylyl cyclase, $G\alpha_s$-insensitive mutants of adenylyl cyclase would not show sensitization of forskolin-stimulated activity. The selected mutations (F379L, R1021Q, and F1093S) are in conserved regions of C_{1a} and C_2 domains that have been shown to interact with $G\alpha_s$ in mutagenesis studies (Yan et al., 1997) and in the crystal structure of a $G\alpha_s$/soluble adenylyl cyclase complex (Tesmer et al., 1997).

We used HSV to express wild-type and mutant ACV in HEK-D_{2L} cells. Whereas wild-type ACV was robustly activated by 1 μM isoproterenol, all three mutants failed to respond to isoproterenol (Fig. 2A), confirming the $G\alpha_s$-insensitive phenotype shown by the mutants in Sf9 cells (Zimmermann et al., 1998). The ability of forskolin to stimulate adenylyl cyclase activity in HEK-D_{2L} cells infected with each of the recombinants was also examined. Wild-type ACV showed marked forskolin-stimulated cyclic AMP accumulation (Fig. 2, B and C). Although F379L also exhibited robust forskolin-stimulated cyclic AMP accumulation, the activity of the mutant was reduced compared with wild-type ACV, which probably reflects its inability to bind $G\alpha_s$ (Zimmermann et al., 1998). Both C_2 mutants showed striking reductions in forskolin-stimulated activity compared with the wild-type ACV (Fig. 2B). This poor activation of the C_2 mutants suggests that the loss of $G\alpha_s$ sensitivity and subsequent loss of synergistic activation is magnified in the intact cell assay, because the same C_2 mutants retained greater forskolin sensitivity in experiments completed in Sf9 cell membranes (Zimmermann et al., 1998). Although the poor responsiveness to forskolin of the C_2 mutants in our expression system precluded our efforts to assess their $G\alpha_{i/o}$ sensitivity, the C_1 mutant F379L retained sensitivity to D_2 receptor activation. Specifically, the addition of quinpirole reduced forskolin-stimulated cyclic AMP accumulation in cells infected with the F379L by greater than 75% (Fig. 3). Additionally, preliminary results indicate each of the mutants is inhibited by $G\alpha_i$ in vitro (R. T., unpublished observations). Thus, these studies indicate that the selective $G\alpha_s$-insensitive phenotype of the ACV mutants is preserved in experiments conducted in mammalian cells and support the use of these mutants to examine the hypothesized requirement for $G\alpha_s$ in heterologous sensitization.

Lack of Sensitization of $G\alpha_s$-Insensitive Mutants of ACV. Cells infected with HSV-ACV were treated with 1 μM quinpirole or vehicle for 2 h before measuring cyclic AMP accumulation. As observed for cells stably expressing ACV (Fig. 1), quinpirole pretreatment enhanced basal cyclic AMP accumulation in cells infected with HSV-ACV from 2.3 ± 0.2

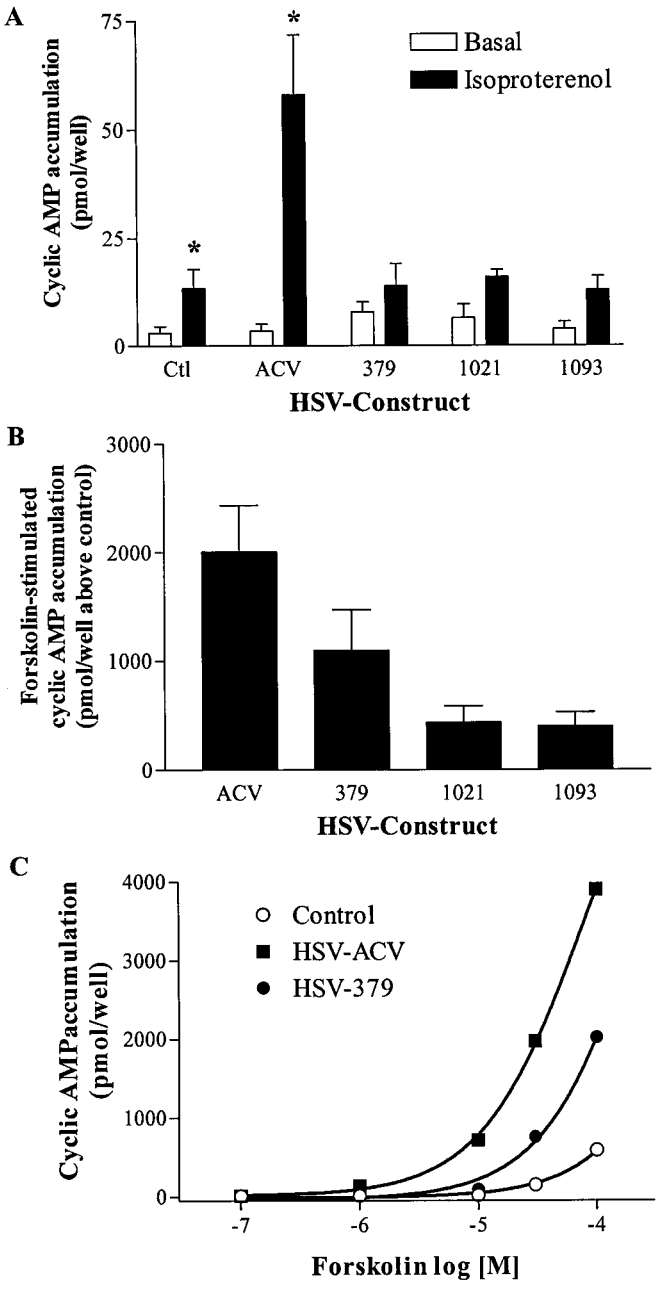

Fig. 2. $G\alpha_s$ and forskolin responsiveness of wild-type and mutant ACV. Basal or drug-stimulated cyclic AMP accumulation was measured in HEK-D_{2L} cells infected with wild-type or mutant AC V as indicated: ACV, wild-type canine adenylyl cyclase type V; 379, the mutant F379L; 1021, R1021Q; and 1093, F1093S. A, basal and 1 μM isoproterenol-stimulated cyclic AMP accumulation. Data shown are the mean ± S.E.M. of three to four independent experiments. *$P < 0.05$ compared with vehicle-treated cells. B, 100 μM forskolin-stimulated cyclic AMP accumulation. Data shown represent cyclic AMP accumulation (pmol/well) above that observed in control cells, and are the mean ± S.E.M. of five independent experiments. C, dose-response curve for forskolin-stimulated cyclic AMP accumulation. Each curve is from a single experiment, assayed in duplicate, representative of three independent experiments.

pmol/well in untreated cells to 4.8 ± 0.3 pmol/well in quinpirole-treated cells (Fig. 4A). In contrast, basal activity was not altered in uninfected cells or cells expressing any of the mutant forms of ACV or β-galactosidase (*lacZ*). Quinpirole pretreatment also increased isoproterenol-stimulated cyclic AMP accumulation from 98 ± 12 pmol/well in untreated ACV-expressing cells to 325 ± 15 pmol/well in cells pretreated with quinpirole (Fig. 4B). Consistent with the observation that the ACV mutants were insensitive to isoproterenol-stimulated activation of $G\alpha_s$ (Fig. 2A), 1 μM isoproterenol caused little increase in cyclic AMP accumulation in either vehicle- or quinpirole-pretreated cells expressing F379L, R1021Q, or F1093S (Fig. 4B). Similarly, forskolin-stimulated activity was greatly enhanced in cells expressing ACV from 5.9 ± 2.4 pmol/well to 49 ± 18 pmol/well in quinpirole-pretreated cells, but was not enhanced in cells expressing F379L, R1021Q, or F1093S (Fig. 4C). Because this concentration of forskolin (100 nM) has little effect on cyclic AMP levels in control HEK-D_{2L} cells or cells infected with HSV-*lacZ* (Fig. 4C), enhanced activity reflects heterologous sensitization of recombinant ACV. Additional studies examined sensitization in cells in expressing wild-type ACV at lower levels (0.25 and 0.5 infectious viral particles/cell) to approximate the level of cyclic AMP accumulation in cells expressing F379L. These experiments revealed that quinpirole pretreatment induced sensitization of ACV under stimulation conditions in which ACV shows little or no response to 100 nM forskolin after vehicle pretreatment (Fig. 5). As in the experiments depicted in Fig. 4C, quinpirole pretreatment had no effect on forskolin-stimulated cyclic AMP accumulation in cells expressing any of the $G\alpha_s$ insensitive ACV mutants or β-galactosidase, supporting our hypothesis that heterologous sensitization requires activation of adenylyl cyclase by $G\alpha_s$. These data also suggest that the enzyme itself is not directly sensitized to forskolin and that the intrinsic activity of adenylyl cyclase is not enhanced as a result of heterologous sensitization.

That responsiveness to $G\alpha_s$ is required for expression of heterologous sensitization of adenylyl cyclase suggests that sensitization reflects altered activity of $G\alpha_s$ or an enhanced interaction between $G\alpha_s$ and adenylyl cyclase. Because rapid heterologous sensitization is not associated with a change in the abundance of $G\alpha_s$ in the cell membrane (Watts et al., 1999), what mechanisms might underlie the enhanced activity of $G\alpha_s$? One possible mechanism involves a change in the distribution of $G\alpha_s$ within the membrane. For example,

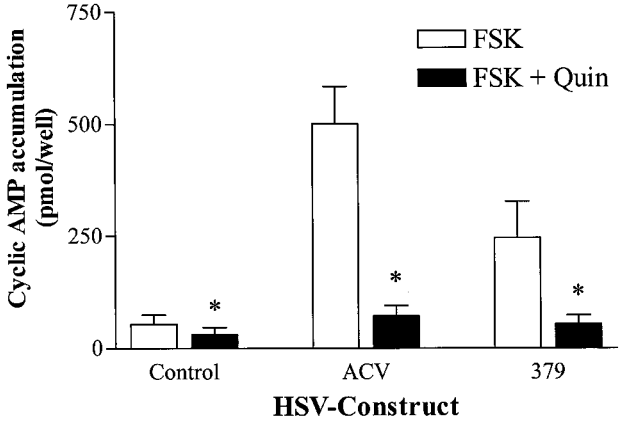

Fig. 3. $G\alpha_{i/o}$ responsiveness of wild-type ACV and F379L. HEK-D_{2L} cells were infected with wild-type ACV or the F397L mutant for 18 h. Cyclic AMP accumulation was measured after 15-min incubation with 10 μM forskolin in the absence (FSK) or presence of 1 μM quinpirole (FSK + Quin). Data shown are the mean ± S.E.M. of four to six independent experiments. *$P < 0.05$ compared with forskolin alone for each condition shown.

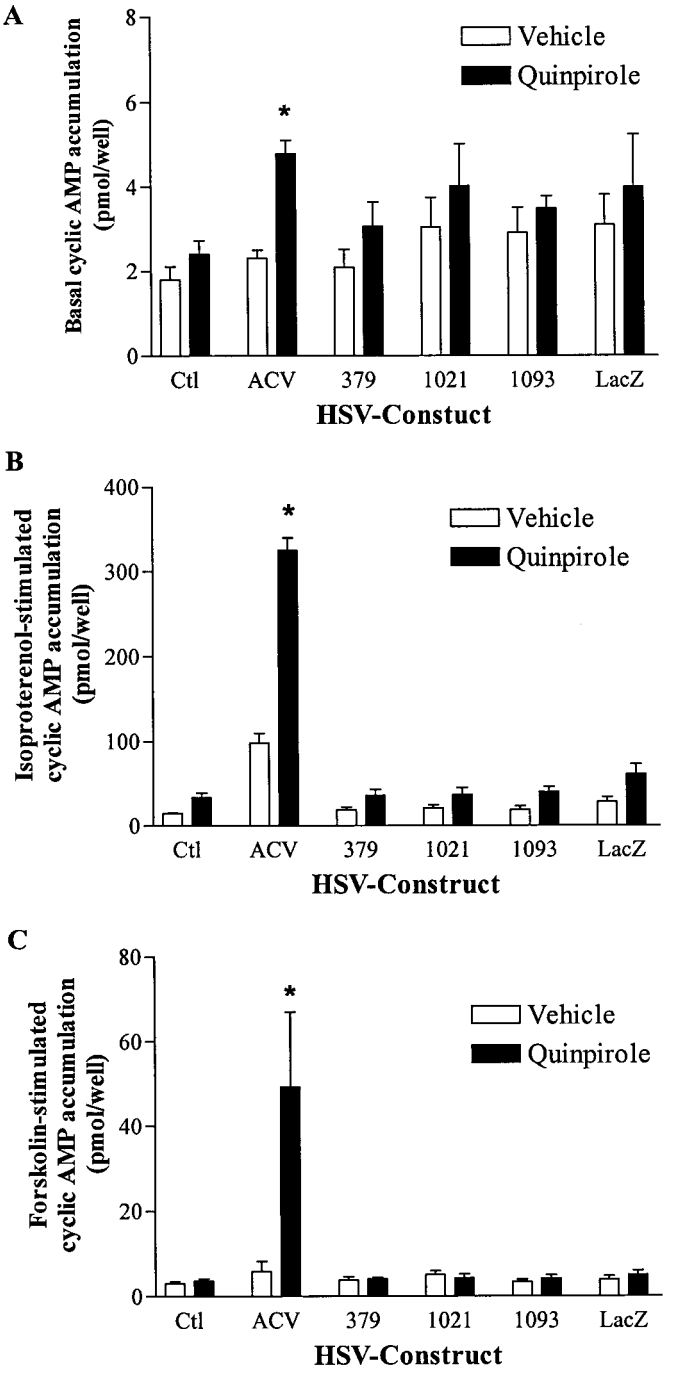

Fig. 4. Heterologous sensitization of wild-type and mutant ACV. Control HEK-D_{2L} cells (Ctl) or cells infected with wild-type or mutant HSV-AC or HSV-*lacZ* were pretreated for 2 h with vehicle (□) or 1 μM quinpirole (■). After pretreatment, cells were washed extensively and subsequently stimulated with vehicle (A), 1 μM isoproterenol (B), or 100 nM forskolin (C). Cyclic AMP accumulation was measured as described under *Materials and Methods*. Data shown are the mean ± S.E.M. of four to six independent experiments. *$P < 0.05$ compared with cells pretreated with vehicle.

chronic treatment of rats or C_6 glioma cells with antidepressant drugs enhances the interactions of $G\alpha_s$ with adenylyl cyclase without changing the abundance of $G\alpha_s$ (Chen and Rasenick, 1995a,b), an effect that is associated with an increase in the proportion of $G\alpha_s$ that can be extracted from the membranes with Triton X-100 and a decrease in the abundance of $G\alpha_s$ in caveolin-enriched domains (Toki et al., 1999). $G\alpha_s$ activity could also increase as a result of reduced activity of a regulator of G protein signaling. There is one report of inhibition of $G\alpha_s$-stimulated adenylyl cyclase activity by an alternatively spliced truncated form of RGS3 (Chatterjee et al., 1997), although the truncated RGS3 does not enhance the GTPase activity of $G\alpha_s$ in vitro (Scheschonka et al., 2000). Another possibility is that adenylyl cyclase is post-translationally modified to change its responsiveness to G proteins. ACV functions as both a GTPase activating protein and an enhancer of GTP/GDP exchange (Scholich et al., 1999), and short-term activation of a $G\alpha_{i/o}$-coupled receptor such as D_{2L} could lead to phosphorylation of the enzyme to alter the binding of $G\alpha_s$ to adenylyl cyclase or the ability of adenylyl cyclase to regulate the activation state of $G\alpha_s$. The identity of the protein kinase that might mediate this effect is unclear; neither protein kinase A nor protein kinase C seems to be involved in D_{2L}-mediated short-term sensitization (Watts and Neve, 1996; V. J. W. and K. A. N., unpublished observations). In conclusion, the finding that responsiveness to $G\alpha_s$ is necessary for the expression of rapid heterologous sensitization by ACV indicates that the mechanism of sensitization is likely to involve a modification of $G\alpha_s$ or of adenylyl cyclase that enhances the sensitivity of the enzyme to $G\alpha_s$.

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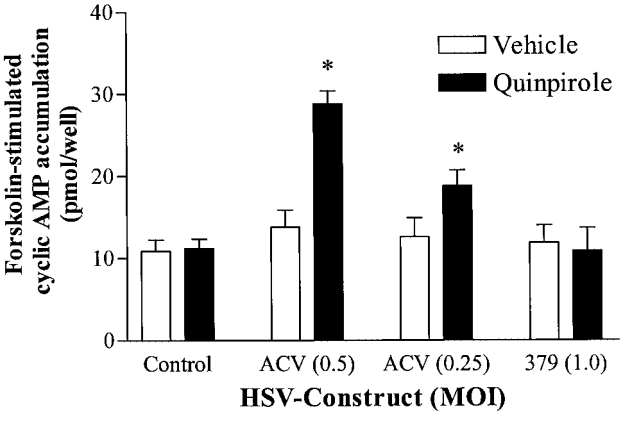

Fig. 5. Heterologous sensitization of wild-type ACV in HEK-D_{2L} cells infected with a reduced multiplicity of infection (MOI). Control HEK-D_{2L} cells or cells infected with the indicated MOI of wild-type ACV or F379L were pretreated for 2 h with vehicle (□) or 1 μM quinpirole (■). After pretreatment, cells were washed extensively and subsequently stimulated with 100 nM forskolin. Cyclic AMP accumulation was measured as described under *Materials and Methods*. Data shown are the mean ± S.E.M. of three to four independent experiments. *$P < 0.05$ compared with cells pretreated with vehicle.

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