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Enhancement of Recombinant γ-Aminobutyric Acid Type A Receptor Currents by Chronic Activation of cAMP-Dependent Protein Kinase

TIMOTHY P. ANGELOTTI, MICHAEL D. UHLER, and ROBERT L. MACDONALD

Departments of Pharmacology (T.P.A.), Biological Chemistry (M.D.U.), Neurology (R.L.M.), and Physiology (R.L.M.) and The Mental Health Research Institute (M.D.U.), University of Michigan Medical School, Ann Arbor, Michigan 48109

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SUMMARY

α1, β1, and γ2S γ-aminobutyric acid (GABA) type A receptor (GABAR) subunit cDNAs were transiently expressed in derivative cell lines of mouse L929 fibroblasts, which possessed different levels of the catalytic subunit of cAMP-dependent protein kinase (PKA). These cell lines included L929 (intermediate levels of kinase), Cα12 (elevated levels of kinase), and RAB10 (low levels of kinase) cells. Pharmacological analysis of GABA-evoked whole-cell currents revealed that, compared with expression in L929 and RAB10 cells, expression of α1β1γ2S GABARs in Cα12 cells produced a selective enhancement of single whole-cell current amplitudes. No other pharmacological properties (Hill slope, EC_{50}, or diazepam sensitivity) of the expressed α1β1γ2S GABARs were modified. The GABAR current enhancement in Cα12 cells was blocked by substitution of a β1 subunit mutated at the PKA consensus phosphorylation site, Ser^{409} [β1(S409A)], for the wild-type β1 subunit. Interestingly, enhancement was specific for GABARs containing all three subunits, because it was not seen after expression of α1β1 or α1β1(S409A) GABAR subunit combinations. Single-channel conductance and gating properties were not different for α1β1γ2S or α1β1(S409A)γ2S GABARs expressed in each cell line, suggesting that PKA did not enhance whole-cell currents by altering these properties of GABARs. These results suggested that unlike acute application of PKA, which has been shown to produce a decrease in GABAR current, chronic elevation of PKA activity can result in enhancement of GABAR currents. More importantly, this effect occurred only with GABARs composed of α1β1γ2S subunits and not α1β1 subunits and was mediated by a single amino acid residue (Ser^{409}) of the β1 subunit.

Rapid modulation of ligand-gated ion channel function by phosphorylation has been shown to occur through several different mechanisms. For example, tyrosine phosphorylation of the nAchR increased the rapid phase of receptor desensitization (1). Enhancement of native glycine (2) and non-NMDA receptor currents by PKA (3, 4) and of recombinant kainate/AMPA and NMDA receptor currents by PKA and PKC, respectively, has also been demonstrated (5–7). In all cases, enhancement was due mainly to an increase in single-channel opening frequency (3, 4, 7) and/or mean open duration (4).

Alternatively, phosphorylation has been shown to affect the biochemistry of receptor assembly and degradation. Claudio and colleagues (8, 9) have provided evidence supporting a role for cAMP, acting through PKA activation, in up-regulating nAchR expression by increasing the efficiency of receptor assembly from its constituent subunits. Increased expression was correlated with phosphorylation of the γ subunit (10). Using chick ciliary ganglion neurons, it was shown that cAMP may also regulate the levels of functional nAchRs on the cell surface (11). Additionally, it has been shown that cAMP can stabilize junctional nAchRs, preventing their degradation (12).

Initial biochemical analysis of GABAR phosphorylation revealed that β subunits could be phosphorylated *in vitro* by PKA (13, 14). Subsequent studies of GABAR modulation by PKA phosphorylation have revealed conflicting outcomes. GABA-gated Cl^- flux in synaptosomes was reported to be decreased by treatment with cAMP analogs and forskolin (15–17). Other experiments showed no effect of activators of PKA on $^{36}Cl^-$ flux desensitization (18). Electrophysiological analysis of GABAR current modulation by externally applied cAMP analogs initially revealed a decrease in whole-cell current amplitudes

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ABBREVIATIONS: nAchR, nicotinic acetylcholine receptor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GABA, γ-aminobutyric acid; GABAR, γ-aminobutyric acid type A receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate.

(17, 19), but subsequent studies revealed that this effect occurred via an extracellular receptor site (20). Demonstration that cAMP analog-mediated inhibition of $^{36}Cl^-$ flux was due to competitive inhibition at the GABA binding site reconciled many of the divergent findings (21). However, it was subsequently shown that incubation of brain microsacs with the active catalytic subunit of PKA also decreased chloride flux (22). In contrast, it has been shown that cerebellar Purkinje cell GABAR currents can be enhanced by acute application of norepinephrine or 8-bromo-cAMP (23, 24).

The first direct electrophysiological study of PKA effects on GABAR currents revealed that application of the purified catalytic subunit of PKA to the intracellular surface of cultured mouse spinal cord neurons or excised outside-out membrane patches reduced current by decreasing single-channel opening frequency (25). However, it was not determined whether PKA was acting indirectly to modulate GABAR currents or directly via phosphorylation of one or more GABAR subunits. Moreover, the subunit composition of GABARs in these cells was not known and, thus, correlation of PKA-mediated phosphorylation of specific subunits with the effect could not be made. Using a recombinant GABAR preparation, Moss *et al.* (26) showed that prolonged intracellular perfusion of cAMP (>20 min) led to a decrease in the rapid desensitization component of $\alpha1\beta1$ GABAR currents and a reduction of whole-cell $\alpha1\beta1\gamma2S$ GABAR currents. Single-channel properties were not studied.

Recently, we have characterized whole-cell and single-channel properties of recombinant GABARs expressed from $\alpha1$, $\beta1$, and $\gamma2S$ subunits after transient expression in mouse L929 cells (27, 28). The studies revealed preferential assembly of $\alpha1\beta1\gamma2S$ GABARs over $\alpha1\beta1$ GABARs, with the former possessing open, closed, and burst duration properties similar to those of native neuronal GABARs. Combining these techniques, we sought to determine the effects of PKA on pharmacological and biophysical properties of a GABAR composed of defined subunits.

Materials and Methods

Sources. Tissue culture dishes were purchased from Corning Glass Works (Corning, NY). Restriction and DNA modification enzymes were obtained from either GIBCO BRL (Bethesda, MD), Boehringer Mannheim Biochemicals (Indianapolis, IN), or New England Biolabs, Inc. (Beverly, MA). Fluorescein-di-β-galactopyranoside was purchased from Molecular Probes, Inc. (Eugene, OR). Diazepam was kindly provided by Hoffmann-LaRoche (Nutley, NJ). All chemicals, drugs, sera, and media were obtained from GIBCO BRL, Boehringer Mannheim Biochemicals, or Sigma Chemical Co. (St. Louis, MO).

cDNA expression plasmids and mutant construction. pCMVNeo-based expression vector constructs encoding bovine $\alpha1$, bovine $\beta1$, and human $\gamma2S$ GABAR subunits and *Escherichia coli* β-galactosidase were described elsewhere (27). In preparation for mutagenesis of the bovine $\beta1$ subunit, the original vector containing the cDNA, pBGR$\beta1$ (obtained from E. Barnard, Medical Research Council, London, UK), was digested with *Bsm*I and *Ppu*MI to release a 1.54-kilobase fragment containing the complete open reading frame (base pairs 2–1539). After blunting of the single-stranded DNA overhangs with T4 polymerase and ligation of *Bam*HI linkers to the insert (New England BioLabs, Beverly, MA), the cDNA was subcloned into the *Bam*HI site of the vector pGem7Z(−) (Promega Inc., Madison, WI) to form the plasmid pG7b$\beta1$S.

The codon specifying Ser^{409} of the mature $\beta1$ subunit protein was changed to code for alanine, to remove the consensus PKA phosphorylation site, using the two-oligonucleotide mutagenesis technique of Zoller and Smith (29). An oligonucleotide was synthesized that spanned the region 5′-GCCgCtCAGCTCAAAGTCA-3′ (base pairs 1297–1315); mutated nucleotides are shown by lower case letters. The 5′ mutation changed the serine codon to alanine, whereas the 3′ mutation introduced a unique restriction site (*Esp*I) to facilitate identification of the mutant cDNA. This mutation did not change the alanine or other codons. The resulting plasmid was named pG7bβK−, and the encoded mutant protein was referred to as $\beta1$(S409A). The mutation was verified by double-stranded DNA sequencing (30). The mutated cDNA was then excised from the plasmid pG7b$\beta1$K− by digestion with *Bam*HI, and the isolated fragment was subcloned into the expression plasmid pCMVNeo (31) to form the plasmid pCMVb$\beta1$K−.

Cell lines and transfections. L929 cells were purchased from the American Type Culture Collection (Rockville, MD). Two L929-derived cell lines with varying levels of catalytically active PKA (Cα12 and RAB10 cells) have been described elsewhere (32). All cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37° in 5% CO_2/95% air. In preparation for transfection and electrophysiological recording, L929 and derivative cell lines were plated as described previously (27). Cells were transfected, using the modified calcium phosphate precipitation method (33), with various combinations of CsCl-banded vectors. Plasmids were mixed in a 1:1:1:1 (α:β:γ:*lacZ*) or 1:1:1 (α:β:*lacZ*) ratio; the total amount of DNA added per 60-mm dish was maintained at 9.6 μg in 300 μl of transfection buffer. Twenty-four hours after transfection, cells were prepared for patch-clamp recording by being replated onto gridded 35-mm dishes (27).

Electrophysiological analysis. GABAR-expressing cells for study were localized before recording by using a fluorescent LacZ substrate, as described previously (27). For whole-cell and single-channel patch-clamp analysis, the cells were washed with external recording medium containing the following, in mM: 142 NaCl, 8.1 KCl, 6 $MgCl_2$, 10 mM glucose, 10 HEPES, pH 7.4. The intrapipette solution contained the following, in mM: 153 KCl, 1 $MgCl_2$, 5 mM EGTA, 10 mM HEPES, pH 7.3. This combination of external and intrapipette solutions produced a chloride equilibrium potential of 0 mV (E_{Cl}) and a potassium equilibrium potential of −75 mV (E_K) across the patch membrane. Electrodes were fashioned from microhematocrit capillary tubing using a model P-87 Flaming Brown micropipette puller (Sutter Instrument Co., San Rafael, CA). Electrodes had a resistance of 3–6 MΩ. Drugs (GABA and diazepam) were diluted with external recording solution, from a stock solution (10 mM or 1 mM, respectively), to the indicated final concentration on the day of the experiment. Drugs were applied with a pressure ejection micropipette (10–15-μm tip diameter; 0.5–1.0 psi) placed next to the cell or patch. Initial drug application was made within 30 sec after rupture of the cell membrane. Whole-cell current recording was performed with methods described previously for mouse spinal cord neuron recordings (25, 34), using a List L/M EPC-7 amplifier (Darmstadt, Germany). All recordings were done at room temperature (22–24°). GABA concentration-response curves and EC_{50} values were fitted with the GraphPAD InPlot program (GraphPAD, San Diego, CA). Statistical analysis of whole-cell current amplitudes was performed using the Student unpaired *t* test.

Whole-cell and single-channel data collection and analysis. Currents were recorded simultaneously with a video cassette recorder (Sony SL-2700, modified to 0–20 kHz) via a digital audio processor (Sony PCM-501 ES, 14 bit, 44 kHz) and with a chart recorder (Gould Inc., Cleveland, OH) for later computer analysis. Whole-cell and single-channel recordings were low-passed filtered (3 dB at 1 kHz, eight-pole Bessel filter; Frequency Devices) before the chart recorder. Peak whole-cell current amplitudes were measured by hand from the chart output. Single-channel open, closed, and burst properties and amplitudes were analyzed using methods described previously for mouse spinal cord neurons and recombinant GABARs (25, 28, 34). At least 1000 openings and 350 bursts were analyzed for each GABAR/cell line combination.

Results

PKA modulation of whole-cell GABAR currents. Effects of PKA on GABAR function were studied by expressing various GABAR subunit combinations in three different cell lines that had varying levels of the active catalytic subunit of PKA. The cell lines included the parental L929 cell line and two stably transfected derivative clones, Cα12 and RAB10 (32). The former cell line overexpressed free activated catalytic subunit and the latter cell line overexpressed mutant mouse type I regulatory subunit of PKA. In the absence of exogenous cAMP, the three cell lines had levels of active kinase that ranged from approximately 5 kinase units/mg of protein (RAB10) to 500 units/mg (Cα12), with L929 cells possessing an intermediate level of activity (100 units/mg) (32).

α1β1γ2S GABARs were expressed in these three cell lines by acute transfection of expression vectors encoding each of the three subunits, and whole-cell currents were elicited from isolated transfected cells. Application of 10 μM GABA to L929, Cα12, and RAB10 cells expressing GABARs revealed a difference in the magnitude of whole-cell current amplitudes (Fig. 1A). Specifically, α1β1γ2S GABAR currents expressed in Cα12 cells were 3–4 times larger in amplitude than GABAR currents expressed in L929 or RAB10 cells. Addition of 50 nM diazepam to 3 μM GABA caused a similar increase in whole-cell GABAR currents expressed in all three cell lines (data not shown). The average enhancement was approximately 60% (Table 1). Although accurate desensitization rates could not be measured with the drug perfusion system utilized, it was noted that α1β1γ2S GABAR currents expressed in Cα12 cells desensitized more rapidly than those expressed in the other two cell lines (Fig. 1A, *arrows*). No differences were noted in the input resistance or membrane capacitance of the different cell lines (data not shown).

Enhancement of GABAR currents in Cα12 cells could have been due to an alteration in the properties of the same receptor expressed in L929 cells or to expression of GABARs composed of a different combination of subunits, with different biophysical properties. The similar diazepam sensitivities present in all three cell lines, however, suggested that the γ2 subunit was likely present in the GABARs expressed in all cell lines. An altered or novel GABAR expressed uniquely in Cα12 cells could produce enhancement of GABAR current due to a shift in agonist affinity for the receptor complex and/or a change in the maximum whole-cell current amplitude.

To investigate these possibilities further, GABA concentration-response curves were constructed for α1β1γ2S GABARs expressed in the three cell lines. The 3–4-fold enhancement of α1β1γ2S GABAR currents seen in Cα12 cells was present at all concentrations of GABA tested (0.3–30 μM), compared with receptors expressed in L929 and RAB10 cells (Fig. 1B). Concentration-response curves were not shifted relative to one another, suggesting that there was no change in GABA affinity for channel activation. The concentration-response curves for the three GABAR/cell line combinations had similar EC_{50} values, ranging from 3.3 to 5.3 μM, and all had Hill slope values greater than 1.5 (Table 1). Data shown represent the pooling of several separate experiments, but similar results were evident after each transfection (data not shown). These data suggest that enhancement of GABAR current in Cα12 cells was due to an increase in maximum whole-cell current without a change in receptor affinity for GABA.

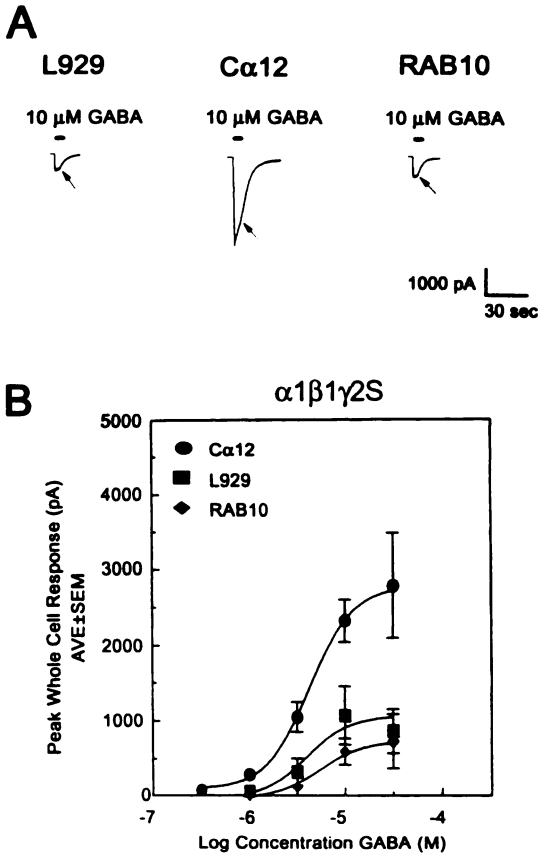

Fig. 1. Whole-cell current analysis of α1β1γ2S GABARs expressed in various cell lines. A, Representative whole-cell currents were recorded from individual cells expressing α1β1γ2S GABARs. A 3-sec application of 10 μM GABA (*bar*) was applied to each cell to elicit a response. Cells were voltage clamped at a holding potential of −75 mV. Downward deflections indicate outward Cl^- conductance. The threshold for detection was 2 pA at a holding potential of −75 mV. Note the amplitude scale. See text for description of different cell lines. These data are representative of 5–8 cells for each receptor/cell line combination. *Arrows*, offset of drug application and thus the extent of current desensitization. B, α1β1γ2S GABARs were expressed in L929, Cα12, and RAB10 cells. Whole-cell currents were evoked with increasing concentrations of applied GABA. Absolute peak whole-cell current values were averaged at each concentration and the best-fit *curve* was drawn. Data represent the summation of two to four transfections. L929, n = 7–11 cells; Cα12, n = 5–20 cells; RAB10, n = 6–8 cells.

Expression of GABARs composed of mutant β1 subunits. The whole-cell GABAR current experiments described above were repeated with mutant β1 subunits [β1(S409A)] substituted for wild-type β1 subunits. This mutant β1 subunit had an alanine residue substituted for serine within the PKA consensus phosphorylation sequence (Ser^{409}) (see Materials and Methods for details). The nucleotide mutations introduced did not affect the steady state levels of transcribed mRNAs, as assessed by Northern blot analysis (data not shown). Expression of wild-type and mutant subunits as bacterial fusion proteins demonstrated that the mutation blocked *in vitro* phosphorylation by purified catalytic subunit of PKA (data not shown), as has been demonstrated by others (35).

Coexpression of α1, β1(S409A), and γ2S subunit cDNAs in either L929, Cα12, or RAB10 cells produced functional GABAR currents, demonstrating that the mutation did not prevent assembly and/or insertion of receptors into the membrane (Fig. 2A). There was no apparent difference in desensitization

TABLE 1

Pharmacological properties of wild-type and mutant GABARs

Whole-cell GABA currents and concentration-response curves were obtained from single LacZ$^+$ transfected cells as described in Materials and Methods. The Hill slope and EC_{50} values were calculated from absolute current values (Figs. 1 and 2).

Receptor type	Peak W-C current (10 μM GABA)[a]	Hill slope[b]	EC_{50}	Diazepam enhancement[c]
			μM	%
α1β1γ2S				
L929	1070 ± 384	1.7 ± 0.2	5.3	56 ± 7 (*n* = 8)
Cα12	2325 ± 283	1.7 ± 0.2	4.3	72 ± 13 (*n* = 6)
RAB10	594 ± 172	1.5–2.0	3.9	64 ± 8 (*n* = 8)
α1β1(S409A)γ2S				
L929	508 ± 189	1.5–2.0	4.0	60 ± 7 (*n* = 6)
Cα12	896 ± 169	1.5–2.0	3.3	64 ± 14 (*n* = 6)
RAB10	1068 ± 297	1.5–2.0	3.3	57 ± 8 (*n* = 8)
α1β1				
L929	200 ± 50	1.1 ± 0.2	1.0	0 (*n* = 9)
Cα12	177 ± 50	0.7–1.3	1.0	0 (*n* = 7)
α1β1(S409A)				
Cα12	218 ± 60	0.7–1.3	2.0	0 (*n* = 4)

[a] Peak whole-cell (W-C) currents elicited by application of 10 μM GABA were measured and are presented as average ± standard error. Expression of wild-type and mutant GABARs in different cell lines were performed in parallel on the same day to limit transfection artifacts.

[b] Hill slope measurements were made from whole-cell GABA concentration-response curves. For most GABARs, the number of applied GABA concentrations was too low to permit an accurate determination. For these cases, the Hill slope is represented by a range estimated from the initial slope of a log-log transformation of the data.

[c] Diazepam (50 nM) was co-applied with GABA (3.0 μM), and the percent enhancement was determined by comparing the peak response to that elicited by GABA (3.0 μM) alone. Values are given as the average ± standard error.

of α1β1(S409A) GABAR whole-cell currents expressed in the three cell lines (Fig. 2A, *arrows*). Expression of α1β1(S409A)γ2S GABARs in all three cell lines produced similar GABA concentration-response curves with respect to maximum current amplitude and agonist affinity (Fig. 2B). The similarity in whole-cell GABAR current amplitudes was present at all concentrations of GABA tested (0.3–30 μM). Again, concentration-response curves were not shifted relative to one another, suggesting no change in GABA affinity. The mutant GABARs had EC_{50} and Hill slope values similar to those of their respective wild-type GABAR/cell line counterparts (Table 1). More importantly, concentration-response curves for α1β1γ2S GABARs expressed in L929 and RAB10 cells (Fig. 1B) and α1β1(S409A)γ2S GABARs expressed in all three cell lines (Fig. 2B) were simlar; no statistically significant differences were seen among them at any concentration of applied GABA (data not shown). The only significant difference in concentration-response curves within a cell line was for wild-type and mutant GABARs expressed in Cα12 cells (Fig. 2B, *inset*). To control for variation within a cell line, parallel plates of cells were transfected with either α1β1γ2S or α1β1(S409A)γ2S GABAR subunits and the majority of recordings were made on the same day (Table 1; Figs. 1B and 2B). Thus, the lack of GABAR current enhancement correlated with either the presence of low levels of active PKA found in L929 and RAB10 cells, relative to Cα12 cells, or the presence of a mutated β1 subunit lacking the PKA phosphorylation sequence and was not a transfection artifact. As seen above, application of 50 nM diazepam and 3 μM GABA caused an increase in all whole-cell currents expressed from α1, β1(S409A), and γ2S subunits, with an average enhancement of approximately 60% (Table 1).

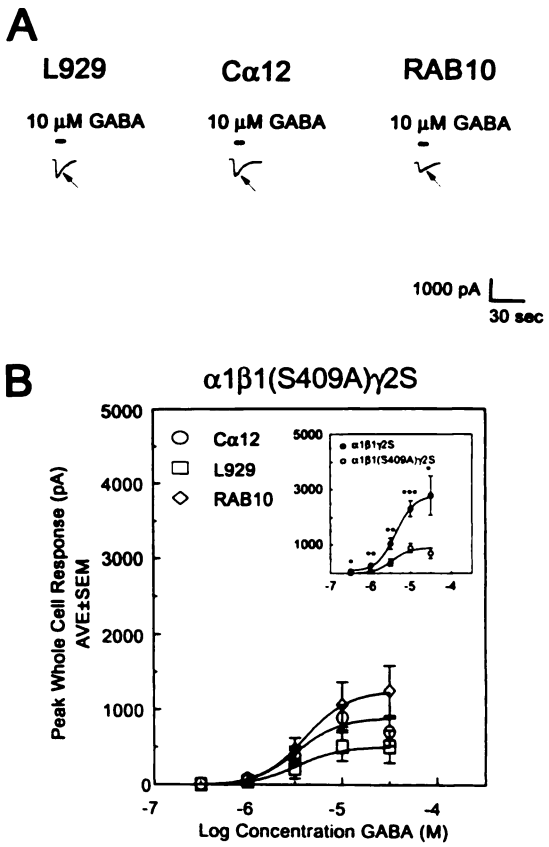

Fig. 2. Whole-cell current analysis of expressed α1β1(S409A)γ2S GABARs. A, α1β1(S409A)γ2S GABARs were expressed in L929, Cα12, and RAB10 cells and GABA-evoked whole-cell currents were obtained as described in the legend to Fig. 1. Representative *tracings* are shown. Notice the amplitude scale. *Arrows*, offset of drug application and thus the extent of current desensitization. B, α1β1(S409A)γ2S GABARs were expressed in L929, Cα12, and RAB10 cells. Whole-cell currents were analyzed as described in the legend to Fig. 1B. Data represent the summation of two to four transfections. L929, *n* = 9–14 cells; Cα12, *n* = 7–28 cells; RAB10, *n* = 4–11 cells. A comparison of the concentration-response curves for α1β1γ2S and α1β1(S409A)γ2S GABARs expressed in Cα12 cells is shown (*inset*). *, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.0001$ (Student's unpaired *t* test).

Enhancement of α1β1γ2S GABAR currents expresssed in Cα12 cells was not reproduced by acute intracellular application of PKA. GABA-elicited whole-cell currents were recorded from L929 cells, expressing either subunit combination, with dialysis of 50 μg/ml concentrations of active catalytic subunit of PKA (plus 2 mM Mg-ATP) into the cell. Over the course of 15 min, the presence of intracellular PKA catalytic subunit did not cause an enhancement of current amplitude in either wild-type or mutant GABARs (data not shown).

PKA effects on α1β1 GABARs. To determine whether the whole-cell current enhancement seen in Cα12 cells occurred for all GABAR subunit combinations containing the β1 subunit, α1β1 and α1β1(S409A) GABARs were expressed in L929 and Cα12 cells. Interestingly, the enhancement seen previously with α1β1γ2S GABARs expressed in Cα12 cells was not evident upon expression of α1β1 subunits. The average amplitudes of the whole-cell currents elicited by 10 μM GABA from α1β1 GABARs expressed in L929 and Cα12 cells and from α1β1(S409A) GABARs expressed in Cα12 cells were similar (Fig. 3). These results demonstrated that the enhancement seen could not be generalized to all GABARs containing a β1

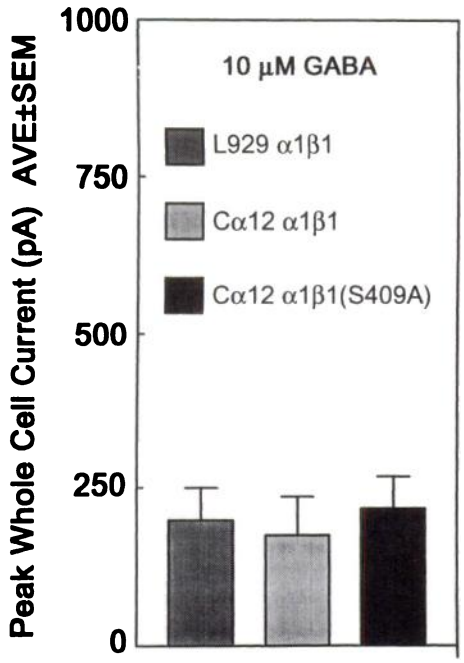

Fig. 3. Whole-cell current analysis of expressed $\alpha1\beta1$ and $\alpha1\beta1$(S409A) GABARs. GABA (10 μM)-elicited peak whole-cell currents of $\alpha1\beta1$ and $\alpha1\beta1$(S409A) GABARs expressed in L929 and Cα12 cells were averaged. Currents were averaged from 9, 7, and 8 cells for L929 ($\alpha1\beta1$), Cα12 ($\alpha1\beta1$), and Cα12 [$\alpha1\beta1$(S409A)] cells, respectively. No statistically significant difference was noted between any cell type or GABAR.

subunit but only GABARs composed of $\alpha1$, $\beta1$, and $\gamma2S$ subunits. Previously, we demonstrated that GABARs composed of $\alpha1\gamma2S$ and $\beta1\gamma2S$ subunits were not functionally expressed in L929 cells (27). Expression of these subunit combinations in Cα12 and RAB10 cells also did not result in functional GABARs (data not shown), suggesting that lack of expression was not due to PKA activity.

GABA concentration-response curves for $\alpha1\beta1$ and $\alpha1\beta1$(S409A) GABARs expressed in L929 and Cα12 cells were also similar, with no discernable differences seen in either the maximum current amplitude or GABA affinity (data not shown). They had similar EC_{50} values (1.0–2.0 μM) and had Hill slope values of approximately 1.0 (Table 1). As expected, because of the lack of the $\gamma2S$ subunit, application of diazepam had no effect on either $\alpha1\beta1$ or $\alpha1\beta1$(S409A) GABARs expressed in L929 or Cα12 cells (Table 1).

$\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABAR single-channel currents. Because enhancement of GABAR whole-cell currents by PKA in Cα12 cells was associated with an increase in maximum response without an alteration in agonist affinity, the enhancement could have been due to changes in single-channel properties of the GABAR, including single-channel conductance or gating properties, or to expression of a different GABAR with dissimilar single-channel biophysical properties but similar whole-cell pharmacological properties. To investigate these possibilities further, single-channel GABAR currents were recorded from excised outside-out membrane patches from L929, Cα12, and RAB10 cells expressing either $\alpha1\beta1\gamma2S$ or $\alpha1\beta1$(S409A)$\gamma2S$ GABARs. Application of 3 μM GABA to the membrane patches elicited openings to two different conductance levels (Fig. 4). No obvious differences were noted in openings, closings, or bursts among the different expressed GABARs. No openings were seen in the absence of GABA for

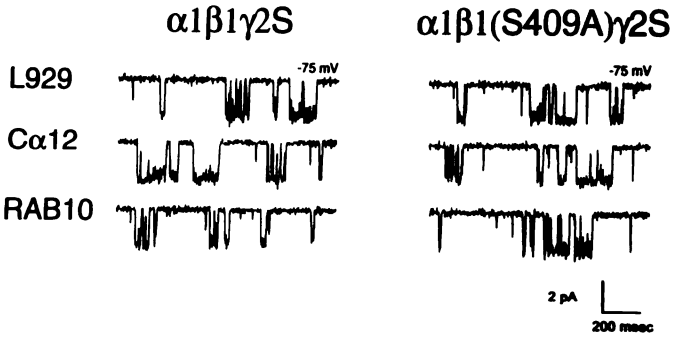

Fig. 4. Raw data *tracings* of single-channel currents of $\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABARs. GABA (3 μM) was applied to excised outside-out membrane patches from L929, Cα12, and RAB10 cells expressing either $\alpha1\beta1\gamma2S$ or $\alpha1\beta1$(S409A)$\gamma2S$ GABARs. Patches were held at −75 mV. Downward deflections indicate outward Cl^- current. Amplitude and time scales are shown at the *bottom right*.

any receptor or any cell type (data not shown), suggesting that PKA phosphorylation did not induce agonist-independent gating of the channel as has been described for some nAchR preparations (36).

To obtain a more accurate measurement of the two conductance levels of wild-type and mutant GABARs expressed in the three different cell lines, complete single-channel current-voltage relationships were constructed. These values were derived from the means of the main conductance and subconductance levels calculated from Gaussian-fitted amplitude histograms constructed at various membrane holding potentials (data not shown). For both main conductance and subconductance level openings of $\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABARs in all cell lines, the current amplitude varied linearly with membrane holding potential and inverted at approximately 0 mV ($E_{Cl} = 0$ mV) (data not shown). The calculated chord conductances ranged from 27.8 to 30.5 pS for the main conductance level and from 19.7 to 21.8 pS for the subconductance level, with no discernible difference being noted between wild-type and mutant GABARs or among cell lines (Table 2). Main conductance level openings accounted for approximately 80% of the total openings in all six GABAR/cell line combinations. The remaining openings were to the subconductance level. In terms of total current, approximately 90% of GABAR current was through the main conductance level for both $\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABARs. The remaining current was through subconductance level openings (Table 2). Thus, in any cell line expression of $\beta1$(S409A) subunits along with $\alpha1$ and $\gamma2S$ subunits, compared with $\alpha1\beta1\gamma2S$ GABARs, did not alter the magnitude of the conductance levels and did not shift the relative amount of current between conductance levels to produce the enhancement of whole-cell GABAR current seen. In addition, few, if any, 10- or 15-pS openings reminiscent of $\alpha1\beta1$ GABARs (28) were found in single-channel traces from any GABAR/cell line combination (<0.1% of openings) (data not shown).

Steady state main conductance and subconductance level openings, closings, and bursts were analyzed for the six different GABAR/cell line combinations, to determine whether dissimilarities in steady state single-channel properties were responsible for the 3–4-fold enhancement of whole-cell GABAR currents in Cα12 cells. Because approximately 90% of single-channel current was through main conductance level openings, any significant change in main conductance level channel prop-

TABLE 2

Single-channel properties of $\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABARs

Single-channel openings were elicited from excised outside-out membrane patches from three different cell lines transiently expressing either $\alpha1\beta1\gamma2S$ or $\alpha1\beta1$(S409A)$\gamma2S$ GABARs. Various concentrations of GABA (1–3 μM) were applied. All values are the average ± standard error.

	Chord conductance[a]		Opening in conductance level[b]		Current in conductance level[c]		Patches
	Subconductance	Main conductance	Subconductance	Main conductance	Subconductance	Main conductance	
	pS		%		%		n
L929							
$\alpha1\beta1\gamma2S$	21.8 ± 0.9	29.3 ± 0.9	18.6 ± 3.4	81.4 ± 3.4	8.6	91.4	13
$\alpha1\beta1$(S409A)$\gamma2S$	21.3 ± 0.4	30.5 ± 0.9	13.5 ± 2.2	86.5 ± 2.2	7.7	92.3	6
Cα12							
$\alpha1\beta1\gamma2S$	20.6 ± 0.6	29.3 ± 0.6	18.3 ± 4.9	81.7 ± 4.9	5.5	94.5	6
$\alpha1\beta1$(S409A)$\gamma2S$	19.7 ± 0.4	27.8 ± 1.0	22.5 ± 6.6	77.5 ± 6.6	16.2	83.8	4
RAB10							
$\alpha1\beta1\gamma2S$	21.6 ± 0.4	28.4 ± 0.4	20.2 ± 3.3	79.8 ± 3.3	13.7	86.3	6
$\alpha1\beta1$(S409A)$\gamma2S$	20.2 ± 0.2	28.2 ± 0.8	16.7 ± 2.9	83.3 ± 2.9	10.6	89.4	6

[a] Chord conductances determined from best-fit current-voltage plots.
[b] The percentage of openings in each conductance level was determined for each GABA application and averaged. Analysis with the Student's unpaired *t* test revealed no statistically significant differences between any of the subconductance or main conductance level percentages.
[c] The percentage of current in each conductance level was calculated from the total average current.

erties responsible for the enhancement in GABAR current should have been evident. However, no distinct changes in any open, closed, or burst duration property for $\alpha1\beta1\gamma2S$ GABARs expressed in Cα12 cells were seen to explain the enhancement of whole-cell currents. Specifically, these included mean open, closed, and burst durations, mean percent open, percentage of analysis time in a burst, and opening and burst frequencies (Table 3).

In summary, no steady state single-channel open, closed, or burst duration property was significantly altered for either $\alpha1\beta1\gamma2S$ or $\alpha1\beta1$(S409A)$\gamma2S$ GABARs expressed in L929, Cα12, and RAB10 cells to account for the enhancement of whole-cell current observed. Thus, expression of either $\alpha1\beta1\gamma2S$ or $\alpha1\beta1$(S409A)$\gamma2S$ GABARs in the three cell lines did not produce unique receptors with different single-channel properties but similar whole-cell pharmacology; all assembled GABARs were homogeneous and electrophysiologically similar.

PKA effects on GABAR expression. Alternatively, chronic PKA activation may result in different steady state levels of functional, cell surface GABARs. Such an effect could account for the whole-cell current enhancement observed without alteration of single-channel properties. This could occur by PKA regulation of GABAR assembly or degradation rates. However, attempts to directly measure levels of expressed GABARs after acute transfection of L929, Cα12, and RAB10 cells were unsuccessful due to low (1–3%) transfection efficiences. Specific binding of [^{3}H]flunitrazepam could not be seen above nonspecific binding, even with 500 μg of membrane protein/binding assay (data not shown). Thus, variations in assembly or degradation rates could not be measured in this expression system with available methods.

Discussion

Transient transfection of L929 cells and derivative cell lines Cα12 and RAB10 (high and low PKA activity, respectively) permitted comparison of the effects of cellular PKA levels on GABAR function and expression. It permitted easy interchange of subunits and cell lines and also substitutition of mutated for wild-type GABAR subunits to determine their role in mediating PKA effects. Recent biochemical experiments have demonstrated that murine $\beta1$ subunits, but neither $\alpha1$ nor $\gamma2S$ subunits, can serve as substrates for PKA phosphorylation and that phosphorylation occurred at Ser^{409} of the $\beta1$ subunit (35). Our present studies utilized transient expression of bovine $\alpha1$, bovine $\beta1$, and human $\gamma2S$ subunits to examine PKA effects on GABARs. However, almost 100% conservation of amino acid residues exists among the different subunit species, suggesting that bovine $\alpha1$ and human $\gamma2S$ subunits also would not be phosphorylated by PKA (37).

The major finding of this study was that expression of $\alpha1\beta1\gamma2S$ GABARs in cells expressing high levels of PKA activity (Cα12 cells) resulted in a 3–4-fold increase in the amplitude of GABAR whole-cell currents, compared with expression of $\alpha1\beta1\gamma2S$ GABARs in cells containing lower levels

TABLE 3

Recombinant $\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABAR main conductance level open and closed properties

All listed parameters were determined from detected main conductance level openings and bursts of $\alpha1\beta1\gamma2S$ and $\alpha1\beta1$(S409A)$\gamma2S$ GABARs transiently expressed in L929, Cα12, and RAB10 cells. Openings were evoked by 3 μM GABA (see Materials and Methods for analysis parameters).

	L929		Cα12		RAB10	
	$\alpha1\beta1\gamma2S$	$\alpha1\beta1$(S409A)$\gamma2S$	$\alpha1\beta1\gamma2S$	$\alpha1\beta1$(S409A)$\gamma2S$	$\alpha1\beta1\gamma2S$	$\alpha1\beta1$(S409A)$\gamma2S$
Mean open duration, msec	5.9	6.6	5.9	6.5	5.4	5.0
Mean closed duration, msec	89	86	115	98	59	50
Mean burst duration, msec	16.4	22.3	17.0	15.8	15.7	14.6
Mean percent open	6.0	6.1	4.0	6.1	7.9	8.8
Percentage of analysis time in burst	7.0	7.9	5.2	6.9	9.5	10.4
Openings/sec	10.2	9.2	6.8	9.4	14.5	17.6
Bursts/sec	4.0	3.0	2.6	4.2	5.7	6.8

of PKA activity (L929 and RAB10 cells). To determine the phosphorylation site responsible for this enhancement, we compared currents obtained after replacement of the wild-type $\beta 1$ subunit with a $\beta 1$ subunit lacking the PKA consensus site, removed by replacement of Ser^{409} with alanine. To determine the mechanism of enhancement, we compared the single-channel properties of wild-type $\alpha 1\beta 1\gamma 2S$ GABARs with those of mutated $\alpha 1\beta 1$(S409A)$\gamma 2S$ GABARs expressed in all three cell lines.

Analysis of $\alpha 1\beta 1\gamma 2S$ and $\alpha 1\beta 1$(S409A)$\gamma 2S$ GABAR whole-cell currents recorded from each of the different cell lines revealed that the mutation of the $\beta 1$ subunit abolished the enhancement of the whole-cell current in Cα12 cells. However, the mutation of the $\beta 1$ subunit did not alter any pharmacological properties of the GABAR whole-cell currents. The wild-type and mutant GABARs had similar Hill slope values for GABA activation, GABA EC_{50} values, and diazepam sensitivities in all three cell types. The only difference found was in the enhancement of peak whole-cell current amplitude in Cα12 cells with a modestly increased rate of desensitization. However, this dissimilarity in desensitization would tend to decrease, not increase, the whole-cell current and thus would not explain the difference in amplitude. It was possible that activation of PKA could be preventing GABAR "run-down," thus causing enhancement of whole-cell current. For instance, chronic activation of PKA may have mimicked the effects of intracellularly applied Mg-ATP or adenosine-5′-*O*-(3-thio)triphosphate to prevent GABAR run-down (38, 39). This seems unlikely, because the enhancement seen was evident even during the first application of GABA to a cell, before run-down usually begins (data not shown).

Curiously, the enhancement of whole-cell current did not occur for all GABAR subunit combinations containing the wild-type $\beta 1$ subunit. Coexpression of only $\alpha 1$ and $\beta 1$ [as well as $\alpha 1$ and $\beta 1$(S409A) subunits] in either L929 or Cα12 cells produced functional GABARs with similar pharmacological properties and, more importantly, similar maximum whole-cell current amplitudes.

The specificity of the effect for only wild-type $\alpha 1\beta 1\gamma 2S$ GABARs and not for $\alpha 1\beta 1$ GABARs or GABARs containing a mutated $\beta 1$ subunit suggested that enhancement of GABAR currents observed after expression in Cα12 cells was mediated via a direct effect on GABAR $\beta 1$ subunits and not an indirect effect of the cell type used for expression. In addition, this effect was not reproduced by acute intracellular application of PKA. Unfortunately, it is experimentally impossible to record whole-cell currents from individual transfected cells and to simultaneously determine the phosphorylation status of cell surface GABARs, to verify the role of phosphorylation in this process.

Because there were no alterations in the pharmacological or biophysical properties of the GABAR whole-cell currents recorded from Cα12 cells, it is likely that the enhancment of current by PKA was due to 1) an increase in the number of functional GABAR channels and/or 2) an alteration in the GABAR single-channel properties. If an increase in the number of GABAR channels with no alteration in the gating properties of the channels is responsible for the increase in whole-cell current, then recordings obtained from a single GABAR channel would be expected to demonstrate no change in single-channel opening frequency or in other single-channel properties. An increase in current due to an alteration in GABAR single-channel properties without an increase in the number of GABAR channels could be produced by an increase in main conductance level, an increase in the mean channel open and/or burst duration, or an increase in opening frequency. Thus, single-channel experiments were performed to determine whether expression of GABARs in Cα12 cells altered any of these single-channel properties.

Single-channel analysis of steady state gating properties of $\alpha 1\beta 1\gamma 2S$ or $\alpha 1\beta 1$(S409A)$\gamma 2S$ GABARs expressed in L929, Cα12, and RAB10 cells revealed no distinct differences in any open, closed, or burst parameters, conductance level, or opening frequency. There were no differences in the main conductance and subconductance levels (29 and 21 pS, respectively) and there were no shifts in the percentage of openings in either conductance level. Therefore, enhancement of $\alpha 1\beta 1\gamma 2S$ currents in Cα12 cells was not explained by an alteration of conductance level or mean open or burst duration or to assembly of GABARs with different single-channel biophysical properties but overall similar whole-cell pharmacological properties.

The only remaining possibility is that more functional GABAR channels with unaltered pharmacological and biophysical properties were present in Cα12 cells. This effect could occur by several mechanisms. Chronic PKA activation could modulate the rate of GABAR assembly or degradation, as recently demonstrated for nAchRs (8–10, 12). Alternatively, chronic PKA-mediated phosphorylation may activate nonfunctional GABARs that are assembled and inserted into the cell membrane (11). Attempts to directly measure levels of GABARs expressed after acute transfection of L929, Cα12, and RAB10 cells were unsuccessful due to nondetectable levels of specific [^{3}H]flunitrazepam binding, most likely due to low transfection efficiencies. Thus, these alternate mechanisms could not be directly tested. These phenomena are not currently amenable to study with electrophysiological techniques and await the development of stable cell lines expressing wild-type and mutant GABARs (40).

Previously our laboratory demonstrated that acute application of the purified catalytic subunit of PKA to cultured fetal mouse spinal cord neurons reduced whole-cell currents by decreasing opening frequency (25). The basis for the differences among these studies is not clear. However, direct comparison of these results with experiments using recombinant GABARs must be interpreted cautiously. First, the exact subunit composition of GABARs in cultured fetal mouse spinal cord neurons was not known. Thus, the reduction in GABAR current seen may be due to an interaction at a non-β subunit or even non-GABAR PKA phosphorylation site. Second, in the present experiments elevated levels of PKA were present chronically for 48 hr before electrophysiological recording and, thus, could alter other aspects of GABARs, as mentioned above.

Using transient expression of $\alpha 1$, $\beta 1$, and $\gamma 2S$ GABAR subunits in HEK 293 cells, Moss *et al.* (26) demonstrated that acute intracellular infusion of cAMP reduced peak whole-cell currents of $\alpha 1\beta 1$ and $\alpha 1\beta 1\gamma 2S$ GABARs, with a maximal effect occurring 30 min after cell membrane rupture. A similar effect was seen after co-transfection of an expression vector encoding the catalytic subunit of PKA along with $\alpha 1$ and $\beta 1$ GABAR subunit cDNAs. Expression of a mutant $\beta 1$ subunit, similar to the one used in our experiments, in place of the wild-type $\beta 1$ subunit blocked all effects. However, unlike intracellular infu-

sion of cAMP, chronic activation of PKA did not drastically alter desensitization properties of $\alpha1\beta1\gamma2S$ GABARs or decrease the whole-cell current amplitude as with $\alpha1\beta1$ GABARs. In addition, the authors stated that the amplitudes of GABA-evoked whole-cell currents expressed in HEK 293 cells varied from cell to cell, thus preventing analysis of the effect of chronic activation of PKA on GABAR whole-cell current amplitudes. Therefore, a phenomenon similar to the one described in this manuscript may have occurred but was not observed due to cell-to-cell variation in GABAR expression. Thus, these results are not inconsistent with our results concerning the role of chronic PKA activation on $\alpha1\beta1\gamma2S$ GABAR function.

Modulation of GABARs by PKA phosphorylation of $\beta1$ subunits may occur by a chronic effect to increase functional receptor levels. The increased GABAR currents in Cα12 cells suggest that chronic activation of PKA could modulate the rate of GABAR assembly or degradation, as recently demonstrated for nAchRs. GABAR $\beta1$ subunits may be similar to nAchR γ subunits, where phosphorylation by PKA was correlated with an increased rate of receptor assembly (8–10). Unlike nAchRs, where phosphorylation correlated with increased assembly of any subunit combination containing γ subunits, expression of $\alpha1\beta1\gamma2S$ and not merely $\alpha1\beta1$ GABAR subunits was necessary to observe the effect. This result may be due to differences in assembly intermediates between nAchRs and GABARs. Alternatively, PKA may increase the number of functional GABARs at the cell surface by changing their intracellular distribution, clustering, or rate of degradation (11, 12). Because $\alpha1\beta1\gamma2S$ GABARs, and not $\alpha1\beta1$ GABARs, are biophysically more similar to native neuronal GABARs (28), these properties may be differentially regulated for these two different GABAR subtypes. There may also be acute effects of PKA phosphorylation on $\beta1$ subunits of GABARs that may reduce or enhance GABAR current, as demonstrated with neuronal and recombinant GABARs (24–26).

Interpretation of these effects is further complicated by the recent finding that Ser^{409} of the $\beta1$ subunit was also a site for PKC phosphorylation (35). PKC effects produced via this site have not yet been reported. Most likely, protein kinases play a complex role in regulating the function of GABARs, with potential interaction among second messenger pathways and an interplay of acute and chronic events. It seems possible that the physiological role of neuronal GABARs may be modified via a chronic effect of PKA to enhance GABAR current, as well as an acute effect of PKA in the down-regulation of GABAR currents. Thus, the complement and activity of postsynaptic GABARs may change with alterations in neuronal cellular properties.

In summary, it is important to note that the opposite directions of alteration of GABAR currents with acute and chronic PKA modulation suggest that acute and chronic phsophorylation of GABAR subunits may give rise to different effects. Acute phosphorylation by PKA may result in an immediate reduction in GABAR function, whereas chronic phosphorylation by PKA may regulate the number of functional GABAR channels. It remains to be seen whether either of these mechanisms is activated under physiological conditions in neurons and how the effects of PKA-mediated phosphorylation of GABARs compare with effects of phosphorylation by other protein kinases such as PKC.

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Send reprint requests to: Robert L. Macdonald, Department of Neurology, 1103 East Huron Street, University of Michigan Medical School, Ann Arbor, MI 48109-1687.