Desensitization of Group I Metabotropic Glutamate Receptors in Rat Sympathetic Neurons

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Kammermeier, Paul J. and Stephen R. Ikeda. Desensitization of group I metabotropic glutamate receptors in rat sympathetic neurons. J Neurophysiol 87: 1669-1676, 2002; 10.1152/jn.00561.2001. Desensitization of heterologously expressed metabotropic glutamate receptor 5a (mGluR5a) was examined in rat sympathetic neurons. Calcium currents in cells expressing mGluR5a exhibited substantial inhibition in response to glutamate exposure. In the continued presence of glutamate, inhibition attenuated rapidly over the course of about a minute. Desensitization was eliminated when a nonhydrolyzable ATP analogue was substituted for ATP in the pipette solution, suggesting that desensitization was mediated by a phosphorylation event. Next, pharmacological agents were used to investigate the nature of the kinase involved in desensitization. Desensitization was sensitive to the nonspecific kinase inhibitor, staurosporine, but not H-7, another nonspecific kinase inhibitor. Inhibitors of myosin light chain kinase and calmodulin-dependent kinase were without effect on desensitization. However, desensitization was sensitive to the protein kinase C inhibitor bisindolylmaleimide. In contrast, Go 6976, a selective inhibitor of PKC, induced rapid desensitization. There is strong evidence that mGluR desensitization is mediated by phosphorylation (Ge- reau and Heinemann 1998; Schoepp and Johnson 1988). Phosphorylation of mGluR1 and 5 has been shown to occur in response to agonist exposure, and prevention of phosphorylation coincides with an attenuation of desensitization (Ciruela et al. 1999; Francesconi and Duvoisin 2000). Several studies have implicated protein kinase C (PKC) in group I mGluR desensitization based largely on pharmacological data and site-directed mutagenesis of putative PKC phosphorylation sites (Francesconi and Duvoisin 2000; Gereau and Heinemann 1998; Herrero et al. 1998; Schoepp and Johnson 1988). However, desensitization of group I mGluRs appears to be complex (Ciruela et al. 1999; Dale et al. 2000; De Blasi et al. 2001). PKC may not be responsible for all of the observed desensitization (Ciruela et al. 1999), and there is evidence that GRKs may be partially involved (Dale et al. 2000). Although PKC appears to play some role in mGluR desensitization, the precise role of PKC and the specific PKC isoform(s) involved are unknown.

The PKC family of serine/threonine kinases is intimately involved in cell signaling (Mahoney and Huang 1994). Most PKCs are activated by diacylglycerol and some require the presence of Ca\(^{2+}\) (see following text). Thus PKCs play the key upstream role in the phospholipase C (PLC)/IP\(_3\), diacylglycerol signaling cascade. Because different forms of PLC can be activated by various messengers including G\(_{a}\), G\(_{b}\), G\(_{y}\) (PLC\(_{b}\)), and tyrosine kinases (PLC\(_{y}\)), the PKC family is involved in a diverse array of signaling cascades (Parker 1994). Currently, there are 12 known members of the PKC family (Way et al. 2000). The family is divided into three groups based on sequence homology and biochemical regulation. Class A, or conventional PKCs (PKC\(_{\alpha}\), \(\beta\), \(\beta\)II, and \(\gamma\) are the well-known, Ca\(^{2+}\)-dependent PKCs. Class B, or novel PKCs (PKC\(_{\epsilon}\), \(\delta\), \(\eta\), \(\theta\), and \(\mu\)), are Ca\(^{2+}\) independent. Finally, Class C PKCs, or atypical PKCs (PKC\(_{\xi}\) and \(\lambda\) are the most divergent class. Atypical PKCs are also Ca\(^{2+}\) independent and do not require diacylglycerol for activation (Way et al. 2000). Group I mGluRs couple to multiple G proteins in heterolo-

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ous (Pin et al. 1995) and native systems (Choi and Lovinger 1996; Hay and Kunze 1994). Commonly, group I mGluRs activate the phospholipase C (inositol 1,4,5-triphosphate (IP3) pathway through activation of G_{o/11} G proteins (Abe et al. 1992; Aramori and Nakaniishi 1992; Joly et al. 1995; Pin et al. 1992). In addition, mGLUR1 and mGLUR5 can activate adenylyl cyclase via G_s or negatively regulate adenylyl cyclase through G_{o/11} (Aramori and Nakaniishi 1992; Joly et al. 1995), depending on the system. Recently, we have shown that group I mGluRs heterologously expressed in isolated rat superior cervical ganglion (SCG) neurons couple to G proteins of the G_{o/11} and G_{o/11a} families (Kammermeier and Ikeda 1999). Activation of these mGluRs in SCG neurons produces strong inhibition of the predominantly N-type calcium current. The relative contributions of the two G protein families on calcium currents can be distinguished by examining the voltage dependence of this inhibition (Kammermeier and Ikeda 1999).

In the present study, we used calcium current inhibition by glutamate in SCG neurons to examine the desensitization of heterologously expressed mGluR5 during continuous exposure to glutamate. The role of phosphorylation in mGluR desensitization was examined using kinase inhibitors and a nonhydrolyzable ATP analogue. The identity of the kinase or kinases involved in desensitization was investigated by examining the effects of several kinase inhibitors. The voltage dependence of calcium current inhibition was tested to determine the G-protein specificity of desensitization. Finally calmodulin, which binds to mGluR5 and regulates phosphorylation (Minakami et al. 1997), was examined for its role in desensitization.

Portions of this study were presented in abstract form (Kammermeier and Ikeda 2000).

**METHODS**

**Cell preparation and microinjection**

Detailed descriptions of the isolation and injection procedures have been previously described (Ikeda 1997). Briefly, the superior cervical ganglia were dissected from adult rats and incubated in Earle’s balanced salt solution (Life Technologies, Rockville, MD) containing 0.45 mg/ml trypsin (Worthington, Freehold, NJ), 0.6 mg/ml collagenase D (Boehringer Mannheim, Indianapolis, IN), and 0.05 mg/ml DNAse I (Sigma, St. Louis, MO) for 1 h at 35°C. Cells were centrifuged twice, transferred to minimum essential medium (Fisher Scientific, Pittsburgh, PA), plated, and placed in an incubator at 37°C for 4 h prior to cDNA injection. Injection was performed with an Eppendorf 5246 microinjector and 5171 micromanipulator (Madison, WI) 4–6 h following cell isolation. Plasmids were stored at −20°C as a 1 μg/μl stock solution in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The mGluR5 insert (from J.-P. Pin) was cloned in the pRK5 vector (Genentech, South San Francisco, CA), and was injected at 0.1 μg/ml. The rat calmodulin (CaM) construct was amplified from an EST clone (Accession No. AA165448) and subcloned into the pCI vector (Promega, Madison, WI). The Ca2+-binding impaired CaM mutants CaM EF 2,3,4 (D 57, 94 and 130 to A) and CaM EF 1,2,3,4 (D 21, 57, 94, and 130 to A) constructs were generated from the rat CaM pCI construct using Gene Editor in vitro site-directed mutagenesis system (Promega). Constructs were sequence verified before injection. All neurons were co-injected with “enhanced” green fluorescent protein cDNA (0.005 μg/μl; PEGFP-N1; Clontech Laboratories, Palo Alto, CA) to facilitate later identification of successfully injected cells. Following injection, cells were incubated overnight at 37°C and patch-clamp experiments were performed the following day.

**Electrophysiology and data analysis**

Patch-clamp recordings were made using 7052 glass (Garnier Glass, Claremont, CA). Pipette resistances ranged from 1 to 3 MΩ, yielding uncompensated series resistances of 2–7 MΩ. Series resistance compensation of >95% was used in all recordings. Data were recorded using an Axopatch 200A patch-clamp amplifier from Axon Instruments (Foster City, CA). Voltage protocol generation and data acquisition were performed using custom data-acquisition software on a Macintosh Quadra series computer with a MacAdios II data acquisition board (G. W. Instruments, Somerville, MA). Calcium currents were sampled at 0.5–5 kHz low-pass filtered at 5 kHz using the 4-pole Bessel filter in the patch-clamp amplifier, digitized, and stored on the computer for later analysis. Experiments were performed at 21–24°C (room temperature). Data analysis was performed using Igor software (WaveMetrics, Lake Oswego, OR). Statistical analyses were performed using Statview software (SAS Institute, Cary, NC).

**Solutions and pharmacological agents**

In all recordings, the external (bath) solution contained (in mM): 145 tetraethylammonium (TEA) methanesulfonate (MS), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 glucose, and 10 CaCl2, and 300 mM N-tetrodotoxin, pH 7.4; osmolality, ~320 mOsm/kg. The internal (pipette) solution contained (in mM): 120 N-methyl-D-glucamine (NMG) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.1 Na2GTP, and 14 Tris creatine phosphate, pH 7.2; osmolality, ~300 mOsm/kg. 1-glutamate (Fisher Scientific, Pittsburgh, PA) was applied at a concentration of 100 μM in all experiments. Bisindolylmaleimide, staurosporine, and 5’-adenylylimidodiphosphate (AMP-PNP), were obtained from Sigma (St. Louis, MO). Gö6976, 1-[N-(2-4-biphenyl)-N-[4-(1-piperazinyl)]-2-benzyladenosine (BPA) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.1 Na2GTP, and 14 Tris creatine phosphate, pH 7.2; osmolality, ~320 mOsm/kg. 1-glutamate (Fisher Scientific, Pittsburgh, PA) was applied at a concentration of 100 μM in all experiments. Bisindolylmaleimide, staurosporine, and 5’-adenylylimidodiphosphate (AMP-PNP), were obtained from Sigma (St. Louis, MO). Gö6976, 1-[N-(2-4-biphenyl)-N-[4-(1-piperazinyl)]-2-benzyladenosine (BPA) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.1 Na2GTP, and 14 Tris creatine phosphate, pH 7.2; osmolality, ~320 mOsm/kg. 1-glutamate (Fisher Scientific, Pittsburgh, PA) was applied at a concentration of 100 μM in all experiments. Bisindolylmaleimide, staurosporine, and 5’-adenylylimidodiphosphate (AMP-PNP), were obtained from Sigma (St. Louis, MO). Gö6976, 1-[N-(2-4-biphenyl)-N-[4-(1-piperazinyl)]-2-benzyladenosine (BPA) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.1 Na2GTP, and 14 Tris creatine phosphate, pH 7.2; osmolality, ~320 mOsm/kg. 1-glutamate (Fisher Scientific, Pittsburgh, PA) was applied at a concentration of 100 μM in all experiments. Bisindolylmaleimide, staurosporine, and 5’-adenylylimidodiphosphate (AMP-PNP), were obtained from Sigma (St. Louis, MO). Gö6976, 1-[N-(2-4-biphenyl)-N-[4-(1-piperazinyl)]-2-benzyladenosine (BPA)
Role of phosphorylation in mGluR desensitization

To test whether the observed desensitization of mGluR5a signaling results from a phosphorylation event, the experiments outlined in Fig. 1 were repeated using cells in which the ATP in the patch pipette (4 mM) was replaced with the nonhydrolyzable ATP analogue, AMP-PNP. In many of these cells, currents exhibited accelerated run-down (Fig. 2A, bottom). Unlike control cells, those containing AMP-PNP exhibited potent calcium current inhibition through the entire duration of the 1-min exposure to glutamate (Fig. 2A). Due to the persistent inhibition and run-down, inhibition did not peak early during glutamate exposure. Therefore calcium current inhibition at ~10 and 60 s after the start of glutamate exposure was used to compare to peak and +50 s values in control cells. Average inhibition of the calcium currents at 10 and 60 s was 49 ± 4 and 58 ± 5% (n = 7), respectively in AMP-PNP-containing cells. These data demonstrate that in the absence of a hydrolyzable analogue of ATP, mGluR5a is unable to desensitize in SCG neurons, supporting a role for phosphorylation as a mediator of desensitization.

Because the preceding evidence indicated that mGluR desensitization was indeed mediated by a phosphorylation event, mGluR desensitization was examined in the presence of two nonspecific kinase inhibitors, staurosporine, and H-7 (Fig. 3). Staurosporine (1 μM) and 100 μM H-7 are both potent inhibitors of various kinases with some overlapping specificity (O’Brian and Kuo 1994). Because PKC is believed to mediate mGluR desensitization, treatment with either drug was expected to ablate desensitization. Surprisingly (Gereau and Heinemann 1998; Schoepf and Johnson 1988), desensitization of mGluR5 persisted following treatment with 100 μM H-7 (Fig. 3, A and C; 100 μM H-7 was in the culture media for ≥1 h prior to the experiment as well as in the bath and intracellular solution during recording). Conversely, similar treatment with 1 μM staurosporine eliminated mGluR5a desensitization (Fig. 3, A and C). Average peak and +50-s inhibitions in control were 32 ± 4 and 11 ± 2%, respectively (n = 13). H-7-treated cells were inhibited 32 ± 6 and 0 ± 3% (n = 7) at peak and +50 s. Cells treated with staurosporine were inhibited 68 ± 5 and 67 ± 6% (n = 9) at peak and +50 s, respectively. Thus the kinase that mediates mGluR desensitization in SCG neurons is eliminated by staurosporine but not by H-7.

Although 1 μM staurosporine inhibits kinase activity relatively nonspecifically, various kinases are affected by staurosporine with different affinities. Therefore a dose-response relation for the effect of staurosporine on mGluR desensitization was constructed (Fig. 3B). For this purpose, the ratio of calcium current inhibition by glutamate 50 s after peak inhibition to that at peak was plotted versus staurosporine concentration and fit to a single-site binding isotherm to determine the IC50. The results of this analysis are shown in Fig. 3B. The data points represent the inhibition ratio at the various staurosporine concentrations and — represents the fit to the single site binding isotherm equation (see Fig. 3 legend). The results indicated that staurosporine inhibited mGluR5a desensitization with an IC50 of ~190 nM. This value is within the expected range for staurosporine inhibition of several kinases in vivo, including calmodulin-dependent protein kinase (CaM kinase), myosin light chain kinase, protein kinase G (PKG), cyclic AMP-dependent protein kinase (PKA), and PKC. However, the ineffectiveness of H-7 at 100 μM strongly suggested
Effects of specific kinase inhibitors

To test for a possible role of myosin light chain kinase and CaM kinase in mGluR desensitization, specific inhibitors ML-7 and KN-62, respectively, were examined. Neither ML-7 nor KN-62 treatment reduced mGluR5a desensitization in SCG neurons. Following treatment (as with staurosporine and H-7, preceding text) with 1 μM ML-7, peak calcium current inhibition and that at +50 s was 23 ± 5 and 10 ± 3% (n = 10), respectively. Cells treated with 1 μM KN-62 were inhibited 28 ± 5 and 14 ± 1% (n = 5), respectively (Fig. 4). These values were similar to inhibition in control (Fig. 4, and see preceding text). Therefore myosin light chain kinase and CaM kinase do not appear to be involved in desensitization of mGluR5.

Although both staurosporine and H-7 are known to inhibit PKC activity (O’Brien and Kuo 1994), neither drug is likely to affect all isoforms of PKC with the same potency. This is particularly true when the nonconventional forms of PKC are considered. It is therefore possible that desensitization of mGluR5 is mediated by a nonconventional PKC isoform with sensitivity to staurosporine but not to H-7. To test this possibility, two specific inhibitors of PKC were examined for their effects on mGluR desensitization. The first, bisindolylmaleimide (BIS) is a selective inhibitor of PKC that does not distinguish well among conventional, atypical and novel isoforms. The second, Go6976, is a selective inhibitor of conventional PKC isoforms (Martiny-Baron et al. 1993). Treatment of neurons with BIS was able to nearly eliminate mGluR desensitization (Fig. 5A, upper). The calcium current inhibition observed in these cells was well maintained throughout the entire duration of the ~1-min application of glutamate. In contrast, Go6976 treatment was without effect on mGluR desensitization (Fig. 5A, bottom). Average peak and +50-s calcium current inhibition in cells treated with BIS was 38 ± 4 and 31 ± 4%, respectively (n = 10; although the inhibitions in BIM were significantly different, the mean difference between groups was only 7%—compared with 21% in control). Cells treated with Go6976 were inhibited 27 ± 4 and 13 ± 3% (n = 5) at peak and +50 s, respectively (Fig. 5C). A dose-response curve was then generated for the effect of BIS on mGluR desensitization (Fig. 5B). The IC50, determined from a fit to a single-site binding isotherm (see Fig. 5 legend), was determined to be ~340 nM (Fig. 5B), a reasonable value for BIS inhibition of PKC in vivo. These data indicate that the kinase responsible for group I mGluR desensitization in SCG neurons is a nonconventional isoform of PKC. Indeed, when similar experiments to those in control were performed after replacing the intracellular 11 mM EGTA with 10 mM bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA), a calcium buffer with similar Ca2+ affinity as EGTA but a much faster on rate, potent desensitization was still observed. In these cells, peak and +50-s inhibition was 20 ± 2 and 2 ± 2% (n = 5), respectively (Fig. 5C). Because conventional forms of PKC are unlikely to be active with this level of calcium buffering, this result further supports a role for a Ca2+-independent, nonconventional PKC isoform in mGluR desensitization.

FIG. 3. Sensitivity of desensitization to nonspecific kinase inhibitors. A: time courses of desensitization in cells expressing mGluR5 without drug treatment (“Control”; top) and those treated with 100 μM H-7 (“H-7”; middle) or 1 μM staurosporine (“Staurosporine”; bottom). B: dose-response relation of the effect of staurosporine on desensitization. Data points represent the average (±SE) ratio of inhibition 50 s after peak inhibition to peak inhibition. For data at 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 μM, n = 5, 7, 3, 6, 3, 3, and 4, respectively; —, a fit to the equation: Bmax [stauro]/EC50 + [stauro] + 1. C: bar graph illustrating average (±SE) peak inhibition (□) and inhibition 50 s after peak in 100 μM glutamate (□) for untreated cells (“Control”), and cells treated with 100 μM H-7 (“H-7”) or 1 μM staurosporine (“Staur”). The numbers of cells are denoted in parentheses. Statistical significance compared with peak inhibition indicated by * (P < 0.05, paired t-test).

FIG. 4. Inhibitors of myosin light chain kinase and calmodulin (CaM) kinase are without effect. Bar graph illustrating average (+SE) peak inhibition (□) and inhibition 50 s after peak in 100 μM glutamate (□) for untreated cells (“Control”), and cells treated with 1 μM KN62 (“KN-62”) or 1 μM ML-7 (“ML-7”). The cell numbers are denoted in parentheses. Statistical significance compared with peak inhibition indicated by * (P < 0.05, paired t-test).
FIG. 5. Desensitization of mGluR5 results from phosphorylation by a non-conventional protein kinase C (PKC) inhibitor. Sensitivity of desensitization to specific PKC inhibitors. A: time courses of desensitization in cells expressing mGluR5 and treated with 10 μM bisindolylmaleimide (“BIM”; top) or 2 μM Go6976 (“Go6976”; bottom). B: dose-response relation of the effect of bisindolylmaleimide (BIS) on desensitization. Data points represent the average (±SE) ratios of inhibition 50 s after peak inhibition to peak inhibition. For data at 0, 0.01, 1, 3, and 10 μM, n = 8, 3, 4, 3, and 4, respectively. —, a fit to the equation: Bref [BIM]/EC50 + [BIM]) + x. C: bar graph illustrating average (+SE) peak inhibition (○) and inhibition 50 s after peak in 100 μM glutamate (●) for untreated cells (“Control”), cells treated with 10 μM bisindolylmaleimide (“BIM”) or 2 μM Go6976 (“Go”), and cells recorded with 10 mM intracellular BAPTA (“BAPTA”). The cell numbers are denoted in parentheses. *, the “+50 s” inhibition was significantly smaller than peak in BIM cells, but the mean difference was only 7%, compared with a mean difference of 21% in control cells (P < 0.05, paired t-test).

G-protein coupling specificity of desensitization

Group I mGluRs can commonly couple to multiple classes of G proteins both in native and heterologous systems (De Blasi et al. 2001). When group I mGluRs are expressed heterologously in SCG neurons, they can activate both the G11 and G111 families of G proteins (Kammermeier and Ikeda 1999). In addition, recent studies have suggested that mGluR desensitization may differentially reduce coupling of G11 and G111 (Francesconi and Duvoisin 2000), or of PTX-sensitive and -insensitive G proteins (Rodriguez-Moreno et al. 1998). It was therefore of interest to determine whether desensitization favored one or the other of the pathways activated in SCG neurons. The G11 and G111 pathways can be distinguished by examining the voltage dependence of calcium current inhibition produced during agonist application (Kammermeier and Ikeda 1999; Kammermeier et al. 2000). Activation of pertussis toxin (PTX)-sensitive, G11 G proteins by mGluR5a produces a predominantly voltage-dependent inhibition that can be partially but strongly reversed by preceding strong depolarizations. Group I mGluRs can also activate G111 G proteins to produce a voltage-independent inhibition of calcium currents, particularly apparent following PTX treatment (Kammermeier and Ikeda 1999). However, calcium current inhibition produced at peak had identical voltage dependence to that 50 s later in the continued presence of glutamate (Fig. 6). When the postpulse inhibition was plotted versus prepulse inhibition for peak inhibition and inhibition at +50 s, the slopes (an objective measure of voltage dependence) (see Kammermeier and Ikeda 1999) were identical. For peak inhibition, the postpulse inhibition versus prepulse inhibition slope was 0.44 ± 0.07 (slope ± 95% confidence interval). For inhibition at +50 s, the slope was 0.43 ± 0.10 (n = 103). These data indicate that desensitization of mGluR5 does not selectively diminish one G-protein pathway in favor of another in SCG neurons.

Role of calmodulin

mGluR5 has been shown to associate with CaM at a site on the intracellular C-terminal tail of the receptor (Minakami et al. 1997). Further, this interaction with calmodulin appears to inhibit phosphorylation of the receptor. Calmodulin was therefore tested to determine whether it could regulate mGluR desensitization by inhibiting phosphorylation at a relevant site. In addition, to offset a possible effect of endogenous calmodulin, two mutant calmodulin constructs with impaired Ca2+ binding (CaM EF 2,3,4 and CaM EF 1,2,3,4 in which 3 and 4 of the 4 EF Hand motifs were mutated, see METHODS) were also examined. Each of these constructs was overexpressed with mGluR5a in SCG neurons and desensitization was examined. Figure 6 illustrates average peak inhibition and that at +50 s in cells expressing mGluR5a alone or with wild-type CaM, CaM EF 2,3,4 or CaM EF 1,2,3,4. No detectable changes in mGluR desensitization were seen with any of the CaM constructs tested (Fig. 7). At peak and +50 s, respectively, calcium currents in control cells were inhibited 32 ± 4 and 11 ± 2% (n = 14). Cells co-expressing wild-type CaM were inhibited 28 ± 3 and 8 ± 2% (n = 9). Those expressing CaM EF 2,3,4 were inhibited 36 ± 13 and 13 ± 2% (n = 3), and cells

FIG. 6. Desensitization of mGluR5 does not favor a specific G-protein pathway. Plot of postpulse inhibition vs. prepulse inhibition as a measure of voltage dependence of modulation. Peak inhibition (●) and inhibition after >50 s of agonist exposure (□) were plotted. A linear regression of each data set was plotted. The slope of the fit from peak inhibition data (——) was 0.44 ± 0.07 (slope ± 95% confidence interval). The fit from inhibition at +50 s (- - -) yielded a slope of 0.43 ± 0.10 (n = 103).
then phosphorylation at this site is not involved in desensitization, it can be inferred that if these constructs inhibit desensitization of these CaM constructs produces no effect on mGluR (n/H11005/100) wild-type CaM or two CaM mutants with diminished Ca²⁺ binding to mGluR5a. Finally, it was shown that overexpression of these inhibitors to those enzymes. It was demonstrated that desensitization of mGluR5a was sensitive to the drug BIS, but not to Go(6976), suggesting that the relevant kinase is in fact in the PKC family but is not likely to be a member of the conventional PKC class. In addition, desensitization was not altered by inclusion of 10 mM BAPTA in the intracellular recording patch pipette, providing further evidence that the Ca²⁺-dependent, conventional PKCs are not involved. Next, by examining the voltage dependence of calcium current inhibition during peak inhibition and after 50 s of desensitization, it was determined that desensitization does not appear to selectively decrease either G-protein pathway coupled to mGluR5a. Finally, it was shown that overexpression of wild-type CaM or two CaM mutants with diminished Ca²⁺-binding capabilities were unable to alter mGluR desensitization. This result suggested that the mGluR phosphorylation that is inhibited by CaM binding to the receptor (Minakami et al. 1997) is not important in mGluR5 desensitization in SCG neurons.

Many previous studies have examined mGluR desensitization and through pharmacological and molecular biological analysis have concluded that PKC is involved in the process (for review see De Blasi et al. 2001). The data presented here support this conclusion. However, further data are presented here to demonstrate that it is a nonconventional (atypical or novel) PKC that mediates this process. This distinction is important when considering the context of conditions under which the desensitization may take place. For example, nonconventional PKCs are not Ca²⁺ dependent, and some members of the atypical PKCs have different sensitivities to phorbol esters and do not require diacylglycerol for activation (Way et al. 2000). Unfortunately, the pharmacological tools to precisely distinguish between the many members of novel and atypical PKCs are not currently available. Finally, it should be noted that some of the conclusions drawn here rely on interpretation of negative results of pharmacological experiments (e.g., the Go(6976) experiment, Fig. 5). In the absence of a positive control for such experiments, interpretation of results should be made cautiously. In the present study, some negative results were presented without positive controls due to the lack of an appropriate assay to assess the inhibitors in our system. However, the main conclusion from these data (that desensitization proceeds through a nonconventional PKC isoform) was supported by the result from an independent experiment (desensitization in 10 mM BAPTA). Therefore this conclusion seems appropriate.

Although the data presented in this study argue strongly for a role of a nonconventional form of PKC in mGluR desensitization, it is unclear why H-7 was without effect. H-7 is known to inhibit PKC activity in vitro with an IC₅₀ of ~6 μM (Quick et al. 1992; Schachtele et al. 1988). However, there is little available data on the differing effects of H-7 on specific isoforms of PKC, particularly on the novel and atypical classes. It would be difficult to argue for a role of a conventional PKC as a mediator of an effect that was not inhibited by 100 μM H-7. However, it is possible that H-7 is an ineffective inhibitor of some nonconventional PKC isoforms. Thus the interpretation that mGluR desensitization is mediated by a nonconventional PKC that is sensitive to inhibition by BIS and staurosporine but not by Go(6976) cannot be refuted by these H-7 data.

Another possible explanation for the apparent lack of effect on desensitization of H-7 is that it may have been producing opposing effects on desensitization that could mask the relief of desensitization due to PKC inhibition. Statistical analysis of data from each pharmacological experiment illustrated that while desensitization was significantly reduced by staurosporine and BIM, a significant enhancement of desensitization was observed only in the H-7-treated cells. At this time it is unclear what the implications of this observation are, but it does suggest that the effects of H-7 treatment may be complex.

Desensitization of group I mGluRs is a dynamic process. In the presence of agonist, desensitization can develop rapidly (~1 min) and last for several minutes. In addition, the degree and rate of desensitization can be variable even under controlled experimental conditions. Therefore the method of measuring desensitization may influence the results obtained in any study. In the present study, desensitization was defined as the loss of calcium current inhibition in the continuous presence of 100 μM glutamate from peak observed inhibition (typically at ~10 s after the introduction of glutamate) to that 50 s later. In
other notable studies (Francesconi and Duvoisin 2000; Gereau and Heinemann 1998), desensitization of mGluRs was defined as the loss of receptor activity on a second application of glutamate applied several minutes after the first. Some differences in results may occur due to this difference in methodology. For example, it is possible that phosphorylation at multiple sites is involved in mGluR5 desensitization and that phosphatase activity at one or more of these sites determines the duration of this loss of activity. Thus accelerated recovery may be mistaken for a decrease in desensitization when using multiple glutamate applications to assess receptor activity. However, Gereau and Heinemann (1998) did observe slowing of desensitization kinetics concurrent with the loss of desensitization measured by repeated applications of glutamate for certain mGluR5 point mutants. Therefore differences in mGluR desensitization in the two studies may result from the different experimental systems (neurons vs. oocytes) and cannot be solely attributed to methodology.

Although a direct phosphorylation of mGluR5 was not demonstrated here, it is likely that the phosphorylation event that mediates desensitization occurs directly on the receptor as indicated in several other studies (Ciruela et al. 1999; Francesconi and Duvoisin 2000; Schoep and Johnson 1988). This is true because the calcium current modulation used here to detect mGluR5a activity is mediated partly through a direct interaction of the channel with Gβγ (DeWard et al. 1997; Herlitze et al. 1996; Ikeda 1996) and partly via activation of Gq/11-GTP (Kammermeier and Ikeda 1999). Because these processes appear to desensitize similarly (see RESULTS) and because similar calcium current modulation can occur in SCG neurons via endogenous receptors without appreciable desensitization (ruling out a phosphorylation of the channel; see Fig. 1B), it is very likely that group I mGluR desensitization in SCG neurons (and thus phosphorylation) occurs at the level of the receptor.

In addition to its effect on mGluR desensitization, treatment with staurosporine also appeared to strongly enhance the magnitude of calcium current inhibition by glutamate (see Fig. 3C). This enhancement effect was seen only when cells were pretreated with staurosporine prior to performing the experiment, although acute exposure to staurosporine was sufficient to eliminate desensitization (data not shown). The staurosporine pretreatment-dependent enhancement may have resulted from relief from tonic receptor desensitization. However, BIS, which was also able to inhibit desensitization, did not produce the same enhancement of calcium current modulation, suggesting that the enhancement effect did not result from inhibition of the same kinase that produced desensitization. Another possibility is that staurosporine inhibited basal kinase activity acting on the N-type calcium channel itself, which can decrease the effectiveness of the G-protein-mediated calcium current modulation (Swartz et al. 1993).

BIS was used in this study as a specific inhibitor of all PKC isoforms. Other agents, such as calphostan C and chelerythrine, can produce similar effects. However, attempts to use these drugs here failed. Cells pretreated with calphostan C or chelerythrine at concentrations necessary to produce PKC inhibition appeared unhealthy and could not be patched due to poor seal formation and excessive leak. The underlying cause of the detrimental effect of calphostan C and chelerythrine was unclear.

Because CaM has been shown to bind to the intracellular C-terminal tail of mGluR5 and inhibit mGluR5 phosphorylation (Minakami et al. 1997), the role of CaM in regulating mGluR5 desensitization was examined. If the phosphorylation that was inhibited by CaM binding were responsible for mGluR desensitization, then CaM would prove to be an important regulator of mGluR function by attenuating this negative feedback control. To test this hypothesis, CaM was co-expressed with mGluR5. However, CaM co-expression did not alter mGluR desensitization. Because CaM is likely to be endogenously expressed in SCG neurons, the lack of effect of heterologously expressed CaM may have been somewhat masked. Therefore the effects of two mutant CaMs with impaired Ca2+ binding were also examined. However, when each CaM construct was co-expressed with mGluR5, calcium current inhibition and mGluR desensitization were indistinguishable from control, suggesting that CaM is not an important regulator of group I mGluR desensitization. Although there was no independent measure of CaM expression in these experiments, the same CaM construct has been used in separate experiments with positive results, suggesting that the construct is expressed (data not shown). Therefore the role of the phosphorylation site(s) competed for by CaM remains unclear.

In summary, the data presented in this study confirm a role for phosphorylation of mGluRs in receptor desensitization and indicate that a form of PKC mediates this phosphorylation. This study also demonstrates that the kinase that mediates mGluR desensitization appears to be a nonconventional form of PKC. This is indicated by the observation that desensitization was blocked by staurosporine and the specific PKC inhibitor BIS, but unaffected by Goi6976, a kinase inhibitor with selectivity for conventional PKC isoforms. This conclusion is further supported by the inability of the fast Ca2+ chelator BAPTA to reduce mGluR desensitization. In addition, mGluR desensitization did not appear to favor either Gq/11 or Go/o coupling to mGluR5. Finally, overexpression of CaM did not alter mGluR desensitization, suggesting that although CaM can inhibit some phosphorylation of mGluR5, this phosphorylation does not appear to be integral to desensitization.

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