Increases in Intracellular Calcium Triggered by Channelrhodopsin-2 Potentiate the Response of Metabotropic Glutamate Receptor mGluR7*

John H. Caldwell, Greta Ann Herin, Georg Nagel, Ernst Bamberg, Astrid Scheschonka, and Heinrich Betz

From the Department of Neurochemistry, Max-Planck-Institute for Brain Research, D-60528 Frankfurt am Main, Germany, the Department of Cell and Developmental Biology, University of Colorado Health Sciences Center, Aurora, Colorado 80045, the Max-Planck-Institute for Biophysics, D-60438 Frankfurt am Main, Germany, and the University of Würzburg, D-97082 Würzburg, Germany

The metabotropic glutamate receptor 7a (mGluR7a), a heptahelical Gαi/o-coupled protein, has been shown to be important for presynaptic feedback inhibition at central synapses and certain forms of long term potentiation and long term depression. The intracellular C terminus of mGluR7a interacts with calmodulin in a Ca2+-dependent manner, and calmodulin antagonists have been found to abolish presynaptic inhibition of glutamate release in neurons and mGluR7a-induced activation of G-protein-activated inward rectifying K+ channel (GIRK) channels in HEK293 cells. Here, we characterized the Ca2+-dependence of mGluR7a signaling in Xenopus oocytes by using channelrhodopsin-2 (ChR2), a Ca2+-permeable, light-activated ion channel for triggering Ca2+-influx, and a GIRK3.1/3.2 concatemer to monitor mGluR7a responses. Application of the agonist (S)-2-amino-4-phosphonobutanoic acid (l-AP4) (1–100 μM) caused a dose-dependent inward current in high K+ solutions due to activation of GIRK channels by G-protein βγ subunits released from mGluR7a. Elevation of intracellular free Ca2+ by light stimulation of ChR2 markedly increased the amplitude of l-AP4 responses, and this effect was attenuated by the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′ ‑tetraacetic acid (NTA), as determined by the endogenous Xenopus Ca2+-activated chloride conductance. Together, these results show that l-AP4-dependent mGluR7a signaling is potentiated by physiological levels of [Ca2+]i, consistent with a model in which presynaptic mGluR7a acts as a coincidence detector of Ca2+ influx and glutamate release.

Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors that play a key role in synaptic transmission. Eight mGluRs have been identified and grouped into three classes (1). mGluR7 is a Gαi/o-protein-linked member of the group III mGluRs that is widely expressed at both glutamatergic and non-glutamatergic synapses and has a crucial role in some forms of hippocampal plasticity (2, 3). In addition, mGluR7−/− mice develop spontaneous seizures (4). Therefore, mGluR7 is a current target for the development of novel antiepileptic drugs.

mGluR7 has been localized to presynaptic terminals, where it inhibits neurotransmitter release via a G-protein-dependent pathway (5). In cortical presynaptic terminals, mGluR7 colocalizes primarily with and inhibits N-type Ca2+ channels. However, in cerebellar granule cells, mGluR7 inhibits P/Q-type Ca2+ channels via a phospholipase C-dependent pathway (6). mGluR7 may act as a presynaptic feedback inhibitor; elevated synaptic glutamate activates mGluR7, thereby decreasing presynaptic Ca2+ influx and reducing glutamate release (7).

Biochemical studies indicate that mGluR7 signaling is regulated by a variety of intracellular proteins. Specifically, the cytoplasmic C-terminal tail of the predominant splice variant mGluR7a has been shown to bind proteins whose properties are directly or indirectly influenced by changes in intracellular Ca2+ levels, such as calmodulin (CaM) (8), protein kinase C (9), and protein interacting with protein kinase C 1 (PICK-1) (10, 11). CaM binds to mGluR7a (and other group III mGluRs) in a Ca2+-dependent manner, and CaM inhibitors have been shown to prevent presynaptic inhibition at glutamatergic autapses and to disrupt mGluR7a signaling in Xenopus oocytes (8). Moreover, inhibition of P/Q voltage-gated calcium channels by l-AP4 is blocked by BAPTA, suggesting that Ca2+ is required for mGluR7-mediated presynaptic inhibition in cerebellar granule cells (6). These data support the view that presynaptic inhibition by mGluR7 depends on [Ca2+]i, likely through regulation of complex protein interactions in the presynaptic nerve terminal (reviews in Refs. 12 and 13).

*This work was supported in part by National Institutes of Health Grant R01 26505 (to J. H. C.). Primary support for the work was from grants from Max-Planck-Gesellschaft, European Community Grant QLG3-CT-2001-00929, and Fonds der Chemischen Industrie (to H. B.) and Deutsche Forschungsgemeinschaft (Grants SFB 472/P1 and DFG-NA207/6)(to G. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed: Dept. of Cell and Developmental Biology, Mail Stop 8315, P. O. Box 6511, Aurora, CO 80045. Tel.: 303-724-3190; Fax: 303-724-3121; E-mail: john.caldwell@uchsc.edu.
3 Present address: Dept. of Biology, Eastern Mennonite University, Harrisonburg, VA 22802.

4 The abbreviations used are: mGluR, metabotropic glutamate receptor; CaM, calmodulin; ChR2, channelrhodopsin-2; GIRK, G-protein activated inward rectifying potassium channel; l-AP4, (S)-2-amino-4-phosphonobutanoic acid; DMSO, dimethyl sulfoxide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′ ‑tetraacetic acid; NFA, niflumic acid; GABA, γ-aminobutyric acid; GABAβ, GABA, type B.
Here, we tested the dependence of mGluR7a activation on submembranous Ca\(^{2+}\) by using the G-protein-activated inwardly rectifying K\(^+\) channel (GIRK; Kiř3.1/3.2) in a heterologous expression system in which we could temporally control Ca\(^{2+}\) influx with channelrhodopsin-2 (ChR2), a light-activated cation channel (14), monitor submembranous Ca\(^{2+}\) levels with the endogenous Ca\(^{2+}\)-activated chloride conductance (15), and study mGluR7a in relative isolation. mGluR7a, the G\(\beta\gamma\) effector GIRK, and ChR2 were co-expressed in Xenopus oocytes, and membrane currents were measured under two-electrode voltage clamp. Our results show that mGluR7a signaling is potentiated with increasing [Ca\(^{2+}\)].

### EXPERIMENTAL PROCEDURES

**Chemicals**—L-AP4 was obtained from Calbiochem (Darmstadt, Germany), and niflumic acid (NFA) was obtained from Cayman Chemical (Ann Arbor, MI). Stock L-AP4 (100 mM) was dissolved in 100 mM NaOH and stored at −20 °C. L-AP4 was diluted 10\(^{-5}\)-fold for experiments (working concentration 1–100 \(\mu\)M). NFA was dissolved in DMSO (300 mM stock), kept frozen at −20 °C, and diluted 1000-fold for a working concentration of 300 \(\mu\)M. The \(K_e\) for NFA block of Ca-activated Cl currents in oocytes is 10 \(\mu\)M. BAPTA-AM (Sigma product number A1076) was dissolved in DMSO, stored under argon at −80 °C, and diluted at a concentration of 100–300 mM, and diluted at least 1000-fold for experiments.

**Expression Constructs and RNA Synthesis**—The mGluR7a cDNA encoding the short isoform of mGluR7 (Ref. 16); (gift from Dr. S. Nakanishi, University of Kyoto, Japan) and the GIRK (Kiř3.1/3.2) construct (gift from Dr. Andreas Karschin, University of Würzburg, Germany) have been described previously (8). The ChR2 construct used was a C-terminally truncated version (amino acids 2–315) that lacks most of the C-terminal cytoplasmic domain (14) and contains a single point mutation (H134R), which results in larger currents but does not change ion selectivity (17). In vitro transcriptions were performed using commercial kits (Stratagene, Agilent Technologies, Santa Clara, CA, or Ambion, Applied Biosystems, Darmstadt, Germany). Synthetic RNAs were stored at −80 °C in aliquots at a concentration of 1 \(\mu\)g/\(\mu\)l.

**Oocyte Expression and Electrophysiological Recordings**—Stage V–VI oocytes were removed from anesthetized Xenopus laevis and enzymatically treated to remove follicular cells. The oocytes were incubated at 18–19 °C in oocyte saline (ND-96, in mM: 96 NaCl, 2 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, pH 7.4) and injected with in vitro transcribed RNA as follows. For recordings from cells injected with mGluR7a and GIRK RNA, 1 \(\mu\)l (25 ng) of mGluR7a RNA was mixed with 1 \(\mu\)l of GIRK RNA (50 pg). Light-activable ChR2 has retinal bound (14). Hence, oocytes were injected on day 1 with a 1:1 mixture of GIRK and mGluR7a RNAs (50 nl/oocyte) and 48 h later with ChR2 RNA (20 ng) followed by incubation in oocyte solution containing trans-retinal (1 \(\mu\)M). Oocytes were used for recordings from day 3 to day 7 after the initial injection using two-electrode voltage clamp recording. The volume of the recording chamber was 15 \(\mu\)l, and this volume was exchanged with 0.5–1 ml of perfusion solution in ~1 s. K\(^+\) was substituted for Na\(^+\) in solutions in which K\(^+\) was elevated. Increases in Ca\(^{2+}\) were achieved by substituting for Na\(^+\). Electrodes had resistances of 0.4–2 megohms and were filled with 3 M KCl in 0.2% (w/v) agar. Data were acquired with an Axon 2B amplifier, Digidata 1200 A/D converter, and pCLAMP 9 software (MDS Inc., Toronto, Canada).

**Photoactivation of ChR2**—ChR2 was activated in electrode-impaled oocytes with a mercury arc lamp and a 450 ± 25-nm band filter (5 milliwatt/mm\(^2\)) as described (14). Neutral density filters with a computer-activated shutter were used to control light intensity.

### RESULTS

**Control of Submembranous Ca\(^{2+}\) with ChR2**—ChR2 is a light-activated eukaryotic cation channel that is permeable to Ca\(^{2+}\) (14). We utilized ChR2 to introduce Ca\(^{2+}\) into the submembranous compartment of Xenopus oocytes to mimic Ca\(^{2+}\) influx through voltage-gated calcium channels into the presynaptic nerve terminal. We took advantage of the well-characterized, endogenous Ca\(^{2+}\)-activated chloride current (15, 18) to monitor free Ca\(^{2+}\) concentrations beneath the oocyte plasma membrane. Fig. 1A shows the voltage clamp protocol and resulting currents in the presence and absence of extracellular Ca\(^{2+}\). ChR2 was activated by illuminating the oocyte immediately before recording, and the oocyte remained illuminated during the record. In the absence of extracellular Ca\(^{2+}\), light-induced currents at +40 mV were small and stable. In the presence of 2 mM extracellular Ca\(^{2+}\), a large Cl\(^-\) current \((I_{\text{Cl(Ca)}})\) was evident during the second pulse to +40 mV (note also the Cl\(^-\) current during the pulse to −140 mV). Thus, Ca\(^{2+}\) entry via the ChR2 channel activates \(I_{\text{Cl(Ca)}}\).

The dependence of \(I_{\text{Cl(Ca)}}\) upon activation of ChR2 was determined by varying the light intensity. In the presence of 2 mM [Ca\(^{2+}\)]\(_o\), peak amplitudes of the Cl\(^-\) current were measured during the second step to +40 mV (Fig. 1B, asterisk) at increasing light intensities, which activate an increasing number of ChR2 channels. The relationship between \(I_{\text{Cl(Ca)}}\) and ChR2 current is plotted in Fig. 1C. The relationship was fit by the Hill equation (Fig. 1C, solid line), with \(k = 0.46 \mu\)A (value of the ChR2 current at the half-maximal response) and \(n = 2.13\). This relationship is very similar to the relationship between [Ca\(^{2+}\)] and \(I_{\text{Cl(Ca)}}\) as determined by Haase and Hartung (18), who applied Ca\(^{2+}\) to the intracellular face of excised inside-out patches of Xenopus oocyte membrane and determined a \(K_d\) value of 500 ± 20 nm and a Hill coefficient of \(n = 2.3 ± 0.12\) (the average of their data is superimposed on our data in Fig. 1C). We assume that the \(K_f\) for Ca\(^{2+}\) determined from excised patches is a constant property of the Ca\(^{2+}\)-activated chloride channel. Thus, by equating the value of \(k\) for the oocyte shown in Fig. 1C with the \(K_d\) of \(I_{\text{Cl(Ca)}}\) for Ca\(^{2+}\) determined by Haase and Hartung (18), we find that \(I_{\text{ChR2}} = 0.46 \mu\)A corresponded to a free [Ca\(^{2+}\)], of 500 nm. The [Ca\(^{2+}\)], produced by smaller and larger currents was assumed to be proportional to the ChR2 current \((\langle \text{Ca}^{2+}\rangle) = (500/K_{f(\text{ChR2})}) \times I_{\text{ChR2}}\). The calibration shown in Fig. 1, B and C, was performed for each oocyte, and each ChR2 current was then converted to free submembranous [Ca\(^{2+}\)].

Submembranous Ca\(^{2+}\) can be sequestered with Ca\(^{2+}\) chelators without altering Ca\(^{2+}\) flux through ChR2. Fig. 1D demon-
**Ca^{2+}** Potentiation of mGluR7 Signaling

strates that extracellular application of the fast, membrane-permeable Ca^{2+} chelator BAPTA-AM largely inhibited $I_{\text{Cl(Ca)}}$ within 5–8 min (69.4 ± 5.7%; average ± S.E., n = 10) and had no effect on ChR2 current (Fig. 1D, squares). The failure to achieve complete block of $I_{\text{Cl(Ca)}}$ raises the possibility that some ChR2 and chloride channels are spatially apposed or colocalize in macromolecular complexes. This block of $I_{\text{Cl(Ca)}}$ corroborates that $I_{\text{Cl(Ca)}}$ reflects [Ca^{2+}], and that BAPTA-AM treatment does not affect ChR2 function and therefore transmembrane Ca^{2+} flux.

To characterize submembranous Ca^{2+} kinetics, we designed protocols to monitor the time course of ChR2-induced changes in [Ca^{2+}]. In oocytes, the increase in submembranous [Ca^{2+}] depends on the rates of both Ca^{2+} entry through ChR2 and Ca^{2+} buffering and removal. Fig. 2A shows the protocol and currents recorded for increasing durations of illumination. The peak Cl^{-} current is plotted versus light duration for a representative cell in Fig. 2B. The current increased with a time constant of 630 ± 125 ms (average ± S.E., n = 5). Thus, in 1–2 s, the intracellular Ca^{2+} concentration reaches a steady state. To determine the time required for [Ca^{2+}], to return to the resting level, the light was turned off during the −140 mV step, and the second step to +40 mV was delayed in 100-ms increments (see the protocol illustrated in Fig. 2C, top). $I_{\text{Cl(Ca)}}$ rapidly declined (peak current during the second step to +40 mV, Fig. 2C) following the termination of illumination. The time course for recovery is shown in Fig. 2D. The time constant for the return to resting [Ca^{2+}], levels was 181 ± 14 ms (n = 4). It is possible that the kinetics of changes in $I_{\text{Cl(Ca)}}$ are limited by the rates of activation and deactivation of the chloride channel. Evidence from excised patches of oocyte membrane where [Ca^{2+}] was increased or decreased rapidly (within a few milliseconds or less) at the cytoplasmic surface have shown that, at the concentrations relevant for our experiments (~0.5 μM), the time constant of $I_{\text{Cl(Ca)}}$ activation is between 50 (15) and 500 ms (18); the latter value is close to the activation time constant we measured (Fig. 2C). Likewise, the deactivation time constants varied from 30 (15) to 100–200 ms (18). Again, the latter values are close to
those measured here for changes in $I_{Cl(Ca)}$ (Fig. 2D). Thus, our ability to measure changes in $[Ca^{2+}]$ could be limited by the inherent kinetics of the chloride channel, and changes in submembranous $[Ca^{2+}]$ may be faster than the time courses shown in Fig. 2, B and D. We conclude that ChR2 channel activation allows us to increase submembranous $[Ca^{2+}]$ in less than 1–2 s to known free $Ca^{2+}$ levels, and once the light is turned off, $[Ca^{2+}]$, returns to basal levels in much less than a second. Thus, the temporal profile of submembranous $[Ca^{2+}]$ can be tightly controlled through illumination.

Potentiation of mGluR7a Agonist Responses by Changes in $[Ca^{2+}]$—Having established a system in which submembranous $[Ca^{2+}]$ can be temporally controlled and accurately measured, we next characterized the $Ca^{2+}$ dependence of mGluR7a signaling. Oocytes co-expressing mGluR7a, GIRK 3.1/3.2, and ChR2 were voltage-clamped, and in each oocyte, the relationship between $I_{ChR2}$ and $I_{Cl(Ca)}$ was examined as described above. Subsequently, the $Ca^{2+}$-activated chloride channel was blocked with NFA (300 $\mu$M). Following calibration of each oocyte and NFA treatment, the group III-specific agonist L-AP4 was applied. Repeated applications of the same concentration of L-AP4 during increasing levels of illumination, and therefore increasing levels of submembranous $[Ca^{2+}]$, were employed to determine changes in mGluR7a function in the presence of $Ca^{2+}$, as described in more detail below. Fig. 3A illustrates a typical experiment. Oocytes were bathed in a solution containing 80 mM K+ (high potassium) to increase the flux of K+ through the inwardly rectifying GIRK channel. Once L-AP4-independent GIRK channel currents were stable, L-AP4 was applied in high potassium. This resulted in a characteristic inward current, indicative of L-AP4-dependent GPR activation of GIRK (19). Currents were recorded with L-AP4 (100 $\mu$M) and all other conditions constant while varying illumination intensity (and thus submembranous $[Ca^{2+}]$). As expected from Figs. 1 and 2, increasing light intensity resulted in larger ChR2 currents, as indicated by the short latency, fast-desensitizing currents immediately following illumination with a smaller maintained ChR2 current (Fig. 3A). L-AP4 was applied during the plateau phase of the ChR2 current. Most notably, as ChR2 current increased, the responses to L-AP4 were progressively potentiated in comparison with the L-AP4 currents obtained in the absence of illumination. Control experiments showed that L-AP4 responses were blocked by Ba2+, which inhibits GIRK currents, and that L-AP4 had no effect on ChR2 current (data not shown).

Results similar to those shown in Fig. 3A were observed in seven out of eight oocytes (one oocyte had no increase in agonist-induced GIRK current). The peak L-AP4-induced current amplitude data for five oocytes are plotted in Fig. 3B as a function of $[Ca^{2+}]$. All responses increased with increasing illumination, and these increases began at very low $[Ca^{2+}]$, (at or below ~100 nM). The relationship between the agonist-induced currents and $[Ca^{2+}]$, could be fitted by binding isotherms, with a mean equilibrium dissociation constant of $K_d$ = 533 ± 201 nM and a mean maximal current of 333 ± 84 nA (means ± S.E.). However, there was considerable variability between individual oocytes. This variability in the sensitivity of the response to $[Ca^{2+}]$, is likely to reflect differences in the expression ratios of GIRK to mGluR7a between oocytes.

To confirm that the potentiation of mGluR7a-stimulated GIRK currents observed upon light illumination is mediated by $Ca^{2+}$, we tested whether BAPTA-AM inhibits light-dependent mGluR7a potentiation (Fig. 3C). BAPTA-AM blocked the ChR2-mediated increase in the mGluR7a response by 65 ± 12% (S.E.; $n$ = 5, $p$ = 0.002). This was similar to the inhibition of $I_{Cl(Ca)}$ (69%, Fig. 1D) seen under the same conditions. These results are consistent with the ChR2-induced potentiation of mGluR7a currents being primarily mediated by an increase in $[Ca^{2+}]$. To further confirm that the increased L-AP4 response seen in panel A is due to $Ca^{2+}$ influx, we repeated the stimulation protocol shown in Fig. 3A with a constant light intensity while varying the extracellular concentration of $Ca^{2+}$. Fig. 3D shows that the peak amplitude of the L-AP4 response increased with increasing $[Ca^{2+}]$. The peak L-AP4 response in panel D and additional data are plotted in panel E (Fig. 3). Two additional points are evident from Fig. 3E. First, BAPTA-AM (filled circles) blocked a large portion of the $Ca^{2+}$-dependent L-AP4 response (open circles). Second, at 10 mM $[Ca^{2+}]$, the response decreased. The smaller agonist response at the highest $[Ca^{2+}]$ is likely caused by direct effects of extracellular Ca2+ on mGluR7 (see below).

Both modeling and experimental studies suggest that in a restricted space such as the synaptic cleft, extracellular Ca2+ can be transiently reduced during periods of high activity, producing short term synaptic depression (20). In addition, mGluRs belong to a superfamily of proteins that bind extracellular $Ca^{2+}$ (21). mGluR1 has been reported to be activated by extracellular $Ca^{2+}$ in the absence of agonist (22), and the agonist responses of the related GABAB receptor are modulated by extracellular $Ca^{2+}$ (23). Thus, mGluR7a signaling might be regulated not only by changes in $[Ca^{2+}]$, but also by extracellular $Ca^{2+}$ levels. We therefore examined whether extracellular $Ca^{2+}$ might activate mGluR7a in the absence of L-AP4 and whether mGluR7a currents elicited by L-AP4 are modulated by changes in the extracellular $Ca^{2+}$ concentration. We found that extracellular $Ca^{2+}$ alone was unable to elicit GIRK currents (Fig. 4A, left half, and 4B, filled circles) and that current responses to L-AP4 varied little between 100 $\mu$M and 2 mM $[Ca^{2+}]$ (Fig. 4A, right half, and Fig. 4B, open diamonds). Although extracellular $Ca^{2+}$ modulates the activation of mGluR7a by agonist (L-AP4), this effect will be important only if extracellular $Ca^{2+}$ is reduced to less than 0.1 mM (Fig. 4B), a value well below the changes predicted or measured under physiological conditions at the synapse. Hence, it appears unlikely that physiological changes in extracellular $[Ca^{2+}]$, influence mGluR7a signaling.

**DISCUSSION**

In this study, we provide direct evidence showing that elevated $[Ca^{2+}]$, modulates agonist activation of mGluR7a. Our results are based on the use of the light-activated cation channel ChR2 (14) for inducing $Ca^{2+}$ influx and of the endogenous calcium-activated chloride channel of *Xenopus* oocytes for estimating free intracellular $Ca^{2+}$ concentrations. The magnitudes of $I_{Cl(Ca)}$ allowed us to determine free $[Ca^{2+}]$, at the inner face...
FIGURE 3. Potentiation of mGluR7a agonist responses upon ChR2 activation. A, continuous recording from an oocyte co-expressing GIRK, mGluR7a, and ChR2. L-AP4 (100 μM) was applied during the periods indicated by bars. Arrows indicate the onset of illumination, and filled circles indicate the time at which the light was turned off. The relative light intensity (percentage of maximum) is indicated above each arrow. Increasing light intensities produced correspondingly larger ChR2 currents, with large initial transients followed by smaller plateau currents. The inset shows the transient and plateau ChR2 currents and the L-AP4 response at an expanded scale (scale bars are 100 nA and 5 s). The same concentration of L-AP4 resulted in larger responses with increasing intensity of illumination. Transients seen in the initial application of L-AP4 were not consistently observed. B, dependence of the L-AP4 response on \([\text{Ca}^{2+}]_i\). Data are presented for five oocytes. \([\text{Ca}^{2+}]_i\) concentrations were calculated from calibrations done individually for each oocyte (as shown in Fig. 1C). Data were fit by with a binding isotherm (\(I_{\text{L-AP4}} = B_{\text{Max}} \times ([\text{Ca}^{2+}]_i)/K_d + [\text{Ca}^{2+}]_i\)); see "Results"). Data for the cell in panel A are plotted as filled circles. Values for \(B_{\text{Max}}\) (in nA) and \(K_d\) (in nM) were 571 and 741 (A); 330 and 233 ( ); 460 and 1205 ( ); 205 and 71 (f); and 99 and 417 ( ). C, inhibition by BAPTA-AM of light-induced mGluR7a potentiation. L-AP4 was applied to Xenopus oocytes expressing mGluR7a, GIRK, and ChR2. Responses to L-AP4 were compared in the absence and presence of ChR2 activation with blue light (left panel). Cells were then incubated with BAPTA-AM (100 μM) for 8–12 min, which is sufficient time for a maximal block of \(I_{\text{Cl(Ca)}}\), and L-AP4 responses were recorded again with and without light (right panel). Thin lines, no light; thick lines, ChR2 activation by light. The thin and thick traces were superimposed at the beginning of the L-AP4 application. The no light response to L-AP4 application tended to increase in the presence of BAPTA-AM, but this was not statistically significant (average increase = 24 ± 18%, S.E., \(n = 5, p = 0.12\)). D, as the inset shown in the lower right of panel A, but repeated illumination with the same light intensity in the presence of variable extracellular \([\text{Ca}^{2+}]_i\) (from 0.1 to 5 mM with the \([\text{Ca}^{2+}]_i\) shown adjacent to each response). L-AP4 responses are superimposed at the beginning of agonist application (note that the responses are small because L-AP4 was 1 μM, rather than 100 μM in panel A, and the driving force for \(K^+\) influx was smaller due to differences in the membrane potential, see below). E, the peak L-AP4 response (maximal response for 0.1 mM, and peaks indicated in panel D by asterisks below the 0.3–5 mM traces) are plotted (with others not illustrated in panel D) along with post-BAPTA-AM responses of the same cell (filled circles). Cells were voltage-clamped at −60 mV (panels C–E) or −100 mV (panels A and B).
of the membrane, where regulation of mGluR7a activity occurs. Light activation of ChR2 provided rapid, reproducible, and readily reversible changes in submembranous [Ca\(^{2+}\)]. Importantly, increases in submembranous [Ca\(^{2+}\)] potentiated the mGluR7a response to the selective agonist L-AP4, as monitored by activation of co-expressed GIRK, and this potentiation could be detected at concentrations close to resting levels of [Ca\(^{2+}\)]. A scheme summarizing the experimental system used and our interpretation of the results obtained are presented in Fig. 5. Accordingly, elevation of [Ca\(^{2+}\)], by light activation of ChR2 results in Ca\(^{2+}\) binding to CaM or other Ca\(^{2+}\)-sensitive proteins that interact with the C-terminal tail of mGluR7a in the presence of Ca\(^{2+}\). This would displace prebound G\(\beta\)\(\gamma\) from mGluR7a and thereby trigger K\(^{+}\) influx via G\(\beta\)\(\gamma\) binding to GIRK. Although not indicated in the figure, it is possible that Ca\(^{2+}\) and Ca\(^{2+}\)-binding proteins act directly on GIRK. This alternative explanation seems unlikely because we have found no reports describing potentiation of GIRK currents by increases of intracellular Ca\(^{2+}\). Conversely, there is mixed evidence for a role of Ca\(^{2+}\) in G-protein-coupled receptor-mediated inhibition of GIRK currents, with some laboratories finding enhanced or accelerated inhibition and others finding no effect of Ca\(^{2+}\) (24–26). We observed no increase in deactivation or inhibition by Ca\(^{2+}\). The scheme in Fig. 5 agrees well with our previous model of presynaptic mGluR regulation by Ca\(^{2+}\)-CaM (8, 12), which was based on the finding that Ca\(^{2+}\)-CaM and G\(\beta\)\(\gamma\) compete for overlapping binding sites in the C-terminal tail of mGluR7a that are conserved in other presynaptic group III mGlRs (27).

What are the implications and predictions of our findings for synaptic physiology? Since mGluR7a is localized in presynaptic terminals of both excitatory and inhibitory synapses and known to inhibit presynaptic Ca\(^{2+}\) channels (6, 28), its sensitivity to [Ca\(^{2+}\)], will provide a gain control for negative feedback inhibition of neurotransmitter release. Active synapses with elevated presynaptic [Ca\(^{2+}\)], will be more potently inhibited upon mGluR7a activation. Such a regulation by Ca\(^{2+}\) implies that inhibition by mGluR7a will be enhanced if the receptor is positioned close to Ca\(^{2+}\) channels. Furthermore, the [Ca\(^{2+}\)], required to modulate mGluR7a should lie in a range that is similar to the [Ca\(^{2+}\)], which triggers synaptic vesicle exocytosis. In the literature, the [Ca\(^{2+}\)], values reported to initiate neurotransmitter release vary widely (reviewed in Ref. 29). In some excitable cells, exocytosis requires such a high Ca\(^{2+}\) concentration (~100 \(\mu\)M) that the fusion machinery is assumed to be within 20 nm of the Ca\(^{2+}\) channels. For other neurons, lower
values are reported, and the [Ca\(^{2+}\)]i necessary for exocytosis appears to reflect a more global concentration estimate within the terminal (neuromuscular junction, 2–4 μM (30); calyx of Held, ~10 μM (31)). Thus, the range of free [Ca\(^{2+}\)]i, over which mGluR7a modulation was observed here is well within or even below that initiating neurotransmitter release from presynaptic terminals. In conclusion, our results are consistent with the concept that presynaptic group III mGluRs act as activity-dependent regulators of neurotransmitter release.

The results described here demonstrate that, at non-saturating agonist concentrations, elevations in [Ca\(^{2+}\)]i result in increased mGluR7a responses. An unusual feature of mGluR7 is its reportedly low affinity for glutamate (K\(d\) ~1 mM; (2, 32)). Therefore, it has been suggested that mGluR7 is only activated during intense synaptic activity or under extreme circumstances, such as epileptic seizures or ischemia. Indeed, l-AP4 has been found to be neuroprotective under conditions of excitotoxicity, e.g. in the presence of elevated extracellular glutamate (33). The enhanced survival of cultured cerebellar neurons observed in the presence of this agonist does not involve large increases in [Ca\(^{2+}\)]i, and appears to depend on mGluR7 (34). Additional studies also have implicated mGluR7, working by inhibition of glutamate release, in neuroprotection. The increased l-AP4 response seen here at elevated [Ca\(^{2+}\)]i (Fig. 3) predicts that concentrations of glutamate well below the K\(d\) would be effective if intracellular Ca\(^{2+}\) levels were raised. Thus, mGluR7 may be important under physiological conditions, rather than solely providing neuroprotection in situations involving excessive stimulation.

The results reported here provide a new perspective for interpreting the phenotypes of mGluR knock-out mice. Synaptic facilitation depends on the [Ca\(^{2+}\)]i level in the presynaptic terminal. Potentiation of mGluR7a by increased [Ca\(^{2+}\)]i as reported here, should enhance feedback inhibition of Ca\(^{2+}\) channels and thus accelerate recovery from facilitation. Consistent with this prediction, mGluR7-deficient mice show a delayed recovery from synaptic facilitation (4). A train of action potentials can increase presynaptic [Ca\(^{2+}\)]i, and under these conditions, mGluR7 responses should be enhanced and their speed increased. Thus, mGluR7 could act as a frequency-dependent filter for synaptic transmission by more effectively reducing release at high frequencies. The magnitude of this effect may be modest since there is evidence at the calyx of Held that presynaptic mGluRs contribute only about 10% to synaptic depression (35). However, even a 10% change may be sufficient to produce susceptibility to epilepsy, and indeed, mGluR7 knock-out mice are prone to epileptic seizures, whereas mice deficient in mGluRs 1, 2, 4, 5, 6, or 8 do not show such a phenotype (4).

Increases in [Ca\(^{2+}\)]i, can result not only from the opening of voltage-gated Ca\(^{2+}\) channels but also from the activation of intracellular Ca\(^{2+}\) stores. Thus, Ca\(^{2+}\) release from intracellular stores might provide a molecular link between different mGluR subtypes because activation of group I mGluRs results in the production of inositol 1,4,5-trisphosphate, and hence, the release of stored Ca\(^{2+}\) (36). Since both group I and III receptors have been shown to be presynaptic at the Schaffer collateral-CA1 synapse in the hippocampus (37), release of intracellular Ca\(^{2+}\) could create another pathway for synergy between mGluRs, as postulated for some aspects of long term potentiation and long term depression (38).

Long term depression is the most obvious form of synaptic plasticity in which mGluR7 might be involved since mGluR7 inhibits Ca\(^{2+}\) channels and synaptic transmission. Indeed, glutamatergic synapses formed by hippocampal mossy fibers on stratum lucidum interneurons, high frequency stimulation has been shown to induce presynaptic long term depression that is mimicked by l-AP4 at concentrations sufficient to activate mGluR7 and is blocked by the group III-specific mGluR antagonist (RS)-α-methylserine-O-phosphate (3). However, mGluR7 is also present at inhibitory nerve terminals, and inhibition of such terminals should produce excitation due to a reduced release of GABA. Consistent with such a mechanism, activation of group III mGluRs has been found to facilitate glutamate release in some layers of the cortex (39). Also, glutamate spillover resulting from mossy fiber stimulation in cerebellar glomeruli has been shown to suppress GABA release via presynaptic mGluR activation (40). All these results are consistent with an important role of group III mGluRs in bidirectional synaptic modifiability. Furthermore, several lines of evidence indicate that the magnitude of the rise in presynaptic [Ca\(^{2+}\)]i following stimulation is a major factor in determining whether a synapse shows potentiation or depression (41, 42). It therefore is tempting to speculate that the sensitivity of mGluRs to [Ca\(^{2+}\)]i, described here for mGluR7a might play an important role in the selection of long term potentiation versus long term depression.

Acknowledgments—We thank Dr. Andreas Karschin (University of Würzburg, Würzburg, Germany) for the GIRQ3.1/3.2 cDNA and Dr. Shigetada Nakanishi (Kyoto University, Kyoto, Japan) for the full-length mGluR7a cDNA.

REFERENCES
1. Conn, P. J., and Pin, J. P. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 205–237
2. Okamoto, N., Hori, S., Akazawa, C., Hayashi, Y., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1994) J. Biol. Chem. 269, 1231–1236
3. Pelkey, K. A., Lavezzi, G., Racca, C., Roche, K. W., and McBain, C. J. (2005) Neuron 46, 89–102
4. Sansig, G., Bushell, T. J., Clarke, V. R., Rozov, A., Burnashev, N., Portet, C., Gasparini, F., Schmutz, M., Klebs, K., Shigemoto, R., Flor, P. J., Kuhn, R., Knoepfel, T., Schroeder, M., Hampson, D. R., Collett, V. J., Zhang, C., Duvoisin, R. M., Collingridge, G. L., and van der Putten, H. (2001) J. Neurosci. 21, 8734–8745
5. Fagni, L., Chavis, P., Ango, F., and Bockaert, J. (2000) Trends Neurosci. 23, 80–88
6. Perroy, J., Prezeau, L., De Waard, M., Shigemoto, R., Bockaert, J., and Fagni, L. (2000) J. Neurosci. 20, 7996–7904
7. Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P. J., Neki, A., Abe, T., Nakanishi, S., and Mizuno, N. (1997) J. Neurosci. 17, 7503–7522
8. O’Connor, V., El Far, O., Bofill-Cardona, E., Nanoff, C., Freissmuth, M., Karschin, A., Airas, J. M., Betz, H., and Boehm, S. (1999) Science 286, 1180–1184
9. Bockaert, J., Marin, P., Dumuis, A., and Fagni, L. (2003) FEBS Lett. 546, 65–72
10. Dev, K. K., Nakajima, Y., Kitano, J., Braithwaite, S. P., Henley, J. M., and Nakanishi, S. (2000) J. Neurosci. 20, 7252–7257
11. El Far, O., Airas, J., Wischmeyer, E., Nehring, R. B., Karschin, A., and Betz, H. (2000) Eur. J. Neurosci. **12**, 4215–4221
12. El Far, O., and Betz, H. (2002) Biochem. J. **365**, 329–336
13. Enz, R. (2007) BioEssays **29**, 60–73
14. Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003) Proc. Natl. Acad. Sci. U. S. A. **100**, 13940–13945
15. Kuruma, A., and Hartzell, H. C. (1999) Am. J. Physiol. **276**, C161–C175
16. Flor, P. J., van der Putten, H., Ruegg, D., Lukic, S., Leonhardt, T., Bence, M., Sansig, G., Knopfel, T., and Kuhn, R. (1997) Neuropharmacology **36**, 153–159
17. Nagel, G., Brauner, M., Liewald, J. F., Adeishvili, N., Bamberg, E., and Gottschalk, A. (2005) Curr. Biol. **15**, 2279–2284
18. Haase, A., and Hartung, K. (2006) Pfluegers Arch. Eur. J. Physiol. **452**, 81–90
19. Saugstad, J. A., Segerson, T. P., and Westbrook, G. L. (1996) J. Neurosci. **16**, 5979–5985
20. Borst, J. G., and Sakmann, B. (1999) *J. Physiol. (Lond.*)** 521**, 123–133
21. Riccardi, D. (2002) *Exp. Physiol.* **87**, 403–411
22. Kubo, Y., Miyashita, T., and Murata, Y. (1998) Science **279**, 1722–1725
23. Wise, A., Green, A., Main, M. J., Wilson, R., Fraser, N., and Marshall, F. H. (1999) *Neuropharmacology* **38**, 1647–1656
24. Sharon, D., Vorobiov, D., and Dascal, N. (1997) *J. Gen. Physiol.* **109**, 477–490
25. Hill, J., and Peralta, E. G. (2001) *J. Biol. Chem.* **276**, 5505–5510
26. Leaney, I. L., Dekker, L. V., and Tinker, A. (2001) *J. Physiol. (Lond.*)** 534**, 367–379
27. El Far, O., Bofill-Cardona, E., Airas, J. M., O’Connor, V., Boehm, S., Freissmuth, M., Nanoff, C., and Betz, H. (2001) *J. Biol. Chem.* **276**, 30662–30669
28. Chavis, P., Fagni, L., Bockaert, J., and Lansman, J. B. (1995) *Neuropharmacology* **34**, 929–937
29. Augustine, G. J., Santamaria, F., and Tanaka, K. (2003) *Neuron* **40**, 331–346
30. Angleson, J. K., and Betz, W. J. (2001) *J. Neurophysiol.* **85**, 287–294
31. Wolfel, M., and Schneggenburger, R. (2003) *J. Neurosci.* **23**, 7059–7068
32. Saugstad, J. A., Kinzie, J. M., Mulvihill, E. R., Segerson, T. P., and Westbrook, G. L. (1994) *Mol. Pharmacol.* **45**, 367–372
33. Graham, M. E., and Burgoyne, R. D. (1994) *Eur. J. Pharmacol.* **288**, 115–123
34. Lafon-Cazal, M., Fagni, L., Guiraud, M. J., Lerner-Natoli, M., Pin, J. P., Shigemoto, R., and Bockaert, J. (1999) *Eur. J. Neurosci.* **11**, 663–672
35. von Gersdorff, H., Schneggenburger, R., Weis, S., and Neher, E. (1997) *J. Neurosci.* **17**, 8137–8146
36. Abdul-Ghani, M. A., Valiante, T. A., Carlen, P. L., and Pennefather, P. S. (1996) *J. Neurosci.* **16**, 5979–5985
37. Gereau, R. W., and Conn, P. J. (1995) *J. Neurosci.* **15**, 6879–6889
38. Cho, K., and Bashir, Z. I. (2002) *Trends Neurosci.* **25**, 405–411
39. Evans, D. I., Jones, R. S., and Woodhall, G. (2000) *J. Neurophysiol.* **83**, 2519–2525
40. Mitchell, S. I., and Silver, R. A. (2000) *Nature* **404**, 498–502
41. Kobayashi, K., Manabe, T., and Takahashi, T. (1999) *Eur. J. Neurosci.* **11**, 1633–1638
42. Dittman, J. S., Kreitzer, A. C., and Regehr, W. G. (2000) *J. Neurosci.* **20**, 1374–1385
43. Peleg, S., Varon, D., Ivanina, T., Dessauer, C. W., and Dascal, N. (2002) *Neuron* **33**, 87–99