A NOVEL, EXTREMELY FAST, FEEDBACK INHIBITION OF GLUTAMATE RELEASE IN THE CRAYFISH NEUROMUSCULAR JUNCTION

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Abstract—Feedback inhibition serves to modulate release when neurotransmitter levels in the synaptic cleft are elevated. The “classical” feedback auto-inhibition of neurotransmitter release is predominantly meditated by activation of presynaptic G-protein-coupled receptors (GPCRs) and exhibits slow kinetics. In cholinergic and glutamatergic synapses and for focal graded depolarization of the axon terminal, feedback inhibition was found to be voltage-dependent. At high depolarizations, such as the one produced by an action potential, low concentrations of neurotransmitter were insufficient to inhibit release. On the other hand, at higher neurotransmitter concentrations, feedback inhibition was observed also for action potential-evoked release. This finding suggests the presence of an additional mechanism of feedback inhibition that operates also at large presynaptic depolarizations. Using the glutamatergic crayfish neuromuscular junction we discovered a novel, extremely fast, form of feedback inhibition which hampers action potential-evoked release. This novel mechanism is pertussis toxin-insensitive, and is activated already 1 ms after flash photolysis producing glutamate concentrations higher than the ones required to activate the classical feedback inhibition. This finding implies that this mechanism is recruited only when glutamate levels in the synaptic cleft are relatively high (after high-frequency activation or in pathological conditions). We show that both the classical and this novel mechanism operate under physiological conditions. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: feedback inhibition, GPCRs, mGluRs, neurotransmitter release, glutamate release, presynaptic modulation.

Neurotransmitter-mediated feedback autoinhibition (FI) is usually a slow process mediated by presynaptic G-protein-coupled receptors (GPCRs) (Starke et al., 1989; Vizi, 2000). The classical FI is implemented by various G-protein-dependent processes, notably inhibition of Ca^{2+} influx (Delcour and Tsien, 1993; Dolphin, 1998; Miller, 1998; Dutar et al., 2000). Other inhibitory mechanisms include increasing K^+ conductance (Miwa et al., 1990; Cochilla and Alford, 1998; Parnas et al., 1994; Dudel, 1981; Kajikawa et al., 2001; Tedford and Zamponi, 2006; Pinheiro and Mulle, 2008). Other inhibitory mechanisms include increasing K^+ conductance (Miwa et al., 1990; Cochilla and Alford, 1998; Miller, 1998; Dutar et al., 2000) or direct action of G_{i/o} on the proteins of the release machinery (Blackmer et al., 2001, 2005; Yoon et al., 2007). The classical G_{i/o}-mediated FI is blocked by pertussis toxin (PTX) (Miwa et al., 1987; Slutsky et al., 2002).

Focal graded depolarizations of presynaptic terminals of the frog neuromuscular junction (NMJ) led to the discovery of two modes of FI of acetylcholine release (Slutsky et al., 2002). At low presynaptic depolarizations FI was produced by low to moderate (<1 μM) agonist concentrations, it was blocked by PTX and was mediated by slow mechanisms (many seconds to minutes). However, this FI obtained at lower transmitter concentrations was curbed at strong depolarizations. At such low transmitter concentrations, action potential-evoked release was not blocked. Yet, at high neurotransmitter concentrations, action potential-evoked release was also inhibited (Slutsky et al., 2002). As pointed out by Slutsky et al. (2002) this latter mode of FI has different characteristics than the classical mode: (1) it is produced only by high neurotransmitter or agonist concentrations; (2) it is PTX-insensitive; and (3) it operates by relatively fast processes (seconds). The relatively shorter time required for its activation found by Slutsky et al. (2002) was limited by the rapidity at which the agonist could be applied and therefore it may even operate on a much faster time scale.

Here we studied whether FI of glutamate (Glu) release consists also of two components; the slower voltage-dependent classical FI and a novel voltage-independent fast mechanism. To characterize a putative rapid form of FI we examined effects of Glu, applied by flash photolysis from a caged compound, on Glu release. We used the crayfish NMJ for this investigation. This system is particularly suitable because the properties of release can be unambiguously determined (Dudel, 1981; Parnas et al., 1994; Kupchik et al., 2008). Also, many attributes that were clearly demonstrated in invertebrates were later shown to exist in vertebrate neurons (Marder, 2006; Pan and Zucker, 2009).

We found that, as for FI of acetylcholine release (Slutsky et al., 2002), GPCR-mediated FI of Glu release comprises, in fact, at least two separate modes of action: the classical voltage-dependent slower FI (Starke et al., 1989), which is accomplished by activation of G-protein, and a novel FI, which is voltage-independent and is ex-
tremely fast. By using flash photolysis of caged Glu we were able to demonstrate that the novel fast FI operates within the time scale of 1 ms.

**EXPERIMENTAL PROCEDURES**

**Animals**

Crayfish (*Procambarus clarkii*) purchased from Naim Bamayim, Israel, were used. The first two walking legs were removed by autotomy. The cephalothorax was crashed rapidly to minimize suffering of the animal and the abdomen was separated. For all experiments (except of Fig. 9), we used the L1 bundle of the deep extensor abdominal muscles (DEAM; Parnas and Atwood, 1966). For the experiments of Fig. 9, the opener muscle (Wojtowicz and Atwood, 1984) of the first two walking legs was used. The isolated muscles were submerged in a modified Van–Harreveld solution (in mM: NaCl 220, KCl 5.4, CaCl₂ 10, MgCl₂ 2.5, Tris maleate 10, pH adjusted to 7.4 with NaOH). When axon action potential amplitude needed to be lowered, [NaCl]₀ was reduced and compensated for by choline chloride ([NaCl]₀ to 55 mM (compensated by choline chloride), K⁺ currents were blocked by adding to the circulating fluid 100 μM 3,4-diaminopyridine and 10 mM tetraethylammonium chloride. Na⁺ currents were blocked only under the electrode rim by 20 μM TTX inside the electrode. This allowed propagation of the action potential to the recording site. Thus, the ENTC consisted of Ca²⁺ currents and leak and capacitative currents. Then 100 μM Cd²⁺ was added to the bathing solution to completely block Ca²⁺ currents, such that the remaining ENTC was composed of leak and capacitative currents. The ENTC with Cd²⁺ was subtracted from the control ENTC to obtain the net Ca³⁺ current.

**Electrophysiology**

Focal depolarization and recording was achieved with a macropatch electrode (–8 μm pore diameter, 200–300 kΩ) (Dudel, 1981). At 10 °C single quanta events are easily detected (Asterisk in Fig. 1A right). The number of quanta released within 10 ms after the stimulus divided by the number of applied pulses provides directly the average number of quanta per pulse, that is the quantal content (Kupchik et al., 2008). The volume of the recording chamber was 1 ml, and the preparation was superfused with a total of 20 ml (reservoir beaker) modified Van–Harreveld solution. For PTX incubation, fluid circulation was stopped. As a result, the temperature increased to about 25 °C. PTX (2 μg/ml) was added directly to the recording chamber. After 3 h, the circulating pump was re-activated and recording was resumed. Three hours of incubation are sufficient to obtain maximal PTX effect (Rathmayer and Djkaj, 2000; Kupchik et al., 2008). Heat-inactivated (80 °C for 30 min) PTX has no effect on release (Kupchik et al., 2008). During incubation with PTX, the macropatch electrode remained on the recording site. If the electrode moved during this time the experiment was discarded. Traces were stored digitally on a PC and analyzed off-line (Kupchik et al., 2008).

Ca²⁺ currents were evaluated from the excitatory nerve terminal current (ENTC) (Brigant and Mallart, 1982; Dudel, 1990; Slutsky et al., 2002; Kupchik et al., 2008). Briefly, the excitatory axon was stimulated by a suction electrode and the ENTC and excitatory postsynaptic current (EPSC) were measured from a release varicosity by a macropatch electrode. In order to avoid saturation of Ca²⁺ currents, the amplitude of the action-potential was reduced by lowering [Na⁺]₀ to 55 mM (compensated by choline chloride). K⁺ currents were blocked by adding to the circulating fluid 100 μM 3,4-diaminopyridine and 10 mM tetraethylammonium chloride. Na⁺ currents were blocked by adding to the electrode rim by 20 μM TTX inside the electrode. This allowed propagation of the action potential to the recording site. Thus, the ENTC consisted of Ca²⁺ currents and leak and capacitative currents. Then 100 μM Cd²⁺ was added to the bathing solution to completely block Ca²⁺ currents, such that the remaining ENTC was composed of leak and capacitative currents. The ENTC with Cd²⁺ was subtracted from the control ENTC to obtain the net Ca³⁺ current.

![Fig. 1](image-url). FI in crayfish NMJ. (A) Two muscle fibers with the excitatory axon are schematically shown. The macropatch electrode (MP), placed over a single axon terminal varicosity (dots), depolarizes it to different levels (pulse amplitudes of −0.5, −0.7, −0.9 μA, above) and records, extracellularly, the postsynaptic currents (traces on the right). The upper trace shows failure of release. The bottom trace shows release of a single quantum (asterisk). When needed, axon action potentials were evoked by the suction electrode (right, SE). Here, and in all figures n corresponds to the number of muscles. (B) The basic experiment. The quantal content of control (◼), after Glu application (●, downward arrow), and following washout (▲, upward arrow). (C–D) Average percent inhibition (±SEM, n=3). (C) Percent inhibition by several [Glu]₀ at three pulse amplitudes (◼, −0.5 μA; ▲, −0.7 μA; ●, −0.9 μA; ---, action potential) presented as dose-inhibition curves. IC₅₀ (μM): 3.06±1.26, 5.38±1.24, 9.84±1.27, and 10.60±1.17, respectively. R²>0.9 for all curves. (D) Average FI as a function of pulse amplitude at different [Glu]₀. For each [Glu]₀, inhibition is normalized to that obtained at −0.5 μA.
Establishing dose-inhibition curves with the alternate stimulation protocol

For every [Glu]o three pulses of different amplitudes were given in an alternate manner (Slutsky et al., 2002). For each pulse amplitude a sigmoid dose-inhibition (DI) curve was fitted to the data by the computer program GraphPad Prism and IC50 was calculated (for all dose-inhibition curves, \( R^2 > 0.9 \)).

Intracellular recordings

Excitatory postsynaptic potentials (EPSPs) were recorded (Axoclamp 2 B amplifier, Axon Instruments, Foster City, CA, USA) from muscle fibers using sharp microelectrodes (–15 M\( \Omega \)) filled with 3 M KCl.

Measurement of muscle-cell membrane resistance

A muscle fiber was impaled with two sharp microelectrodes (–15 M\( \Omega \), filled with 3 M KCl). One microelectrode injected current steps (–10 nA to –40 nA) and the second recorded the consequent voltage drop (Fig. 2).

Flash-photolysis

For the uncaging experiments we used 4-methoxy-7-nitroindolyl-caged L-glutamate (MNI-Glu) (Matsuzaki et al., 2001). The xenon lamp (100 J), an ensemble of a Strobex Power Pack (model 238) and a Strobex lamp model 278 (Chadwick–Helmuth), was positioned vertically over the preparation. The light was focused with an elliptical mirror (Pichel, CA, USA) to give an image of ~7 mm at the focal plane (Hochner et al., 1989). For the macropatch experiments we circulated 20 ml of the extracellular solution (that contained MNI-Glu), while the bath volume was 1 ml. Therefore, the amount of MNI-Glu in the extracellular solution sufficed for several repeated flashes. Because the efficacy of the uncaging was ~10%, Glu did not accumulate within the circulating fluid. For the macropatch experiments, where 90 depolarization pulses were required, a test pulse was applied every 10 s. The flash was applied before every other test pulse (i.e. every 20 s). By this, we measured control release and flash-inhibited release alternately. For the intracellular measurements, the artifact of the flash alone was recorded in each experiment and then subtracted off line from the EPSP recordings.

Oocyte experiments

We used the same techniques as described before (Ohana et al., 2006). Xenopus laevis oocytes were isolated and incubated in ND96 solution composed of ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 5 Hapes-NaOH, pH 7.5), with the addition of 2.5 mM Na\(^+\) pyruvate, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin (Dascal and Lotan, 1992). A day after their isolation, the oocytes were injected (Picospritzer, PLI-100; Medical Systems Corporation) with the mGluR3 and G-protein-coupled inwardly rectifying K\(^+\) (GIRK) channel-related cRNAs.

In vitro synthesis of RNA transcripts from the cloned cDNA was performed using standard procedures (Dascal and Lotan, 1992). The amounts of cRNA injected per oocyte were as follows: GIRK1 and GIRK2, 0.2 ng; \( \text{G}_{\text{i3}} \), 2 ng (to decrease the basal GIRK current (\( I_K \)) that is produced by free endogenous \( \text{G}_{\text{i3}} \); Dascal, 1997 and thus to

Fig. 2. Glu, at the concentrations used had no postsynaptic effects. (A) The average size of 50 single quanta in control (black) and in the presence of 50 \( \mu \)M Glu (grey) was the same. (B) 30 \( \mu \)M Glu did not affect the time constant of decay of the EPSP. Two sample traces of normalized-to-peak EPSPs recorded in control (black) and with [Glu*]local=30 \( \mu \)M (grey). The EPSPs are the same ones depicted in Fig. 8D. (C) The average time constant of the decay phase of the EPSP (\( \tau_{\text{decay}} \)), calculated by adjusting a single exponential decay curve to the decaying phase of the EPSP, was not altered by Glu* (control, \( \tau_{\text{decay}} \) = 10.66 ± 0.9 ms, [Glu*]local=30 \( \mu \)M, \( \tau_{\text{decay}} \) = 10.61 ± 0.8 ms, \( n = 4 \) muscles). (D) Recordings of membrane resistance of a single muscle cell using two intracellular microelectrodes. Microelectrode 1 (upper panel) injected current steps of variable amplitude and microelectrode 2 (lower panel) recorded the consequent voltage drop. Recordings were performed in control (black) and with 50 \( \mu \)M Glu (grey). (E) IV curves produced from the experiment performed in (D). Black line–control, grey line–after adding 50 \( \mu \)M Glu.
improve the relative activation of the GIRK channels by the agonist; Peleg et al., 2002; mGluR3, 5 ng. Currents were measured 3–5 days following cRNA injection.

The mGluR3-induced GIRK currents were recorded using the standard two-electrode voltage clamp technique (Axoclamp 2 B amplifier, Axon Instruments, Foster City, CA, USA). The oocyte was impaled with two electrodes pulled from 1.5 mm borosilicate glass capillaries (Hilgenberg GmbH, Malsfeld, Germany). Both electrodes were filled with 500 mM KCl solution. The recording and the current-passing electrode resistances were 15 and 1 MΩ, respectively, pCLAMP8 software (Axon Instruments) was used for data acquisition and analysis.

The mGluR3-induced GIRK currents served to calibrate the local concentration of the uncaged Glu produced by the flash ([Glu]local). To measure the mGluR3-induced GIRK currents, oocytes were voltage-clamped at –80 mV in ND96 solution. To measure K⁺ currents, ND96 was replaced by 24 mM K⁺ solution (similar to ND96 but with 72 mM NaCl, 24 mM KCl, and pH adjusted with KOH), and Iᵥ appeared. Then [Glu]o was elevated stepwise to produce the dose-response (DR) curve, without washout between steps, and the corresponding GIRK currents (Iᵥ) were measured for the various [Glu]o. Iᵥ was terminated upon Glu washout. For calibration of [Glu]local a 50 µl drop of MNI-Glu in one of three concentrations (10, 100, or 300 µM) was positioned under the flash lamp, so that the entire drop would undergo flash-photolysis. After the flash was administered, the entire drop was transferred to 50 ml (×1000 dilution) 24 mM K⁺ solution and this new solution was applied to the oocyte. The active fraction of Glu that was produced following the flash (Glu*) produced Iᵥ. By comparing Iᵥ with the dose-response curve of Glu-induced GIRK currents we could estimate the efficiency of the flash (% uncaging) throughout the flash experiments and hence [Glu]local at the various [MNI-Glu]o (Fig. 3). The dose-response curve was fitted with Equation 1, a Michaelis–Menten type equation assuming two agonist binding sites (Ohana et al., 2006),

\[
Y = \frac{B_{\text{max}} X}{(K_a + X)^2}
\]

Where Y is the fractional amplitude of the current at any agonist concentration, B_{\text{max}} is the response to saturating concentration of agonist defined as 100%, X is the concentration of the agonist (Glu), and K_a denotes the dissociation constant.

**Data and statistical analysis**

Dose-inhibition curves were fitted (goodness of fit $R^2>$0.9) to a sigmoid curve (Slutsky et al., 2002), and IC₅₀ was evaluated using a standard least squares-sum fit. Significance was checked by paired or unpaired two-tailed t-tests. All analyses were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software.

**Chemicals**

All salts, l-glutamate and PTX, were purchased from Sigma (Rehovot, Israel). LY379268, LY341495, and MNI-Glu were purchased from Tocris Cookson, UK. TTX was purchased from Alomone Laboratories (Jerusalem, Israel).

**RESULTS**

**Glu induced both voltage-dependent and voltage-independent Fls**

The experimental procedure is depicted in Fig. 1A. A macropatch electrode (Dudel, 1981) was placed over a single presynaptic varicosity (see Fig. 4 in Ravindran et al., 1997). The varicosity was depolarized either focally, to different levels, by passing negative current pulses of different amplitudes (Fig. 1A, top) or by stimulating the nerve bundle by a suction electrode (SE in Fig. 1A). At each focal depolarization, control quantal content (each point –180 pulses at 3 Hz) was established (Fig. 1B, filled squares). Then, Glu was added to the reservoir beaker and the quantal content was measured every minute (Fig. 1B, empty squares). It took more than 6 min to reach maximal inhibition, reflected in the lower steady-state level. It took about 20 min of wash for complete recovery (Fig. 1B, filled triangles).

Dose-inhibition curves were constructed using five Glu concentrations (in µM: 0.1, 1, 5, 10, 50) and at three depolarization levels (in –µA: 0.5, 0.7, 0.9). The results are depicted in Fig. 1C. Two observations should be noted. (i) Percent inhibition, for the same Glu concentration, is higher at the lower depolarization level. For example, at 5 µM Glu, percent inhibition was 58% at –0.5 µA, 42% at –0.7 µA and only 19% at –0.9 µA. (ii) At the higher depolarization the dose-inhibition curve shifted to the right, that is the Glu concentration required to produce 50% inhibition (IC₅₀) increased as depolarization increased. The IC₅₀ was 3.06±1.26 µM at a –0.5 µA pulse (filled squares) and it increased to 9.84±1.27 µM at –0.9 µA (filled circles, n=3). It is interesting to note that for action potential-evoked release the IC₅₀ was 10.60±1.17 µM, similar to that obtained at –0.9 µA (Fig. 1C, dashed line). In order to show the effect of depolarization on FI, the results of Fig. 1C were plotted in a different way (Fig. 1D). For each Glu concentration, percent inhibition was normalized to that obtained at –0.5 µA (taken as 100%). For example, at 1 µM Glu the inhibition seen when the depolarizing pulse was –0.9 µA was only 10% of that obtained at –0.5 µA. It is clear that the lower the Glu concentration the higher is the dependence of FI on the level of depolarization. Thus, at low Glu concentrations strong depolarization prevents the FI. At high Glu concentration (50 µM) the level of inhibition obtained at –0.9 µA was almost as high as that obtained at –0.5 µA. Thus, the inhibition
produced by high Glu concentrations is voltage-independent. These results suggest that the classical slow voltage-dependent FI operates already at low [Glu]o, but it is effective only at low presynaptic depolarization. At high [Glu]o (>10 μM) a novel FI is unraveled. The novel FI operates also at high depolarizations, such as those produced by full-blown action potentials.

Both the novel and the classical FIs operate via the same group II–like metabotropic Glu receptors

To examine which receptor(s) activates the two forms of FI we used specific agonists and antagonists. In selecting those we were guided by the following observations. Presynaptic metabotropic Glu receptors (mGluRs) were shown to exist in crustaceans, such as the crayfish (Parnas et al., 1996; Schramm and Dudel, 1997; Kupchik et al., 2008) and the lobster (Miwa et al., 1987, 1990). Furthermore, the Drosophila melanogaster mGluR was found to resemble the group II mGluRs (Parmentier et al., 1996). Therefore, we used an agonist and an antagonist of group II mGluRs (although it should be noted that also group III mGluRs were found to inhibit release throughout the nervous system; Pinheiro and Mulle, 2008; Nakajima et al., 2009). Fig. 4A shows the effect of the group II mGluR specific antagonist (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) on Glu-induced inhibition of Glu release. First, a control quantal content of release was obtained (0.53±0.04, filled squares). Then, 50 μM Glu (a concentration sufficient to activate also the novel FI) was added and the quantal content decreased to 0.09±0.02 (83% inhibition, P<0.0001, filled circles). Washing out the Glu restored the control level of release (0.49±0.06, filled triangles). Application of 0.1 μM LY341495 blocked the inhibitory effect of 50 μM Glu; the quantal content remained as in the control (Fig. 4A, 0.51±0.07, x symbols). This result suggests that the specific antagonist blocked both the classical and the novel FIs as 50 μM Glu suffices to activate both types of FIs.

Fig 4B shows the effect of the group II mGluR specific agonist (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) on release. Application of 1 μM LY379268 reduced the quantal content of release from 0.42±0.01 in control (empty squares) to 0.11±0.02 (74% inhibition, P<0.0001, empty circles), similar to the Glu effect seen in Fig. 4A. Washing out the agonist restored the quantal content almost to the control level (Fig. 4B, 0.37±0.03, empty triangles). When the experiment in Fig. 4B was repeated but with three LY379268 concentrations and with three different depolarization levels we found (Fig. 4C) that, like Glu (Fig. 1D), low concentrations of the specific agonist inhibited Glu release in a voltage-dependent manner and this dependence declined as the agonist concentration increased. Note that even at high depolarizations, such that the classical FI was only slightly activated or even not manifested, LY379268 still inhibited release. Thus, it seems that both the classical and the novel FI operate via the same presynaptic inhibitory
mGluR, which like in Drosophila (Parmentier et al., 1996) shows characteristics of the mammalian group II mGluRs. Addition of PTX reveals a PTX-resistant, voltage-insensitive FI mediated by group II-like mGluRs

Group II mGluRs are Gi/o coupled (Conn and Pin, 1997). Since, as shown above, the crayfish receptors are similar to group II mGluRs we expected that PTX, known to uncouple GPCRs from their G\textsubscript{i/o} (Fields and Casey, 1997; Tedford and Zamponi, 2006), will abolish the FI mediated by activation of G\textsubscript{i/o} proteins. To check for this, PTX (2 \textmu g/ml) was added and the muscles were incubated in this solution for 3 h. After the PTX was washed out the effect of various Glu concentrations on release evoked by three different depolarizing pulses was examined. As was found also in the cholinergic synapse of the frog NMJ (Slutsky et al., 2002), FI persisted after PTX incubation, but with altered characteristics: it was voltage-independent, and its IC\textsubscript{50} rose to ~28 \textmu M (Fig. 5A, B). To check whether also the PTX-resistant FI was accomplished by group II-like mGluRs we tested whether the specific agonist of group II mGluRs, LY379268, induced FI in a PTX-treated preparation. Fig. 5C shows that indeed the agonist LY379268 induced PTX-resistant, voltage-independent FI.

The classical and the novel form of FIs are not produced by reduction in Ca\textsuperscript{2+} currents

To check whether any of the FIs shown in Figs. 1 and 5 target the Ca\textsuperscript{2+} channel, we measured the effect of 50 \textmu M Glu, a concentration sufficient to activate both the classical and the novel FIs, on presynaptic Ca\textsuperscript{2+} currents and on release. To do so, the excitatory axon was stimulated by a suction electrode (see Fig. 1) and the EPSC was measured with a macropatch electrode placed over a presynaptic varicosity (see Fig. 4 in Ravin et al., 1997). The arrival of the action potential to the terminal was detected by the appearance of the ENTC just before the EPSC. In the control, the ENTC is composed of sodium, potassium, calcium, leak and capacitative currents. After blocking the different ionic currents the Ca\textsuperscript{2+} currents were derived from the ENTC as described in the Experimental procedures. Fig. 6 shows examples of Ca\textsuperscript{2+} currents and the corresponding release obtained before and after 50 \textmu M Glu was applied. While release (reflected by the amplitude of the EPSC) was reduced drastically (84% inhibition, Fig. 6 right) the Ca\textsuperscript{2+} currents (left), derived from the ENTC (see Experimental Procedures), were not affected. A representative experiment—similar results were obtained in three other experiments. Thus, neither the classical nor the novel forms of FIs were produced by a reduction in Ca\textsuperscript{2+} currents.

Addition of PTX reveals a PTX-resistant, voltage-insensitive FI mediated by group II-like mGluRs

mGluR, which like in Drosophila (Parmentier et al., 1996) shows characteristics of the mammalian group II mGluRs.

Fig. 5. The PTX-resistant FI is voltage-independent and is mediated by group II-like mGluRs. All experiments were done following 3 h incubation with 2 \textmu g/ml PTX. (A) % inhibition as a function of pulse amplitude (n=4) for several [Glu]. (B) The data of (A), symbols as in A, as dose-inhibition curves. IC\textsubscript{50} (n=4): 27.15±1.43, 27.54±1.47, 24.81±1.34, and 29.63±1.69, \textmu M, respective to pulse amplitudes in Fig. 1C. (C) The group II mGluR agonist LY379268 inhibited Glu release in a voltage-independent manner. 1 \textmu M LY379268 (A) produced on average 49±1% inhibition, while 10 \textmu M LY379268 (V) produced 68±2% inhibition (n=4).

Fig. 6. Application of 50 \textmu M Glu did not affect Ca\textsuperscript{2+} currents. To avoid saturation of Ca\textsuperscript{2+} currents the size of the action potential was reduced by lowering [Na\textsubscript{1}]_o to 55 mM. 50 \textmu M Glu (dashed line) inhibited the quantal content, reflected in the amplitude of the EPSC, by 84% (right) while the Ca\textsuperscript{2+} currents (left), derived from the ENTC (see Experimental Procedures), were not affected. A representative experiment—similar results were obtained in three other experiments.
entry, in line with earlier findings where other neurotransmitters that inhibit release were used (Dale and Kandel, 1990; Przywara et al., 1991; Gray et al., 1999; Blackmer et al., 2001, 2005). These results suggest that the two FIs operate downstream to Ca^{2+} entry or that in parallel to Ca^{2+} entry they directly affect the release machinery.

The novel FI is extremely rapid

The results, so far, showed that in the glutamatergic NMJ of the crayfish, similar to the cholinergic NMJ of the frog (Slutsky et al., 2002), there are at least two modes of FI. The classical one is G_{i/o}-protein-mediated as it was blocked by PTX, and the novel one is G_{i/o}-protein-independent, as it persisted following PTX treatment. It is thus possible that one and the same GPCR acts by more than one mechanism. Namely, the classical and the novel FI may operate via the same receptor which activates two different G-proteins (Hermans, 2003). Thus, the novel FI might operate by an interaction of the GPCR with a PTX-insensitive G-protein. Alternatively, the GPCR, in addition to the activation of a G_{i/o}-protein to produce the classical voltage-dependent FI, may act also by a mechanism that does not involve activation of any G-protein. It is quite complicated to discern between these possibilities (Hermans, 2003). However, if the second possibility holds then the novel FI may act by a much faster mechanism than usually found when G-proteins are activated. Slutsky et al. (2002) already showed, for the frog NMJ, that indeed the PTX-insensitive (novel) FI has a faster time-course (still a few seconds) than the PTX-sensitive one, but the time resolution in those experiments was still in the seconds range. Thus, in that case, the PTX-insensitive FI could still be mediated by activation of G-proteins. Yet, if the novel FI acts by a very rapid mechanism in the millisecond range the chance that a G-protein is involved is lessened. To test for faster kinetics we used in the present experiments flash-photolysis of MNI-Glu which enables instant (microseconds) elevation of [Glu]_{o} (Ellis-Davies, 2007). The local concentration of free Glu produced by the flash, [Glu]_{local}^{*}, was estimated using Xenopus oocytes expressing mGluR3 and GIRK channels (Ohana et al., 2006). [Glu]_{local}^{*} was found to be ~10% of the [MNI-Glu] in the bathing solution (see Experimental procedures and Fig. 3).

Before addressing the flash experiments it should be noted that, in the crayfish NMJ, presynaptic Glu receptors acquire much higher affinity toward Glu than the postsynaptic receptors (Tour et al., 1995). This enables activation of the presynaptic receptors by low [Glu]_{o} without activating postsynaptic receptors. Also, the presynaptic receptors do not show desensitization in the continuous presence of 10 μM Glu (Parnas et al., 1996) and this Glu concentration does not desensitize the postsynaptic receptors (Tour et al., 2000). In order to minimize the fraction of FI produced by the classical slow voltage-dependent mechanism in the flash experiments, release was evoked by focal large depolarization. An example of such an experiment is depicted in Fig. 7. First, test pulses were given alone (Fig. 7A, left column) or were preceded (1 ms) by a flash but without addition of MNI-Glu to the bath (Fig. 7A, middle column). Then, MNI-Glu was added and again release was measured without (not shown) or with a flash preceding the test-pulse by 1 ms (Fig. 7A, right column). The quantal content was determined either by the direct counting the number of quanta or by counting the number of failures (Martin, 1966). In four experiments such as the one shown in Fig. 7A, when the flash was given without MNI-Glu (middle column), the quantal content did not change; the quantal content of control was 100% and with the preceding flash it was 99.75±2% of control (no inhibition) using the method of direct counting of quanta or 96.5±2% of control when counting the number of failures. This result shows that the flash, by itself, had no effect on release. After addition of 100 μM MNI-Glu and giving the flash 1 ms before the test pulse, the quantal content was reduced by 41±3% and 43±4% by the direct quanta counting and the failure counting methods, respectively (P<0.0001, Fig. 7A, right column). Thus, Glu acted very rapidly, within 1 ms, to inhibit release. To find how long the effect of [Glu]_{local}^{*} lasted we gave the flash with different delays before the test pulse. Fig. 7B depicts the average results of another set of four experiments. When the flash preceded the test pulse by 1 ms the inhibition was somewhat lower than in the previous experiments, only 30±5%. With 4 ms interval
the inhibition was only 7±5%. No inhibition was seen at longer intervals (10, 20 and 50 ms). Thus, the effect of the uncaged Glu was short-lived. This result indicates that even though $[\text{Glu}]_{\text{local}}$ is uniformly increased in the entire illuminated region, the uncaged Glu was removed very rapidly most probably by rapid diffusion or by very rapid buffering by binding to Glu transporters which are available in large quantities (Lehre and Danbolt, 1998; Beart and O’Shea, 2007; Furness et al., 2008). This may explain why there was no accumulation of the uncaged Glu despite giving 90 flashes. It should be remembered that the interval between flashes was 20 s, a sufficient time for the removal of Glu from the circulating solution.

We next examined whether the effects seen in Fig. 7 apply also to physiological conditions. We, thus, tested for effects of the flash on action potential-evoked release. In these experiments the EPSP, which monitors release from many varicosities, was measured. The amplitude of the EPSP reflects the quantal content as long as other postsynaptic parameters are constant. The flash was given 1 ms before the beginning of the EPSP. While the flash alone, without MNI-Glu, had no effect (Fig. 8A), in the presence of MNI-Glu, it reduced the amplitude of the EPSP in a dose-dependent manner (Fig. 8B–D). Note that the reduction in EPSP amplitude cannot be attributed to a decrease in muscle membrane resistance or to a reduction in quantum size (Fig. 2). Importantly, the inhibition cannot be attributed to slow processes activated by the GPCR because the flash did not induce any long-term or slow inhibition and no late inhibition was observed during the 30 s following the flash (Fig. 8E).

We next tested whether this rapid inhibition produced by $[\text{Glu}]_{\text{local}}$ is voltage-dependent like the classical FI or voltage-independent like the novel FI. Since release was evoked by an action potential we had to vary the amplitude of the action potential. This was done by changing $[\text{Na}^+]_o$ (Parnas et al., 1982; Kupchik et al., 2008). Percent inhibition was found to be the same, for a given $[\text{Glu}]_{\text{local}}$, at three action potential amplitudes (Fig. 8F), implying that the fast inhibition produced by the flash, unlike the classical one, is voltage-independent.

The cumulative results in Figs. 7 and 8 suggest the existence of a very rapid FI that is manifested already within 1 ms following application of Glu. Shorter intervals could not be measured because of the artifact produced by the flash. This rapid inhibition is voltage-independent and, unlike the classical FI, can operate also at high presynaptic depolarization.

**Physiological variations of presynaptic depolarization affect FI**

An interesting question is whether the amplitude of the action potential reaching the nerve terminal is of importance in determining the efficacy of FI. The crayfish opener muscle enables examination of this question. In this muscle, which is innervated by a single excitatory axon (Atwood and Morin, 1970; Wojtowicz and Atwood, 1984), the action potential decays passively in the axon branches that innervate the distal muscle fibers (Dudel, 1963), but is carried actively in the axon branches that innervate the proximal part of the muscle (Zucker, 1974). Thus, the action potential’s amplitude is probably higher at the latter. Dose-inhibition curves produced for nerve stimulation, show that, as for focal depolarization (Fig. 1C), the IC$_{50}$ is higher at the proximal fibers (full action potential) than at the distal fibers (smaller action potential) (Fig. 9A). Remarkably, the IC$_{50}$ at the proximal fibers is similar to that obtained at a focal depolarization of −0.9 μA (9.78±1.61 and 9.84±1.27 μM, respectively), while that of the distal fibers resembles the IC$_{50}$ obtained with focal depolarization of −0.5 μA (3.91±1.2 and 3.06±2.16 μM). The ratio between % inhibition at the distal and proximal fibers (% inh.$_{\text{dist.}}$/%inh.$_{\text{prox.}}$) decreased as $[\text{Glu}]_o$ increased (Fig. 9B), implying that also physiologically the classical FI is dominant only at low $[\text{Glu}]_o$ while at higher $[\text{Glu}]_o$ the novel FI prevails. The reduction in the ratio cannot be attributed to...
saturation of inhibition since the ratio approaches 1 already at low [Glu]o (Fig. 9B). Following PTX treatment, the dose-inhibition curves of the proximal and distal fibers overlapped and the IC50 rose to ~30 µM (Fig. 9C), similar to the results obtained with focal depolarization (Fig. 5). Overall, the results of Fig. 9 indicate that also under physiological conditions, at a lower action potential amplitude, the (classical) slow, high-affinity, voltage-dependent FI dominates, whereas the (novel) fast, low-affinity, voltage-independent FI dominates at full-blown action potential and high [Glu]o.

DISCUSSION

In this work we unravelled in the crayfish glutamatergic NMJ a novel fast mechanism of FI. The classical FI differs from the novel FI in three ways, namely voltage-dependence, affinity, and kinetics. The classical FI is activated already at low concentrations (hundreds nanomolars) of neurotransmitter. Such concentrations can be achieved after a train of action potentials (Scanziani et al., 1997). The classical FI activates G-protein-dependent processes and is blocked by PTX (Miwa et al., 1987; Slutsky et al., 2002). But, it is not effective in inhibiting action potential-evoked release (Fig. 1). The novel fast mechanism requires for its activation neurotransmitter levels of 10–30 µM, concentrations that may be achieved under very high stimulation rates or under pathological conditions, for example, stroke and it is equally effective at all levels of depolarization.

What could be the mechanism underlying the novel extremely fast FI? As indicated by the MNI-Glu experiments (Figs. 7 and 8), this FI exerts its effect only if the flash is given shortly (1–4 ms) before the depolarizing pulse. Thus, slow activation of second messengers by G-protein cascades is less likely for this type of FI. Also, it is unlikely that Gβγ is involved in this extremely fast inhibition. This is because the production of Gβγ requires 20 ms to hundreds of milliseconds (Gerachshenko et al., 2005; Lohse et al., 2008), while release here was inhibited already 1 ms after the flash. Furthermore, the novel fast FI revealed here cannot be attributed to a direct interaction of Gβγ with Ca2+ channels (Dolphin, 1998) as Ca2+ currents were not reduced (Fig. 6). It is also unlikely that the fast inhibition of release seen here is produced by rapid interaction of Gβγ with the soluble NSF attachment protein receptor (SNARE) complex (Yoon et al., 2007) as this type of inhibition was detected only about 20 ms after the administration of serotonin by flash photolysis to produce Gβγ (Gerachshenko et al., 2005). In contrast, Fig. 7B shows that the flash-induced fast FI seen here is manifested only for a few milliseconds after the flash and no inhibition is seen at longer intervals.

The finding that the flash was active only when given few milliseconds before depolarization suggests that the neurotransmitter must be present during the depolarizing pulse in order to produce inhibition of release. What, then, can be the mechanism for such a rapid FI? We previously showed that, in the presynaptic membrane, two independent processes occur during the action potential: (1) the well established Ca2+ influx (Augustine, 2001); and (2) a shift of the GPCR to a low affinity state (Ilouz et al., 1999; Ben-Chaim et al., 2003; Ohana et al., 2006). This, in turn,
promotes release (Parnas and Parnas, 2007). Since Glu did not reduce Ca$^{2+}$ currents (Fig. 6) we hypothesize that the novel FI hampers, by as yet an unknown rapid mechanism, the depolarization–induced shift of the receptor to a low affinity state, thus keeping the release machinery under tonic block (Parnas and Parnas, 2007). This suggestion is compatible with the extremely fast kinetics of the novel FI, and with the finding that the FI does not involve a reduction in Ca$^{2+}$ current.

Our results suggest a novel role for the amplitude of the action potential in recruiting mechanisms of FI. It is possible that in synapses where the action potential is graded (Rengathan et al., 2001; Alle and Geiger, 2006; Clark and Hauser, 2006; Shu et al., 2006), both FI types coexist, while in synapses encountering a full-blown action potential mainly the novel fast FI may operate.

Finally, since GPCRs mediate most signal-transduction processes, our findings that GPCRs can operate on a millisecond timescale probably without activation of the G-protein cascade are of utmost importance for all normal and pathological processes mediated by GPCRs.

Acknowledgments—We thank Dr. B. Hochner for assistance in the flash photolysis experiments. We are grateful to Dr. Kenneth Stein and the Goldie-Anna fund for their continuous support.

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