Fast Inhibition of Glutamate-Activated Currents by Caffeine

Nicholas P. Vyleta, Stephen M. Smith*

Division of Pulmonary & Critical Care Medicine, Oregon Health & Science University, Portland, Oregon, United States of America

Abstract

Background: Caffeine stimulates calcium-induced calcium release (CICR) in many cell types. In neurons, caffeine stimulates CICR presynaptically and thus modulates neurotransmitter release.

Methodology/Principal Findings: Using the whole-cell patch-clamp technique we found that caffeine (20 mM) reversibly increased the frequency and decreased the amplitude of miniature excitatory postsynaptic currents (mEPSCs) in neocortical neurons. The increase in mEPSC frequency is consistent with a presynaptic mechanism. Caffeine also reduced exogenously applied glutamate-activated currents, confirming a separate postsynaptic action. This inhibition developed in tens of milliseconds, consistent with block of channel currents. Caffeine (20 mM) did not reduce currents activated by exogenous NMDA, indicating that caffeine block is specific to non-NMDA type glutamate receptors.

Conclusions/Significance: Caffeine-induced inhibition of mEPSC amplitude occurs through postsynaptic block of non-NMDA type ionotropic glutamate receptors. Caffeine thus has both pre and postsynaptic sites of action at excitatory synapses.

Introduction

The popular stimulant, caffeine, modulates intracellular calcium signaling in many cell types [1]. The ryanodine receptor (RyR) is one target for caffeine. Millimolar concentrations (5–20 mM) of caffeine stimulate calcium-induced calcium release (CICR) from intracellular stores. Caffeine sensitizes RyR so that low nanomolar concentrations of cytosolic calcium activate RyR leading to calcium efflux into the cytoplasm [2]. In addition, caffeine also acts on the other primary endoplasmic reticulum calcium release channel, the inositol triphosphate receptor (IP₃R), over the same concentration range. Caffeine inhibited IP₃R single-channel openings with a half-maximal inhibition of 1.6 mM [3]. Therefore, depending on the relative densities of RyR and IP₃R in a particular cell, caffeine can either stimulate or block calcium release from intracellular stores.

Caffeine has been used extensively to study calcium signaling in neurons. Caffeine increases spontaneous glutamate release from nerve terminals [4] and can induce presynaptic long-term potentiation [5]. Synaptically activated AMPA receptor-mediated CICR has also been described using caffeine [6].

Here, we demonstrate that caffeine decreases the size of miniature excitatory postsynaptic currents (mEPSCs) recorded from neocortical neurons by directly inhibiting postsynaptic glutamate receptor currents. Consistent with other reports, we also demonstrate an increase in mEPSC frequency indicating a caffeine-induced presynaptic increase in glutamate release. Thus, caffeine modulation of glutamatergic transmission involves both pre and postsynaptic targets.

Results

Caffeine decreases mEPSC size in neocortical neurons

We examined the effects of caffeine (20 mM) on mEPSCs recorded from neocortical neurons. Sodium channels were blocked by tetrodotoxin (TTX) and spontaneous synaptic events recorded at −70 mV. Caffeine was applied for 300 seconds. The amplitude of mEPSCs decreased during the first 100 seconds of caffeine application (Fig. 1A). The insets show representative mEPSCs from the beginning and end of their respective traces on an expanded time scale. The inhibition of mEPSC amplitude was reversible (Fig. 1A). Amplitude histograms of all events during 200 seconds of recording directly before caffeine application and all events recorded during the last 200 seconds of caffeine application confirmed this inhibition (Fig. 1B). Average mEPSC amplitude is shown for seven cells before, during, and after caffeine application (Fig. 1C). Average mEPSC amplitude was 27.6 ± 2.3 pA before and 15.7 ± 1.0 pA during caffeine application (P < 0.001; n = 7), demonstrating a 43 ± 4% inhibition by caffeine. Miniature EPSC amplitude was reversibly decreased by caffeine in all seven cells. Fig. 1D shows average normalized diary plots of mEPSC frequency (top) and amplitude (bottom) for seven cells. Caffeine increased mEPSC frequency in all seven cells, consistent with previous work [4]. The increase in mEPSC frequency was slower than the decrease in amplitude, suggesting different sites of action. These data confirm that caffeine increases mEPSC frequency in neocortical neurons, and demonstrate a reduction in mEPSC amplitude not previously described.
Caffeine blocks currents activated by exogenous glutamate

We next asked whether caffeine exerted its effects on mEPSC amplitude by a presynaptic or postsynaptic mechanism. We applied glutamate (1 mM) to test for a direct postsynaptic effect of caffeine on glutamate-activated currents (I_{glu}). Glutamate was applied with a piezoelectric-controlled perfusion device to neurons voltage-clamped at -70 mV. Glutamate was applied for 100 milliseconds and co-application with caffeine reversibly decreased peak I_{glu} amplitude (Fig. 2A). In these experiments glutamate applications were made after perfusion with caffeine containing solutions for 30 seconds (top 20 mM and bottom 50 mM). Bar graphs show the average results from glutamate application in the presence of 20 mM caffeine (Fig. 2B). Glutamate-activated currents were 3.79 ± 0.61 nA and reduced to 1.63 ± 0.35 nA in the presence of caffeine (n = 7; P = 0.002). Currents recovered after caffeine wash to 3.84 ± 0.72 nA (P = 0.10 compared to control).

Thus, caffeine (20 mM) reversibly reduced the magnitude of I_{glu} by 57 ± 5%. The action of caffeine was dose-dependent (Fig. 2C). Inhibition of I_{glu} by caffeine increased during the application (scaled traces, Fig. 2A). Consequently the steady state concentration-effect relationship was left-shifted relative to that for peak glutamate-activated currents (Fig. 2C). Curves represent the equation:

\[ I/I_{\text{max}} = 1/(1 + ([\text{caffeine}]/IC_{50})^n) \]

where IC_{50} was 7 mM and 10 mM for inhibition of the steady-state and peak glutamate-activated currents, respectively.

Caffeine blocks glutamate-activated currents rapidly

In previous experiments (Fig. 2) glutamate was applied after >30 seconds of caffeine application. We next measured the kinetics of caffeine block of glutamate-activated currents by applying caffeine (20 mM) simultaneously with glutamate (1 mM). Fig. 3 shows I_{glu} with and without caffeine. Each trace is the average I_{glu} elicited by 5 applications of glutamate recorded with a 0.33 Hz duty cycle. Traces did not change substantially between applications. Caffeine block of I_{glu} developed during a 500 ms co-application of glutamate and caffeine. Inhibition is absent during the initial rising phase of I_{glu}, partial during the peak, and reaches steady-state by ~200 ms. The effect was reversible. The time course of caffeine block was quantified by dividing the glutamate-activated current in the presence of caffeine (I_{caf}) by the previous current elicited by glutamate alone. This ratio (I_{caf}/I_{glu}) is plotted as a function of time for the
representative recording starting 10 ms after initiation of glutamate application (black, Fig. 3 inset). This decay was fit with a single exponential function with a time constant of 57 ms. The average time constant for inhibition was 52 ± 6 12 ms (n = 3). The dashed line represents the steady-state inhibition of Iglu and crosses the y-axis at 0.27 for this recording. The average steady-state value for Icaff/Iglu was 0.29 ± 0.02, which agrees well with the inhibition of steady-state Iglu in the presence of steady-state caffeine concentration (0.28, Fig. 2C). Thus, caffeine action is rapid, and Iglu blockade develops during glutamate application (Fig. 2). These data demonstrate that caffeine inhibits glutamate receptor current by a fast mechanism, consistent with simple channel block but not consistent with more complex forms of regulation such as changes in receptor expression.

**Discussion**

Caffeine modulates intracellular calcium and can stimulate neurotransmitter release from nerve terminals [4]. We determined that the frequency of spontaneous transmitter release was increased in recordings from neocortical neurons. Surprisingly, caffeine also produced an accompanying decrease in the amplitude of quantal events. Caffeine inhibited currents activated by direct application of glutamate to cortical neurons, confirming a postsynaptic site of action. This unexpected form of inhibition developed over tens of milliseconds and was independent of NMDA receptors, consistent with non-NMDA receptor block.

**Figure 2.** Caffeine inhibits postsynaptic glutamate-activated currents (Iglu). (A) Glutamate (1 mM) was applied to neurons in whole-cell voltage clamp in the presence of TTX. Individual traces from a representative recording of Iglu before (black), during (blue) and after (red) the addition of 20 mM (top) or 50 mM (bottom) caffeine. (B) Average peak Iglu for 5 consecutive applications of glutamate (3 second interval) before, during, and after 20 mM caffeine application. (C) Normalized peak (open circles) and steady-state (closed triangles) Iglu for 1.25, 5, 20, and 50 mM caffeine. Values normalized to average peak or steady-state Iglu before caffeine for each recording. Curves represent Equation 1.

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Caffeine modulates synaptic transmission by both pre- and postsynaptic mechanisms

We found that caffeine increased the frequency of mEPSCs, consistent with a previous report [4] (Fig. 1). A change in the frequency of spontaneous neurotransmitter release events is a clear indicator of a presynaptic change in the probability of vesicle fusion. A change in quantal size could result from either pre or postsynaptic mechanisms [7] – variation in mEPSC size was recently shown to depend on vesicular glutamate concentration [8]. We postulated that caffeine-mediated changes in mEPSC amplitude and frequency (Fig. 1) both resulted from presynaptic modulation. However, the distinct time courses of both changes pointed to different mechanisms of action (Fig. 1D). Caffeine inhibited currents activated by exogenous glutamate and quantal events by a similar amount confirming a postsynaptic action. We postulated two mechanisms by which caffeine mediated these effects: by directly blocking the glutamate activated channels or by reducing postsynaptic glutamate receptor density. However,
of caffeine stimulates synaptic transmission is by inhibiting the adenosine binding, relieving the inhibition of voltage-gated calcium channels and producing higher probability of release of neurotransmitter.

However, it is possible that excess use of caffeine tablets may lead to brain levels at which glutamate-activated channel modulation occurs. For instance, acute caffeine toxicity has been blamed for the death of a 22-yr old woman who experienced serum caffeine levels of ~8 mM following overdose with diet pills [16].

**Conclusions**

We have demonstrated a novel action of caffeine on excitatory transmission in the central nervous system. We show that, in addition to increasing the probability of spontaneous release of neurotransmitter, caffeine inhibits postsynaptic AMPA-type glutamate-activated channels. This occurred at caffeine concentrations regularly used for studying intracellular calcium signaling. Furthermore, our results are consistent with the hypothesis that caffeine-mediated glutamate receptor blockade may only occur under extreme conditions of toxicity.

**Methods**

**Neuronal preparation**

Neocortical neurons were isolated from P1-2 mouse pups. All animal procedures were approved by OHSU I.A.C.U.C. in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the N.I.H. Guide for the Care and Use of Laboratory Animals. Animals were deeply anesthetized with isoflurane before decapitation and removal of cortices. Cortices were then incubated in trypsin and DNAse and then dissociated with a heat polished pipette. Dispersed cells were cultured in MEM plus 5% FBS on glass coverslips. ARAC (4 μM final concentration) was added 48 hours after plating to limit glial division. Cells were used after at least 14 days in culture. Eight different neuronal culture preparations from eight different mice were used for these experiments.

**Electrophysiological recordings**

Cells were visualized with an Olympus IX70 inverted microscope. Recordings were made in whole-cell voltage clamp. Holding potential was ~75 mV. Extracellular solutions contained (in mM) 150 NaCl, 4 KCl, 10 HEPES, 10 glucose, pH 7.35. NaCl was substituted with either caffeine or sucrose to maintain osmolarity. Recordings of mEPSCs were made in the presence of TTX (1 μM) and bicuculline (10 μM) to block Na channels and GABA-activated currents, respectively. TTX was also used during glutamate application experiments. Intracellular solution consisted of (in mM) 140 K+ gluconate, 9 EGTA, 10 HEPES, 4 MgCl2, 1 CaCl2, 4 NaATP, 0.3 NaGTP, 1.4 phosphocreatine, pH 7.2. Electrode tips had final resistances of 3–6 MΩ. Currents were recorded with a HEKA EPC9/2 amplifier. For mEPSC recordings, currents were filtered at 1 kHz using a Bessel filter and sampled at 10 kHz. For recordings of currents evoked by applied glutamate, currents were filtered at 3 kHz and sampled at 20 kHz. Series resistance (Rs) was monitored, and recordings were discarded if Rs changed by more than 10% during recording. For glutamate-application experiments, Rs was usually compensated by ~70%. We estimate that in a typical experiment our inhibition of Ibg by caffeine (20 mM) is underestimated by ~9% [17].

**Solution application**

For mEPSC recordings, solutions were bath applied through a perfusion pipette placed ~1 mm from the patch pipette tip. Local...
solution equilibration occurred in <10 seconds as measured by open-tip conductance changes. For fast application of glutamate, a custom-built piezolectric-driven perfusion system was used. A piece of theta glass containing four small perfusion tubes (two in each barrel) was mounted to a piezoelectric bimorph (Piezo Systems, Inc., product # T234-A360D-203X) which was mounted to a plastic rod controlled by a micromanipulator. A high-voltage stimulus isolator (World Precision Instruments, Sarasota, Florida, product # A360D or A365D) was used to stimulate the bimorph. A TTL pulse supplied by one of the digital-to-analog outputs on the EPC9/2 was used to drive the stimulus isolator. Theta glass was pulled and broken to a tip diameter of approximately 300 microns. A smooth break of barrels and septum was achieved by pulling and breaking the theta tube characteristic of a cell soma. Total solution change in this configuration occurred in ~5 ms.

Analysis

Data were acquired on a PIII computer and analyzed with IgorPro (Wavemetrics, Lake Oswego, OR) and Minianalysis (Synaptosoft, Decatur, GA) software. For all experiments, statistical significance was determined using Student’s t-test as appropriate (Microsoft EXCEL, Richmond, WA). Averaged data values are reported as means ± SEM.

Author Contributions

Conceived and designed the experiments: NPV SMS. Performed the experiments: NPV. Analyzed the data: NPV SMS. Contributed reagents/materials/analysis tools: NPV SMS. Wrote the paper: NPV SMS.

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