3,4-Methylenedioxymethamphetamine (Ecstasy) Activates Skeletal Muscle Nicotinic Acetylcholine Receptors

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ABSTRACT

Adverse 3,4-methylenedioxymethamphetamine (MDMA; ecstasy) effects are usually ascribed to neurotransmitter release in the central nervous system. Since clinical features such as fasciculations, muscle cramps, rapidly progressing hyperthermia, hyperkalemia, and rhabdomyolysis point to the skeletal muscle as additional target, we studied the effects of MDMA on native and cultured skeletal muscle. We addressed the question whether malignant hyperthermia (MH)-susceptible (MHS) muscle is predisposed to adverse MDMA reactions. Force measurements on muscle strips showed that 100 μM MDMA, a concentration close to that determined in some MDMA users, regularly enhanced the sensitivity of skeletal muscle to caffeine-induced contractures but did not cause contractures on its own. The left-shift of the dose-response curve induced by MDMA was greater in normal than in MHS muscle. Furthermore, MDMA did not release Ca2+ from isolated sarcoplasmic reticulum vesicles. These findings do not support the view of an MH-triggering effect on muscle. However, MDMA induced Ca2+ transients in myotubes and increased their acidification rate. Surprisingly, α-bungarotoxin, a specific antagonist of the nicotinic acetylcholine receptor (nAChR), abolished these MDMA effects. The nAChR agonistic action of MDMA was confirmed by patch-clamp measurements of ion currents on human embryonic kidney cells expressing nAChR. We conclude that the neuromuscular junction is a target of MDMA and that an activation of nAChR contributes to the muscle-related symptoms of MDMA users. The drug may be of particular risk in individuals with abundant extrajunctional nAChR such as in generalized denervation or muscle regeneration processes and may act on central nAChR.

3,4-Methylenedioxymethamphetamine (MDMA) is a synthetic amphetamine derivative commonly abused by young people. The desired psychedelic and mood-altering effects of MDMA have been assigned to an inhibition of glutamate-evoked firing of cells in the nucleus accumbens, which is an important interface between the striatum and the limbic system. This inhibitory effect on neuronal activity is predominantly mediated by serotonin and dopamine. An MDMA-induced increase in cytosolic [Ca2+] in neuronal terminals and subsequent exocytosis of vesicles is a major mechanism for the neurotransmitter release (Crespi et al., 1997). Furthermore, MDMA activates monoamine transporters and inhibits serotonin reuptake. Besides its affinity to serotonin recognition sites (e.g., reuptake sites), MDMA exhibits dopaminergic, cholinergic, histaminergic, and adrenergic effects (Battaglia and De Souza, 1989; Mechan et al., 2002).

The most common side effects of MDMA include fasciculations, muscle pain, muscle cramps, and trismus (Henry, 1992; Nimmo et al., 1993). More severe complications such as rapidly progressing hyperthermia, hyperkalemia, metabolic acidosis, excessive creatine kinase (CK) elevation, and rhabdomyolysis have been reported rarely but even at low MDMA dosages (Screaton et al., 1992; Hall et al., 1996). These adverse effects point to the skeletal muscle or the neuromuscular junction as a target of MDMA outside the central nervous system. Metabolic myopathies have been postulated to be the cause of these severe crises (Henry et al., 1992), and recently,
MDMA has been proposed to uncouple oxidative phosphorylation in skeletal muscle mitochondria by an indirect mechanism (Rusyniak et al., 2005). Toxic rhabdomyolyses have been reported in healthy individuals for a variety of substances, including statins, amphetamines, opiates, and succinylcholine (SCh). Despite different initiation, muscle breakdown finally is linked to membrane destabilization and ATP depletion, leading to cellular hypoxia, rhabdomyolysis, and edema. Muscle swelling in the tight envelope of fascia can damage the nerves and blood vessels by exerting direct pressure, i.e., cause a compartment syndrome (Martyn et al., 1992; Breucking et al., 2000; Evans and Rees, 2002). In genetically disposed swine, MDMA has been reported to trigger malignant hyperthermia (MH) in vivo (Fiege et al., 2003).

The purpose of our study was to determine whether MDMA can cause some of its adverse effects peripherally via the skeletal muscle. Therefore, we tested whether MDMA increases cytoplasmic [Ca\textsuperscript{2+}] and triggers contractures in isolated skeletal muscle of normal and MHS individuals. When we identified that MDMA and SCh had similar effects on muscle and that the effects were blocked by \(\alpha\)-bungarotoxin, we extended the study in a previously unexpected direction.

**Materials and Methods**

**Patients.** Informed consent was obtained from 17 individuals who were referred to the Ulm and Cork MH centers because of a previous anesthetic event indicative of MH in the history or in the family. EDTA-treated blood was drawn for genetic analysis, and vastus lateralis muscle samples excised under regional anesthesia were taken for the in vitro contracture test (IVCT), which permits to diagnose or to exclude MH susceptibility. The procedures were approved by the ethics committee of Ulm University (Ulm, Germany) and University College Cork (Cork, Ireland). Averaged data on MDMA serum levels of 35 illicit drug users were included in the study. Data were obtained from drug screening in anonymous form. The individuals were suspected of illicit drug taking and had a compulsory blood withdrawal, a legal requirement under German law. The samples were sent by the police to the Department of Forensic Medicine (Ulm University).

**MDMA Analysis by Gas Chromatography (GC)/Mass Spectrometry.** Centrifuged serum samples were mixed with deuterized MDMA (d\textsubscript{3}-MDMA) as internal standard before solid-phase extraction (HASS 11; Sepapartis, Grenzach-Wyhlen, Germany). Homogenized muscle tissue of normal and MHS individuals was filtered through a 20-μm nylon mesh. Myoblasts were seeded on poly-L-ornithine-coated glass coverslips. Cells were first kept in growth medium containing 5% fetal calf serum (C-23060; PromoCell, Heidelberg, Germany) and pure, i.e., preservative-free, 2% SCh was from Curamed Pharma (Karlsruhe, Germany). Corresponding to MDMA concentrations applied in other nonperfused tissue preparations (Leonardi and Azmitia, 1994; Carvalho et al., 2002; Rusyniak et al., 2005), we used 100 to 1000 (4000) μM.

**Mutation Analysis.** For mutational screening, MH susceptibility hot-spot exons 17, 38, 39, 40, and 45 of the gene encoding the ryanodine receptor type 1 of skeletal muscle (RyR1), the Ca\textsuperscript{2+} channel of the SR, were directly sequenced using primers as described previously (Klingler et al., 2002).

**Cell Culture.** Human muscle samples (50–200 mg) were mechanically teased and treated for 1 h at 37°C with collagenase (330 U/ṁ) (C8685; Sigma Chemie) and dissociated in Ham’s F-12 medium (F0815; Biochrom, Berlin, Germany). The resulting suspension was filtered through a 20-μm nylon mesh. Myoblasts were seeded on poly-i-ornithine-coated glass coverslips. Cells were kept in growth medium containing 5% fetal calf serum (C-23060; PromoCell, Heidelberg, Germany) at 37°C and 5% CO\textsubscript{2}. After 4 to 5 days, cell differentiation was induced by reducing serum content. Within 1 week, the myoblasts became confluent and started to fuse. Myotubes were identified by their polynucleated appearance.

**Ca\textsuperscript{2+} Fluorometry.** Changes of intracellular [Ca\textsuperscript{2+}] were measured by use of fura-2. For dye loading, the myotubes were incubated for 30 min with 2 μM fura-2 acetylmethyl ester (Calbiochem, Bad Soden, Germany) in standard external solution (140 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl\textsubscript{2}, 6.0 mM glucose, 1.5 mM CaCl\textsubscript{2}, and 12.0 mM HEPES, pH 7.3) at 37°C. Uptake was facilitated by addition of 0.02% Pluronic acid (F-127; Sigma Chemie). For the relative calibration of Ca\textsuperscript{2+} signals, the fluorescence ratio during alternating excitation at 340 and 380 nm was obtained.

MDMA was administered to the bath using a superfusion system (L/M-SPS-8; List Electronics, Darmstadt, Germany) that permitted drug application to a single cell or a small group of cells in a highly reproducible manner. Selection among the eight supply vessels connected to the multibarreled inlet pipette was controlled with magnetic valves. Pressure to the supply vessels was adjusted using a
multipressure control unit (MPCU-3; Lorenz, Lindau, Germany). All Ca^{2+} fluorometry measurements were performed at room temperature (20°C).

Measurements on Isolated SR. Heavy SR was prepared from hind limb muscles of five sacrificed rats by homogenization and differential centrifugation as reported previously (O'Sullivan et al., 2001). The final SR pellet was resuspended in 0.1 M KCl solution and cryopreserved in liquid nitrogen. For spectrophotometry, the isolated SR was incubated with the Ca^{2+} chelometric dyes antipyrilazo III in a total volume of 2 ml using a ground glass-stoppered glass cuvette and a medium containing 19 mM MOPS, 93 mM KCl, 7.5 mM sodium pyrophosphate, 1 mM MgATP, 5 mM creatine phosphate, 20 ml of CK, and 250 mM antipyrilazo III at pH 7.0. Flux was monitored continuously with a Hewlett Packard 8452A diode-array spectrophotometer operating in dual wavelength mode at 710 and 790 nm at 37°C and constant cuvette stirring. The rate of Ca^{2+} uptake was calculated from the first seven 20-nmol pulses of Ca^{2+} added to the cuvette with a Hamilton dispensing microliter syringe. When the SR was maximally loaded with Ca^{2+}, usually after 15 pulses, putative releasing agents were added at varying concentrations to establish whether Ca^{2+} release occurred. Halothane was added from a concentrated stock solution made up in pure ethanol. SR protein concentration was measured as reported previously (O’Sullivan et al., 2001).

Proton Release Measurements. The metabolism of cultured myotubes was monitored using a pH-sensitive microphysiometer (Molecular Devices, San Diego, CA) as described previously (Klinger et al., 2002). Briefly, the cells in the measuring chamber were superfused (37°C; 50 μl/min) using a computer-controlled rolling pump that alternately was switched on and off. The medium (F-12 medium; N6760; Sigma Chemie, with equimolar substitution of sodium bicarbonate by NaCl, total osmolality 290 mOsm, pH 7.35) was only weakly buffered. During the pump-on interval the proton concentration in the chamber equilibrated with that of the medium. After stopping the perfusion, the biosensor voltage decreased (i.e., pH increase) at varying concentrations to establish whether Ca^{2+} release occurred. Halothane was added from a concentrated stock solution made up in pure ethanol. SR protein concentration was measured as reported previously (O’Sullivan et al., 2001).

Whole-Cell Patch-Clamp Recordings. Transformed human embryonic kidney (HEK) 293 cells were transfected with cDNA of mouse α, β, δ, and ε NaChR subunits. Patch-clamp measurements were performed on small cells lifted from the bottom for rapid application experiments using standard methods. The patch pipettes contained 140 mM KCl, 11 mM EGTA, 10 mM HEPES, 10 mM glucose, and 2 mM MgCl2. HEK293 cells were superfused with an extracellular solution containing 162 mM NaCl, 5.3 mM KCl, 2 mM CaCl2, 0.67 mM NaH2PO4, 0.22 mM KH2PO4, 15 mM HEPES, and 5.6 mM glucose. The pH of both solutions was adjusted to 7.3. MDMA and 500 μM SCh up to 8 mM did not elicit contractures in MHS or in MHN muscle strips.

Muscle Strip Contractures Induced or Potentiated by MDMA and SCh. A first set of experiments tested the potency of cumulative MDMA to elicit muscle contractures in vitro. Muscle strips from three of eight MHS patients reacted to 500 μM MDMA: strips of the R614C carrier contracted at 500 μM (Fig. 1A); strips of two patients with unknown RyR1 mutations at 1 and 2 mM, respectively; and the strips of the MH-equivocal patient at 2 mM MDMA (not shown). Also, eight MHN muscle strips were exposed to stepwise increasing concentrations of MDMA. None of the eight MHN individuals exhibited contractures even when the MDMA concentration reached 4 mM. Cumulative SCh up to 8 mM did not elicit contractures in MHS or in MHN muscle strips.

Further experiments tested whether the presence of MDMA in the organ bath lowered the concentrations of caffeine (Fig. 1, B versus C) or halothane required to cause a contracture. In normal muscle, the lowest MDMA concentration of 1 μM shifted the dose-response curve for caffeine on average from 3 to 1.5 mM (Fig. 2A), but 1 mM MDMA was necessary to lower the contracture-inducing halothane concentration from 4 to 2% (Fig. 2B). MHS muscle developed contractures at 1.5 mM caffeine in the absence of MDMA (Fig. 2C) and at 1.0 mM caffeine in the presence of 500 μM MDMA (Fig. 2D). Similar shifts to lower caffeine concentrations were found with 500 μM SCh for MHN (Figs. 1D and 2A) and MHS muscle strips (Fig. 2C). Although 500 μM SCh elevated basal force with increasing halothane concentration steps in MHN muscle, this increase was not high enough to meet the IVCT criteria of a contracture (Fig. 2B). In MHS muscle strips, the effects of 500 μM SCh on halothane-induced contractures were similar to those of 500 μM MDMA (Fig. 2D).

Statistical analysis of the dose-response relationships shows that the shifts of the curves to lower caffeine concentration were similar for 100 μM MDMA and 500 μM SCh (Fig. 3, top), except for halothane that always caused smaller contractures than caffeine did. The similarity of the effects of 100 μM MDMA and 500 μM SCh on MHN and MHS muscle is also evident when comparing the increase in muscle contracture amplitudes (Fig. 3, middle) and in twitch force amplitudes (Fig. 3, bottom).

Intracellular Ca^{2+} Level in Myotubes. Muscle contractures result from a myoplasmic [Ca^{2+}] exceeding the mechanical threshold. Caffeine and halothane are known to raise myoplasmic [Ca^{2+}] (Herrmann-Frank et al., 1999). This increase can be determined by measuring fluorescence changes of Ca^{2+}-specific dyes in myotubes. Transient MDMA stimulation resulted in all 40 myotubes in a phasic Ca^{2+}
signal characterized by a rapid increase, dose-dependent peak values and slow return to the resting level (Fig. 4). In an additional 10 myotubes, the nAChR was blocked with α-Bgt (0.1 μM; 15 min), leading to a nondetectable amplitude or at least to a drastically reduced signal as in Fig. 4.

**Metabolic Activation of Myotubes by MDMA.** An enhanced cell metabolism, e.g., originating from an increased myoplasmic \([\text{Ca}^{2+}]\), can be studied by measuring cell proton secretion (Klingler et al., 2002). The use of such a pH-sensitive biosensor allowed us to determine the effects of MDMA on the metabolic activation of myotubes. Transient exposure of myotubes cultured on micronets from three MHN individuals to 500 μM MDMA resulted in a phasic-tonic increase in the acidification rate. This effect was also almost abolished when the cells were exposed to α-Bgt (Fig. 5).

**No Effects of MDMA and SCh on Isolated SR Vesicles.** Caffeine and halothane are reagents known to release \([\text{Ca}^{2+}]\) from the terminal cisternae of the SR, the most important myoplasmic \([\text{Ca}^{2+}]\) store. We measured the effects of up to 170 μM ryanodine, 1200 μM halothane, 1000 μM SCh, and 700 μM MDMA on \([\text{Ca}^{2+}]\) flux rates. In this preparation, which was taken from rat muscle, \([\text{Ca}^{2+}]\) was released only upon incubation with halothane or ryanodine but not upon incubation with MDMA or SCh. This suggests that MDMA and SCh do not directly act upon RyR1 (Fig. 6).

**nAChR Agonistic Action of MDMA.** To further elucidate a possible agonistic effect of MDMA on nAChR patch-clamp experiments were performed on HEK293 cells expressing recombinant nAChR channels of the adult type. nAChR were stimulated by 1 mM acetylcholine (ACh) or MDMA applied to single cells lifted from the bottom. The current reached −1687 pA after application of ACh and decayed in presence of the agonist due to desensitization (Fig. 7A). After application of 100, 300, and 1000 μM MDMA, the peak current amplitude reached −95.9, −163.6, and −305.3 pA, respectively. As shown in the dose-response curve (Fig. 7B) the maximal current activated by MDMA was 0.17 ± 0.03 (n = 8) of that induced by 1 mM ACh. The current activated by MDMA was completely but reversibly antagonized by pancuronium, a competitive inhibitor of nAChR channels (Löwenick et al., 2001) (Fig. 7C).
Chemical Analysis of Ecstasy Pills and MDMA Serum Levels. Of the 30 ecstasy pill samples confiscated by the police, free MDMA base content varied from 0.4 to 62.5%, with 25.5% being the mean MDMA base content. Three samples were found to be supplemented with caffeine. Of the 54 non-MDMA samples, the mean pure drug content of other amphetamine derivatives was 9.8%, and six were mixed with caffeine. MDMA levels of ecstasy users varied from 0.06 to 2.28 mg/l (0.3–11.7 μM) in 35 serum samples and were on average 0.40 mg/l (0.1 μM). MDMA has a clearance of 12 h and a distribution volume of 4 l/kg. Peak concentration in muscle tissue may reach higher levels than in serum, especially if there is increased muscle perfusion (e.g., dancing). After intravenous administration of 1 to 2 mg/kg SCh during intubation, peak concentrations of SCh have been estimated to be 20 mg/l (100 μM). Because of the hydrophilic properties of SCh, muscular concentration may be somewhat lower (Hoshi et al., 1993).

Discussion

Actions of MDMA on Skeletal Muscle. Ecstasy street preparations often contain several other amphetamine derivatives in addition to MDMA as well as paracetamol and caffeine (Milroy et al., 1996; O’Connell and Heffron, 2000). In our analysis, about 10% of all pills (ecstasy and other amphetamines) were supplemented with caffeine, which acts as adenosine receptor antagonist and phosphodiesterase inhib-
formed at a holding potential of -40 mV. This may aggravate not only the stimulatory central but also the peripheral effects. This was obvious in our in vitro tests on normal adult skeletal muscle: although the highest concentration (1000 μM) was not able to induce a contracture by its own, the lowest [MDMA] used in our study, 100 μM, regularly increased its sensitivity for caffeine-induced contractures. This observation may be of clinical relevance because MDMA is often ingested in combination with fashion drinks (“energy drinks”), which contain up to 1 mg/ml caffeine. MDMA applied to cultured muscle cells induced intracellular Ca^{2+} peaks and acidification signals. The prevention of these effects by α-Bgt, which binds with a high specificity and high affinity to the endplate, points to a direct interaction of MDMA with nAChR. The agonistic effect was verified by current measurements on HEK293 cells expressing αβδε nAChR. Interestingly, a curare-like block by amphetamine overdosage was identified to cause paralysis (Skau and Ger- ald, 1978; Liu et al., 2003), pointing to the same target. In contrast to the effects of amphetamine, weakness has not yet been reported for illicit ecstasy users.

Both MDMA’s inability to induce contractures on its own and its potentiating effect on caffeine- and halothane-induced contractures resemble the in vitro action of α-Bgt (Galloway and Denborough, 1986; Ørding and Skovgaard, 1987; this study), one of the classical nAChR activators. Hence, we conclude that skeletal muscle is a target for MDMA outside the central nervous system that is affected via the nAChR of the neuromuscular junction. Mostly, strips taken from the quadriceps muscle do not contain an endplate. Nevertheless, MDMA can exert its activating effect on extrajunctional nAChR. Although the receptors of the adult type are less dense than at the endplate, they are frequent enough to produce measurable macroscopic currents (Koltgen and Franke, 1992). Also, primary myotube cultures express nAChR and their characteristics resemble the embryonic type (Lorenzon et al., 2002).

**Are MHS Individuals Predisposed to Adverse MDMA Effects?** Some of the clinical features such as metabolic acidosis, hyperkalemia, CK elevation, hyperthermia, and rhabdomyolysis in illicit MDMA users (Henry, 1992; Screaton et al., 1992; Hall et al., 1996) could be the result of a primary damage of skeletal muscle, e.g., MH (O’Leary et al., 2001). The response of such adverse MDMA effects to the MH antidote dantrolene supported this view (Hall et al., 1996), but the beneficial effects such as reduced myoplasmic Ca^{2+} and heat production are not restricted to MH crises (Hadad et al., 2005). We have tested the effects of MDMA on MHS muscle: None of the MHS muscles exhibited an in vitro contracture at 100 μM MDMA. This corresponds approximately to the highest MDMA serum value ever reported for an ecstasy user (De Letter et al., 2004), whereas the usual MDMA serum concentration ranged from 0.5 to 6 μM (Henry et al., 1992; this study). MDMA caused a smaller left-shift of the dose-response curve for caffeine- and halothane-induced contractures in MHS than in MHN muscle. Thus, MDMA increased the sensitivity of MHN muscle more than that of MHS. However, muscle bundles from three of eight MHS patients developed a contracture after exposure to 500 μM (n = 1), 1000 μM (n = 1), or 2000 μM (n = 1) MDMA. The smaller sensitivity increase for MHS than MHN muscle, the inconsistency, and the required high concentrations of MDMA-induced contractures do not support the view that muscle of MHS individuals is more sensitive to adverse MDMA reactions than normal muscle. Furthermore, we have excluded an MDMA-induced facilitation for the release of Ca^{2+} from the SR, the pathogenetic mechanism of MH (MacLennan and Phillips, 1992). To our knowledge there is no evidence that Sch triggers MH crises in humans in the absence of volatile anesthetics (Klingler et al., 2005).

In contrast to our results on excised human muscle, in vivo experiments on MHS pigs showed that intravenously injected MDMA (8–12 mg/kg b.wt. corresponding to a serum concentration of ~7 μM) caused hyperthermia, acidosis and rhabdomyolysis (Fiege et al., 2003). Since MDMA leads to a serotonergic overstimulation, the MH-like symptoms in the pigs could be explained by a central serotonin syndrome (Gerbershagen et al., 2003).

Taking into account the central stimulation that activates the motoneuron system, an additional direct effect on nAChR may lead to relevant muscle symptoms even at low doses of MDMA. Since muscle fasciculations, muscle cramps, and muscle ache also have been typically reported by illicit MDMA users (Henry, 1992; Screaton et al., 1992; Hall et al., 1996), the underlying cause should be proximal to muscle, that means at the neuromuscular junction. nAChR are present in both the postsynaptic and at presynaptic parts, and from the latter, electric activity can spread out in retrograde direction along the motor unit and cause fasciculations and cramps (Guiloff and Modarres-Sadeghi, 1992). And all other muscle-related symptoms and signs of MDMA ingestion could be the result of postsynaptic nAChR stimulation, e.g., hyperkalemia, metabolic acidosis, muscle fiber swelling, and rhabdomyolysis could be simply explained by maldistribution of electrolytes due to K^{+} efflux and Na^{+} and Ca^{2+}
influX through junctional and extrajunctional nAChR (Klinger et al., 2005).

**Susceptibility to Adverse MDMA Reactions.** MDMA may cause adverse effects in muscles abnormally reacting to nicotinic drugs. Particularly gross muscle with a high density of extrajunctional nAChR will be predisposed: generalized denervation processes such as in spinal muscular atrophies (Martyn et al., 1992; Lefebvre et al., 1998) or generalized muscle regeneration such as in progressive muscular dystrophies (Breucking et al., 2000; Gattenloher et al., 2002). Ingestion of ecstasy also may be hazardous in patients with myotonic syndromes because the hyperexcitable muscle fiber membrane increases the effect of nicotinic agents on muscle (Lehmann-Horn and Jurkat-Rott, 1999).

In summary, MDMA 1) resembles the in vitro action of SCh on skeletal muscle strips, 2) induces Ca^{2+} transients in myotubes that are inhibited by α-Bgt, 3) increases acidification and metabolism in myotubes, 4) has no effect on SR vesicles, and 5) activates nAChR dose dependently. Thus, the nAChR of the neuromuscular junction is one of the physiological targets of MDMA. This result was highly unexpected, although MDMA was reported to exert cholinergic effects in addition to serotonergic, dopaminergic, adrenergic, and histaminergic actions (Battaglia and De Souza, 1989; Crespi et al., 1997; Mechan et al., 2002; Gerbershagen et al., 2003).

However, these cholinergic effects were ascribed to the muscarinic receptor types that are highly different from the nAChR at the neuromuscular junction. Our report draws attention to adverse ecstasy reactions particularly in patients with a generalized up-regulation of extrajunctional nAChR such as in some hereditary neuromuscular diseases. Retro- and prospective clinical observations will help reveal these potential pharmacogenetic reactions.

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