

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

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Received March 21, 2005; accepted June 2, 2005

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 μ M 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. Further-

more, MDMA did not release Ca^{2+} from isolated sarcoplasmic reticulum vesicles. These findings do not support the view of an MH-triggering effect on muscle. However, MDMA induced Ca^{2+} 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 $[Ca^{2+}]$ 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; Mehan 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,

Our network on excitation-contraction coupling and calcium signaling in health and disease was supported by European Community HPRN-CT-2002-00331 (Ulm, Germany), Improving Human Potential Programs, and the Southern Health Board (Cork, Ireland). This research was presented in part at the 22nd Meeting of the European Malignant Hyperthermia Group, 2003 June 11-14; Brunnen, Switzerland.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.105.086629.

ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; CK, creatine kinase; SCh, succinylcholine; MH, malignant hyperthermia; MHS, malignant hyperthermia-susceptible; IVCT, in vitro contracture test; GC, gas chromatograph; TFA, trifluoroacetic anhydride; MHN, malignant hyperthermia-negative; RyR1, ryanodine receptor type 1; SR, sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEK, human embryonic kidney; nAChR, nicotinic acetylcholine receptor(s); α -Bgt, α -bungarotoxin; ACh, acetylcholine.

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 $[Ca^{2+}]$ 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 α -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 muscle strips 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 committees 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_5 -MDMA) as internal standard before solid-phase extraction (HX 11; Separtis, Grenzach-Wyhlen, Germany). Homogenized ecstasy pills were extracted with methanol. Both types of extracts were derivatized with trifluoroacetic anhydride (TFA) and evaporized (60°C; 30 min). The residues were dissolved in ethyl acetate for GC/mass spectrometry analysis performed on a Hewlett Packard 5890 GC interfaced with a Hewlett Packard 5971 mass selective detector. The carrier gas was helium. Injector and detector temperatures were 250 and 280°C, respectively. Oven temperature was set at 100°C, held for 2 min, and then increased to 280°C at 15°C/min and maintained for 1 min. The total run time was 15 min. Data were acquired with a Hewlett Packard G1034C MS ChemStation. The TFA derivatives were identified with the following masses: 135, 154, and 162 for TFA-MDMA; and 158 and 164 for TFA- d_5 -MDMA. In addition, caffeine was qualitatively determined.

Contracture Measurements. The IVCT was performed according to the European protocol (Ørding et al., 1997). This test determines the sensitivity of fresh muscle strips separately exposed to caffeine and halothane at cumulative concentrations in an organ bath containing Krebs-Ringer solution (118 mM NaCl, 3.4 mM KCl, 0.8 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 11.1 mM glucose, 25.0 mM $NaHCO_3$, and 2.5 mM $CaCl_2$, pH 7.4). Muscle strip force was measured with a mechano-electrical transducer (FT03; Grass Instru-

ments, Quincy, MA). An increase in force by active shortening induced by drugs was considered as a contracture if a threshold value of ≥ 2 mN was reached. According to the protocol, contractures were considered as pathological if they occurred at concentrations of ≤ 2 mM caffeine or $\leq 2\%$ halothane. Patients with pathological contractures to both substances were classified as MHS, individuals whose strips pathologically reacted to only one test reagent were classified as MH-equivocal, and the absence of pathological contractures was classified as MH-negative (MHN). Muscle strips were considered as viable if their twitch amplitudes to supramaximal electrical stimulation (pulse frequency, 0.2 Hz; pulse width, 1 ms) were >10 mN.

The effects of MDMA and SCh were also tested on muscle strips, either alone or in combination with caffeine or halothane. In this case, MDMA or SCh were added to the organ baths 10 min before the caffeine/halothane challenge. Shifts in the dose-response curves for caffeine or halothane in the presence of MDMA or SCh were determined as the difference between the lowest concentration (e.g., with and without MDMA) at which contractures of ≥ 2 mN occurred. This is in contrast to the usual chemical procedure at which concentrations leading to half-maximum contractures are determined and subtracted from each other. The reason for the different protocol is the experience that application of concentrations that usually cause maximum contractures often results in rupture at the tied ends of the muscle fiber segments. The disadvantage of the European protocol is that a shift of the steepness of the dose-response relationship is not taken into account for determination. Here, we compensate for this lack by comparing contracture amplitudes as shown in Fig. 3B.

Stock solutions of caffeine (100 mM) and MDMA (10 mM) were prepared in double distilled water. Halothane was purchased from Zeneca (Plankstadt, Germany) and applied by a vaporizer (Vapor 19.1; Draeger, Lübeck, Germany). Caffeine was purchased from Merck (Darmstadt, Germany), MDMA and ryanodine were from Sigma Chemie (Deisenhofen, 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^{2+} release 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/ml) (C6885; Sigma Chemie) and dissolved 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-L-ornithine-coated glass coverslips. Cells were first kept in growth medium containing 5% fetal calf serum (C-23060; PromoCell, Heidelberg, Germany) at 37°C and 5% CO_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^{2+} Fluorometry. Changes of intracellular $[Ca^{2+}]$ 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 $MgCl_2$, 6.0 mM glucose, 1.5 mM $CaCl_2$, 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^{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 dye 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. Ca^{2+} 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 (Klingler et al., 2002). Briefly, the cells in the measuring chamber were superfused (37°C; 50 $\mu\text{l}/\text{min}$) 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 osmolarity 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 decreased) because protons that were extruded by the cells accumulated in the chamber until the pump was switched on again. The slope of the pH decrease gives the acidification rate corresponding to the metabolic activation of the myotubes. Pump cycle control, data acquisition, and rate calculation were carried out with the system's microcomputer (Macintosh Power PC 7600/132) and the Cytosoft program supplied with it (Molecular Devices, San Diego, CA).

Whole-Cell Patch-Clamp Recordings. Transfected 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 MgCl_2 . HEK293 cells were superfused with an extracellular solution containing 162 mM NaCl, 5.3 mM KCl, 2 mM CaCl_2 , 0.67 mM NaH_2PO_4 , 0.22 mM KH_2PO_4 , 15 mM HEPES, and 5.6 mM glucose. The pH of both solutions was adjusted to 7.3. MDMA and pancuronium were obtained from Sigma Chemie. Data were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc., Union City, CA). Membrane currents were sampled with 20 kHz using a Digidata 1200 Interface and the pCLAMP6 software suite on a PC (Axon Instruments Inc.). Data were filtered at 5 kHz for further analysis. The holding potential of the cells was kept at -40 mV. A piezo-driven, double-barreled ultrafast perfusion system was used for application of the agents to excised outside-out membrane patches or small cells. The time for solution exchange was regularly <100 μs (Krampfl et al., 2002). For the quantitative evaluation, four to 12 current traces were averaged for each experiment.

Statistical Analysis. Values showing normal distribution are presented as means and S.D. if not indicated otherwise. Values without normal distribution and discrete data are given as median, and 95% percentiles as indicated. The significance of differences

between groups was evaluated by use of Wilcoxon matched pairs signed rank tests. *P* values less than 0.05 are considered significant.

Results

MH Classification of the Patients and Genetic Results. Of our 17 samples, eight individuals were MHS, eight individuals were MHN and served as controls, and one person was classified as MH-equivocal. For genetic confirmation of the IVCT, mutation screening in RyR1 was performed. Known hot-spot RyR1 mutations were identified in four of the eight MHS patients (R614C and G2434R, each in a single patient, and T2206M in two patients) and none in the others (MHN and MH-equivocal).

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 $\geq 500 \mu\text{M}$ 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 100 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 evaluation 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 $[\text{Ca}^{2+}]$ exceeding the mechanical threshold. Caffeine and halothane are known to raise myoplasmic $[\text{Ca}^{2+}]$ (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+}

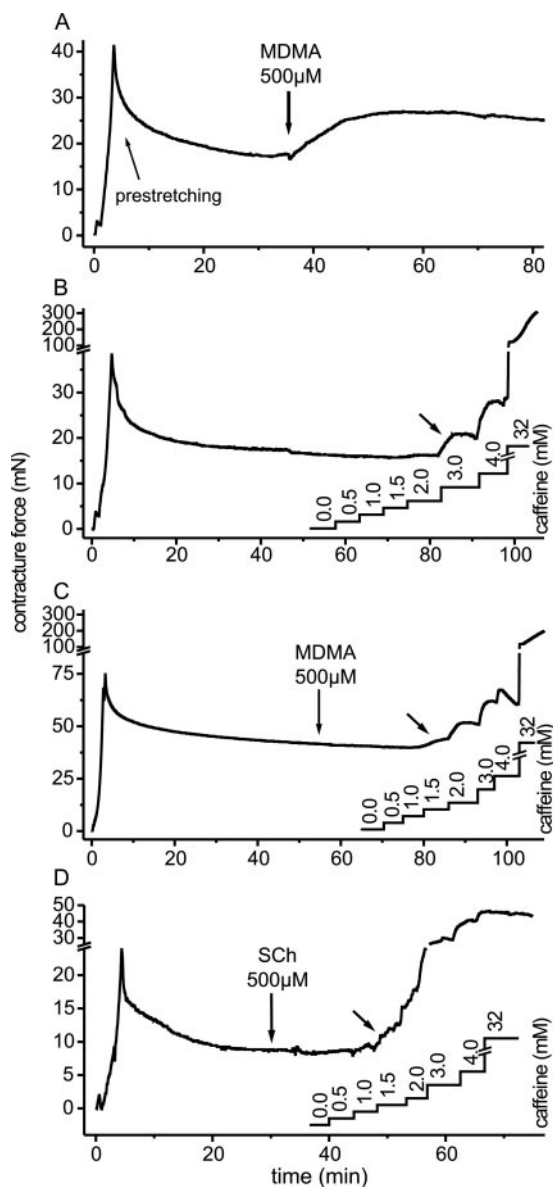


Fig. 1. Effects of MDMA and Sch on the caffeine dose-response curve. Original recordings of basal force and contractures of MHS and MHN muscle strips over time. The initial peaks reflect the passive prestretching of the strips from which they relax to the range of optimal alignment of the contractile filaments. Drug-induced contractures develop from this basal force. A, muscle strip of an MHS patient developed a 9-mN contracture after addition of 500 μ M MDMA to the bath solution. The MHS classification resulted from the IVCT performed on additional strips from this patient who was later identified to carry the frequent RyR1 mutation R614C. B, recording shows the European IVCT protocol for caffeine. Note that the muscle strip developed a contracture (defined as increase in force by ≥ 2 mN) at 3 mM caffeine corresponding to a normal muscle reaction. C, presence of 500 μ M MDMA shifted the reaction to a caffeine concentration of 1.5 mM in another muscle strip of the same MHN individual. This shift was much greater than the interstrip variability at a given concentration. D, 500 μ M Sch had the same effect as MDMA in C.

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 μ M; 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 $[Ca^{2+}]_i$, can be studied by measuring cell proton

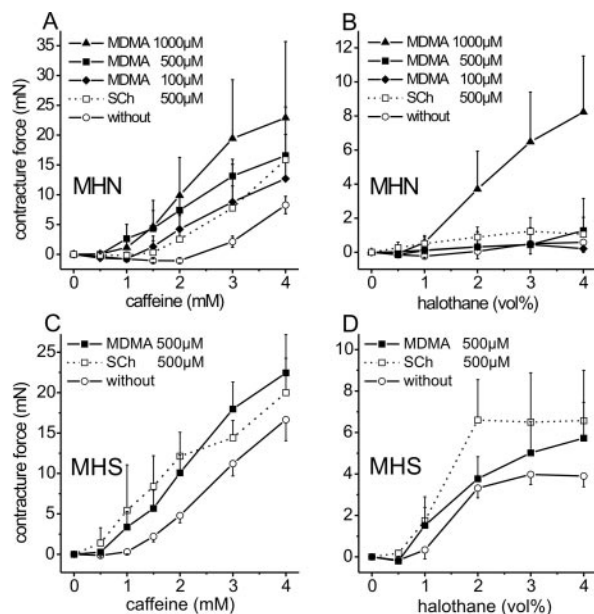


Fig. 2. Left-shift of dose-response curves by MDMA and Sch. Dose-response relationships are given for muscle strips from individuals with MHN (A and B) and MHS IVCT results (C and D). The test was performed with caffeine (A and C) and halothane (B and D) on different strips. One hundred to 1000 μ M MDMA or 500 μ M Sch was added to the organ baths 10 min before the caffeine/halothane challenge. Both MDMA and Sch shifted the curves to the left. According to the European protocol (Ørding et al., 1997), this shift was determined at the 2-mN level. Each curve is calculated from five to seven strips. Error bars represent S.D.

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 exposition of myotubes cultured on micronets from three MHN individuals to 500 μ M 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 Ca^{2+} from the terminal cisternae of the SR, the most important myoplasmic Ca^{2+} store. We measured the effects of up to 170 μ M ryanodine, 1200 μ M halothane, 1000 μ M Sch, and 700 μ M MDMA on Ca^{2+} flux rates. In this preparation, which was taken from rat muscle, 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 μ M 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).

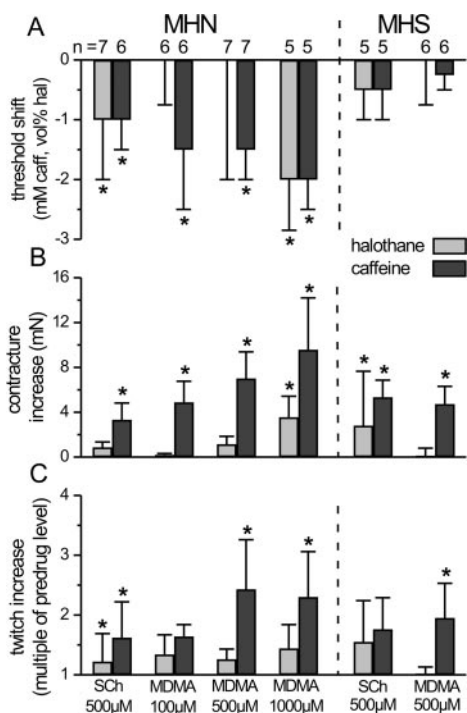


Fig. 3. Evaluation of changes induced by MDMA and SCh. MDMA and SCh lowered the caffeine and halothane concentrations that triggered a contracture (A), increased contracture (B), and twitch force amplitudes (C). A, concentration shifts were determined according to the European protocol, i.e., the lowest concentrations (e.g., with and without MDMA) at which contractures of ≥ 2 mN occurred were determined and the difference was made. Histograms indicate median values and bars 95% percentiles. B and C, amplitudes measured at 2 mM caffeine and 2% halothane, respectively, were evaluated. Each result was individually compared with the corresponding bundle without MDMA or SCh. Wilcoxon matched pairs signed rank test was performed and significant differences ($p < 0.05$) are labeled by asterisks. The number of muscle strips is also given in the diagram.

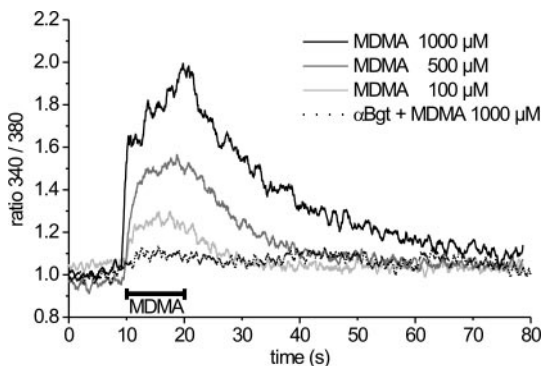


Fig. 4. MDMA-induced Ca^{2+} transients. This graph shows representative traces of measurements on a single myotube. The phasic fluorescence signals were induced by various MDMA concentrations added to the bath solution. The 340 nm/380 nm ratio indicated the averaged $[\text{Ca}^{2+}]$ signals from microscopically selected areas of 40 myotubes from four MHN individuals.

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

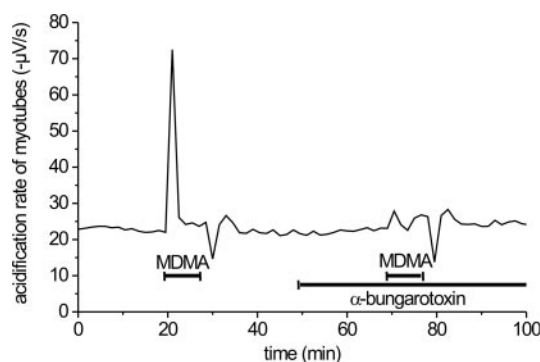


Fig. 5. MDMA-induced acidification. The metabolic activity of cells is given by the proton production per time. MDMA strongly activated the acidification rate of myotubes (600 μ V; ~ 0.1 pH units). The signal consisted of a rapid up- and down-stroke followed by a plateau. The washout was followed by a transient change of the acidification rate. α -Bgt (0.1 μ M) drastically reduced the MDMA-induced activation. This example is one of three MHN preparations tested. A signal from about 30 to 50 myotubes in the proton-sensitive part of the experimental chamber is displayed.

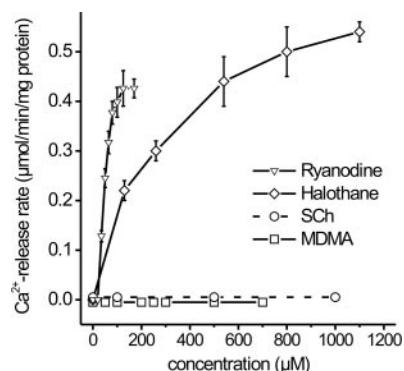


Fig. 6. Ca^{2+} release from SR vesicles. Rat muscle heavy SR was preloaded maximally with Ca^{2+} before stimulation of release with ryanodine, halothane, MDMA, or SCh. This current is conducted by the Ca^{2+} release channel of skeletal muscle, the RyR1, in which many mutations are causative for MH susceptibility in human and several animals. Note the much higher affinity of ryanodine to RyR1 than that of halothane. In contrast to ryanodine and halothane, MDMA and SCh did not release Ca^{2+} from SR vesicles. Each value is mean \pm standard error from three to five separate muscle preparations. Each determination was carried out in triplicate.

2.28 mg/l (0.3–11.7 μ M) in 35 serum samples and were on average 0.40 mg/l (~ 2 μ M). 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 μ M). 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-

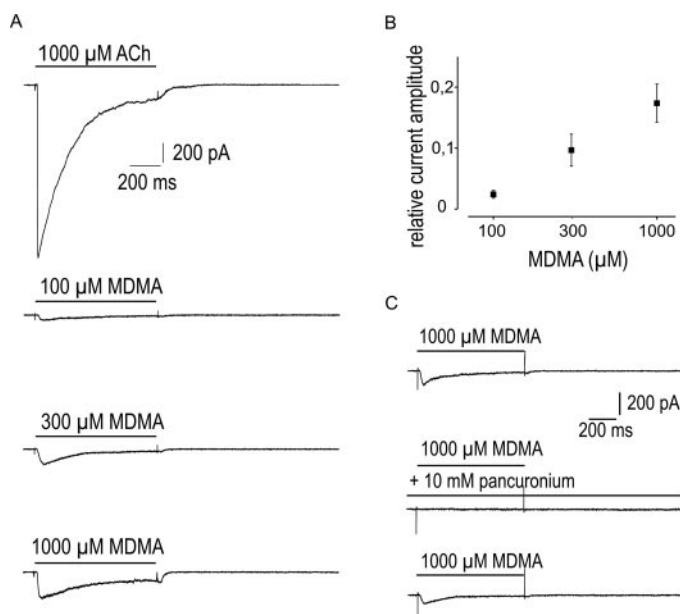


Fig. 7. Agonistic effect of MDMA at nAChR channels. Agonistic effect of MDMA at nAChR channels shown by patch clamp experiments with fast application of the agonists to recombinant adult-type nAChR channels expressed from HEK293 cells. A, average currents activated by 1000 μM ACh (top) and different concentrations of MDMA (bottom). B, dose-response curves of the MDMA-activated currents (100 μM , $n = 12$; 300 μM , $n = 6$; and 1000 μM , $n = 8$). C, complete and reversible inhibition of the agonistic effect of MDMA by pancuronium. All experiments were performed at a holding potential of -40 mV.

itor. 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 $\alpha_2\beta\delta\epsilon$ nAChR. Interestingly, a curare-like block by amphetamine overdosage was identified to cause paralysis (Skau and Gerald, 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 Sch (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; Sreaton 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; Sreaton 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 (Klingler 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; Gattenlohner 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 Ca²⁺ 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; Mehan 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.

Acknowledgments

We thank Drs. Reinhardt Rüdél, Karl Föhr, and Michael Gschwend for support on Ca²⁺ experiments and discussion; Dr. Christoph Baur for patient documentation; and Ursula Mohr and Erhard Schoch for technical assistance (all Ulm University).

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