Depolarization Initiates Phasic Acetylcholine Release by Relief of a Tonic Block Imposed by Presynaptic M₂ Muscarinic Receptors

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Parnas, H., I. Slutsky, G. Rashkovan, I. Silman, J. Wess, and I. Parnas. Depolarization initiates phasic acetylcholine release by relief of a tonic block imposed by presynaptic M₂ muscarinic receptors. J Neurophysiol 93: 3257–3269, 2005. First published February 9, 2005; doi:10.1152/jn.01131.2004. The role of presynaptic muscarinic autoreceptors in the initiation of phasic acetylcholine (ACh) release at frog and mouse neuromuscular junctions was studied by measuring the dependency of the amount (m) of ACh release on the level of presynaptic depolarization. Addition of methoctramine (a blocker of M₂ muscarinic receptors), or of acetylcholinesterase (ACHE), increased release in a voltage-dependent manner; enhancement of release declined as the depolarizing pulse amplitude increased. In frogs and wild-type mice the slope of log m/log pulse amplitude (PA) was reduced from about 7 in the control to about 4 in the presence of methoctramine or AChE. In M₂ muscarinic receptor knockout mice, the slope of log m/log PA was much smaller (about 4) and was not further reduced by addition of either methoctramine or AChE. The effect of a brief (0.1 ms), but strong (≈ 1.2 µA) depolarizing prepulse on the dependency of m on PA was also studied. The depolarizing prepulse had effects similar to those of methoctramine and AChE. In particular, it enhanced release of test pulses in a voltage-dependent manner and reduced the slope of log m/log PA from about 7 to about 4. Methoctramine + AChE occluded the prepulse effects. In knockout mice, the depolarizing prepulse had no effects. The cumulative results suggest that initiation of phasic ACh release is achieved by depolarization-mediated relief of a tonic block imposed by presynaptic M₂ muscarinic receptors.

INTRODUCTION

Presynaptic G-protein–coupled receptors (GPCRs) are known to modulate neurotransmitter release by various mechanisms (MacDermott et al. 1999), one of which involves direct modulation of proteins of the release machinery (for example, Blackmer et al. 2001; Capogna et al. 1996; Scholz and Miller 1992; Silinsky 1984; Trudeau et al. 1996; for review see Miller 1998).

We recently proposed that control of the time course of neurotransmitter release, both of initiation and termination, is achieved by a direct effect of presynaptic inhibitory autoreceptors on key proteins of the release machinery (Parnas et al. 2000). In particular, we proposed that at rest (i.e., at resting potential and resting low level of transmitter in the cleft) the release machinery is under tonic block. The block is achieved as a result of an interaction of the transmitter-occupied inhibitory autoreceptor with the core proteins of the release machin-
manipulations known to affect Ca$^{2+}$ entry and removal. In contrast, in M$_2$R knockout mice, these same experimental manipulations significantly altered the time course of ACh release; release was briefer when Ca$^{2+}$ removal was accelerated and the duration of release was prolonged when more Ca$^{2+}$ had entered (Slutsky et al. 2003). Furthermore, it was shown that these changes in the time course of release were not the result of changes in Ca$^{2+}$-current kinetics (Slutsky et al. 2003). These results were interpreted by Slutsky et al. (2003) as meaning that in the M$_2$R knockout mice, in which the tonic block by the receptor cannot be imposed, the release machinery is permanently free. Thus the time course of ACh release is determined by the second limiting factor, that is, influx and removal of Ca$^{2+}$, whereas in WT mice it is determined by the M$_2$R. This interpretation is further supported by data showing that release in M$_2$R knockout mice starts earlier and lasts longer than that in WT mice (Slutsky et al. 2003).

Here we subject our hypothesis concerning initiation of release to a further test. Specifically, we examine whether depolarization indeed plays 2 roles in the initiation of phasic release: opening of voltage-gated Ca$^{2+}$ channels and relief of a tonic block imposed by the transmitter-occupied inhibitory autoreceptor M$_2$R in the preparations studied. To reveal this additional role of depolarization, the terminal was depolarized to different levels; the relationship between quantal content and the depolarizing pulse amplitude (PA) was then measured under control conditions and under conditions in which the M$_2$R-imposed tonic block had been drastically reduced or completely abolished.

The rationale underlying these experiments is as follows: One of the means of establishing the role of Ca$^{2+}$ in promoting release has been to measure, at constant depolarization, the relationship between the quantal content $m$ and Ca$^{2+}$ ([Ca$^{2+}]_o$, Dodge and Rahamimoff 1967; $I_{Ca}$, Augustine et al. 1985; [Ca$^{2+}]_o$, Bollmann et al. 2000; Raven et al. 1999; Schneggenburger and Neher 2000). The log/log plot of quantal content versus [Ca$^{2+}]_o$ had a slope of about 4, indicating that 4 Ca$^{2+}$ ions are required for the release of one quantum (Dodge and Rahamimoff 1967). To establish the role of depolarization in promoting release, in the experiments below we measure the dependency of $m$ on depolarization at fixed [Ca$^{2+}]_o$. If depolarization indeed plays the above-mentioned 2 roles in promoting neurotransmitter release then this relationship, expressed as the maximal slope of log $m$ versus log PA, should reflect the quantitative contributions of both roles of depolarization, that is, in Ca$^{2+}$ entry and in relief of the tonic block. It is thus predicted that the slope of log $m$/log PA should be reduced when the additional role of depolarization, relieving the tonic block, is abolished. It should then reflect only the role of depolarization in permitting Ca$^{2+}$ influx.

Such experiments were performed both on the frog neuromuscular junction (nmj) and on those of wild-type and M$_2$R knockout mice, and the predictions made above were confirmed.

**METHODS**

**Preparations and solutions**

**FROGS.** Frogs (*Rana ridibunda*) were killed by stunning and double pitting in accordance with institutional guidelines and the Israel animal protection law. The cutaneous pectoris neuromuscular preparation was isolated and pinned in a chamber with a Sylgard bottom and shallow walls (0.4 × 1.5 × 4 cm$^3$). The chamber was secured on the stage of an upright microscope (Axioskope, Zeiss), which was modified to hold the micromanipulators also. The chamber was continuously perfused (Gilson Minipulse 3 pump) with a bathing solution that passed through a cooling device. The temperature was controlled at 8–10 ± 1°C. The standard Ringer solution contained (in mM): NaCl, 116; KCl, 2.5; MgCl$_2$, 1; CaCl$_2$, 3; HEPES, 10; glucose, 8, bubbled with 95% O$_2$-5% CO$_2$. The pH was adjusted to 7.4 with NaOH. (Small changes in CaCl$_2$ or MgCl$_2$ were not compensated for.)

**MICE.** M$_2$R knockout mice (M$_2$-KO) (Gomez et al. 1999), 1.5–3 mo of age, were used. They had a mixed genetic background (129/J X CF-1; 50%/50%). Age-matched wild-type (WT) mice of the same genetic background served as controls. Mouse colonies were amplified at Taconic Farms (Germantown, NY). Mice were anesthetized with CO$_2$ and decapitated, in accordance with institutional guidelines and the Israel animal protection law. Hemidiaphragm neuromuscular preparations were isolated and pinned in the chamber described above. The standard bathing solution contained (in mM): NaCl, 160; KCl, 2.5; MgCl$_2$, 1; CaCl$_2$, 3; HEPES, 10; glucose, 8, bubbled with 95% O$_2$-5% CO$_2$. The temperature (30 ± 1°C) was maintained by circulating (Gilson Minipulse 3 pump) the fluid through a heat exchanger. The pH was adjusted to 7.4 with NaOH.

**Stimulation and recording**

The macropatch technique (Dudel 1981) was used for local depolarization of a small region of the terminal and for concomitant recording of single quanta events. With this technique depolarization is produced by shifting the extracellular potential to more negative values. This is done by passing constant negative current pulses, which can vary in amplitude and duration, through the macropatch electrode. The seal resistance (180–200 kΩ) determines the maximal possible depolarization of the membrane of the terminal. The time constant of the depolarization thus produced does not depend on the resistance–capacitance (RC) of the membrane, but rather on the time constant of the amplifier (10 kHz, 0.1 ms); thus for brief current pulses of 0.1 ms, the shift in external potential is truncated (Dudel 1981).

The macropatch technique suffers from a major drawback, inasmuch as the depolarization level is not known. The current that passes through the electrode is shunted and not all of it reaches the extra-cellular space around the terminal. One way to roughly estimate the level of depolarization is to measure what level of current pulse produces a similar quantal content to that produced by an action potential. However, the amplitude and duration of the action potential at the nerve terminal are not known. Nevertheless, because the frog nerve terminal is excitable (Katz and Miledi 1965a), we estimate the duration of the action potential and its amplitude to be 1–1.2 ms and 100 mV, respectively. At the same recording site [after addition of 0.2 μM tetrodotoxin (TTX)] we applied current pulses of 1–1.2 ms duration and varying amplitudes. In the example given in Fig. 1, the quantal content (m) for nerve stimulation (NS) was 0.66. A pulse of 1.2 ms and −0.5 μA [direct stimulation (DS)] produced a similar quantal content (m = 0.68). In 2 additional experiments, for a pulse of 1 ms, −0.7 μA was required to produce release similar to that of an action potential. Because the seal resistance was 200 kΩ the maximal possible depolarization for the 3 experiments was 100–140 mV. Thus there must be some shunting (≤40%) of the current pulse. Qualitatively, we can assume that larger current pulses produce stronger depolarization and greater release (Fig. 2). (For further technical details see Dudel 1981; Raven et al. 1997; see also Katz and Miledi 1965b.)

To visualize the very fine axon terminals, a long-distance objective (×40) with 1.8-mm working distance was used. This necessitated...
horizontal positioning of the macropatch electrode (Ravin et al. 1997). The fire-polished tip (about 8 μm) was slightly bent to allow positioning of the macropatch electrode over a branch of the endplate in the small space between the objective and the preparation. Pulse duration was 0.3 ms for mice and 0.7 ms for frogs, pulse amplitude usually varied between 0.3 and 0.7 A, and frequency of stimulation was 1–3 Hz. TTX (0.2 μM) was present in the solution to prevent sodium excitability, thus permitting graded depolarization of the axon terminal (Dudel 1981).

**Determination of quantal content**

At 8–10°C (frog experiments), and even at 30°C (mouse experiments), the quanta appeared after the stimulus artifact, and could be easily discerned and counted even when 2 or 3 quanta were released concomitantly (Figs. 1 and 2). Figure 2 shows, for the mouse nmj, that for any pulse, irrespective of its amplitude, the number of quanta released after each depolarizing pulse varies. Furthermore, for a pulse of a constant duration (0.4 ms), the quantal content increased with current pulse amplitude. To determine the quantal content, the quanta were counted for a period of 10 ms after the beginning of each depolarization pulse (2,000 pulses for m <0.25 and 512 for m >0.3). The total number of quanta divided by the number of applied pulses yields the quantal content (the average number of quanta released per pulse). Such a procedure was used for several pulse amplitudes (given randomly) at the same recording site (Fig. 2). The number of spontaneously released quanta was counted for the period starting 10 ms after the pulse until the following pulse. The number of spontaneous releases varied in the various preparations and was higher in M$_2$-KO mice than that in WT mice. It also varied according to the experimental conditions. However, in all cases the probability of spontaneous release occurring during the period of 10 ms after each pulse was <0.0001 (2–3 quanta for 200 s), precluding the need to subtract spontaneous from evoked release. In the experiment depicted in Fig. 2, 4 pulse amplitudes, given in a random manner, were used. The quantal content for each pulse amplitude is given below each column of traces (m was determined for 2,000 pulses). Figure 2B shows that the slope of log m/log PA was 6.8. Current traces were digitized using a Neurodata (Neuro-Corder DR-484) A/D converter at 50 kHz and transferred to a Pentium III-500 computer using a Labview (AT-MIO-16F-5, NIDAQ 4.9.0 driver software) interface.

**Preparation of acetylcholinesterase (AChE)**

The G$_2$ dimeric form of AChE was purified from *Torpedo californica* electric organ by affinity chromatography, after solubilization with phosphatidylinositol-specific phospholipase C (Futerman et al. 1985). Its specific activity was about 3,000 units per mg protein, one unit corresponding to hydrolysis of 1 μmol min$^{-1}$ of acetylthiocholine, assayed according to Ellman et al. (1961) (see also Slutsky et al. 2001).
Statistical evaluation

Significance was checked by the Student’s paired (the same experiment) and unpaired (different experiments) 2-tail t-test. Results are given as means ± SD throughout.

Results

Methoctramine reduces the maximal slope of log m/log PA at the frog neuromuscular junction

We begin by testing whether depolarization does indeed play 2 roles in initiation of phasic release, that is, opening of voltage-gated Ca²⁺ channels and relief of a tonic block of release imposed by ACh-occupied M₄R. To do so, we compared the dependency of the quantal content (m) on depolarizing pulse amplitude (PA) at fixed [Ca²⁺]₀ (expressed as the slope of log m/log PA), both under control conditions and in the presence of the selective M₄R/M₅R antagonist methoctramine. As an M₄R antagonist, methoctramine is expected to reduce or even abolish, the M₄R-imposed tonic block of ACh release, resulting in reduction of the slope of log m/log PA.

Figure 3A shows the average results for 10 such experiments. Because both M₁R and M₄R are present at frog nerve terminals, and M₁R enhances release (Slutsky et al. 1999), all experiments were conducted in the presence of the M₁R antagonist pirenzepine (10 μM). First, the control quantal content was measured at several low-to-medium PAs administered in a random manner. After establishing the control curve (Fig. 3A, filled squares), methoctramine (1 μM) was applied and the experimental protocol was repeated. As shown earlier (Slutsky et al. 1999), methoctramine increased the quantal content in a voltage-dependent manner (Fig. 3A, open squares). At a low-pulse amplitude (−0.3 μA) methoctramine increased the quantal content by 251%, but at a larger amplitude (−0.5 μA), by only 3%. Thus the slope of log m/log PA declined in the presence of methoctramine (see slope values below). An M₄R agonist should compete with methoctramine for binding to M₄R, thus diminishing or abolishing its effect. Indeed, when 100 μM muscarine was added to the methoctramine, the curve relating m to PA was similar to that obtained in the control (Fig. 3A, open circles).

The control slope was 7.2 ± 0.2 and was reduced to 4.7 ± 0.3 (35% reduction, P < 0.0001) after addition of 1 μM methoctramine. The value recovered to 6.9 ± 0.4 (insignificant difference, P = 0.54) after the addition of 100 μM muscarine.

AChE also reduces the maximal slope of log m/log PA

Another way to diminish occupancy of the M₂R by ACh, and thereby to reduce the tonic inhibition, is to reduce the concentration of ACh in the synaptic cleft by adding the potent ACh-hydrolyzing enzyme AChE (Slutsky et al. 2001). Thus the experiments described in Fig. 3A were repeated, but in the presence of 80 μg/ml AChE (Fig. 3B). After establishing the control curve (Fig. 3B, average of 3 experiments, filled squares) AChE was added and 5 min later the dependency of m on PA was measured (Fig. 3B, open squares). AChE, similarly to methoctramine, increased the quantal content in a depolarization-dependent manner: an increase of 277% at −0.3 μA versus an increase of 1% at −0.45 μA. Thus AChE, like methoctramine, reduced the slope of log m/log PA (see following text). Again, addition of muscarine restored the slope to control values.

Specifically, the slope of log m/log PA was 7.0 ± 0.2 in the control (Fig. 3B, filled squares); it was reduced to 4.2 ± 0.3 after addition of AChE (Fig. 3B, open squares; extremely significant, P < 0.0001) and increased to the control slope (7.0 ± 0.44, P > 0.142; Fig. 3B, open circles) after subsequent application of muscarine.

Two initial conclusions may be drawn: 1) methoctramine and AChE indeed reduce a tonic block of release imposed by the M₄R; 2) high levels of depolarization completely relieve the tonic block. This second conclusion derives from the finding that neither methoctramine nor AChE produce elevation of release at high pulse amplitudes.

The slope of log m/log PA is higher in WT than in M₂-KO mice

Experiments similar to those shown in Fig. 3 were performed on diaphragm muscles taken from 2 mouse strains: wild-type (WT) mice possessing functional M₂R and knockout mice lacking functional M₂R (M₂-KO) (Gomeza et al. 1999; Slutsky et al. 2003). We predicted that in the M₂-KO mice, in which the M₄R is not functional, the release machinery should constantly be in a “free” state. Thus the additional role of depolarization in relieving the tonic block should not be observed and, consequently, the behavior of the M₂-KO mice should be similar to that obtained in both the frog and in WT mice in the presence of methoctramine or AChE. In particular, release should be higher in M₂-KO than in WT mice, but only at the low-pulse amplitudes and, consequently, the slope of log m/log PA should be lower in M₂-KO mice than in WT mice.

The data presented in Fig. 4 confirm this prediction. Figure 4 presents average quantal contents measured in WT (n = 13, Fig. 4A, filled squares) and in M₂-KO mice (n = 10, Fig. 4A, open squares) at 4 (low-to-medium) depolarizing pulse ampli-
The average slope of log m/log PA in WT mice was \( 7.47 \pm 0.56 \) (black column), declining to 4.35 \( \pm 0.36 \) (extremely significant, \( P < 0.0001 \)) in the presence of 1 \( \mu \)M methoctramine (\( n = 12 \)) (striped column). In M2-KO mice the control slope of 4.34 \( \pm 0.31 \) (\( n = 10 \)) remained unaltered after addition of methoctramine (4.3 \( \pm 0.21 \), \( P = 0.23 \), \( n = 8 \)) (striped column).

In an attempt to at least partially distinguish between the 2 possibilities, we took into account that a relatively long prepulse, in the range of a few milliseconds, is required for disinhibition of Ca\(^{2+}\) channels. Furthermore, the time constant of reinhibition on repolarization is also long, in the range of 50–100 ms (Arnot et al. 2000; Kasai 1992). We therefore tested whether a strong, but very brief depolarizing prepulse, that would not be likely to cause disinhibition of Ca\(^{2+}\) channels or to detachment of the release machinery from the M2R, or to both.

In frog, a strong and brief depolarizing prepulse increases test pulse (low-to-medium amplitude) release and reduces the slope of log m/log PA

The results presented thus far are compatible with the hypothesis according to which the agonist-occupied M2R imposes a tonic block, at resting potential, and that for initiation of ACh release to take place, this block must be alleviated. Furthermore, the reduction in the slope of log m/log PA on addition of methoctramine, or in the presence of AChE, is in accordance with the notion that it is strong depolarization that alleviates the M2R-imposed tonic block, thus permitting release of ACh to occur.

Because this last notion is our principal contention in the present study, we sought to substantiate the role of depolarization in relieving the block by an independent set of experiments. Slutsky et al. (1999, 2002) previously showed that the M2R-imposed inhibition of ACh release is Ca\(^{2+}\)-independent but voltage-dependent, where inhibition is strong at low depolarizations, declining as depolarization increased, and being completely abolished at high depolarizations. It follows that, if a depolarizing test pulse (of low-to-medium PA) is preceded by a strong depolarizing prepulse, it will produce release higher than a test pulse alone. This is because a depolarizing test pulse (of low or medium amplitude) administered shortly after a strong depolarization should encounter release machinery that had been “freed” by the strong depolarization that preceded the test pulse. We thus conducted experiments similar to those used to study the mechanism of membrane-delimited inhibition of Ca\(^{2+}\) channels mediated by G-protein–coupled receptors (GPCRs) (see, e.g., Kuo and Bean 1993; for reviews, see Arnot et al. 2000; Bertram and Behan 1999; Dolphin 1998; Hille 1994; Ikeda 1996; Jarvis et al. 2000; Zamponi and Snutch 1998). Accordingly, we compared release produced by a low test pulse alone with release when it was preceded by a strong depolarizing prepulse.

Considerations underlying choice of the experimental protocol for prepulse administration

A strong depolarization both relieves inhibited Ca\(^{2+}\) channels (see references above) and activates the proteins of the release machinery, either by dissociating them from M2R (Ilouz et al. 1999; Linial et al. 1997) or by an unknown Ca\(^{2+}\)-independent mechanism (Slutsky et al. 1999, 2002). Thus if we observe prepulse-mediated enhancement of release it may be attributable either to disinhibition of Ca\(^{2+}\) channels or to detachment of the release machinery from the M2R, or to both.

In an attempt to at least partially distinguish between the 2 possibilities, we took into account that a relatively long prepulse, in the range of a few milliseconds, is required for disinhibition of Ca\(^{2+}\) channels. Furthermore, the time constant of reinhibition on repolarization is also long, in the range of 50–100 ms (Arnot et al. 2000; Kasai 1992). We therefore tested whether a strong, but very brief depolarizing prepulse, that would not be likely to cause disinhibition of Ca\(^{2+}\) channels, would nevertheless enhance release by a subsequent test pulse.

Accordingly, the experiments shown in Figs. 3 and 4 were repeated, but with a strong and brief depolarizing prepulse (\( -1.2 \mu \)A, 0.1-ms duration, denoted hereafter as the “standard prepulse”) preceding the test pulse.

The alternating stimulus regime (Slutsky et al. 2003) was used. As a control, we randomly administered test pulses of various amplitudes, such that the control (no prepulse, Fig. 5A, middle column) and the experiments (with prepulse, Fig. 5A, right column) were given successively. This ensured that the quantal contents of all test pulses, with and without a prepulse, would be determined over the same period of time.

Before describing the prepulse experiments, the choice of the duration and amplitude of the prepulse, and of the interval between the pre- and test pulses, need to be considered. The prepulse should be very large but sufficiently brief so as not to produce release by itself. The shortest duration possible is 0.1 ms because the time constant of the amplifier is about 0.1 ms. Indeed, the rise time to peak current was found to be 95 \( \mu \)s. Concerning its amplitude, we can infer from Fig. 1 that a pulse of \(-0.5\) to \(-0.7 \mu \)A (1- to 1.2-ms duration) produces release...
As an action potential. Thus a pulse of $-1.2 \mu A$ (despite some truncation) will produce depolarization similar to, or larger than, that of an action potential. Indeed, using a prepulse of $-1.2 \mu A$, of 0.1-ms duration, we found that it alone did not produce any release (Fig. 5A, left column). Figure 5A shows samples of recordings from one site. Fifteen consecutive traces are shown without any selection. In the middle column a test pulse of $-0.5 \mu A$, 0.7 ms produced release with a quantal content of 0.2. When the test pulse was preceded by the prepulse (in this case with 1-ms delay), the same test pulse produced release with $m = 2.4$, a 12-fold increase (Fig. 5A, right column).

To obtain a maximal effect of the prepulse, it should ideally be administered with zero interval before the test pulse. Under such conditions, however, the prepulse should be considered as being composed of 2 components, each having a different effect on the test pulse. The first component, having the same amplitude as that of the test pulse (Fig. 5, B and C, inset, gray column), should not be expected to relieve the tonic block more than the test pulse itself (Slutsky et al. 1999; Yusim et al. 1999; Zamponi and Snutch 1998), but should increase $\text{Ca}^{2+}$ influx during the test pulse simply by increasing its duration. We denote this effect of the prepulse as the “prolongation” effect. The prepulse is indeed very brief (0.1 ms), but so is the test pulse (0.7 ms for frog and 0.3 ms for mouse). Consequently, by combination with the prepulse, the test pulse is prolonged by 15 and 25% in frog and mouse, respectively.

The second component, the higher amplitude of the prepulse (Fig. 5, B and C, inset, empty column), is responsible for relief of the tonic block, and it is in this component that we are interested. Figure 5, B and C presents an experimental protocol designed to distinguish between the 2 components.

Figure 5, B and C shows the average results for 5 experiments. Figure 5B shows the control curve for log $m$/log PA (filled squares). Application of the standard prepulse ($-1.2 \mu A$, 0.1 ms) with zero interval before the test pulses (open squares) increased $m$ in a voltage-dependent manner; thus $m$ increased 11-fold at a PA of $-0.3 \mu A$ and 2.4-fold at $-0.7 \mu A$. When the prepulse had an amplitude identical to that of each of the test pulses (shaded squares), its effect was significantly different from that of the strong ($-1.2 \mu A$) standard prepulse. It increased test-pulse release in a voltage-independent manner. Furthermore, the increase was much smaller (average increase about 2-fold) than the maximal increase (at low-pulse amplitudes) produced by the $1.2-\mu A$ prepulse (11-fold).

The same experiment, at the same recording site, was repeated, but with a 1-ms interval between the pre- and test pulses. Figure 5C shows that with 1-ms interval the “prolongation” effect (shaded squares) was completely abolished; however, the strong ($-1.2 \mu A$) standard prepulse (empty squares) still significantly increased release, with the increase being largest at the low-pulse amplitudes, as in Fig. 5B. The data presented in Fig. 5, B and C thus indicate that the prepulse does indeed enhance release by 2 mechanisms. One, arising from prolongation of the test pulse, is short-lived, dissipating within about 1 ms. The other mechanism, relief of the tonic block, is primarily responsible for enhancement of test-pulse release and persists for several milliseconds (see following text).

The actual prepulse experiments

To avoid possible distortion of the results arising from the “prolongation” effect of the prepulse, in the following experiments we used an interval of 1 ms or more between the pre- and test pulses. Figure 6 depicts the results obtained, with the experimental protocol being shown in the inset of Fig. 6A. Figure 6A shows that the standard prepulse ($-1.2 \mu A$, 0.1 ms), administered with intervals of $-1$, $-2$, and $-4$ ms before the test pulses, increased the quantal content of test-pulse release in a depolarization-dependent manner, similarly to methoctramine and AChE. For example, with a 1-ms interval, $m$ increased 16-fold for $-0.4-\mu A$ test pulse, but only 2.5-fold for a $-0.7-\mu A$ test pulse. Furthermore, the increase in release produced by the prepulse declined as the interval between the

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**FIG. 5.** Effects of prepulse depolarization on test pulse release in frog. A: samples of 15 consecutive traces recorded at the same release site. Left column: prepulse alone did not produce any evoked release. Actually in this experiment 20,000 prepulses were given without producing any evoked release. Middle column: release produced by the test pulse alone. Pulse parameters are given above. Right column: with the prepulse (1-ms delay) release of the test pulse increased 12-fold. Quantal content (m) is given below each column. B: average of 5 experiments. Standard prepulse ($-1.2 \mu A$, 0.1 ms) was given at zero delay from each of the test pulses (inset). “Prolongation” effect was evaluated by applying a prepulse of the same amplitude as that of each of the test pulses (gray column in inset); ■, control, test pulse alone; □, with the “prolongation”; and □, with the full standard prepulse. Log m/log PA slope of the control was $7.6 \pm 0.93$; with the “prolongation,” the slope was $7.7 \pm 0.89$ (an insignificant change, $P = 0.7$). Slope with the standard prepulse was $4.67 \pm 0.5$ (extremely significant difference, $P = 0.0008$). C: same as B, but the prepulses for both the “prolongation” and the standard prepulse preceded the test pulses by 1 ms (inset in B): ■, slope of the control was $7.6 \pm 0.93$; □, with the prolongation, there was no increase in $m$, and consequently no change in slope ($7.58 \pm 0.76$, $P = 0.25$); □, with the standard prepulse the slope decreased to $4.98 \pm 0.45$ ($P = 0.004$).
The results presented in Fig. 6, showing that relief of the tonic block of release is accomplished by an extremely brief depolarizing prepulse, suggest that the tonic block is not produced by M2R-induced inhibition of Ca$^{2+}$ channels.

Although the results displayed in Fig. 6 fulfill our theoretical predictions that the prepulse enhances test-pulse release resulting from a relief of a tonic block, it is still necessary to consider the possibility that, rather than relieving a tonic block, the prepulse (even though it did not produce release itself) admitted a small amount of Ca$^{2+}$, a residual amount of which produced facilitation (Katz and Miledi 1968) of the test-pulse release. To check for this possibility, we applied a train of 20,000 pulses at 100, 200, and 300 Hz, with the parameters of the standard prepulse. It would be expected that if some Ca$^{2+}$ were to enter during the prepulse, it would accumulate. Thus it would further be expected that increases in evoked and spontaneous release should be seen. Furthermore, the rates of both modes of release should increase progressively along the time course of the train.

The results obtained do not confirm these expectations. In one such experiment only 3 spontaneously released quanta (no stimulation) were counted for a period of 200 s. On stimulation at the same recording site, the number of quanta recorded for 20,000 pulses at 100, 200, and 300 Hz were 3, 2, and 3, respectively, and these quanta were not “locked” to the stimulus. Thus the “quantal content” was the same as the rate of spontaneous release, about 0.0001. Similar results were obtained in an additional 3 experiments in which 10,000 pulses were administered. These results, together with those displayed in Fig. 6, indicate that no significant entry of Ca$^{2+}$ occurred during the standard prepulse. Thus the prepulse-mediated enhancement of release seen in Fig. 6 cannot be ascribed to entry of Ca$^{2+}$ during the prepulse. The results presented in Fig. 9, which show that the standard prepulse did not affect twin-pulse facilitation, further corroborate this conclusion.

**Perfusion with methoctramine + AChE occludes the effect of the standard prepulse at the frog neuromuscular junction**

Irrespective of the exact mechanism, the data shown in Fig. 6 are compatible with the notion that the strong and brief depolarizing prepulse, similarly to methoctramine and AChE, relieves a tonic block of release imposed by the transmitter-occupied M2R. To further test this conclusion we examined whether application of methoctramine + AChE would occlude the effect of the prepulse. Figure 7 shows that this is indeed the case. Methoctramine + AChE increased release, as before, in a depolarization-dependent manner and, consequently, reduced the slope of log m/log PA from 7.7 ± 0.81 (filled squares) to 5.34 ± 0.35 (filled diamonds, n = 7). Subsequent application of the prepulse did not further increase release and did not further reduce the slope (open diamonds). We may thus conclude that both the standard prepulse and either methoctramine or AChE affect release by the same mechanism, which is likely to be relief of a tonic block imposed by the M2R.

**In M2-KO mice the standard prepulse does not enhance release produced by a test pulse**

Another way to confirm whether the depolarizing prepulse indeed relieves a tonic block imposed by the M2R is to study its effect in M2-KO mice lacking functional M2R. Accordingly, we repeated the experiments done in frog for both WT (6 experiments) and M2-KO mice (3 experiments). The results obtained in WT mice closely resemble those obtained in frog (Fig. 8, A–C). In particular, the standard prepulse, when given at zero delay, exhibits both the “prolongation effect” (Fig. 8A, shaded squares) and the relief from tonic block (Fig. 8A, open squares). As in frog, the control slope of 7.6 ± 0.2 decreased to 4.59 ± 0.15 when the standard prepulse was applied. Furthermore, as in the frog, the prolongation effect is both
The following experiments attempt to elucidate the mechanism that underlies tonic inhibition of release. As mentioned before, one possibility that must be considered is that the tonic block is attributed to M2-R-mediated voltage-dependent membrane-delimited inhibition of Ca\(^{2+}\) channels (Arnot et al. 2000; Jarvis and Zamponi 2001a,b; Jarvis et al. 2000; Zamponi and Snutch 1998). Another possibility is that the tonic block is achieved by a Ca\(^{2+}\)-independent, but voltage-dependent mechanism (Slutsky et al. 1999, 2002). Such a mechanism might involve physical interaction of the M2-R with SNARE proteins (Iloz et al. 1999; Linial et al. 1997; Parnas et al. 2000).

An optimal way to distinguish between these 2 putative mechanisms would be by direct measurement of Ca\(^{2+}\) currents in the nerve terminal, at various pulse amplitudes, both before and after application of a depolarizing prepulse as done, such as in a transfected cell line (Arnot et al. 2000). This is currently impossible. The available techniques for focal measurement of Ca\(^{2+}\) currents (Brigant and Mallart 1982; Dudel 1990; Slutsky et al. 2003). This result further supports our earlier report that properties of Ca\(^{2+}\) channels are similar in M2-KO and WT mice.

The second component of the prepulse effect, relief from a tonic block, is completely absent in the M2-KO mice (Fig. 8, D and E, open squares). Moreover, as already shown, methoctramine has no effect on release in M2-KO mice. The average control slope was 4.5 ± 0.27 and it was 4.4 ± 0.14 and 4.3 ± 0.1 after application of the “prolongation” and standard prepulses, respectively. Furthermore, neither methoctramine + AChE nor the standard prepulse had any effect on test-pulse release. Thus the average control slope was 4.5 ± 0.18, remaining the same in the presence of methoctramine + AChE (4.4 ± 0.17, P = 0.38) and after subsequent application of the standard prepulse (4.5 ± 0.1, P = 0.52).

In summary, the data displayed in Fig. 8 further support the notion that the strong and brief depolarizing prepulse indeed relieves a tonic block imposed by the M2-R.

The strong and brief depolarizing prepulse does not affect twin-pulse facilitation in all three preparations

Figure 8B further shows that the time course of decay of the prepulse-mediated enhanced release is similar to that seen in the frog (despite the temperature difference). When the standard prepulse preceded the test pulse by 1 ms, the control slope of 7.5 ± 0.26 (filled squares) declined to 4.7 ± 0.13 (open squares). If the interval was 4 ms the slope increased to 5.32 ± 0.33 (open triangles).

Finally, Fig. 8C shows that, as in frog, the prepulse had no additive effect when administered after perfusion with methoctramine + AChE. The control slope (filled squares) of 7.44 ± 0.16 decreased to 4.54 ± 0.11 in the presence of methoctramine + AChE (shaded diamonds), and remained at 4.55 ± 0.14 after subsequent application of the standard prepulse with a 1-ms interval (open diamonds).

The observations for M2-KO mice (n = 3) were radically different. In the M2-KO mice only the prolongation effect of the prepulse persisted (Fig. 8D, shaded squares) and this effect, as in both frog and WT mice, disappeared when the prepulse preceded the test pulse by 1 ms (Fig. 8E, shaded squares; Slutsky et al. 2003). This result further supports our earlier report that properties of Ca\(^{2+}\) channels are similar in M2-KO and WT mice.

Relatively small and independent of pulse amplitude (slope = 7.59 ± 0.12). Also, as in the frog, the prolongation effect was abolished if there was a 1-ms interval between the pre- and test pulses (Fig. 8B, shaded squares).

FIG. 8. Effects of depolarizing prepulses on release of test pulses in WT (n = 6) and M2-KO mice (n = 3). A–C: results in WT mice. D–F: results in M2-KO mice. A: ■, control slope = 7.6 ± 0.2; □, prolongation effect, slope = 7.59 ± 0.12 (an insignificant change, P = 0.34); ◇, with the standard prepulse, slope decreased to 4.59 ± 0.15 (extremely significant, P < 0.0001). B: standard prepulse given 1 and 4 ms before the test pulses; ■, control, slope = 7.5 ± 0.26; □, with 1-ms delay the slope decreased to 4.7 ± 0.13 (extremely significant, P < 0.0001); ◇, with 4-ms delay the slope already increased to 5.32 ± 0.33 (P = 0.24); ◇, no prolongation effect with 1-ms delay. C: a cocktail of 10 μM methoctramine + 80 μg/ml AChE occludes the effect of the standard prepulse; ■, control, slope = 7.44 ± 0.16; ◇, in the presence of methoctramine + AChE, slope = 4.54 ± 0.11 (extremely significant, P < 0.0001); ◇, standard prepulse, 1-ms delay, together with methoctramine + AChE. The control slope remained 4.55 ± 0.14 (P = 0.35). D: “prolongation” effect is observed in M2-KO mice, but the standard prepulse has no additional effect: ■, control, slope = 4.5 ± 0.27; □, with the “prolongation,” the slope remains 4.4 ± 0.14 (insignificant change, P = 0.52); ◇, standard prepulse has no additional effect beyond that of the prolongation, the slope remaining 4.3 ± 0.1 (P = 0.25). E: prepulse was given −1 and −4 ms before the test pulse: ■, control, slope = 4.5 ± 0.27; □, with a 1-ms interval, the slope remains 4.3 ± 0.1 and has the same value if the interval is 4 ms (4.5 ± 0.33) (insignificant changes, P = 0.30 and 0.35, respectively); ◇, prolongation effect with −1-ms interval; F: in the presence of a cocktail of methoctramine + AChE. Concentrations as in C: ■, control, slope = 4.5 ± 0.18; ◇, with the drugs, slope = 4.4 ± 0.17 (P = 0.38); and ◇, with the standard prepulse, slope = 4.5 ± 0.1 (P = 0.52).

The time-course decay of the prepulse-mediated enhanced release is similar to that seen in the frog (despite the temperature difference). When the standard prepulse preceded the test pulse by 1 ms, the control slope of 7.5 ± 0.26 (filled squares) declined to 4.7 ± 0.13 (open squares). If the interval was 4 ms the slope increased to 5.32 ± 0.33 (open triangles).
The quantal content of the first and second test pulses as $m_1$ and $m_2$, respectively; when preceded by a prepulse, as $m_{1p}$; and the quantal content of the second test pulse, when the first is preceded by a prepulse, as $m_{2p}$. The rationale underlying these experiments is as follows: If the increase in release during the first test pulse is attributed to increased entry of Ca$^{2+}$, the residual Ca$^{2+}$ encountered by the second test pulse should also be larger, and twin-pulse facilitation should be enhanced. If, in contrast, the depolarizing prepulse does not relieve a block of Ca$^{2+}$ channels, but rather a block of the release machinery, and in addition, this effect is brief (4–5 ms), then the twin-pulse facilitation should not be affected by the prepulse. Such experiments can distinguish between these two possibilities only if the time course of the decline of prepulse-enhanced release differs significantly from the time course of facilitation.

Figure 9, A and B shows that this is indeed the case for both frog and WT mice. For clarity we denote the prepulse as $pp$; the quantal content of the first and second test pulses as $m_1$ and $m_2$, respectively; the quantal content of the first test pulse, when preceded by a prepulse, as $m_{1p}$; and the quantal content of the second test pulse, when the first is preceded by a prepulse, as $m_{2p}$. With the standard prepulse, the decline in prepulse-enhanced release of the first test pulse ($m_{1p}/m_1$), both in frog (Fig. 9A, open circles) and in WT mice (Fig. 9B, open circles), is rapid. The enhancing effect disappears, similarly to the effect on the slope (Fig. 6), after about 5 ms. The ratio $m_{1p}/m_1$ was 1.3 after 4 ms and 1.16 after 5 ms. In the M$_2$-KO mice the standard prepulse, as expected, did not enhance release of the first pulse (open circles in C). Twin-pulse facilitation ($m_{2p}/m_1$), in contrast, lasted about 20 ms in all three preparations (Fig. 9, A–C, filled squares). The results in Fig. 9 clearly show that even for these low test pulses (small entry of Ca$^{2+}$), the time course of twin-pulse facilitation is much slower (by about an order of magnitude) than the time course of decline in prepulse-enhanced release.

We thus measured the effect of the standard prepulse, applied 1 ms before the first test pulse, on release produced by the second test pulse (the ratio $m_{2p}/m_1$), that is, on twin-pulse facilitation. It is seen that even though $m_{2p}/m_1$ was 16 in the frog (Fig. 9A, open circles), twin-pulse facilitation ($m_{2p}/m_1$) was not affected (Fig. 9A, open squares on the facilitation curve). A 16-fold increase of release of the first pulse, all other parameters being equal, requires that entry of Ca$^{2+}$ during the pulse is at least doubled (in exhibiting a power of 4 relationship to Ca$^{2+}$) (Dodge and Rahamimoff 1967). We expected that such an increase in Ca$^{2+}$ influx, had it existed, would be detected in the facilitation experiments (see confirmation for this expectation below).

The same behavior was seen in WT mice (Fig. 9B). In this case, $m_{2p}/m_1$ was 8.2 (Fig. 9B, open circles) and, as in the frog, twin-pulse facilitation was not affected (Fig. 9B, open squares on the facilitation curve). In M$_2$-KO mice, prepulse-mediated enhancement of release was, as before, completely lacking (open circles) and, naturally, the standard prepulse did not affect facilitation (open squares on the facilitation curve).

![Figure 9](http://jn.physiology.org/)

**FIG. 9.** Effect of different prepulses on facilitation in frog (A), WT mice (B), and M$_2$-KO mice (C). A: twin test pulses (0.7 ms, −0.7 μA) were given with different intervals at a rate of 1 Hz. Facilitation (■), defined as $m_{2p}/m_1$, is denoted on the right ordinate. Facilitation could not be measured at intervals <4 ms between the pulses because of overlap of the release generated by the 2 pulses. Note that facilitation lasted >20 ms. Horizontal dashed line at 1 indicates no facilitation ($m_{2p}/m_1 = 1$). At the same 3 recording sites the standard prepulse (0.1 ms, −1.2 μA) given at different delays from the first of the twin pulses increased the release of the first test pulse. This is given on the left ordinate as $m_{1p}/m_1$ (○); that is, the quantal content of the first test pulse when it followed the prepulse ($m_{1p}$) divided by $m_1$, the quantal content of the first test pulse alone. Note that the effect of the prepulse is short lived (about 4 ms). Standard prepulse increased the release of the first pulse, on average 16-fold, but it did not increase facilitation ($m_{2p}/m_1$), and the control facilitation curves (■) overlapped. When a wider but lower prepulse (0.4 ms, −0.5 μA) was used, the release of the first test pulse increased 13-fold (+). In this case, facilitation $m_{2p}/m_1$ is larger (□). B and C: same experiments for WT and M$_2$-KO mice, respectively. Test pulse 0.3 ms, −0.5 μA. Lower and longer prepulse 0.3 ms, −0.4 μA.

Together, these results indicate that, even though release in the first of the twin pulses increased in frog and WT mice when preceded by a prepulse, the increase was not produced by prepulse-induced higher Ca$^{2+}$ influx during the first of the twin pulses. It may be argued that twin-pulse facilitation is not sufficiently sensitive to detect small changes in Ca$^{2+}$ entry. To test this possibility, we changed the parameters of the
prepulse such that it admitted Ca$^{2+}$ by itself. To this end, we lowered the amplitude of the prepulse and prolonged its duration. In selecting the final stimulation parameters of the new prepulse, we aimed at achieving comparable enhancement of release ($m_{p}/m_{1}$) to that produced by the standard prepulse. With this new prepulse we repeated the experiment described before. For the frog, the release produced by the new prepulse (0.4 ms, −0.5 μA) plus the first test pulse was increased, where $m_{p}/m_{1}$ was 13 (Fig. 9A, asterisk), slightly less than the increase produced by the standard prepulse ($m_{p}/m_{1} = 16$). However, in contrast to the case of the standard prepulse, facilitation was significantly increased (Fig. 9A, open squares with asterisks).

In WT mice, the longer and lower-amplitude new prepulse (0.3 ms, −0.4 μA) enhanced release produced by the first of the twin-test pulses 10-fold (Fig. 9B, asterisk), a little more than the standard prepulse. Here also, as in the frog, this prepulse increased twin-pulse facilitation (Fig. 9B, squares with asterisk). As mentioned earlier, both in frog and in WT mice we adjusted the parameters of the longer and lower-amplitude prepulse such that it produced enhancement of release similar to that produced by the standard prepulse. In the case of M$_2$-KO mice, such a criterion could not be applied because the standard prepulse did not enhance release. Consequently, for the longer and lower-amplitude new prepulse we used the same parameters that were used for WT mice (0.3 ms, −0.4 μA). Figure 9C shows that, as for frog and WT mice, also in the M$_2$-KO mice the test pulse preceded by the new prepulse produced release 17-fold higher than the test pulse alone (Fig. 9C, asterisk), and twin-pulse facilitation increased concomitantly (Fig. 9C, open squares with asterisks).

The data presented in Fig. 9 show that when either the prepulse admits Ca$^{2+}$, or the prepulse increases Ca$^{2+}$ influx during the first of the twin pulses, the increase in Ca$^{2+}$ influx can be detected as residual Ca$^{2+}$ by twin-pulse facilitation. Therefore both the lack of effect of the standard prepulse on twin-pulse facilitation, and the increase in facilitation produced by the lower-amplitude but longer new prepulse, support the conclusion that the standard prepulse alone does not admit Ca$^{2+}$, nor does it increase Ca$^{2+}$ influx during the first of the twin pulses. This conclusion is further strengthened by the following observation. In the frog, with the longer new prepulse, release was increased 13-fold (Fig. 9A), whereas the standard prepulse increased test-pulse release 16-fold. If the level of release is proportional only to the level of [Ca$^{2+}$], these results mean that the broader prepulse, together with the test pulse, admitted less Ca$^{2+}$ than the standard prepulse together with the test pulse. Yet, only the broader prepulse affected facilitation. Finally, the results of Fig. 9 further corroborate an earlier conclusion (see above and Slutsky et al. 2003) that the mechanisms of Ca$^{2+}$ influx and removal, and thus of twin pulse-facilitation, are not altered in M$_2$-KO mice. What is lacking is the M$_2$R-imposed tonic block of release. Thus depolarization plays only one role in M$_2$-KO mice, that of opening voltage-gated Ca$^{2+}$ channels.

**DISCUSSION**

In the present study we provide evidence to support the notion that depolarization, in addition to its role in opening voltage-gated Ca$^{2+}$ channels, relieves a tonic block imposed by the ACh-occupied M$_2$R, resulting in initiation of ACh release. Furthermore, the M$_2$R-imposed tonic block of ACh release, which is relieved by a very brief prepulse, does not appear to involve M$_2$R-mediated inhibition of Ca$^{2+}$ channels.

The evidence in support of the above conclusions derives from experiments in which the quantal content (m) was measured at various presynaptic depolarizations [achieved by varying the depolarizing pulse amplitude (PA)], and establishing the slope of log m/log PA under various experimental conditions. The main evidence is as follows: 1) The slope of log m/log PA was 7–8 in frog and in WT mice, and only about 4 in M$_2$-KO mice. 2) Addition of methoctramine, or AChE, treatments previously shown to abolish the M$_2$R-imposed block (see references in INTRODUCTION), increased release in a voltage-dependent manner in both frog and WT mice. Enhancement became stronger as the depolarizing pulse decreased. In M$_2$-KO mice, neither methoctramine nor AChE had any effect on release. As a corollary to the above, both methoctramine and AChE reduced the slope of log m/log PA in both frog and WT mice, but had no effect on the slope in M$_2$-KO mice. 3) Administration of a brief (0.1-ms) but strong (−1.2 μA) depolarizing pulse before a test pulse enhanced release of the test pulse similarly to methoctramine. Thus it enhanced release in a voltage-dependent manner in frog and in WT mice but did not affect release in M$_2$-KO mice. 4) A cocktail of methoctramine + AChE occluded the effect of the depolarizing pulse. 5) The prepulse-enhanced release decayed in about 4–5 ms in frog and WT mice, at 8 and 30°C, respectively, whereas the duration of twin-pulse facilitation was >20 ms in all 3 preparations. 6) The strong and brief prepulse did not affect the amplitude of twin-pulse facilitation in any of the 3 preparations. In contrast, a prepulse lower in amplitude and of longer duration increased twin-pulse facilitation in all 3 preparations.

**Relation between the dependency of m on depolarization and on Ca$^{2+}$ entry**

The slopes of log m/log [Ca$^{2+}$]$_{i}$, or I$_{Ca}$, or [Ca$^{2+}$], have values of 3–5 (Augustine et al. 1985; Dodge and Rahamimoff 1967; Ravin et al. 1999). We show here that the slope of log m/log PA is about 7–8 in both frog and WT mice. We have interpreted the higher slope of log m/log PA, relative to log m/log [Ca$^{2+}$], as meaning that for release to be initiated, depolarization must fulfill 2 roles: relief from a tonic block and opening of voltage-gated Ca$^{2+}$ channels (Parnas et al. 2000). However, other explanations should be kept in mind. For example, the higher slope of log m/log PA might result from a nonlinear relationship between Ca$^{2+}$ currents and depolarization. In particular, Simon and Llinas (1985) suggested that for identical I$_{Ca}$ at 2 levels of depolarization, the Ca$^{2+}$ concentration at the release site is expected to be higher at the higher depolarization. This is because the number of open channels increases with depolarization, and thus overlapping of Ca$^{2+}$ diffusing from adjacent channels is expected. Also, other mechanisms for a supralinear relationship between Ca$^{2+}$ influx and depolarization could exist. It should be noted that a nonlinear relationship between Ca$^{2+}$ currents and depolarization cannot explain the lower slope in the M$_2$-KO mice. We argue, however, that even if some nonlinearity of the types mentioned above does exist, it is the lower slope in the M$_2$-KO
mice and the reduction in the slope in frog and WT mice on addition of methoctramine or applying a brief depolarizing prepulse that constitute the key points. These findings provide evidence that, irrespective of the exact mechanism, depolarization does have an additional role (in initiation of release) over and above opening of Ca²⁺ channels.

Possible mechanism of the tonic block

The main thrust of this study had been to demonstrate that for initiation of ACh to take place, depolarization must relieve a tonic block imposed by the M₂R. Our experiments were not aimed at directly studying the mechanism that underlies the tonic block. Nevertheless, we discuss 2 possible mechanisms. One possibility is that under resting conditions the voltage-gated Ca²⁺ channels are tonically inhibited by a mechanism that involves M₂R-mediated voltage-dependent membrane-delimited inhibition (see, e.g., Jarvis and Zamponi 2001a,b; Jarvis et al. 2000; Zamponi and Snutch 1998). The second possibility is that the tonic inhibition of release is achieved by the M₂R interacting with SNARE proteins at resting potential (Ilouz et al. 1999; Linial et al. 1997), thereby blocking the release machinery (Parnas et al. 2000). In both cases the block would be relieved by depolarization (see references above).

Although we cannot completely rule out the first suggestion, we consider this possibility less likely. This assertion is based on the following considerations. 1) Slutsky et al. (2002) showed that muscarine, at concentrations as high as 70 μM, does not reduce Ca²⁺ currents produced by the physiological action potential. It is thus hard to imagine that the low concentration of ACh (about 10 nM; Katz and Miledi 1977) present in the synaptic cleft at rest would be capable of maintaining the Ca²⁺ channels in a blocked configuration. 2) The brief, but strong, depolarizing prepulse-enhanced release evoked by the first of the twin-test pulses, but did not affect facilitation. In contrast, a longer but lower-amplitude prepulse enhanced release of the first test pulse, but concomitantly increased twin-pulse facilitation (Fig. 9). 3) The time constant observed here for inhibition (decline of prepulse enhanced test-pulse release) is of the order of a few milliseconds (4–5 ms, Fig. 9), whereas the time constant for inhibition of voltage-gated Ca²⁺ channels is significantly slower [e.g., about 50 ms for a depolarizing prepulse >20 mV and even slower for lower prepulse depolarizations (Arnot et al. 2000; Kasai 1992)]. 4) In the preceding studies, for the strongest depolarizing prepulse (>20 mV) to be able to relieve inhibition of the Ca²⁺ channels, its duration had to be about 4 ms. We find that even an extremely brief (0.1-ms) depolarizing prepulse substantially relieves the block.

We should note, however, that under physiological conditions the action potential is wider than 0.1 ms, and it is quite possible that the action potential also relieves, in addition to the M₂R imposed but Ca²⁺-independent block, an M₂R-imposed membrane-delimited inhibition of voltage-gated Ca²⁺ channels [but recall point 1) above].

It is interesting to note that the short time course of reinstatement of the M₂R-imposed block (Fig. 9) is similar (but somewhat longer) to that of termination of action potential-evoked release. This is compatible with the suggestion that termination of ACh release occurs because on membrane repolarization the M₂R rapidly shifts to its high-affinity state, rebinds ACh, and reimposes the tonic block (Slutsky et al. 2001, 2003). If so, then why is termination of release faster than the relaxation of disinhibition after a 0.1-ms prepulse observed here? One reason could be that because the prepulse did not induce release, the ACh concentration in the cleft encountered by the M₂R remained very low (about 10 nM; Katz and Miledi 1977). Thus the first step in reinstatement of the block, after the prepulse (i.e., binding of ACh) is slower. In contrast, under physiological conditions, ACh concentration in the cleft is high because of release induced by the action potential and binding of ACh to the M₂R is fast.

Termination of action potential induced release may be even faster than anticipated based on the considerations mentioned above. This is because elevation of transmitter concentration in the cleft, attributed to release induced by the action potential, may enable even the low-affinity M₂R (prevailing during depolarization and shortly thereafter) to bind ACh and reinstate the block even sooner. Thus termination of release could begin on membrane repolarization, even before the M₂R shifts eventually back to its high-affinity state. This interpretation is consistent with findings of Slutsky et al. (2002) showing very fast inhibition of ACh release (which coexists with the classical slow second-messenger–dependent inhibition). This fast inhibition, which occurs at all levels of depolarization, requires a high concentration of muscarine.

An alternative explanation of our data

It is possible that the involvement of the M₂R in controlling release that had been demonstrated here involves M₂R activation of G-protein–activated inwardly rectifying K⁺ (GIRK) channels. The scenario could be as follows. At resting potential and resting concentration of ACh (about 10 nM; Katz and Miledi 1977) the GIRK channels are constantly activated and thus hyperpolarize the nerve terminal. These channels would then have a strong effect in reducing Ca²⁺ currents at a small test-pulse depolarization and their effect will decrease with higher depolarization. Consequently, addition of methoctramine, which blocks M₂R activation of GIRK channels, will indeed reduce the log m/log PA slope as seen here. Also, the lower slope obtained in M₂-KO mice can be accounted for by the above hypothesis.

Several considerations render the GIRK channel hypothesis less likely. The effect of the brief depolarizing prepulse was occluded by methoctramine, suggesting that the prepulse acts on the same target as methoctramine. According to the above hypothesis, this would be the M₂R activated GIRK channel. If this is the case, then one expects that the brief depolarizing prepulse will somehow deactivate the GIRK channels and will thus increase Ca²⁺ influx at the test pulse that follows the prepulse. Under such conditions, in contrast to observations (Fig. 9), a higher twin-pulse facilitation is expected.

Another possible difficulty with the GIRK channel hypothesis stems from reports concerning their location. In the brain where location of GIRK channels was studied, most studies emphasize that GIRK channels were found postsynaptically with no sign for presynaptic existence (e.g., Drake et al. 1997; Inanobe et al. 1999; Luscher et al. 1997; Miyashita et al. 1997). Only a few studies report presynaptic location of GIRK channels in some areas in the brain (Morishige et al. 1996; Ponce et
al. 1996). As far as we know there are no similar studies concerning neuromuscular junctions.

**How can our hypothesis be reconciled with experiments where release was induced by Ca\(^{2+}\) alone without depolarization?**

We provided here further support, in addition to earlier evidence (Slutsky et al. 1999, 2001, 2003), for the notion that both initiation and termination of ACh release are governed, respectively, by depolarization relieving and repolarization reinstating a tonic block of the release machinery imposed by the M\(_2\)R.

Despite all this supporting evidence, the above notion appears to be contradicted by experiments in which release was induced by Ca\(^{2+}\) alone (Ca\(^{2+}\)-induced release), without concomitant depolarization (Bollman et al. 2000; Schneggenburger and Neher 2000). In a theoretical study, Parnas et al. (2002) showed that Ca\(^{2+}\)-induced release can be directly derived from the molecular scheme describing depolarization-induced release (Yusim et al. 1999). In particular, Parnas et al. (2002) provided evidence that shows that spontaneous release is a subset of the molecular scheme of depolarization-induced release. These authors argued that Ca\(^{2+}\)-induced release may be viewed as a manifestation of spontaneous release at relatively high [Ca\(^{2+}\)], conditions that were achieved when release was induced by Ca\(^{2+}\)-uncaging (Bollman et al. 2000; Schneggenburger and Neher 2000). As discussed in Parnas et al. (2002), the properties of Ca\(^{2+}\)-uncaging–induced release are different from those of action potential–induced release. Notable among them is the dependency of the duration, time to peak, and minimal delay of Ca\(^{2+}\)-uncaging–induced release on Ca\(^{2+}\) concentration. In physiological release all those facets of release are independent of Ca\(^{2+}\) concentration.

Also relevant to this discussion is the finding of Slutsky et al. (2003). These authors showed that in M\(_2\)R knockout mice, where the release machinery is not blocked, Ca\(^{2+}\) entry induces release and this release resembles a physiological one in most aspects, but differs from it in one critical aspect—the precise timing of release and the dependency of the time course of release on Ca\(^{2+}\) concentration. In M\(_2\)-KO mice release starts sooner and lasts longer than in WT mice. In addition, only in M\(_2\)-KO mice, but not in WT mice, the time course of release depends on Ca\(^{2+}\) concentration.

A further challenge to the notion that depolarization, per se, is required for initiation of release comes from experiments in which depolarization was applied after induction of release by Ca\(^{2+}\)-uncaging (Felmy et al. 2003; Hochner et al. 1989; Mulkey and Zucker 1991; Zucker and Haydon 1988). In these experiments depolarization did not increase release above the level produced by Ca\(^{2+}\) alone (excluding the experiments of Hochner et al. 1989, where depolarization moderately increased release; but see dispute of this interpretation by Mulkey and Zucker 1991). It was shown (Sela et al. 2005) that the lack of an effect of the long depolarization, when applied after Ca\(^{2+}\)-uncaging, can also be readily explained in the framework of the molecular scheme of depolarization-induced release (Yusim et al. 1999). In particular, during the course of release produced by Ca\(^{2+}\)-uncaging, the concentration of transmitter in the cleft increases. When a depolarizing pulse is subsequently administered, depolarization-induced release occurs, but at an already high transmitter concentration in the synaptic cleft. It was argued theoretically (Yusim et al. 1999), and subsequently shown experimentally (Slutsky et al. 2002), that phasic ACh release is rapidly inhibited, even at high depolarizations, at a high concentration of muscarine. Based on these results, Sela et al. (2005) attributed the lack of effect of depolarization after Ca\(^{2+}\)-uncaging to the fast inhibition of release produced by the elevated transmitter concentration, an elevation that occurred before depolarization.

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