Use of Knockout Mice Reveals Involvement of M₂-Muscarinic Receptors in Control of the Kinetics of Acetylcholine Release

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Use of knockout mice reveals involvement of M₂-muscarinic receptors in control of the kinetics of acetylcholine release. J Neurophysiol 89: 1954–1967, 2003; 10.1152/jn.00668.2002. We have previously suggested that presynaptic M₂-muscarinic receptors (M₂R) are involved in the control of the time course of evoked acetylcholine release in the frog neuromuscular junction. The availability of knockout mice lacking functional M₂R (M₂-KO) enabled us to address this issue in a more direct way. Using the phrenic diaphragm preparation, we show that in wild-type (WT) mice experimental manipulations known to affect Ca²⁺ entry and removal, greatly affected the amount of acetylcholine released (quantal content). However, the time course of release remained unaltered under all these experimental treatments. On the other hand, in the M₂-KO mice, similar experimental treatments affected both the quantal content and the time course of release. In general, a larger quantal content was accompanied by a longer duration of release. Similarly, the rise time of the postsynaptic current produced by axon stimulation was sensitive to changes in [Ca²⁺]₀ or [Mg²⁺]₀ in M₂-KO mice but not in WT mice. Measurements of Ca²⁺ currents revealed that the shorter rise time of the postsynaptic current seen in high [Mg²⁺]₀ in M₂-KO mice was not produced by a shorter wave of the presynaptic Ca²⁺ current. These results support our earlier findings and provide direct evidence for the major role that presynaptic M₂-muscarinic receptors play in the control of the time course of evoked acetylcholine release under physiological conditions.

INTRODUCTION

It is well accepted that Ca²⁺ plays a major role in promoting the physiological depolarization-induced release of neurotransmitter from nerve terminals (Del Castillo and Katz 1954; Katz 1969; Neher 1998; Silinsky 1985). Furthermore, it is generally accepted that the entry of Ca²⁺ serves as the trigger for release initiation (Katz 1969 and revs. Augustine 2001; Llinás 2001; Martin-Serrano 1994; Van der Kloot and Molgo 1994; Zucker 1969) and the fast feedback inhibition of acetylcholine (ACh) release (Slutsky et al. 1999) prolonged the time course of ACh release (Slutsky et al. 2001).

Drugs, however, often interact with multiple targets and hence may exhibit nonspecific effects. Therefore a more direct approach is needed to further consolidate the involvement of the M₂R in control of initiation and termination of transmitter release (reviewed by Parnas et al. 2000).

Support for this hypothesis was recently obtained. It was shown that addition of methoctramine, a selective antagonist of the M₂ muscarinic receptor (M₂R), the receptor that mediates feedback inhibition of ACh release (Slutsky et al. 1999) prolonged the time course of ACh release (Slutsky et al. 2001).

Recently, a line of knockout mice lacking functional presynaptic M₂R (M₂-KO) was isolated (Gomeza et al. 1999). The availability of such knockout mice enabled us to compare the time course of release under different experimental conditions in wild-type (WT) and M₂-KO mice. These experiments allow us to assess more critically the role of the M₂R in controlling the kinetics of ACh release under physiological conditions.

We found, as was shown in other systems (see preceding experimental manipulations known to affect Ca²⁺ entry (Andreau and Barrett 1980; Borst and Sakmann 1996; Datyner and Gage 1980; Isaacson and Walmsley 1995; Parnas et al. 1986; Van der Kloot 1988) or Ca²⁺ removal (Arechiga et al. 1990; Hochner et al. 1991).

In contrast to the preceding text, when release of transmitter was induced by Ca²⁺ alone, by an abrupt elevation of [Ca²⁺]₀, without a concomitant depolarization, the amount but also the time course of release were both sensitive to the level of [Ca²⁺]₀ (Bollmann et al. 2000; Heidelberger et al. 1994; Schneggenburger and Neher 2000). The different behavior of depolarization-induced release and calcium-induced release may suggest that in the former, another factor, rather than [Ca²⁺]₀, may be involved in initiation and termination of release. (An explanation for the different behavior of the 2 modes of release is provided by Parnas et al. 2002, but see Meinrenken et al. 2002.)

Recent data have led to the hypothesis that presynaptic inhibitory autoreceptors, in addition to their well-known role in mediating feedback inhibition of transmitter release, are also involved in the control of initiation and termination of transmitter release (reviewed by Parnas et al. 2000).
references), that in WT mice, experimental manipulations that are known to affect Ca$^{2+}$ entry and removal strongly affected the amount of release. These same treatments, however, had no effect on the time course of release. In the M$_2$-KO mice, the same experimental treatments affected the amount of release but also the time course of release. In particular, increased Ca$^{2+}$ entry was accompanied by longer duration of release and a faster removal of Ca$^{2+}$ was associated with shorter duration of release.

**METHODS**

**Mice**

The M$_2$R mutant mice (M$_2$-KO) used for these studies had a mixed genetic background (129J1 × CF-1:50%/50%). The generation of the M$_2$-KO mice and the phenotype of these mice were described earlier (Gomeza et al. 1999). We could not discern any sluggish movements or other abnormalities in the M$_2$-KO mice. Mouse colonies were maintained at Taconic Farms (Germantown, NY). We used the mice at the age of 1.5–3 mo.

**Preparation and solutions**

Mice were anesthetized with CO$_2$ and decapitated, in accordance with institutional guidelines and Israel animal protection law. Hemidiaphragm neuromuscular preparations were isolated. The standard bathing solution was composed of (in mM) 160 NaCl, 2.5 KCl, 1 MgCl$_2$, 3 CaCl$_2$, 10 HEPES, and 8 glucose, bubbled with 95% O$_2$–5% CO$_2$. The temperature was kept at 20 ± 1°C and was controlled using a heat exchanger. The pH was adjusted to 7.4 by adding NaOH.

**Recording and stimulation**

Two modes of stimulation were used. For local depolarization of a small region of the terminal, near a release site, and recording of single quanta events, the macropatch technique (Dudel 1981) was employed. The end plates were visualized with a long-distance objective (Zeitz-Instruments, Munich). The fire-polished tip (∼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, pulse amplitude varied between −0.3 and −1.0 μA, and frequency of stimulation ranged between 3 and 30 Hz. TTX (0.2 μM) was present in the solution to prevent sodium excitation and, hence, enabling graded depolarization of the axon terminal (Dudel 1981).

To obtain action-potential-evoked nerve terminal currents (ENTCs) and releases (Figs. 2, 4, and 5; no TTX), the nerve was stimulated with a suction electrode. Pulse duration was 0.2 ms, and the pulse amplitude was twice threshold. Stimulation frequency was 1 Hz, d-tubocurarine chloride (dTc, 1–2 μM) was added to avoid muscle contraction. The ENTC and the excitatory postsynaptic current (EPSC) were recorded with a macropatch electrode located over a release region (Dudel 1990).

**Quantal content and time course of release**

At 20°C, quanta appeared after the stimulus artifact and could be easily discerned and counted (Fig. 3A) even when two or three quanta were released together. The total number of quanta (measured during 10 ms after the depolarizing pulse) divided by the number of applied pulses yields the quantal content (the average number of quanta released per pulse). Current traces were digitized using Neurodata (Neuro-Corder DR-484) A/D converter at 50 kHz and transferred to a Pentium II computer (450 MHz) using the Labview (AT-MIL-16F-5, NI-DAQ 4.9.0 driver software) interface.

**TREATMENTS CHANGING [Ca$^{2+}$]$_i$**

<table>
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<tr>
<th>Treatments</th>
<th>WT Mice</th>
<th>M$_2$-KO Mice</th>
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<tr>
<td>PA, μA</td>
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<tr>
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<td>0.17 ± 0.007</td>
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Values are means ± SD. For all D values, $R^2 > 0.99$. PA, pulse amplitude; SF, stimulation frequency; C, control; BA, BAPTA-AM; m, quantal content; WT, wild type; M$_2$-KO, knockout mice lacking functional presynaptic muscarinic receptor.
possible sodium inward current below the electrode, the electrode contained 20 µM TTX. Addition of 10–40 µM dTC to the bath solution largely blocked the postsynaptic current as well as prevented contractions and distortion of the ENTC. Potassium channel blockers tetrathyrammonium chloride (TEA, 10 mM) and 3,4-diaminopyridine (3,4-DAP, 100 µM) were then added to the bath solution, diffusing also into the space below the electrode, and blocked the potassium currents. Largely positive capacitative and leak currents, as well as the Ca2+ current, remained. In Fig. 1A (—), the respective current component is almost purely positive. In other sites, also positive/negative (Fig. 1C) or positive/negative/positive traces were obtained representing a different balance of positive and negative current components; simple positive traces were typical of recordings from the tip of a terminal (Brigant and Mallart 1982). When Cd2+ (100 µM) now was added, inward Ca2+ currents were blocked, and the recorded traces became more positive (Fig. 1A, —). Subtraction of the two traces revealed the Ca2+ current (Fig. 1B).

A second example of such a “pealing” process is given in Fig. 1, C and D. In Fig. 1C, the remaining response after addition of TTX, dTC, and the potassium channel blockers was biphasic (Fig. 1C, ——). Addition of Cd2+ reduced the negative phase (Fig. 1C, ——) and subtraction gave the Ca2+ current shown in Fig. 1D.

The described “pealing” process introduced by Brigant and Mallart (1982) was used to isolate the presynaptic Ca2+ current component also in the experiments of Figs. 5 and 6. If the recordings under the conditions of Fig. 1A contained a relatively large negative component, the finally isolated Ca2+ currents often showed a positive component either before (Fig. 5H) or also after the negative Ca2+ current (Fig. 5, D1 and H1).

The Cd2+ concentration used here was very high to ensure complete block; 20 µM Cd2+ blocked >90% of the Ca2+ current and 50 µM blocked the Ca2+ current completely (see also Dudel 1990).

**Na+ current measurement**

When we elicited release from terminals by graded depolarizing pulses, it had been necessary to add TTX to the electrode to prevent all-or-nothing responses at a depolarization threshold. This suggested that the axon terminals in the diaphragm contained also voltage-dependent Na+ channels in difference from axon terminals of the trignusalar muscle of the mouse (Brigant and Mallart 1982).

To clarify this point, we “pealed” a presynaptic Na+ current component in a similar manner to that described in the preceding text for Ca2+ currents. In this case, however, TTX could not initially be present in the electrode. We therefore used the perfused macropatch electrode (Dudel 1989; Dudel and Heckmann 2002), which allows addition of TTX into the electrode after a control ENTC has been recorded. Nerve stimulation produced vigorous contractions, and these were prevented by eliminating Ca2+ from the bath solution. The electrode solution, on the other hand, contained 3 mM Ca2+, enabling release of neurotransmitter just from the terminal region below the electrode (Fig. 2A). This stage was essential to ensure that the electrode recorded an ENTC near a release site. From time to time, a threshold for a muscle action potential was reached as a result of electrotonically and retrogradely to produce the late positive current in the region below the electrode.

Pealing the ENTC by subtraction from a recorded current shown in Fig. 2C. Subtraction of Fig. 2C from B, yielded the Na+ current shown in D. In 10 recording sites, we observed similar Na+ currents. The motor nerve terminals of the diaphragm thus seem to be excitable. In this preparation there is no indication of depolarization-elicited regenerative Ca2+ currents. This is because we did not encounter threshold jump in synaptic currents in axon terminals block with TTX.

The excitability of the terminals explains the often-observed late positivity of some ENTCs (see for example Figs. 5 and 6). An action potential in the axon invades the region below the electrode (which does not produce inward Na+ current), generating a positive current phase. If the axon terminal continues somewhat beyond the recording electrode, a further Na+ regenerative response is elicited, propagating electrotonically and retrogradely to produce the late positive current in the region below the electrode.

In some experiments (e.g., Fig. 6), we had to add 100 µM procaine (Dreyer and Penner 1987) to prevent repetitive firing of the axon in the presence of K+ channel blockers. Such repetitive firing never
distorted the ENTCs because the refractory period of the axon was >2 ms, but sometimes spontaneous and local muscle contractions endangered stability of the recording. Addition of procaine reduced the Na\(^{+}\) current component of the ENTC by \(\sim 30\%\), as expected for a local anesthetic but had no obvious further effects (not shown).

**Alternating pulse amplitude protocol**

In some of the experiments (Fig. 3), it was necessary to establish the quantal content at different pulse amplitudes. Because it takes quite a long time to establish the quantal content for one pulse amplitude, it follows that if the quantal content at the various pulse amplitudes would be measured in a sequential order, a long time would elapse between the time of measuring the first and the last quantal content, which may distort the true dependence of the quantal content on the pulse amplitude. To ensure that this is not the case and that the quantal contents at the various pulse amplitudes are measured roughly at the same time, the alternating pulse amplitude protocol had been employed.

Four depolarizing pulses of different amplitudes (-0.3, -0.5, -0.7, and -0.9 \(\mu\)A) were administered in a random manner, and the control quantal contents were determined. The interval between pulses was 300 ms. Stimulation was continuous to allow accumulation of re-

**Drugs and chemicals**

TTX, muscarine, and methoctramine were purchased from RBI (Natick, MA); d-TC, TEA, 3,4-DAP, and procaine were purchased from Sigma (St. Louis, MO).

**Statistical evaluation**

Significance was checked by paired (the same experiment) and unpaired (different experiments) Student’s two-tail \(t\)-test. Average results are given as means \(\pm SD\) throughout.

**RESULTS**

**Lack of \(M_2\)R-mediated inhibition of ACh release in knockout mice**

\(M_2\)R is known to act as an inhibitory autoreceptor in cholinergic synapses (see references in introduction). We first examined whether phrenic diaphragm preparations from the \(M_2\)-KO mice indeed lacked autoinhibition of ACh release. Specifically, we compared effects of muscarine (a nonhydro-

**Time course of action-potential-evoked release in \(M_2\)-KO mice**

We first compared the behavior of the time course of release in WT and \(M_2\)-KO mice evoked by the physiological stimulus (action potential), at 2 \([Ca^{2+}]_o\). In these experiments, we recorded the nerve terminal current (ENTC) and the EPSC the rise time of which reflects the time course of release (Aumann and Parnas 1991; Schneegubergen and Neher 2000). Figure 4A shows responses obtained at WT mice with 1 and 3 mM \([Ca^{2+}]_o\). The ENTCs obtained at the 2 \([Ca^{2+}]_o\) were completely superimposable (Fig. 4B, inset), indicating that there were no gross changes in the shape of the presynaptic action potential. Figure 4B shows superposition of the normalized
To our knowledge, it is the first time that changing \([\text{Ca}^{2+}]_0\) produced changes in the time course of single action-potential-evoked release, and this was seen only in the M2-KO mice (but see effects of residual \(\text{Ca}^{2+}\) on the slower transient and delayed release: Chen and Regehr 1999).

To present these data in a more quantitative way, we measured \(t_{\text{rise}}\) [defined as \(t\) (90% of peak EPSC) – \(t\) (10% of peak EPSC)] (Franke et al. 1991). For the example given in Fig. 4B, in WT mice, \(t_{\text{rise}}\) was 0.52 ms for \([\text{Ca}^{2+}]_0 = 1 \text{ mM}\) and 0.54 ms for \([\text{Ca}^{2+}]_0 = 3 \text{ mM}\). On average, in WT mice, elevation of \([\text{Ca}^{2+}]_0\) from 1 to 3 mM increased the EPSC amplitude 2.9 ± 0.5-fold (\(n = 4, P < 0.001\)) but did not affect \(t_{\text{rise}}\) (\(n = 4, P > 0.8\)).

In the M2-KO mice, for the example given in Fig. 4D, the amplitude of the EPSC increased 2.8-fold for the same changes in \([\text{Ca}^{2+}]_0\). Concomitantly, \(t_{\text{rise}}\) was prolonged; in 1 mM \([\text{Ca}^{2+}]_0 \) \(t_{\text{rise}}\) was 0.56 and it increased to 0.74 ms in 3 mM \([\text{Ca}^{2+}]_0\). It should be noted that due to the significant change in the width of the peak EPSC on changes in \([\text{Ca}^{2+}]_0\) (Fig. 4D), \(t_{\text{rise}}\) of the EPSC, as defined in the preceding text, understimates the changes that had occurred in the time course of release. In four experiments in the M2-KO mice, the EPSC amplitude increased 2.7 ± 0.4-fold (\(n = 4, P = 0.001\)) and \(t_{\text{rise}}\) was prolonged from 0.54 ± 0.03 to 0.71 ± 0.04 (\(n = 4, P = 0.001\), an increase of ∼32%). Thus in the M2-KO mice where the M2R is not functional, the time course of release is sensitive to changes in \([\text{Ca}^{2+}]_0\).

**Measurement of presynaptic \(\text{Ca}^{2+}\) current and EPSCs in WT and M2-KO mice**

One way to explain the results of Fig. 4 is as follows. It is possible, that in the M2-KO mice, the lack of functional M2R somehow affects the properties of the \(\text{Ca}^{2+}\) channels, such that not only the amplitude, but also the kinetics of the \(\text{Ca}^{2+}\) current varies with changes in \([\text{Ca}^{2+}]_0\). It is, then, this change that produces the prolongation in the time course of release. We checked for this possibility by measuring \(\text{Ca}^{2+}\) currents and their corresponding EPSCs at two extracellular \(\text{Mg}^{2+}\) concentrations, \([\text{Mg}^{2+}]_0\).

First, we measured the EPSCs at two levels of \([\text{Mg}^{2+}]_0\) (1 and 4 mM in the presence of 2 \(\mu\text{M} \text{ dTC}\)) in WT and M2-KO mice. It is seen that in both WT (Fig. 5A) and M2-KO (Fig. 5E) mice, the amplitude of the EPSC was reduced on increase in \([\text{Mg}^{2+}]_0\). The normalized EPSCs are shown at a higher resolution in Fig. 5B. Note that only in the M2-KO mice was the lower amplitude accompanied by a shorter rise time of the EPSC (Fig. 5F), reflecting a shorter duration of release in the presence of 4 mM \([\text{Mg}^{2+}]_0\), than in 1 mM \([\text{Mg}^{2+}]_0\). In contrast, in the WT mice, the superposition of the two EPSCs is good (Fig. 5B).

Next, after washing and full recovery of the control EPSC, we measured \(\text{Ca}^{2+}\) currents at the same release regions (Fig. 5, C and G) but in the presence of 10 \(\mu\text{M} \text{ dTC}\) to achieve a complete block of the postsynaptic current.

Figure 5, C and G, shows the net \(\text{Ca}^{2+}\) current (as obtained by current subtraction, see Fig. 1 in METHODS) in WT and M2-KO mice. Both in WT mice (Fig. 5C) and in M2-KO mice (Fig. 5G) increasing \([\text{Mg}^{2+}]_0\) decreased the amplitude of the \(\text{Ca}^{2+}\) currents (negative peaks) but did not change appreciably their kinetics. Normalization of the waves seen in Fig. 5,
Different from those seen in Fig. 5, D and H. (The precise shape depends on the location of the electrode, as well as on the precise experimental conditions, see Methods for further details.) But, the effect of changing [Mg ²⁺] o, on the Ca²⁺ current measured at the same site is the same. Increasing [Mg ²⁺] o decreased the amplitude of the Ca²⁺ currents (not shown) but did not change appreciably their kinetics, as evident from the normalized currents (Fig. 5, D1 and H1).

On average, in WT mice, increasing [Mg ²⁺] o, from 1 to 4 mM, decreased the amplitude of the Ca²⁺ current 0.57 ± 0.1-fold (n = 4, P < 0.001) without an appreciable change in the shape of the Ca²⁺ current. Concomitantly, increasing [Mg ²⁺] o, decreased the amplitude of the EPSC 0.5 ± 0.1-fold (n = 4, P < 0.001) but did not affect the t rise of the EPSC (n = 4, P > 0.9). In the M₂-KO mice, the situation was different. The same increase in [Mg ²⁺] o, similarly to the WT mice, decreased the amplitude of the Ca²⁺ current 0.5 ± 0.12-fold (n = 4, P < 0.001) without affecting the kinetics of the Ca²⁺ current. However, in contrast to the WT mice, the concomitant decrease in the amplitude of the EPSC (0.55 ± 0.1-fold, n = 4, P < 0.001) was now accompanied by a 0.80 ± 0.05-fold (n = 4, P < 0.001) decrease in t rise.

It may be argued that the isolation of the Ca²⁺ current component by “peeling” did not preserve the time course of the Ca²⁺ current. To show that our technique is sensitive to changes in the time course of the Ca²⁺ currents, we conducted the experiment described in Fig. 6. Specifically, no Ca²⁺ was added to the bath superfusate that also contained 100 µM procaine to prevent repetitive firing of the nerve. Under these conditions, a normal short action potential reached the space below the electrode and elicited a corresponding Ca²⁺ current (isolated by “peeling”, as shown in Fig. 1 and used in Fig. 5). Indeed, this Ca²⁺ current was brief (0.5-ms duration measured at a level of 10% of the peak current, Fig. 6, - - -). Then, the Cd²⁺ in the electrode was washed out and 2 mM TEA and 50 µM DAP were added to the bath superfusate, which, as is well known, prolongs the action potential in the axon. The prolonged action potential reaching the space below the electrode is expected to elicit a longer Ca²⁺ current as indeed was the case. The duration of the Ca²⁺ current was now 1.2 ms (Fig. 6, A).

C and G (to their corresponding negative peaks), shows that changing [Mg ²⁺] o, did not cause meaningful changes in the shape of the Ca²⁺ currents, neither in the WT nor in the M₂-KO mice (Fig. 5, D and H). Figure 5, D1 and H1, shows another example of normalized Ca²⁺ currents (another experiment) in WT and M₂-KO mice. It is seen that the shapes of the Ca²⁺ currents, both in WT and M₂-KO mice, are somewhat different from those seen in Fig. 5, D and H.

**FIG. 5.** Presynaptic Ca²⁺ currents and EPSCs in WT (A–D) and M₂-KO (E–H) mice at [Mg ²⁺] o, = 1 mM (—) and [Mg ²⁺] o, = 4 mM (-----). A: ENTC and EPSC in control (—, normal [Mg ²⁺] o, 1 mM) and in 4 mM [Mg ²⁺] o, (-----). Note the reduction in the EPSC, by −50%. B: higher resolution of the rise time of A, curves normalized to peak. For convenience, the current is shown as positive. The WT preparation was then washed with normal saline until the EPSC recovered to the control level, and 10 µM dTC was then added to block completely the EPSC. C: Ca²⁺ current was then measured, as described in Methods; —, the Ca²⁺ current in the control; --, after adding again 4 mM Mg²⁺. Note that the negative peak, corresponding to the Ca²⁺ current, was reduced by 40%. D: normalization of the wave forms presented in C to the negative peak. E–H: same as in A–C but for M₂-KO mouse. Note in F, the shorter t rise, at the higher Mg²⁺ concentration. t rise values at 1 and 4 mM [Mg ²⁺], were 0.72 and 0.58 ms, respectively. G: the negative peak Ca²⁺ current was reduced by 45%. D1 and H1: normalized Ca²⁺ currents (as in D and H) from a different experiment.

**FIG. 6.** Ca²⁺ currents obtained for a “short” and a “long” nerve action potential. The experiment was done with a perfused macropatch electrode. The electrode contained 0.6 µM TTX, 40 µM dTC, 10 µM TEA, and 100 µM 3,4-DAP. The Ca²⁺ current was obtained by adding 20 µM Cd²⁺, and subtraction. The Cd²⁺ was washed out and 2 mM TEA and 50 µM 3,4-DAP were added to the bath solution to establish a control current. Cd²⁺ (20 µM) was then added again to the electrode, and subtraction gave the Ca²⁺ current (——).
The results of Fig. 6 thus confirm that the technique used is sensitive to detect changes in the time course of the Ca$^{2+}$ currents if they do occur.

The results of Figs. 5 and 6 are thus compatible with the conclusion that the changes in the time course of release seen in the M$_2$-KO mice (Fig. 4, C and D) are not due to marked changes in the kinetics of the Ca$^{2+}$ current.

In M$_2$-KO mice, but not in WT mice, the synaptic delay histograms are sensitive to entry of Ca$^{2+}$

We also examined whether modulation in Ca$^{2+}$ entry affects the time course of ACh release measured directly by constructing synaptic delay histograms (Katz and Miledi 1965). To do so, synaptic delay histograms, for each population separately (Fig. 7 for WT mice and Fig. 8 for M$_2$-KO mice), were measured for their response to experimental manipulations known to affect Ca$^{2+}$ entry.

Increase in Ca$^{2+}$ influx was achieved either by increasing the amplitude of the depolarizing pulse or by increasing [Ca$^{2+}$]$_o$. Synaptic delay histograms (presented by continuous lines) obtained in WT mice, when the same terminal was depolarized by three levels of depolarization, are seen in Fig. 7A. The inset shows the delay histogram constructed by bins from which the continuous lines were formed. It is clear that as the pulse amplitude increased from $-0.5$ to $-0.7$ $\mu$A, the quantal content (reflected by the area of the histogram) increased. At $-0.5$ $\mu$A, the quantal content was $0.04$, and at $-0.7$ $\mu$A, it was $0.3$, an approximately sevenfold increase. Normalization of these three histograms (Fig. 7B) shows complete superposition. The $\tau_D$'s (see METHODS) of the three histograms were very similar (Table 1, WT mice). Another way to affect Ca$^{2+}$ entry is by changing [Ca$^{2+}$]$_o$. Figure 7, C and D, shows that the quantal content increased from $0.07$ in $1$ mM [Ca$^{2+}$]$_o$ to $0.17$ in $3$ mM [Ca$^{2+}$]$_o$. Normalization of the histograms shows no meaningful changes in the time course of release (Table 1 and Fig. 7D). Thus in WT mice, the quantal content increased with treatments known to increase Ca$^{2+}$ entry, but the time course of release was not altered.

In several such experiments, the same trend was obtained; the quantal content varied with the different experimental manipulations, while the time course of release remained rather constant (Table 2).

The M$_2$-KO mice exhibited basic different behavior in response to the same experimental treatments. Figure 8A shows delay histograms obtained at three levels of depolarization. As for the WT mice, the quantal content increased as the pulse amplitude increased, but different from the WT mice, the histograms became longer the higher the depolarization was.

![Fig. 7](https://example.com/fig7.png)
Normalization of the histograms (Fig. 8B) shows that the decay of the histograms was prolonged (Table 1). The observation that the time course of release is not sensitive to pulse amplitude in WT mice (Fig. 7A), whereas release is prolonged as the pulse amplitude increases in M2-KO mice (Fig. 8A) weakens the possibility that M2R-mediated membrane delimited inhibition of Ca2+/H11001 channels, which is voltage dependent (reviews by Hille 1994; Jarvis and Zamponi 2001a; Zamponi and Snutch 1998), underlies the prolongation of release seen in M2-KO mice. This is because in M2-KO mice, such inhibition is expected to be lacking altogether, and hence, contrary to the observations, the time course of release is expected to be the same at all pulse amplitudes.

Differences in behavior of the M2-KO mice and WT mice were also seen when delay histograms obtained in 1 and 3 mM [Ca2+]/H11001 were compared (compare Figs. 7, C and D, and 8, C and D, and see Table 1). The average results of several such experiments are given in Table 2. The cumulative results show that the dependence of the delay histograms on treatments known to affect Ca2+/H11001 entry is markedly different in WT and M2-KO mice.

In M2-KO mice, but not in WT mice, the synaptic delay histograms are sensitive to treatments known to affect accumulation of Ca2+.

Increasing the frequency of stimulation was shown to elevate the level of intracellular Ca2+ concentration and simulta-

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<td>m2/m1</td>
</tr>
<tr>
<td>1.4-fold change in PA</td>
<td>7</td>
<td>12 ± 4**</td>
</tr>
<tr>
<td>3-fold change in [Ca2+]o</td>
<td>5</td>
<td>3.2 ± 0.9**</td>
</tr>
<tr>
<td>10-fold change in SF</td>
<td>6</td>
<td>7.3 ± 1.3**</td>
</tr>
<tr>
<td>Before and after BAPTA-AM</td>
<td>5</td>
<td>0.12 ± 0.1**</td>
</tr>
</tbody>
</table>

Values are means ± SD. ** P < 0.001; * P > 0.9.
neously the quantal content (Delaney et al. 1989; Ravin et al. 1997, 1999). We therefore checked whether stimulation at three frequencies affects differently the time course of release in WT and M₂-KO mice. Figure 9A shows that in WT mice increasing the frequency of stimulation (3, 10, and 30 Hz) increased the quantal content from 0.07 (3 Hz) to 0.55 (30 Hz). Normalization of the corresponding histograms shows that the histograms are very similar, and so are the $\tau_{1/2}$s (Table 1 and Fig. 9B). Thus in WT mice, repetitive firing at frequencies of $\leq 30$ Hz showed different quantal contents, but the time course of release was not altered.

Note, that under our experimental conditions, repetitive stimulation, even at 30 Hz, did not produce a transient or delayed release (Barrett and Stevens 1972; Chen and Regehr 1999; Miledi and Thies 1971; Rahamimoff and Yaari 1973; Van der Kloot and Molgo 1994) as evident from the excellent fit of the decay of the histograms by a single exponential.

In the M₂-KO mice, the behavior of the time course of release was different at the same three frequencies. Figure 9C shows the three delay histograms obtained at 3, 10, and 30 Hz. The respective quantal contents were 0.12, 0.73, and 1.1. Here, however, normalization of the corresponding histograms (Fig. 9D) shows that the decay phase of the delay histogram was prolonged (see also Tables 1 and 2). These results emphasize again the difference in behavior of the time course of release in WT and M₂-KO mice.

In M₂-KO mice, but not in WT mice, the synaptic delay histograms are sensitive to treatments known to affect the removal of Ca$^{2+}$.

Faster than normal removal of Ca$^{2+}$ could be achieved by superfusion of the terminal with the fast Ca$^{2+}$ chelator, BAPTA-AM (Angelson and Betz 2001). Figure 10A shows a delay histogram obtained in WT mice before (---) and after 10 min of incubation with BAPTA-AM (- - -). It is reasonable to conclude that Ca$^{2+}$ was removed faster as the quantal content was reduced from 1.1 in the control to 0.08 (Table 1, a

**FIG. 9.** The synaptic delay histograms from M₂-KO mice, but not from WT mice, are sensitive to treatment known to affect steady-state [Ca$^{2+}$]. A: delay histograms from WT mice (1,000 pulses, $-0.5$ μA, [Ca$^{2+}$]$_o$ = 3 mM) at stimulation frequencies of 3 Hz (---, $m = 0.07$), 10 Hz (···, $m = 0.41$), and 30 Hz (- - -, $m = 0.55$). B: peak normalization of the histograms presented in A. B and D, insets: exponential fits of the decay phases of the normalized histograms ($R^2 > 0.99$), 3 Hz; 3 Hz; 3 Hz; 3 Hz; 3 Hz. C: delay histograms from M₂-KO mice (1,000 pulses, $-0.5$ μA, [Ca$^{2+}$]$_o$ = 3 mM) at stimulation frequencies of 3 Hz (---, $m = 0.12$), 10 Hz (···, $m = 0.73$), and 30 Hz (- - -, $m = 1.1$). D: peak normalization of the histograms presented in C.
reduction of 93%). Yet, when normalized, the two histograms superimpose nicely (Fig. 10). A similar behavior was seen in four additional experiments (Table 2). It should be noted that incubation with BAPTA-AM for longer periods than those that were used here (up to an hour) shorten the duration of release in frog neuromuscular junction (results not shown). But concerning the experiments described here, there was no point in prolonging the duration of incubation with BAPTA-AM, as a reduction of ~90% in release was already achieved after 10 min of incubation (see Table 1).

The results seen here are similar to those obtained in crayfish neuromuscular junction, where injection of BAPTA, or a BAPTA-like compound (nitr-5) greatly decreased the quantal content without affecting the time course of release (Hochner et al. 1991). Injection of BAPTA into the presynaptic terminal of the squid giant synapses reduced release (Adler et al. 1991), but the time course of release was not measured.

When such experiments were repeated in M₂-KO mice, similar to the WT mice, the quantal content declined from 1.0 to 0.1 (90% decline), but now normalization of the histograms shows that in the presence of BAPTA-AM, release stopped sooner and the \( \tau_D \) was shorter (Fig. 10, C and D). Three additional experiments showed similar results (Table 2). Clearly, the lack of functional M₂R receptors caused the delay histogram in M₂-KO mice to be sensitive to the rate at which \( \text{Ca}^{2+} \) was removed from the vicinity of the release sites.

Analysis of the pooled results of the different experimental treatments described in the preceding text (Figs. 7–10) showed that there was no correlation between \( \tau_D \) and the quantal content (\( m \)) in WT mice. \( m \) varied greatly, between 0.06 and 1.2, whereas \( \tau_D \) remained constant and short. In contrast, in M₂-KO mice, \( \tau_D \) became longer as \( m \) increased, showing a positive correlation between \( m \) and \( \tau_D \) (Fig. 11A). These results indicate that in the M₂-KO mice a common mechanism controls both the quantal content and the time course of ACh release, whereas, in the WT mice, the mechanism that determines the quantal content differs from the mechanism that controls the time course of release (Parnas and Parnas 1999).
Release starts sooner and lasts longer in M2-KO mice than in WT mice

To further resolve the question of whether or not a common mechanism underlies the kinetics of ACh release in WT and M2-KO mice, we compared the time course of summed delay histograms of the two populations (Fig. 11B). The individual delay histograms for either WT (n = 11) or M2-KO (n = 13) mice were summed, and a normalized (to peak) delay histogram was obtained. It is seen that release in M2-KO mice starts sooner, and as a result, the rise time of release is longer than in WT mice. Furthermore, release lasts longer in the M2-KO mice than in WT mice. The cumulative results support the notion that in WT mice it is the M2R that controls the time course of ACh release, while in M2-KO mice some other factor controls it. The results of Figs. 4–10 suggest that in the M2-KO mice this other factor is entry and the rate of removal of Ca2+.

DISCUSSION

The Ca2+ hypothesis for neurotransmitter release asserts that Ca2+ is the only limiting factor in the release process, at least for single action-potential-evoked release, as long as other mechanisms such as depletion (Schneeggenburger et al. 1999; Stevens and Wesseling 1998; Wang and Kaczmarek 1998) or as-yet undefined inactivation of the release process (Yamada and Zucker 1992) do not take place under the experimental conditions used. It follows that the entry of Ca2+ triggers initiation of release, and its fast removal from the vicinity of the release sites causes termination of release (see references in INTRODUCTION).

The results presented here are not compatible with the assertion that influx of Ca2+ and its removal govern initiation and termination of release. This is because we showed that in WT mice (similarly to other preparations, see INTRODUCTION for references) the time course of release was not sensitive to treatments that affect Ca2+ influx and its rate of removal. This suggests that under a wide range of physiological conditions, Ca2+ entry and removal are not the rate limiting steps in determining the time course of release.

It was argued that the often-observed insensitivity of the time course of release to changes in Ca2+ influx (see INTRODUCTION for references) might be explained in the framework of the Ca2+ hypothesis. Accordingly, such treatments produce only a small change (in the tens of microseconds range) in the kinetics of Ca2+ transients, and this, in practice, does not produce measurable changes in the time course of release (Meinrenken et al. 2002). The sensitivity of the time course of release in the M2-KO mice to changes in Ca2+ influx (Figs. 4, 5, 8, and 9) is not compatible with this explanation. These results show that the methods used here to measure the time course of release, either synaptic delay histograms or the rise time of the EPSC, are sufficiently sensitive to detect changes in the time course of release, if indeed they do occur. These results further suggest that when Ca2+ is the only limiting factor for release, as probably is the case in the M2-KO mice, the time course of release does become sensitive to changes in Ca2+ influx and its rate of removal.

It could be argued that our results may still be explained in the framework of the Ca2+ hypothesis. Specifically, the lack of functional M3R in the M2-KO mice could affect the kinetics of the Ca2+ currents in these mice, and this in turn would affect the time course of release. The cumulative results, in general, and the results seen in Figs. 5 and 6, in particular, render this possibility unlikely.

Fif not Ca2+, what is the limiting factor in control of the kinetics of ACh release?

Slutsky et al. (2001) showed that the time course of ACh release is determined by the M2R. Acknowledging the key role that Ca2+ plays in control of release, this would suggest that during initiation of release the process(es) mediated by the M3R should be slower than the process(es) governed by the influx of Ca2+: and during termination of release, the M3R mediated process(es) should be faster than the removal of Ca2+.

Indeed, we found that, on average, in M2-KO mice, release starts sooner and lasts longer than in WT mice. Our results thus indicate that in the mouse neuromuscular junction, both initiation and termination of the physiological action-potential-evoked ACh release are controlled by mechanisms that involve the presynaptic M3R receptors.

The limiting factor for termination of release could artifically become the removal of Ca2+. This would be the case if the preparation is subjected to long periods of incubation with BAPTA-AM. Under these nonphysiological conditions, the removal of Ca2+ could become faster than the M3R-mediated process that normally governs termination of release.

The M3R-controlled mechanisms operate for single action-potential-evoked release or for release observed at frequencies of <30 Hz. This conclusion is reached because under such experimental conditions the time course of release in WT mice is robust and independent of Ca2+. However, at high-frequency stimulation (>50 Hz), the time course of ACh release becomes sensitive to Ca2+ as evident from the observation that at these high frequencies (but not at lower ones, see Fig. 10),

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BAPTA-AM shortened the time course of release also in WT mice (results not shown). Similar sensitivity to Ca\(^{2+}\) was seen in cerebellar synapses where delayed release was eliminated after application of EGTA (Chen and Regen 1999). Note, however, that under the experimental conditions employed here, delayed release was not seen in the mouse neuromuscular junction.

Possible physiological significance of two limiting processes in control of release

It may be asked, why did nature “invent” separate processes to control the time course and the amount of neurotransmitter release? Our data show that when the same process governs both the quantal content and the time course of release, as in the M\(_2\)-KO mice, modulation of the amount of release is accompanied by changes in the time course of release. The existence of two separate processes, one controlling the amount of release, and the other controlling the time course of release, may guarantee the robust and reproducible time course of release required for reliable neuronal communication, and concomitantly, enable synaptic plasticity by modulation of the amount of release. The following example illustrates the preceding arguments. Twin pulse facilitation is governed mainly by residual Ca\(^{2+}\) (Katz and Miledi 1968). The duration of facilitation ranges between tens of milliseconds to seconds (Mallart and Martin 1967; Rahamimoff 1968), whereas the time course of release in these cases is constant (Datyner and Gage 1980) and brief, in the millisecond range.

What may be the mechanism that underlies the M\(_2\)R-mediated control of the kinetics of ACh release?

In this work, we consolidated earlier findings (Slutsky et al. 2001), indicating that the time course of ACh release is determined by the M\(_2\)R. Unraveling the mechanism by which the M\(_2\)R controls initiation and termination of release is beyond the scope of this work. Nevertheless, based on earlier work (reviewed by Parnas et al. 2000), the following working hypothesis may be suggested. At rest (resting potential and resting concentration of transmitter in the synaptic cleft), release is tonically blocked. This block is achieved by a physical interaction between the transmitter occupied inhibitory autoreceptor and key proteins of the exocytotic machinery. Initiation of release is achieved on relief of the tonic block by depolarization. The free release machinery, together with the Ca\(^{2+}\) that had entered, then initiates release. Release terminates when this block is reinstated on membrane repolarization.

At present, this hypothesis relies mainly on studies concerning release of ACh. The suggestion that at rest, the release machinery is under tonic block, is supported by the finding that release of ACh was enhanced by addition of muscarinic receptor antagonists (D’Agostino et al. 1986; Morita et al. 1982; Peteris and Ogren 1988; Slutsky et al. 1999; Wessler 1988). The notion that this block may be achieved by association of the M\(_2\)R with proteins of the exocytic machinery gains support from the following findings. It was shown that the M\(_2\)R co-precipitates with SNARE proteins and with synaptotagmin in rat brain synaptosomes using Ca\(^{2+}\)-depleted solutions. Furthermore, this association is voltage dependent and takes place only if the M\(_2\)R is bound to its agonist (Ilouz et al. 1999; Linial et al. 1997). Finally, it was recently shown that termination of release involves binding of ACh to the M\(_2\)R, as retardation of binding of ACh to the M\(_2\)R prolonged the time course of ACh release (Slutsky et al. 2001). Recall that it is the M\(_2\)R that mediates feedback inhibition of ACh release (Allen and Brown 1993; Rouse et al. 1997; Slutsky et al. 1999, 2002).

The precise molecular mechanism by which the M\(_2\)R exerts its controlling effect on the kinetics of ACh release is not known. Before we detail possible mechanisms, it should be borne in mind that most, if not all, studies (see also the two examples discussed in the following text) that examined effects of G-protein-coupled receptors (GPCRs), as the M\(_2\)R is, measured their effects on the quantal content. In contrast, we are concerned here mainly with effects of the M\(_2\)R on the kinetics of ACh release. We will, nevertheless, consider two possible molecular mechanisms. One would involve M\(_2\)R-mediated G-protein modulation of voltage-gated Ca\(^{2+}\) channels. In this case, the tonic inhibition of release could be achieved by membrane-delimited block of Ca\(^{2+}\) channels (Jarvis and Zamponi 2001b; Jarvis et al. 2000), for example, by cysteine string proteins, which interact with both G proteins and N-type Ca\(^{2+}\) channels (Magga et al. 2000). Our results showing that only in the M\(_2\)-KO mice, where the M\(_2\)R is not functional, the time course of release was sensitive to changes in Ca\(^{2+}\) influx and removal, renders this possibility unlikely.

Another possibility that is more compatible with our results is the following. Blackmer et al. (2001) showed that G protein \(\beta\)y subunits mediate presynaptic inhibition of transmitter release by a Ca\(^{2+}\)-independent mechanism. These authors, hence, suggested that this inhibition takes place “downstream of Ca\(^{2+}\) entry.” However, the preceding suggested mechanism could easily underlie some aspects of our hypothesis. Accordingly, the transmitter occupied M\(_2\)R was shown to interact with the SNARE proteins (Linial et al. 1997). This interaction could be achieved by the mechanism suggested by Blackmer et al. (2001), i.e., by means of G protein \(\beta\)y subunits. This interaction, in turn, would, as suggested by us, cause tonic inhibition of the release machinery.

It seems, therefore that with minor modification in interpretation, the molecular mechanism suggested by Blackmer et al. (2001) could be embodied in our hypothesis. Accordingly, the mechanism that was suggested for Ca\(^{2+}\)-independent presynaptic inhibition (Blackmer et al. 2001) could underlie the tonic inhibition suggested by us. Furthermore, this Ca\(^{2+}\)-independent inhibition could occur prior, and in parallel, to Ca\(^{2+}\) influx (Parnas et al. 2000) rather than downstream to Ca\(^{2+}\) influx (Blackmer et al. 2001). But, irrespective of the precise mechanism, our results justify the conclusion that the kinetics of ACh release under physiological conditions is governed by the M\(_2\)R. Only when the M\(_2\)R is not functional, as is the case in the M\(_2\)-KO mice, the kinetics of release is governed by influx and removal of Ca\(^{2+}\).

Finally, we suggest that regulation of the time course of release by presynaptic inhibitory autoreceptors may be a general mechanism serving also other peripheral and central fast synapses (e.g., glutamatergic and GABAergic). The type of presynaptic autoreceptors will obviously vary according to the released transmitter.

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REFERENCES


Franke K, Hatt H, Parnas H, and Dudel J. Kinetic constants of the acetylcholine (ACH) receptor reaction deduced from the rise in open probability after steps in ACh concentration. *Biophys J* 60: 1008–1016, 1991.


