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Mitochondrial Involvement in Post-Tetanic Potentiation of Synaptic Transmission

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Summary

Posttetanic potentiation (PTP) is an essential aspect of synaptic transmission that arises from a persistent presynaptic $[Ca^{2+}]_i$ following tetanic stimulation. At crayfish neuromuscular junctions, several inhibitors of mitochondrial Ca^{2+} uptake and release (tetraphenylphosphonium or TPP^+, carbonyl cyanide *m*-chlorophenylhydrazone or CCCP, and ruthenium red) blocked PTP and the persistence of presynaptic residual $[Ca^{2+}]_i$, while endoplasmic reticulum (ER) Ca^{2+} pump inhibitors and release channel activators (thapsigargin, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone or BHQ, and caffeine) had no effects. PTP apparently results from the slow efflux of tetanically accumulated mitochondrial Ca^{2+}.

Introduction

At most synapses, extensive high-frequency or tetanic presynaptic activity leads to an enhancement of synaptic transmission lasting many minutes, called posttetanic potentiation (PTP) (Zucker, 1989). This is a critical process in the nervous system, since it shapes the responses and information processing functions of neural circuitry, can modify motor activity, and is involved in behavioral modifications including simple forms of learning (Fisher et al., 1997). Following a briefer period of presynaptic activity, transmission is enhanced for a shorter period (less than one min), a process called augmentation. In addition, a third process called facilitation increases transmission within about 1 s, and decays just as quickly after stimulation. PTP, augmentation, and facilitation are all generated by the continuing action of residual presynaptic calcium ions, whose concentration ($[Ca^{2+}]_i$) remains elevated after the conditioning tetanus and increases the number of transmitter quanta released by each presynaptic action potential (Delaney et al., 1989; Delaney and Tank, 1994; Kamiya and Zucker, 1994; Regehr et al., 1994; Brain and Bennett, 1995; Atluri and Regehr, 1996). The long duration of PTP is due to a prolonged residual $[Ca^{2+}]_i$ following a strong tetanus, but the origin of this persistent $[Ca^{2+}]_i$ has remained elusive.

Neuronal $[Ca^{2+}]_i$ is regulated (Brinley, 1978; McBurney and Neering, 1987; Tsien and Tsien, 1990; Miller, 1991; Pozzan et al., 1994) by plasma membrane extrusion pumps, cytoplasmic buffers, and at least two intracellular organelles: mitochondria (Thayer and Miller, 1990; Al-Baldawi et al., 1993; Friel and Tsien, 1994; Wang et al., 1994; Werth and Thayer, 1994; White and Reynolds, 1995; Herrington et al., 1996) and ER (Brorson et al., 1991; Reber and Reuter, 1991; Friel and Tsien, 1992; Irving et al., 1992; Hua et al., 1993; Reber et al., 1993; Llano et al., 1994; Tse et al., 1994; Kano et al., 1995). We have investigated whether these organelles are involved in maintaining posttetanic presynaptic $[Ca^{2+}]_i$ elevated at levels sufficient to activate PTP. We used the excitatory (glutamatergic) neuromuscular junctions on crayfish leg opener muscles to study the effects of specific pharmacological inhibitors on presynaptic $[Ca^{2+}]_i$ levels imaged with the fluorescent Ca^{2+} indicator fura-2, and on transmitter release monitored by postsynaptic electrophysiological recording.

Results

We produced PTP by stimulating the motor axon for 7–10 min at 20–33 Hz. EJPs were recorded before and after the tetanus at 0.2–0.33 Hz, as well as during the tetanus. Presynaptic $[Ca^{2+}]_i$ was measured simultaneously in most experiments. The left-hand traces in Figures 1A and 1B show measurements of $[Ca^{2+}]_i$ and peak EJP amplitudes before, during, and after a tetanus. The increase in EJP amplitude from 0.2 mV to 15 mV during the tetanus includes the combined effects of augmentation, potentiation, and facilitation. PTP is revealed by the posttetanic decay in EJP amplitude back to pretetanic levels.

We examined effects of drugs on PTP and presynaptic $[Ca^{2+}]_i$ changes by waiting 1.5 hr for PTP to decay fully and then repeating the EJP and $[Ca^{2+}]_i$ measurements evoked by a second tetanus delivered 10 min after adding the drug to the bathing medium. We first examined the response to two tetani in the absence of drugs (Figures 1A and 1B). We quantified PTP by fitting an exponential function to the slowly decaying component of the posttetanic decay in EJP amplitude. In three preparations, PTP amplitude was 0.9 ± 0.8 mV (mean $\pm$ SD) after the first tetanus and 0.8 ± 0.3 mV after the second, showing no difference ($P > 0.5$). Similarly, the peak amplitude of the EJP during the first tetanus (11.6 ± 2.3 mV) was identical to the amplitude during the second tetanus (11.4 ± 2.5 mV, $P > 0.5$). An example of the response of $[Ca^{2+}]_i$ to repeated tetani is shown in Figure 1A. These also were unchanged.

Mitochondrial Ca^{2+} Sequestration Is Required for PTP

Mitochondrial Ca^{2+} sequestration and release were blocked by each of three drugs: TPP^+, CCCP, or ruthenium red. TPP^+ is a lipophilic cation known to block Na^+-dependent ($K_I = 0.2$ μM) and Na^+-independent ($K_I = 10$ μM) mitochondrial Ca^{2+} efflux with little effect on ATP production (Aiuchi et al., 1985; Karadjov et al., 1986; Wingrove and Gunter, 1986; Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994). Figures 1C–1F show effects of 10 μM TPP^+ on tetanic and posttetanic Ca^{2+} accumulation and EJP amplitude. No effects on pretetanic $[Ca^{2+}]_i$ or synaptic strength were observed (Table 1, left column, lines 11 and 12), which also rules out nonspecific postsynaptic effects. However, significant increases in peak $[Ca^{2+}]_i$ and EJP amplitude during tetanic

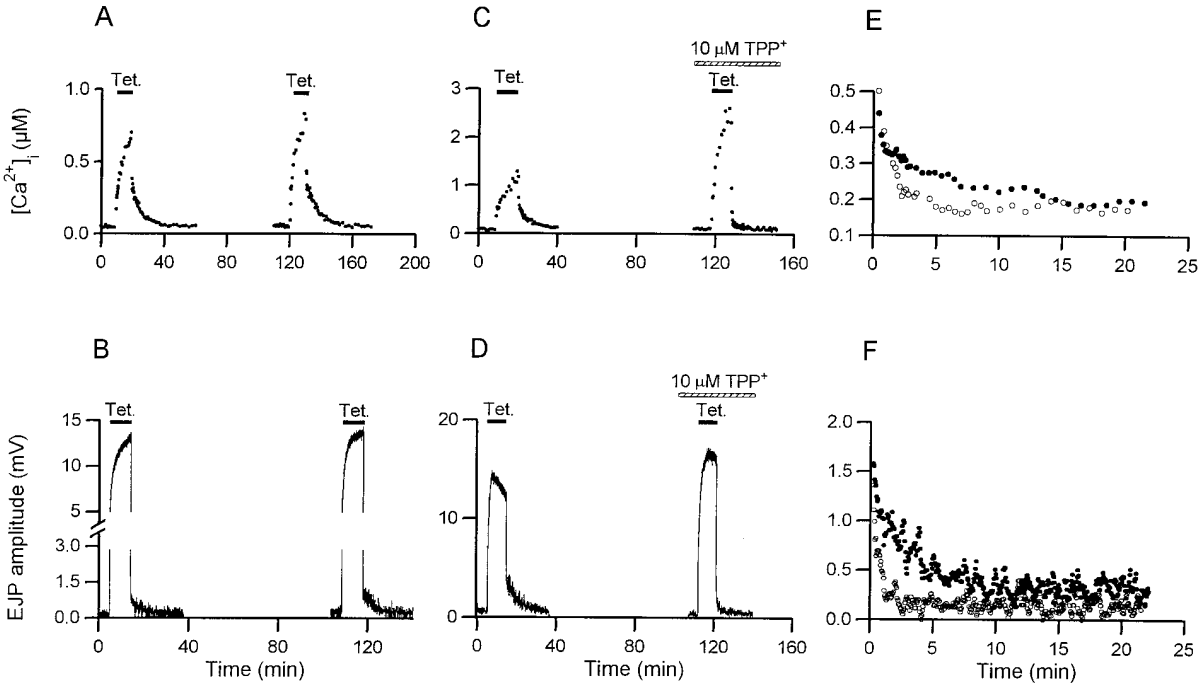

Figure 1. Effects of TPP^+ on $[Ca^{2+}]_i$ and PTP

(A and B) Control experiment showing unchanged $[Ca^{2+}]_i$ and EJP amplitudes in response to 2 tetani (Tet.: 20 Hz for 10 min).

(C and D) The first tetanus is given in normal saline and the second tetanus is given in the presence of 10 μM TPP^+.

(E) Running averages of 5 successive posttetanic $[Ca^{2+}]_i$ measurements in control saline (closed circles) and in the presence of TPP^+ (open circles).

(F) Running averages of 9 successive posttetanic EJP amplitudes from the same preparation as in (E), obtained simultaneously with the $[Ca^{2+}]_i$ measurement.

stimulation occurred (Table 1, lines 1 and 2), and the slow posttetanic components of both $[Ca^{2+}]_i$ and potentiated EJP amplitudes were significantly reduced (Table 1, lines 3, 4, 6, and 7). Similar results were obtained in eight such experiments (Table 1). PTP amplitude was estimated in two ways: as the EJP amplitude 1 min after the tetanus (Table 1, line 3), or as the extrapolated magnitude of the slowly decaying exponential component of EJP amplitude (Table 1, line 4). A faster component of EJP decay, corresponding to augmentation, was detected in seven of the eight preparations and was increased by TPP^+ (Table 1, line 9). The decay time constants of augmentation and potentiation were not affected by TPP^+ (Table 1, lines 5 and 10). Owing to muscle movements at the end of the tetanus, we were unable to measure the augmentation phase of posttetanic $[Ca^{2+}]_i$, but a slowly decaying phase corresponding to PTP was reduced significantly by TPP^+ (Table 1, line 8).

TPP^+ appeared to selectively block PTP *after* a tetanus while increasing posttetanic augmentation. We therefore tried to determine how changes in potentiation, augmentation, or facilitation contributed to the overall increase in EJP amplitude *during* stimulation caused by TPP^+. In both the presence and absence of drugs, tetanic potentiation was sometimes obscured by a slowly developing depression (Figures 1 and 2), and so could not be measured unambiguously as a separate process during stimulation. But the early plateau in EJP amplitude should be the sum of augmentation and facilitation. This sum was increased by 19 ± 24% ($P \simeq 0.05$, $n = 8$). TPP^+ apparently has no effect on facilitation; the average of 5 EJPs 1 s after the start of a 20–33 Hz tetanus, when augmentation remained negligible, was the same after adding 10 μM TPP^+ (6 ± 51 % increase, $n = 8$, $P > 0.9$). Therefore, tetanic augmentation was increased by TPP^+, consistent with the increase in the augmentation component of posttetanic EJP decay (Table 1, line 9). Since this increase in augmentation is similar to the overall increase in EJP amplitude during tetanic stimulation (Table 1, line 1), it appears to be the main process enhanced during the tetanus.

CCCP is an uncoupler of oxidative phosphorylation that dissipates the mitochondrial membrane potential driving uniporter Ca^{2+} uptake (Heytler and Pritchard, 1962; Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994); it also blocks Ca^{2+} efflux ($K_l = 300$ nM). Unlike TPP^+, CCCP often caused a progressive depression of EJP amplitude to appear during tetanic stimulation, which interfered with PTP measurement. We therefore restricted our analysis to 5 preparations (out of 19) in which CCCP caused little or no depression.

Like TPP^+, 2 μM CCCP increased the tetanic EJP amplitude and peak level of $[Ca^{2+}]_i$ without affecting pretetanic levels (Figures 2A and 2B; Table 1, middle column, lines 1, 2, 11, and 12). Although the lack of

Table 1. Effects of TPP^+, CCCP, and Ruthenium Red on Presynaptic $[Ca^{2+}]_i$ and Synaptic Plasticity. Various Measures of Augmentation, Potentiation, Baseline Synaptic Transmission, and Levels of Presynaptic $[Ca^{2+}]_i$ Measured Before and After Tetanic Stimulation Are Shown Before and After Exposure to the Indicated Concentration of Drug

	TPP^+		CCCP		Ruthenium Red	
Measurement	Control	10 μM	Control	2 μM	Control	10–25 μM*
1. Peak EJP amplitude during tetanus (mV)	24.1 ± 4.0	27.0 ± 4.3 †(8)	15.4 ± 5.3	20.2 ± 6.6 †(5)	19.1 ± 4.7	23.8 ± 5.7 (5)
2. Peak $[Ca^{2+}]_i$ during tetanus (nM)	1030 ± 340	2050 ± 890 †(8)	660 ± 260	2830 ± 1070 †(6)		
3. Potentiated EJP amplitude 1 min after tetanus (mV)	1.5 ± 0.9	0.8 ± 0.4 †(8)	1.1 ± 0.7	0.4 ± 0.2 †(5)	0.9 ± 0.8	0.4 ± 0.4 (5)
4. Extrapolated amplitude of PTP after tetanus (mV)	1.5 ± 1.0	0.5 ± 0.4 †(8)	0.8 ± 0.5	0.3 ± 0.6 ‡(5)	0.8 ± 0.7	0 †(5)
5. PTP decay time constant (min)	3.49 ± 2.02	3.63 ± 1.69 (6)§	5.02	1.12 (1)§	§	§
6. $[Ca^{2+}]_i$ 1 min after tetanus (nM)	340 ± 80	240 ± 90 ‡(8)	380 ± 130	840 ± 130 †(6)		
7. Extrapolated amplitude of slow $[Ca^{2+}]_i$ decay after tetanus (nM)	264 ± 91	123 ± 133 †(8)	390 ± 170	620 ± 340 (6)		
8. Slow $[Ca^{2+}]_i$ decay time constant (min)	2.53 ± 1.15	3.32 ± 1.93 (5)§	4.27 ± 1.26	3.23 ± 0.69 †(6)		
9. Extrapolated amplitude of augmentation after tetanus (mV)	14.5 ± 7.4	21.9 ± 12.6 †(7)			5.1 ± 3.9	4.8 ± 2.9 (4)
10. Augmentation decay time constant (s)	5.4 ± 1.9	5.4 ± 1.3 (7)			8.7 ± 1.9	5.3 ± 2.4 (4)
11. Pretetanic EJP amplitude (mV)	0.3 ± 0.2	0.2 ± 0.1 (8)	0.3 ± 0.1	0.4 ± 0.2 (5)	0.2 ± 0.2	0.3 ± 0.2 (5)
12. Pretetanic $[Ca^{2+}]_i$ (nM)	90 ± 30	90 ± 30 (8)	80 ± 40	100 ± 30 (6)		

* Estimated concentration range in nerve terminals.
Significant changes by drug normalized to controls (Student's *t* test): †, $P < 0.01$; ‡, $P < 0.05$.
Data mean ± SD; numbers of measurements in parentheses.
§ Time constant averages, but not extrapolated amplitudes, exclude instances when PTP or augmentation was not measurable after drug application.

effect on pretetanic EJP is a test for nonspecific effects of CCCP, we also measured CCCP's effect on postsynaptic input resistance, which remained unchanged (0 ± 30% change, $n = 10$, $P > 0.9$). PTP was reduced or eliminated (Table 1, lines 3–5), similar to the effect of TPP^+. The slow phase of posttetanic $[Ca^{2+}]_i$ often decayed more rapidly than in controls to a permanently elevated level after CCCP treatment (Table 1, line 8); there was no significant difference in the amplitude of this phase (Table 1, line 7). We currently have no explanation for the more rapid decay.

If mitochondria become loaded with Ca^{2+} during a tetanus, then CCCP should release this Ca^{2+}. Figure 3A shows the result of one such experiment, where CCCP applied shortly after tetanic stimulation caused a large transient increase in $[Ca^{2+}]_i$. Similar effects were observed in four of five experiments in which the average amplitude of the $[Ca^{2+}]_i$ transient was 470 ± 260 nM ($n = 4$). We did not record EJP amplitudes in these experiments, but we have reported previously that CCCP increases transmitter release 2–4 fold at this synapse (Zucker and Landò, 1986; Zucker et al., 1986).

The synaptic depression and persistently elevated $[Ca^{2+}]_i$ sometimes observed after exposure to CCCP might be consequences of the reduction in ATP production caused by oxidative uncouplers, since ATP is required for vesicle docking and loading vesicles with transmitter (Molgo and Pecot-Dechavassine, 1988; Holz et al., 1989; Llinás et al., 1989; Hay and Martin, 1992; Parsons et al., 1995; Schiavo et al., 1995), as well as Ca^{2+} extrusion (Brinley, 1978; McBurney and Neering, 1987; Tsien and Tsien, 1990; Miller, 1991; Pozzan et al., 1994). However, these effects could not be prevented by oxygenation and addition of 10 mM glucose to the saline (data not shown). Furthermore, we observed no greater tendency for tetanic depression after exposure to 20 μg/ml of oligomycin, which should block mitochondrial ATP production. We also sometimes observed tetanic depression in untreated preparations (e.g., Figure 1D), and it may be unrelated to changes in [ATP]. In seven experiments, oligomycin also showed no significant effect or even a tendency to affect the magnitude or time constant of either PTP or augmentation, or the pretetanic EJP amplitude (data not shown). Thus, CCCP's effects cannot easily be attributed to changes in ATP production. The small but persistent posttetanic elevation in $[Ca^{2+}]_i$ without concurrent persistent enhancement of synaptic transmission is also somewhat puzzling. Perhaps CCCP has additional small side effects both on processes controlling Ca^{2+} regulation and on synaptic transmission following prolonged stimulation; no such effects were observed in TPP^+ experiments. We have checked for an effect of CCCP on fluorescence of fura-2 solutions, and found none.

Ruthenium red is a more specific blocker of the mitochondrial Ca^{2+} uptake uniporter ($K_I = 30$ nM) (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994), with little effect on ATP production. Since ruthenium red is membrane impermeable, it was injected presynaptically. Like CCCP, 10 μM ruthenium red increased tetanic enhancement of EJPs (Table 1, right column, line 1) and blocked PTP (Table 1, lines 3 and 4). Augmentation and pretetanic levels of transmission were unaffected (Table 1, lines 9 - 11). Because of the difficulty of sequential presynaptic injections of fura-2 and ruthenium red, we have $[Ca^{2+}]_i$ measurements from only one experiment using this drug (Figures 2C and 2D), so no summaries of such measurements appear in Table 1. Ruthenium red also led to a modest tetanic depression in EJP amplitude in five experiments (Figures 2C and 2D). In crayfish,

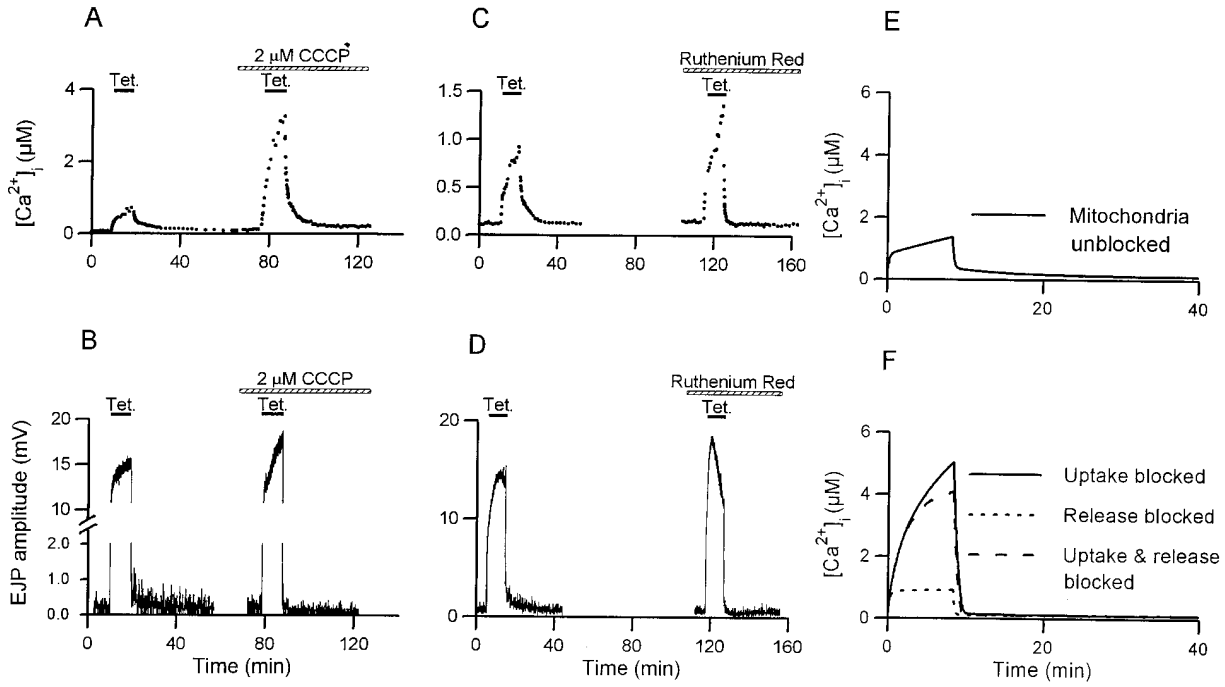

Figure 2. Effects of CCCP or Ruthenium Red on $[Ca^{2+}]_i$ and PTP

(A–D) The first tetanus is given in control saline and the second is given either in the presence of 2 μM CCCP (A and B) or after presynaptic injection of ruthenium red (C and D, 0.5 mM ruthenium red in the injection electrode). (A) and (B) show simultaneous $[Ca^{2+}]_i$ and EJP amplitudes from a single preparation exposed to CCCP. (C) shows $[Ca^{2+}]_i$ from one preparation and (D) shows EJP amplitudes from a different preparation to demonstrate the effects of ruthenium red. EJP and $[Ca^{2+}]_i$ measurements were suspended while it was pressure-injected presynaptically (final concentration about 20 μM). Tet.: stimulation at 20 Hz for 10 min.

(E and F) Computer simulations of $[Ca^{2+}]_i$ in a nerve terminal regulated by Ca^{2+} influx, Ca^{2+} extrusion pumps, cytoplasmic Ca^{2+} buffers, and mitochondrial Ca^{2+} uptake and release. (E) shows control simulation; (F) shows effects of reducing Ca^{2+} uptake by 95%, release by 95%, or both. CCCP and ruthenium red, and probably also TPP^+, block Ca^{2+} uptake; their effects resemble those of simulations blocking uptake.

ruthenium red had no direct effect on transmission, such as increasing spontaneous release rate as reported at hippocampal synapses (Trudeau et al., 1996).

Endoplasmic Reticulum Is Not Involved in PTP

We searched for a role for ER in synaptic plasticity by blocking the Ca^{2+}-ATPase responsible for Ca^{2+} influx into ER with either 2 μM thapsigargin ($K_I < 1$ nM) or 10 μM BHQ ($K_I = 400$ nM) (Brinley, 1978; McBurney and Neering, 1987; Tsien and Tsien, 1990; Miller, 1991; Pozzan et al., 1994). Neither drug affected synaptic transmission or $[Ca^{2+}]_i$ before, during, or after tetanic stimulation (Table 2). Caffeine releases Ca^{2+} from ER stores through a Ca^{2+} channel associated with ryanodine receptors (with a half-maximal effective concentration of 2.5 mM) (Weber and Herz, 1968; Endo et al., 1970; Brinley, 1978; McBurney and Neering, 1987; Tsien and Tsien, 1990; Miller, 1991; Pozzan et al., 1994) and prevents accumulation of Ca^{2+} in this class of ER. We observed no effect of 2 mM caffeine on tetanic enhancement of EJP or on PTP (Table 2). Higher concentrations of caffeine caused depression of synaptic transmission. Caffeine also had no effect on presynaptic $[Ca^{2+}]_i$ level, even when it was applied after tetanic stimulation and should have loaded ER with Ca^{2+} (Figure 3B). We did not record EJPs in these experiments, because it has been observed that caffeine has complex effects on crayfish neuromuscular transmission that appear to be unrelated to actions on presynaptic Ca^{2+} stores (K. Judd et al., 1996, Soc. Neurosci., abstract).

Discussion

Our results indicate that mitochondria play a critical role in the generation of PTP, while the ER does not. CCCP and ruthenium red are known to block Ca^{2+} uptake and release in mitochondria (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994). TPP^+ is known to block mitochondrial Ca^{2+} efflux; its effect on uptake has not been reported, but its enhancement of cytoplasmic Ca^{2+} accumulation and tetanic EJPs indicates that it is likely to block uptake as well. CCCP appears to have some effects on synaptic transmission unrelated to reducing mitochondrial Ca^{2+} transport, such as the likelihood of observing tetanic depression during stimulation. Since oligomycin does not have similar effects, ATP production is not likely to be responsible. Ruthenium red is difficult to use because it requires presynaptic pressure injection of an uncharged substance. For these reasons, TPP^+ is our preferred blocker of mitrochondrial Ca^{2+} transport, since it shares none of these defects.

All three agents could be seen to clearly reduce PTP and the posttetanic slow phase of $[Ca^{2+}]_i$ reduction, and to increase the accumulation of cytoplasmic Ca^{2+} during

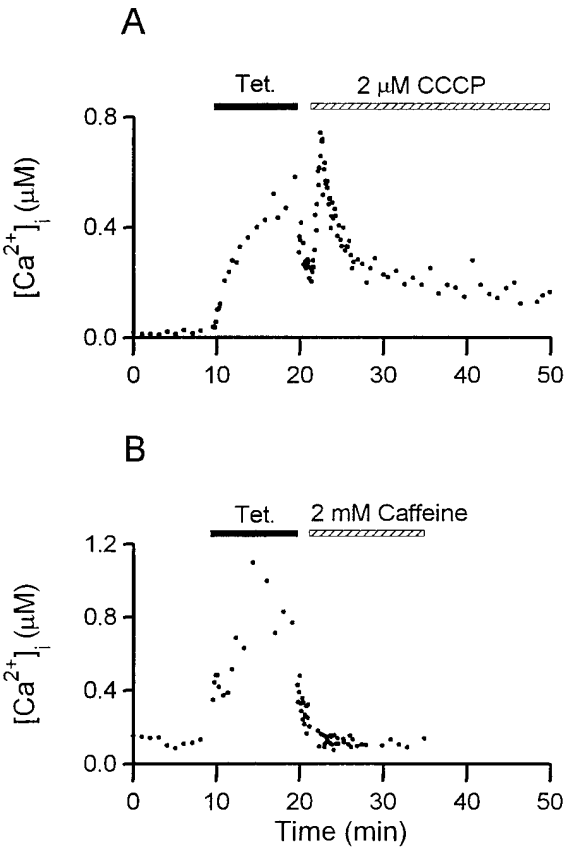

Figure 3. CCCP Can Release Ca^{2+} Stored in Mitochondria

(A) 2 μM CCCP was applied 2 min after a 20 Hz tetanus lasting 10 min, causing a transient rise in $[Ca^{2+}]_i$.

(B) Caffeine (2 mM) had no such effect.

stimulation, as well as the overall growth in tetanic synaptic transmission. The only biochemical effect shared by all three agents is an inhibition of mitochondrial Ca^{2+} transport. Our results therefore are most consistent with PTP being produced by a mechanism involving mitochondrial Ca^{2+} sequestration during tetanic stimulation and subsequent posttetanic efflux into cytoplasm. This slow mitochondrial efflux of Ca^{2+}, balanced with plasma membrane extrusion pumps, then leads to a minutes-lasting plateau of $[Ca^{2+}]_i$, which in turn acts at a site distinct from those causing exocytosis and facilitation to induce PTP (Delaney et al., 1989; Delaney and Tank, 1994; Kamiya and Zucker, 1994; Regehr et al., 1994; Brain and Bennett, 1995).

When mitochondrial Ca^{2+} uptake is blocked, $[Ca^{2+}]_i$ rises to higher levels during tetanic stimulation. This Ca^{2+} appears to act primarily at the common Ca^{2+} target triggering augmentation and potentiation (Kamiya and Zucker, 1994) to enhance transmitter release more than when mitochondria are active. The continual rise in $[Ca^{2+}]_i$ during a tetanus, while EJPs increase only slightly, may reflect a saturation of facilitation and potentiation at $[Ca^{2+}]_i$ levels exceeding 1–2 μM. The release process may also be approaching saturation, especially if release is a binomial process limited by a finite number of release sites (Zucker, 1974). However, the concurrent involvement of other factors, such as depletion of vesicles available for release, cannot be excluded.

After a tetanus, cytoplasmic Ca^{2+} is more readily extruded when mitochondria have not been loaded with Ca^{2+} and so cannot release it. Then cytoplasmic $[Ca^{2+}]_i$ decays with a time constant of seconds as determined by membrane extrusion pumps; PTP is thus blocked, and the higher initial level of transmission decays with the characteristic time constant of augmentation. Figure 4 is a cartoon of the role of mitochondria in generating PTP. In addition to loading mitochondria with Ca^{2+}, tetanic stimulation has been shown to load terminals with Na^+, reducing transmembrane Na^+ gradients and slowing the efflux of cytoplasmic Ca^{2+} dependent on Na^+/Ca^{2+} exchange. This process also contributes to the prolongation of residual Ca^{2+} responsible for PTP (Mulkey and Zucker, 1992).

As a further test of our hypothesis for mitochondrial function in PTP, we performed computer simulations of Ca^{2+} regulation in nerve terminals. We included provisions for: 1) Ca^{2+} influx through voltage-gated Ca^{2+} channels in a tetanus (40 fA average Ca^{2+} current, corresponding to 25 flickering Ca^{2+} channels open for 0.5 ms during each action potential in a 20 Hz tetanus), 2) Ca^{2+} extrusion through surface membrane pumps (first-order

Table 2. Effects of ER-Targeting Drugs on Presynaptic $[Ca^{2+}]_i$ and PTP. Since Thapsigargin and BHQ Effects Were Identical; Their Data Have Been Grouped Together

	2 μM Thapsigargin (3) and 10 μM BHQ (3–4)		Caffeine (3)	
Measurement	Control	Drug	Control	Drug
1. EJP amplitude during tetanus (mV)	18.4 ± 9.8	19.0 ± 10.0	22.3 ± 15.6	21.5 ± 14.1
2. $[Ca^{2+}]_i$ during tetanus (nM)	810 ± 340	890 ± 180		
3. Potentiated EJP amplitude 1 min after tetanus (mV)	1.9 ± 1.9	2.3 ± 2.2	2.5 ± 3.3	3.5 ± 5.0
4. Extrapolated amplitude of PTP after tetanus (mV)	1.9 ± 2.0	2.1 ± 2.3	2.0 ± 2.4	2.8 ±4.1
5. PTP decay time constant (min)	4.10 ± 0.61	3.66 ± 1.08	4.01 ± 1.18	3.78 ± 0.36
6. $[Ca^{2+}]_i$ 1 min after tetanus (nM)	310 ± 130	310 ± 130		
7. Extrapolated amplitude of slow $[Ca^{2+}]_i$ decay after tetanus (nM)	250 ± 140	270 ± 150		
8. Slow $[Ca^{2+}]_i$ decay time constant (min)	4.70 ± 2.54	4.05 ± 1.62		
9. Pretetanic EJP amplitude (mV)	0.3 ± 0.2	0.4 ± 0.3	0.5 ± 0.6	0.9 ± 1.2
10. Pretetanic $[Ca^{2+}]_i$ (nM)	90 ± 30	100 ± 10		

No measurements show significant effects of drugs ($P > 0.12$ for all comparisons). Data mean ± SD; numbers of measurements in parentheses following name of drug.

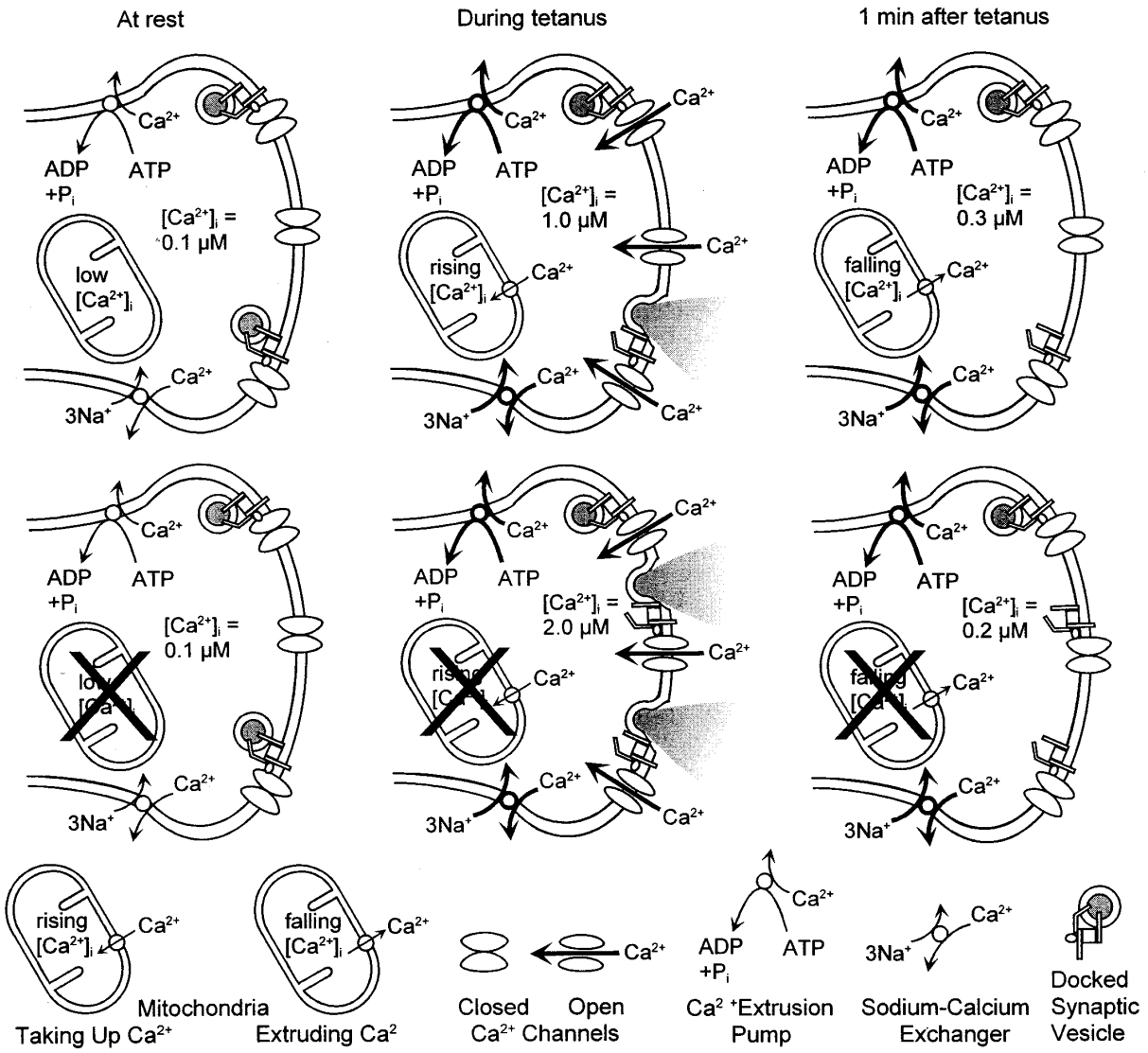

Figure 4. Pictorial Diagram of Mechanism of PTP

At rest, $[Ca^{2+}]_i$ is held to 0.1 μM by plasma membrane Ca^{2+} pumps. During a tetanus, Ca^{2+} influx raises $[Ca^{2+}]_i$ to 1 μM, regulated by the extrusion pumps and mitochondrial Ca^{2+} uptake operating at high rates. After a tetanus, Ca^{2+} efflux from mitochondria and Ca^{2+} extrusion holds $[Ca^{2+}]_i$ to 0.3 μM. Blocking mitochondrial Ca^{2+} transport has no effect at rest, allows $[Ca^{2+}]_i$ to reach a higher level (at least 2 μM) during tetanic stimulation, and reduces the late plateau elevation of $[Ca^{2+}]_i$ responsible for PTP.

extrusion with maximum efflux rate of 500 fM/cm^{2}s and half maximal extrusion at $[Ca^{2+}]_i$ = 500 nM), 3) binding of Ca^{2+} to cytoplasmic buffers (1 mM buffer concentration, K_D = 10 μM), and 4) Ca^{2+} uptake into and release from mitochondria. Mitochondria were represented as a slow buffer with the capacity for absorbing 30 mM total Ca^{2+} from cytoplasm, forward (uptake) rate constant 125 /M.s, and backward (efflux) rate constant 0.0025 /s. Ca^{2+} influx, extrusion, and buffering parameters are based on our unpublished measurements and are similar to those of Tank et al. (1995). Mitochondrial parameters were varied freely until simulated changes in tetanic and posttetanic $[Ca^{2+}]_i$ (Figure 2E) resembled experimental results without drugs (Figures 1A, 1C, 2A, and 2C). When mitochondrial uptake is blocked, the simulations (Figure 2F) reproduce the effects of mitochondrial Ca^{2+} transport inhibitors—$[Ca^{2+}]_i$ reaches higher levels during the tetanus, and the posttetanic plateau phase of residual $[Ca^{2+}]_i$ is greatly reduced.

Our results could be the first to directly implicate mitochondria in PTP. Several studies have shown the involvement of either mitochondria or ER in presynaptic $[Ca^{2+}]_i$ regulation (Alnaes and Rahamimoff, 1975; Blaustein et al., 1978; McGraw et al., 1980; Rasgado-Flores and Blaustein, 1987; Fossier et al., 1994; Stuenkel, 1994; Kobayashi et al., 1995; Grohovaz et al., 1996), but none specifically addressed synaptic plasticity. Further study will be necessary to determine whether mitochondria or ER play essential roles in synaptic plasticity at other synapses.

Experimental Procedures

Animals, Solutions, and Drugs

All experiments were performed on the opener muscle of the first walking leg of crayfish (Procambarus clarkii, 2–2.5 inches) obtained from Atchafalaya Biological Supplies (Raceland, LA) or California Golden Egg Co. (West Sacramento, CA) and kept in accordance with institutional guidelines. Autotomized legs were pinned in a continuously perfusing saline containing (in mM) 195 NaCl, 13.5 $CaCl_2$, 5.4 KCl, 2.6 $MgCl_2$, and 10 Na-HEPES (pH 7.4). The temperature of the preparation was maintained at 15–18°C. Other drugs were obtained from the following suppliers: Thapsigargin, CCCP, oligomycin, and caffeine from Sigma (St. Louis, MO); BHQ from Calbiochem (La Jolla, CA); ruthenium red from Research Biochemicals International (Natick, MA); TPP^+ chloride from Aldrich (Milwaukee, WI). Since thapsigargin, oligomycin, and BHQ were without effects, care was taken to perform most experiments using these agents with fresh solutions that were at most a few days old.

Electrophysiology

EJPs were recorded from central-proximal or proximal muscle fibers. Electrophysiological data were digitized and acquired by the EGAA data acquisition system (R. C. Electronics, Santa Barbara, CA). EJP amplitudes during a tetanus were measured by subtracting peak amplitude from the extrapolated falling phase of the previous EJP and correcting for the nonlinear relationship between postsynaptic potential and transmitter release (Martin, 1955), assuming an EJP reversal potential of 0 mV (Taraskevich, 1971).

Dye Injection

The excitatory axon was penetrated near the Y branch with a microelectrode filled with 22 mM fura-2 pentapotassium salt (Molecular Probes, Eugene, OR) in 200 mM KCl. Fura-2 was iontophoresed into the axon using 10–15 nA of continuous hyperpolarizing current for approximately 30 min. The motor nerve was stimulated by a 50 Hz train of 5–10 pulses every 30 s during the injection while both nerve action potentials and excitatory junction potentials (EJPs) were monitored. Injection was stopped when a slight decrease of EJP amplitude was observed, as high fura-2 concentrations can alter calcium transients in neurons (Tank et al., 1995). Using methods outlined in Mulkey and Zucker (1992), we estimate the concentration of fura-2 in the boutons was about 250 μM.

Imaging $[Ca^{2+}]_i$ in Presynaptic Terminals

Terminals that synapsed onto the fiber from which EJPs were recorded or an immediately adjacent fiber were chosen for measuring $[Ca^{2+}]_i$. An intensified CCD camera (Quantex Corp., Sunnyvale, CA) was used for imaging through either a 40X Zeiss (Thornwood, NY) or a 40X Olympus (Lake Success, NY) water immersion objective. Fluorescence was alternately excited through filters of 350 ± 10 nm and 385 ± 5 nm (Omega Optical, Brattleboro, VT). A dichroic mirror (450 nm) separated excitation and emission lights, and a barrier filter (530 ± 20 nm) restricted interference from autofluorescence. An area near the imaged bouton with uniform intensity similar to that around the bouton was chosen for obtaining tissue background. Background subtraction and shading correction were performed automatically in an image processor (Gould FD 5000, Fremont, CA). Averages of 32 sequential 350 nm- and 385 nm-excited images were stored on an optical disk recorder (Panasonic, TQ-2028F, Secaucus, NJ). The imaging processor, optical disk recorder, and filter changer were under the control of a Scientific Microsystems SMS 1000 computer (Mountain View, CA), using software written by Dr. Roger Tsien (Pharmacology Department, University of California at San Diego).

Fura-2 images were calibrated by measuring the fluorescence ratio obtained with 50 μM fura-2 in solutions at 280 mM ionic strength, resembling crayfish cytoplasmic solution (250 mM K-gluconate, 15 mM NaCl, 15 mM K-HEPES [pH 7.02]) with zero-calcium (10 mM K_2EGTA), 5 mM Ca^{2+}, or $[Ca^{2+}]$ buffered to 500 nM with 10 mM K_2EGTA and 5 mM $CaCl_2$, as calculated from the measured affinity of EGTA (Grynkiewicz et al., 1985). Ratios measured in terminals were converted to $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985) after application of a viscosity correction corresponding to a 30% reduction in the minimum and maximum 350 nm/385 nm fluorescence ratios (Almers and Neher, 1985).

Because of movement due to muscle contraction during and following PTP-inducing stimulation, images of nonsuperimposable terminals at 350 and 385 nm were usually observed within 20 s following the onset and offset of tetanus. These were easily identified and were excluded from further analysis, leaving a single exponential component of $[Ca^{2+}]_i$ decay. All reported values for $[Ca^{2+}]_i$, including means and SD, are reported to the nearest 10 nM.

Ruthenium Red Injection

The excitatory axon was penetrated by a second electrode, which was filled with ruthenium red (0.5 mM) dissolved in 200 mM KCl. Ruthenium red was injected by applying 40-ms pressure pulses of 1.3 bar to the pipette at 0.33 Hz for 1 hr. We estimate final concentration at about 20 μM. The electrode was inserted into the excitor at the outset of the experiment, but ruthenium red was not injected until after control data were acquired.

Data Analysis

Two data analysis programs, IGOR (Wavemetrics, Eugene, OR) and Prism (GraphPad Software, San Diego, CA), were used to determine the decay time constants of PTP, augmentation, and $[Ca^{2+}]_i$. EJP amplitudes normally showed double exponential decay. When no slow component was detectable (after drug application), the amplitude of that component was considered to be zero. A two-sided Student's *t* test on percentage changes from control in each pair was used to estimate statistical significance.

Computational Modeling

Computer simulations were performed on a Pentium computer using a program similar to those described in previous publications (Smith and Zucker, 1980; Nowycky and Pinter, 1993), except that intraterminal diffusion was ignored because it reaches equilibrium on the time scale of our simulation.

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