Residual Ca^{2+} and short-term synaptic plasticity

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At many synapses, the amount of transmitter released by action potentials increases progressively during a train of spikes. This enhancement of evoked transmitter release grows during tetanic stimulation with several time constants, each bearing a different name (facilitation: tens to hundreds of milliseconds; augmentation: several seconds; potentiation: several minutes), and the enhancement of release to test spikes after a tetanus decays with similar time constants. All these processes depend on presynaptic Ca^{2+} influx during the conditioning tetanus[1]. It has often been proposed that these forms of synaptic plasticity are due to residual Ca^{2+} present in nerve terminals following conditioning activity[2]. We tested this idea directly by using photolabile Ca^{2+} chelators to reduce residual Ca^{2+} following conditioning stimulation or to generate an artificial elevation in Ca^{2+} concentration, and observed the effects on synaptic transmission at crayfish neuromuscular junctions. We found that facilitation, augmentation and potentiation are caused by the continuing action of residual Ca^{2+}. Augmentation and potentiation seem to arise from Ca^{2+} acting at a separate site from facilitation, and these sites are different from the molecular target triggering neurosecretion.

We used the photolabile Ca^{2+} chelator diazo-2 (ref. 3) to lower residual Ca^{2+} concentration ($[Ca^{2+}]_i$) suddenly after conditioning stimulation and observed effects on short-term plasticity at excitor neuromuscular junctions in crayfish. Presynaptic injection of diazo-2 had little effect on the excitatory junctional potentials (e.j.ps) or their facilitation in a short train (Fig. 1*a*), as expected from the low buffer power of the unphotolysed chelator. Flash photolysis with ultraviolet light produces a high-affinity photoproduct which rapidly reduced facilitated e.j.ps tested 10 ms after photolysis nearly to the size of unfacilitated e.j.ps, while leaving unaffected the control e.j.ps recorded after facilitation that would normally have dissipated (Fig. 1*b*). The virtual abolition of facilitation within milliseconds of photolysis producing a step reduction in $[Ca^{2+}]_i$ shows that facilitation is caused by residual Ca^{2+} acting at a site with fast kinetics which is in rapid equilibrium with residual Ca^{2+}.

We used a similar protocol to determine the role of residual Ca^{2+} in augmentation generated by a longer conditioning train and measured when facilitation had fully decayed. Figure 2*a* illustrates the post-tetanic decay of e.j.p. amplitudes tested twice per second. Photolysis of diazo-2 10 ms before an augmented e.j.p. had little immediate effect; later e.j.ps, however, were reduced nearly to control (non-augmented) levels (Fig. 2*b*). When photolysis preceded the test stimulus by 50 ms, augmentation was significantly reduced, and when the interval was set to 100 ms, the effect was even greater. The summary of these results (Fig. 2*c*) indicates that augmentation is blocked by reduction of residual $[Ca^{2+}]_i$ with a time constant of ~350 ms. A faster (50 ms time constant) component may also be present. Augmentation seems to be generated by residual Ca^{2+} acting at a slowly responding site different from that generating facilitation.

Next we looked at the role of residual Ca^{2+} in post-tetanic potentiation (PTP). Using an even longer conditioning train,

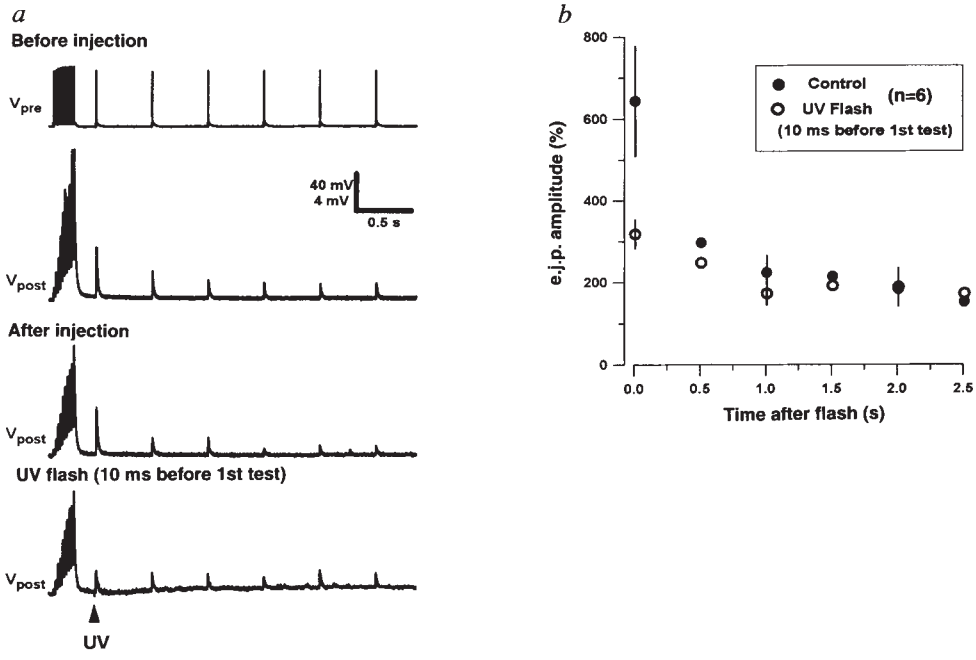

FIG. 1 Rapid reduction of residual $[Ca^{2+}]_i$ quickly eliminates synaptic facilitation. *a*, Facilitation is established by 10 presynaptic stimuli at 50 Hz, and tested at 2 Hz beginning 200 ms after the end of the conditioning tetanus, when the first component of facilitation has decayed but the second component is still strong[29]. Top trace, action potentials recorded from a preterminal axon branch. Second trace, e.j.ps facilitate during the tetanus, and facilitation decays within 1.5 s. Third trace, after presynaptic injection of diazo-2, e.j.ps are slightly reduced. Bottom trace, a UV flash to photolyse diazo-2 occurs 10 ms before the first post-tetanic test response; production of presynaptic high-affinity Ca^{2+} buffer substantially eliminates facilitation. *b*, Rapid removal of facilitation by diazo-2 photolysis. Averaged e.j.p. amplitudes (±s.e., $n=6$) are expressed as percentages of unfacilitated e.j.ps before photolysis and plotted against time after flash. Similar results were obtained when the first post-tetanic e.j.p. followed the train by 10 ms as in these experiments or by 50 ms, indicating that both fast and slow components of facilitation are dependent upon residual Ca^{2+}.

METHODS. Dactyl opener muscles and motor nerves of isolated crayfish (*Procambarus clarkii*) walking legs were prepared as described previously[12]. The excitor motor neuron was penetrated near the first major Y branch with a bevelled 60–80 MΩ microelectrode containing 50 mM diazo-2, 10 mM fluorescein (both from Molecular Probes, Eugene, OR) and 100 mM K–HEPES, pH 7.3, and backfilled with 3 M KCl. E.j.ps were recorded at 14–16 °C with 4–10 MΩ microelectrodes filled with 3 M KCl from proximal-central fibres on the ventral surface near the presynaptic electrode. Diazo-2 was injected iontophoretically until e.j.p. amplitudes just began to fall in muscles excited by terminals showing detectable fluorescein fluorescence; the final diazo-2 concentration was in the low millimolar range. A modified Chadwick-Helmuth (El Monte, CA) flashlamp was used to photolyse diazo-2, producing ~200 μM photoproduct with 150 nM Ca^{2+} affinity. This should have little effect on the $[Ca^{2+}]_i$ peaks causing secretion[25], but should readily absorb residual $[Ca^{2+}]_i$ of a few μM[14]. In some experiments, diazo-4 (ref. 3) was used instead of diazo-2, with similar results. In control experiments, diazo-3 (ref. 3) was photolysed to produce photoproducts and a pH change similar to those of diazo-2 and diazo-4, but without a change in Ca^{2+} affinity; e.j.p. amplitudes were unaffected.

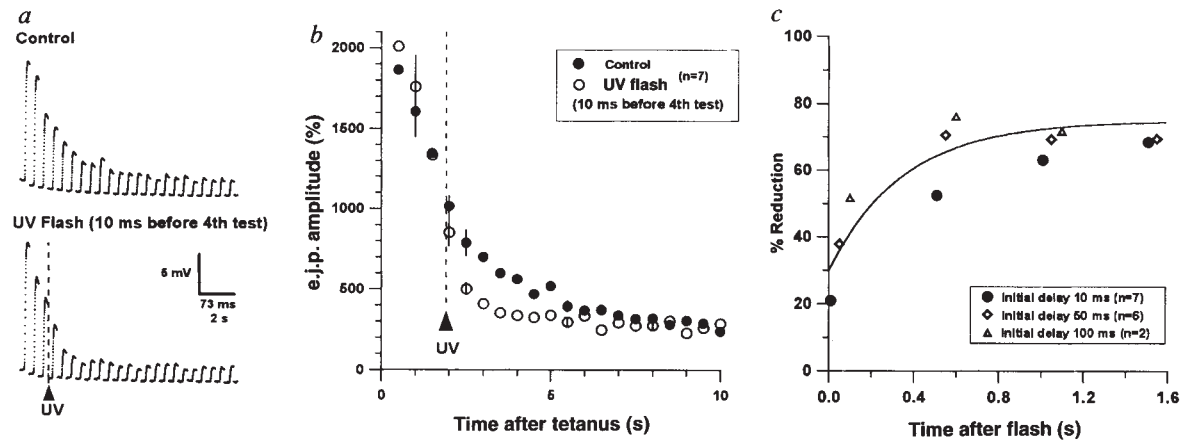

FIG. 2 Rapid reduction of residual $[Ca^{2+}]_i$ eliminates synaptic augmentation after about 0.5 s. *a*, Augmentation is established by stimulating the motor neuron at 50 Hz for 4 s, and tested at 2 Hz beginning 500 ms after the end of the conditioning tetanus. Only postsynaptic responses are shown. Each response is displayed using a fast time scale (73 ms time calibration), but successive responses occur at 0.5 s intervals (2 s time calibration). In the lower trace, presynaptically injected diazo-2 is photolysed with a UV flash 10 ms before the fourth test response. This response is similar to the fourth response in the upper control trace without photolysis (but after diazo-2 injection); however, later responses are markedly reduced after photolysis. *b*, Average reduction ($n=7$) of augmentation by diazo-2 photolysis. e.j.p. amplitude is expressed as percentage of responses at 0.5 Hz. Photolysis causes little reduction of augmentation at 10 ms, but substantial reduction by 510 ms. *c*, Time course of block of augmentation expressed as percentage reduction of e.j.p. at different times after diazo-2 photolysis; 100% means that e.j.p. has been reduced to the final level 10 s after the tetanus, when augmentation is almost fully decayed. The flash preceded the first response by 10 ms, 50 ms, or 100 ms. The line is an exponential with time constant of 350 ms.

and waiting 1 min after the tetanus for facilitation and augmentation to decay fully, we observed the effect of diazo-2 photolysis on potentiated e.j.ps. When the flash preceded an e.j.p. by 10 ms, little effect was observed, but PTP was nearly eliminated by the next e.j.p. 0.5 s later (Fig. 3*a*). The averaged results (Fig. 3*b*, *c*) show that PTP is blocked with kinetics similar to augmentation when residual Ca^{2+} is eliminated. As augmentation and potentiation are linearly correlated with similar sensitivities to micromolar levels of residual Ca^{2+} following presynaptic activity[4,5], it is likely that these processes are caused by Ca^{2+} acting at one site with high affinity and slow kinetics, perhaps involving biochemical intermediaries.

Figures 2 and 3 show one difference between PTP and augmentation. Following diazo-2 photolysis, PTP always recovered to its previous level within ~30 s, whereas augmentation never did. Perhaps there is a recovery of residual Ca^{2+} after diazo-2 photolysis in PTP. This might occur if the intense long tetani required to establish PTP load intracellular Ca^{2+} stores, and slow leakage of Ca^{2+} from these organelles, as well as a reduction in Ca^{2+} removal by Na^+/Ca^{2+} exchange due to presynaptic Na^+ accumulation[6], produce the normal slow recovery of PTP. Leakage of Ca^{2+} from stores after diazo-2 photolysis might eventually saturate the small amount of photoproduct produced, leading to a rise in residual $[Ca^{2+}]_i$ and the reappearance of PTP. We

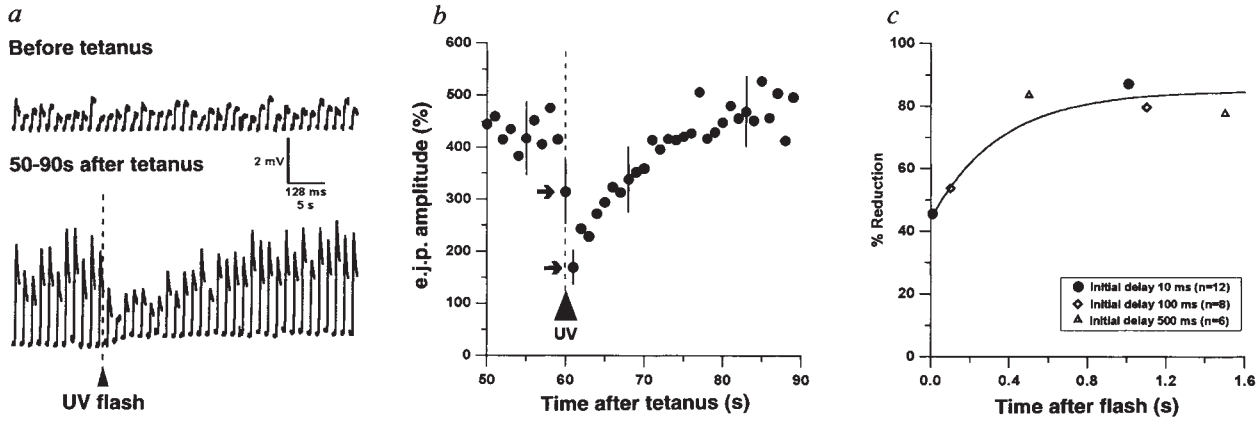

FIG. 3 After rapid reduction of residual $[Ca^{2+}]_i$, PTP dissipates at a similar rate as augmentation, but then recovers within 0.5 min. *a*, Potentiation is established by stimulating the motor neuron at 50 Hz for 5 min, and tested at 1 Hz after the tetanus. Forty postsynaptic responses are displayed in each trace; 25 ms of individual responses are displayed at fast sweep speeds as in Fig. 2. Upper trace, responses before the tetanus; lower trace, responses 50–90 s after the end of the tetanus, with a UV flash delivered 10 ms before the 61st post-tetanic response to photolyse diazo-2. At 1 min after the tetanus, facilitation and augmentation have decayed fully, but e.j.ps are ~3 times their initial amplitude due to PTP. The first response after the flash is within the range of responses before photolysis, but the next few responses are markedly reduced. PTP gradually recovers to its pre-existing level after photolysis. *b*, Average reduction ($n=12$) of PTP by diazo-2 photolysis and subsequent recovery. e.j.p. amplitude is expressed as percentage of pre-tetanic control responses at 1 Hz. The arrows point to responses recorded 10 and 1,010 ms after the UV flash. *c*, Time course of block of PTP expressed as percentage reduction of e.j.p. at different times after diazo-2 photolysis. The flash preceded the first response by 10 ms, 100 ms, or 500 ms. The line is an exponential with time constant of 350 ms. As with augmentation, block of PTP by diazo-2 photolysis was not always complete, but the block shown at 10 ms intervals was not significantly different between PTP and augmentation (two-tailed *t* test, $P>0.05$).

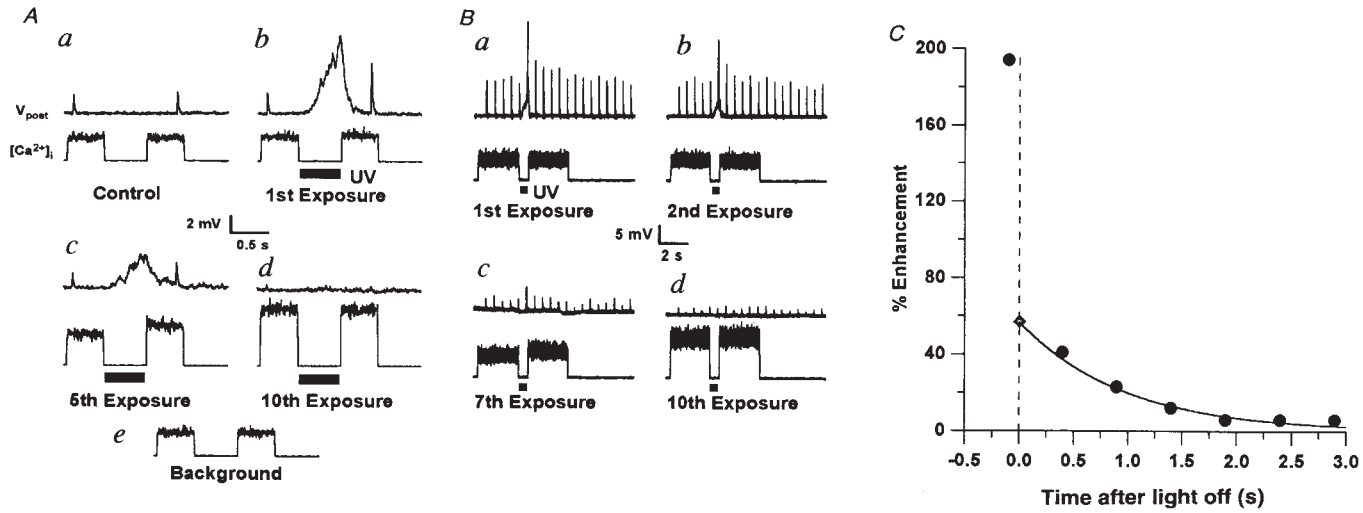

FIG. 4 Effect of reversible elevation of presynaptic $[Ca^{2+}]_i$ on synaptic transmission. *A*, Steady photolysis of DM-nitrophen for 0.5 s elevates $[Ca^{2+}]_i$ and evokes transmitter release during illumination (13 experiments). In each panel, top traces show postsynaptic potential; bottom traces monitor fluorescence of fluo-3 in terminals. In *a*, two 0.5-s $[Ca^{2+}]_i$ measurements are made, indicated by a noisy departure of the $[Ca^{2+}]_i$ trace from baseline. Evoked e.j.ps are relatively constant, and $[Ca^{2+}]_i$ is low, so that fluo-3 fluorescence is close to background levels (shown in *e*). In *b*, the photolysis light is tuned on between the $[Ca^{2+}]_i$ measurements: transmitter release occurs at an accelerating rate during light exposure, but ceases rapidly afterwards. The $[Ca^{2+}]_i$ returns to low levels after photolysis, but e.j.ps are enhanced. By the 4th exposure $[Ca^{2+}]_i$ has begun to rise after extinguishing the light, and rises more with the 5th (*c*) and successive exposures; transmitter release now persists at modest levels between illuminations. By the 10th exposure (*d*), $[Ca^{2+}]_i$ has been in the μM range for over 1 min, and transmitter release is exhausted. In *B*, a similar experiment with 2 Hz stimulation shows the time course of the decay of enhanced e.j.ps after $[Ca^{2+}]_i$ returns to a low level. In *C*, the decay in enhancement in e.j.ps amplitude (filled circles) is plotted against time following photolysis of DM-nitrophen for 0.5 s in a different preparation. This decay resembles that of augmentation and potentiation after diazo-2 photolysis. It is extrapolated to the e.j.p. enhancement that would be seen at the end of the photolysis period (◇). This is substantially less than the enhancement shown by an e.j.p. during photolysis (first ●). An additional facilitation-like process enhances spike-evoked release while $[Ca^{2+}]_i$ is elevated, and it decays rapidly when photolysis stops. In six experiments, the extrapolated increase in e.j.p. amplitude after photolysis was 52 ± 10% (mean ± s.e.), whereas the increase in an e.j.p. measured 100 ms before stopping photolysis was significantly greater—144 ± 24% ($P < 0.05$, two-sided *t*-test).

METHODS. The presynaptic electrode contained 45 mM DM-nitrophen (Calbiochem, San Diego), 13.5 mM $CaCl_2$, 0.9 mM Fluo-3 (Molecular Probes), and 57 mM KHEPES, pH 7.3. DM-nitrophen was injected by pressure to a final concentration in terminals of a few mM. Fluo-3 fluorescence was excited at 460–500 nm and monitored at 510–560 nm with a shuttered photomultiplier tube on the microscope's camera port. Photolysis was produced by a shuttered 150 W collimated Xe arc lamp. Calculations[9,11,12] suggest that $[Ca^{2+}]_i$ increased to ~2 μM during light exposure.

tried to test this hypothesis by measuring $[Ca^{2+}]_i$ in nerve terminals filled with diazo-2, but our efforts were thwarted by serious quenching of fluorescent indicators[7] by diazo-2.

If residual Ca^{2+} acts at low levels at separate sites to activate facilitation and augmentation/potentiation, elevation of Ca^{2+} to micromolar levels should elicit both forms of synaptic plasticity without conditioning stimulation. We injected nerve terminals with DM-nitrophen[8], a photolabile high-affinity Ca^{2+} buffer which produces low-affinity photoproducts on photolysis. Exposure of DM-nitrophen partially loaded with Ca^{2+} to steady ultraviolet illumination causes $[Ca^{2+}]_i$ to rise while the light remains on, and then return to near its initial level when the light is extinguished, so long as unphotolysed chelator remains in excess of total $[Ca^{2+}]_i$ (ref. 9). Figure 4*A* shows the effect of 0.5 s illumination on presynaptic $[Ca^{2+}]_i$ (measured with fluo-3, ref. 10), and postsynaptic potential. Asynchronous transmitter release increases during the light exposure to a rate of a few quanta per millisecond, causing several millivolts of postsynaptic depolarization[11]. This is intermediate between the resting miniature e.j.p. frequency (a few quanta s^{-1}) and the maximal rate of secretion during an e.j.p. (~250 quanta ms^{-1}). We cannot measure $[Ca^{2+}]_i$ during exposure to the photolysing radiation, but calculate a rise to 1–3 μM[9,11,12]. When illumination stops, secretion ceases and the fluorescence of fluo-3 is unchanged, indicating that $[Ca^{2+}]_i$ has returned to a low level. Subsequent exposures evoke diminishing secretion, perhaps due to exhaustion of releasable transmitter stores or docked vesicles, or a side effect of DM-nitrophen photoproducts. By the fifth exposure, fluo-3 fluorescence has begun to increase and secretion continues after the light turns off, indicating that insufficient unphotolysed DM-nitrophen remains to buffer the Ca^{2+} released by photolysis.

The reversible rise in $[Ca^{2+}]_i$ increases e.j.ps evoked by action potentials (Fig. 4*A*). At 400 ms after the first exposure, when $[Ca^{2+}]_i$ and transmission have returned to baseline levels, evoked transmission is still enhanced. Figure 4*B* shows that this enhancement decays with a time constant of several hundred milliseconds, similar to the decay of augmentation/potentiation after photolysis of diazo-2. This post-exposure enhancement of transmission is likely to be due to Ca^{2+} released from DM-nitrophen acting at the augmentation/potentiation site. The gradual rise in miniature e.j.p. frequency during illumination and a small tail of release lasting ~1 s afterwards may also be due to Ca^{2+} acting at this site. Moreover, e.j.ps evoked during photolysis are increased even more than expected from the decay of the post-illumination enhancement (Fig. 4*C*). This effect, which decays rapidly on extinguishing the light, is probably due to Ca^{2+} released from DM-nitrophen acting at the site responsible for facilitation.

A popular hypothesis[1,2] attributes all forms of presynaptically enhanced transmission to the effect of a residual elevation in $[Ca^{2+}]_i$, summating with the change in $[Ca^{2+}]_i$ accompanying action potentials to generate a higher peak $[Ca^{2+}]_i$ transient acting on the molecular trigger for transmitter release. The nonlinear dependence of transmitter release on $[Ca^{2+}]_i$ (ref. 12) can generate a large enhancement of transmission in the face of little transmitter release due to residual Ca^{2+} itself. The different time constants of facilitation, augmentation and potentiation then reflect the complex time course of decay of residual Ca^{2+}. This idea is supported qualitatively by measurements of residual Ca^{2+} in nerve terminals associated with augmentation and

potentiation[4,5,13]. But recorded[4,5,13] or calculated[14] levels of residual $[Ca^{2+}]_i$, the rates of accumulation and decay of enhanced release[2,15,16], and differential effects of Ba^{2+} and Sr^{2+} (refs 17, 18) do not accord quantitatively with predictions of the simple hypothesis of residual Ca^{2+} summating with Ca^{2+} influx to act at a single molecular target with nonlinear Ca^{2+} dependency.

Experimental tests of this hypothesis have focused on effects of presynaptically administered EGTA or BAPTA[13,16,19–24]; these tests have had variable results, sometimes blocking a component of enhancement of synaptic transmission, and sometimes not. Negative results may arise from exogenous chelator becoming saturated with Ca^{2+} during conditioning stimulation, whereas positive results could indicate that the exogenous chelator prevented Ca^{2+} from reaching sites causing synaptic plasticity that are distinct from the release trigger. Enhanced transmitter release could then be due either to actions of residual free Ca^{2+}, to Ca^{2+} remaining bound to sites of action, or to after-effects of Ca^{2+} that are independent of the continued presence of residual Ca^{2+}.

Our results show that facilitation, augmentation and potentiation are all due to the continuing action of residual free Ca^{2+} following neuronal activity. These processes are abolished by diazo-2 photolysis and activated by $[Ca^{2+}]_i$ elevation. The similarity of the kinetics and calcium sensitivities of augmentation and potentiation suggest that these processes are due to Ca^{2+} acting at one site, with the prolonged duration of PTP reflecting the longer time it takes to remove residual Ca^{2+} after prolonged stimulation. The fast equilibration of the facilitation process with residual Ca^{2+} clearly distinguishes it from augmentation and potentiation, and suggests that Ca^{2+} acts at a separate site. It is also possible that facilitation arises from a more direct action of Ca^{2+} on some target, whereas augmentation and potentiation arise from subsequent reactions dependent on that same target. One question is whether facilitation can arise from the same low-affinity fast kinetic site as secretion[25], as proposed by the original residual Ca^{2+} hypothesis. Attempts to simulate such a process have met with difficulty[14], and led to the suggestion that facilitation is due to Ca^{2+} acting at a separate site from that causing secretion. Either this is a higher affinity site that is somewhat distant (at least a few hundred nanometres) from the local high peaks of Ca^{2+} very near Ca^{2+} channels, or its kinetics are not as fast as those causing secretion; otherwise it would be saturated by a single action potential. The recent observation of a delay in the development of facilitation at 0 °C (ref. 26) further distinguishes the facilitation site from the exocytosis site.

An important outstanding problem is the identification of the molecular targets of Ca^{2+} action in secretion and short-term plasticity. Many candidates exist among vesicular, plasma membrane, and cytoplasmic proteins, with little evidence to select between them[27]. A popular candidate for a target of short-term synaptic plasticity is synapsin I[28], which can be phosphorylated by Ca^{2+}/calmodulin-dependent kinase II (CamKII). We measured the effects of presynaptic injection of calmodulin-binding domain (at 1 mM in the pipette with 10 mM fluorescein to monitor progress of injection), as well as bath application or presynaptic injection of the calmodulin inhibitor calmidazolium (up to 50 μM in 0.5% DMSO in the bath or 0.2 mM in 2% DMSO in the pipette) and the CamKII inhibitor KN-62 (3 μM added to 0.15% DMSO in the bath or 1 mM in 20% DMSO in the pipette); DMSO was added to the bath to prevent precipitation of hydrophobic substances in injected terminals. All treatments were without effect on transmission, facilitation, augmentation and potentiation. Such results do not favour calmodulin-mediated reactions as responsible for short-term synaptic plasticity. Identification of the molecular targets of Ca^{2+} awaits the development of more selective molecular probes.

Note added in proof: A recent study of augmentation and PTP at hippocampal mossy fibre synapses[30] describes Ca^{2+}-dependent kinetics similar to, but somewhat slower than, those reported here for crayfish neuromuscular junctions. □

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Insulin-promoter-factor 1 is required for pancreas development in mice

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THE mammalian pancreas is a mixed exocrine and endocrine gland that, in most species, arises from ventral and dorsal buds which subsequently merge to form the pancreas. In both mouse and rat the first histological sign of morphogenesis of the dorsal pancreas is a dorsal evagination of the duodenum at the level of the liver at around the 22–25-somite stage, and shortly thereafter a ventral evagination appears as a derivative of the liver diverticulum[1–3]. Low levels of insulin gene transcripts are already present and restricted to the dorsal foregut endoderm at 20 somites, suggesting that pancreas- or insulin gene-specific transcriptional factors are present in this region before the onset of morphogenesis[4]. Insulin-promoter-factor 1 (IPF1) is a homeodomain protein which, in the adult mouse pancreas, is selectively expressed in the β-cells and binds to and transactivates the insulin promoter[5]. In mouse embryos, IPF1 expression is restricted to the developing pancreatic anlagen and is initiated when the foregut endoderm is committed to a pancreatic fate[5]. We now show that mice homozygous for a targeted mutation in the *Ipf1* gene selectively lack a pancreas. The mutant pups survive fetal development but die within a few days after birth. The gastrointestinal part and all other internal organs were normal in appearance. No pancreatic tissue and no ectopic expression of insulin or pancreatic amylase could be detected in mutant embryos and neonates. These findings show that IPF1 is needed for the formation of the pancreas and suggest that it acts to determine the fate of common pancreatic precursor cells and/or to regulate their propagation.

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