Developmental upregulation of presynaptic NCKX underlies the decrease of mitochondria-dependent posttetanic potentiation at the rat calyx of Held synapse

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Lee JS, Kim MH, Ho WK, Lee SH. Developmental upregulation of presynaptic NCKX underlies the decrease of mitochondria-dependent posttetanic potentiation at the rat calyx of Held synapse. J Neurophysiol 109: 1724–1734, 2013. First published January 2, 2013; doi:10.1152/jn.00728.2012.—The sensitivity of posttetanic potentiation (PTP) to high-frequency stimulation (HFS) steeply decays during the first 2 postnatal weeks. We investigated the underlying mechanisms for the developmental change of PTP induced by HFS (100 Hz, 2 s) at postnatal days 4–6 and 9–11 at the rat calyx of Held synapse. Low-concentration tetraphenylphosphonium (2 μM), an inhibitor of mitochondrial Na+/Ca2+ exchanger, suppressed the amount of posttetanic residual Ca2+ and PTP to a larger extent at the immature calyx synapse, indicating a developmental reduction of mitochondrial contribution to PTP. The higher amount of mitochondrial Ca2+ uptake during HFS and consequent posttetanic residual Ca2+ at the immature calyx of Held was associated with higher peak of HFS-induced Ca2+ transients, most likely because the mitochondrial Ca2+ uptake during HFS was supralinearly dependent on the presynaptic resting Ca2+ level. Probing into the contribution of Na+/Ca2+ exchangers to Ca2+ clearance, we found a specific upregulation of the K+-dependent Na+/Ca2+ exchanger (NCKX) activity in the mature calyx of Held. We conclude that the upregulation of NCKX limits the Ca2+ buildup and inhibits mitochondrial Ca2+ uptake during HFS, which in turn results in the reduction of posttetanic residual Ca2+ and PTP at the mature calyx of Held synapse.

The calyx of Held synapse undergoes a variety of structural and functional changes during postnatal development (Felny and Schneggenburger 2004; Taschenberger et al. 2002; Wang et al. 2008; Wimmer et al. 2006). The immature calyx of Held synapses exhibit higher sensitivity for PTP induction compared with more mature ones (Korogod et al. 2005), but the underlying mechanisms are not well defined. Previously, we have reported that Na+/Ca2+ exchanger (Na/CaX) clears Ca2+ loads more readily than mitochondria at the calyx of Held and that mitochondria take part in Ca2+ clearance only when Na/CaX is inhibited or its function is saturated (Kim et al. 2005). Ca2+ clearance becomes faster as the calyx of Held matures (Chuhma and Ohmori 2001), but it has not been investigated whether developmental changes in Ca2+ clearance are causally related to developmental changes in PTP. Given that presynaptic mitochondria compete with Na/CaX for incoming Ca2+ (Kim et al. 2005), we hypothesized that mitochondrial Ca2+ uptake during HFS could be reduced by a developmental increase in the activity of other Ca2+ clearance mechanisms such as Na/CaX or plasma membrane Ca2+-ATPase (PMCA). Such developmental decrease in the mitochondrial Ca2+ uptake may in turn suppress posttetanic Ca2+ res, and thus PTP, in mature calyx of Held synapses.

We studied whether the developmental change of Ca2+ clearance mechanisms underlies that of PTP at the calyx of Held synapse. To this end, we compared PTP, the peak of HFS-induced Ca2+ transients (HFS-CaTs), mitochondrial Ca2+ uptake during HFS, and posttetanic Ca2+res at the calyx of Held synapse at two developmental stages, postnatal days 4–6 (P4–6) and 9–11 (P9–11). We found that the peak Ca2+ levels ([Ca2+]peak) of HFS-CaTs are closely correlated with the amount of mitochondrial Ca2+ uptake and posttetanic Ca2+res and that the developmental upregulation of the K+-dependent Na+/Ca2+ exchanger (NCKX) activity limits the intracellular [Ca2+] buildup during HFS to reduce mitochondrial Ca2+ uptake during HFS, resulting in the reduction of posttetanic Ca2+res and PTP.

METHODS

Preparation of brain stem slices. Calyx of Held synapse preparations were prepared from transverse 200-μm-thick brain stem slices containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body. P4–6 or P9–11 Sprague-Dawley rats were decapitated, and brain stems were chilled containing the medial nucleus of trapezoid body.
incubated at 37°C for 30 min in normal ACSF, the constituents of which are the same as low-Ca\(^{2+}\) ACSF except 1 mM MgCl\(_2\) and 2 mM CaCl\(_2\), and thereafter maintained at room temperature (23–25°C) until required. Protocols were approved by the Animal Care Committee of Seoul National University.

**Electrophysiological recording and reagents.** Slices were transferred to a recording chamber in an upright microscope (BX50WI; Olympus, Tokyo, Japan). Whole cell patch-clamp recordings from calyces of Held were made under visual control using differential interference illumination. Presynaptic whole cell patch abolishes the PTP and reduces the Ca\(^{2+}\) release at the calyx of Held synapse (Korogod et al. 2005; Lee et al. 2010). Thus, for estimation of PTP and presynaptic [Ca\(^{2+}\)], we preload Ca\(^{2+}\) indicator dye to calyx terminals by a brief presynaptic whole cell patch-clamp recording for about 90 s with the K-glucurate-containing pipette solution composed of (mM) 120 K-glucurate, 30 KCl, 20 HEPS, 4 Na\(_2\)H-phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP and 0.2 fura-4F (Figs. 2, 4, and 5) or fura-FF (Fig. 3) (pH 7.3 adjusted with KOH). Postsynaptic recordings were made in the voltage-clamp mode at a holding potential of −70 mV. Series resistance (R\( \text{s}\); range 4–15 M\( \Omega \)) was regularly checked and partially compensated by up to 75% for remaining uncompensated R\( \text{s}\) value of 3–4 M\( \Omega \). Experiments were discarded when uncompensated R\( \text{s}\) exceeded 15 M\( \Omega \). Patch pipettes with a resistance of 3.5–4.5 M\( \Omega \) were used for recordings. Postsynaptic patch pipettes contained (in mM) 110 Cs-glucurate, 20 tetraethylammonium (TEA)-Cl, 10 NaCl, 20 HEPS, 10 EGTA, 4 Mg-ATP, 5 Na\(_2\)H-phosphocreatine, and 5 lidocaine N-ethyl bromide (QX314; an intracellular blocker of Na\(^{+}\) currents) (pH 7.3 adjusted with CsOH). For measuring presynaptic Ca\(^{2+}\) transients under presynaptic whole cell patch-clamp recording conditions, K-glucurate and KCl in the K-glucurate pipette solution were replaced with equimolar tetramethylammonium (TMA)-glucurate and TEA-Cl, respectively. Presynaptic Ca\(^{2+}\) current was recorded in the presence of 1 \( \mu \)M tetrodotoxin (TTX) and 10 mM TEA-Cl using a HFS-CaT composed of n action potential-induced Ca\(^{2+}\) transients (AP-CaTs), each of which occurs with a uniform interpulse interval (\( \Delta t \)).Because the Ca\(^{2+}\) build-up during HFS is the difference between the sum of AP-induced Ca\(^{2+}\) rises and the sum of Ca\(^{2+}\) cleared during HFS, the peak amplitude of the HFS-CaT [\( \Delta Ca(n) \)] can be described as

\[
\Delta Ca(n) = \sum_{i}^{n} A_{i} - \frac{1}{\Sigma \kappa} \sum_{i}^{n} [(dCa/dt)_{i}] \times \Delta t, \tag{1}
\]

where \( A_{i} \) is the amplitude of the \( i \)th AP-CaT and the second term with a minus sign represents the Ca\(^{2+}\) decay exerted by Ca\(^{2+}\) clearance mechanisms during the interval \( \Delta t \). Equation 1 can be rewritten in terms of total calcium (free plus bound form, Ca\( T \)) as

\[
\Delta Ca(n) = \Delta Ca(n) \times (1 + \Sigma \kappa) = \sum_{i}^{n} A_{i} \frac{\Delta t}{\kappa} \Delta t - \frac{1}{\Sigma \kappa} \sum_{i}^{n} [(dCa/dt)_{i}] \times \Delta t, \tag{2}
\]

where \( A_{i} \Delta t \) is an increment of total calcium induced by the \( i \)th pulse, and \( \Sigma \kappa \) represents the linear Ca\(^{2+}\) binding ratio of exo- and endogenous Ca\(^{2+}\) buffers. The linearized \( \kappa \) value of a buffer, \( B \), between two Ca\(^{2+}\) concentrations \( x_{0} \) and \( x_{0} \) is defined by \( K_{\text{d}} \times (B_{\text{free}}/[B]) \) where \( K_{\text{d}} \) is a dissociation constant of \( B \). The \( K_{\text{d}} \) value of fura-FF and the endogenous Ca\(^{2+}\) binding ratio were assumed to be 3.14 \( \mu \)M and 40, respectively (Helmchen et al. 1996).

The second term of the right-hand side of Eq. 2 can be split into contributions of mitochondria and other Ca\(^{2+}\) clearance mechanisms under control conditions. Thus

\[
\Delta Ca(n)_{\text{control}} = \sum_{i}^{n} A_{i} \frac{\Delta t}{\kappa} - \frac{1}{\Sigma \kappa} \sum_{i}^{n} [(dCa/dt)_{i}]_{\text{others}} \times \Delta t. \tag{3}
\]

Assuming that Ru360 blocks only the mitochondrial Ca\(^{2+}\) uptake with no off-target effect, \( \Delta Ca(n) \) in the presence of Ru360 [\( \Delta Ca_{\text{Ru360}} \)] is

\[
\Delta Ca_{\text{Ru360}} = \sum_{i}^{n} A_{i} \frac{\Delta t}{\kappa} - \frac{1}{\Sigma \kappa} \sum_{i}^{n} [(dCa/dt)_{i}]_{\text{others}} \times \Delta t. \tag{4}
\]

Subtraction of \( \Delta Ca(n)_{\text{control}} \) from \( \Delta Ca(n)_{\text{Ru360}} \) yields

\[
\Delta Ca_{\text{Ru360}} - \Delta Ca(n)_{\text{control}} = \sum_{i}^{n} [(dCa/dt)_{i}]_{\text{mito}} \times \Delta t + e, \tag{5}
\]

where \( e = \sum_{i}^{n} [(dCa/dt)_{i}]_{\text{others}} - (dCa/dt)_{i}]_{\text{others}} \times \Delta t \). Since the activity of other Ca\(^{2+}\) clearance mechanisms is saturated at high [Ca\(^{2+}\)] (see DISCUSSION), the error term, \( e \), would not be large. If the
error term is negligible, the cumulative total amount of mitochondrial Ca\textsuperscript{2+} uptake (\(\Phi_{\text{mito}}\)) at the \(n\)th pulse can be calculated as

\[
\Phi_{\text{mito}}(n) = \Delta [\text{Ca}^{2+}]_{\text{res}}(n) - \Delta [\text{Ca}^{2+}]_{\text{res}}(n)\text{control}.
\]

Two terms in the right-hand side of Eq. 6 were measured at the same calyx terminal preloaded with fura-FF before and after the second patch with the pipette solution containing fura-FF and Ru360. The change in fura-FF concentration was estimated from fluorescence uptake (\(\Delta [\text{Ca}^{2+}]_{\text{res}}\)) of mitochondrial Na\textsuperscript{+}-efflux, although it dissipated mitochondrial membrane potential at higher concentration (>10 \(\mu\text{M}\)) (Aiuchi et al. 1985; Karadjoj et al. 1986; Wingrove and Gunter 1986). Previously, we have shown that 2 \(\mu\text{M}\) TPP\textsuperscript{+} abolishes the posttetanic \([\text{Ca}^{2+}]_{\text{res}}\) with little effect on the peak of HFS-Ca\textsubscript{T} (100 Hz, 4 s) at the calyx of Held (Lee et al. 2008). We assessed the effects of TPP\textsuperscript{+} on PTP induced by HFS (100 Hz, 2 s). The amplitude of the baseline EPSC was not altered by TPP\textsuperscript{+} treatment (P5, 1.33 ± 0.19 nA, \(n = 7\), \(P = 0.19\); P10, 1.80 ± 0.40 nA, \(n = 6\), \(P = 0.13\), paired \(t\)-test). In both age groups, the peak amplitude of PTP was significantly reduced by bath application of 2 \(\mu\text{M}\) TPP\textsuperscript{+}, from 123.8 ± 18.9 to 64.4 ± 6.7\% in P5 (\(n = 7\), \(P = 0.014\), paired \(t\)-test, Fig. 1, Aa and Ab) and from 55.4 ± 8.3 to 38.8 ± 5.9\% in P10 (\(n = 6\), \(P = 0.012\), paired \(t\)-test, Fig. 1, Ac and Ad). As the calyx synapse matured, the TPP\textsuperscript{+}-sensitive component of PTP (denoted by \(\Phi_{\text{mito}}\)) was reduced to a larger extent (59.4 ± 17.4\% at P5, 16.6 ± 4.3\% at P10, \(P = 0.048\), Fig. 1Ag) than the TPP\textsuperscript{+}-insensitive component (\(\Phi_{\text{mito, non-mito}}\), 64.4 ± 6.7\% at P5 and 38.8 ± 5.9\% at P10, \(P = 0.017\), Fig. 1Ah). In addition to the peak of PTP, the time courses of PTP were compared between control conditions and in the presence of 2 \(\mu\text{M}\) TPP\textsuperscript{+} using two-way ANOVA test. Whereas the time course of PTP at P5 was significantly different between these two conditions (\(F_{1, 300} = 19.22\), \(P < 0.001\)), that at P10 was not different (\(F_{1, 250} = 2.409\), \(P = 0.122\)). These results indicate that the reduced contribution of mitochondria to PTP is primarily responsible for the different magnitude of PTP at P5 and P10.

The \([\text{Ca}^{2+}]\) elevation at axon terminals during HFS is essential for the induction of PTP and critically depends on temperature (David and Barrett 2000). We tested whether the developmental change of PTP\textsubscript{mito} also occurs in near physiological temperature. We did the same experiments at the bath temperature of 32–34 °C. The baseline EPSC amplitudes were not different between P5 (3.55 ± 0.31 nA, \(n = 7\)) and P10 (3.79 ± 0.84 nA, \(n = 6\), \(P = 0.78\), Fig. 1Be). The peak amplitude of PTP was significantly lower at 32–34°C than at room temperature in both age groups (P5, 13.8 ± 3.91\%at \(n = 7\), \(P < 0.01\); P10, 14.8 ± 4.14\%, \(n = 6\), \(P < 0.01\)). Nonetheless, the PTP amplitude at P5 was significantly higher than at P10 (\(P = 0.012\), Fig. 1Bf). Furthermore, bath-applied TPP\textsuperscript{+} significantly reduced the PTP amplitude at P5 (17.07 ± 2.29\%, \(n = 7\), \(P < 0.01\), Fig. 1, Ba and Bb) but not at P10 (15.91 ± 3.07\%, \(n = 6\), \(P = 0.836\), paired \(t\)-test, Fig. 1, Be and Bd). Accordingly, PTP\textsubscript{mito} was significantly higher at P5 than at P10 (P5, 14.76 ± 2.49\%; P10, –1.10 ± 2.31\%, \(P < 0.01\), Fig. 1Bg), but PTP\textsubscript{mito, non-mito} was not different between two age groups (P5, 17.07 ± 2.29\%; P10, 15.91 ± 3.07\%, \(P = 0.76\), Fig. 1Bh). Comparing the time course of PTP, we found that it was significantly affected by TPP\textsuperscript{+} at P5 (\(F_{1, 300} = 5.29\), \(P = 0.022\)) but not at P10 (\(F_{1, 250} = 0.292\), \(P = 0.59\)). Such different effects of TPP\textsuperscript{+} on PTP between two age groups indicate the developmental decrease in the mitochondrial contribution to PTP at physiological temperature. TPP\textsuperscript{+} had no effect on the baseline EPSC amplitude in both age groups (P5, 3.51 ± 0.31 nA, \(n = 7\), \(P = 0.19\); P10, 3.85 ± 0.76 nA, \(n = 6\), \(P = 0.13\)). Next, we focused on the developmental changes of Ca\textsuperscript{2+} dynamics responsible for the developmental decrease of PTP\textsubscript{mito} at postnatal calyx of Held synapse. All subsequent experiments were performed at room temperature.

Developmental changes in posttetanic residual Ca\textsuperscript{2+} and PTP. The smaller effect of TPP\textsuperscript{+} on PTP at the P10 calyx of Held implies a smaller amount of posttetanic Ca\textsubscript{res} at P10 than at P5. To test this prediction, we examined presynaptic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) and EPSC simultaneously at the same synapse. We loaded the calyx axon terminal with a Ca\textsuperscript{2+}
Preloading the calyx terminal with fura-4F significantly reduced the baseline EPSC amplitude (to 0.61 ± 0.08 nA, n = 5, P = 0.017) and enhanced PTP induced by HFS (100 Hz, 2 s) (180.8 ± 5%, n = 5, P = 0.03) at the P5 calyx synapses, whereas it had no significant effect on the baseline EPSC amplitude (0.96 ± 0.26 nA, n = 6, P = 0.1) and on PTP (58.9 ± 5.9%, n = 6, P = 0.74) at the P10 synapses (Fig. 2, A and B). These differential effects of preloading on the baseline EPSC amplitude and PTP are consistent with the idea that the spatial coupling between synaptic vesicles and Ca⁡\textsuperscript{2+} channels becomes tighter as the calyx synapse matures (Wang et al. 2008). As expected from the smaller PTP⁡\textsubscript{mito} at P10 synapses, the amount of posttetanic \(\text{Ca}_{\text{res}}\) was significantly smaller at P10 synapses (0.40 ± 0.07 \(\mu\text{M·s}\), \(P < 0.01\)) than at P5 (2.48 ± 0.18 \(\mu\text{M·s}\)) (Fig. 2, A and B).

We used 2 \(\mu\text{M} \text{TPP}^+\) to inhibit the mitochondrial Na⁺-dependent Ca⁡\textsuperscript{2+} efflux without disturbing mitochondrial membrane potential and Ca⁡\textsuperscript{2+} uptake (Lee et al. 2008). As expected, \(\text{TPP}^+\) did not alter the peak of HFS-CaTs (P5, \(P = 0.54\); P10, \(P = 0.80\), Fig. 2, Ae and Be), but it significantly suppressed PTP and posttetanic \(\text{Ca}_{\text{res}}\) in both age groups (PTP, \(P < 0.01\) for P5, \(P = 0.014\) for P10; posttetanic \(\text{Ca}_{\text{res}}\), \(P < 0.01\) for P5, \(P = 0.019\) for P10, paired \(t\)-test, Fig. 2, A and B). For both age groups, the time course of PTP was significantly different between control and in the presence of \(\text{TPP}^+\) [P5, \(F_{(4, 200)} = 33.55, P < 0.001\); P10, \(F_{(1, 250)} = 7.70, P = 0.006\)]. The developmental reduction of \(\text{TPP}^+\)-sensitive PTP, denoted by \(\text{PTP}\text{\_}\text{mito}\), paralleled the mitochondrial-dependent \(\text{Ca}_{\text{res}}\) amount (Fig. 2C), indicating that the reduced \(\text{TPP}^+\)-sensitive PTP at P10 can be ascribed to the smaller amount of mitochondria-dependent \(\text{Ca}_{\text{res}}\).

Previously, we have reported that mitochondrial Ca⁡\textsuperscript{2+} uptake occurs at high [Ca⁡\textsuperscript{2+}]i levels, where Na/CaX is saturated (Kim et al. 2005). Thus we compared the peak of HFS-CaTs (denoted by \([\text{Ca}^2+]_\text{peak}\)) was associated with a higher amount of posttetanic \(\text{Ca}_{\text{res}}\) (Fig. 2D). We further investigated the relationship between \([\text{Ca}^2+]_\text{peak}\) and mitochondrial Ca⁡\textsuperscript{2+} uptake.
Developmental change in mitochondrial Ca$^{2+}$ uptake during HFS. The smaller amount of posttetanic Ca$_{pre}$ at P10 suggests a lower mitochondrial Ca$^{2+}$ uptake during HFS. To test this idea, we compared HFS-CaTs in each age group before and after inhibition of mitochondrial Ca$^{2+}$ uptake using Ru360. It has been shown that Ru360 specifically inhibits the mitochondrial Ca$^{2+}$ uniporter with little effect on L-type Ca$^{2+}$ current and other Ca$^{2+}$ clearance mechanisms including PMCA and Na/CaX (Matlib et al. 1998). The presynaptic terminal was preloaded with a K-gluconate internal solution containing 200 μM fura-FF, a low-affinity Ca$^{2+}$-indicator dye. After withdrawal of the pipette, we recorded Ca$^{2+}$ transients evoked by HFS of afferent fibers (100 Hz for 2 s). The [Ca$^{2+}$]$_{peak}$ of HFS-CaT was 3.85 ± 0.47 μM in control conditions at P5 (n = 5). Subsequently, we repatched the same axon terminal using a patch pipette containing 200 μM fura-FF and 20 μM Ru360. After a brief (~30 s) whole-cell patch, we pulled out the pipette and recorded the HFS-CaT again. After intracellular application of Ru360, an inhibitor of the mitochondrial Ca$^{2+}$ uniporter, the [Ca$^{2+}$]$_{peak}$ was significantly elevated to 21.2 ± 2.0 μM at same axon terminal (n = 5, P < 0.01, paired t-test, Fig. 3A). In contrast, at P10 the [Ca$^{2+}$]$_{peak}$ increased from 1.42 ± 0.1 μM to 3.5 ± 0.3 μM (n = 5, P < 0.01, paired t-test, Fig. 3B). The increment of the [Ca$^{2+}$]$_{peak}$ caused by Ru360 was significantly higher at P5 than at P10 (17.4 ± 1.70 μM and 2.1 ± 0.15 μM, respectively, P < 0.01). We plotted the [Ca$^{2+}$]$_{peak}$ values of HFS-CaTs in the presence of Ru360 as a function of the control [Ca$^{2+}$]$_{peak}$ in Fig. 3C, showing the high correlation between these two parameters.

Figure 3C implies that the higher mitochondrial Ca$^{2+}$ uptake may be related to the higher elevation of [Ca$^{2+}$]$_i$ during HFS. To test this idea, we estimated the cumulative mitochondrial Ca$^{2+}$ uptake (denoted by Φ$_{mito}$) during HFS according to Eq. 6 and plotted these values as a function of [Ca$^{2+}$]$_i$ under control conditions in Fig. 3D. This plot shows a supralinear dependence of mitochondrial Ca$^{2+}$ uptake on the presynaptic Ca$^{2+}$ level. Furthermore, we found that the mitochondrial
Ca\(^{2+}\) uptake at P5 is not different from that at P10 as long as the [Ca\(^{2+}\)]\(_{\text{mit}}\) level is the same (Fig. 3D), arguing against the possibility that the lower posttetanic Ca\(_{\text{res}}\) at P10 is caused by lower mitochondrial function. These results indicate that the higher [Ca\(^{2+}\)]\(_{\text{peak}}\) of a HFS-CaT leads to a higher mitochondrial Ca\(^{2+}\) uptake (Fig. 3C), which in turn results in a higher mitochondria-dependent Ca\(^{2+}\) clearance (Fig. 2D) and PTP\(_{\text{mito}}\) (Fig. 2E).

Finally, we tested whether the amount of posttetanic mitochondrial Ca\(^{2+}\) extrusion (\(J_{\text{mito}}\)) estimated by Eq. 7 is close to the mitochondrial Ca\(^{2+}\) uptake estimated at the end of HFS according to Eq. 6. To estimate the former (\(J_{\text{mito}}\)), the time integral of TPP\(_{\text{Na}}\)-sensitive Ca\(_{\text{res}}\) was calculated from the data in Fig. 2, and the \(\gamma\) value for Ca\(^{2+}\) clearance was calculated according to Eq. 8 using the Ca\(^{2+}\) decay rate constant \(r_{\infty}\) measured from CaTs evoked by double APs (interspike interval = 5 ms, 4.1/s for P5 and 4.9/s for P10, Fig. 4). The two estimates were not significantly different from each other in both age groups (Fig. 3E), suggesting that most of the Ca\(^{2+}\) uptake by mitochondria during HFS is extruded at the end of HFS.

The estimates for mitochondrial Ca\(^{2+}\) uptake are valid when Ru360 has no effect on the Ca\(^{2+}\) current, but bath-applied Ru360 has been reported to reduce the amplitude of an AP-induced CaT in motor nerve terminal (David 1999). To test if intracellularly applied Ru360 has any effect on Ca\(^{2+}\) current, we compared Ca\(^{2+}\) currents induced by HFS (short depolarizing pulses at 100 Hz for 2 s) with or without 20 \(\mu\)M Ru360 in the pipette solution at the calyx of Held terminals of P10. Figure 3F shows that Ru360 has no significant effect on Ca\(^{2+}\) current (\(P > 0.4\) at all time points). The total amounts of charge influx elicited by the HFS was not different between the two conditions either (411.8 \pm 39.8 pC, \(n = 7\); 394.3 \pm 23.5 pC, \(n = 6\), \(P = 0.724\)). Despite little effect of Ru360 on Ca\(^{2+}\) influx, higher elevation of [Ca\(^{2+}\)] in the presence of Ru360 may enhance diffusion of Ca\(^{2+}\) from the terminal to the attached axon, resulting in underestimation of mitochondrial Ca\(^{2+}\) uptake. Nevertheless, such Ca\(^{2+}\) clearance by diffusion may be limited because most afferent axon fibers are truncated during slice preparation.

Developmental upregulation of Ca\(^{2+}\) clearance mechanisms. The lower peak of HFS-CaTs at P10 can be ascribed to a smaller AP-induced Ca\(^{2+}\) influx or a higher Ca\(^{2+}\) clearance. The amount of AP-induced Ca\(^{2+}\) influx at P10 is thought to be only lower by about 5% than that at P5 because of the shorter AP duration at P10 (Taschenberger et al. 2002), implying that faster Ca\(^{2+}\) clearance rather than smaller Ca\(^{2+}\) influx is responsible for the lower peak of HFS-CaTs at P10. We investigated developmental changes of Ca\(^{2+}\) clearance at the calyx of Held. We have previously reported that Na/CaX is a major Ca\(^{2+}\) clearance mechanism, whereas sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) contributes little to Ca\(^{2+}\) clearance at the calyx of Held (Kim et al. 2005). The Na/CaX activity can be further separated into the K\(^+-\)independent form of Na/CaX (NCX) and NCKX according to the dependence on intracellular K\(^+\) (Lee et al. 2002). NCX and NCKX can be specifically inhibited by intracellular perfusion of exchanger inhibitory peptide (XIP) and K\(^+-\)free TMA\(^+\) solution, respectively (Kim et al. 2005; Lee et al. 2002, 2007b; Li et al. 1991), whereas both of them (denoted by Na/CaX) can be inhib-
We investigated the developmental changes of NCX and NCKX activities at the calyx of Held loaded with 50 μM fura-4F via a whole cell patch pipette. Because the Na/CaX activity is activated at higher [Ca^{2+}]_i than PMCA (Kim et al. 2005), we examined the inhibitory effects on Ca^{2+} transients of two different amplitudes evoked by 2 or 20 repetitive depolarizing pulses at 200 Hz (denoted by CaT2 or CaT20, respectively; pulse duration, 2 ms). From a holding potential of −70 mV, the depolarization step was adjusted between −20 and 0 mV such that the peak of the resulting CaT20 was near 2 μM (in the range of 1.5–2.5 μM). The statistical means of the decay rate constants under various conditions are summarized in Table 1. Under control conditions, that is, in the presence of normal concentrations of intracellular K^+ ([K^+]_i) and extracellular Na^+ ([Na^+]_o), the decay rate constant of CaT2 was not different between P5 and P10 (Fig. 4Aa). However, the decay rate constant r_w of CaT20 was significantly faster at P10 than at P5 (P < 0.01, Fig. 4Ab). Next, we compared the decay rate constant using the TMA-internal solution for selective inhibition of NCKX. Under this condition, both decay rate constants, CaT2 and CaT20, were not significantly different between P5 and P10 (Fig. 4Bb), suggesting that NCKX is responsible for the developmental acceleration of the decay rate. To study the contribution of NCX, we intracellularly perfused the calyx terminal with K-gluconate internal solution containing 10 μM XIP. Under this condition, the decay rate constant of CaT20 was faster at P10 than at P5 (Fig. 4Cb), whereas that of CaT2 was not affected at either age (Fig. 4Ca). Furthermore, the difference in the decay rate constant of CaT20 between P5 and P10 in the presence of XIP (4.7/s) was comparable to that under control conditions (5.3/s), suggesting that NCX is not...
DEVELOPMENTAL CHANGES IN Ca\(^{2+}\) CLEARANCE AND SHORT-TERM PLASTICITY

Table 1. Statistical mean values of decay rate constant and peak Ca\(^{2+}\) amplitude of CaT2 and CaT20 under various conditions

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<td>2</td>
<td>8</td>
<td>0.24 (\pm) 0.02</td>
</tr>
<tr>
<td>TMA(_i)</td>
<td>20</td>
<td>11</td>
<td>2.07 (\pm) 0.10</td>
</tr>
<tr>
<td>XIP</td>
<td>2</td>
<td>5</td>
<td>0.28 (\pm) 0.03</td>
</tr>
<tr>
<td>Li(_{ext})</td>
<td>20</td>
<td>6</td>
<td>2.16 (\pm) 0.10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8</td>
<td>2.18 (\pm) 0.17</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE \((n = \text{no. of synapses analyzed})\) for the decay rate constant \((r_w)\) and the peak Ca\(^{2+}\) amplitude \((\Delta[Ca^{2+}]\) of Ca\(^{2+}\) transients evoked by 2 or 20 repetitive depolarizing pulses (CaT2 or CaT20, respectively) under various conditions.

developmentally upregulated. To exclude possible developmental changes of Ca\(^{2+}\) clearance mechanisms other than NCX and NCKX, we compared Ca\(^{2+}\) transients at the two age groups after suppression of the Na/CaX by replacement of external 125 mM Na\(^+\) with equimolar Li\(^+\). Under this condition, neither the decay rate constant of CaT2 and nor that of CaT20 was significantly different between P5 and P10 (Fig. 4D), indicating little developmental changes of Ca\(^{2+}\) clearance mechanisms other than the Na/CaX. Furthermore, within the same age group, the decay rate constant of CaT2 was not affected by any of the above inhibitors \((P > 0.05)\), implying no developmental change of PMCA.

Finally, we estimated contributions of NCKX and NCX to Ca\(^{2+}\) clearance from difference in decay rate constants between K\(^+\) and TMA\(^+\) (for NCKX) and between K\(^+\) and XIP (for NCX) at P5 and P10 (Fig. 4, E and F). The contribution of NCKX was significantly increased during development (P5, 2.13 \(\pm\) 0.52; P10, 6.32 \(\pm\) 0.83, \(P < 0.01\)), whereas the NCX activity was not different between P5 and P10 (P5, 3.82 \(\pm\) 0.65; P10, 4.37 \(\pm\) 1.33, \(P = 0.71\)). These results suggest that the developmental upregulation of the NCKX activity is responsible for the faster Ca\(^{2+}\) clearance at mature calyx of Held terminals.

**Shaping of a HFS-induced Ca\(^{2+}\) transient by Ca\(^{2+}\) clearance mechanisms.** We tested how such developmental changes of NCX and NCKX shape the HFS-CaT evoked by a train of depolarizing pulses (100 Hz for 4 s; denoted by CaT400) under whole cell patch-clamp recording conditions. We used the same step depolarizing pulse as in Fig. 4. First, we confirmed that the \([Ca^{2+}]_{\text{peak}}\) of CaT400 under whole cell patch-clamp recording conditions is not different from that evoked by afferent fiber stimulation without a presynaptic whole cell patch at each age group (Fig. 5A). Next, the averaged trace of control CaT400s was compared with that of CaT400s recorded under each condition that inhibits NCKX or NCX (Fig. 5B for P5 and Fig. 5C for P10). In addition, averaged traces of CaT400s at P5 and P10 were compared under different conditions in Fig. 5D. When either NCKX or NCX was inhibited using the TMA-based internal solution (for NCKX) or XIP (for NCX), the averaged CaT400 at P5 was significantly different from that at P10 [TMA, \(F_{(1,1600)} = 34.64, P < 0.001\), Fig. 5Da; XIP, \(F_{(1,2000)} = 1.771.8, P < 0.001\), Fig. 5Db]. Despite the overall difference in the CaT400 trace between P5 and P10 under the TMA condition, the second half of the Ca\(^{2+}\) rising phase plus the decay phase were not different between two age groups [\(F_{(1,012)} = 0.88, P = 0.35\), Fig. 5Da]. The Ca\(^{2+}\) charge transfer elicited by the same train of depolarizing pulses (100 Hz, 4 s) was not significantly different between the two age groups (Fig. 5E). These results together with those of Fig. 4 support the idea that upregulation of NCKX is primarily responsible for the increased Ca\(^{2+}\) clearance at P10. We concluded that such a developmental increase of Ca\(^{2+}\) clearance prevents mitochondria from absorbing Ca\(^{2+}\) during HFS, resulting in the reduction of posttetanic Ca\(_{\text{res}}\) and PTP.

**DISCUSSION**

In the present study, we found that the lower amplitude of PTP at P10 is caused in part by the decrease in mitochondria-derived Ca\(_{\text{res}}\) (Fig. 2C). The lower posttetanic Ca\(_{\text{res}}\) at P10 is closely related to the lower \([Ca^{2+}]_{\text{peak}}\) of the HFS-CaT (Fig. 2D) because a lower \([Ca^{2+}]_{\text{peak}}\) buildup leads to lower mitochondrial Ca\(^{2+}\) uptake and thus lower posttetanic Ca\(_{\text{res}}\) (Fig. 3, C and D). This finding is consistent with our previous reports that mitochondrial Ca\(^{2+}\) uptake is activated at high \([Ca^{2+}]_w\) where Na/CaX is saturated (Kim et al. 2005). We showed that NCKX is most prominently upregulated at P10 (Fig. 4) and that the higher NCKX activity results in the lower \([Ca^{2+}]_{\text{peak}}\) of HFS-CaTs at P10 (Fig. 5). Therefore, we conclude that the reduced mitochondrial Ca\(^{2+}\) uptake during HFS at P10 can be ascribed to developmental upregulation of NCKX.

During the second postnatal week, the calyx of Held undergoes drastic developmental changes not only in Ca\(^{2+}\) clearance but also in its morphology, electrophysiological properties, short-term plasticity of EPSCs and synaptic ultrastructures (Chuhma and Ohmori 2001; Lee et al. 2012a; Taschenberger et al. 2002; Taschenberger and von Gersdorff 2000; Wang et al. 2008), implying that the developmental increase in NCKX occurs in parallel with other changes which optimize the calyx of Held for precise synaptic transmission. Similar rapid changes in Ca\(^{2+}\) clearance have been reported in other parts of the mammalian brain during early postnatal weeks. For example, SERCA and NCX activities greatly increase in supraoptic magnocellular neurons within a few days during the weaning period (Lee et al. 2007c). The mRNA expression levels of NCKX2, NCKX3, and NCX2 are strongly upregulated during the second week in cultured hippocampal neurons (Kip et al. 2006).

**Mechanisms for PTP at the calyx of Held synapse.** Although the present study focused on mitochondrial Ca\(_{\text{res}}\) as a mechanism for PTP at the calyx of Held synapse, other mechanisms for PTP have been reported: posttetanic increase in Ca\(^{2+}\) influx...
Previously, we have reported that the posttetanic increase in release probability was mediated by mitochondria-dependent Ca\(_{\text{res}}\) (Lee et al. 2008). Consistent with this view, Fioravante et al. (2011) showed that the posttetanic release probability increase in the calyx synapses of PKC k/o mice, where posttetanic Ca\(_{\text{res}}\) was not altered, is not different from that in the wild-type synapses. The present study shows that mitochondria-dependent mechanisms play a minor role in PTP at the mature calyx of Held. The experiments in Fioravante et al. (2011; P11–14) and Korogod et al. (2007; P7–10) have been performed in the relatively mature calyx of Held (P11–14) compared with Lee et al. (2008, P6–8). The minor role of mitochondrial Ca\(_{\text{res}}\) in PTP at the mature calyx of Held may explain why most but not all of the PTP was suppressed at the calyx of Held in PKC k/o mice, given that PKC is involved in the posttetanic increase in the readily releasable vesicle pool size.

The postnatal calyx of Held synapses are developmentally heterogeneous. Numerous parameters involving synaptic transmission are developmentally regulated (Taschenberger et al. 2002). Therefore, synaptic mechanisms underlying synaptic plasticity may be heterogeneous to result in seemingly inconsistent findings at the early postnatal calyx of Held synapses.

Interpretation of presynaptic Ca\(_{\text{2+}}\) buildup during HFS. A Ca\(_{\text{2+}}\) buildup during HFS depends on the net flux of Ca\(_{\text{2+}}\) into and out of the cytosolic compartment. In Fig. 3D, we estimated the cumulative mitochondrial Ca\(_{\text{2+}}\) uptake during HFS from the difference of Ca\(_{\text{2+}}\) buildup between the control and in the presence of Ru360. This estimation is valid when the activity of other Ca\(_{\text{2+}}\) clearance mechanisms and Ca\(_{\text{2+}}\) influx is not altered by internal application of Ru360 (Matlib et al. 1998) (Fig. 3F). Even if Ru360 has no direct effect on other Ca\(_{\text{2+}}\) clearance mechanisms, higher [Ca\(_{\text{2+}}\)] buildup itself can increase the activity of other Ca\(_{\text{2+}}\) clearance mechanisms (or dCa\(_{\text{2+}}$/dt), and thus the error term in Eq. 5 may not be negligible, which can invalidate Eq. 6. But this concern is unlikely. The slope of the [Ca\(_{\text{2+}}\)] buildup during HFS depends on Ca\(_{\text{2+}}\) influx and clearance. If Ca\(_{\text{2+}}\) clearance increases linearly as [Ca\(_{\text{2+}}\)] increases in the presence of Ru360, the slope of Ca\(_{\text{2+}}\) buildup should become diminished, because Ca\(_{\text{2+}}\) influx is rather reduced at the late period of HFS (Fig. 5E). Figure 3, A and B, shows that the slope of the [Ca\(_{\text{2+}}\)] buildup did not lessen during the [Ca\(_{\text{2+}}\)] buildup phase in the presence of Ru360, implying that Ca\(_{\text{2+}}\) clearance might be saturated at such high [Ca\(_{\text{2+}}\)] level. Therefore, dCa\(_{\text{2+}}$/dt may be little dependent on [Ca\(_{\text{2+}}\)], at least in the range where mitochondrial Ca\(_{\text{2+}}\) uptake is active, and thus the error term in Eq. 5 may not seriously affect the F\(_{\text{m,free}}\) estimates calculated according to Eq. 6 (Fig. 3D). This argument is further supported by the statistical similarity between estimates for mitochondrial Ca\(_{\text{2+}}\) uptake during HFS and TPP\(^{+}\)-sensitive posttetanic Ca\(_{\text{2+}}\) extrusion from mitochondria (Fig. 3, E and F).

This way of estimation of Ca\(_{\text{2+}}\) clearance, however, cannot be used for blockers of Ca\(_{\text{2+}}\) clearance mechanisms other than mitochondria. Because the higher buildup of [Ca\(_{\text{2+}}\)] caused by inhibition of NCX or NCKX invoke new participation of mitochondria in Ca\(_{\text{2+}}\) clearance (Fig. 3D), the [Ca\(_{\text{2+}}\)] buildup in the presence of a Ca\(_{\text{2+}}\) clearance mechanism blocker can be alleviated by mitochondria. This idea can explain why the increase in the peak Ca\(_{\text{2+}}\) of CaT400s caused by XIP is larger in P10 than that in P5 (Fig. 5, B and C), despite that the NCX activity estimated from CaT20s was not different between at P5 and at P10 (Fig. 4). Given that NCX is the major Ca\(_{\text{2+}}\) clearance mechanism in the moderate [Ca\(_{\text{2+}}\)] range and that the [Ca\(_{\text{2+}}\)] dynamic range of the control CaT400 at P5 is higher than that of P10, the higher Ca\(_{\text{2+}}\) buildup caused by XIP may recruit mitochondrial Ca\(_{\text{2+}}\) uptake more readily at P5 than P10, which in turn can alleviate excessive rise of [Ca\(_{\text{2+}}\)], during HFS at P5. In contrast, inhibition of NCKX may invoke little such mitochondrial Ca\(_{\text{2+}}\) uptake at P5 because NCX is still active and intrinsic NCKX activity is low at P5.
Finally, it should be noted that the dependence of mitochondrial contribution to Ca\(^{2+}\) clearance on [Ca\(^{2+}\)]\(_i\) in the present study is different from our previous results (Kim et al. 2005). When the CaT was induced by a short depolarizing pulse (50 ms), mitochondrial Ca\(^{2+}\) uptake started at [Ca\(^{2+}\)]\(_i\) > 2.5 \(\mu\)M. In contrast, it started at [Ca\(^{2+}\)] > 1 \(\mu\)M when CaTs were induced by HFS (Fig. 3D). Assuming that more homogeneous Ca\(^{2+}\) elevation is induced by HFS than by a short depolarizing pulse, this discrepancy may result from difference in the spatial distances from Ca\(^{2+}\) channels to mitochondria or NC(\(K\))X. The second possibility is suppression of NC(\(K\))X during HFS. It is well known that Na/CaX activity is steeply dependent on the membrane potential (\(V_m\) (Earm et al. 1990)). The \(V_m\) of the calyx of Held is not completely repolarized during repetitive firing at 100 Hz, but the trough of \(V_m\) is kept depolarized by ~20 mV (Kim et al. 2007). Such 20-mV depolarization can profoundly reduce the Na/CaX activity (Earm et al. 1990). Given that NC(\(K\))X competes with mitochondria for Ca\(^{2+}\) (Kim et al. 2005), such suppression of NC(\(K\))X may provide more chance for mitochondria to take up cytosolic Ca\(^{2+}\). Third, a shift of the threshold for the mitochondrial Ca\(^{2+}\) uptake in Kim et al. (2005) may be caused by a washout of cytosolic factors that facilitate mitochondrial Ca\(^{2+}\) uptake such as polyamines under the whole cell configuration (Jensen et al. 1987).

Ca\(^{2+}\) clearance mechanisms in the axon terminal. The present study showed that NCX is the major Ca\(^{2+}\) clearance mechanism, and NCKX plays a minor role at P5 (Fig. 4, E and F). As the calyx matures, the NCKX activity increases by a factor of 3, whereas the NCX activity is relatively stable, resulting in NCKX and NCX contributions to total Ca\(^{2+}\) clearance of 42% and 29% at P10. These values are comparable to 41% and 28% that we have previously reported for contributions of NCKX and NCX (Kim et al. 2005). It should be noted, however, that these estimates are valid for the [Ca\(^{2+}\)]\(_i\) elevation of about 2 \(\mu\)M. As shown in Fig. 4, neither NCKX nor NCX contributes to Ca\(^{2+}\) clearance in the decay phase of lower amplitude Ca\(^{2+}\) transients induced by two APs. Furthermore, NCX might be saturated at lower cytosolic [Ca\(^{2+}\)] than NCX. The comparison of Ca\(^{2+}\) build-up phases of CaT400s under TMA and XIP conditions implies that the Ca\(^{2+}\) build up is more effectively suppressed by NCKX than NCX at the early phase of the CaT400 where [Ca\(^{2+}\)] is ~2 \(\mu\)M (red trace in Fig. 5C), consistent with higher contribution of NCKX in this [Ca\(^{2+}\)] range (Fig. 4, E and F). The Ca\(^{2+}\) build up under the XIP condition (red trace), however, crossed over that under the TMA condition (blue trace in Fig. 5C) at the time point when the 300th AP invaded and the [Ca\(^{2+}\)]\(_i\) was ~4 \(\mu\)M, implying that the NCKX activity might be not so strong as NCX at such high [Ca\(^{2+}\)] range.

The present study demonstrates that the major developmental change in Ca\(^{2+}\) clearance is the increase in the NCKX activity at calyx of Held axon terminal. Among the NCKX family, NCKX2 may mediate the NCKX activity at axon terminals because NCKX at axon terminals exhibits low affinity for K\(^+\) (Lee et al. 2002) and PKC-dependent enhancement (Lee et al. 2006), which are characteristics of NCKX2. More importantly, NCKX2 has been shown to be transported to the axon terminal via KIF21A, whereas it undergoes endocytosis in somatodendritic regions (Lee et al. 2012b). These results are consistent with functional studies that have shown a lack of the NCKX activity in somatodendritic regions (Kim et al. 2003; Lee et al. 2007b, 2009).

Fast Ca\(^{2+}\) clearance enables an axon terminal to keep the presynaptic global [Ca\(^{2+}\)] low during HFS. High Ca\(^{2+}\) buildup promotes asynchronous neurotransmitter release (Chuhma et al. 2001; Otsu et al. 2004), which in turn dissipates the reluctant synaptic vesicles that can be readily recruited for synchronous release (Lee et al. 2012a). Furthermore, presynaptic axon terminals, which have a higher surface-to-volume ratio, undergo much higher [Ca\(^{2+}\)] elevation during HFS than soma, which renders involvement of mitochondria inevitable without other efficient Ca\(^{2+}\) clearance mechanisms. As shown in the present study, mitochondrial Ca\(^{2+}\) uptake is closely associated with PTP. Therefore, efficient Ca\(^{2+}\) clearance is essential for precise synaptic transmission and avoiding excessive depression. The developmental upregulation of NCKX might be one of the mechanisms that optimize the calyx of Held for fast and accurate synaptic transmission.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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