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SHORT COMMUNICATION
Syntaxin I modulation of presynaptic calcium channel inactivation revealed by botulinum toxin C1

Elise F. Stanley
Cellular and Molecular Biology Division, MP14-320, Toronto Western Research Institute, University Health Network, 399 Bathurst Street, Toronto ON, Canada

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Abstract

The chick ciliary ganglion calyx-type nerve terminal was used to examine voltage-sensitive inactivation of presynaptic N-type Ca^{2+} channels and to test if this inactivation is modulated by the transmitter release-associated protein syntaxin I. We tested the role of this protein with botulinum toxin C1 (BtC1) which cleaves syntaxin I close to its membrane anchor. The presynaptic Ca^{2+} current inactivated as two distinct populations with ~75% inactivating at a depolarized potential, $V_{1/2}$ ~−15 mV, with the remainder inactivating at ~−75 mV. BtC1 had no detectable effect on the latter component but resulted in a ~7 mV positive shift in the $V_{1/2}$ of the −15 mV inactivating component. These results confirm that the bulk of presynaptic N-type Ca^{2+} channels are in general resistant to voltage dependent inactivation and provide the first direct evidence that the physiological properties of presynaptic nerve terminal Ca^{2+} channels are subject to modulation by release site-associated proteins.

Syntaxin I is an integral surface membrane protein that plays a key role in neurosecretion at the presynaptic nerve terminal and that has multiple interactions with both release site and secretory vesicle proteins (O'Connor *et al*., 1993; Lévêque *et al*., 1994). The finding that this protein also interacts directly with the presynaptic N-type Ca^{2+} channel (Sheng *et al*., 1994) raised the possibility that syntaxin I might also modulate Ca^{2+} influx. Indeed, when N-type Ca^{2+} channels are coexpressed with syntaxin I in oocyte or cell-line models, the channels are inhibited by a mechanism that involves a prominent negative shift in the voltage range of inactivation (Bezprozvanny *et al*., 1995; Wiser *et al*., 1996; Degtiar *et al*., 2000; Jarvis & Zamponi, 2001a). In control cells the half-inhibition potential ($V_{1/2}$) ranged from −80 to −45 mV but with syntaxin I the inactivation curve was hyperpolarized by about −20 mV. On the basis of these observations, it has been suggested that syntaxin I may modulate Ca^{2+} dynamics at the nerve terminal by down regulating release site-associated Ca^{2+} channels (Bezprozvanny *et al*., 1995). Ca^{2+} channels at intact nerve terminals have characteristics, however, that differ substantially from the same channel types in oocyte or cell line expression systems. At the few nerve terminals where Ca^{2+} currents can be recorded directly, the channels have generally been found to be markedly resistant to voltage-dependent inactivation. Attempts to detect a syntaxin I-dependent effect on the Ca^{2+} current inactivation properties have proved negative (Marsal *et al*., 1997; Stanley & Mirotznik, 1997) but a recent study was suggestive of such an effect, with the finding that syntaxin I cleavage caused a delayed potassium-dependent Ca^{2+} uptake through Ca^{2+} channels into synaptosomes (Bergsman & Tsien, 2000).

We have re-examined the role of syntaxin I in modulating presynaptic Ca^{2+} channel inactivation *in situ* using the calyx presynaptic nerve terminal preparation at the chick ciliary ganglion (Stanley & Goping, 1991). We find that the presynaptic Ca^{2+} channels fall into two distinct populations with respect to their inactivation properties and that only one of these is syntaxin I sensitive. The chick calyx nerve terminal has the experimental advantage that virtually all the Ca^{2+} channels are of the N-type ($Ca_V2.2$) (Stanley, 1991; Yawo & Momiyama, 1993; Stanley, 1997). The involvement of syntaxin I in the kinetics of these channels was examined with botulinum toxin C1 (BtC1) which is known to specifically cleave syntaxin I at amino acids 253/4, close to its transmembrane anchor, and results in a loss of staining for syntaxin I at the calyx nerve terminal (Stanley & Mirotznik, 1997).

The experimental preparation of the chick ciliary ganglion calyx for patch clamp recording has been described (Stanley & Goping, 1991; Stanley, 1991; Stanley & Mirotznik, 1997). Fifteen day chick embryos were used. Chicks were removed from the egg and immediately beheaded prior to the dissection of both ciliary ganglia. The external recording medium was (in mM): sodium-N-tris[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES), 115; $CaCl_2$, 10; $MgCl_2$, 1; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 10; tetraethylammonium chloride, 10; with 1 μM tetrodotoxin. The patch (internal) solution was: Cs^+ gluconate, 120; CsCl, 30; ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) , 10; $MgCl_2$, 3; HEPES, 10; ATP, 2; adjusted to pH 7.3 with CsOH. All recordings were carried out at room temperature, 22–24 °C. Recordings and data analysis was carried out with the pClamp suite of programs (Axon Instruments). Data was filtered at 2 kHz on line and was sampled at 0.1 ms per point. Leak subtraction was carried out on line with the P/4 protocol, using a positive leak pulse with the leak train following the corresponding test pulse. Normalized current amplitudes were subjected to a least squares fit (Origin, Microcal) with the Boltzmann relation:

$$I_2 + [(I_1 - I_2)/\{1 + \exp[(V - V_{1/2})/K]\}]$$

Correspondence: Dr Elise F. Stanley, as above.
E-mail: estanley@uhnres.utoronto.ca

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where V is the interpulse holding potential, I_1 is the estimated peak current at $V_{-\infty}$, I_2 is the estimated peak current at $V_{+\infty}$, $V_{1/2}$ is the midpoint holding potential voltage and k is a slope constant.

Ca^{2+} current inactivation was assayed by holding the membrane potential at a fixed value (V_H), initially −80 mV, and then delivering a short test pulse to measure the evoked Ca^{2+} current amplitude at that V_H. The V_H was then stepped by a +10 mV increment and, after waiting for inactivation to stabilize, the test pulse was repeated (protocol, Fig. 1A, upper panel). We first determined the necessary holding duration for V_H to stabilize between trials by evoking Ca^{2+} currents at 5 s intervals after a step in V_H from −80 to −40 mV. The decline in current amplitude could be fit with a single time constant of ~6 s with a maximum (asymptotic) inactivation of 23 ± 1% ($N = 4$). As whole-calyx recordings are difficult to maintain in excess of 5 min, it was necessary to minimize the experiment duration by limiting each holding potential step to 5 s. While the Ca^{2+} current did not stabilize in this time period with the single step example above, this did not greatly affect the results as in the actual protocol, sequential V_H steps of +10 mV, inactivation at any one V_H is additive to that during the prior step. Thus, when V_H was held for 5 s at each 10 mV increment across the −80 to −40 range, the cumulative inhibition was 20 ± 2 (see Fig. 1B) which is not significantly different from the asymptotic inhibition observed a single step from −80 to −40 above. However, due to the limitations in recording duration the results presented here reflect 'prepulse' rather than true 'steady state' Ca^{2+} channel inactivation characteristics. We examined Ca^{2+} channel inactivation with +10 mV increments in V_H from −80 to +10 mV. Across the −80 to −40 mV range the amplitude of the test current pulse underwent a gradual decline but beyond −30 mV there was a precipitous fall (Fig. 1B).

A visual inspection of the data suggests two populations of Ca^{2+} channels, one that inactivates at relatively negative potentials, the 'inactivation-sensitive' population, and a second 'inactivation-resistant' pool. This interpretation was supported by Boltzmann fits to the data. While a very poor fit was obtained with a single Boltzmann across the entire data set, the fit was excellent when applied only to the inactivation-resistant −40 to +20 mV subset (Fig. 1B; $V_{1/2} = -13.6 \pm 0.7$ mV, $k = 5.4 \pm 0.6$ mV, $N = 4$). When this fit was subtracted from the total data, a second Boltzmann was fit to the residual data across the −40 to −80 mV range. This fit described the inactivation-sensitive population (Fig. 1C; $V_{1/2} = -73.0 \pm 1.2$ mV, $k = 14.5 \pm 0.9$ mV, $N = 4$). Thus, at the baseline −80 mV holding potential, the inactivation-resistant component comprises 80.9 ± 1.7% of the total Ca^{2+} current while the remainder comprises the inactivation-sensitive component. However, projecting the two fits to their negative asymptotes indicate that the ratio of inactivation-resistant to inactivation-sensitive Ca^{2+} channels is 75% : 25%. We used botulinum C1 toxin (BtC1) to test if syntaxin I affects N-type Ca^{2+} channel inactivation at the intact

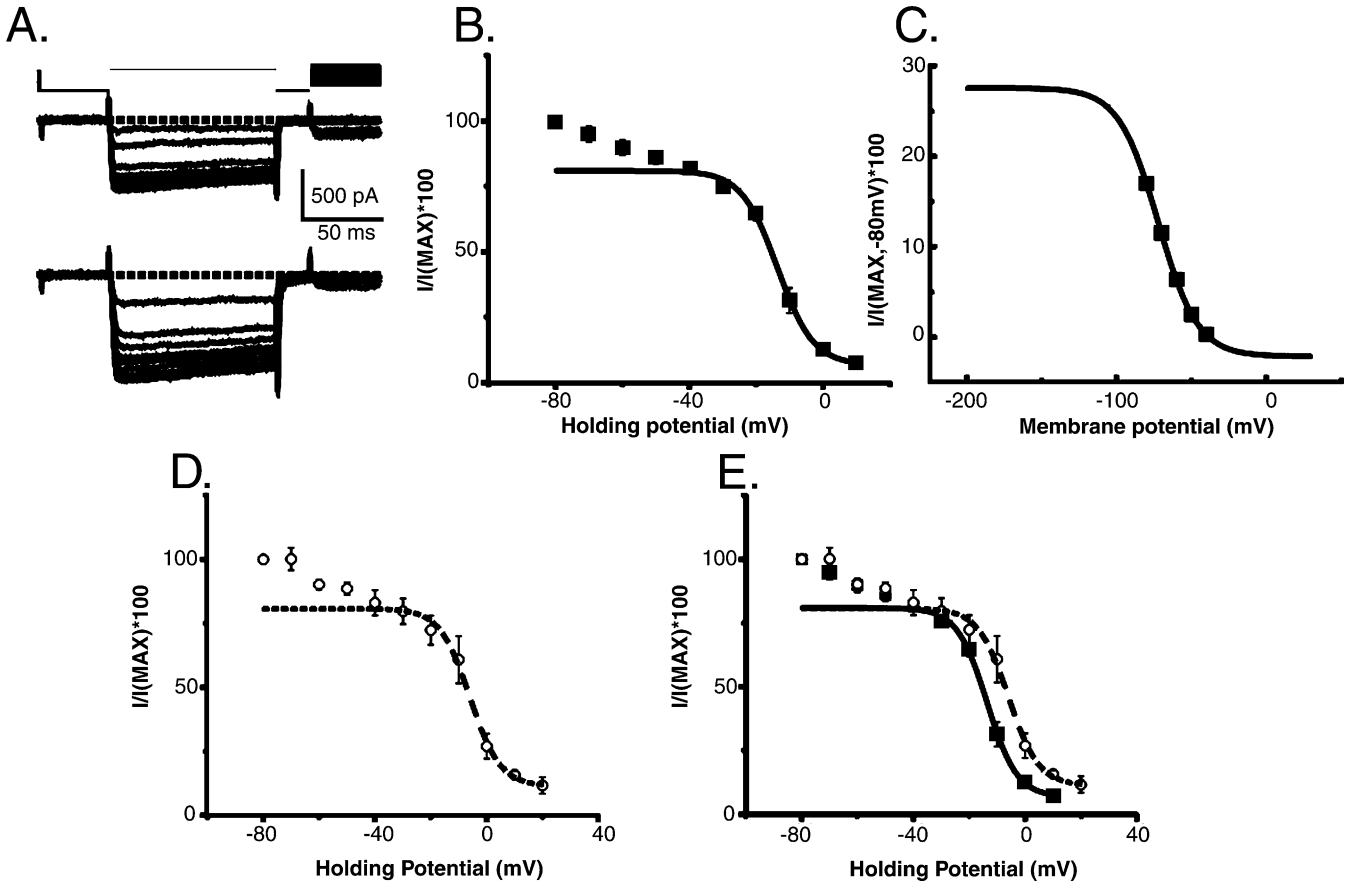

FIG. 1. (A) Current traces recorded from control (middle panel) and BtC1 -treated (lower panel) calyx nerve terminals by a test pulse to +20 mV. The dashed line indicates the 0 current level. The protocol is shown in the top panel. (B) Plot of mean ± SEM peak current amplitude in 4 control terminals. The data points from holding potentials of −40 to +10 mV have been fit by a Boltzmann relation which was extrapolated to −80 mV (see text). (C) Plot of the residual current from part B after subtracting the Boltzmann fit from the current amplitude data in the −40 to −80 mV range. The subtracted data set was fitted by a second Boltzmann relation and extrapolated to its negative asymptote. The vertical-axis intercept is an estimate of the low-voltage-inactivating current component as a fraction of the total current at −80 mV. (D) Effect of BtC1 on the nerve terminal calcium current-to-voltage relation ($N = 4$). Analysis as in B. (E) Superimposition of B and D.

nerve terminal. Chicks were paralysed by an overnight treatment *in ovo* with toxin (Stanley & Mirotznik, 1997) and calyces were isolated for presynaptic Ca^{2+} current analysis. As in the control calyces, the bulk of the current inactivated over a very positive range, consistent with a distinct population (Fig. 1D). The Boltzmann relation provided an excellent fit across the -40 to $+20$ mV range ($V_{1/2} = -5.9 \pm 1.1$, $k = 5.4 \pm 1.0$ mV, $N = 4$, $P < 0.01$, compared with controls, *t*-test). The residual low-voltage-inactivating Ca^{2+} current could be superimposed on that of the control data (Fig. 1E).

Thus, BtC1 treatment had no obvious effect on the low-voltage inactivating component while it shifted the high-voltage inactivating components to a more positive inactivation range. This shift occurred without an obvious change in the slope factor and is likely the antithesis of the negative shift observed when syntaxin I is coexpressed with N-type channels. This finding validates the cellular expression system model in the analysis of Ca^{2+} channel–release-site interactions at the nerve terminal. However, a future challenge for the expression system approach is to explain the molecular basis of the markedly more positive, typically 20 to 70 mV, Ca^{2+} channel inactivation range in the normal intact presynaptic nerve terminal. Possibilities include a modified alpha subunit, a different accessory subunit or modulation of channel properties by an associated presynaptic terminal protein. We do not know as yet if the inactivation voltage-sensitive, and hence, syntaxin I independent, population represents Ca^{2+} channels that are not linked to the release sites or comprise an entirely different channel pool. It should be noted that syntaxin I removal actually has rather a small effect on presynaptic Ca^{2+} channel activity compared with that reported for cell expression systems. Nonetheless, the observed inactivation shift provides the first direct physiological evidence that presynaptic Ca^{2+} channels are modulated by an interaction that involves syntaxin I. Previous studies indicate that at least in cellular expression system models a number of release site-associated proteins are also able to modulate the Ca^{2+} channel or can interfere with the action of syntaxin I (Atlas, 2001; Jarvis & Zamponi, 2001b). Together these findings are suggestive of a complex intermolecular conversation between the proteins of the vesicular fusion mechanisms and the Ca^{2+} channels in their immediate vicinity.

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Abbreviation

BtC1, botulinum toxin C1.

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