Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel

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Abstract The voltage sensitive N-type calcium channel interacts functionally and biochemically with synaptotagmin (p65). N-type channel interaction with p65 is demonstrated in the *Xenopus* oocyte expression system, where p65 alters the steady state voltage inactivation of the N-channel, and fully restores the syntaxin-modified current amplitude and inactivation kinetics in a calcium dependent manner. In agreement with the functional results, GST-p65 fusion protein binds to a cytosolic region, amino acids 710–1090 of the N-type channel (N-loop$_{710-1090}$). The results of the combined approach provide a functional and biochemical basis for proposing that p65 interaction with the N-type channel brings p65 into a close association with a syntaxin-coupled channel. In turn, calcium entry through the liberated channel initiates fusion of the primed vesicles with the cell membrane at a short distance from the site of calcium entry.

Key words: N-type calcium channel; Transmitter release; Synaptotagmin; Syntaxin; SNAP-25

1. Introduction

Synaptotagmin (p65) is a vesicular calcium-binding protein initially proposed to play an essential role in the process of regulated secretion [1,2]. Association of the N-type channel with p65 [3] was demonstrated in immunoprecipitation experiments which also suggested the formation of a ternary complex with syntaxin [3–8]. A functional role for p65 has been indicated by genetic studies [9,10], microinjection into squid neurons [11] and PC12 cells [12], interaction with neurexins [13] and analysis of mutation in the p65 gene in mice [14,15]. Further support for the role of p65 in the transmitter release process was inferred from its ability to bind phospholipids [16–19]. The calcium binding sites of p65 are localized at the C2 domains [20,21] and recently, the three-dimensional structure of the C2A domain was elucidated to establish a new calcium binding motif [22]. p65 binding to syntaxin is calcium dependent and is confined to the C2A domain [18,23].

Previously, we and others have shown that co-expression of the N-type Ca^{2+} channel with cRNA of syntaxin in the *Xenopus* oocyte expression system modifies current properties and channel kinetics [24,25]. The modified channel properties are further modulated by SNAP-25 [25]. Syntaxin interaction with the N-type calcium channel was demonstrated as inward current inhibition and a decrease in the rate of inactivation [24,25]. Coexpression of SNAP-25 with syntaxin 1A restores current amplitude but does not recover the time constant of inactivation [25]. To explore a putative functional N-type channel interaction with p65 and to further establish channel/syntaxin/SNAP-25/p65 relationships, we combined physiological studies using the oocyte expression system with in vitro binding of recombinant cytosolic N-channel fragment to GST-p65 recombinant fusion protein.

Here we show by utilizing the oocyte expression system that p65 modifies the kinetic properties of both the N-type channel and an N-type channel/syntaxin putative complex. In vitro binding assays of recombinant fusion proteins demonstrate a direct Ca^{2+} dependent interaction of p65 with a cytosolic loop separating repeats II and III of the N-type channel (N-loop$_{710-1090}$). These results provide a functional and biochemical basis for a close association of the N-type channel with synaptic vesicle. A sequential interaction of the N-type channel with syntaxin and p65 may be important for the efficient synaptic transmission.

2. Materials and methods

2.1. Materials

The following plasmids were kindly provided: GST-syntaxin(amino acids 4–267), GST-SNAP-25 (full length) and anti-p65 antibodies by M.K. Bennett. GST-p65(1–3; amino acid 96–265), p65(1–5; amino acid 96–421) and p65(3–5; amino acid 248–421) by R.H. Scheller and T.C. Südhof; full lengths: α2/δ subunits by A. Schwartz, β2A by X. Wei and L. Birnbaumer and rbB-I by T.P. Snutch. Expression vector pGEX-KG was from Pharmacia and pQE was from Qiagen. Glutathione-agarose-4B (GSH) beads, IPTG, G(5′)ppp(5′)G were from Pharmacia (USA).

2.2. Expression in Xenopus oocytes and cRNA injection

Xenopus laevis oocytes, stage V–VI, were removed surgically from the ovaries of anesthetized animals and transferred to a Ca^{2+} free ND96 buffer: 96 mM NaCl; 2 mM KCl; 1 mM $MgCl_2$; 5 mM HEPES, pH 7.4 containing 2 mg/ml collagenase (154 U/mg, Worthington Biochem., USA). The follicular cell layer was removed by shaking the oocytes in this buffer for 1.5–2 h at 23°C [25]. Plasmid DNAs were linearized, treated with proteinase K and transcribed with T7 polymerase (Stratagene kit), in the presence of the cap analog G(5′)ppp(5′)G. Before injection, cRNA samples were examined on ethidium bromide-stained denatured agarose gel to verify the correct size of a single undegraded band, and cRNA concentrations were determined by absorbance at 260 nm.

2.3. Electrophysiological assays

Whole cell currents were recorded by applying standard two-microelectrode voltage clamp using a Dagan 8500 amplifier. Voltage and current agar cushioned electrodes (0.3–0.6 MΩ tip resistance) filled with 3 M KCl were used [28]. Oocytes were injected with 40 nl of 50 mM BAPTA [1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid], or EGTA [ethyleneglycol-bis(β-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid], pH 7.0 prior to recording, as indicated. Current-voltage relationships were determined as follows: the oocytes were impaled in ND96 buffer and inward current changes were monitored by voltage steps from −80 to +60 mV of 500 ms duration, with 30 s intervals, in Ba^{2+} solution: 40 mM $Ba(OH)_2$; 50 mM NMDG; 1 mM KOH; 5 mM HEPES pH 7.5; or Ca^{2+} solution: 5 mM $Ca(OH)_2$; 85 mM NMDG; 1 mM KOH; 5 mM HEPES, pH 7.5. The buffers were

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titrated to pH 7.5 with methyl sulfonate. Voltage dependent inactivation was carried out as previously described [25]. The rate of inactivation was analyzed by a single exponential decay fit = A exp[t/τ]+B, where A = current amplitude; τ = rate constant; t = ms and B = non-inactivating current. Steady state voltage dependent inactivation was fitted by a single Boltzmann distribution with normalized current = $C/\{1+\exp[(E_{50}-E_m)/k]\}+(1-C)$, where C = maximal steady-state inactivating current, E_{50} = midpoint of inactivation, E_m is the conditioning voltage and k = slope parameter. The software packages are pClamp 6.0 (Axon Instruments, CA), and Origin 3.73 (MicroCal).

2.4. Construction and expression of recombinant GST and His fusion proteins

N-channel fragment was prepared from rat brain α_{1B} cDNA (rbB-1) excised by *Bst*XI (2128 bp) and *Sfc*I (3371 bp), filled in and ligated to pQE30 (Qiagen).

All constructs were transformed into the protease deficient strain BL21pLysS of *Escherichia coli* (Novagen). Fusion proteins were prepared essentially according to Guan and Dixon [29]. Free syntaxin and SNAP-25 were prepared by thrombin cleavage of the corresponding purified GST fusion proteins.

Purification of His_6-N-$loop_{710-1090}$ was carried out according to Qiagen protocols.

2.5. In vitro binding assays and Western blotting

GST fusion proteins (100 pmol), determined by Coomassie blue evaluation on SDS-PAGE, were bound to GSH beads (25 μl) in PBST: 140 mM NaCl; 2.7 mM KCl; 10.1 mM Na_2HPO_4; 1.8 mM KH_2PO_4 pH 7.3 and 0.05% Tween 20, for 30 min at 23°C. Then the beads were washed with TBST (0.3 ml×2) and binding to purified His_6-N-$loop_{710-1090}$ (100 pmol) was carried out in PBS buffer containing 1 mg/ml bovine serum albumin, for 1 h with gentle mixing at 23°C. Beads were washed (1 ml×3) in PBS containing 0.1% Triton X-100 at 4°C. Bound proteins were eluted with 15 mM glutathione in 50 mM Tris-HCl, pH 8.0, removed from the beads by 1 min centrifugation (3500 rpm) and applied to SDS-PAGE for analysis. Immunoblots were probed using affinity purified antibodies generated against a peptide sequence, RHHRHRDRDKTSAST [30], at the N-loop region and visualized by enhanced chemiluminescence (ECL) system.

3. Results

3.1. Functional interaction of synaptotagmin (p65) with the N-type Ca^{2+} channel

Inward currents generated by N-type calcium channel subunits (α1B/α2/δ/β2A) expressed in *Xenopus* oocytes are significantly modified when coexpressed with syntaxin and SNAP-25 [25]. In the present study we have examined the effect of p65 on N-type calcium channel. Inward Ba^{2+} currents (α1B/α2/δ/β2A), evoked from a holding potential of −80 mV to various test potentials in 10 mV increments, were tested in *Xenopus* oocytes coexpressing the channel subunits with p65 (Fig. 1A–C). As shown in the current-voltage relationship, current amplitude is not affected by p65 (< 5% at test pulse to +20 mV) (Fig. 1A). Similarly, there is no significant effect of p65 on the rate of inactivation, measured at the range of −10 to +50 mV (Fig. 1B). However, analysis of the steady state voltage dependent inactivation shows a 10 mV shift in the midpoint of inactivation (E_{50}) toward more depolarizing potentials by expressing p65 (−19 ± 1 to −9 ± 2 mV), and the slope parameter changes from 10 ± 1 to 21 ± 6 (Fig. 1C).

As previously shown, when syntaxin is co-expressed with the channel there is a strong reduction in current amplitude and a decrease in the rate of inactivation [25]. This reduction in evoked current amplitude imparted by syntaxin 1A is hardly affected by p65 (Fig. 2A). However, the reduced rate of inactivation is partially reversed by p65, from τ = 3.2 ± 0.3 s in the presence of syntaxin to 2.5 ± 0.3 s when both syntaxin and p65 are coexpressed (Fig. 2B,C).

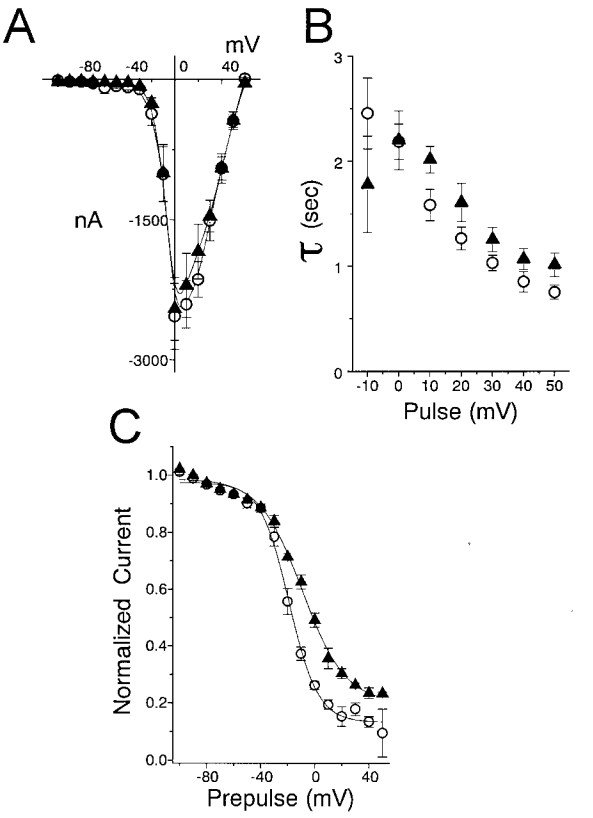

Fig. 1. Interaction of syntaxin and p65 with the N-type Ca^{2+} channel. N-type channel subunits α1B/α2/δ/β2A were expressed in *Xenopus* oocytes in the absence and in the presence of p65. A: Ba^{2+} currents were evoked from a holding potential of −80 mV in response to a 500 ms pulse to various test potentials in 10 mV increments are presented in a leak subtracted peak current-voltage relationship. Data were collected from oocytes expressing the three channel subunits alone (○) and with p65 (▲). B: Inactivation rate constants (τ) of currents generated by α1B/α2/δ/β2A alone, plotted against a 4.5 s test pulse to various potentials in 10 mV increments (○), and with p65 (▲). C: Inactivation curves of N-type calcium channel. Steady-state normalized Ba^{2+} currents generated by α1B/α2/δ/β2A (○) and with p65 (▲). Peak normalized currents were fitted to a Boltzmann inactivation curve represented by the smooth curve. Two-sample Student's *t*-tests assuming unequal variance were applied, and *P* values < 0.01 were obtained. cRNA injected (per oocyte): α1B (16 ng); α2/δ (6 ng); β2A (10 ng); p65 (10 ng). The channel subunits were injected 2 days prior to the injection of p65. Currents were recorded 7–9 days after injection; BAPTA was injected prior to recording. The data points correspond to the mean ± S.E.M. of currents, $n = 6$–10.

In the experiments presented above, syntaxin-modified N-channel properties were determined with Ba^{2+} as the charge carrier and BAPTA, the calcium chelator. Substituting Ca^{2+} for Ba^{2+} and EGTA for BAPTA shows that similar to Ba^{2+} currents, the Ca^{2+} current amplitude is not affected by p65 (Fig. 3A). Conversely, current amplitude inhibited by syntaxin 1A (55% at test pulse to +20 mV), is fully reinstated by p65 (Fig. 3A). Similarly, the time constant of inactivation (τ) of the syntaxin associated channel (5.7 ± 0.7 s), is fully recovered to the τ value of the channel alone (3.3 ± 0.2 s; Fig. 3B). Reversal of the syntaxin reduced rate of inactivation by p65 is not affected by the presence of SNAP-25 (τ = 5.3 ± 0.5 to 3.1 ± 0.5 s [25]; Fig. 3B).

Hence, p65 relaxes the channel from the syntaxin clamp, possibly by competing with the channel for syntaxin binding; this 'liberation' of the channel appears to be calcium sensitive. While both Ca^{2+} and Ba^{2+} support p65 relief of the syntaxin-channel clamp Ba^{2+} appears to be less effective perhaps due to the change in p65 specificity for Ba [15,21] (see below).

3.2. N-loop$_{710-1090}$ binding to synaptic proteins

The functional modifications of the N-type channel's properties by p65 prompted our in vitro binding studies of recombinant p65 fusion protein to a cytosolic region of the N-type channel. Equivalent concentrations (100 pmol/300 μl) of GST-p65(1–5; amino acids 79–421) containing C2A and C2B domains, GST-p65(1–3; amino acids 96–265) containing C2A domain, GST-p65(3–5; amino acids 248–421) containing C2B domain, and GST alone, were immobilized onto GSH

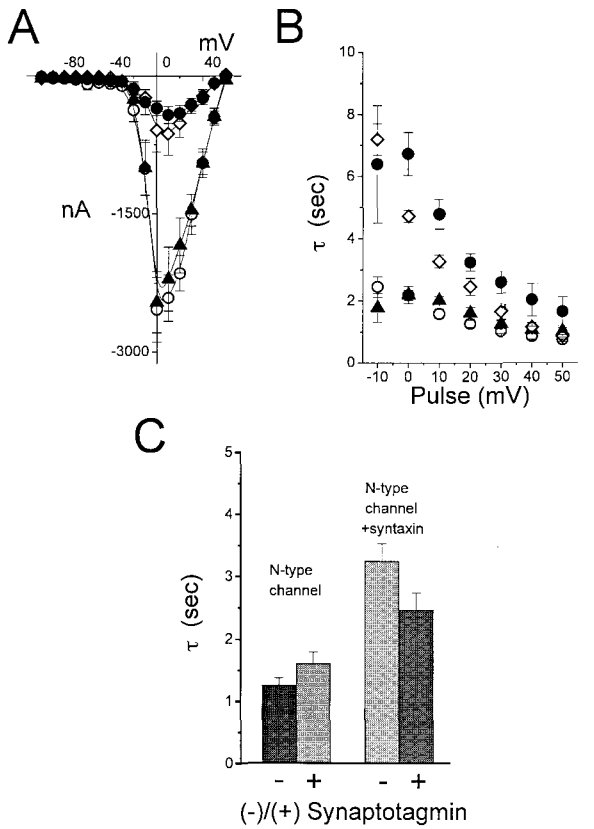

Fig. 2. Modification of the N-type calcium channel expressed in *Xenopus* oocytes by p65, syntaxin 1A and both combined. N-type channel subunits α1B/α2/δ/β2A were expressed in *Xenopus* oocytes either alone or in combination with synaptic proteins as indicated. A: Ba^{2+} currents were evoked from a holding potential of −80 mV in response to a 500 ms pulse to various test potentials in 10 mV increments and are presented in a leak subtracted peak current-voltage relationship. Data were collected from oocytes expressing α1B/α2/δ/β2A (○), with syntaxin 1A (●), with p65 (▲), and both combined (◇). B: Inactivation rate constants (τ) of currents generated by α1B/α2/δ/β2A (○) with syntaxin 1A (●), with p65 (▲) and both combined (◇), plotted against a 4.5 s test pulse to various potentials in 10 mV increments. C: Inactivation rate constants in response to a test potential of +20 mV (see B). Oocytes were injected with BAPTA prior to recording. The data points correspond to the mean ± S.E.M. of currents ($n = 5$–7). Two-sample Student's *t*-test assuming unequal variance was applied, and *P* values < 0.01 were obtained. cRNA injected, see legend to Fig. 1; syntaxin 1A (7 ng/oocyte).

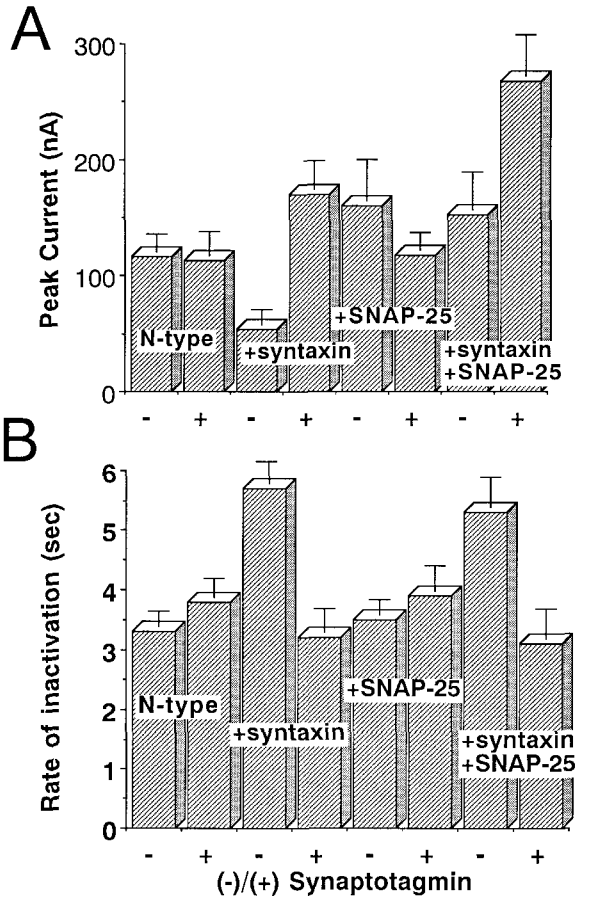

Fig. 3. Functional interaction of p65 with the N-type channel is sensitive to calcium ions. N-type channel subunits α1B/α2/δ/β2A were expressed in *Xenopus* oocytes either alone or together with synaptic proteins as indicated. A: Ca^{2+} currents were evoked from a holding potential of −80 mV in response to a 500 ms pulse to a +10 mV test potential. Peak currents (mean+S.E.M.) were recorded from oocytes co-expressing syntaxin 1A, SNAP-25, p65, and their combinations, as indicated. B: Rate of inactivation (τ) was determined for α1B/α2/δ/β2A Ca^{2+} currents evoked to a test potential of +20 mV for a duration of 5 s in oocytes co-expressing syntaxin 1A and SNAP-25 with or without p65. Oocytes were injected with EGTA prior to recording. The data points correspond to the mean ± S.E.M. of currents ($n = 6$–10). Two-sample Student's *t*-test assuming unequal variance was applied, and *P* values < 0.01 were obtained. cRNA injected, see legend to Fig. 1.

beads and incubated with recombinant His_6-N-loop$_{710-1090}$ (N-loop; 100 pmol). Recombinant N-loop binds to p65(1–5), p65(1–3) and p65(3–5), as detected by affinity purified antibodies prepared against the N-loop peptide (Fig. 4A). Interaction specificity is demonstrated since GST protein itself does not bind N-loop (Fig. 4A, right lane). The sequence similarity and folding of the two halves of p65 may determine the almost equipotent binding of both C2 regions to the N-loop. In addition, His_6-N-loop$_{710-1090}$ binds to syntaxin 1A and SNAP-25 (Tobi et al., unpublished results), confirming N-channel interaction with SNAP-25 and syntaxin [26,27].

3.3. Characterization of N-loop binding to p65: calcium dependence

The Ca^{2+} dependence of N-loop$_{710-1090}$ binding to p65 is demonstrated by the effect of increasing Ca^{2+} concentration (Fig. 4B, upper panel). N-loop$_{710-1090}$ binding to GST-p65(1–

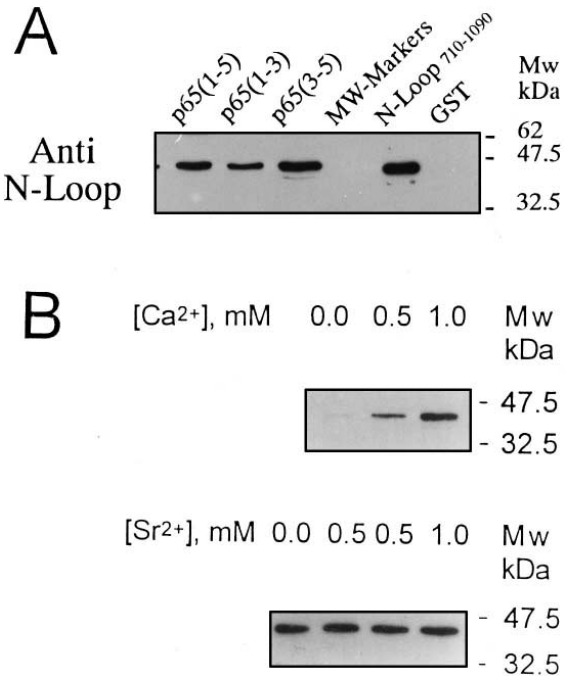

Fig. 4. Binding of N-loop$_{710-1090}$ to p65; calcium sensitivity. A: Recombinant GST fusion proteins (100 pmol): GST, SNAP-25, GST-syntaxin 1A, GST-p65(1–5), GST-p65(1–3), GST-p65(3–5) and GST alone were immobilized on GSH beads, and reacted for 90 min at 23°C with recombinant His$_6$-N-loop$_{710-1090}$ fusion protein (100 pmol). Bound His$_6$N-loop$_{710-1090}$ and the corresponding synaptic proteins were specifically eluted with 15 mM glutathione and separated on SDS-PAGE (see Section 2). Synaptic GST fusion proteins were visualized at the nitrocellulose membrane by staining with Ponceau S, and His$_6$-N-loop$_{710-1090}$ by specific affinity purified antibodies. B: Recombinant His$_6$-N-loop$_{710-1090}$ (100 pmol) was added to GSH immobilized: GST-p65(1–5) and incubated with increasing Ca^{2+} (upper panel) or Sr^{2+} concentrations (lower panel), as indicated. The presence of equal amounts of the GST-fusion proteins transferred to the nitrocellulose membrane was confirmed by Ponceau S staining.

5) in a Ca^{2+} free solution (defined as a solution with no added Ca^{2+}), is greatly enhanced (~30-fold) by [Ca^{2+}] rise (Fig. 4B, upper panel). The enhanced p65(1–5) binding to N-loop$_{710-1090}$ occurs at a [Ca^{2+}] range of 0.5–1.0 mM, much higher than observed for N-loop$_{718-963}$ binding to syntaxin (12 μM [27]), or phospholipid binding to p65-C2A domain (4 μM [18]). It is ~2–5-fold higher than predicted for the secretion process (~200 μM [31]), but is in the range exhibited for syntaxin/p65 binding (0.6 mM [18]). Alterations in the electrostatic charges by Ca^{2+} at the C2A domain [22] may account for a stronger N-loop$_{710-1090}$ binding to p65(1–5) in its calcium bound form.

N-loop$_{710-1090}$ binding to p65(1–5) is detected also in the presence of Sr^{2+} (Fig. 4B, lower panel). However, unlike Ca^{2+}, N-loop binding to p65 is not influenced by increasing Sr^{2+} concentrations [32]. This result implies Ca^{2+} specificity of N-loop$_{710-1090}$/p65(1–5) binding and may account for the reduced ability of p65 to restore the syntaxin modified channel properties when Ba^{2+} substitutes for Ca^{2+} (Fig. 2A–C).

4. Discussion

4.1. Functional interaction of N-type calcium channel with p65

Previously it was shown that purified ω-conotoxin sensitive calcium channel is quantitatively immunoprecipitated with anti syntaxin antibodies [3–5] suggesting that p65 can form a ternary complex with syntaxin and the N-type channel [3–8]. Recently, it was shown that p65 interacts to generate a stabilized activated complex with syntaxin and SNAP-25 and upon Ca^{2+} binding may drive the fusion reaction [33]. To further examine these possibilities we explored N-type channel functional interactions with various synaptic proteins.

The expression of p65 with the channel alters significantly the rate of inactivation suggesting an interaction with the channel in the absence of the other components of the exocytotic complex. A direct p65 interaction with the channel is reflected by recombinant p65 binding to His6-N-loop$_{710-1090}$ fusion protein.

p65 interaction with the channel is manifested by reversal of the kinetics and current amplitude of a syntaxin associated N-type channel, further supporting a p65 and syntaxin crosstalk with the N-type channel. When syntaxin-induced inhibition of the channel has already been relaxed by SNAP-25, p65 further increases the amplitude of the current making it more than twice larger than the 'free' channel. A less prominent but apparently significant increase above 'free channel' level is observed even in the absence of SNAP-25. These facts may support an allosteric effect of p65 on the N-channel/syntaxin interaction, where the functional effect of syntaxin is reversed by p65 while the physical association is preserved. Hence, these results may predict the formation of a ternary complex of channel, p65 and syntaxin.

The calcium specific reversal of syntaxin induced inhibition of inward current and reduced rate of inactivation could be interpreted by the formation of a high affinity syntaxin/p65 complex [4,18,23] which does not bind to the channel. Furthermore, the Ca^{2+} specific p65 interaction with syntaxin [18,23] could explain the less efficient rescue of the syntaxin clamped channel by p65 when Ba^{2+} is substituted for Ca^{2+}.

In addition, the fast component of neurotransmitter release in p65 knockout mice is strongly depressed [14] and cannot be stimulated by Sr^{2+} [15]. These results further support our proposed hypothesis that a distinct Ca^{2+} specific p65/syntaxin interaction restores the channel properties, and that the calcium sensitive N-loop interaction with p65 may be related to the fast component of regulated secretion. The likelihood of a syntaxin/p65/channel ternary complex formation was examined by in vitro binding of N-loop to p65(1–5). When both N-loop and free syntaxin are present, syntaxin binds to p65, while the distinct N-loop binding to p65(1–5) is lost (Tobi et al., unpublished results). Hence, in addition to targeting of the vesicle to the plasma membrane via a direct p65 interaction, p65 dissociates the N-channel from a syntaxin 'grasp', possibly by forming a p65/syntaxin complex. The liberated channel provides calcium ions leading to vesicle fusion with the cell membrane.

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