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Nicotinic Acid Adenine Dinucleotide Phosphate Enhances Quantal Neurosecretion at the Frog Neuromuscular Junction: Possible Action on Synaptic Vesicles in the Releasable Pool

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ABSTRACT

Inositol 1,4,5-trisphosphate (IP_3) and cyclic adenosine diphosphate-ribose (cADPR) are second messengers that enhance neurosecretion by inducing Ca^{2+} release from smooth endoplasmic reticulum (SER). The putative intracellular messenger, nicotinic acid adenine dinucleotide phosphate (NAADP), releases Ca^{2+} from stores that are distinct from SER. Evidence is presented here that NAADP causes a concentration-dependent increase in quantal output that is associated with an increase in probability of transmitter release at the frog neuromuscular junction. This effect is mimicked by A23187, a Ca ionophore that promotes Ca^{2+} entry at the plasmalemma. The response to NAADP is potentiated by IP_3 but antagonized by cADPR. Thapsigargin completely blocks IP_3 and cADPR responses and decreases but does not prevent the response to NAADP. We conclude that NAADP, whose receptors are widely distributed in the brain, enhances neurosecretion by releasing Ca^{2+} from an internal store near the plasmalemma, possibly from synaptic vesicles in the releasable pool. These data also support the hypothesis of a two-pool model for Ca^{2+} oscillations at the presynaptic site.

Ca^{2+} signaling within cells involves endogenous second messengers that mobilize Ca^{2+} from internal stores (Berridge, 1993; Shuttleworth, 1997). Two well-accepted messengers are inositol 1,4,5-trisphosphate (IP_3; Berridge, 1993) and cyclic adenosine diphosphate-ribose (cADPR; Lee, 1997). A more recent candidate, found in systems ranging from plants to humans, is nicotinic acid adenine dinucleotide phosphate (NAADP) (Lee, 1997, 1999, 2000). NAADP is effective in the nanomolar range and is thus one of the most potent Ca^{2+} mobilizers known to date (Lee, 2001). The existence of three such converging pathways (Cancela et al., 2000) could explain the complex temporal and spatial interaction in Ca^{2+} signaling described by some investigators (Hirose et al., 1999).

NAADP has been shown to be synthesized biochemically by ADP-ribosyl cyclase, the same enzyme that catalyzes the cyclization of NAD to produce cADPR (Lee, 1999). Several studies have shown that NAADP causes Ca^{2+} release from an internal store (Clapper et al., 1987; Albrieux et al., 1998; Thomas et al., 1998; Cancela et al., 1999). This effect is blocked by L-type Ca^{2+} channel blockers (Genazzani et al., 1996) but not by antagonists for cADPR or IP_3 receptors (Lee, 2000).

The location of the NAADP-sensitive site is not known, but it is clearly separate from SER [i.e., NAADP is able to induce Ca^{2+} release in microsomal preparations that have been treated with thapsigargin, an agent that normally blocks release of Ca^{2+} from SER (Genazzani and Galione, 1996)]. Injection of NAADP into the center of sea urchin eggs produces waves of Ca^{2+} emanating from just beneath the plasma membrane (Genazzani et al., 1996; Genazzani and Galione, 1997), which suggests that this novel Ca^{2+} pool may be located on "an as yet unidentified but distinct organelle" (Genazzani and Galione, 1997).

Major calcium stores in the nerve terminal include SER (Grohovaz et al., 1996), mitochondria (David and Barrett, 2000), Golgi (Pinton et al., 1998), and secretory vesicles (Grohovaz et al., 1996; Fossier et al., 1998). There is some evidence for the ability of second messengers to activate or mobilize these stores: 1) injection of cADPR into the presynaptic site of *Aplysia* enhances transmitter output (Mothet et al., 1998); 2) liposomal delivery of IP_3 or cADPR into frog motor nerve endings increases the release of quantal transmitter (Brailoiu and Miyamoto, 2000); and 3) introduction of caged IP_3 into corticotrophs increases exocytosis on flash photolysis (Tse and Lee, 2000). This suggests that IP_3 and

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ABBREVIATIONS: IP_3, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; cADPR, cyclic adenosine diphosphate ribose; MEPPs, miniature endplate potentials; SER, smooth endoplasmic reticulum; *p*, mean probability of release; *n*, number of operational transmitter release sites; *m*, quantal frequency; var_s *p*, spatial variance in p.

cADPR may be endogenous second messengers and play a role in modulating neurosecretion (Csordás et al., 1999).

In view of the results with IP_3 and cADPR, we were interested in the possibility that NAADP might also affect transmitter release. NAADP had been shown to release Ca^{2+} from brain microsomes (Bak et al., 1999), suggesting that NAADP might be a second messenger that modulates neural activity (Petersen and Cancela, 1999). We used liposomal techniques to deliver compounds into the intracellular compartment and unbiased estimates of quantal release parameters to determine effects on the components of neurosecretion. We found that NAADP increases neurosecretion by a mechanism distinct from that of IP_3 or cADPR and probably involves Ca^{2+} release from an organelle near the active zone.

Materials and Methods

Isolated sciatic-sartorius nerve-muscle preparations from frogs (*Rana pipiens*) were used for these experiments. The experimental procedures were reviewed and approved by the East Tennessee State University Committee for Animal Care. Animals were decapitated followed by rapid double pithing. Muscles were removed and mounted in a 5-ml Sylgard-lined Petri dish bath. The bath was continuously perfused with Ringer solution using a dual-chambered roller pump. The concentration of K^+ in the control Ringer solution was increased (equimolar substitution of KCl for NaCl) to raise the basal frequency of miniature endplate potentials (MEPPs), which increases the likelihood of binomial (versus Poisson) release of transmitters. The control Ringer solution contained 100.0 mM NaCl, 12.5 mM KCl, 1.8 mM $CaCl_2$, 2.0 Tris, to pH 7.2, and 5.6 mM glucose. Preparations were equilibrated for 30 min before use. The Ca^{2+}-free Ringer solution was prepared by omitting $CaCl_2$ and adding 2.5 mM EGTA.

Encapsulation of agents in liposomes is an accepted procedure for delivering small and heavy molecules into the motor nerve terminal (de Paiva and Dolly, 1990; Silinsky et al., 1995; Brailoiu and van der Kloot, 1996; Brailoiu et al., 1999). Multilamellar liposomes were prepared with 60 mg/ml egg phosphatidylcholine (Sigma, type X-E), as previously described (Brailoiu and van der Kloot, 1996; Brailoiu and Miyamoto, 2000). Chemicals to be incorporated into liposomes were dissolved in 140 mM KCl solution at pH 6.9. Liposome batches were dialyzed (Sigma dialysis sacs) against control Ringer solution [1/600 (v/v), 150 min] to remove nonincorporated agent, and the Ringer solution changed every 30 min. Control liposomes contained 140 mM KCl solution, pH 6.9, only. Liposome suspensions were administered by continuous perfusion after 1/20 (v/v) dilution in control Ringer solution. Potassium D-*myo*-inositol 1,4,5-trisphosphate, cyclic adenosine diphosphate-ribose phosphate, sodium nicotinic acid adenine dinucleotide phosphate, A23187, thapsigargin, and EGTA were all obtained from Sigma Chemical Co. (St. Louis, MO).

Miniature endplate potentials (MEPPs) were recorded with standard intracellular microelectrode (3 M KCl; 10–15 MΩ resistance) techniques. Selection was made from impalements showing focal recording, large MEPP size (>0.4 mV), moderately high frequency (>300/min), and good signal-to-noise ratio (<5% of the signal). The muscle resting potential was between −50 and −60 mV and remained stable throughout the course of the experiment. Only one trial was carried out on each preparation. Experiments were conducted at the ambient room temperature (21–23°C). Bioelectric signals were fed into a high impedance preamplifier and viewed on a Tektronix oscilloscope. Signal-to-noise ratio was increased using a band-pass filter (0–1 kHz), and results were recorded on magnetic tape using a modified videocassette recorder. Signals were boosted 20-fold by a rear-output amplifier of the oscilloscope to allow interfacing with an analog-digital data acquisition unit. Results were stored on magnetic tape for off-line analysis. MEPP amplitudes were measured with a grid template on a flat screen monitor, and 100 determinations made for each time point.

A detailed description of the quantal analysis is described elsewhere (Provan and Miyamoto, 1993). Briefly, the number of quanta released by one nerve impulse (m) was replaced by the number of MEPPs in a 50-ms interval (bin), and 500 sequential bins were used for each quantal estimate. Data were divided into subgroups of 100 before analysis to minimize nonstationarity, and results that were nonstationary according to statistical test were discarded. Unbiased estimates of m, n, p, and $var_s\ p$ were then computed using equations previously reported (Miyamoto, 1986). The slightly negative *estimates* for $var_s\ p$ were due to a systematic underestimation from the use of 50-ms bins (use of smaller bins eliminates this but raises problems in counting). This issue is addressed fully in a previous report (Provan and Miyamoto, 1993).

Data for each experiment (except those for $var_s\ p$) were expressed as percentages of the value at time 0, and the results from six experiments were averaged (points indicate means ± S.E.M.). Analysis of statistical differences was made by comparing each point with points obtained in control Ringer solution, with $P < 0.05$ indicating significant differences (paired t test).

Results

Effect of NAADP on Quantal Release Parameters. To examine whether NAADP could mobilize internal Ca^{2+} and affect neurosecretion, we prepared liposomes containing 10^{-5}, 10^{-4}, and 10^{-3} M NAADP, using methods described previously (Brailoiu and van der Kloot, 1996; Brailoiu and Miyamoto, 2000). The final concentration within the nerve terminal was estimated to be 100-fold lower than that in the liposome vesicle, for molecules of the size of NAADP (Brailoiu et al., 1999). Bath application of liposomes containing NAADP produced an increase in m (the number of quanta released) (Provan and Miyamoto, 1993) that was progressive with time during continual delivery of the compound (Fig. 1, A-C; Fig. 2A). The amount of increase was concentration-dependent over the range tested, with the data falling on the linear portion of the log [NAADP]-response curve (results not shown). The increase in m was caused by a concentration-dependent increase in p (the mean probability of release) (Fig. 2C), because there was no significant change in n (the number of operational transmitter release sites) (Fig. 2D). NAADP also caused a small but significant decrease in $var_s\ p$ (the spatial variance in p) that was concentration-dependent (Fig. 2B). All effects were reversed with 40 min of wash. Control liposomes, filled with 140 mM KCl only, produced no significant changes in any of the quantal release parameters. NAADP had no significant effect on MEPP size at any of the concentrations tested.

Effect of A23187 on Quantal Release Parameters. The above results differed from those found with IP_3 and cADPR; i.e., the increase in m with NAADP was due to an increase in p (Fig. 2, A and C), whereas the increase in m with IP_3 and cADPR was due to an increase in n (Brailoiu and Miyamoto, 2000). Because increases in p were associated with Ca^{2+} influx at the plasmalemma (Provan and Miyamoto, 1993), we examined the effect of the Ca ionophore A23187 to obtain a template response for Ca^{2+} entry at the plasmalemma and to compare the response with that found with NAADP. Bath application of A23187 (final concentration, 5 μM) produced a 20% increase in m that was sustained during 30 min of exposure (Fig. 3A). The increase was associated primarily

with an increase in p (Fig. 3C), because there was only a small and statistically insignificant decrease in n (Fig. 3D) and $var_s\ p$ (Fig. 3B) over the 30-min period. The effects were reversed with 30 min of wash. The effects of NAADP on quantal release parameters were thus similar to those found with A23187, suggesting that NAADP may mobilize non-endoplasmic reticulum stores.

Effect of NAADP in the Presence of IP_3. It is clear that IP_3 and cADPR release Ca^{2+} from SER (Berridge, 1993) and that NAADP releases Ca^{2+} from a store other than SER (Lee, 1997, 2000). Administration of IP_3 (or cADPR) and NAADP would thus be expected to release Ca^{2+} from both stores and increase both n and p. To test this, we prepared liposomes that contained NAADP and IP_3 in the same phospholipid vesicle. This was to ensure that the two agents would be delivered to the nerve terminal in equimolar amounts. As shown by Figs. 4A and 1E, administration of liposomes containing 10^{-4} M IP_3 and 10^{-4} M NAADP caused an increase

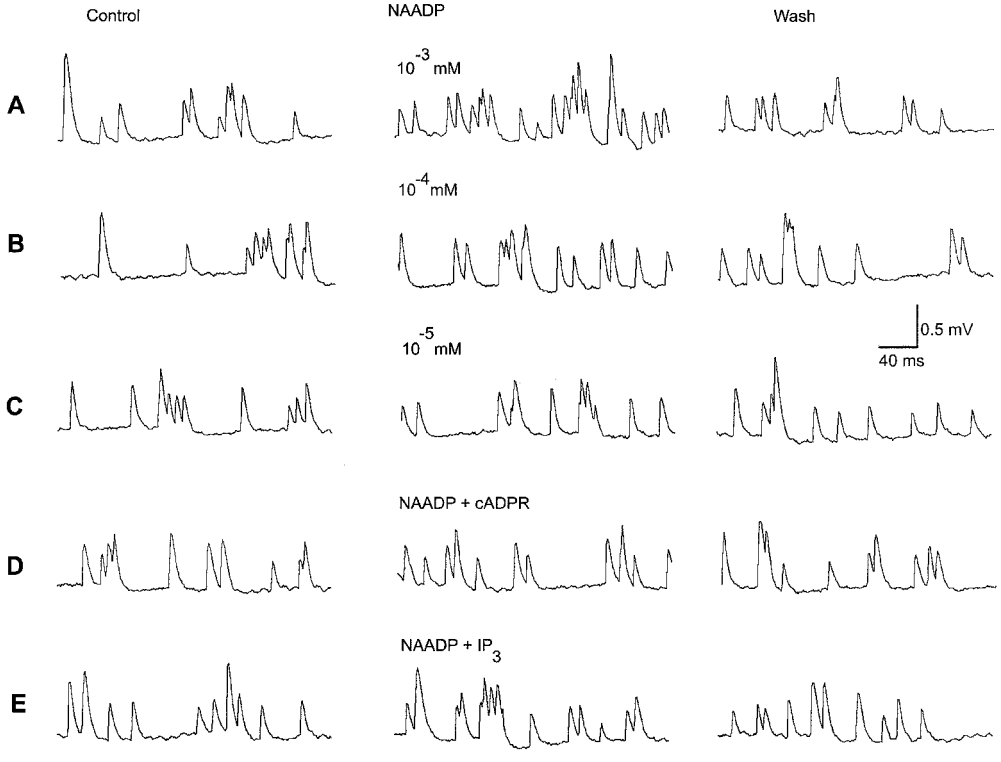

Fig. 1. Representative traces of MEPPs recorded at the frog neuromuscular junctions in various concentrations of NAADP packaged into the liposomes. Administration of liposomes containing 10^{-5} M (C), 10^{-4} M (B), or 10^{-3} M (A) NAADP show a concentration-dependent increase in spontaneous transmitter release. Coadministration of NAADP (10^{-4} M) and cADPR (10^{-4} M) (D) causes a small increase in MEPP frequency, whereas coadministration of NAADP (10^{-4} M) and IP_3 (10^{-4} M) produces an additive effect.

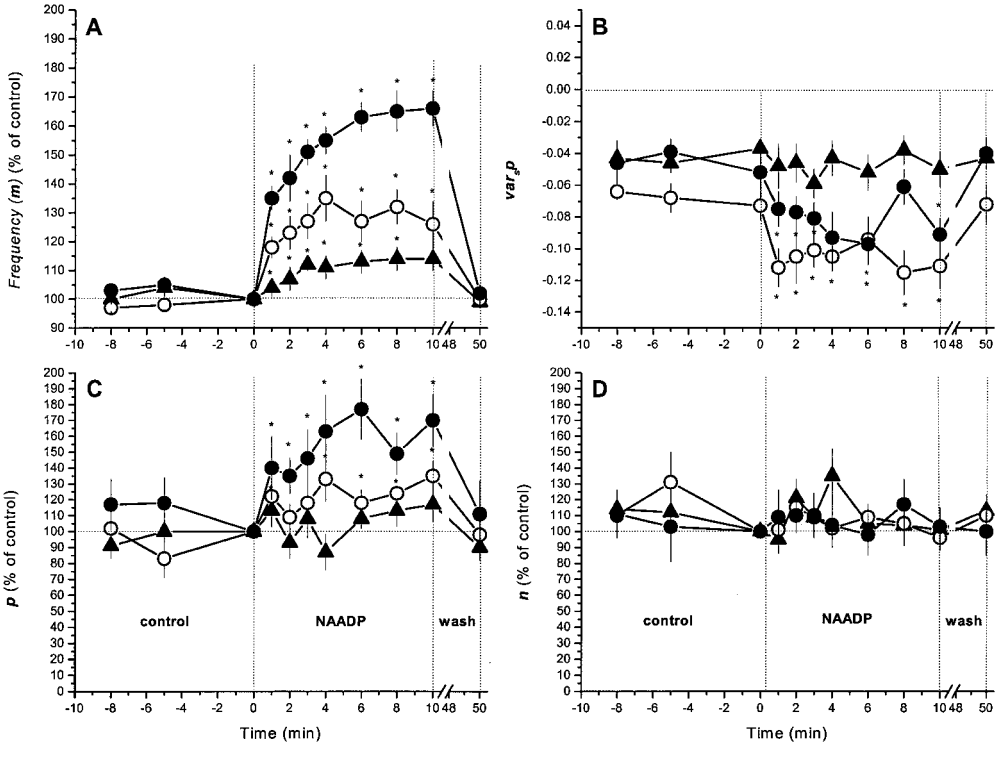

Fig. 2. NAADP produces a concentration-dependent increase in quantal transmitter release. NAADP encapsulated in liposomes was delivered at time 0 and washed out after 10 min. Shown are the effects of 10^{-5} M (▲), 10^{-4} M (○) and 10^{-3} M (●) NAADP on m (A), $var_s\ p$ (B), p (C), and n (D). Asterisks indicate significant differences between specific points and points in control period ($n = 6$). The concentration-dependent increase in frequency (m) is caused primarily by an increase in p, because there is no significant change in n. Asterisks in B indicate significant decreases in $var_s\ p$ for 10^{-4} and 10^{-3} M NAADP. Stated concentrations refer to [NAADP] encapsulated in liposomes. [NAADP] in the nerve terminal cytoplasm is about 1/100 of the liposomal [NAADP]. Quantal parameters were determined as indicated under *Materials and Methods*.

in m (48%) that was greater than the increase produced by NAADP (34%, Fig. 2A) or IP_3 (25%) alone (Brailoiu and Miyamoto, 2000). As anticipated, the increase in m was associated with increases in both p (Fig. 4C) and n (Fig. 4D). There was again a significant decrease in var_s p (Fig. 4B).

Effect of NAADP in the Presence of cADPR. In this series of experiments, liposomes containing NAADP (10^{-4} M) and cADPR (10^{-4} M) in the same vesicle were prepared. In contrast to the above results, coadministration of NAADP and cADPR produced an increase (8%) in m (Fig. 5A; Fig. 1D) that was much smaller than that found with NAADP alone (34%, Fig. 2A). There was no significant increase in either p (Fig. 5C) or n (Fig. 5D). There was also no significant change in var_s p (Fig. 5B); i.e., the expected *decrease* in var_s p with NAADP (Fig. 2B) may have been antagonized by the presence of cADPR.

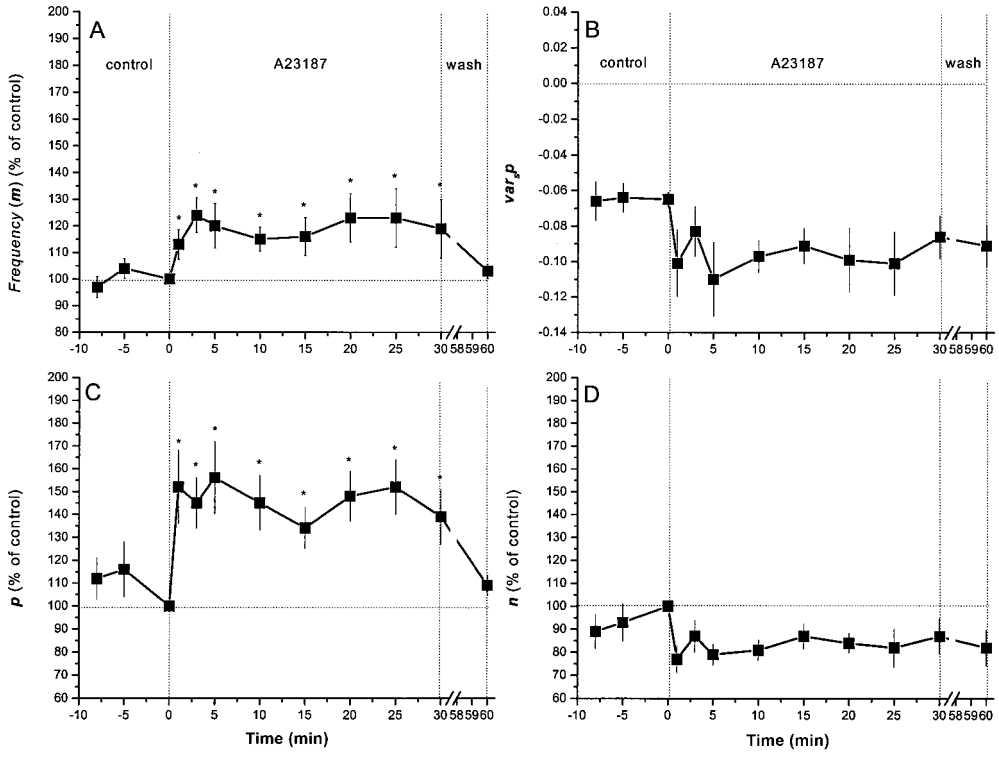

Fig. 3. Calcium ionophore A23187 increases quantal output by increasing probability of release. Administration of 5 μM (final concentration in bath) A23187 causes a sustained increase in m (A) that is associated with a sustained increase in p (C). The apparent decreases in n (D) and var_s p (B) with A23187 are not significant over the full 30-min recording period. Plots and quantal parameters are as in Fig. 2.

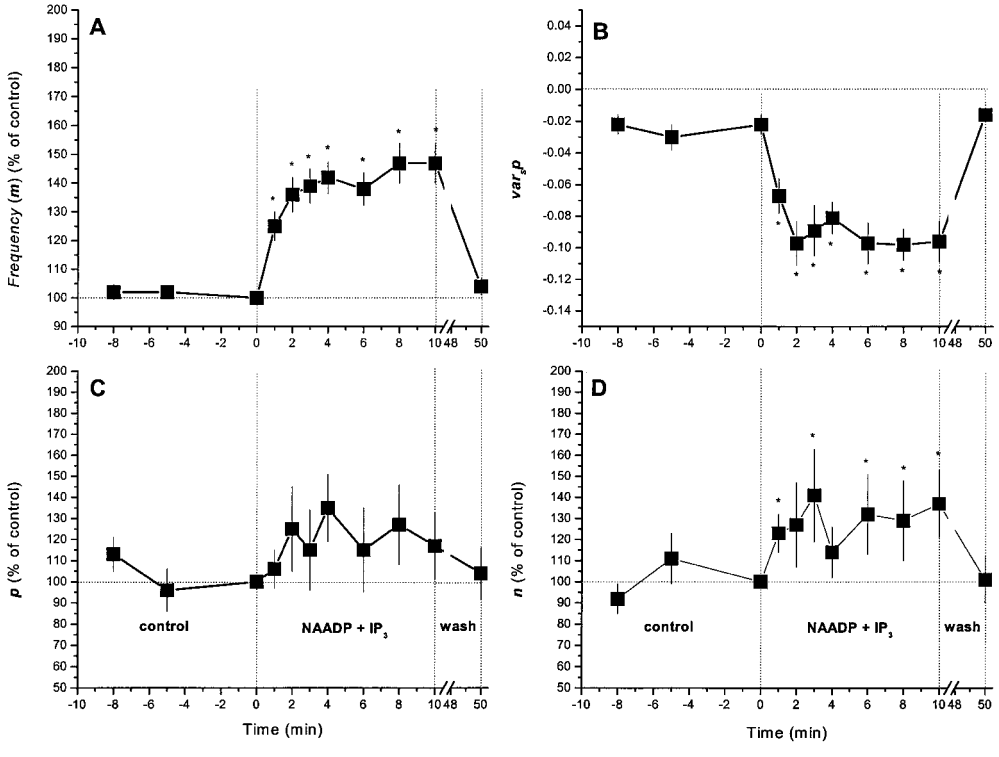

Fig. 4. Coadministration of NAADP and IP_3 (both 10^{-4} M in liposomes) produces a superimposed but less than additive increase in quantal output. The increase in m (A) is only 80% of the sum of the separate increases to NAADP and IP_3. The increase in m is associated with an apparent increase in p (C) and a significant increase in n (D). There is a significant decrease in var_s p that is consistent with the decrease in var_s p with NAADP alone (Fig. 2B). Plots and quantal parameters are as in Fig. 2.

Effect of NAADP after Pretreatment with Thapsigargin. To see whether thapsigargin (functional removal of SER) affected the NAADP response in our system, we incubated muscles in Ca^{2+}-free Ringer solution plus 2.5 mM EGTA and 1 μM thapsigargin for 30 min, followed by restoration of muscles to Ca^{2+}-containing Ringer solution for another 30 min. This treatment completely blocked the effects of liposomes containing IP_3 and cADPR (both 10^{-4} M in aqueous phase) (Fig. 6). Addition of liposomes containing 10^{-4} M NAADP produced an attenuated but significant increase in m (14%) (Fig. 6A). The increase expected with 10^{-4} M NAADP was about 34% (from Fig. 2A); therefore, the NAADP-operated Ca^{2+}-releasing mechanism was about 20% resistant to thapsigargin in this case. The effect on n and p was complex- the increase in m was associated with a small and insignificant decrease in p (Fig. 6C) and a significant increase in n (Fig. 6D). Finally, pretreatment with thapsigargin abolished the expected decrease in var_s p with NAADP (Fig. 6B).

Discussion

Our results show that NAADP is able to modulate transmitter release at the frog motor nerve terminals. This is the first demonstration of an action of this novel intracellular mediator in intact nerve endings. In view of a widespread distribution of NAADP receptors in the brain (Patel et al., 2000), these results suggest that NAADP may be an important intracellular messenger in neural tissue.

The increase in quantal output with NAADP (Fig. 2A) is similar to that seen with IP_3 and cADPR (Brailoiu and Miyamoto, 2000). However, there are important differences in the responses that support the idea of two distinct mechanisms: 1) the increase in m with NAADP is progressive with time (Fig. 2A), whereas the increase with IP_3 or cADPR is almost immediate (Brailoiu and Miyamoto, 2000). 2) The increase in m with NAADP is caused by an increase in p (Fig. 2C), whereas the increase in m with IP_3 or cADPR is caused by an increase in n (Brailoiu and Miyamoto, 2000). 3) NAADP causes a decrease in var_s p (Fig. 2B), whereas IP_3 and cADPR have no effect (Brailoiu and Miyamoto, 2000) or produce an increase in var_s p with higher concentrations (E. Brailoiu, M. D. Miyamoto, and N. J. Dun, manuscript in preparation).

Coadministration of NAADP and IP_3 (Fig. 4A) produced an increase in m (48%) that was slightly less than the sum of the increases in m produced separately (59%). Genazzani and Galione (1997) described a less than additive effect for any two agonists (of IP_3, cADPR, and NAADP) that were coadded. This is compatible with the notion of Ca^{2+} release from two separate pools; for example, the attenuation may be caused by overlapping Ca^{2+} domains rather than interference of IP_3 signals (Genazzani et al., 1996; Cancela et al., 2000) or binding (Billington and Genazzani, 2000) by NAADP. In support of this, NAADP and IP_3 given together produced increases in p and n (Fig. 4, C and D), whereas NAADP alone increased only p (Fig. 2, C and D), and IP_3 alone increased only n (Brailoiu and Miyamoto, 2000).

Coadministration of NAADP and cADPR resulted in a much smaller increase in m; i.e., the effect of NAADP seemed to be antagonized by cADPR (Fig. 5A). cADPR displaces NAADP at 1 μM (Billington and Genazzani, 2000). The smaller increase may be explained by possible interference of Ca^{2+} oscillations induced by the concomitant presence of NAADP and cADPR, although we cannot exclude the possibility that cADPR may be a competitive antagonist at the NAADP receptor and vice versa. The alternative explanation, that cADPR binds NAADP when entrapped in liposomes, seems less likely from a teleological standpoint, because

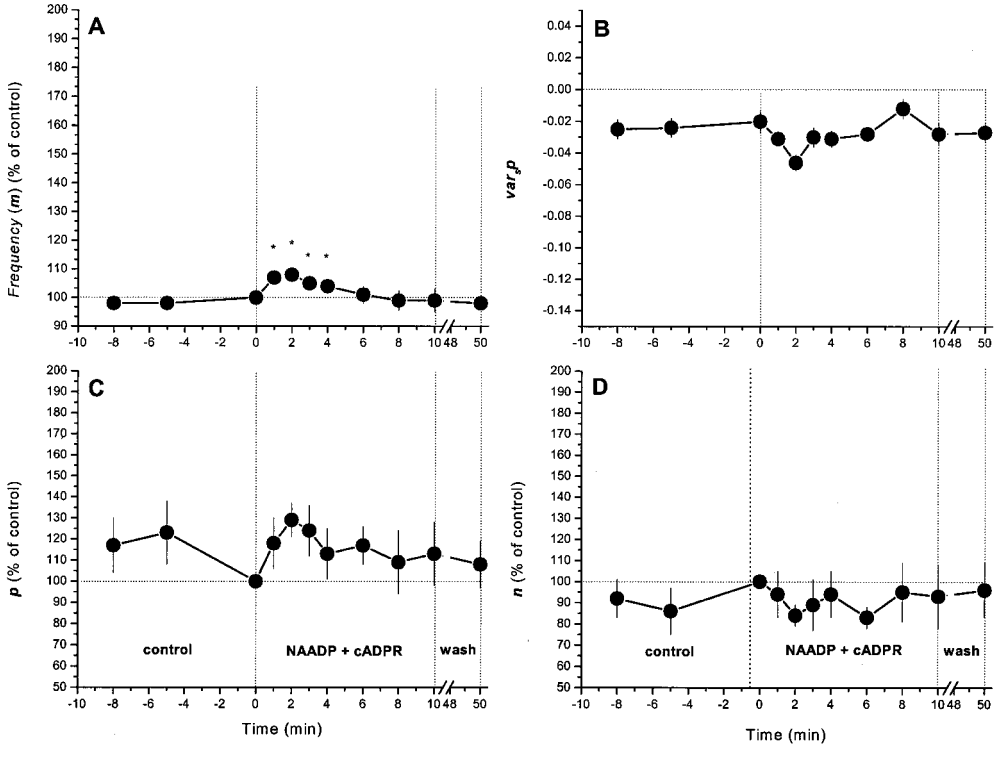

Fig. 5. Coadministration of NAADP and cADPR (both 10^{-4} M in liposomes) produces an increase in quantal output that is much smaller than that produced by either agent alone. The much attenuated increase in m (A) is not associated with a significant increase in p (C) and n (D). NAADP does not produce a significant decrease in var_s p (B) in the presence of cADPR. Plots and quantal parameters are as in Fig. 2.

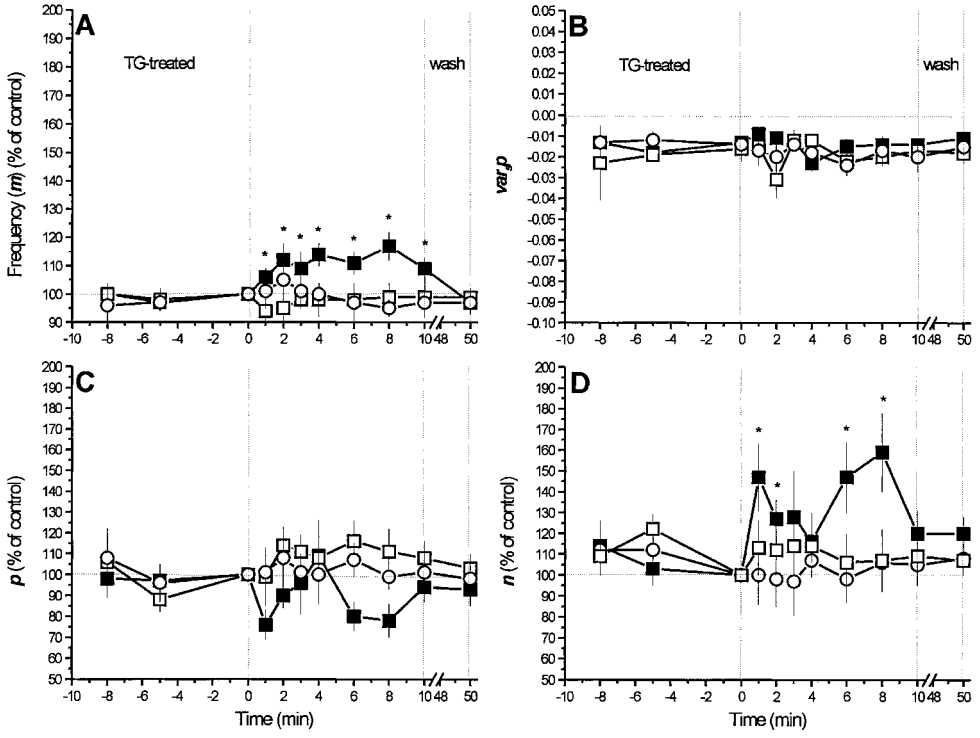

Fig. 6. Functional elimination of SER by thapsigargin (TG, 1 μM) reduces but does not eliminate the ability of NAADP (■) to increase m (A). The increase in m is associated primarily with an increase in n (D), although there is also a small but statistically insignificant decrease in p (C). The ability of NAADP to decrease var$_s$ p is effectively abolished (B). Effects of IP_3 (□) and cADPR (○) are completely blocked by thapsigargin.

NAADP and cADPR are synthesized by the same enzyme (Aarhus et al., 1995).

The NAADP-sensitive Ca^{2+} store can be distinguished from the IP_3 and cADPR-sensitive stores by its relative resistance to thapsigargin (Lee, 1999; Billington and Genazzani, 2000). Possible candidates for the NAADP-sensitive Ca^{2+} store are Golgi apparatus and synaptic vesicles, both of which contain Ca^{2+}-ATPases that are about 50% resistant to thapsigargin (Salvador and Mata, 1998; Lee, 2001). In our system, the NAADP-induced increase in m was only 20% resistant to thapsigargin (Fig. 6A). The difference in amount of inhibition may be because we used Ca^{2+}-free Ringer solution, which would be expected to intensify the effect of thapsigargin.

Because the Golgi apparatus, which has been shown to be an IP_3-sensitive system (Pinton et al., 1998), may be excluded as a possible candidate, the vesicle seems to be the major candidate for the NAADP-sensitive store. Another indication that NAADP may act at vesicles comes from electrophysiologic findings: Ca^{2+} influx at the plasmalemma is associated with an increase in p and no increase in var$_s$ p (Provan and Miyamoto, 1993), whereas Ca^{2+} release from distal organelles is associated with increases in n and var$_s$ p (Provan and Miyamoto, 1993). The response with NAADP (increase in p and no increase in n or var$_s$ p) is thus consistent with an action at the plasmalemma, conceivably at vesicles in the releasable pool, which are Ca^{2+}-loaded (Fossier et al., 1998).

NAADP-induced Ca^{2+} release from synaptic vesicles may be analogous to the situation in oocytes. In unfertilized eggs, injection of NAADP triggers a Ca^{2+} wave from just beneath the plasma membrane (Genazzani and Galione, 1997) in the region of the secretory vesicles (cortical granules). One possibility is that fertilization generates NAADP (Genazzani and Galione, 1997), which releases Ca^{2+} from the granules (Schuel, 1978) to trigger exocytosis and formation of the fertilization envelope.

Ca^{2+} released from distal organelles is viewed as: 1) acting on synapsin (Llinás et al., 1991) to mobilize vesicles to the releasable pool (increase in n) (Brailoiu and Miyamoto, 2000) and 2) creating "Ca^{2+} waves" that increase [Ca^{2+}] variation at the active zone (increase in var$_s$ p) (Provan and Miyamoto, 1993, 1995). The decrease in var$_s$ p with NAADP (Figs. 2B and 4B) and A23187 (Fig. 3B) is thus unexpected and implies a decrease in [Ca^{2+}] variation. This may mean that elevation of Ca^{2+} at the plasmalemma reduces [Ca^{2+}] variation at the active zone.

At the frog neuromuscular junction, calcium-induced calcium release mechanism has been shown to be involved in transmitter release (Narita et al., 2000). Churchill and Galione (2001) showed in sea urchin eggs the first direct evidence for the "two-pool model" of Ca^{2+} oscillation; i.e., thapsigargin-insensitive and thapsigargin-sensitive. In the context of this "two-pool model", NAADP may act at the thapsigargin-insensitive pool first and its effects are subsequently amplified by the calcium-induced calcium release pathway, whereas cADPR and IP_3 may be involved in the later cycles of calcium dynamics (Brailoiu and Miyamoto, 2000).

In conclusion, our study indicates that NAADP increases quantal neurosecretion at the frog motor nerve terminal, possibly by releasing Ca^{2+} from tethered or docked synaptic vesicles. Also, these data support the hypothesis of a two-pool model for Ca^{2+} oscillations at the frog nerve terminal. If this were the primary function of NAADP in neural systems, its action would be limited to the presynaptic site. This is in contrast to IP_3 and cADPR, which may be involved in modulation of Ca^{2+} pre- or postsynaptic sites.

Acknowledgments

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