commentary

ACCELERATED PUBLICATION

Modulation of spontaneous transmitter release from the frog neuromuscular junction by interacting intracellular Ca^{2+} stores: critical role for nicotinic acid–adenine dinucleotide phosphate (NAADP)

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Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a recently described potent intracellular Ca^{2+}-mobilizing messenger active in a wide range of diverse cell types. In the present study, we have investigated the interaction of NAADP with other Ca^{2+}-mobilizing messengers in the release of transmitter at the frog neuromuscular junction. We show, for the first time, that NAADP enhances neurosecretion in response to inositol 1,4,5-trisphosphate (IP_3), cADP-ribose (cADPR) and sphingosine 1-phosphate (S1P), but not sphingosylphosphorylcholine. Thapsigargin was without effect on transmitter release in response to NAADP, but blocked the responses to subsequent application of IP_3, cADPR and S1P and their potentiation by NAADP. Asynchronous neurotransmitter release may therefore involve functional coupling of endoplasmic reticulum Ca^{2+} stores with distinct Ca^{2+} stores targeted by NAADP.

Key words: Ca^{2+} stores, Ca^{2+} mobilization, endoplasmic reticulum, neurotransmitter release, nicotinic acid–adenine dinucleotide phosphate (NAADP), thapsigargin.

INTRODUCTION

Cytosolic Ca^{2+} changes are intimately involved in the regulation of a host of cellular processes in many, if not all, cells [1]. These changes can derive from influx of Ca^{2+} across the plasma membrane as well as mobilization of intracellular Ca^{2+} pools by second-messenger-activated Ca^{2+} channels [1]. Inositol 1,4,5-trisphosphate (IP_3), a prototypical intracellular Ca^{2+}-mobilizing messenger, is synthesized in a wide variety of cells in response to an equally wide variety of extracellular cues and stimulates Ca^{2+} mobilization via activation of specific IP_3 receptor/Ca^{2+} channels complexes located on the endoplasmic reticulum (ER) [2,3]. cADP-ribose (cADPR) [4–6], an NAD^+ metabolite, mediates Ca^{2+} mobilization in an analogous manner to IP_3 by activation of ryanodine receptors [7], a family of Ca^{2+} channels related to IP_3 receptors. In addition to mediating cellular effects through activation of cell-surface receptors, sphingomyelin metabolites, such as sphingosine 1-phosphate (S1P) [8,9] and sphingosylphosphorylcholine (SPC) [10], are also thought to act as intracellular Ca^{2+}-mobilizing messengers, but less is known about the Ca^{2+} channels activated by these molecules.

Recently, much attention has focused on the Ca^{2+}-mobilizing properties of nicotinic acid–adenine dinucleotide phosphate (NAADP) [4,6,11,12]. This metabolite of NADP, the most potent of the Ca^{2+}-mobilizing messengers described to date, displays some unique defining features. Receptors for NAADP, although ill-defined at the molecular level, appear particularly prone to desensitization whereby inactivation of Ca^{2+} release can, depending on ligand concentration, precede activation [4,6,11,12]. The Ca^{2+} stores targeted by NAADP also appear novel. From cellular fractionation studies, NAADP-sensitive Ca^{2+} stores do not co-migrate with markers for the ER [13], an established Ca^{2+} store for IP_3, cADPR and S1P. Accordingly, NAADP-induced Ca^{2+} release is demonstrable after inhibition of ER Ca^{2+} pumps by thapsigargin [14,15]. In sea urchin eggs, NAADP-sensitive Ca^{2+} stores may correspond to reserve granules [16], a lysosomal-like organelle, whereas in pancreatic β cells, secretory vesicles seem a likely candidate [17,18]. These studies raise the possibility that NAADP may target acidic stores of Ca^{2+}.

Several independent lines of evidence indicate a critical role for NAADP in coordinating the activity of other intracellular Ca^{2+} channels [11]. This is based primarily on the apparently discrepant pharmacology of NAADP-induced Ca^{2+} release in broken cells compared with intact cells. In sea urchin eggs, for example, antagonists of IP_3- and cADPR-induced Ca^{2+} release have little effect on Ca^{2+} release mediated by NAADP from egg homogenates, consistent with the activation of a dedicated Ca^{2+} channel [19]. In contrast, the same antagonists markedly attenuate Ca^{2+} increases in intact eggs [20]. Similarly, global increases of Ca^{2+} mediated by NAADP in intact pancreatic acinar [21] and smooth muscle [22] cells are also sensitive to inhibitors of IP_3 and/or ryanodine receptors. One possibility to explain these data is if NAADP provides a 'trigger' release of Ca^{2+} that is then amplified by other Ca^{2+} channels [17,21]. This may arise through recruitment of IP_3 or ryanodine receptors by the process of Ca^{2+}-induced Ca^{2+} release [21] and/or from enhanced filling of ER Ca^{2+} stores fuelled by the mobilization of NAADP-sensitive Ca^{2+} stores [15]: effects that would be observable only in intact cells where the correct arrangement of intracellular Ca^{2+} channels and pools is preserved. Indeed, NAADP appears particularly well suited to 'chatter' [11] as highlighted recently where the combinatorial roles of NAADP, IP_3 and cADPR in evoking Ca^{2+} signals were systematically studied

Abbreviations used: cADPR, cADP-ribose; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; MEPP, miniature end-plate potential; NAADP, nicotinic acid–adenine dinucleotide phosphate; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine.

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in pancreatic acinar cells [23]. Clearly, with multiple mechanisms for intracellular Ca^{2+} release expressed within the same cell, understanding which of these pathways are engaged upon stimulation of cells by different stimuli [24,25] and, moreover, their interaction, is crucial as it may provide mechanistic insight into how Ca^{2+} signals encode functional specificity.

Mounting evidence implicates a role for intracellular Ca^{2+} stores in neuronal transmitter release [26]. Presynaptic introduction of IP_3, cADPR and NAADP has been demonstrated to stimulate neurotransmitter release at cholinergic synapses in *Aplysia* [27]. Our own analyses using the frog neuromuscular junction confirm these findings [28,29]. In addition, we show that S1P and SPC also enhance transmitter release [30,31], making this preparation a particularly useful model for examining the role of intracellular Ca^{2+}-release pathways in neurosecretion. In the present study, we have examined the interaction of NAADP with other intracellular Ca^{2+}-mobilizing messengers in the stimulation of transmitter release. Our data reveal that neurosecretion can be regulated by functionally distinct, but interacting, intracellular Ca^{2+} stores.

MATERIALS AND METHODS

Animals

Frogs (*Rana pipiens*) were decapitated, rapidly double-pithed and sciatic–sartorius nerve–muscle preparations were isolated. Every effort was made to use the minimum number of animals required for valid statistical analyses. Procedures were reviewed and approved by the East Tennessee State University Committee for Animal Care.

Liposome preparation

Delivery of cell-impermeant Ca^{2+}-mobilizing messengers into the frog neuromuscular junction was achieved using the liposome technique [32]. Agents were incorporated into either multilammellar liposomes (for NAADP, cADPR and IP_3) [28,29] or reverse-phase evaporation vesicles (for S1P and SPC) [30,31] as described previously. The concentrations of NAADP (100 μM) and cADPR, IP_3, S1P and SPC (10 μM) referred to correspond with those during incorporation (performed in the presence of 140 mM KCl and 60 mg/ml egg phosphatidylcholine, pH 6.9) which, following delivery, result in intracellular concentrations of approx. 1 μM and 100 nM respectively (for details see [29]). For messenger mix experiments, NAADP was incorporated with the appropriate test messenger within the same liposomes. Control liposomes were prepared in the presence of 140 mM KCl only.

Perfusion

Muscles were mounted in a 3 ml Sylgard-lined Petri dish bath, which was continuously perfused with Ringer's solution [2.0 mM Tris/HCl (pH 7.2), 110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 5.6 mM glucose] using a dual-chambered roller pump. Ryanodine (100 μM) was included in some experiments 30 min prior to recording. In experiments examining the effects of thapsigargin, preparations were perfused with the drug (1 μM) for 60 min, first in Ringer's solution without added calcium and supplemented with 2.5 mM EGTA (30 min), followed by normal Ringer's solution (30 min). Both drugs were present during recording. Liposomes were diluted (1:20) into Ringer's solution prior to delivery.

Electrophysiological recording

Miniature end-plate potentials (MEPPs) were recorded using conventional microelectrode (3 M KCl, 5–15 MΩ) techniques similar to those described previously [28]. These postsynaptic events are due to membrane depolarization resulting from the release of acetylcholine following fusion of single synaptic vesicles at the nerve terminal. Secretion of neurotransmitter is critically dependent upon cytosolic Ca^{2+} increases, which can be derived from either Ca^{2+} influx across the plasma membrane or from mobilization of intracellular Ca^{2+} stores [26]. Although the amplitude of MEPP can be modulated at the postsynaptic level, the frequency of these events is a faithful reporter of vesicle fusion and thus an accurate, be it indirect, measure of presynaptic cytosolic Ca^{2+} concentration. Moreover, liposomal contents will be preferentially delivered into the nerve endings at least at the relatively dilute liposomal preparations used in the present study [32]. Selection of recordings were made from impalements that showed large MEPP size (> 0.3 mV), good signal-to-noise ratio (baseline peak-to-peak noise < 0.1 mV) and high and stable muscle resting membrane potential (> -80 mV, with < 3 mV decline during the control period). Resting potentials ranged between -80 and -90 mV in different fibres. Impaled muscle fibres that showed more than a 10 % decrease in the resting membrane potential during an experiment were not used. Experiments were conducted at room temperature (21–22 °C), and only one trial was carried out on each muscle. Preparations were equilibrated for at least 30 min before use.

Data analysis

In all experiments, MEPP frequency represents the number of events recorded within a 60 s interval. To minimize the effects of junction-to-junction variation, data for each experiment were expressed as a percentage of values at time zero and results from six independent experiments were averaged (plots show means $\pm$ S.E.M.). Student's t test, followed by ANOVA and Bonferonni's test, were used to test differences between different conditions ($P < 0.05$ indicating significant differences).

RESULTS

We monitored spontaneous transmitter release at the frog neuromuscular junction by measuring MEPP frequency. Example recordings are shown in Figure 1(A). Perfusion of the preparation with normal Ringer's solution supplemented with liposomes containing NAADP (100 μM) caused a transient increase in MEPP frequency which peaked (71 ± 3.7 %, $n = 6$) 3 min following the start of liposome perfusion (Figure 1 and Table 1). The effects of NAADP on neurosecretion were concentration-dependent and unaffected by ryanodine (100 μM; Table 1). MEPP frequency was also increased by liposomes containing cADPR (10 μM) and IP_3 (10 μM) (Figure 1B). In these experiments, the peak responses (35 ± 2.2 % for IP_3 and 48 ± 2.6 % for cADPR, $n = 6$) were observed following 4 min of liposome perfusion. These results indicate that intracellular delivery of NAADP, IP_3 and cADPR enhances quantal output under basal conditions. Confirming our previous studies [30,31], S1P and SPC (10 μM within liposomes) stimulated neurosecretion in normal Ringer's solution at 4 min by 39 ± 2.6 % ($n = 6$) and 48 ± 3.2 % ($n = 6$) respectively (Figure 1B). In contrast, MEPP frequency was unaffected by perfusion with liposomes prepared in the presence of buffer only (Table 1).

Having established the effects of various intracellular messengers on spontaneous neurotransmitter release under similar

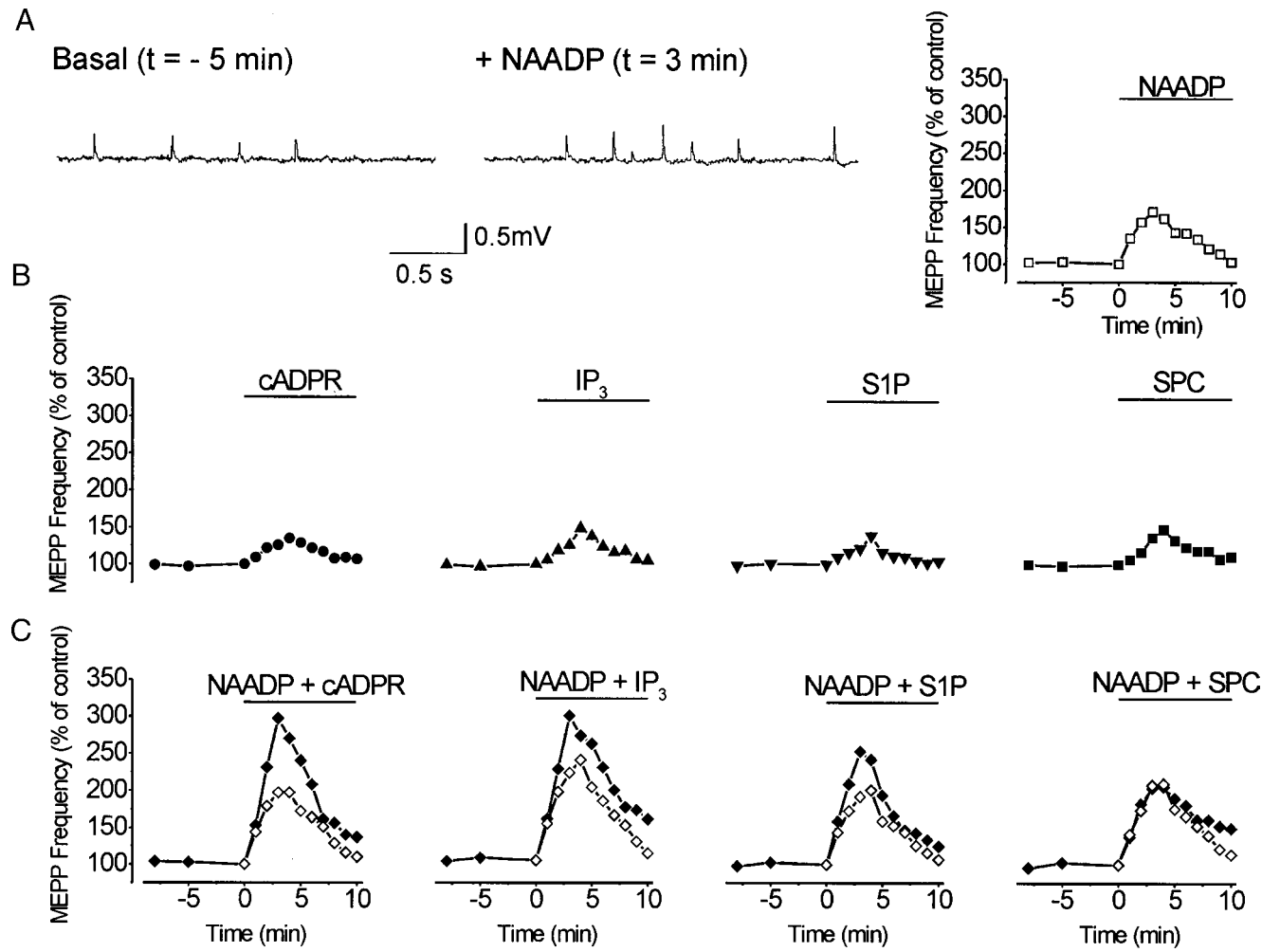

Figure 1 NAADP potentiates neurotransmission in response to cADPR, IP_3, S1P, but not SPC

MEPP frequency was monitored at the frog neuromuscular junction during perfusion with liposomes containing various intracellular messengers alone and in combination. (**A**) Change in MEPP frequency in response to NAADP (□, 100 μM). Example recordings taken 5 min prior to (left panel) and 3 min after (middle panel) liposome perfusion. Right panel, the full normalized time course. (**B**) Change in MEPP frequency in response to cADPR (●), IP_3 (▲), S1P (▼) and SPC (■) (10 μM). (**C**) Change in MEPP frequency in response to messenger mixes. Observed responses to liposomes containing NAADP + cADPR, NAADP + IP_3, NAADP + S1P and NAADP + SPC (◆; same concentrations as above) were compared to the sum of the responses to the individual messenger alone (◇) calculated from the data in (**A**) and (**B**). Values are means ± S.E.M. from six independent preparations. Error bars are smaller than the symbols. Bar indicates 10 min.

Table 1 Modulation of neurotransmitter release by NAADP

Frog neuromuscular junction preparations were perfused with Ringer's solution alone or Ringer's solution supplemented with ryanodine (100 μM) or thapsigargin (1 μM) as described in the Materials and methods section. The effect of liposomes on MEPP frequency was calculated 1 min prior to (Basal) and 3 min after (Peak) perfusion with Ringer's solution containing liposomes prepared in the absence (Control liposomes) or presence of the indicated concentrations of NAADP. Values are means ± S.E.M of six independent preparations.

Treatment	Basal frequency (s^{-1})	Peak frequency (s^{-1})	Increase (%)
Control liposomes	1.43 ± 0.09	1.39 ± 0.03	−3 ± 2.6
NAADP			
10 μM	1.52 ± 0.08	2.11 ± 0.11	39 ± 5.2
100 μM	1.55 ± 0.12	2.65 ± 0.09	71 ± 3.7
1 mM	1.32 ± 0.06	2.83 ± 0.16	114 ± 5.6
+ Ryanodine			
100 μM NAADP	1.15 ± 0.22	2.02 ± 0.12	76 ± 5.9
+ Thapsigargin			
100 μM NAADP	2.43 ± 0.19	4.06 ± 0.12	67 ± 2.9

experimental conditions, we next examined their possible interaction. Comparison of the effect of perfusing liposomes containing both NAADP (100 μM) and cADPR (10 μM) with the sum of the response elicited by the messengers alone revealed that the response to the combination of messengers was markedly potentiated (Figure 1C). NAADP and cADPR alone increased MEPP frequency by 71 ± 3.7% ($n = 6$) and 26 ± 3.2% ($n = 6$) respectively, following 3 min of liposome perfusion, whereas the increase elicited by the messenger mix (at the same time point) was 197 ± 4.3% ($n = 6$; Figure 1C, ◆), more than 2-fold higher than that expected if the effects of NAADP and cADPR were simply additive (71 + 26 = 97%; Figure 1C, ◇). Similar potentiation was observed with mixes of NAADP + IP_3 (160 ± 3.7% compared with 97%; $n = 6$) and NAADP + S1P (153 ± 4.1% compared 92%; $n = 6$) (Figure 1C). In contrast with the potentiated effects of cADPR, IP_3 and S1P when co-applied with NAADP within the liposomes, the effects of delivery of liposomes containing both NAADP and SPC on spontaneous transmitter release were largely additive (Figure 1C). The increase in MEPP frequency to this messenger

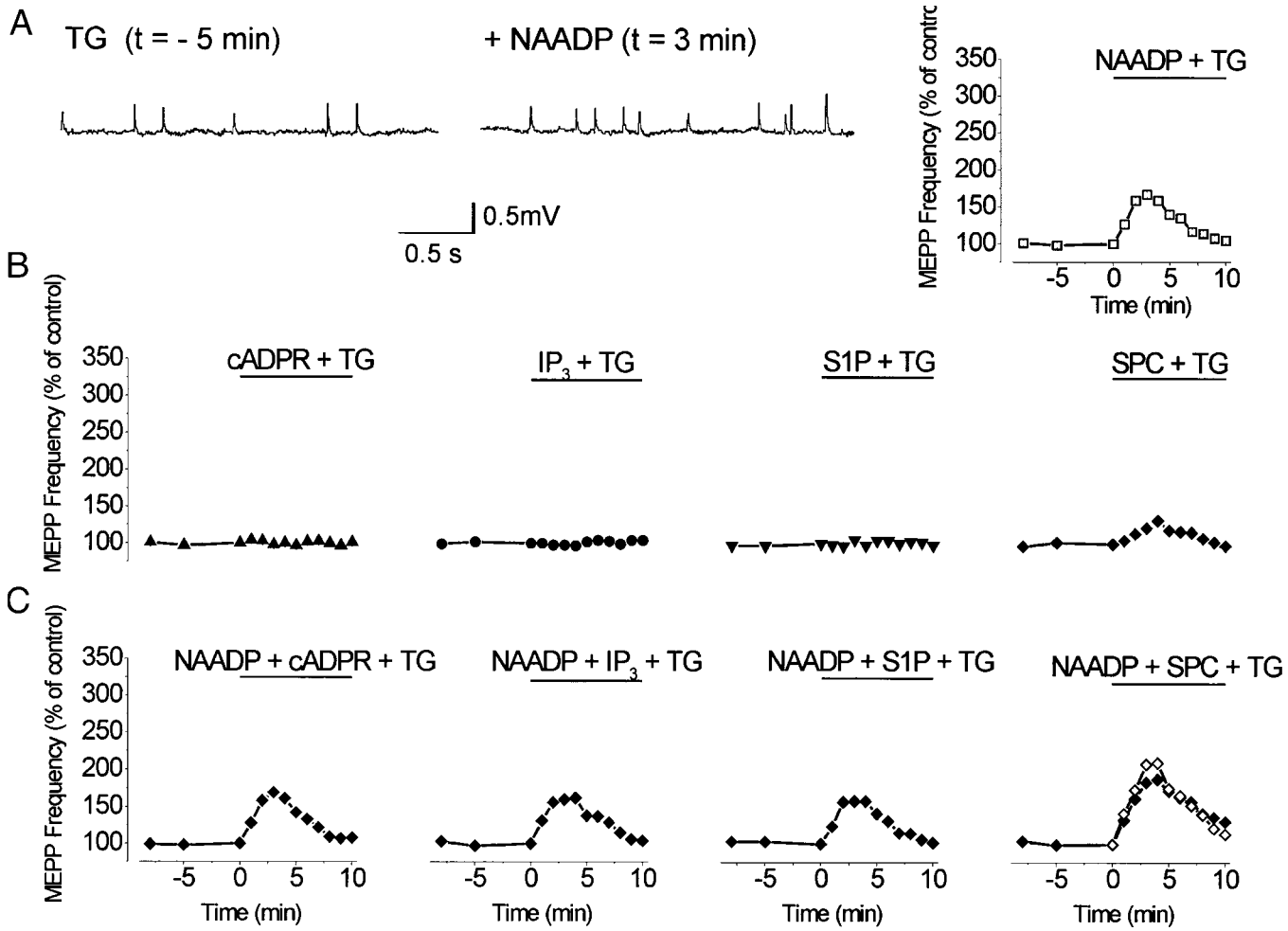

Figure 2 Potentiating effects of NAADP on neurotransmission by cADPR, IP_3 and S1P are blocked by thapsigargin

ER Ca^{2+} stores were depleted by perfusing preparations with thapsigargin (TG, 1 μM) in Ca^{2+}-free Ringer's solution for 30 min, followed by thapsigargin in normal Ringer's solution for a further 30 min. MEPP frequency in response to liposome perfusion containing the various messengers (**A** and **B**) and messenger mixes (**C**) was subsequently determined as in the legend to Figure 1. Values are means $\pm$ S.E.M. from six independent preparations. Error bars are smaller than the symbols.

mix was not significantly different to the sum of the responses to NAADP and SPC alone ($P > 0.05$).

We next examined the effect of thapsigargin treatment on messenger-evoked neurosecretion in normal Ringer's solution (Figure 2 and Table 1). Thapsigargin caused a modest (< 2-fold) increase in basal MEPP frequency (Figure 2A and Table 1). We found that the increase in MEPP frequency by NAADP (100 μM) in control preparations ($71 \pm 3.7\%$, at 3 min; Figure 1A) and preparations treated with thapsigargin ($67 \pm 2.9\%$, $n = 6$, at 3 min; Figure 2A and Table 1) were not significantly different ($P > 0.05$). In contrast, similar treatment with thapsigargin eliminated the increase in MEPP frequency induced by cADPR, IP_3 and S1P (Figure 2B). The response to SPC (Figure 2B) was largely insensitive to thapsigarigin. Thapsigargin treatment abolished the potentiation of neurotransmitter release by liposome perfusion of messenger mixes containing NAADP + cADPR, NAADP + IP_3 and NAADP + S1P (Figure 2C). Following 3 min of perfusion with NAADP + cADPR, the increase in MEPP frequency in preparations pretreated with thapsigargin was $61 \pm 4.1\%$ ($n = 6$), and thus not significantly different from the increase in MEPP frequency elicited by NAADP alone ($67 \pm 2.9\%$, $n = 6$; Figure 2A). The corresponding values for NAADP + IP_3 ($62 \pm 3.5\%$, $n = 6$) and NAADP + S1P ($58 \pm 3.5\%$, $n = 6$) were also not significantly different (Figure 2C). In contrast, thaspigargin had little effect on the combined effects of NAADP + SPC (Figure 2C). NAADP (100 μM) and SPC (10 μM) alone elicited at 3 min a $67 \pm 2.9\%$ ($n = 6$; Figures 2A and 2B) and $37 \pm 3.2\%$ increase in MEPP frequency respectively, the sum of which (104 %) was similar to the observed increase in MEPP frequency in response to liposomes containing both messengers at the same concentration ($106 \pm 4.7\%$, $n = 6$; Figure 2C).

DISCUSSION

The major findings of the present study are that neurosecretion can be stimulated by multiple Ca^{2+}-mobilizing messengers, that these messengers target distinct Ca^{2+} stores and, most importantly, that these Ca^{2+} stores interact.

Based on the differential sensitivity to thapsigargin of the various Ca^{2+}-mobilizing messengers in stimulating neurotransmitter release (Figure 2), there probably exist at least two pools of intracellular Ca^{2+} at the nerve terminal (Figure 3). Our present data indicate that, whereas cADPR, IP_3 and S1P mediate their effects through mobilization of Ca^{2+} from the ER,

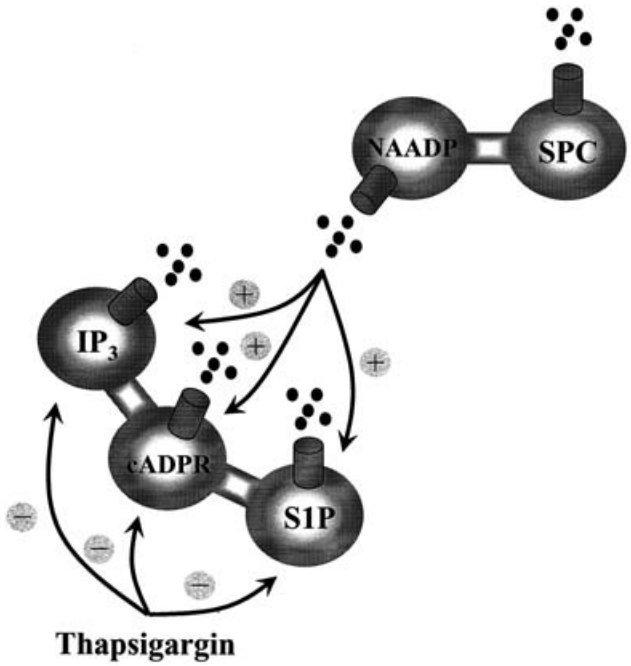

Figure 3 Proposed two-pool model for regulation of transmitter release by intracellular Ca^{2+}-mobilizing messengers

The finding that neurotransmission in response to cADPR, IP_3 and S1P is abolished by thapsigargin suggests that these messengers target Ca^{2+} stores associated with the ER. In contrast, the responses to NAADP and SPC are probably mediated by functionally distinct thapsigargin-insensitive Ca^{2+} stores. Enhanced neurotransmission observed in the presence of NAADP and ER-based messengers may result from local amplification of NAADP-induced Ca^{2+} release by Ca^{2+}-induced Ca^{2+} release from the ER and/or priming of the ER via uptake of Ca^{2+} released from thapsigargin-insensitive stores. See the text for more details.

NAADP and SPC appear to target distinct Ca^{2+} stores. Lack of effect of thapsigargin on asynchronous transmitter release in response to NAADP reported in the present study corresponds well with direct measurements of NAADP-induced Ca^{2+} release in the presence of thapsigargin in sea urchin eggs [14,15] and, more recently, in smooth muscle [22] and pancreatic β [17] cells. Direct potentiation of neurotransmitter release by thapsigargin alone (Table 1) is consistent with elevation of cytosolic Ca^{2+} through Ca^{2+} influx mediated by depletion of ER Ca^{2+} stores.

We show that NAADP potentiated the effects of cADPR, IP_3 and S1P, but not SPC, suggesting that NAADP sensitizes neurotransmitter release stimulated by thapsigargin-sensitive Ca^{2+}-release pathways only (Figure 3). That this potentiation was eliminated in the presence of thapsigargin indicates that neurotransmitter release enhancement by messenger mixes requires replete ER Ca^{2+} stores and is thus not a direct effect of cADPR, IP_3 or S1P on NAADP-induced Ca^{2+} release. Although recent studies have suggested that NAADP may directly activate ryanodine receptors [33,34], ryanodine (100 μM) did not affect neurotransmission in response to NAADP (Table 1). Our data are therefore consistent with those of Copello et al. [35] who, using similar methods as Mojzisova et al. [33] and Hohenegger et al. [34], failed to show any effect of NAADP on skeletal or cardiac muscle ryanodine receptors [35].

Extending our previous observations performed in modified Ringer's solution containing increased KCl [29], we show that intracellular delivery of NAADP (and also IP_3 and cADPR) enhances quantal output under basal conditions (Figures 1 and 2). However, important differences were also observed between the two studies. Of interest is the partial inhibition of NAADP-induced transmitter release by thapsigargin noted previously [29]. These data are in contrast with the present finding where thapsigargin was without effect on the responses to NAADP under basal conditions (Figures 1A and 2A, and Table 1). This may reflect recruitment of ER-based Ca^{2+} channels by NAADP in the absence of exogenous introduction of their agonists in the former study [29], possibly as a result of priming of the ER with Ca^{2+} through evoked Ca^{2+} influx. Indeed, NAADP-induced Ca^{2+} release in pancreatic acinar cells appears absolutely dependent upon activation of ER Ca^{2+} channels [21]. Differences in the filling state of intracellular Ca^{2+} stores may also in part underlie the less than additive effects of messenger mixes observed previously [29]. Our unpublished data show that concentration-effect relationships for neurotransmission in response to IP_3 and cADPR are biphasic such that, as the concentration of the messenger within the liposome is increased beyond 100 μM, stimulation of transmitter decreases (E. Brailoiu, S. Patel and N. J. Dun, unpublished work). A probable explanation for this observation is that Ca^{2+} signals generated during liposome perfusion are more prolonged at lower messenger concentrations than at higher concentrations, resulting in greater release of transmitter. This could be due to regenerative Ca^{2+} release in the form of Ca^{2+} oscillations stimulated by lower messenger concentrations, a well established phenomenon in response to submaximal (but not maximal) stimulation in many other cells [1]. Thus, under the 'sensitized' conditions of our previous analysis (in the presence of high extracellular K^+) [29], NAADP may have potentiated Ca^{2+} release from the ER to an extent where the Ca^{2+} signal is less effective in stimulating transmitter release, i.e. to a point on the descending phase of the concentration–effect relationship for the co-messenger. Although beyond the scope of the present study, direct measurement of cytosolic Ca^{2+} concentration at different concentrations of messengers and messenger mixes would be clearly informative.

The exact nature of the NAADP-sensitive Ca^{2+} store at the nerve terminal and the mechanism of coupling between this store and the ER remain to be investigated. Recent studies in other systems, however, suggest that NAADP may mobilize acidic Ca^{2+} stores [16,17]. This raises the intriguing possibility that, in neurons, NAADP may directly target synaptic vesicles in the releasable pool [29], as these organelles are also acidic stores of Ca^{2+} filled in a thapsigargin-insensitive manner. Unfortunately, although agents such as bafilomycin A1 (a vacuolar proton pump inhibitor) have been used to ascertain the acidic nature of NAADP-sensitive Ca^{2+} stores in sea urchin eggs [16], the absolute dependence of proton gradients for neurotransmitter uptake into synaptic vesicles precludes the use of such agents in determining whether stimulation of neurosecretion by NAADP in our preparation proceeds by mobilization of acidic Ca^{2+} stores.

In summary, our data in the present study provide further evidence that NAADP plays a central role in the generation of Ca^{2+} signals through the recruitment of other intracellular Ca^{2+}-release pathways and that such coupling may be important in the modulation of transmitter release at the neuromuscular junction. A more widespread signalling role for NAADP in neurons is consistent with recent demonstrations of NAADP-induced Ca^{2+} release from brain microsomes [36], specific binding sites for NAADP throughout the brain [37] and regulated metabolism of NAADP by brain membranes [38].

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