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Influence of stimulation on Ca^{2+} recruitment triggering [^{3}H]acetylcholine release from the rat motor-nerve endings

Paulo Correia-de-Sá [a,*], M. Alexandrina Timóteo [a], J. Alexandre Ribeiro [b]

[a] *Laboratório de Farmacologia, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS) — Universidade do Porto, L. Prof. Abel Salazar, 2, 4099-003 Porto, Portugal*

[b] *Laboratory of Neurosciences, Faculty of Medicine — University of Lisbon, 1649-028 Lisbon, Portugal*

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Abstract

The influence of rat phrenic nerve stimulation frequency (5–50 Hz) and of pulse duration (0.04–1 ms) on Ca^{2+} mobilization triggering [^{3}H]acetylcholine release was investigated. The P-type voltage-dependent Ca^{2+} channel (VDCC) blocker, ω-agatoxin IVA (100 nM), decreased [^{3}H]acetylcholine release evoked by pulses of 0.04-ms duration delivered at 5 Hz frequency. When the stimulus pulse duration was increased to 1 ms (5 Hz frequency) or the stimulation frequency to 50 Hz (0.04-ms duration), inhibition of [^{3}H]acetylcholine release became evident after blockade of L-type VDCC, with nifedipine (1 μM), and/or depletion of thapsigargin-sensitive internal stores. The inhibitory effect of thapsigargin (2 μM) was still observed in Ca^{2+}-free medium. Neither ω-conotoxin GVIA (1 μM) nor ω-conotoxin MVIIC (150 nM) modified neurotransmitter release. The results suggest that, depending on the stimulus paradigm, both internal (thapsigargin-sensitive) and external (either P- or L-type channels) Ca^{2+} pools can be mobilized to promote acetylcholine release from motor nerve terminals.

Keywords: Ca^{2+} channel blocker; Thapsigargin; Stimulus pattern; Acetylcholine release; Neuromuscular junction

1. Introduction

It is known that multiple subtypes of voltage-dependent Ca^{2+} channels (VDCC) are co-localized in nerve terminals, and that they can be activated synergistically to regulate the Ca^{2+} entry needed to trigger transmitter release (Wheeler et al., 1994). Brief depolarizations trigger calcium currents which are apparently different from the normally quiescent "facilitatory" currents that are activated by large pre-depolarizations or by repetitive action potentials (cf. Artalejo et al., 1992). Changes in the duration of cell depolarization have been proposed as an elegant method to modify neuronal Ca^{2+} influx (Dudel, 1989; Van der Kloot and Molgó, 1994). Furthermore, we and others showed that the physiological adaptation of neuromuscular transmission to nerve stimulation paradigms (frequency, pulse duration, number of pulses) depends on the balance between tonic activation of several facilitatory presynaptic receptors (e.g. nicotinic, muscarinic, peptidergic, purinergic) (see, e.g. Wessler, 1989; Correia-de-Sá et al., 1996, 1997a) which might trigger the mobilization of independent Ca^{2+} pools.

At the mature mammalian neuromuscular junction stimulated with a low-frequency pattern (0.2–0.5 Hz), the P-type Ca^{2+} channels seem to have a dominant influence on evoked transmitter release (Van der Kloot and Molgó, 1994). Although a clear role for L-type channels in peripheral synaptic transmission has been difficult to demonstrate (Bean, 1989; Van der Kloot and Molgó, 1994), it has been argued that these channels need more intense/prolonged stimulation to be activated (Miller, 1987). The purpose of this work was to investigate if other VDCCs (non-P type) could also be of physiological significance in triggering [^{3}H]acetylcholine release from rat motor nerve terminals. Road et al. (1995) demonstrated that the firing rate of phrenic motoneurons changed from relatively slow (0–30

* Corresponding author. Tel.: +351-2-206-2243; fax: +351-2-206-2232.

E-mail address: farmacol@icbas.up.pt (P. Correia-de-Sá).

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Hz) to relatively high (40–50 Hz) during recovery from apnea and inspiratory resistive loading, respectively. Adaptation of neurosecretion during manipulation of the stimulus frequency (5–50 Hz) and pulse duration (0.04–1 ms) has also been shown (Wessler, 1989; Correia-de-Sá et al., 1996, 1997a). Thus, using similar stimulation conditions, we characterized the type(s) of Ca^{2+} channel(s) involved in evoked [^{3}H]acetylcholine release by testing the pharmacological effects of several VDCC blockers. The drugs used were ω-agatoxin IVA, which blocks P-type channels in the nanomolar concentration range (Zhang et al., 1993), ω-conotoxin GVIA, which selectively blocks N-type channels (Olivera et al., 1994; Dunlap et al., 1995), ω-conotoxin MVIIC, a blocker of both P-, Q- and N-type channels (Wheeler et al., 1994), and nifedipine, a blocker of L-type Ca^{2+} channels (Olivera et al., 1994; Dunlap et al., 1995). The action of the specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (Thastrup et al., 1990), was also studied to determine whether recruitment of Ca^{2+} from internal stores also participates in evoked transmitter release.

A preliminary account of some of the results has already appeared (Correia-de-Sá et al., 1997b).

2. Methods

2.1. Preparation and experimental conditions

The experiments were carried out on left phrenic nerve-hemidiaphragm preparations (4–6-mm width) taken from Wistar rats of either sexes of about 200 g in weight (from Gulbenkian Institute of Science, Oeiras — Portugal). The procedures used for labelling the preparations and for measuring evoked [^{3}H]acetylcholine release have previously been described (Correia-de-Sá et al., 1991) and were used with minor modifications. Briefly, the preparations were superfused (3 ml min^{-1}) in 3-ml organ baths at 37°C with Tyrode solution continuously gassed with 95% O_2 and 5% CO_2, containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30-min equilibration period, the perfusion was stopped and the nerve endings were labelled for 40 min with 1 μM [^{3}H]choline (specific activity 2.5 μCi $nmol^{-1}$) under electrical stimulation at 1-Hz frequency. After the end of the labelling period, the preparations were again superfused (15 ml min^{-1}) and nerve stimulation was stopped. From this time onwards, hemicholinium-3 (10 μM) was present to prevent the uptake of choline. After a 60-min period of washout, the perfusion was stopped, and 2-ml bath samples were collected every 3 min by emptying and refilling the organ bath with the solution in use, using a fraction collector (Gilson, FC 203B) coupled to a peristaltic pump (Gilson, Minipuls3)-programmed device. Tritium content of the samples was measured by liquid scintillation spectrometry (%Tritium efficiency: 40 ± 2%).

2.2. Nerve stimulation conditions

The left phrenic nerve was stimulated with an extracellular glass-platinum suction electrode placed near its first division branch, to avoid direct stimulation of muscle fibers. [^{3}H]Acetylcholine release was evoked by electrical stimulation of the phrenic motor nerve endings with 750 pulses with a duration of 0.04–4 ms and a current strength of 8 mA. Supramaximal intensity rectangular pulses were used to achieve synchronization of phrenic motoneuron firing (as observed during brief inspiratory resistive loading, see, e.g. Road et al., 1995), thus reducing the number of silent units that might make interpretation of the release data difficult. The stimulation frequency changed within the physiological range from 5 to 50 Hz, applied over a period of 2.5 min. Five bursts of 150 pulses, applied with a 20-s interburst interval, were delivered when 50-Hz stimulation was used, in order to achieve a similar amount of [^{3}H]acetylcholine release as that observed when 5-Hz frequency stimuli (keeping 0.04-ms pulse duration) were used (see Table 1). Two stimulation periods were applied: at 12 min (S_1) and at 39 min (S_2) after the end of washout (zero time). The pulses were delivered by a Grass S48 stimulator coupled to a stimulus isolation unit (Grass SIU5), operating in a constant current mode. The stimulation parameters were continuously monitored on an oscilloscope (Meguro, MO-1251A) and were within the same range used in previous studies with this preparation (Wessler and Kil-

Table 1
Influence of the stimulation paradigm on evoked [^{3}H]acetylcholine release from rat motor nerve endings

Stimulation conditions (ms)	S_1 (10^3 dpm/g)	Control S_2/S_1
5-Hz frequency:		
0.04	102 ± 5 (19)	0.81 ± 0.03 (8)
0.50	115 ± 4 (8)[a]	0.81 ± 0.05 (4)
1.00	128 ± 6 (21)[a]	0.82 ± 0.02 (5)
4.00	168 ± 3 (7)[a]	0.83 ± 0.06 (3)
50-Hz frequency:		
0.04	105 ± 5 (11)	0.84 ± 0.04 (8)

The phrenic nerve was stimulated at the 12th and 39th min after the end of the washout period with 750 supramaximal intensity pulses delivered at frequencies of 5 and 50 Hz. The duration of the pulses varied from 0.04 to 1 ms when the 5-Hz stimulation frequency was used. S_1 represents the average (± S.E.M.) evoked [^{3}H]acetylcholine release during the first period of stimulation. Number of experiments is shown in parentheses. S_2/S_1 is the ratio between evoked [^{3}H]acetylcholine release during the second period of stimulation and evoked [^{3}H]acetylcholine release during the first period of stimulation.

[a] $P < 0.05$ (Student's t-test) as compared with the average evoked [^{3}H]acetylcholine release obtained with pulses of 0.04-ms duration.

binger, 1986; Wessler et al., 1995; Correia-de-Sá et al., 1996).

Electrical stimulation of the phrenic nerve increased only the release of [^{3}H]acetylcholine while the output of [^{3}H]choline remained unchanged during the stimulation periods (Wessler and Kilbinger, 1986) (see also Fig. 1). Neither muscular (Tucek, 1982; Molenaar et al., 1987) nor non-quantal neuronal (Katz and Miledi, 1977) acetylcholine components seemed to be released upon electrical stimulation of the phrenic nerve under the present experimental conditions. These assumptions are based on the findings that (a) upon blocking muscle potentials and twitches with a supramaximal concentration (5 μM) of tubocurarine (e.g. Correia-de-Sá et al., 1991), evoked [^{3}H]acetylcholine release by pulses up to 4-ms duration was not significantly reduced, and (b) it has been shown that the spontaneously releasable neuronal pool of acetylcholine is not labelled with [^{3}H]choline nor it is released by electrical nerve stimulation (Molenaar et al., 1987), and it is completely exhausted (within minutes) in the presence of hemicholinium-3 (Nikolsky et al., 1991). Additionally, the prevention of release observed in the absence of external Ca^{2+} (CaO + EGTA, 1 mM) plus tetrodotoxin (1 μM) (see Section 3) suggests that evoked [^{3}H]acetylcholine outflow comes mainly from vesicle exocytosis from depolarized nerve terminals. Therefore, evoked [^{3}H]acetylcholine release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (Correia-de-Sá et al., 1991).

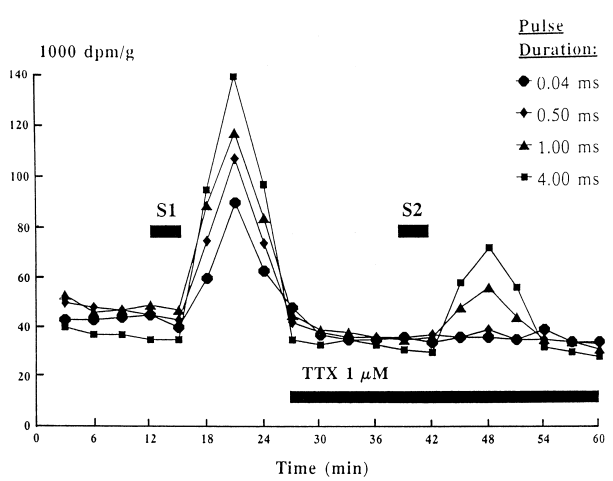

Fig. 1. Effect of tetrodotoxin (1 μM) on [^{3}H]acetylcholine release from the rat motor nerve terminals evoked by stimulation pulses of variable duration (0.04–4 ms). Time course of tritium outflow from rat hemidiaphragms in the presence of tetrodotoxin (1 μM) taken from typical experiments. After the labelling and washout periods, [^{3}H]acetylcholine release was elicited by electrical stimulation of the phrenic nerve (5 Hz, 750 pulses) at the indicated times (S_1 and S_2). The stimulus pulse duration ranged from 0.04 to 4 ms, and the corresponding value is indicated for each experiment. Tetrodotoxin (1 μM) was applied 15 min before S_2 (horizontal bar). Note that no significant changes in spontaneous tritium outflow were detected. Average tritium outflow (dpm/g) collected during S_1 is shown in Table 1.

2.3. Quantification of drug effects

Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Their effects were expressed by the ratios S_2/S_1, i.e. the ratio between evoked [^{3}H]acetylcholine release during the second stimulation period (in the presence of the test drug) and evoked [^{3}H]acetylcholine release during the first stimulation period (without the test drug). Average evoked [^{3}H]acetylcholine release during S_1 (S_1 average, dpm/g of wet weight of preparation) and S_2/S_1 under control conditions, i.e. without addition of drugs, is shown in Table 1. Percentage values shown in figures correspond to percentage changes in S_2/S_1 ratios as compared with the S_2/S_1 ratio in control experiments, using the same stimulation paradigm. Zero percent represents identical ratios; positive and negative values represent facilitation and inhibition of evoked [^{3}H]acetylcholine release, respectively. None of the drugs used significantly ($P > 0.05$) changed basal tritium outflow.

2.4. Materials and solutions

Cadmium chloride ($CdCl_2$), choline chloride, ω-conotoxin GVIA, ω-conotoxin MVIIC, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), hemicholinium-3, nifedipine, tetrodotoxin and thapsigargin were from Sigma. ω-Agatoxin IVA was from Peptide Institute. [Methyl-^{3}H]choline chloride (ethanol solution, 80 Ci mmol^{-1}) was obtained from Amersham. All other reagents were of the highest purity available. Nifedipine was made up in a 10-mM stock solution in ethanol, which was kept protected from the light to prevent photodecomposition. Thapsigargin was made up as a 2-mM stock solution in dimethyl sulphoxide. All stock solutions were stored as frozen aliquots at −20°C. Aqueous dilutions of these stock solutions were made daily and appropriate solvent controls were used. The pH of the superfusion solution was not changed by the addition of the drugs in the maximum concentrations applied to the preparations.

2.5. Statistics

The data are expressed as means ± standard error of the mean, from *n* observations. The significance of the differences was evaluated by Student's *t*-test. $P < 0.05$ was considered to represent significant differences.

3. Results

Electrical stimulation (5-Hz frequency, 750 pulses) of the phrenic nerve increased the release of [^{3}H]acetylcholine in a pulse duration-dependent manner (Fig. 1 and

Table 2
Influence of the stimulus pulse duration on the inhibitory effects of TTX (1 μM), $CdCl_2$ (500 μM) and external calcium withdrawal (CaO + EGTA, 1 mM) on evoked [^{3}H]acetylcholine release from rat motor nerve endings: role of thapsigargin-sensitive intracellular calcium pools

Stimulation pulse duration	0.04 ms		1.00 ms	
	% Inhibition	S_2 (10^3 dpm/g)	% Inhibition	S_2 (10^3 dpm/g)
Control		83 ± 4 (8)		105 ± 5 (5)
Tetrodotoxin (1 μM)	98 ± 1 (5)	2 ± 1 (5)	65 ± 6 (4)[a]	37 ± 7 (4)[b]
+ Thapsigargin (2 μM)			59 ± 4 (6)[a]	43 ± 4 (6)[b]
+ CaO + EGTA (1 mM)			90 ± 2 (4)	11 ± 1 (4)[b]
+ Cadmium (500 μM)			99 ± 1 (4)	1 ± 1 (4)
CaO + EGTA (1 mM)	95 ± 3 (4)	4 ± 2 (4)	69 ± 1 (4)[a]	33 ± 1 (4)[b]
+ Thapsigargin (2 μM)			91 ± 4 (4)	9 ± 5 (4)
Cadmium (500 μM)	90 ± 5 (4)	8 ± 6 (4)	62 ± 6 (5)[a]	40 ± 6 (5)[b]
+ Thapsigargin (2 μM)			88 ± 8 (6)	13 ± 9 (6)

The evoked [^{3}H]acetylcholine release was elicited twice (S_1 and S_2) by nerve stimulation with 750 supramaximal intensity pulses of 0.04- or 1-ms durations, delivered at a frequency of 5 Hz. Test drugs or calcium-free solution (+EGTA, 1 mM) was added to the incubation media 15 min before S_2. Percentage inhibition corresponds to reductions in the S_2/S_1 ratio as compared with the S_2/S_1 ratio in control experiments, using the same stimulation paradigm (see Table 1); 0% indicates that the two ratios were identical. S_2 represents the average (±S.E.M.) evoked [^{3}H]acetylcholine release during the second period of stimulation. Number of experiments is shown in parentheses.

[a] $P < 0.05$ (Student's *t*-test) as compared with the percentage inhibition.

[b] $P < 0.05$ (Student's *t*-test) as compared with the average evoked [^{3}H]acetylcholine release obtained with pulses of 0.04-ms duration.

Table 1). [^{3}H]Acetylcholine release evoked with pulses up to 0.5-ms duration was abolished in the absence of extracellular Ca^{2+} ions (Ca^{2+}O + EGTA, 1 mM), in the presence of the non-selective VDCCs blocker, $CdCl_2$ (500 μM), or after pretreatment with tetrodotoxin (1 μM) (Fig. 1 and Table 2). With pulses of 1-ms duration, the evoked [^{3}H]acetylcholine release persisted as 40 ± 7% ($n = 4$) of the control values in the presence of a supramaximal concentration of tetrodotoxin (1 μM) (Correia-de-Sá et al., 1996). Removal of external Ca^{2+} ions (Ca^{2+}O + EGTA, 1 mM) or the presence of $CdCl_2$ (500 μM) suppressed the tetrodotoxin-insensitive component of [^{3}H]acetylcholine release (Table 2). Table 2 also shows that the inhibition of evoked [^{3}H]acetylcholine release upon removal of external Ca^{2+} (CaO + EGTA, 1 mM) or after addition of $CdCl_2$ (500 μM) became less effective when the stimulation pulse duration was increased to 1 ms (keeping the stimulation frequency at 5 Hz).

Depletion of Ca^{2+} internal stores by a high concentration (2 μM) of thapsigargin (Thastrup et al., 1990) did not significantly change [^{3}H]acetylcholine release evoked by 5-Hz frequency, 0.04-ms depolarizing pulses (Fig. 2 and Table 3). In contrast, evoked [^{3}H]acetylcholine release induced by longer (5 Hz, 1 ms) depolarizing pulses or by high-frequency bursts (50 Hz, 0.04 ms) was significantly ($P < 0.05$) inhibited by thapsigargin (2 μM) (Fig. 2 and Table 3). Recruitment of Ca^{2+} from intracellular pools might alternatively participate in transmitter release when extracellular Ca^{2+} is unavailable, because thapsigargin (2 μM) almost abolished the evoked [^{3}H]acetylcholine release (5 Hz, 1 ms) remaining (30–40%) after removal of external Ca^{2+} (Ca0 + EGTA, 1 mM) or in the presence of $CdCl_2$ (500 μM) (Table 2). The inhibitory effect of thapsigargin (2 μM) on [^{3}H]acetylcholine release was no longer observed after pretreatment with tetrodotoxin (1 μM) Table 2), thus indicating that intracellular Ca^{2+} mobilization is triggered by a nerve action potential-dependent mechanism.

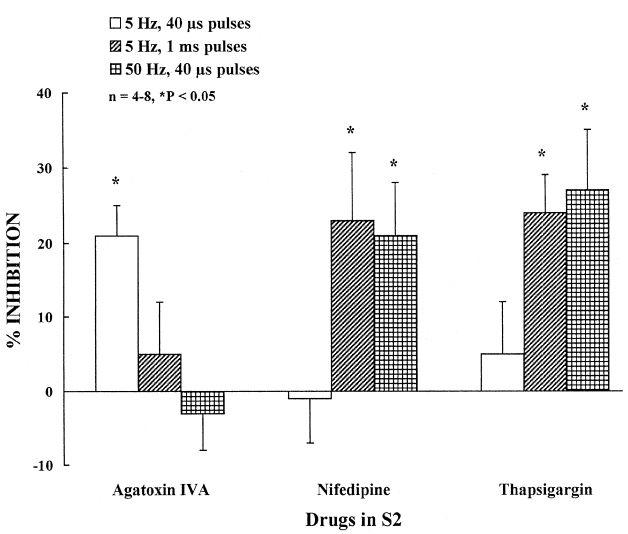

Fig. 2. Influence of the stimulation frequency and pulse duration on Ca^{2+} mobilization triggering [^{3}H]acetylcholine release from rat motor nerve endings. Evoked [^{3}H]acetylcholine release was elicited twice (S_1 and S_2) by nerve stimulation with 750 supramaximal intensity pulses of either 0.04- or 1-ms duration. The stimulation frequency was either 5 or 50 Hz. Five bursts of 150 pulses, applied with a 20-s interburst interval, were delivered when the 50-Hz frequency (0.04-ms pulse duration) was used. Thapsigargin (2 μM), nifedipine (1 μM), and ω-agatoxin IVA (100 nM) were applied 15 min before the end of S_2. Percentage inhibition is the reduction of S_2/S_1 ratio in the presence of test drug as compared with the S_2/S_1 ratio in control experiments using a similar stimulation protocol (see Table 1). Zero percent indicates that the two ratios were identical. Each column is the mean of four to eight experiments. The vertical bars represent ± S.E.M. * $P < 0.05$ (Student's *t*-test) when compared with control experiments using the same stimulus paradigm.

Table 3
Influence of the stimulation parameters on the amount of [^{3}H]acetylcholine released (S_2 values, 1000 dpm/g) from rat motor nerve endings: role of VDCC and thapsigargin-sensitive intracellular stores

Stimulation conditions	Control	Thapsigargin (2 μM)	Nifedipine (1 μM)	ω-Agatoxin IVA (100 nM)	ω-Conotoxin MVIIC (150 nM)	ω-Conotoxin GVIA (1 μM)
5-Hz frequency:						
0.04 ms	83 ± 4 (8)	79 ± 7 (6)	84 ± 6 (8)	66 ± 4 (4)[a]	85 ± 7 (6)	80 ± 6 (4)
1.00 ms	105 ± 5 (5)	80 ± 5 (5)[a]	81 ± 8 (4)[a]	100 ± 7 (4)	107 ± 7 (4)	106 ± 5 (5)
50-Hz frequency:						
0.04 ms	88 ± 6 (8)	64 ± 6 (8)[a]	69 ± 5 (4)[a]	91 ± 5 (4)	87 ± 8 (4)	84 ± 7 (4)

The phrenic nerve was stimulated at the 12th and 39th min after the end of the washout period with 750 supramaximal intensity pulses delivered at frequencies of 5 and 50 Hz. The duration of the pulses varied from 0.04 to 1 ms when the 5 Hz stimulation frequency was used. Data represent the average (±S.E.M.) evoked [^{3}H]acetylcholine release during the second period of stimulation (S_2, 1000 dpm/g of wet tissue), after a 15-min incubation period with test drugs. Number of experiments is shown in parentheses. Control S_2 values, in the absence of test drugs, are shown for comparison.

[a] $P < 0.05$ (Student's *t*-test) as compared with the average evoked [^{3}H]acetylcholine release obtained in control conditions.

The effects of the type-specific blockers of VDCCs, nifedipine (1 μM), ω-agatoxin IVA (100 nM), ω-conotoxin GVIA, (1 μM) and ω-conotoxin MVIIC (150 nM), on evoked [^{3}H]acetylcholine release were studied. [^{3}H]Acetylcholine release was not significantly ($P > 0.05$) affected by ω-conotoxin GVIA (1 μM) or ω-conotoxin MVIIC (150 nM) in any of the stimulation conditions used (Table 3). ω-Agatoxin IVA, applied in a concentration (100 nM) that selectively blocked P-type VDCC (Zhang et al., 1993), inhibited evoked [^{3}H]acetylcholine release by 21 ± 4% ($n = 4$) when the phrenic nerve was stimulated with pulses of 0.04-ms duration delivered at a frequency of 5 Hz. This inhibitory effect was significantly attenuated to 5 ± 9% ($n = 4$) when the pulse duration was increased to 1 ms (5-Hz frequency). Ca^{2+} influx through P-type channels also became less relevant during high-frequency (50 Hz, 0.04 ms) bursts, since ω-agatoxin IVA (100 nM) failed to inhibit [^{3}H]acetylcholine release under these conditions (Fig. 2 and Table 3). In contrast, nifedipine (1 μM) abolished the facilitation of neurotransmitter release (23 ± 9%, $n = 4$) detected when the phrenic nerve was stimulated with pulses of 1-ms duration (5-Hz frequency); the amount of evoked [^{3}H]acetylcholine release ($S_2 = 83 \pm 4$ 10^3 dpm/g, $n = 8$) in control (5-Hz frequency, 0.04-ms pulses) conditions was of similar magnitude to the total tritium outflow ($S_2 = 81 \pm 8$ 10^3 dpm/g, $n = 4$), in the presence of nifedipine (1 μM) when 1-ms pulses were used (Table 3). A similar effect was observed when nifedipine was applied in a higher concentration (10 μM, data not shown). Although nifedipine (1 μM) failed to modify acetylcholine release evoked by 5-Hz frequency short (0.04-ms) depolarizing pulses, the increase in the stimulation frequency within the physiological range to 50 Hz (0.04-ms pulse duration) revealed a significant inhibitory effect of 21 ± 7% ($n = 4$) (Fig. 2).

Note that the magnitude of inhibition of neurotransmitter release induced by ω-agatoxin IVA (100 nM), nifedipine (1 μM) or thapsigargin (2 μM) was not dependent on the total amount of [^{3}H]acetylcholine released during each stimulation condition, as can be seen by comparison of the S_2 values in controls and those in the presence of test drugs (Table 3). For instance, although similar amounts of evoked tritium outflow were measured in controls when both 5-Hz ($S_2 = 83 \pm 4$ 10^3 dpm/g, $n = 8$) and 50-Hz ($S_2 = 88 \pm 6$ 10^3 dpm/g, $n = 8$) frequencies (keeping 0.04-ms pulse duration) were used, ω-agatoxin IVA (100 nM) inhibited [^{3}H]acetylcholine release at the 5-Hz frequency ($S_2 = 66 \pm 4$ 10^3 dpm/g, $n = 4$), whereas both nifedipine (1 μM, $S_2 = 69 \pm 5$ 10^3 dpm/g, $n = 4$) and thapsigargin (2 μM, $S_2 = 64 \pm 6$ 10^3 dpm/g, $n = 8$) caused inhibition only after the stimulation frequency was increased to 50 Hz.

It has been suggested that thapsigargin could also block L-type VDCC when used in concentrations higher than 1 μM (Buryi et al., 1995). Since the inhibitory effect of

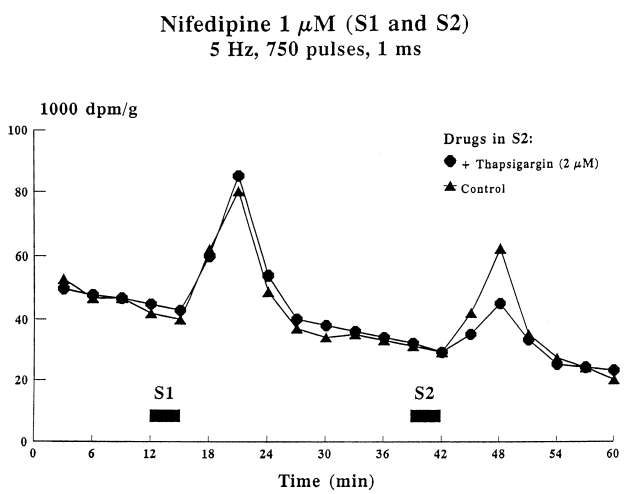

Fig. 3. Inhibitory effect of thapsigargin (2 μM) on electrically evoked (5 Hz, 750 pulses, 1-ms duration) [^{3}H]acetylcholine release during L-type VDCC blockade with nifedipine (1 μM). Time course of tritium outflow from the rat motor nerve terminals taken from typical experiments in the presence of nifedipine (1 μM), applied in both stimulation periods (S_1 and S_2). Thapsigargin (2 μM) was added 15 min before S_2. Note that no significant changes in spontaneous tritium outflow were detected. A control experiment, in the absence of thapsigargin, is shown for comparison.

thapsigargin (2 μM) on evoked [^{3}H]acetylcholine release was detected only when either the stimulus pulse duration was increased to 1 ms or the frequency of stimulation was increased to 50 Hz, i.e. under circumstances similar to those that were used to reveal inhibition of release by nifedipine (1 μM), experiments were designed to study whether the inhibition by thapsigargin was related to L-type VDCC blockade. In these experiments, nifedipine (1 μM) was added to the incubation media 15 min before S_1 and S_2, whereas thapsigargin (2 μM) was applied together with nifedipine (1 μM) 15 min before S_2. Note that under these conditions, the S_2/S_1 ratio (0.88 ± 0.06, $n = 6$), when only nifedipine (1 μM) was added, was not different from control (5 Hz frequency, 1-ms pulses) values in the absence of any drug (Table 1). The inhibitory effect of thapsigargin (2 μM) on [^{3}H]acetylcholine release, evoked with pulses of 1 ms in the presence of nifedipine (1 μM), was even higher ($47 \pm 7\%$, $n = 4$) than that observed when it was applied alone (Fig. 3). This indicates that both thapsigargin and nifedipine inhibit transmitter release from rat motor nerve terminals by blocking independent and even synergistic mechanisms involved in the release process.

4. Discussion

The results show that the stimulation pattern influences Ca^{2+} mobilization from either extracellular or intracellular pools, triggering acetylcholine release from rat motor nerve endings. The release of [^{3}H]acetylcholine depends exclusively on the influx of Ca^{2+} through P-type VDCCs when low-frequency/short-duration stimulation pulses were used, whereas high-frequency and/or long-duration depolarizing stimuli were required to reveal the functional importance of either Ca^{2+} influx through nifedipine-sensitive L-type channels or Ca^{2+} recruitment from thapsigargin-sensitive intracellular pools, which might act synergistically to increase acetylcholine release. Previous studies showed that high-frequency nerve stimulation could promote recruitment of additional VDCCs (Hong et al., 1996) as well as intraneuronal Ca^{2+} stores (Smith and Cunnane, 1996) that play an important role in the facilitation of transmitter release in the autonomic nervous system.

It is well established that quantal neurotransmitter release is graded with the duration of presynaptic depolarization applied in the presence of tetrodotoxin (Katz and Miledi, 1966; Dudel, 1989). Here, we showed that (a) the amount of acetylcholine release from the rat neuromuscular junction was dependent on the stimulation pulse duration, and (b) during long depolarizing pulses (> 1 ms), evoked [^{3}H]acetylcholine release can be triggered by a tetrodotoxin-insensitive focal depolarization phenomena, requiring Ca^{2+} influx through VDCCs. The quantal release of acetylcholine by direct depolarization of motor nerve terminals, measured via an extracellularly placed suction electrode, has been described previously (Van der Kloot and Molgó, 1994). Although the physiological relevance of focal depolarization in the control of transmitter release is still a matter of debate in the literature, its role in the balance between activation of cholinergic, peptidergic and purinergic receptors modulating acetylcholine release from the rat neuromuscular junction has been demonstrated (Correia-de-Sá et al., 1997a).

Recruitment of Ca^{2+} from intracellular pools becomes functionally relevant during high-frequency bursts or long-duration depolarizing pulses. Mobilization of thapsigargin-sensitive internal stores, which depends on the generation of action potentials (tetrodotoxin sensitivity), can provide an alternative source of Ca^{2+} to trigger [^{3}H]acetylcholine release during prolonged depolarizations in the absence of extracellular Ca^{2+} or in the presence of the non-selective Ca^{2+} channel blocker, $CdCl_2$ (500 μM). In contrast to previous data indicating a blocking action of L-type Ca^{2+} currents by thapsigargin in the micromolar concentration range (Buryi et al., 1995), our findings showed that thapsigargin (2 μM) further inhibited evoked [^{3}H]acetylcholine release in the presence of nifedipine (1 μM). This suggests that both thapsigargin and nifedipine inhibit transmitter release by blocking synergistic mechanisms. Experiments from various laboratories indicate the existence of another depolarization-dependent factor that combines with Ca^{2+} influx to control facilitation of transmitter release, i.e. depolarization can trigger quantal release when Ca^{2+} entry is blocked and intracellular Ca^{2+} is held at elevated levels (for a review, see Parnas et al., 2000).

Knowledge of the differential co-localization, biophysical properties and inactivation mechanisms that determine the relative contribution of neuronal VDCCs to Ca^{2+} influx, elicited by a given stimulus, is important to anticipate their involvement in the regulation of neurotransmitter release under different pathophysiological conditions. Using a frequency of stimulation (5 Hz) that mimics the mean physiological respiratory rhythm in non-anasthetized rats (Monteiro and Ribeiro, 1987), we showed that the P-type channel blocker, ω-agatoxin IVA (100 nM), was the only drug tested able to inhibit [^{3}H]acetylcholine release evoked by brief (0.04 ms) stimulation pulses. Neither ω-conotoxin GVIA (1 μM) nor ω-conotoxin MVIIC (150 nM) modified neurotransmitter release. These results fully agree with previous findings (e.g. Wessler et al., 1995), suggesting that, at the rat neuromuscular junction, the P-type family have a dominant role in nerve-evoked transmitter release. Other Ca^{2+} channels, namely Q- and N-type, have been implicated in acetylcholine release from motor nerve terminals, though the experiments were performed in different species (mouse and frog) with low-frequency stimulation patterns (0.2–1 Hz), and after reducing the safety margin of neuromuscular transmission by tubocurarine or high Mg^{2+}/low Ca^{2+} (e.g. Bowersox et al., 1995; Katz et al., 1995, 1996). Some of the differences

might also be explained by the use of ω-conotoxin MVIIC in the micromolar concentration range, as compared to its IC_{50} of < 150 nM (used in this work) to block Q-type channels in physiological studies (see Olivera et al., 1994; Wheeler et al., 1994).

Clustering of P-type VDCCs has been demonstrated near the active zones of vertebrate nerve terminals (Stanley, 1997). The amount of Ca^{2+} influx triggering transmitter release through these channels per action potential has a much greater influence on release during short stimulation pulses (Miller, 1987), but it declines rapidly as a consequence of a Ca^{2+}-dependent inactivation system. Inactivation of P-type channels may be enhanced by additional Ca^{2+} influx via nearby located VDCCs (for a review, see, e.g. Tareilus and Breer, 1995). During high-frequency or prolonged depolarizing stimuli, it can be anticipated that the synchronized opening of several VDCC subtypes (namely, P- and L-types) with different biophysical properties might occur, giving rise to spatiotemporal gradients in close proximity to the channels domains which trigger exocytosis. These conditions favour Ca^{2+}-dependent inactivation of P-type channels (e.g. Luebke et al., 1993) while increasing ω-agatoxin IVA-insensitive Ca^{2+} currents (probably of the L-type) (Hong et al., 1995). Our data clearly demonstrate that facilitation of [^{3}H]acetylcholine release, evoked by high-frequency (50 Hz) long-lasting (> 1 ms) depolarizing pulses, is mediated by increases in L-type Ca^{2+} influx which can act synergistically to Ca^{2+} recruitment from internal stores. According to the literature, the influx of Ca^{2+} through L-type channels requires strong depolarizations to become functionally apparent (Miller, 1987). This might happen because L-type channels are distributed away from the active zones (Tsien et al., 1988; Robitaille et al., 1990), are more resistant to inactivation in neurons, and possess a high conductance capacity (~ 25 pS) able to saturate intraterminal buffers which limit Ca^{2+} diffusion to the release apparatus (for a review, see, e.g. Tareilus and Breer, 1995). The pathophysiological significance of the L-type calcium currents triggering acetylcholine release from motor nerve terminals is difficult to demonstrate. However, L-type VDCCs may play a modulatory role (a) at developing neuromuscular junctions, when P-/Q-type channels are still functionally immature (Sugiura and Ko, 1997), (b) in reinnervating endplates after nerve damage (Katz et al., 1996), (c) during high-frequency motoneuron firing in recovery from resistive inspiratory syndromes (Road et al., 1995), and (d) in Lambert–Eaton myasthenic syndrome, where spared L-type currents can be activated by repetitive nerve stimulation or by agents (e.g. 4-aminopyridine) that prolong membrane depolarization (see, e.g. McEvoy 1994), to compensate for the downregulation of P-/Q-type channels (Garcia and Beam, 1996). Thus, besides its ontogenic importance, facilitation of Ca^{2+} influx through "quiescent" L-type VDCC might have a putative role in re-establishing neuromuscular transmission under pathological conditions.

In conclusion, the results indicate that the modulation of acetylcholine release, depending on the nerve stimulation paradigm, involves the regulation of Ca^{2+} recruitment from both internal (thapsigargin-sensitive) and/or external Ca^{2+} pools. The fine-tuning of the involvement of Ca^{2+} influx through either P-/L-type VDCCs on acetylcholine release, evoked by depolarizing pulses of variable frequency and duration, might be relevant to regulate synaptic efficacy in several pathophysiological conditions.

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