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Short communication

P-type Ca^{2+} channels trigger stimulus-evoked [^{3}H]acetylcholine release from mammalian motor endplates

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Abstract

In the present experiments it was tested whether ω-agatoxin-IVA, a peptide blocking P-type voltage-dependent Ca^{2+} channels, inhibits the evoked release of newly synthesized [^{3}H]acetylcholine from the rat phrenic nerve. Release of [^{3}H]acetylcholine was evoked by electrical stimulation of the isolated phrenic nerve (100 or 750 pulses at 5 Hz). ω-Agatoxin-IVA inhibited evoked [^{3}H]acetylcholine release in a concentration-related manner; inhibition started at a concentration of 30 nM with complete block occurring at 500 nM. In conclusion, the present experiments demonstrate that ω-agatoxin-IVA-sensitive P-type Ca^{2+} channels are critically involved in the regulation of stimulus-induced transmitter release at mammalian motor endplates.

Keywords: Motor endplate; ω-Agatoxin-IVA; Ca^{2+} channel, P-type; Acetylcholine release

1. Introduction

The influx of Ca^{2+} ions through voltage-dependent Ca^{2+} channels is the critical signal to trigger evoked transmitter release in central and peripheral neurones. Based on the sensitivity of specific toxins five different voltage-dependent Ca^{2+} channels have been discriminated in electrophysiological studies, the T-, L-, N-, P- and Q-channels (Tsien et al., 1988; Zhang et al., 1993; Olivera et al., 1994). N-type Ca^{2+} channels, which can be blocked by ω-conotoxin-GVIA, have been found to regulate stimulus-induced release of acetylcholine and of noradrenaline from central neurones and from neuroeffector junctions within the peripheral autonomic nervous system (Dooley et al., 1987; Wessler et al., 1990; Olivera et al., 1994). L-type Ca^{2+} channels are involved in the regulation of catecholamine release from chromaffin cells (Cena et al., 1983). In contrast, ω-conotoxin-GIVA or L-type Ca^{2+} channel inhibitors (nifedipine, verapamil) did not affect acetylcholine release from the motoneurones excluding a major regulatory role of N-type Ca^{2+} channels on stimulus-induced acetylcholine release at the motor endplates (Wessler et al., 1990).

In addition to the L- and N-type Ca^{2+} channels a third high-threshold voltage-dependent Ca^{2+} channel has been detected in the mammalian central nervous system. Ca^{2+} conductance mediated by these channels could be blocked by the purified fraction of the venom of the funnel-web spider *Agelenopsis aperta* whereas ω-conotoxin or nifedipine were ineffective at these P-type Ca^{2+} channels (Bertolino and Llinas, 1992; Olivera et al., 1994). The purified toxin and its synthetic analog have recently been found to inhibit evoked endplate potentials in the mouse diaphragm and presynaptic Ca^{2+} channels recorded from the nerves innervating the mouse levator auris muscle (Uchitel et al., 1992). So far, the chemical conterpart is lacking, demonstrating directly an inhibition by ω-agatoxin-IVA of transmitter release from the motoneurones. Therefore, in the present experiments it was tested whether ω-agatoxin IVA actually inhibits the electrically stimulated release of [^{3}H]acetylcholine from the rat phrenic nerve.

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2. Materials and methods

2.1. Tissue preparation and incubation protocol

The experimental protocol has been described in detail elsewhere (Wessler et al., 1986). In short, Spraque-Dawley rats of either sex were killed by stunning followed by exsanguination. Left hemidiaphragms were removed and small muscle strips (wet weight 25–30 mg) containing the endplate region together with the phrenic nerve were dissected and placed in 2 ml organ baths. The endplate preparations were superfused (2 ml/min) with oxygenated physiological salt solution of the following composition (given in mM): NaCl 125, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 24, D-glucose 11.2, choline 0.001. The medium was gassed with 5% CO_2 (v/v) in O_2. The pH of the gassed solution was 7.30. After a 30 min superfusion period the endplate preparations were incubated (40 min) with 0.37 MBq [^{3}H]choline to label neuronal transmitter stores. During the incubation period the phrenic nerve was electrically stimulated (square wave pulses of 0.2 ms duration, current strength 8 mA, 1 Hz). After a subsequent washout period (60 min) and the addition of 10 μM hemicholinium-3 (to prevent re-uptake of choline) tritium efflux from the organ bath was measured in 3 min intervals. Release of [^{3}H]acetylcholine was elicited by two periods (S_1, S_2) of electrical nerve stimulation; S_1 and S_2 started 12 and 72 min after the end of the washout period, respectively. During both stimulation periods 100 pulses (or 750 pulses, see Fig. 1B) were applied at 5 Hz. The current flow was determined by the voltage drop across a resistance measured by an oscilloscope. In the respective experiments ω-agatoxin-IVA was added 45 min before the second stimulation period.

2.2. Calculations, statistical analysis and special chemicals

Tritium outflow is expressed as dpm g^{-1} 3 min^{-1}. Stimulated tritium outflow was calculated by subtracting the basal from the total tritium outflow (Wessler et al., 1986). Results are given as means ± S.E. The ratios S_2/S_1, i.e. the tritium outflow evoked by S_2 related to that evoked by S_1 were calculated and the respective ratios obtained in the presence and absence of ω-agatoxin-IVA were compared. The significance of dif-

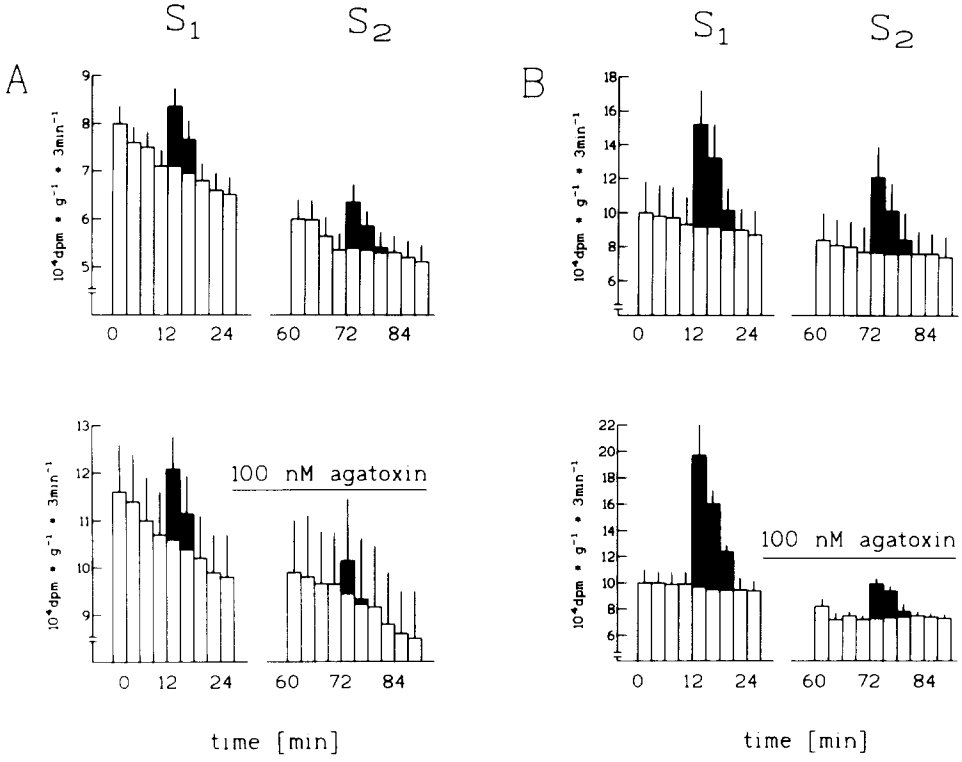

Fig. 1. Effect of ω-agatoxin-IVA on the basal and stimulated tritium outflow from endplate preparations of the rat left hemidiaphragm. After the incubation with [^{3}H]choline and the subsequent washout tritium outflow was measured in 3 min intervals. Tritium outflow was stimulated by two periods (S_1, S_2) of electrical nerve stimulation, 100 (A) or 750 pulses (B) were applied at 5 Hz during both stimulation periods. ω-Agatoxin-IVA (100 nM) was added 45 min before S_2 (indicated). The samples collected between 27 and 60 min of incubation were discarded. Under control conditions ratios S_2/S_1 of 0.83 ± 0.06 (n = 12) and 0.61 ± 0.07 (n = 4) were obtained with 100 and 750 pulses, respectively. In the presence of 100 nM ω-agatoxin-IVA ratios S_2/S_1 declined to 0.40 ± 0.07 (n = 5; 100 pulses) and 0.28 ± 0.02 (n = 2). The filled parts of the columns represent the stimulated release of [^{3}H]acetylcholine.

ferences was evaluated by Student's t-test (unpaired and paired data); for multiple comparison the modified t-test according to Bonferroni was used (Wallenstein et al., 1980). P values < 0.05 were regarded as significant. It had already been demonstrated that the basal tritium outflow consists of [^{3}H]phosphorylcholine and [^{3}H]choline, whereas the electrically evoked tritium outflow consists exclusively of [^{3}H]acetylcholine (Wessler, 1992). Keeping this in mind the specific tritium signal is high, because the basal release is zero. Therefore, the stimulated increase in tritium outflow represents a useful and highly significant signal ($P < 0.01$, paired t-test) to estimate the evoked release of [^{3}H]acetylcholine, although in absolute amounts (dpm g^{-1} 3 min^{-1}) the ratio between the basal and evoked tritium efflux is poor.

The following chemicals were used: hemicholinium-3 bromide (Sigma Chemie); [*methyl*-^{3}H]choline (80 Ci/mmol; NEN, Germany); synthetic ω-agatoxin-IVA (Peptide Inst., Osaka, Japan).

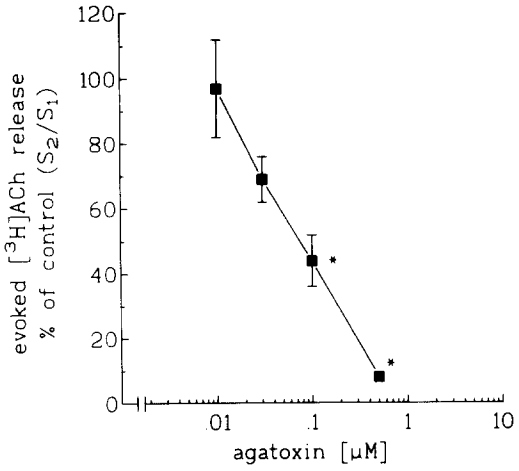

Fig. 2. Concentration-response curve of the inhibitory effect of ω-agatoxin-IVA on the release of [^{3}H]acetylcholine from the isolated rat phrenic nerve. The experimental protocol is shown in Fig. 1A. Given are the means ± S.E. of 4–5 experiments for each individual concentration. Significance of differences from the control: $^*P < 0.01$.

3. Results

Fig. 1 shows the basal and stimulated tritium outflow obtained from [^{3}H]choline-labelled endplate preparations. It had already been demonstrated that the electrically stimulated increase in tritium outflow reflects exclusively the Ca^{2+}-dependent release of [^{3}H]acetylcholine from the nerve terminals (Wessler et al., 1986). Under control conditions both periods of electrical nerve stimulation (S_1, S_2) caused a comparable increase in tritium efflux (Fig. 1, upper part; ratio S_2/S_1: 0.83 ± 0.06, for experiments with 100 pulses ($n = 12$) and ratio S_2/S_1: 0.61 ± 0.07 for experiments with 750 pulses ($n = 4$)). ω-Agatoxin-IVA inhibited the stimulated increase in tritium outflow very effectively. For example, 100 nM ω-agatoxin-IVA added 45 min before S_2 reduced the stimulated increase in tritium outflow by about 50% irrespective of the number of pulses applied (Fig. 1). A concentration of 500 nM ω-agatoxin-IVA abolished the tritium outflow evoked by 100 pulses (see Fig. 2). The complete concentration-response curve (stimulation with 100 pulses) is shown in Fig. 2, the IC_{50} value obtained for the inhibitory, presynaptic effect was 75 ± 25 nM.

4. Discussion

Electrophysiological and release studies have shown that different types of voltage-dependent Ca^{2+} channels are involved in regulating transmitter release from different neurones. For example, evoked release of acetylcholine and noradrenaline from central neurones and neuroeffector junctions within the peripheral autonomic system are dominantly controlled by N-type Ca^{2+} channels, whereas catecholamine release from chromaffin cells and substance P release from dorsal root ganglia are strongly inhibited by dihydropyridines (Cena et al., 1983; Cazalis et al., 1987). Using the same technique as in the present experiments it has already been demonstrated that neither ω-conotoxin-GVIA nor high concentrations of nifedipine or verapamil inhibited evoked [^{3}H]acetylcholine release from the phrenic nerve at a physiological extracellular Ca^{2+} concentration (Wessler et al., 1990). Thus, N- or L-type Ca^{2+} channels are not essential to trigger evoked transmitter release from motoneurones (see also Olivera et al., 1985; Atchison, 1989). In the present experiments, however, the stimulated release of [^{3}H]acetylcholine was inhibited and even completely suppressed by ω-agatoxin-IVA indicating P-type Ca^{2+} channels to be critically involved in regulating the release of acetylcholine from the motoneurones. This conclusion is substantiated by the concentration-related effect observed with ω-agatoxin-IVA and is in line with the respective electrophysiological experiments showing a suppression of evoked endplate potentials by the toxin (Uchitel et al., 1992). In a Ca^{2+} reduced medium (0.9 mM) ω-conotoxin-GVIA slightly reduced [^{3}H]acetylcholine release (Wessler et al., 1990) which suggests the existence of N-type Ca^{2+} channels at motor nerve terminals. The N-type Ca^{2+} channels, however, are not involved in the regulation of exocytosis at physiological Ca^{2+} concentrations.

In the present experiments ω-agatoxin-IVA suppressed evoked transmitter release very potently; rather low concentrations of ω-agatoxin-IVA (30–500 nM) were already effective in inhibiting acetylcholine release from the phrenic nerve terminals. In contrast,

rather high concentrations of ω-agatoxin-IVA (0.2–1000 μM) were required to reduce glutamate release from hippocampal neurones or Ca^{2+} uptake in cortical neurones and the maximal effect did not exceed 50% inhibition (Uchitel et al., 1992; Burke et al., 1993). This difference may indicate that in the central neurones additional types of Ca^{2+} channels are functionally important and closely involved in the process of transmitter exocytosis whereas at the motor endplate P-type Ca^{2+} channels play an exclusive role in triggering stimulus-induced release of acetylcholine. In one study, however, ω-agatoxin-IVA suppressed the potassium-induced Ca^{2+} uptake by rat neocortical slices with a similar potency as observed in the present study (Geer et al., 1993). The high efficacy of ω-agatoxin-IVA to block transmitter release at motor endplates raises the possibility of developing selective P-type Ca^{2+} channel modulators. Whether P-type Ca^{2+} channel blockers can be developed as useful drugs to block neuromuscular transmission for general anesthesia or to reduce enhanced skeletal muscle tone depends on the possibility to find drugs which do not penetrate the blood-brain barrier but invade to and also escape from the motor endplate rapidly.

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