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Calcium channels involved in the inhibition of acetylcholine release by presynaptic muscarinic receptors in rat striatum

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1 The mechanism of the inhibitory action of presynaptic muscarinic receptors on the release of acetylcholine from striatal cholinergic neurons is not known. We investigated how the electrically stimulated release of [^{3}H]-acetylcholine from superfused rat striatal slices and its inhibition by carbachol are affected by specific inhibitors of voltage-operated calcium channels of the L-type (nifedipine), N-type (ω-conotoxin GVIA) and P/Q-type (ω-agatoxin IVA).

2 The evoked release of [^{3}H]-acetylcholine was not diminished by nifedipine but was lowered by ω-conotoxin GVIA and by ω-agatoxin IVA, indicating that both the N- and the P/Q-type (but not the L-type) channels are involved in the release. The N-type channels were responsible for approximately two thirds of the release. The release was $>97\%$ blocked when both ω-toxins acted together.

3 The inhibition of [^{3}H]-acetylcholine release by carbachol was not substantially affected by the blockade of the L- or P/Q-type channels. It was diminished but not eliminated by the blockade of the N-type channels.

4 In experiments on slices in which cholinesterases had been inhibited by paraoxon, inhibition of [^{3}H]-acetylcholine release by endogenous acetylcholine accumulating in the tissue could be demonstrated by the enhancement of the release after the addition of atropine. The inhibition was higher in slices with functional N-type than with functional P/Q-type channels.

5 We conclude that both the N- and the P/Q-type calcium channels contribute to the stimulation-evoked release of acetylcholine in rat striatum, that the quantitative contribution of the N-type channels is higher, and that the inhibitory muscarinic receptors are more closely coupled with the N-type than with the P/Q-type calcium channels.

Keywords: Calcium channels; acetylcholine release; conotoxins; agatoxin; nifedipine; presynaptic receptors; muscarinic receptors; cholinergic neurons; striatum

Introduction

A number of G protein-coupled receptors located on presynaptic nerve terminals are known to inhibit the release of neurotransmitters, and it is generally believed that they act mainly by diminishing the opening of voltage-operated calcium channels in the terminals, although evidence is also available for more direct ('post-calcium-entry') effects on the release apparatus (reviews Wu & Saggau, 1997; Miller, 1998; Haydon & Trudeau, 1998). The interaction between the receptors and the relevant calcium channels is mediated by the β subunits of the G proteins (Ikeda, 1996; Herlitze *et al*., 1996; Delmas *et al*., 1998; review Dolphin 1998). Six types of voltage-operated calcium channels (L, N, P, Q, R, T) have been described in neurons (Randall & Tsien, 1995; reviews Olivera *et al*., 1994; Dunlap *et al*., 1995), but most observations indicate that only three of them (N, P, Q) are involved in the nerve impulse-evoked neurotransmitter release.

It is not yet possible to draw a simple and definite picture of the relative roles of individual channel types in the release of various neurotransmitters from various neurons, and in the receptor-mediated regulation of the release. Remarkable differences have been found between different neurons (reviews Dunlap *et al*., 1995; Miller, 1998). The N-type channels are mainly if not exclusively responsible for calcium influx initiating neurotransmitter release from sympathetic neurons (Boehm & Huck, 1996; Smith & Cunnane, 1997), but they play no role in motor nerve terminals (Wessler *et al*., 1990; Katz *et al*., 1997). Within the cerebrum and cerebellum, both the N-type and the P- and Q-type calcium channels are involved in evoked neurotransmitter release (review Olivera *et al*., 1994), with indications that the P- or Q-type channels are more closely coupled to neurotransmitter exocytosis (Luebke *et al*., 1993; Cousin *et al*., 1997; Doughty *et al*., 1998; Wu *et al*., 1999; but see Stanley, 1997).

Some observations suggest that the role of individual types of calcium channels is different in neurons secreting different neurotransmitters; e.g., Turner *et al*. (1993) concluded that the P-channels alone are responsible for the release of glutamate from striatal synaptosomes, whereas both the P- and the N-channels play a role in the release of dopamine. On a similar vein, Cunningham *et al*. (1998) found that, in the ileum, the release of noradrenaline is mediated by the N-type channels, whereas that of acetylcholine depends on both the N- and the P-channel types. Different types of channels are probably responsible for the release of the excitatory and the inhibitory neurotransmitters in the cerebellum (Doroshenko *et al*., 1997).

Variable answers have been also obtained to the question of which channel type is responsible for the modulation of neurotransmitter release by presynaptically located G protein-coupled receptors. We are interested in the control of the release of acetylcholine from striatal cholinergic neurons (Doležal & Tuček, 1990; Doležal & Wecker, 1990), and we have shown recently that the M_4 subtype of muscarinic receptors is mainly responsible for the autoinhibition of acetylcholine release from these neurons (Doležal & Tuček, 1998). In the present work, we used electrically stimulated

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superfused slices of rat striatum and measured (1) how the evoked release of acetylcholine is affected by various antagonists of calcium channels, and (2) how various antagonists of calcium channels interfere with the inhibitory effect of carbachol on acetylcholine release. In a third group of experiments, slices were pretreated with an irreversible inhibitor of cholinesterases. In these experiments, the evoked release of acetylcholine became inhibited by the endogenous acetylcholine accumulating in the intercellular fluid, and the inhibition of the release could be overcome by the addition of atropine, and evaluated according to the atropine-induced enhancement of the release. The data which we obtained suggest that the N-type calcium channels play a dominant role both in the stimulation-evoked release of acetylcholine from striatal cholinergic neurons, and in its modulation by presynaptic muscarinic receptors.

Methods

Arrangement of experiments

Experiments were performed on single superfused slices prepared from striata of 2–3-month-old male rats. The arrangement of experiments (i.e., the preparation and handling of the slices, the superfusion system, the stimulation parameters and the determination of the release of [^{3}H]-acetylcholine) was as described by Doležal & Tuček (1998).

In brief, slices were prepared by McIlwain's tissue chopper set at 0.35 mm slice thickness, and were loaded with [^{3}H]-choline (19–32 nM) during a preincubation lasting 30 min at 37°C. Where specifically mentioned, 50 μM paraoxon (an irreversible inhibitor of cholinesterases) was also present in the incubation medium during the preincubation with [^{3}H]-choline. The preincubation with [^{3}H]-choline was finished by washing off the labelled compound. In some experiments, it was followed by another 30 min preincubation period with one of two toxins (100 nM ω-conotoxin GVIA or 300 nM ω-agatoxin IVA) known for their ability to irreversibly block specific populations of voltage-operated calcium channels. Hemicholinium-3 (10 μM) was added to the superfusion fluid at the end of the preincubation with [^{3}H]-choline, to prevent the reuptake of [^{3}H]-choline arising from the hydrolysis of the released [^{3}H]-acetylcholine.

Individual slices were placed in superfusion chambers and superfused (0.5 ml min^{-1}) with a medium consisting of (mM) NaCl, 123; KCl, 3; $CaCl_2$, 1.3; $MgSO_4$, 1; NaH_2PO_4, 1.2; $NaHCO_3$, 25 and glucose, 10, and saturated with 95% O_2/5% CO_2. After 1 h, the superfusate started to be collected in 4 min fractions, and the fractions were designated as F1 (0–4 min), F2 (4–8 min) etc. until F12. In the standard experimental arrangement, the two-stimulation S_2/S_1 paradigm was applied and the slices were electrically stimulated twice for 20 s, at the beginning of the third and the ninth collection periods. The stimulation was by 60 unipolar square pulses lasting 2 ms at a frequency of 3 Hz, with a voltage gradient of 10 V across the inter-electrode distance of 5 mm. The radioactivity released by stimulation was taken to correspond to [^{3}H]-acetylcholine and was expressed as fractional release, i.e. as per cent of the total radioactivity present in the slice at the start of the first or the second stimulation. The stimulated release of radioactivity was computed as the difference between the fractional release of radioactivity in (F3 + F4) and that in (F2 + F5) (for S_1, i.e. the effect of the first stimulation), or between the fractional release of radioactivity in (F9 + F10) and that in (F8 + F11) (for S_2, i.e. the effect of the second stimulation). Drugs to be evaluated for their effect on the release of [^{3}H]-acetylcholine evoked by the second stimulation were added to the superfusion medium 12 min before the start of the second stimulation.

Source of materials

ω-Conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC were from Alomone Laboratories (Jerusalem), nifedipine was from Sigma (St. Louis, MO, U.S.A.).

Statistical evaluation

Data are expressed as means ± s.e.mean. Statistical significance of differences was evaluated by ANOVA followed by Dunnett's test, or by two-tailed *t*-test.

Results

Experiments with atropine, haloperidol and domperidone

Since the striatum is richly innervated by dopaminergic nerve fibres, the evoked release of acetylcholine from the nerve terminals of striatal cholinergic neurons might be inhibited not only by the released acetylcholine itself (acting *via* the presynaptic muscarinic autoreceptors), but also by dopamine (acting *via* the presynaptic D_2 dopamine receptors). We wanted to know whether, under the conditions of our experiments, an accumulation of endogenous acetylcholine or dopamine in the intercellular fluid has any effect on the stimulation-evoked release of [^{3}H]-acetylcholine. Figure 1 shows that the evoked release of [^{3}H]-acetylcholine was not altered by 1 μM atropine (blocking the muscarinic receptors), 1 or 10 μM haloperidol (blocking the D_1 and D_2 dopamine receptors), and 1 μM domperidone (blocking the D_2 dopamine receptors). Apparently, the concentrations of acetylcholine and dopamine which accumulated in the superfused tissue during stimulation were sufficiently low under the conditions of our experiments so that they did not measurably affect [^{3}H]-acetylcholine release.

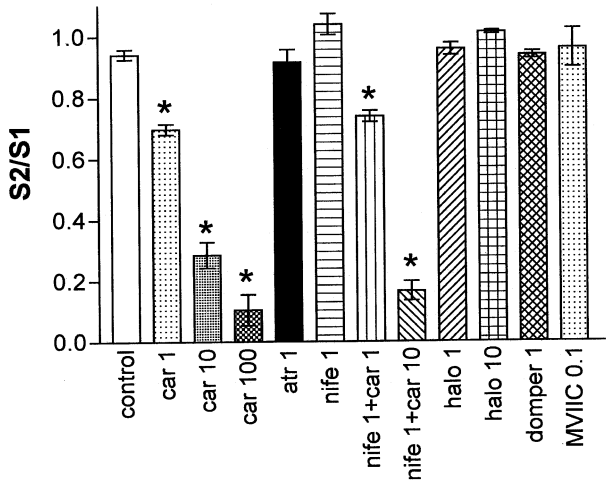

Figure 1 Effect of drugs on the stimulation-induced release of [^{3}H]-acetylcholine. The two-stimulation (S_2/S_1) paradigm was used. Columns from the left to the right: control; carbachol 1 μM; carbachol 10 μM; carbachol 100 μM; atropine 1 μM; nifedipine 1 μM; nifedipine 1 μM with carbachol 1 μM; nifedipine 1 μM with carbachol 10 μM; haloperidol 1 μM; haloperidol 10 μM; domperidone 1 μM; and ω-conotoxin MVIIC 0.1 μM. Ordinate: S_2/S_1 ratio. Data are means (± s.e.mean) of measurements on 3–26 slices. * = significantly different ($P<0.05$) from control.

Experiments with nifedipine, ω-conotoxin MVIIC and carbachol

Nifedipine (1 μM; blocking the L-type calcium channels) and ω-conotoxin MVIIC (100 nM; with a complex action on calcium channels – see Discussion) had no effect on the evoked release of [^{3}H]-acetylcholine (Figure 1). The release was strongly diminished by carbachol, known to act *via* the muscarinic receptors. The inhibitory effect of 10 μM carbachol was slightly stronger when nifedipine was present simultaneously, but the difference was not significant statistically (70% inhibition without and 82% inhibition with nifedipine; Figure 1).

Experiments with ω-conotoxin GVIA, ω-agatoxin IVA and carbachol

The stimulated release of [^{3}H]-acetylcholine was diminished by 44% in slices preincubated with 300 nM ω-agatoxin IVA and by 75% in slices preincubated with 100 nM ω-conotoxin GVIA. The inhibitory effects of the two toxins on the release of [^{3}H]-acetylcholine were additive. In slices preincubated with both toxins, the release of [^{3}H]-acetylcholine was diminished by more than 97% (Figure 2).

The inhibitory effect of 10 μM carbachol was unchanged in slices pretreated with ω-agatoxin IVA (73% inhibition, compared to 70% in controls), but it was diminished in slices pretreated with ω-conotoxin GVIA (50% inhibition, compared to 70% in controls).

Experiments on slices pretreated with paraoxon

Paraoxon inhibits cholinesterases and brings about an accumulation of acetylcholine in the tissue. The accumulated extracellular acetylcholine is expected to inhibit the stimulated release from the nerve terminals. In correspondence with this expectation, the stimulated fractional release of ^{3}H was diminished from 1.80% in control slices (see Figure 2) to 0.35% in the slices pretreated with paraoxon (control in Figure 4), i.e. by 81%. In slices pretreated with ω-agatoxin IVA, fractional release was lowered from 1.01 to 0.28% after the exposure to paraoxon, i.e. by 72%, and in slices pretreated with ω-conotoxin GVIA, fractional release diminished from 0.45% without to 0.11% with paraoxon, i.e. by 76% (compare corresponding columns in Figures 2 and 4).

The inhibitory effect of the accumulated acetylcholine can be removed by atropine (Figure 5). At a concentration of 1 μM, atropine augmented the stimulated release of [^{3}H]-acetylcholine from the slices pretreated with paraoxon 4.2 fold. Atropine-induced enhancement of the release of [^{3}H]-acetylcholine from slices pretreated with paraoxon and ω-agatoxin IVA (6.1 fold) was significantly higher ($P<0.01$) than that from slices pretreated with paraoxon and ω-conotoxin GVIA (2.8 fold; Figure 5).

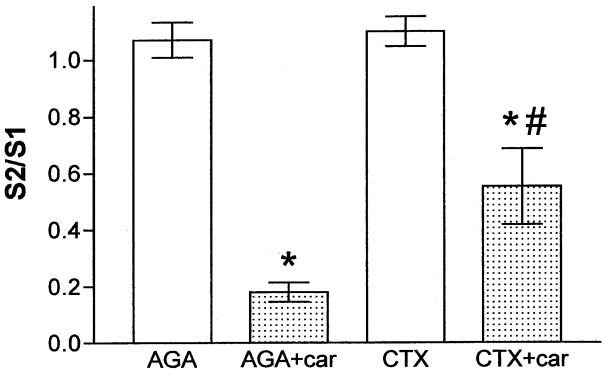

Figure 3 Effect of carbachol on the stimulated release of [^{3}H]-acetylcholine from slices pretreated with ω-agatoxin IVA (AGA) and ω-conotoxin GVIA (CTX). The first stimulation (S_1) was on superfused slices which had been preincubated for 30 min with 300 nM AGA or 100 nM CTX. The second stimulation (S_2) was on the same slices in the same medium without any further addition (columns denoted as AGA or CTX), or with the addition of 10 μM carbachol (columns denoted as AGA + car or CTX + car). Ordinate: S_2/S_1 ratio. Data are means (± s.e.mean) of measurements on six slices. * = significantly different from corresponding control without carbachol ($P<0.01$). # = significantly different ($P<0.01$) from AGA + CAR.

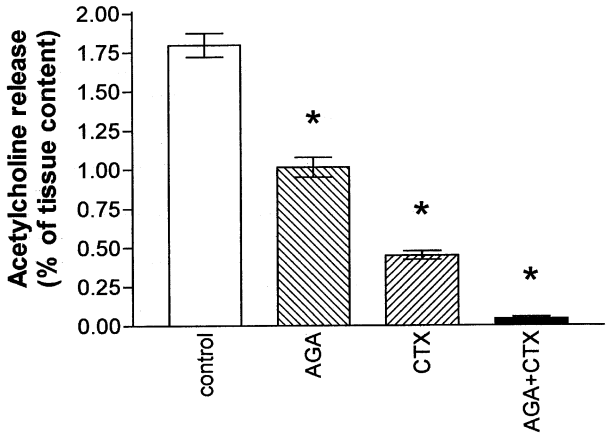

Figure 2 Stimulated release of [^{3}H]-acetylcholine from slices pretreated with toxins. Columns from the left to the right: control; ω-agatoxin IVA (AGA; 300 nM for 30 min), ω-conotoxin GVIA (CTX; 100 nM for 30 min); both toxins (CTX + AGA). Ordinate: Stimulated release of radioactivity expressed as per cent of total radioactivity present in the slice. Data are means (± s.e.mean) of measurements on 6–40 slices. * = significantly different ($P<0.01$) from control.

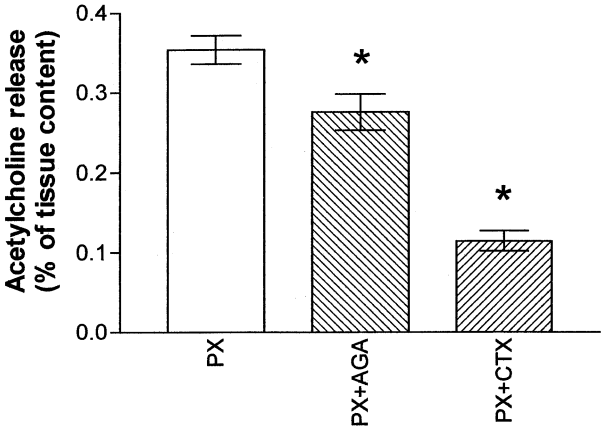

Figure 4 Effects of ω-conotoxin GVIA and ω-agatoxin IVA on the stimulated release of [^{3}H]-acetylcholine from slices pretreated with paraoxon. Columns from the left to the right: slices pretreated with paraoxon (50 μM for 30 min; PX); slices pretreated with paraoxon and ω-agatoxin IVA (300 nM for 30 min; PX + AGA); slices pretreated with paraoxon and ω-conotoxin GVIA (100 nM for 30 min; PX + CTX); Ordinate: Stimulated release of radioactivity, expressed as per cent of total radioactivity in the slices. Data are means (± s.e.mean) of measurements on 11–23 slices. * = significantly different from PX ($P<0.05$).

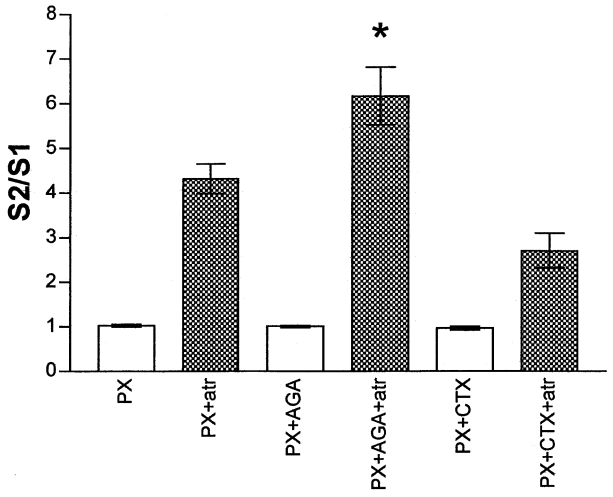

Figure 5 Effects of 1 μM atropine (atr) on the stimulated release of [^{3}H]-acetylcholine from slices preincubated with paraoxon (PX) alone, or with paraoxon in combination with ω-agatoxin IVA (AGA) or ω-conotoxin GVIA (CTX). Preincubations lasted 30 min and the applied concentrations were 50 μM for paraoxon, 100 nM for CTX, and 300 nM for AGA. Ordinate: S_2/S_1 ratios. Data are means ($\pm$s.e.mean) of measurements on 5–6 slices. The 6.1 fold increase in the release induced by atropine from slices pretreated with PX + AGA was significantly higher ($^*P<0.05$) than the 4.2 fold and 2.8 fold increases from slices pretreated with PX or PX + CTX, respectively.

Discussion

We used three antagonists which are highly specific in their action on individual types of calcium channels: the dihydropyridine compound nifedipine, inhibiting the L-type (review Kazda & Knorr, 1990), and the peptides ω-conotoxin GVIA and ω-agatoxin IVA, inhibiting the N-type and the P- and Q-types of calcium channels, respectively (reviews Miljanich & Ramachandran, 1995; Tareilus & Breer, 1995; Uchitel, 1997). In addition, we used 100 nM ω-conotoxin MVIIC, which is less selective in its inhibitory action on high-voltage activated calcium channels (Olivera *et al*., 1994; McDonough *et al*., 1996). ω-Conotoxin MVIIC was found to bind to specific sites in brain membranes with extremely high affinity (1 nM and less; Hillyard *et al*., 1992; Adams *et al*., 1993) and was proposed to be an antagonist of putative O channels (Adams *et al*., 1993). In experiments on cDNA-injected oocytes (Sather *et al*., 1992), low concentrations (150 nM) of ω-conotoxin MVIIC inhibited specific calcium channels which were subsequently designated as the Q-type of channels (Randall & Tsien, 1995). The negative result which we obtained with 100 nM ω-conotoxin MVIIC (Figure 1) indicates that the high-affinity target for the binding of this toxin (the putative O channels – Adams *et al*., 1993) is not important for acetylcholine release.

Striatal nuclei are rich in dopaminergic nerve terminals, and we were concerned with the possibility that the stimulated release of acetylcholine measured in our experiments on striatal slices might be influenced by the release of endogenous dopamine, and by the inhibitory action which dopamine exerts on acetylcholine release *via* the D_2 dopamine receptors (Drukarch *et al*., 1990; Doležal *et al*., 1992). For this reason, we checked in preliminary experiments whether the evoked release of acetylcholine is influenced by haloperidol (a non-selective antagonist of dopamine receptors) or domperidone (a D_2-selective antagonist of dopamine receptors) in our experimental paradigm. The answer we obtained (Figure 1) was negative, indicating that the released dopamine did not have a measurable influence of the release of acetylcholine under the conditions of our experiments.

The finding that 1 μM nifedipine had no effect on the stimulated release of acetylcholine (Figure 1) was in line with other data indicating that the L-type calcium channels are of low importance for the evoked release of neurotransmitters (Doležal & Tuček, 1987; Tarelius & Breer, 1995; Cousin *et al*., 1997) – except in situations in which their role becomes enhanced by special treatments (e.g., by 4-aminopyridine – see Doležal *et al*., 1996). On the other hand, the effects of ω-toxins (Figure 2) indicated that both the ω-agatoxin IVA-sensitive and the ω-conotoxin GVIA-sensitive channels are important for the stimulated release of acetylcholine. It has been demonstrated previously that the two toxins are very selective in their targets, with no overlap and excellent additivity of their actions (Mintz *et al*., 1992; Adams *et al*., 1993; Wu & Saggau, 1994a; Randall & Tsien, 1995; Yan & Surmeier, 1996). In agreement with this, the evoked release of acetylcholine was virtually completely arrested in slices pre-exposed to both toxins. Data in Figure 2 suggest that the N-type channels were responsible for approximately 2/3 and the P/Q-type channels for approximately 1/3 of stimulated acetylcholine release. Although it has been reported that ω-conotoxin GVIA also inhibits a subpopulation of the L-type channels, its action on these channels is reversible (in contrast to that on the N-channels) (Williams *et al*., 1992; Dolphin, 1995) and therefore unlikely to occur long after the washout of the toxin.

We cannot reliably distinguish between the contributions of the P- and Q-types of channels because our experiments were performed on slices that had been preincubated with a single high concentration of ω-agatoxin IVA and then superfused in the absence of the toxin. It seems more likely that the P-type channels were the main target of ω-agatoxin IVA in view of the fact that, after the wash-out of the toxin, the block of the Q-type channels fades away much faster than the block of the P-type channels (Randall & Tsien, 1995; see also Mintz *et al*., 1992; Protti & Uchitel, 1993), and also in view of the total absence of any effect of 100 nM ω-conotoxin MVIIC (Figure 1), which had been described to produce half-maximum block of the Q-type channels at a concentration of <150 nM (Sather *et al*., 1993).

The release of acetylcholine was strongly diminished in the presence of carbachol (Figure 1), which inhibits the release by a membrane-delimited pathway involving muscarinic M_2/M_4 receptors and Gi/Go proteins (see Doležal *et al*., 1989; Doležal & Tuček, 1998). The inhibition by carbachol was not diminished in the presence of 1 μM nifedipine (Figure 1), which was expected because the L-type channels do not play any role in acetylcholine release. The inhibition by carbachol was also fully preserved in slices pretreated with ω-agatoxin IVA, in which only the N-type channels were functional (Figure 3). This indicates that there is firm coupling between the inhibitory muscarinic receptors and the N-type calcium channels. On the other hand, the inhibitory effect of carbachol was smaller in slices pretreated with ω-conotoxin GVIA, suggesting looser coupling between muscarinic receptors and the P/Q-type channels.

It is tempting to associate our finding of the major role of the N-type channels in the inhibition of acetylcholine release with observations of specific direct links between the N-type calcium channels and the components of the exocytotic release apparatus in neurons (Leveque *et al*., 1994; Mochida *et al*., 1996; review Stanley, 1997). It seems relevant to note that the N-type channels are selectively inhibited by the D_2 dopamine receptors in the cholinergic neurons of the striatum (Yan *et al*.,

1997), and that in the hippocampus, the adenosine A_1 (Wu & Saggau, 1994b) and muscarinic receptors (Qian & Saggau, 1997) inhibit the release of an excitatory neurotransmitter by selectively modulating the N- (rather than the P/Q-) type of calcium channels. The N-type calcium channels are alone responsible for the regulation of the release of noradrenaline from sympathetic neurons by several presynaptic receptors (Boehm & Huck, 1996; Doležal *et al*., 1996; Koh & Hille, 1997).

In a set of experiments performed on slices pretreated with paraoxon (Figures 4 and 5), we chose to utilize the endogenously accumulating acetylcholine (after the inhibition of cholinesterases) as the agonist for muscarinic receptor activation. Evoked fractional release of [^{3}H]-acetylcholine was 5.1 fold diminished after paraoxon pretreatment, but it could be 4.2 fold enhanced by atropine. The fact that atropine (expected to completely block the presynaptic autoinhibition of acetylcholine release) did not fully restore the extent of the release, may be explained on the assumption that the intraterminal [^{3}H]-acetylcholine (the release of which was measured) was more diluted with unlabelled acetycholine in paraoxon-treated than in control slices, so that a part of the observed 'inhibition' of release was not real. Paraoxon produced similar changes in slices pre-exposed to ω-agatoxin IVA (3.7 fold lower fractional release, compared to experiments without paraoxon) or to ω-conotoxin GVIA (3.9 fold lower fractional release, compared to experiments without paraxon).

Two features seem important in data showing the effects of atropine on the release of [^{3}H]-acetylcholine from slices pretreated with paraoxon plus ω-toxins (Figure 5). (1) Muscarinic inhibition of the release (as revealed by the atropine-induced enhancement) was preserved both in the slices in which the P/Q channels had been blocked by ω-agatoxin IVA, and in the slices in which the N channels had been blocked by ω-conotoxin GVIA. (2) The relative inhibition of the release diminished in the order (slices with functional N-type channels) > (slices not exposed to any calcium channel antagonist) > (slices with functional P/Q-type channels). It appears that muscarinic receptors have the best chance to inhibit the release of acetylcholine in the slices in which the P/Q channels had been obstructed by ω-agatoxin IVA and in which the release depends solely on calcium influx *via* the N-type channels. This agrees with the results of experiments performed without an inhibitor of cholinesterases and supports the idea of a preferential role of the N-type channels in the muscarinic modulation of acetylcholine release from striatal cholinergic neurons.

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