The Journal of Neuroscience, July 1, 1996, *16*(13):4155–4161

Multiple Subtypes of Voltage-Gated Calcium Channel Mediate Transmitter Release from Parasympathetic Neurons in the Mouse Bladder

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Multiple subtypes of voltage-gated calcium channels are coupled to transmitter release from central neurons; however, only N-type channels have been shown to play a role in autonomic neurons. The aim of the present study was to investigate potential roles for other channel subtypes in transmitter release from parasympathetic neurons in the mouse bladder using calcium channel toxins alone and in combination. Transmitter release was measured indirectly by recording the contraction of bladder dome strips in response to electrical stimulation of the neurons by single pulses or trains of 20 pulses at 1–50 Hz. ω-Conotoxin-GVIA (GVIA) and ω-conotoxin-MVIIC (MVIIC) inhibited contractions in a concentration-dependent manner, with IC_{50} values of ~30 and 200 nM, respectively, at low stimulation frequencies. ω-Agatoxin-IVA (agatoxin) alone did not have any significant effect up to 300 nM. Cumulative addition of the toxins demonstrated that 300 nM agatoxin had a significant effect after N-type channels were blocked with 100 nM GVIA. MVIIC (3 μM) reduced the contraction amplitude further. Testing the toxins on the cholinergic or purinergic component of the contraction separately showed that acetylcholine release depends primarily on N-type channels and, to a lesser extent, on P- and Q-type channels, whereas ATP release involves predominantly P- and Q-type channels. In conclusion, parasympathetic neurons in the mouse bladder, like central neurons, use multiple calcium channel subtypes. Furthermore, the release of the two main transmitters in these neurons has differing dependencies on the calcium channel subtypes.

Key words: acetylcholine; ATP; agatoxin; autonomic; urinary bladder; conotoxin; parasympathetic; voltage-gated calcium channels

Calcium influx into the nerve terminal through voltage-gated channels is essential for neurotransmitter release. In recent years, a combination of pharmacological, electrophysiological, and molecular biological studies has identified numerous subtypes of voltage-gated calcium channels (VGCCs). N-type channels are blocked by ω-conotoxin-GVIA (GVIA). P-type channels are blocked by low concentrations of ω-agatoxin-IVA (agatoxin) and by ω-conotoxin-MVIIC (MVIIC) (Hillyard et al., 1992; Mintz et al., 1992). Q-type channels are blocked by MVIIC and by higher concentrations of agatoxin (Wheeler et al., 1994). Another subtype of VGCC that is resistant to GVIA, agatoxin, and MVIIC has been called the R-type channel. This is likely to represent a heterogeneous population of channels, some of which are sensitive to ω-grammotoxin-SIA (Piser et al., 1995; Turner et al., 1995).

Recently, studies in the CNS have demonstrated that multiple subtypes of VGCCs are colocalized in nerve terminals, where they act synergistically to cause transmitter release (Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994). However, studies on transmitter release from autonomic neurons have found that N-type channels play a major role, with no evidence for a role of P- or Q-type channels (Lundy and Frew, 1993, 1994; Boot, 1994). Nevertheless, in many cases, transmitter release is not entirely blocked by GVIA, suggesting the involvement of other calcium channel subtypes.

The detrusor muscle of the bladder dome is innervated by postganglionic parasympathetic neurons that release acetylcholine and ATP, producing contraction via muscarinic and P_{2X} purinoceptors, respectively (Hoyle and Burnstock, 1993). These transmitters are assumed to be released from the same neurons. Previous studies have demonstrated that the combined contraction produced by acetylcholine and ATP is inhibited by up to 50, 75, and 88% by GVIA in the rat, guinea-pig, and rabbit bladder, respectively (Maggi et al., 1988; De Luca et al., 1990; Maggi, 1991; Zygmunt et al., 1993; Lundy and Frew, 1994). The combined contraction was found to be more sensitive to GVIA than the purinergic contraction (in the presence of atropine to block muscarinic receptors) (Maggi, 1991; Zygmunt et al., 1993). It was thus concluded that acetylcholine release depends more on N-type channels than ATP release. A single study has examined the effect of agatoxin on rat bladder, but found no effect at a concentration of 100 nM (Lundy and Frew, 1994). It was concluded therefore that P-type VGCCs do not play a role in transmitter release in the rat bladder. The nature of the VGCCs mediating GVIA-resistant transmitter release in the bladder therefore is unknown.

The aim of the present study was to investigate systematically the effects of GVIA, agatoxin, and MVIIC on the combined contraction of the mouse bladder produced by acetylcholine and ATP and on the separate cholinergic and purinergic components to determine (1) whether P- and/or Q-type VGCCs are involved in transmitter release, in addition to the well-established role of N-type VGCCs, and (2) whether the release of each transmitter depends to the same extent on the different VGCC subtypes.

Received Nov. 15, 1995; revised April 18, 1996; accepted April 23, 1996.

This work was supported by The Queen's Trust of Australia, the Nuffield Foundation, and Jesus College, Oxford. I am grateful to Prof. J. Newsom-Davis and Dr. B. Lang for critical discussion of this manuscript.

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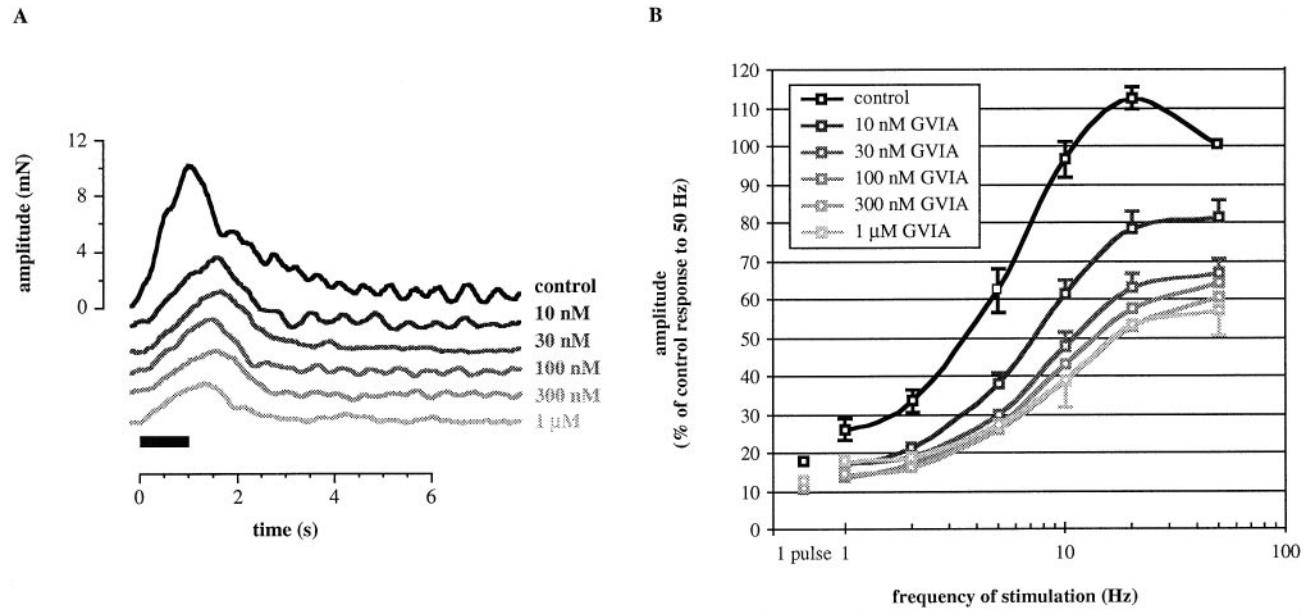

Figure 1. Effect of GVIA on whole contraction of bladder dome. Strips of bladder dome were stimulated with single pulses or 20 pulses at frequencies of 1–50 Hz. Toxin was added cumulatively at concentrations of 10, 30, 100, 300, and 1000 nM. Thirty minutes were allowed between periods of stimulation for the toxin to equilibrate. *A*, Example of the effect of the toxin on contractions evoked by 20 Hz stimulation. The *bar* shows the period of electrical stimulation. *B*, Summary of the effect of 10–1000 nM toxin on eight preparations from different animals. Two-way ANOVA indicated that each concentration produced significant inhibition compared with control (10 nM, $p < 0.005$; all other concentrations, $p < 0.001$); n = eight preparations from separate animals.

MATERIALS AND METHODS

Male albino (MF1) mice (20–30 gm) were killed by raised atmospheric CO_2 followed by cervical dislocation. The whole urinary bladder was excised and placed in Krebs' solution at room temperature, gassed with 95% O_2/5% CO_2, pH 7.4, at 37°C. The composition of the Krebs' solution was (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, D-glucose 11.0, and $CaCl_2$ 2.5. The dome of the urinary bladder was separated from the base and pinned flat in a Petri dish with the mucosa uppermost. The mucosa was gently dissected away and the remaining muscle layers cut into two strips. The strips were mounted in 5 ml organ baths and connected to Kent TRN001/TRN002 isometric transducers (ADInstruments, Sydney, Australia) under an initial tension of 10 mN. Contractions were recorded on a Macintosh computer using Chart version 3.4s software and a MacLab/8s data acquisition system (ADInstruments). Electrical field stimulation (EFS) was made by means of platinum wire electrodes placed at the top and bottom of the organ baths and connected to a digital stimulator (Applegarth Electronics, Oxford, UK). Single pulses or trains of 20 square wave pulses (pulse duration 0.3 msec, supramaximal voltage) were delivered at frequencies of 1, 2, 5, 10, 20, and 50 Hz at intervals of 90 sec. An interval of 30 min was left between consecutive frequency–response curves. Toxins were thus incubated for 30 min before measuring their effect. This incubation time is sufficient to reach a steady-state concentration (Turner et al., 1993). Preliminary experiments showed that consecutive control curves were superimposable.

Preparations were allowed to equilibrate for 20 min before capsaicin (10 μM) was added for 20 min to desensitize sensory nerves (Holzer, 1991). The preparations then were washed. Guanethidine (3 μM) was added to inhibit transmitter release from sympathetic neurons, and hexamethonium (500 μM) was added to block nicotinic ganglionic transmission. Responses to EFS, which were tetrodotoxin-sensitive, thus were considered to be mediated by transmitter release from postganglionic parasympathetic neurons.

Some preparations were desensitized to α,β-methylene ATP (α,β-MeATP) in the following manner: α,β-MeATP (10 μM) was added to the preparation, and this produced a rapid contraction. Once the tension returned to baseline, an additional 10 μM α,β-MeATP was added. There usually was no response to the second dose; however, to ensure complete desensitization, a third dose was added after an additional 10 min.

Drugs. Drugs used were α,β-MeATP, atropine, capsaicin, guanethidine, hexamethonium, tetrodotoxin (Sigma, St. Louis, MO), bethanechol (RBI, Natick, MA), GVIA (RBI, Natick, MA; Peninsula Laboratories, Belmont, CA; Peptide Institute, Osaka, Japan), agatoxin (Peptide Institute; Alomone Laboratories, Jerusalem), and MVIIC (Peptide Institute). Agatoxin was dissolved per instructions in 0.9% NaCl. Capsaicin was dissolved in ethanol. Ethanol at a final concentration of 0.1% did not alter the contraction amplitude significantly. All other drugs were dissolved in distilled water.

Analysis of results. Data were analyzed using two-way ANOVA, followed by preplanned comparisons of frequency–response curves in the presence of a drug versus control or comparisons of sequential frequency–response curves in experiments involving the cumulative addition of different toxins. Probabilities < 0.05 were considered significant.

RESULTS

Strips of mouse bladder dome contracted in a frequency-dependent manner, with half-maximal contractions occurring at a frequency of ~4.5 Hz. Contractions at all frequencies were blocked by 0.1–0.3 μM tetrodotoxin. Contractions of the bladder dome are mediated by a combination of acetylcholine and a purine, probably ATP (see introductory remarks and Components of the contraction of mouse bladder dome, below). Initially, the effects of calcium channel-blocking toxins were examined on the whole contraction produced by the combined effects of acetylcholine and ATP.

Effect of GVIA on the whole contraction

Frequency–response curves were constructed of the whole contraction in the absence of toxin and in the presence of 10, 30, 100, 300, and 1000 nM of the N-type VGCC blocker GVIA (Fig. 1). The contraction amplitude was reduced significantly by GVIA at each concentration. Maximal inhibition occurred at ~30 nM; higher concentrations did not produce a significantly greater effect. To ensure maximal blockade of N-type channels, however, 100 nM was selected for the remaining experiments. Contractions produced by the muscarinic receptor agonist bethanechol (10 μM) and the P_{2X} receptor agonist α,β-MeATP (10 μM), both of which act at receptors on the smooth muscle, were unaffected by GVIA, indicating that the toxin acts prejunctionally. Therefore, these

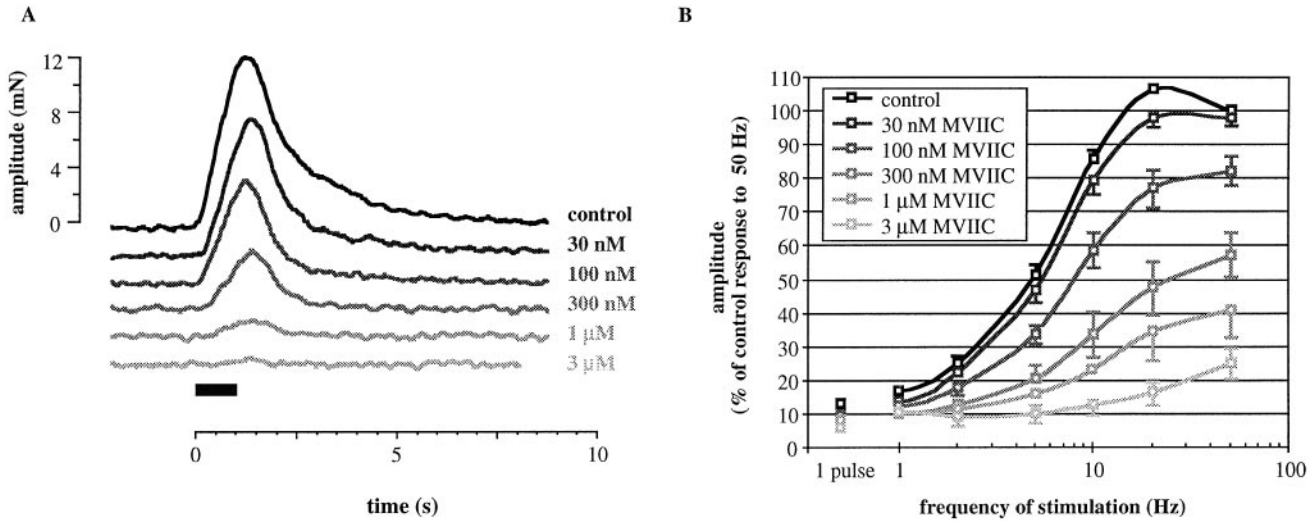

Figure 2. Effect of MVIIC on whole contraction of bladder dome. Strips of bladder dome were stimulated with single pulses or 20 pulses at frequencies of 1–50 Hz. Cumulative concentrations of 30, 100, 300, 1000, and 3000 nM were tested. Thirty minutes were allowed between periods of stimulation for the toxin to equilibrate. *A*, Example of the effect of the toxin on contractions evoked by 20 Hz stimulation. The *bar* shows the period of electrical stimulation. *B*, Summary of the effect of 30–3000 nM toxin on eight preparations from different animals. The effect of 30 nM toxin was not significant compared with control ($p > 0.05$). The contraction amplitude was reduced significantly by each of the higher concentrations (100 nM, $p < 0.05$; 300 nM, $p < 0.0001$; 1000 nM, $p < 0.0001$; 3000 nM, $p < 0.0001$).

experiments demonstrate that N-type VGCCs play a major role in transmitter release from parasympathetic nerve terminals in the mouse bladder. However, GVIA did not abolish nerve-evoked contractions, suggesting that other subtypes of VGCC also may be involved.

Effect of MVIIC on the whole contraction

The effect of the N-, P-, and Q-type VGCC blocker MVIIC was investigated on the whole contraction using the same experimental design as above. The contraction amplitude was not altered significantly by the lowest concentration of MVIIC, 30 nM (Fig. 2). However, contractions at all frequencies of stimulation were greatly reduced by each of the other concentrations. At stimulation frequencies less than 10 Hz, responses were blocked completely by 3 μM MVIIC. Small contractions could be elicited at higher stimulation frequencies in the presence of 3 μM MVIIC. The remaining experiments used 3 μM MVIIC. Contractions produced by 10 μM bethanechol and by 10 μM α,β-MeATP were not altered by MVIIC, confirming that the site of action of the toxin is prejunctional.

Table 1 shows the approximate concentrations at which GVIA and MVIIC produced 50% inhibition of the contraction. These values were interpolated from concentration–response graphs at a single stimulation frequency (i.e., the data shown in Figs. 1, 2 but plotted according to frequency instead of toxin concentration). GVIA was a more potent inhibitor than MVIIC for all stimulation frequencies except 50 Hz. For each stimulation frequency, MVIIC produced greater inhibition than GVIA. MVIIC can block N-, P-, and Q-type VGCCs and because its effect exceeded that of GVIA, it is likely to be acting at least partly on P- and/or Q-type channels. Previous studies have not found any evidence for the existence of these channel subtypes in autonomic nerve terminals, so we investigated this possibility further.

Effect of agatoxin on the whole contraction

To investigate whether P-type VGCCs are present in this preparation and are involved in mediating transmitter release, we tested the effect of agatoxin. In an initial experiment, 1 and 3 nM toxin did not have any effect. We therefore tested the effects of 10, 30, 100, and 300 nM agatoxin in four preparations. Figure 3 demonstrates that the toxin did not have a significant effect at these concentrations. Furthermore, the toxin did not alter the response to 10 μM bethanechol.

Table 1. Effect of GVIA and MVIIC on whole contraction

Stimulus	GVIA IC_{50} (nM)	MVIIC IC_{50} (nM)	Maximum % inhibition by GVIA	Maximum % inhibition by MVIIC
5 Hz	30	200	53.4 ± 6.66	83.4 ± 5.50
10 Hz	30	200	54.8 ± 5.26	85.5 ± 3.74
20 Hz	125	225	48.6 ± 4.08	80.1 ± 6.99
50 Hz	>1000	470	36.3 ± 2.88	76.1 ± 4.06

The concentration of toxin required to reduce the amplitude of bladder dome contractions by 50% was interpolated from graphs of toxin concentration versus response at a particular frequency. These values are listed for GVIA and MVIIC in columns 2 and 3. The maximum inhibition produced by GVIA, obtained at concentrations >30 nM, is shown in column 4. The maximum effect of MVIIC obtained in these experiments, at a concentration of 3 μM, appears in column 5. Each calculation is based on the results of eight experiments on preparations from different animals.

Effect of sequential addition of GVIA, agatoxin, and MVIIC on the whole contraction

Although agatoxin alone did not have any effect on the contraction amplitude, it is possible that N-type channels are sufficient to mediate a maximal contraction and that a role for P-type channels will be demonstrated only after the N-type channels are blocked. We therefore performed a series of experiments in which agatoxin was added after GVIA. Having blocked N- and P-type channels with GVIA and agatoxin, respectively, MVIIC was added to test whether Q-type channels mediate a proportion of the transmitter release.

Figure 4 shows a summary of experiments in which the three toxins were added cumulatively. As expected from the results described above, 100 nM GVIA reduced the amplitude of con-

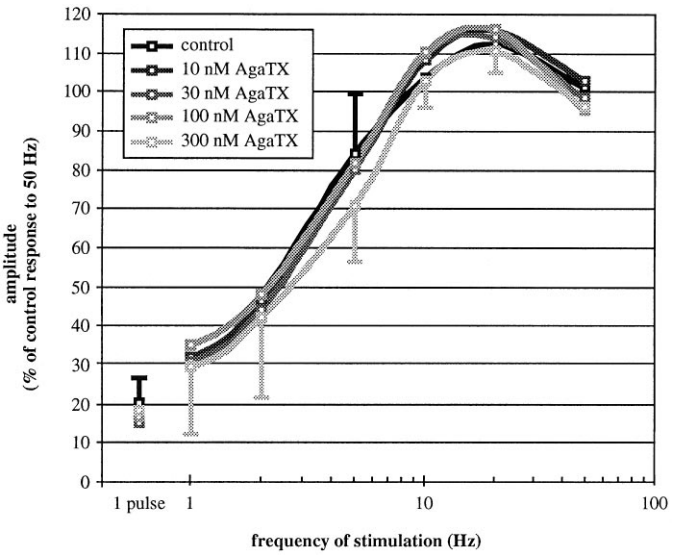

Figure 3. Effect of agatoxin on whole contraction of bladder dome. Experiments were performed as in Figures 1 and 2. Agatoxin did not have any effect on contraction amplitude at concentrations of 10–300 nM ($p > 0.05$ for each concentration); n = four preparations from different animals.

tractions significantly. More importantly, subsequent addition of 300 nM agatoxin reduced the amplitude of the remaining contraction. This effect was greatest at stimulation frequencies of 5–20 Hz (35–50% inhibition vs 10–25% inhibition at the lower stimulation frequencies and 50 Hz). This suggests that P-type VGCCs are present on parasympathetic nerve terminals in the mouse bladder and play a role in transmitter release. Finally, MVIIC was added in the continued presence of GVIA and agatoxin. This produced additional significant inhibition of the contraction, suggesting a role for Q-type channels in transmitter release.

The addition of all three toxins did not alter significantly the response to 10 μM bethanechol (6.69 ± 0.84 mN before and 8.34 ± 1.39 after; $n = 13$, $p > 0.05$, paired t test).

Two transmitters largely mediate contraction of the bladder in response to stimulation of parasympathetic neurons, acetylcholine, and ATP (Hoyle and Burnstock, 1993). Having investigated the nature of the calcium channels involved in transmitter release in the whole contraction, we investigated next which calcium channels are coupled to the release of each transmitter. First, we characterized the components of the whole contraction.

Components of the contraction of mouse bladder dome

Desensitization of bladder strips to α,β-MeATP reduced the amplitude of contractions at all frequencies of stimulation (Fig. 5*A*). Subsequent addition of 1 μM atropine virtually abolished the remaining response. Similarly, 1 μM atropine alone reduced significantly the amplitude of the contraction, and subsequent desensitization of P_{2X} receptors using α,β-MeATP virtually abolished the remaining response (Fig. 5*B*). The amplitude of either the cholinergic or purinergic components of the contraction was calculated once the effects of ATP and acetylcholine, respectively, had been inhibited. Thus, the cholinergic component of the contraction was calculated as the difference between the second and third curves shown in Figure 5*A*, and the purinergic component was calculated as the difference between the second and third curves shown in Figure 5*B*. Figure 5*C* shows the average values for each stimulation frequency obtained from four experiments of each type. These experiments demonstrate that cholinergic and purinergic transmission each account for ~50% of the contractile response, except at 50 Hz stimulation, when the cholinergic component represents 70% of the total.

The role of different neuronal VGCC subtypes in mediating the cholinergic and purinergic contraction (after desensitization to α,β-MeATP and in the presence of atropine, respectively) was investigated by adding sequentially GVIA, agatoxin, and MVIIC as described above for the whole contraction.

Effect of sequential addition of GVIA, agatoxin, and MVIIC on the cholinergic contraction

Figure 6*A* shows the effect of the toxins on the cholinergic contraction. Addition of 100 nM GVIA reduced the contraction

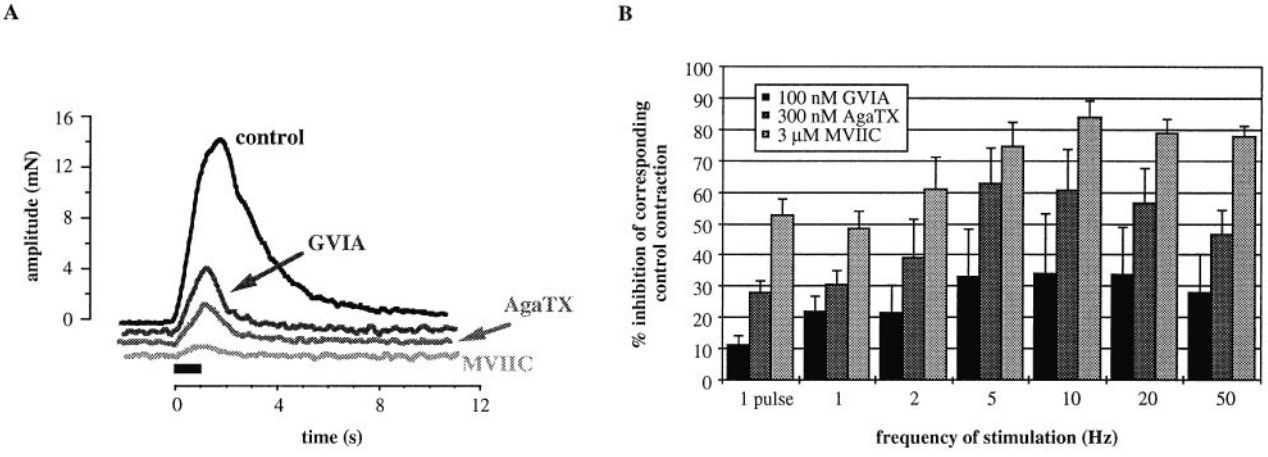

Figure 4. Effect of sequential addition of GVIA, agatoxin, and MVIIC on whole contraction. *A*, Examples of raw traces in which the bladder dome strips were stimulated with 20 pulses at 20 Hz. The *bar* shows the period of electrical stimulation. *B*, Summary of the effect of the toxins on four strips from separate animals. For each stimulus parameter, the first column represents the percentage inhibition produced by GVIA (*GVIA*) alone. The second column shows the combined effect of GVIA and agatoxin (*AgaTX*), and the third column, the effect of all three toxins. Contractions were reduced significantly in amplitude by 100 nM GVIA ($p < 0.0001$). In the continued presence of GVIA, 300 nM AgaTX caused an additional significant decrease in contraction amplitude ($p < 0.0001$ compared with responses in presence of GVIA alone). Addition of 3 μM MVIIC further decreased the contraction amplitude in the presence of the other two toxins ($p < 0.0001$ compared with responses in presence of AgaTX and GVIA).

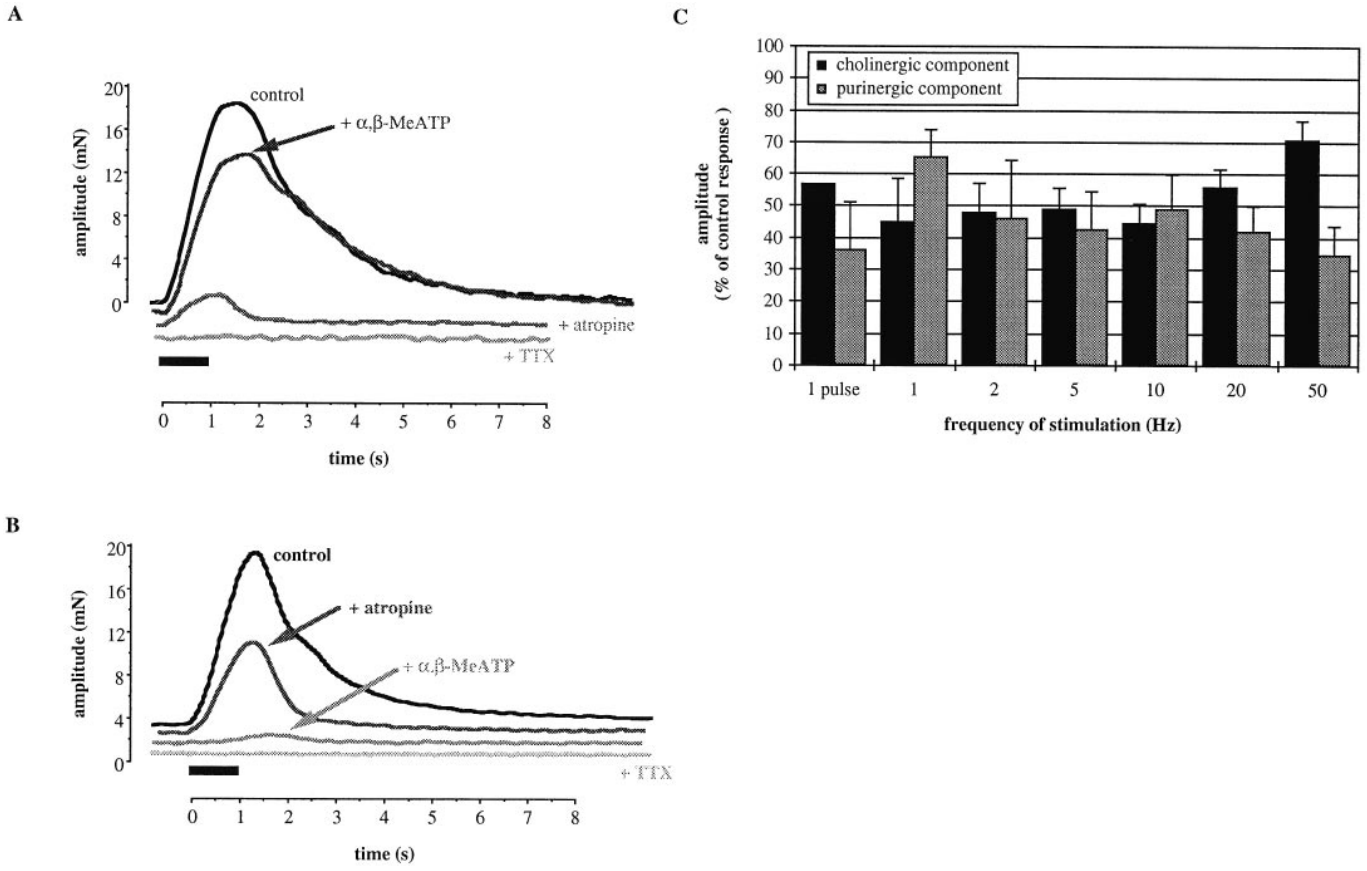

Figure 5. Components of bladder dome contractions. *A*, Example of control contractions in response to 20 pulses delivered at 20 Hz and the effect of desensitization to α,β-MeATP and subsequent addition of atropine. The *bar* shows the period of electrical stimulation. *B*, Example of control contractions in response to 20 pulses delivered at 20 Hz and the effect of atropine and the subsequent desensitization to α,β-MeATP. The *bar* shows the period of electrical stimulation. *C*, Summary of eight experiments on tissues from separate animals. The whole bladder dome contraction was reduced significantly by desensitization of the preparations to α,β-MeATP, which blocks postjunctional P_{2X} purinoceptors ($p < 0.001$). Subsequent addition of 1 μM atropine almost blocked completely the remaining response ($p < 0.001$). The residual response was blocked by 0.1 μM tetrodotoxin ($p < 0.001$). The cholinergic response was calculated as the difference between contraction amplitude after desensitization to α,β-MeATP and after the addition of 1 μM atropine and expressed as a percentage of the control response (i.e., the difference between the second and third curves in *A*). Similarly, the purinergic component was calculated as the difference in contraction amplitude after the addition of 1 μM atropine and after desensitization to α,β-MeATP (i.e., the difference between the second and third curves in *B*).

amplitude in response to all stimulus parameters by 32–56%. Addition of 300 nM agatoxin and 3 μM MVIIC each produced a small but significant inhibition of 6–9% of control. This suggests that acetylcholine release from parasympathetic neurons in the mouse bladder depends primarily on N-type channels and, to a lesser extent, on P- and Q-type channels.

Effect of sequential addition of GVIA, agatoxin, and MVIIC on the purinergic contraction

The purinergic contraction was inhibited significantly by GVIA (Fig. 6*B*), although the magnitude of this inhibition was much less than the inhibition of the cholinergic contraction. Thus, at stimulation frequencies up to 5 Hz, GVIA produced 7–15% inhibition. The maximal effect of the toxin, 25–41% inhibition, occurred at stimulation frequencies of 10–50 Hz. In the continued presence of GVIA, agatoxin produced an additional reduction in contraction amplitude. At frequencies up to 10 Hz, agatoxin produced an additional 15–27% inhibition and 7–11% inhibition at 20–50 Hz. The remaining purinergic contraction was greatly reduced by MVIIC (24–38% inhibition at all stimulation frequencies). This suggests that Q-type channels play a dominant role in ATP release from parasympathetic neurons in the mouse bladder. At low stimulation frequencies, P-type channels play an important role, with little involvement of N-type channels.

DISCUSSION

The present study has demonstrated that multiple subtypes of VGCCs are required for neurotransmitter release from parasympathetic nerve terminals in the mouse bladder.

The present experiments were performed using tissues that had been desensitized to capsaicin *in vitro*, and guanethidine and hexamethonium were present throughout. Thus, nerve-mediated responses could be attributed to activation of postganglionic parasympathetic neurons. Each of the toxins acted prejunctionally, because they did not alter responses to agonists acting directly on the muscle. VGCCs are present on the soma and dendrites of neurons as well as nerve terminals. Although the toxins used in this study may have blocked channels in the soma and dendrites, this would not be expected to be detected because EFS would bypass this blockade. Thus, effects of the toxins in these experiments can be attributed to actions on VGCCs at or near the nerve terminals of postganglionic parasympathetic neurons.

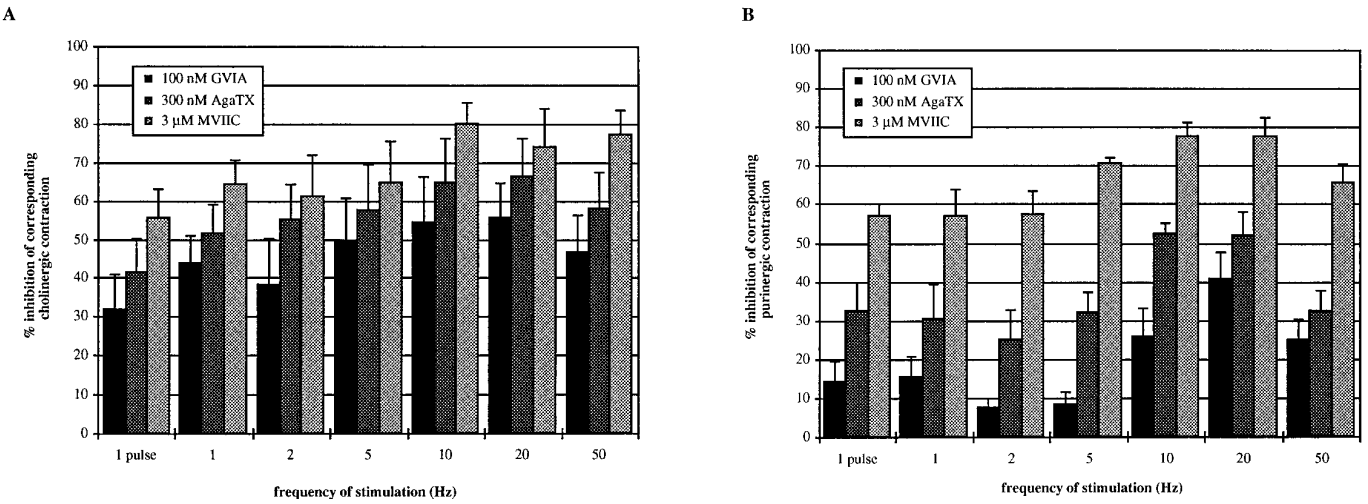

Figure 6. Effect of sequential addition of GVIA, agatoxin, and MVIIC on the cholinergic and purinergic components of the bladder contraction. *A*, Summary of the effect of the toxins on the cholinergic contraction, and *B*, the effect on the purinergic component. For each stimulus parameter, the first column represents the percentage inhibition produced by GVIA (*GVIA*) alone. The second shows the combined effect of GVIA and agatoxin (*AgaTX*), and the third column, the effect of all three toxins. GVIA had significant effects on both the cholinergic and purinergic contractions ($p < 0.0001$ for each), as did agatoxin ($p < 0.0005$ and $p < 0.0001$, respectively; comparison with GVIA curve). MVIIC reduced significantly the remaining cholinergic and purinergic contractions ($p < 0.0005$ and $p < 0.0001$, respectively); n = five preparations from different animals.

Calcium channel subtypes mediating the whole contraction

Toxins were used alone or in combination to deduce the involvement of different VGCC subtypes in transmitter release from parasympathetic neurons. Transmitter release was measured indirectly by recording the muscle contraction.

GVIA specifically blocks N-type channels (Dunlap et al., 1995). Reduction in bladder contraction amplitude by GVIA therefore implies a role for these channels in transmitter release, in agreement with previous studies on the bladder (Maggi et al., 1988; De Luca et al., 1990; Maggi, 1991; Zygmunt et al., 1993; Lundy and Frew, 1994). The IC_{50} for GVIA in this preparation was ~30 nM at stimulation frequencies up to 10 Hz, which is consistent with the IC_{50} obtained in other studies involving functional measurements on tissues innervated by the autonomic nervous system (Boot, 1994; Hong and Chang, 1995).

Agatoxin blocks P-type channels and, at higher concentrations, Q-type channels (see introductory remarks). In these experiments, we used a saturating concentration for P-type channels (300 nM) (Dunlap et al., 1995), thus any P-type channels present should be blocked. MVIIC can act at N-, P-, and Q-type channels, although not all N-type channels appear to be sensitive (Boot, 1994). By first adding GVIA and agatoxin to block N- and P-type channels, respectively, we can assume that an additional effect of MVIIC is most likely attributable to an action at Q-type channels. At the concentration used in these experiments, agatoxin may have an effect on Q-type channels in addition to P-type channels. However, two observations suggest that in the conditions used in the present study, agatoxin and MVIIC are acting on different channels. If 300 nM agatoxin and 3 μM MVIIC were acting on the the same channels, one would expect their effects to occur in parallel. However, agatoxin had a large effect on the purinergic contraction evoked at low stimulation frequencies and little effect at high frequencies; MVIIC produced a similar effect at all stimulation frequencies. Second, antibodies against VGCCs produced by patients with Lambert–Eaton myasthenic syndrome are able to decrease the component of transmitter release blocked by 300 nM agatoxin, yet increase the component blocked by MVIIC (Waterman et al., 1996). Because agatoxin and MVIIC appear to act at different channels, the simplest explanation is that they block P- and Q-type channels, respectively, in this preparation. Thus, transmitter release from parasympathetic neurons in the mouse bladder involves a combination of N-, P-, and Q-type channels. During preparation of this manuscript, evidence for a role of Q-type channels in transmitter release from parasympathetic neurons in the rat bladder has been published (Frew and Lundy, 1995).

The IC_{50} for MVIIC on currents mediated by a complex containing the α_{1B} subunit (N-type channel) is in the order of 10 nM (Grantham et al., 1994), and its IC_{50} for P-type currents is 1–10 μM (Hillyard et al., 1992). In a functional study on the guinea-pig ileum longitudinal muscle (Boot, 1994), the IC_{50} for MVIIC was 26 nM. In the latter study, it was concluded that MVIIC was acting on N-type channels, because the effect was abolished by previous incubation with GVIA. The IC_{50} obtained for MVIIC in the present study in the absence of other toxins was an order of magnitude greater than that reported by Boot. This is consistent with our conclusion that in the mouse bladder, MVIIC acts on neuronal P- and Q-type channels, in addition to N-type channels.

Calcium channel subtypes mediating the cholinergic and purinergic components of the contraction

Having tested the role of different VGCC subtypes in the contraction mediated by the combined effect of released acetylcholine and ATP, the relative importance of the channels in the release of each transmitter individually was investigated. This was tested indirectly by measuring the effect of the toxins on the contraction produced by acetylcholine alone (after desensitization of the P_{2X} purinoceptors to α,β-MeATP) and that produced by ATP alone (after addition of the muscarinic receptor antagonist, atropine). These experiments indicated that N-type channels play a dominant role in acetylcholine release but a much lesser role in ATP release. Conversely, P-type channels were important in ATP release but had little involvement in acetylcholine release. Q-type

channels played a major role in ATP release but not in acetylcholine release.

If acetylcholine and ATP were localized in the same synaptic vesicles in all parasympathetic neurons, one would anticipate a parallel effect of the toxins on the cholinergic and purinergic contractions. The fact that the effects differed suggests that in at least some parasympathetic neurons in the mouse bladder, the transmitters are not contained in the same vesicles and that they even may be released from different subpopulations of neurons. Because this study involved measuring the effects of transmitter release from populations of neurons, we cannot eliminate the possibility that the two transmitters may be colocalized in the same vesicles in some parasympathetic nerve terminals.

Because acetylcholine and ATP release depend to differing extents on calcium influx through different VGCC subtypes, one may predict that the effects of any disease process involving calcium channels would not be the same for the two transmitters.

In conclusion, N-, P-, and Q-type channels are involved in transmitter release from postganglionic parasympathetic neurons in the mouse bladder. Acetylcholine release requires N-type channels and, to a lesser extent, P- and Q-type channels, whereas ATP release requires predominantly P- and Q-type channels. Thus, as is the case with neurons in the CNS, autonomic neurons use multiple subtypes of VGCCs.

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