N-Type Ca\(^{2+}\) Channels Trigger Release of Excitatory and Inhibitory Neurotransmitter from Nerve Endings in Canine Bronchi

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ABSTRACT
We set out to characterize the types of Ca\(^{2+}\) channels that mediate release of the predominant excitatory (acetylcholine) and inhibitory (norepinephrine) neurotransmitters in canine bronchi, using electrically evoked contractions and relaxations, respectively, as indicators of this release. We found that the selective N-type Ca\(^{2+}\) channel blocker \(\omega\)-conotoxin GVIA eliminated electrically evoked contractions in a dose-dependent fashion (half-maximal inhibition in the presence of 1–5 nM) but had no significant effect on those evoked by exogenously added acetylcholine. Selective blockers of P-type Ca\(^{2+}\) channels (\(\omega\)-agatoxin TK; 10\(^{-8}\) to 10\(^{-7}\) M) or of L-type Ca\(^{2+}\) channels (nifedipine; 10\(^{-8}\) to 10\(^{-6}\) M) had no significant effect on the responses to neurally released or exogenously added acetylcholine. Likewise, electrically evoked relaxations were blocked by \(\omega\)-conotoxin GVIA (10\(^{-7}\) M) but not by \(\omega\)-agatoxin TK (10\(^{-7}\) M) or nifedipine (10\(^{-7}\) M); none of these Ca\(^{2+}\) channel blockers had a significant inhibitory effect on isoproterenol-triggered relaxations. We conclude that excitatory and inhibitory neurotransmission in canine bronchi is mediated predominantly by N-type Ca\(^{2+}\) channels, with little or no contribution from L-, P-, Q-, or T-type channels.

The predominant excitatory innervation to the airway smooth muscle (ASM) of all species is cholinergic in nature (Barnes, 1987); in some species, there may also be a minor contribution from excitatory noncholinergic nonadrenergic nerves. As such, electric field stimulation (EFS) of isolated canine ASM tissues evokes a twitch contraction that is completely abolished by the neural blocker tetrodotoxin (TTX), by nonspecific muscarinic receptor antagonists (atropine), or by \(M_3\)-selective muscarinic receptor antagonists, indicating these to be mediated by neurally derived acetylcholine (ACh) acting on \(M_3\) receptors (Janssen and Daniel, 1990b). Inhibitory neural responses, on the other hand, are somewhat more difficult to study and are much more variable between species. Canine ASM at rest exhibits no basal tone and therefore must first be precontracted, after which EFS evokes a twitch contraction followed by a relaxation that develops and resolves much more slowly than the contraction (Janssen and Daniel, 1990b, 1991a). To facilitate study of the latter, the contractions can be prevented by atropine, but this requires that a noncholinergic agonist be used to precontract the tissue. Using this approach, the relaxations were shown to be mediated by the action of neurally released norepinephrine (NE) on \(\beta_1\) receptors (Janssen and Daniel, 1991a).

To further complicate the study of neurotransmitter release in ASM, a number of autacoids have been found to modulate such release via receptors on the nerve endings. For example, ACh release in canine ASM is inhibited in this way by prostaglandin \(E_2\) (Abela and Daniel, 1995) or ACh itself (i.e., “autoinhibition”; Janssen and Daniel, 1990b) but enhanced by thromboxane \(A_2\) (Janssen and Daniel, 1991c). The neurotransmitters ACh and NE are contained within vesicles in the parasympathetic and sympathetic nerve endings, respectively (Whittaker, 1971). EFS causes opening of TTX-sensitive Na\(^+\) channels in the axolemma, thereby depolarizing the nerve ending and leading to the opening of voltage-dependent Ca\(^{2+}\) channels. The Ca\(^{2+}\) that enters through the latter then induces fusion of the vesicles with the axolemma and subsequent release of neurotransmitter (Catsicas et al., 1994).

Voltage-dependent Ca\(^{2+}\) channels have been classified into a growing list of subtypes that presently include N, L, T, P, Q, and R subtypes (Olivera, 1994). These different channel subtypes exhibit unique profiles of electrophysiological characteristics (voltage and time dependence of activation, inactivation, and deactivation), which are best examined using patch-clamp techniques (Olivera, 1994). However, they can also be distinguished on the basis of pharmacological sensi-

ABBREVIATIONS: ASM, airway smooth muscle; AgTX, agatoxin TK; \(\omega\)-CTX, \(\omega\)-conotoxin GVI A; EFS, electrical field stimulation; ACh, acetylcholine; S1, S2, S3, first, second, and third series of electrical field stimulation pulse trains, respectively; NE, norepinephrine; TTX, tetrodotoxin.

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The image contains a page of a scientific text discussing the characterization of smooth muscle and the pharmacological properties of neurotransmitters in bronchial tissue. The text describes the preparation of bronchial ring segments from canine bronchial nerve endings and the use of electrophysiological techniques to study the release of acetylcholine (ACh) and norepinephrine (NE) in response to electrical field stimulation (EFS). The study also examines the role of various blockers, such as dihydropyridines and muscarinic antagonists, in understanding the release mechanisms of neurotransmitters in airway smooth muscle. The text is a scientific abstract discussing the mechanisms underlying neurotransmitter release in airway smooth muscle and the potential implications for asthma therapy. The page includes a representative tracing showing contractile responses to isotonic KCl (100 mM) and EFS in canine bronchi. The tracing includes a graph with labeled axes and markers indicating the time course of responses. The bottom of the page includes the author information: Johri and Janssen, Vol. 290.
Statistical Analysis. EFS-evoked responses were expressed as a percentage of the maximum response to 100 mM KCl measured at the beginning of each experiment. The mean responses after treatment (S2 and S3) were compared with the mean responses before treatment (S1) by one-way ANOVA. If overall differences among means were found, then pairs of means were compared using Newman-Keuls post-hoc test to reveal the frequencies at which significant differences occurred before and after treatment. These comparisons were also made in tissues "treated" with vehicle to account for time-related changes in EFS-evoked responses (e.g., receptor desensitization). The logarithmic value of the effective concentration of the channel blocker producing 50% of the maximal inhibition of responses (IC50) was derived by fitting to a sigmoid equation provided by Fig P software.

EFS-evoked relaxations were expressed as a percentage of the tone existing immediately before each electrical stimulation. The ratio of the responses before and after treatment with Ca2+ channel blockers or vehicle (i.e., S2/S1) were compared using one-way ANOVA, as described above. If overall differences were found among the mean values, then pairs of mean values were compared using a Newman-Keuls post hoc test.

All data are presented as mean ± S.E.M., with n indicating the number of animals used (generally speaking, two ring segments per animal were used for each experimental condition). Values of p < .05 were accepted as statistically significant.

Drugs. ω-CTX and AgTX (Alomone Labs, Jerusalem, Israel) were prepared as aqueous solutions, whereas nifedipine (Sigma Chemical Co., St. Louis, MO) was prepared in 50% ethanol; these stock solutions were stored at −20°C, and a fresh aliquot of each was made up in distilled water on the day of the experiment. All other chemicals were obtained from Sigma Chemical Co and were prepared as aqueous solutions.

Results

Contractions Evoked by KCl, EFS, and ACh. KCl (100 mM) evoked a sustained contraction in these tissues (Fig. 1), the mean magnitude of which was 4.8 ± 1.2g (n = 17). This tone reached a peak within 3 to 5 min after addition of KCl and persisted until KCl was washed out of the bath.

EFS, on the other hand, evoked contractile responses that were transient in nature, reversing back to baseline level within 1 min after the end of the pulse train (Fig. 1), and that reached a maximum magnitude of 2.6 ± 0.7g, or 52.3 ± 10.3% of the response to KCl (n = 17). The magnitudes of these responses were frequency dependent, as shown in Fig. 2A, with maximal stimulation occurring at 5 pps. The lowest frequency tested (0.5 pps) generally gave >50% of the maximal response. More importantly, the maximal magnitude and frequency dependence of these responses did not change over the course of several hours with repeated stimulation, as shown in Fig. 2A. EFS-evoked contractions in canine ASM have been shown elsewhere to be mediated by neurally derived ACh (Janssen and Daniel, 1990b).

ACh evoked contractions that were larger than those evoked by KCl, reaching an overall maximum of 7.1 ± 1.3g above baseline (or 154.8 ± 22.2% of the response to KCl) at a concentration of 10−3 M (n = 6). The time course of these responses was intermediate between those triggered by KCl or by EFS, reaching a peak within 1 to 2 min after the addition of ACh and then beginning to reverse even in the maintained presence of ACh (Fig. 1). Dose-response curves were generated by the cumulative addition of increasing concentrations of ACh once each response reached a plateau (Fig. 2B): the interpolated concentration of ACh that caused half-maximal excitation (EC50) was 5.4 × 10−7 M.

Effects of ω-CTX on EFS-Evoked Contractions. ω-CTX, the specific blocker for N-type Ca2+ channels (Olivera, 1994), had no direct effect on basal tone but inhibited EFS-evoked contractions in a dose-dependent fashion. The latter were markedly reduced in the presence of 10−8 M ω-CTX (n = 4) and essentially abolished in the presence of 10−7 M ω-CTX (n = 4): a representative example of this is given in Fig. 3A, and the mean frequency-response relationships are given in Fig. 3B. We noticed that the sensitivity of these responses to ω-CTX varied with frequency. That is, responses to low-frequency stimulation were somewhat more sensitive to ω-CTX than those triggered by higher frequencies. For example, we took the data shown in Fig. 3B and replotted the responses at each frequency as a fraction of the control responses at those same frequencies (Fig. 3C): reexpression of the data in this way reveals the frequency dependence of the ω-CTX-imposed inhibition. From these data, we calculated the concentration of ω-CTX that produced half-maximal inhibition (IC50) of the responses at each frequency, finding this to range from 1 to 6 nM for responses to field stimulation frequencies of 0.5 to 20 pps (Fig. 3D).
\(\omega\)-CTX had no effect, however, on the contractile responses to exogenously added ACh (Fig. 3, A and E; \(n = 54\)). Even in the presence of \(10^{-7} M\) \(\omega\)-CTX, ACh evoked contractions of 7.5 ± 1.2% above baseline, equivalent to 154.8 ± 15.6% of the response to KCl (Fig. 3E); likewise, the ACh EC\(_{50}\) value was \(1.4 \times 10^{-2} M\) in the presence of \(\omega\)-CTX (Fig. 3E). These values for peak magnitude and half-maximally effective concentration were not significantly different from those obtained in the absence of \(\omega\)-CTX (above).

**Effects of Agatoxin on EFS-Evoked Contractions.**

AgTX (\(10^{-8}\) to \(10^{-7}\) M), a specific blocker for P-type Ca\(^{2+}\) channels (Adams et al., 1992), had no significant effect on the magnitude of contractions evoked by EFS at any frequency (Fig. 4A; \(n = 4\)), nor did it significantly alter the EC\(_{50}\) value (\(9.7 \times 10^{-7} M\)) or the maximal response (145.2 ± 25.2% of KCl response) to ACh (Fig. 4B; \(n = 4\)).

**Effects of Nifedipine on EFS-Evoked Contractions.**

Nifedipine, like other dihydropyridines, acts selectively to block L-type Ca\(^{2+}\) channels at submicromolar concentrations but can also antagonize T-type Ca\(^{2+}\) channels at higher concentrations (Tsien et al., 1988; Olivera et al., 1994; Janssen, 1997). At a concentration of \(10^{-9}\) M, nifedipine had no effect on EFS-evoked contractions (Fig. 5A; \(n = 4\)). At \(10^{-8}\) and \(10^{-7}\) M, however, the responses to low-frequency stimulation (i.e., 0.5 and 2 pps) were slightly but significantly reduced (Fig. 5A; \(n = 4\)). In separate experiments, we tested the effects of \(10^{-6}\) M nifedipine and found that the responses were not significantly different from the responses obtained at the lower concentrations of nifedipine tested. As shown elsewhere (Janssen et al., 1998), nifedipine (\(10^{-7}\) M) partially suppressed the contractile responses to exogenously added ACh (\(n = 4\)).

**Relaxations Evoked by EFS or Isoproterenol.** To examine EFS-evoked relaxant responses, tissues were precontracted with a submaximal concentration (\(10^{-7}\) M) of the thromboxane A\(_2\) analog U46619. These responses have been shown to be somewhat sustained, with a slow decrease in tone over the course of 30 to 60 min (Janssen and Daniel, 1991c). EFS at frequencies of 2 or 20 pps elicited transient relaxations, the magnitudes of which were 52.5 ± 7.2% and 64.0 ± 5.4% of U46619-induced tone (Fig. 6B; open bars); responses to lower-frequency EFS were too variable and were not systematically studied (data not shown). These relaxations reached a peak within 30 s after delivery of the EFS pulse train, after which tone returned to prestimulation levels over the course of 1 to 2 min. These inhibitory responses were less reproducible over time than the excitatory ones described above, which prevented us from characterizing the
full frequency-response relationship of the relaxations in great detail, as well as from investigating the same range of concentrations of Ca\(^{2+}\) channel blockers as was used above. These relaxations have been shown previously to be mediated by neurally derived NE acting on \(\beta\)-adrenoceptors.

The nonselective \(\beta\)-adrenoceptor agonist isoproterenol evoked sustained relaxations in all U46619-precontracted tissues \((n = 34)\). These relaxations were sustained and dose dependent, with a threshold concentration of \(10^{-9}\) M and complete reversal of tone occurring after the addition of \(10^{-7}\) M isoproterenol (Fig. 6A and B).

**Effects of Ca\(^{2+}\) Channel Blockers on EFS-Evoked Relaxations.** \(\omega\)-CTX \((10^{-7}\) M\) had no effect on U46619-induced tone \((n = 7)\) or on the relaxant responses evoked by isoproterenol \((n = 7)\) but eliminated EFS-evoked relaxations \((n = 7)\). AgTX \((10^{-7}\) M\), on the other hand, had no significant effect on either EFS-or isoproterenol-evoked relaxations \((n = 5)\).

Our examination of the effect of nifedipine on EFS relaxations was complicated by the fact that nifedipine itself directly antagonized U46619-induced tone by 20 to 66%. Despite this, we found that the magnitudes of EFS- and isoproterenol-evoked relaxations in the presence of nifedipine, expressed as a percent of tone existing immediately before delivery of each electrical pulse, were not significantly different from those recorded in control tissues \((\text{Fig. 6B; } n = 6)\).

**Discussion**

We set out to pharmacologically characterize the Ca\(^{2+}\) channels that mediate release of the primary excitatory and inhibitory neurotransmitters \((\text{ACH} and \text{NE}, \text{respectively})\) in canine bronchial smooth muscle. Although many previous examinations of neurotransmitter release, particularly the earlier ones, describe a predominant role for N-type Ca\(^{2+}\) channels, recent studies using more highly selective blockers have found exceptions to this rule. For example, P- and Q-type Ca\(^{2+}\) channels contribute in part to nerve-mediated responses in vas deferens smooth muscle \((\text{Westfall et al., 1996; \text{Wright and Angus, 1996; \text{Waterman, 1997}}})\) and seem to be exclusively involved in the hemidiaphragm muscle \((\text{Wright and Angus, 1996})\). L-type Ca\(^{2+}\) channels play a major role in facilitation of neurotransmitter release \((\text{but are not essential for release per se})\) in the urinary bladder \((\text{Somogyi et al. 1997})\). L-, N-, P-, Q-, and R-type Ca\(^{2+}\) channels are found in neurons dissociated from rat paratracheal ganglia \((\text{Murai et al., 1998})\). Furthermore, the relative involvement of these different channels may be frequency or activity dependent \((\text{Altieri et al., 1991; \text{Wright and Angus, 1996; \text{Lenicker and Hong, 1997; \text{Waterman, 1997}}})\) and, within a given tissue type, vary between species \((\text{discussed below})\).

We therefore examined the effects of agents that block N-, L-, T-, and P-type Ca\(^{2+}\) channels on excitatory and inhibitory responses triggered by low- and high-frequency neural stimulation. In canine bronchial smooth muscle, \text{ACH} and \text{NE} are the sole mediators of excitatory and inhibitory responses, respectively \((\text{Russell, 1980; \text{Janssen and Daniel, 1990, 1991}})\); the only previous studies of the Ca\(^{2+}\) channels mediating neurotransmission in ASM were done using guinea pig and rat tissues, in which there is evidence for the additional involvement of nonadrenergic noncholinergic neurotransmitters of various types.

**Release of Excitatory Neurotransmitter.** In canine ASM, electrically evoked contractions serve as a useful indirect index of \text{ACH} release because they are blocked completely by atropine or \text{TTX} \((\text{Janssen and Daniel, 1990a})\). Although HPLC analysis of \text{ACH} overflow into the bathing medium provides a more direct measure of such release, this approach is less sensitive, requiring relatively high frequencies and/or prolonged durations of stimulation to provide a useful signal-to-noise ratio \((\text{e.g., continuous stimulation for 10 min at 5 pps for a total of 3000 pulses, repeated several times during the course of a study; \text{Baker et al., 1993})}\). Contractions, on the other hand, can be evoked with even a single electrical pulse \((\text{Janssen and Daniel, 1990})\).

We found that in canine bronchial smooth muscle, excitatory responses evoked by both low- and high-frequency stimulations were unaffected by \(10^{-7}\) M AgTX, whereas \(10^{-6}\) M nifedipine had only a slight (but statistically significant) effect against low-frequency stimulation. These data suggest that P-, L-, and T-type Ca\(^{2+}\) channels play little or no role in mediating \text{ACH} release; the slight inhibitory effect of nifedipine may be due to a direct effect on the smooth muscle \((\text{Farley and Miles, 1977, 1978})\).

\(\omega\)-CTX, on the other hand, suppressed excitatory responses, particularly those at lower frequencies, when used...
at a concentration of $10^{-8}$ M and essentially abolished all responses when used at $10^{-7}$ M, suggesting that N-type Ca$^{2+}$ channels are primarily responsible for ACh release. The frequency dependence of antagonism produced by $10^{-8}$ M $\omega$-CTX does not necessarily indicate that some other channel type might mediate release at higher frequencies. This submaximal concentration of $\omega$-CTX would block only a fraction of the N-type Ca$^{2+}$ channels available; thus, more aggressive electrical stimulation would allow the remaining unblocked channels to elevate [Ca$^{2+}$] above the threshold required for fusion of the secretory vesicles in the nerve endings with the axolemma. The complete inefficacy of $\omega$-CTX against contractions evoked by exogenously added ACh argues against any postreceptor action of $\omega$-CTX.

The role of Ca$^{2+}$ channels in excitatory neurotransmission was first examined in guinea pig airway preparations (Altieri et al., 1991); despite the use of a supramaximal concentration ($10^{-6}$ M) of $\omega$-CTX, this group found that the sensitivity of electrically evoked contractions to $\omega$-CTX decreased as stimulation frequency increased, with complete abolition at frequencies $<3$ pps but little or no effect on responses to 30 to 100 pps stimulation. Later, ACh overflow evoked by prolonged stimulation at a single frequency (5 pps for 10 min) was measured directly in guinea pig trachealis (Baker et al., 1993) and again shown to be largely, but not completely, reduced by $10^{-6}$ M $\omega$-CTX. Finally, $\omega$-CTX reduced electrically evoked and capsaicin-induced contractions in guinea pig airways in vivo and in vitro, but even $10^{-5}$ M, $\omega$-CTX did not abolish these responses (Fujii, 1996). Electrically evoked contractions in rat bronchial smooth muscle are also reduced by $\omega$-CTX (Walday et al., 1992), but a significant portion is resistant to $\omega$-CTX at concentrations well above its accepted $K_i$ value (e.g., $\approx 25\%$ and $\approx 10\%$ in the presence of $10^{-7}$ and $10^{-6}$ M $\omega$-CTX, respectively). This may be due to the additional presence of L-, P/Q-, and R-type Ca$^{2+}$ channels in these tissues (Murai et al., 1998). These data suggest that N-type Ca$^{2+}$ channels play an important but not exclusive role in mediating excitatory neurotransmission in guinea pig and rat ASM. To our knowledge, there are no other published data regarding the contributions of various Ca$^{2+}$ channels to ACh release in ASM of any other species.

**Release of Inhibitory Neurotransmitter.** We used electrically evoked relaxations as an indirect index of NE release because these are blocked by propranolol or TTX, indicating that they are predominantly $\beta$-adrenoceptor-mediated neurogenic responses (Russell, 1980; Janssen and Daniel, 1991). Atropine was used to block the effects of electrically evoked ACh because there is evidence for negative interactions between muscarinic excitatory and adrenergic inhibitory responses in ASM (Fernandes et al., 1992). Of course, this necessitated the use of a noncholinergic spasmogen, the thromboxane A$_2$ mimetic U46619 (Janssen and Daniel, 1991c), to preconstrict the tissues and thereby allow relaxant responses to be visualized. The transient nature of these responses (slow but steady decay over the course of the experiment) prevented us from using a wide range of stimulation frequencies or concentrations of Ca$^{2+}$ channel blockers in our examination of NE release.

We found that neither AgTX nor nifedipine inhibited EFS-evoked relaxations, whereas $\omega$-CTX abolished them without having any significant effect on relaxant responses to exog-
enously added isoproterenol, suggesting that N-type channels mediate the release of NE.

In one previous study of inhibitory neurotransmission in ASM, electrically evoked relaxations in porcine tracheal tissues (which are insensitive to propranolol and therefore do not involve NE release) were found to be abolished by TTX but only partially inhibited by ω-CTX (3 × 10⁻⁷ M) (Kannan and Johnson, 1992).

**Study Implications.** In the past, the finding that the Ca²⁺ channels on ASM cells were predominantly of the L-type led to the suggestion that blockers of these channels might be useful as therapeutic agents for the treatment of asthma, much as they are used to treat hypertension. Unfortunately, it was found that they were almost ineffective in this respect because contraction of ASM is largely driven by release of internally sequestered Ca²⁺; the precise role for the Ca²⁺ channels on the sarcolemma is still the focus of much research and may include refilling of the internal Ca²⁺ store.

In like fashion, given that neurotransmitter release in ASM is predominantly mediated by N-type Ca²⁺ channels, it is possible that these channels could be pharmacologically targeted to modify neurotransmission and thereby treat asthma in the same way that cholinergic antagonists are used. Based on positive results in animal models of global and focal ischemia, synthetic analogs of ω-CTX are being tested in clinical trials for preventing neuronal degeneration after ischemic insult (Miljanich and Ramachandran, 1995).

The airways are ideally suited to such an approach because inhalation allows for the deposition of a relatively high concentration of agents on the airway wall, where the innervation is found; although the agents may then become absorbed into the circulation and distributed throughout the body, they are diluted well below their effective concentrations. One caveat to the use of such agents, however, is that ongoing release of inhibitory neurotransmitter (possibly occurring as a compensatory mechanism to counteract the spasmodenic influence of inflammatory mediators) would also be suppressed, thereby leading to marked increase in bronchoconstriction. A better understanding of the differences between the Ca²⁺ channels on the excitatory and inhibitory innervations (e.g., structural, coupling to second messenger systems, regulation by various endogenous hormonal autacoids, sensitivity to exogenous agents, and so on) may allow a more selective therapeutic approach to suppression of excitatory neurotransmission.

**Summary and Conclusions.** We examined the Ca²⁺ channels that mediate the release of excitatory and inhibitory neurotransmitters in canine ASM, finding both to be mediated predominantly (perhaps exclusively) by N-type Ca²⁺ channels. In contrast, the few studies that have been performed using guinea pig, rat, or porcine tissues indicate a significant role for non-N-type Ca²⁺ channels in the airways of those species, although the nature of those channels remains unclear.

**References**


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