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THE PHARMACOLOGY OF γ-AMINOBUTYRIC ACID AND ACETYLCHOLINE RECEPTORS AT THE ECHINODERM NEUROMUSCULAR JUNCTION

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Summary

This review describes the various subtypes of γ-aminobutyric acid (GABA) receptors found at the echinoderm neuromuscular junction (NMJ), based on pharmacological and physiological studies. The review focuses mainly on holothurian GABA receptors at the NMJ located between the radial nerve and longitudinal muscle of the body wall (LMBW) and compares them to GABA receptors described at other echinoderm NMJs. Since a primary action of GABA on the holothurian LMBW is to modulate contractile responses to the excitatory neurotransmitter, acetylcholine (ACh), the pharmacology of echinoderm nicotinic ACh receptors (nAChRs) and muscarinic ACh receptors (mAChRs) is also addressed.

GABA responses have been described in the asteroids, echinoids and holothuroids but not in the other echinoderm classes. Some actions of GABA on echinoderm muscle include regulation of basal tone and spontaneous rhythmic contractions and modulation of cholinergic responses. Both GABA A and B receptor subtypes are present at the echinoderm NMJ, a feature also common to the arthropods, molluscs and chordates. Echinoderm GABA A receptors may mediate the excitatory responses to GABA. The GABA A receptor antagonist bicuculline has a paradoxical effect on contractility, stimulating large protracted contractions of the LMBW. The GABA A agonist muscimol potentiates cholinergic contractions of the holothurian LMBW. Another population of GABA receptors is inhibitory and is sensitive to the GABA B agonist baclofen and GABA B antagonists phaclofen and 2-OH-saclofen. The pre- and/or postsynaptic location of the GABA A and B receptors is not currently known. The folded GABA analogue 4-cis-aminocrotonic acid has no effect on the contractility of the holothurian LMBW so GABA C receptors are probably lacking in this preparation.

Pharmacological studies have shown that distinct nAChRs and mAChRs are colocalized in numerous echinoderm muscle preparations. Most recently, nAChR agonists were used to characterize pharmacologically receptors at the holothurian LMBW that bind ACh. Nicotinic AChRs with unique pharmacological profiles are localized both pre- and postsynaptically at this NMJ, where their physiological action is to enhance muscle tone. Muscarinic agonists also have excitatory actions on the LMBW but their action is to stimulate phasic, rhythmic contractions of the muscle. The location of mAChRs at the echinoderm NMJ, however, is unknown.

Since most of the studies described in the present review have used whole-mount preparations consisting largely of a combination of muscle fibers, neurons and connective tissue, it is extremely difficult to determine pharmacologically the exact location of the various receptor subtypes. Additional electrophysiological studies on isolated neurons and muscle fibers are therefore required to clearly define extra-, pre- and/or postsynaptic sites for the receptor subtypes at the echinoderm NMJ.

Key words: echinoderm, holothuria, γ-aminobutyric acid receptor, nicotinic acetylcholine receptor, muscarinic acetylcholine receptor, bicuculline, muscimol, baclofen, phaclofen, 2-hydroxy-saclofen, longitudinal muscle of the body wall, neuromuscular junction.

Introduction

Since the pioneering work of Bacq (Bacq, 1935a; Bacq, 1935b; Bacq, 1935c; Bacq, 1939) and Reisser (Reisser, 1931), the evidence for an excitatory, cholinergic component to the motoneurons of the longitudinal muscle of the body wall (LMBW) has been steadily growing (see reviews by Binyon, 1972; Hill, 1993; Pentreath and Cobb, 1972; Welsh, 1966). A number of 'classical' neurotransmitters (5-hydroxytryptamine, norepinephrine, octopamine, dopamine, GABA; Leake and Walker, 1980), a growing family of neuropeptides (Melarange and Elphick, 2001) and nitric oxide (Billack et al., 1998; Melarange and Elphick, 2001; C. L. Devlin, unpublished data) have been implicated in the control of echinoderm muscle, but the evidence for an excitatory cholinergic component of the echinoderm neuromuscular system is most compelling. γ-

aminobutyric acid (GABA) acts as a classic inhibitory neurotransmitter in the holothurian cloaca (Hill, 1970) and LMBW, where it causes muscle relaxation, suppresses spontaneous rhythmicity and inhibits cholinergic contractions (Devlin and Schlosser, 1999). In addition to GABA, a growing list of echinoderm neuropeptides, such as the SALMFamides, act as potent muscle relaxants in various echinoderm muscle preparations (Melarange and Elphick, 2001).

Mammalian GABA receptors are classified as A, B and C subtypes as defined by their distinct membrane effectors, ion conductances and pharmacology. Both the GABA A and C receptor subtypes are associated with a Cl^- channel complex that mediates hyperpolarization of the cell, whereas the GABA B receptor subtype is linked to a G-protein/adenylase cyclase transduction pathway that ultimately results in K^+ efflux (Costa, 1998). Though our knowledge of GABA subtypes in the Echinodermata is extremely limited, recent studies have shown that both GABA receptor subtypes A and B coexist at the echinoderm neuromuscular junction (NMJ) and mediate either excitatory or inhibitory responses (Devlin and Schlosser, 1999).

This review focuses primarily on the pharmacological features of echinoderm GABA A and B receptors and speculates on their physiological action as a gain-control mechanism for the cholinergic motor system. At the center of our attention in this review is the holothurian LMBW, the classic echinoderm model for the study of comparative neuromuscular pharmacology since the early 1930s. From an evolutionary point of view, GABA modulation of cholinergic responses in the holothurian LMBW is remarkably similar to the dual GABAergic–cholinergic innervation of the vertebrate nerve plexus–ileum smooth muscle preparation. Therefore, we argue that the holothurian LMBW is an evolutionary precursor to later emerging deuterostome smooth muscle systems that are innervated by antagonistic neurotransmitters. The holothurian LMBW serves as an excellent invertebrate model to investigate dual GABAergic and cholinergic control of smooth muscle in vertebrate systems.

Evidence for GABA in the echinoderms

An early suggestion that an inhibitory neurotransmitter substance existed in echinoderms came from the work of Ernst Florey (for a fascinating history of Factor I/GABA, see Florey, 1991). His extract, ‘Factor I’, isolated from both mammalian brain and arthropod nervous tissues, caused opposing actions in two echinoderm muscle preparations tested. Factor I evoked a contraction in the longitudinal muscle of the holothurian *Actinopyga agassizi* that mimicked the action of acetylcholine (ACh), while causing paralysis of the musculature mediating spine movements of the sea urchin *Centrechinus antillarum (*Florey, 1956). At that time, GABA had not yet been identified in echinoderm tissues, and Florey remained skeptical that ‘Factor I’ was GABA; however, further studies would prove that ‘Factor I’ and GABA were one and the same (Florey, 1991).

Immunocytochemical studies have since localized GABA throughout the echinoderm nervous and muscular systems. GABA was localized in both echinoid (*Echinus esculentes*) and asteroid (*Asterias rubens*) radial nerve preparations (Osborne, 1971) and detected in *Echinus esculentes* tube feet extracts (Florey et al., 1975). More recently, Newman and Thorndyke (Newman and Thorndyke, 1994) located GABAergic networks in ectoneural fibers of the superficial and deep portions of the radial nerve cord, in the basal nerve ring, longitudinal nerve and basi-epithelial nerve plexus of the tube feet, and in the basi-epithelial plexus and mucosal perikarya of various digestive structures of *Asterias rubens*. Because of the presence of GABA-immunoreactivity in the ectoneural fibers in particular, Newman and Thorndyke (Newman and Thorndyke, 1994) suggested a possible role for GABA in modulating motor activities.

Excitatory GABA A receptors at the echinoderm NMJ?

The immunocytochemical findings are supported by growing pharmacological evidence suggesting that GABA modulates echinoderm muscle contractility. Both GABA and ACh cause the contractions of muscles of the isolated tube feet of three different sea urchin species (*Strongylocentrotus franciscanus*, *Arbacia lixula* and *Echinus esculentus*) (Florey et al., 1975) and the starfish *Asterias amurensis* (Protas and Muske, 1980). The excitatory GABA responses of sea urchin tube musculature, but not excitation by ACh, are blocked by bicuculline or picrotoxin (Florey et al., 1975), indicating that GABA and ACh operate at distinct receptor sites. On the basis of their sensitivity to both bicuculline and picrotoxin, the tube feet musculature of sea urchins has receptors with a similar pharmacology to the GABA A receptor (using the mammalian GABA receptor classification). Though the GABA A receptor is a Cl^- channel-associated protein and usually mediates hyperpolarizing responses, Florey et al. (Florey et al., 1975) reported that this anomalous, excitatory effect of GABA is caused by an enhanced membrane permeability to Na^+ and a reduced permeability to K^+ and Cl^-. Furthermore, since GABA responses in sea urchin tube feet are inhibited by treatment with cholinergic receptor blockers (Banthine, decamethonium, gallamine triethiodide, hexamethonium or Mytolon) and potentiated by cholinesterase inhibitors (physostigmine and neostigmine), Florey et al. (Florey et al., 1975) concluded that GABA acts presynaptically on cholinergic nerve fibers that innervate the muscles rather than directly on the muscle.

In contrast, Protas and Muske (Protas and Muske, 1980) concluded that the excitatory effect of GABA on the starfish (*Asterias amurensis*) tube foot preparation is not mediated through excitation of cholinergic motoneurons. This is based on their observation that GABA responses are not inhibited by cholinergic antagonists (Mytolon and BTM-15). Instead, they proposed a postsynaptic localization of the GABA receptors directly on the muscle itself. Elphick (Elphick, 1991) similarly found that GABA contractions in starfish tube feet are not blocked by cholinergic antagonists. Excitatory responses to GABA have also been reported in the protractor muscle of a holothurian *Cucumaria japonica* (Kobzar, 1984). In contrast,

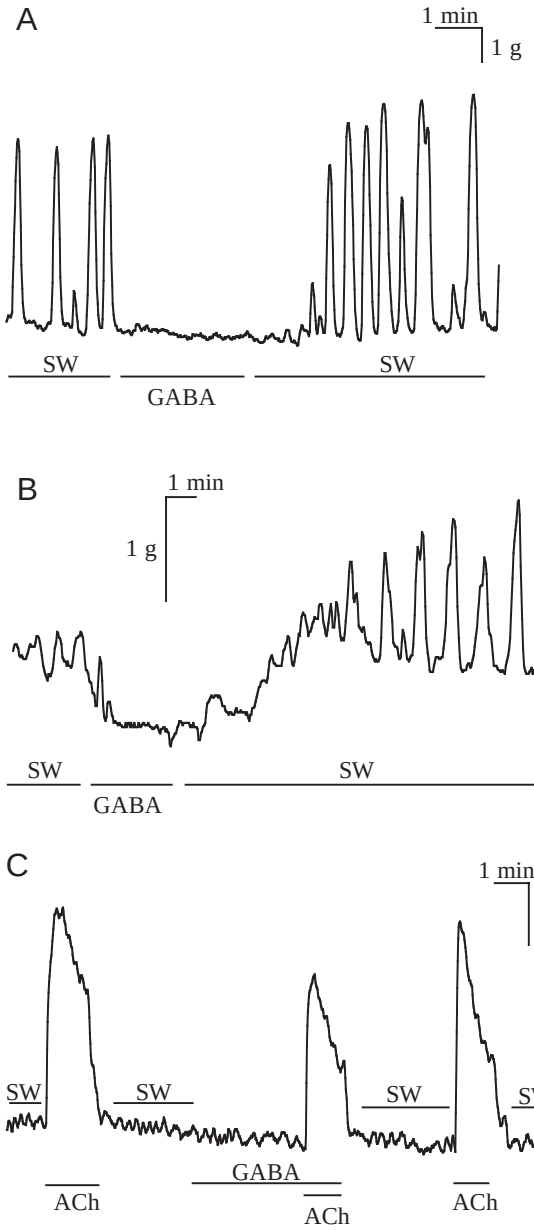

Fig. 1. γ-aminobutyric acid (GABA) has three inhibitory actions on the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. (A) Spontaneous contractions of the holothurian LMBW bathed in artificial sea water (SW) are completely blocked by treatment with GABA (10^{-5} mol l^{-1}). (B) A LMBW exhibiting some rhythmic activity is relaxed by treatment with GABA (10^{-4} mol l^{-1}). (C) GABA (10^{-5} mol l^{-1}) inhibits acetylcholine (ACh)-induced contractions by approximately 20 %. The inhibitory GABA effect in each example is completely reversible.

GABA acts as a relaxant on the cloaca of the holothurian *Isostichopus badionotus* (Hill, 1970) and the LMBW from *Sclerodactyla briareus* (Fig. 1A-C**)** (Devlin and Schlosser, 1999).

Contractility of the LMBW is enhanced by treatment with either the GABA A agonist muscimol or antagonist bicuculline

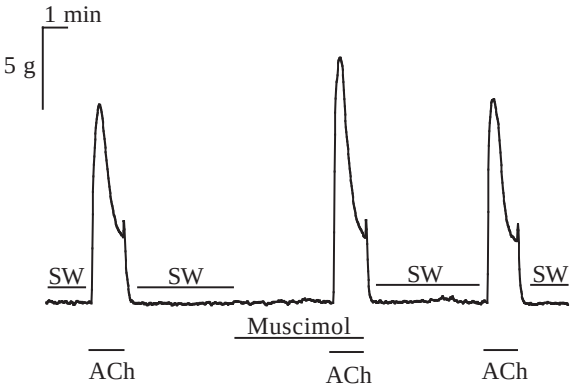

Fig. 2. Muscimol, a γ-aminobutyric acid (GABA) A receptor agonist, potentiates an acetylcholine (ACh)-induced contraction of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus.* First contraction: ACh control is caused by perfusion with ACh (10^{-4} mol l^{-1}) in artificial sea water (SW) only. The ACh is washed from the muscle with SW. The muscle is then treated with muscimol (10^{-4} mol l^{-1}) for 5 min. Second contraction: an enhanced contraction is caused by ACh (10^{-4} mol l^{-1}) in muscimol (10^{-4} mol l^{-1}). Third contraction: ACh post-control (10^{-4} mol l^{-1} ACh in SW only) shows that the ACh contraction is restored to the control level when muscimol is washed from the muscle.

(Devlin and Schlosser, 1999). This finding is quite curious in light of the fact that 'traditional pharmacology' dictates that the agonist (muscimol) and antagonist (bicuculline) should have opposite effects on muscle contractility. This also serves as a reminder that invertebrate neuropharmacology has its own idiosyncrasies.

Since GABA inhibits the contractility of the LMBW, we originally expected muscimol, a GABA A agonist, to mimick the inhibitory action of GABA. Oddly, muscimol treatment (10^{-4} mol l^{-1}) potentiates ACh-induced contractions of the LMBW by approximately 20 % (Fig. 2) and increases the force of spontaneously rhythmic contractions (Devlin and Schlosser, 1999). Excitatory muscimol-sensitive receptors have been described in other invertebrate neuronal preparations such as crustacean stretch receptors (Florey, 1991) and molluscan neurons (Kim and Takeuchi, 1990). Many signal transduction inhibitors were without effect on the inward current conducted by excitatory muscimol-sensitive receptors, indicating the response of an ionotropic rather than a metabotropic GABA receptor (Zhang et al., 1998). Depolarizing effects mediated by GABA A receptors can also explained by Cl^- efflux resulting from a transmembrane Cl^- gradient shift, not from the activation of a signal transduction pathway (Costa, 1998).

Excitatory responses to muscimol have been reported in vertebrates as well. Muscimol stimulates the release of radiolabelled ACh from neurons of the myenteric plexus, causing a tension increase in ileal smooth muscle of a guinea pig (Kleinrok and Kilbinger, 1983; Taniyama et al., 1988), a process that is Ca^{2+}-dependent and tetrodotoxin (TTX)-sensitive (Taniyama et al., 1988). Since these muscimol-induced responses are blocked by bicuculline, picrotoxin and

TTX, these findings indicate a prejunctional action of muscimol at GABA A receptors upstream in the nerve plexus (Taniyama et al., 1988). If these excitatory muscimol receptors are also present on the cholinergic neurons innervating the LMBW, then their activation (depolarizing effects) could result in enhanced ACh release and a subsequent enhanced contraction.

Bicuculline, a competitive antagonist of GABA A receptors, causes large contractions of holothurian LMBW from *Sclerodactyla briareus* (Devlin and Schlosser, 1999). In contrast, picrotoxin, which blocks GABA A chloride channels, fails to alter either basal tone or ACh-induced contractions of the LMBW. Bicuculline causes dose-dependent contractions of the LMBW only at high concentrations (10^{-5}–10^{-4} mol l^{-1}). In spontaneously contracting muscles or quiescent muscle preparations, bicuculline stimulates a quick phasic contraction followed by a sustained tone (Fig. 3). Bicuculline completely cross-desensitizes the LMBW to subsequent exposures to GABA, ACh or bicuculline, which suggests some common site of action for GABA and ACh.

There are a few putative mechanisms by which bicuculline could stimulate contraction. The first mechanism assumes that there are GABAergic neurons upstream in the radial nerve cord that exert a tonic suppression of the cholinergic motoneurons innervating the LMBW. If bicuculline blocks GABA receptors on cholinergic motoneurons, increased ACh release will result in an enhanced contraction. This model argues for a presynaptic location of the GABA A receptors and also assumes them to be inhibitory in their action; it also runs contrary to the argument that echinoderm GABA A receptors are excitatory.

Bicuculline contractions in the holothurian LMBW are partially blocked by atropine or d-tubocurarine (dTC), which raises the possibility that bicuculline may act on postsynaptic GABA A receptors, their locus in some protostome groups (Walker et al., 1993). Bicuculline increases the force of spontaneous contractions and the basal tone of mammalian smooth muscle, a response not blocked by treatment with TTX, providing additional evidence for a postsynaptic site of action (Minocha and Galligan, 1993).

GABA A antagonists can also bind to nicotinic acetylcholine receptors (nAChRs) because of a structural homology between the nAChR and GABA A receptor (Walker and Holden-Dye, 1991). Both bicuculline and picrotoxin block insect and nematode nAChRs but not mammalian nAChRs (Walker and Holden-Dye, 1991). Since bicuculline causes contractions of the LMBW, it is possible that bicuculline is stimulating nAChRs directly (and provides the possible mode by which dTC partially inhibits bicuculline-induced contractions). If so, the effects of bicuculline on nAChRs are unlike those in the protostome invertebrates (i.e. the insects and nematodes) and those on mammalian nAChRs, and are unique to echinoderms.

Bicuculline also acts as an acetylcholinesterase inhibitor and so prolongs the action of ACh in the synapse (Breuker and Johnston, 1975), providing yet another explanation for how bicuculline enhances contractions. The location of bicuculline-sensitive receptors at the echinoderm NMJ is completely unresolved and they cannot yet be characterized as exclusively excitatory or inhibitory in their mode of action.

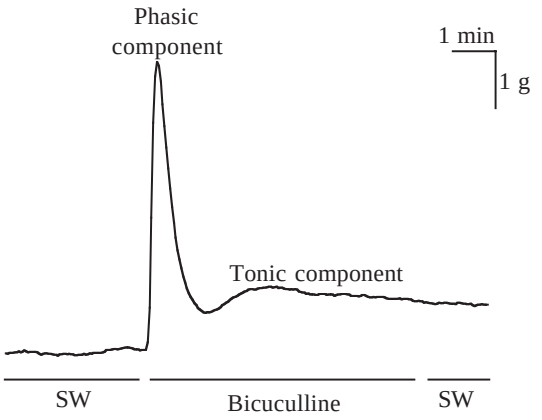

Fig. 3. A bicuculline-induced (10^{-4} mol l^{-1}) contraction of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus* consists of a phasic contraction followed by a sustained tone. Upon washing out the bicuculline with artificial sea water (SW), tone still persists.

Evidence for inhibitory GABA B receptors at the echinoderm NMJ

Unlike the excitatory GABA responses reported from sea urchin and starfish tube foot muscles, GABA has three inhibitory actions on the holothurian *Sclerodactyla briareus* LMBW (Devlin and Schlosser, 1999): (1) it blocks spontaneous rhythmic contractions (Fig. 1A), (2) it produces muscle relaxation (Fig. 1B) and (3) it reduces ACh-induced contractions by approximately 20 % (Fig. 1C). GABA similarly blocks spontaneous rhythmic contractions of an isolated cloaca from *Isostichopus badionotus* (Hill, 1970) and reduces light-induced contractions of the lantern retractor muscle of *Parechinus angulosus* (Boltt and Ewer, 1963). This more ‘classic’ inhibitory action of GABA is reported from other invertebrate (annelid and nematode) body wall muscle preparations where GABA activates postsynaptic GABA A receptors that are bicuculline- and/or picrotoxin-sensitive (depending on the specific invertebrate group) and mediate Cl^- influx (see review by Walker et al., 1993). Since GABA A receptors in the echinoderms appear to be excitatory in nature, it is unlikely that they are involved in GABA-induced muscle relaxation. Therefore, the GABA A receptors of the echinoderms (deuterostomes) are quite dissimilar to the GABA A receptors of the helminth protostomes.

GABA B receptors have not been reported in the helminth groups (Walker et al., 1993) but are present in the echinoderms (Devlin and Schlosser, 1999), molluscs (Rubakhin et al., 1995; Azatian et al., 1997) and arthropods (Fischer and Parnas, 1996; Miwa et al., 1990). Activation of GABA B receptors mediates inhibitory responses in the holothurian LMBW (Devlin and Schlosser, 1999). Baclofen, a GABA B agonist, causes similar

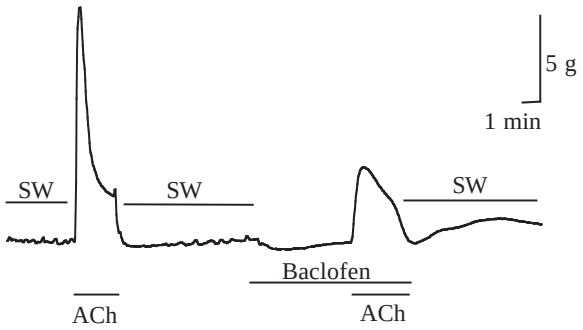

Fig. 4. Baclofen, a γ-aminobutyric acid (GABA) B receptor agonist, has an inhibitory effect on acetylcholine (ACh)-induced contractions of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. First contraction: ACh control is caused by perfusion with ACh (10^{-4} mol l^{-1}) in artificial sea water (SW) only. The ACh is washed from the muscle with SW. The muscle is then treated with baclofen (10^{-4} mol l^{-1}) for 5 min, during which time it relaxes the muscle. Second contraction: a reduced and attenuated contraction is caused by ACh (10^{-4} mol l^{-1}) in the presence of baclofen (10^{-4} mol l^{-1}).

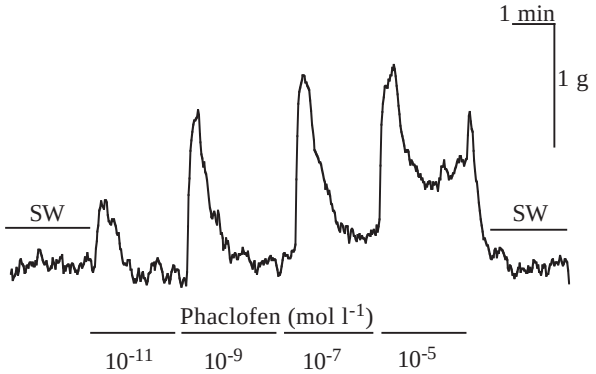

Fig. 5. Phaclofen, a γ-aminobutyric acid (GABA) B receptor antagonist, causes a concentration-dependent increase in phasic contractions and tone (from 10^{-11} to 10^{-5} mol l^{-1}) of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. SW, artificial sea water.

responses to GABA: a relaxation of spontaneous tone, a reduction in size of ACh-induced contractions and a complete block of spontaneous rhythmicity of the LMBW. Baclofen is a more potent inhibitor than GABA, blocking ACh-induced contractions by approximately 50 % (Fig. 4).

The effects of baclofen in other invertebrate preparations are puzzling and often contradictory. Baclofen causes depolarization in stretch receptor neurons of the crayfish *Orconectes limosus* (Florey, 1991), whereas it mediates inhibitory responses at the crayfish (*Procambarus clarkii*) opener NMJ (Fischer and Parnas, 1996) and the spiny lobster (*Palinurus japonicus*) NMJ (Miwa et al., 1990). Underlying the baclofen-induced hyperpolarization is activation of an inhibitory G-protein/adenylase cyclase system that results in enhanced K^+ currents (Miwa et al., 1990). In contrast to the crustacean NMJ, GABA and baclofen reduce K^+ A-currents, which increases action potential duration and reduces the after-hyperpolarizing phase of the action potential in molluscan neurons (Rubakhin et al., 1995). This effect may explain the ACh-induced contractions of the holothurian LMBW, which are slow to return to resting tone after GABA or baclofen treatment (Fig. 4). Both GABA and baclofen suppress voltage-gated Ca^{2+} currents in molluscan neurons (Rubakhin et al., 1995), which, if occurring in the holothurian NMJ, would diminish ACh release from the motoneuron. Suppression of the Ca^{2+} current postsynaptically would limit the amount of Ca^{2+} available for the contractile apparatus of the muscle cells and so reduce the force of the contraction.

Both the GABA B antagonists phaclofen (Fig. 5) and 2-OH-saclofen have potent excitatory effects on the holothurian LMBW, inducing large concentration-dependent contractions that are reversed by the addition of either baclofen or GABA (Devlin and Schlosser, 1999). Phaclofen (10^{-5} mol l^{-1}) doubles the amplitude of ACh-induced (10^{-4} mol l^{-1}) contractions. ACh is unable to elicit contractions, nor is GABA able to relax the muscle after prior 2-OH-saclofen treatment; this again suggests cross-densensitization at a common site, a response paralleling that observed after bicuculline treatment.

The physiological action of phaclofen in vertebrate smooth muscle is inhibition of the K^+ current normally induced by GABA or baclofen, so prolonging excitation of the cell (Minocha and Galligan, 1993). A reduction in the K^+ current leads to increased ACh release from the presynaptic neuron, and drives the muscle potential closer towards the threshold, so enhancing excitability of the postsynaptic cell. 2-OH-saclofen blocks presynaptic inhibition caused by either baclofen or GABA at the NMJ of the crayfish *Procambarus clarkii* (Fischer and Parnas, 1996).

The GABA C receptor agonist cis-4-aminocrotonic acid has no effect the spontaneous contractility of the holothurian LMBW or on contractions elicited by ACh, so there is little evidence to suggest the presence of GABA C receptors in the echinoderms (C. L. Devlin and W. Schlosser, unpublished data). Table 1 summarizes the occurrence of GABA in the various echinoderm classes where it is reported to have a physiological action. Since GABA clearly exerts some control over cholinergic responses, it is also of interest to investigate the pharmacology of the AChRs present at the echinoderm NMJ.

Echinoderm cholinoreceptors

The early pharmacological and histological investigations that provided the initial evidence that ACh is the excitatory neurotransmitter mediating motor responses in the echinoderms has been extensively reviewed (Binyon, 1972; Pentreath and Cobb, 1972; Welsh, 1966). Even in these early studies, the search was on for the AChR subtypes that underlie the contractile responses of echinoderm muscles. In addition to being one of the first to extract ACh from echinoderm tissues (holothurian LMBW and intestine) (Bacq, 1935a), Bacq also conducted one of the first pharmacological experiments on the

Table 1. *Occurrence of GABA in neuromuscular preparations in the echinoderm classes*

Class	Role of GABA	Genus	Muscle preparation	Reference
Asteroidea	E	*Asterias*	Tube feet	Protas and Muske, 1980
	E	*Asterias*	Tube feet	Elphick, 1991
Crinoidea	No data			
Echinoidea	I	*Centrechinus*	Spines	Florey, 1956
	I	*Parechinus*	Lantern retractor muscle	Boltt and Ewer, 1963
	E	*Strongylocentrotus*	Tube feet	Florey et al., 1975
	E	*Arbacia*	Tube feet	Florey et al., 1975
	E	*Echinus*	Tube feet	Florey et al., 1975
Holothuroidea	I	*Stichopus*	Cloaca	Hill, 1970
	I	*Sclerodactyla*	LMBW	Devlin and Schlosser, 1999
	E	*Actinopyga*	LMBW	Florey, 1956
	E	*Cucumaria*	Protractor muscle	Kobzar, 1984
Ophiuroidea	No data			

GABA, gamma-aminobutyric acid; LMBW, longitudinal muscle of the body wall.
E indicates an excitatory response to GABA; I indicates an inhibitory response to GABA.

LMBW of *Stichopus regalis*, finding it to be sensitive to nicotine (Bacq, 1939). Subsequent investigators found numerous other echinoderm muscle tissues to be sensitive to nicotine and its antagonist dTC (see review by Welsh, 1966), providing initial evidence for the presence of nAChRs at the echinoderm NMJ. Other early pharmacological studies showed that mAChRs are also present because ACh-induced contractions are blocked by the muscarinic antagonist atropine (Bacq, 1939; Wyman and Lutz, 1930). Later studies surveyed the responses of numerous echinoderm muscles to a range of both nicotinic and muscarinic agonists and antagonists (Florey et al., 1975; Florey and Cahill, 1980; Shelkovnikov et al., 1977). It was generally concluded that both types of cholinoreceptor are present in the echinoderm muscle preparations tested (Florey et al., 1975; Florey and Cahill, 1980; Shelkovnikov et al., 1977; Kobzar and Shelkovnikov, 1985a; Protas and Muske, 1980).

The pharmacology of excitatory nicotinic receptors

The following is a combined list of effective nicotinomimetics on echinoderm muscle: nicotine, suberyldicholine, butyrylcholine, arecoline methiodide, propionylcholine and benzoylcholine (Florey et al., 1975; Florey and Cahill, 1980; Shelkovnikov et al., 1977; Kobzar and Shelkovnikov, 1985a; Mendes et al., 1970; Protas and Muske, 1980). Some effective nicotinic antagonists are: dTC, gallamine triethiodide, Mytolon and sebacoyldicholine (Shelkovnikov et al., 1977; Mendes et al., 1970). The most recent study on nicotinic agonists was conducted on the holothurian LMBW (C. L. Devlin and W. Schlosser, manuscript in preparation): a series of nAChR antagonists, α- and β-bungarotoxins (α-BGT and β-BGT), dTC, mecamylamine, methyllycaconitine (MLA), succinylcholine, the choline uptake blocker, hemicholinium-3, and the neuronal Na^+ channel blocker, tetrodotoxin (TTX) were used to identify sites where the agonists (cytisine, epibatidine, nicotine) may act at the holothurian NMJ. These experiments showed that distinct subpopulations of nAChRs exist both pre- and postsynaptically at this NMJ preparation.

Nicotine

Nicotine causes concentration-dependent, slowly-rising contractions in the holothurian LMBW. It acts predominantly at muscle-type nAChRs on the LMBW, since both dTC and succinylcholine significantly potentiate the nicotine response. Similar potentiating effects of dTC on cholinergic responses have been described in other invertebrate preparations (Alvarez et al., 1969; Mattisan et al., 1974). Kehoe (Kehoe, 1972a; Kehoe, 1972b; Kehoe and McIntosh, 1998) found that three current components underlie the cholinergic response in *Aplysia californica* neurons: an inhibitory rapid response (mimicked by nicotine and blocked by dTC or α-BGT), an inhibitory slow response, and an excitatory response that is activated by nicotine or carbacol. Potentiation of nicotine contractions by dTC in the holothurian LMBW could be explained by suppression of this dTC-sensitive inhibitory rapid response (Kehoe, 1972a; Kehoe, 1972b; Kehoe and McIntosh, 1998), which would serve to prolong excitation and result in a protracted contraction of the LMBW. dTC alone causes a slight increase in basal tone of the holothurian LMBW, a response also observed in *Parechinus angulosa* muscle (Boltt and Ewer, 1963). The effect of dTC on ACh-induced contractions in various echinoderm muscle preparations is extremely variable. ACh-induced contractions are blocked by dTC treatment in the LMBW of *Holothuria grisea* (Ambache and Sawaya, 1953) and in the radial muscle from sea urchin *Asthenosoma ijimai* (Tsuchiya and Amemiya, 1977). However, it has no effect on the responses to ACh of the LMBW of *Thyone briareus* (DuBuy, 1936) and of *Sclerodactyla briareus* (Devlin et al., 2000), or on ACh-induced contractions of sea urchin tube feet (Florey et al., 1975).

Responses to nicotine are also partially inhibited by

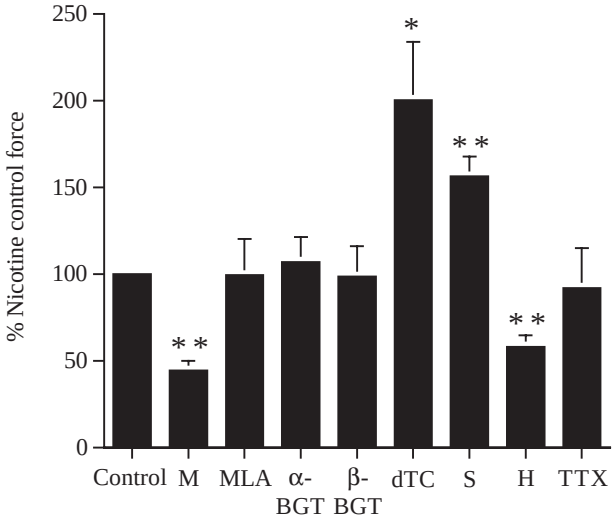

Fig. 6. The effects of nicotinic antagonists on 10^{-6} mol l^{-1} nicotine-induced contractions of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. Antagonists tested were: 10^{-6} mol l^{-1} methyllycaconitine (MLA, N=4), 10^{-6} mol l^{-1} mecamylamine (M, N=3), 10^{-7} mol l^{-1} α-bungarotoxin (α-BGT, N=3), 10^{-7} mol l^{-1} β-bungarotoxin (β-BGT, N=6), 10^{-6} mol l^{-1} d-tubocurarine (dTC, N=3), 10^{-6} mol l^{-1} succinylcholine (S, N=4), 10^{-6} mol l^{-1} hemicholinium-3 (H, N=4) and 5×10^{-7} mol l^{-1} tetrodotoxin (TTX, N=4). Values (means ± S.D.) are expressed as a percentage of the control nicotine-induced contractions prior to antagonist treatment. Asterisks indicate a significant difference from the control value: *P<0.01; **P<0.005.

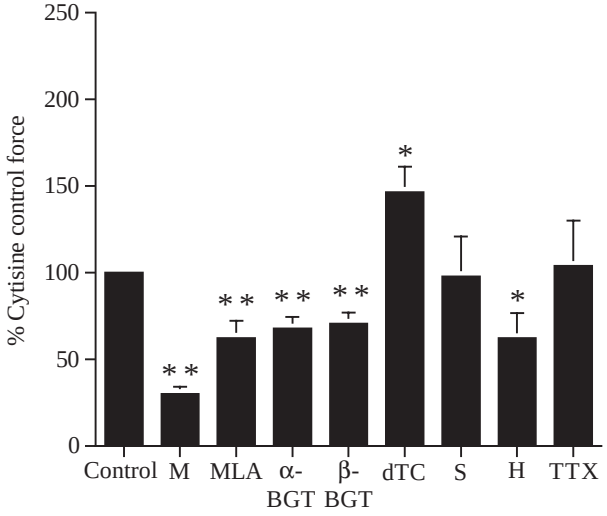

Fig. 7. The effects of antagonists on 10^{-6} mol l^{-1} cytisine-induced contractions of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. Antagonists tested were: 10^{-6} mol l^{-1} methyllycaconitine (MLA, N=5), 10^{-6} mol l^{-1} mecamylamine (M, N=4), 10^{-7} mol l^{-1} α-bungarotoxin (α-BGT, N=6), 10^{-7} mol l^{-1} β-bungarotoxin (β-BGT, N=6) and 10^{-6} mol l^{-1} d-tubocurarine (dTC, N=3), 10^{-6} mol l^{-1} succinylcholine (S, N=4), 10^{-6} mol l^{-1} hemicholinium-3 (H, N=3) and 5×10^{-7} mol l^{-1} tetrodotoxin (TTX, N=4). Values (means ± S.D.) are expressed as a percentage of the control cytisine-induced contractions prior to antagonist treatment. Asterisks indicate a significant difference from the control value: *P<0.01; **P<0.005.

hemicholinium-3 (a choline uptake blocker) and mecamylamine (an antagonist selective for neuronal nAChRs), indicating that it may be operating at presynaptic sites, though only as a weak agonist. The fact that TTX, MLA, α-BGT or β-BGT fail to block nicotine responses provides additional evidence that nicotine has a minimal effect on neuronal nAChRs. These pharmacological data are summarized in Fig. 6.

Cytisine

Cytisine is another non-specific agonist at the holothurian NMJ acting on both muscle-type nAChRs and neuronal nAChRs. Cytisine produces contractions that are blocked significantly by treatment with α-BGT, MLA, mecamylamine, β-BGT and hemicholinium-3, indicating a presynaptic site of action on the motoneurons that innervate the LMBW (Fig. 7). Cytisine may be acting on autoreceptors involved in the feedback control of ACh release that have been described in other invertebrate (Fossier et al., 1988) and vertebrate (Tian et al., 1997; Wilkie et al., 1996) neuronal preparations. Cytisine-induced contractions are generated during TTX-treatment, indicating that this agonist also acts on muscle-type nAChRs, stimulating contraction regardless of the fact that the motoneurons upstream are shutdown by the toxin. Like nicotine, cytisine responses are also potentiated by dTC.

Epibatidine

Of all the nicotinic agonists tested, epibatidine generates the largest contractions of the LMBW. Epibatidine has primarily a presynaptic site of action on the motoneurons of the radial nerves since its responses are significantly reduced by treatment with hemicholinium-3, β-BGT and TTX (Fig. 8). Since some neuronal nAChRs act as autoreceptors, it is possible that epibatidine-sensitive receptors are involved in (positive) feedback control of ACh release at the motoneuron terminal. Responses to epibatidine in the holothurian LMBW are also blocked by dTC and MLA, but most potently by mecamylamine. Epibatidine responses of the LMBW are not sensitive to block by α-BGT; likewise, α-BGT is unable to block ACh-induced responses in sea urchin dentis retractor muscle (Kobzar and Shelkovnikov, 1985a).

The pharmacology of muscarinic ACh receptors

The following is a combined list of effective muscarinomimetics on echinoderm muscle preparations: acetyl-β-methylcholine, carbacol, methylfurmethide, F-2268, dioxolane and arecoline bromhydrate (Florey et al., 1975; Florey and Cahill, 1980; Shelkovnikov et al., 1977; Kobzar and Shelkovnikov, 1985a; Kobzar and Shelkovnikov, 1985b; Mendes et al., 1970; Protas and Muske, 1980). Some effective antagonists of echinoderm mAChRs included: atropine, benzilylcholine, propanthelium, benactyzine and dibenamine (Shelkovnikov et al., 1977). In a recent survey, Devlin et al. (Devlin et al., 2000) used more selective muscarinic agonists and antagonists to determine whether there are

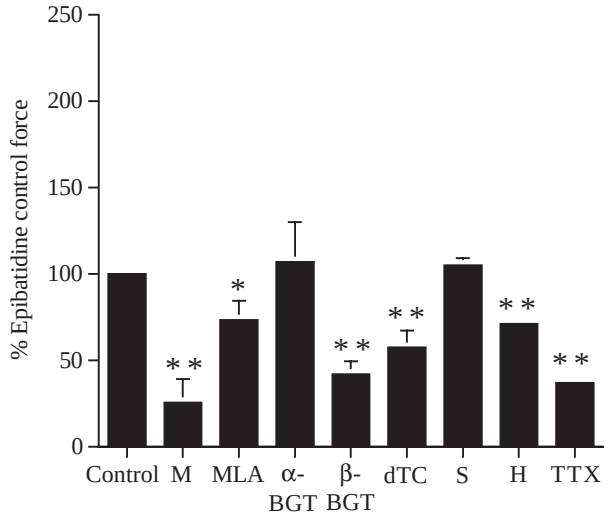

Fig. 8. The effect of various antagonists on 10^{-6} mol l^{-1} epibatidine-induced contractions of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. Antagonists tested were: 10^{-6} mol l^{-1} methyllycaconitine (MLA, *N*=3), 10^{-6} mol l^{-1} mecamylamine (M, *N*=4), 10^{-7} mol l^{-1} α-bungarotoxin (α-BGT, *N*=6), 10^{-7} mol l^{-1} β-bungarotoxin (β-BGT, *N*=4), 10^{-6} mol l^{-1} d-tubocurarine (dTC, *N*=3), 10^{-6} mol l^{-1} succinylcholine (S, *N*=4), 10^{-6} mol l^{-1} hemicholinium-3 (H, *N*=3) and 5×10^{-7} mol l^{-1} tetrodotoxin (TTX, *N*=3). Values (means ± S.D.) are expressed as a percentage of the control epibatidine-induced contractions prior to antagonist treatment. Asterisks indicate a significant difference from the control value: **P*<0.01; ***P*<0.005.

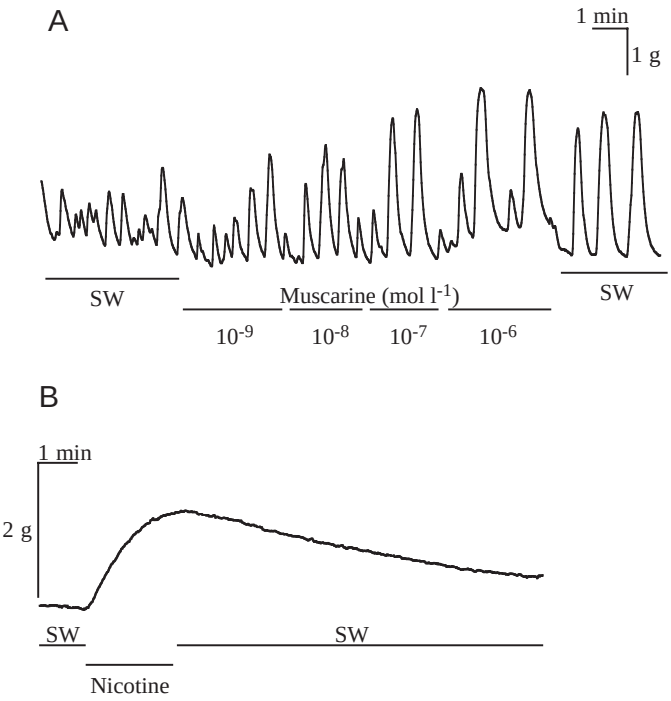

Fig. 9. Different effects of muscarinic and nicotinic agonists on the contractility of the longitudinal muscle of the body wall (LMBW) from the holothurian *Sclerodactyla briareus*. (A) Muscarine (and other muscarine agonists) have the typical effect of initiating or enhancing rhythmic contractions of the LMBW. In the present example, muscarine increases rhythmic contractions in a dose-dependent manner (range tested, 10^{-9}–10^{-6} mol l^{-1}). (B) Nicotine (and other nicotinic agonists) elicits broad, sustained contractions of the LMBW, but never stimulates rhythmicity. SW, artificial sea water.

pharmacological similarities between echinoderm mAChRs and the M1-M5 receptor subtypes found in the vertebrates.

A general effect of the muscarinic agonists is either an initiation or amplification of rhythmic contractions (Fig. 9A). This response is never observed during treatment with the nicotinic agonists, which stimulate only broad tonic contractions (Fig. 9B). The agonists muscarine and oxotremorine and the antagonist pirenzepine all have a common action of initiating rhythmic contractions and enhancing the basal tone of the LMBW (Devlin et al., 2000). Oxotremorine (M2/M4 agonist) and pirenzepine (M1 antagonist) both act as dual 'antagonists/agonists,' relaxing the tone of the holothurian LMBW at lower concentrations while stimulating phasic activity at higher concentrations. Oxotremorine, however, is completely without effect on the dentis retractor muscle of the sea urchin *Strongylocentrotus intermedius* (Kobzar and Shelkovinkov, 1985a) and on the pharynx protractor muscle of holothurian *Cucumaria japonica* (Shelkovinkov et al., 1977). The M2/M4 antagonist methoctramine has two distinct inhibitory actions on the holothurian LMBW. First, it causes the ACh-induced contraction to relax and return more quickly to resting tone, and second, it decreases the maximum amplitude of the ACh-induced contraction.

The M5 agonist pilocarpine is the most potent of the agonists tested in potentiating ACh-induced responses in the LMBW. Alone, pilocarpine does not initiate rhythmic waves or stimulate tonic contractions, as do some of the other muscarinic agonists tested. Similarly, pilocarpine has no effect on the isolated cloaca and LMBW of the holothurian *Stichopus moebii* (Wyman and Lutz, 1930), on the lantern retractor muscles from *Echinometra lucunter* (Mendes et al., 1970), or on the pharynx protractor muscle of holothurian *Cucumaria japonica* (Shelkovinkov et al., 1977).

These pharmacological data reveal the presence of distinct mAChR subtypes in the echinoderms that bear some resemblance to, and are perhaps precursors of, the M1-M5 receptors of the vertebrates. The location of mAChRs at the echinoderm NMJ has not been investigated. In other invertebrate and vertebrate preparations, both pre- and postsynaptic actions of various muscarinic agonists have been reported. The presynaptic action of the muscarinic agonists includes depolarization and stimulated burst activity in arthropod nerve preparations (crustacean, Braun and Mulloney, 1993; insect, David and Pitman, 1995; insect, Parker and Newland, 1995) and enhanced ACh release from molluscan neurons (Fossier et al., 1988). In contrast, presynaptic mAChRs (sensitive to the M2/M4-selective antagonists methoctramine) in another arthropod (insect) nerve preparation are autoreceptors that the inhibit release of ACh

onto the postsynaptic effector cells (Trimmer, 1995). Postsynaptic mAChRs in the insect central nervous system mediate excitatory, depolarizing responses (Trimmer, 1995). Postsynaptic mAChRs also stimulate contraction in locust foregut (Wood et al., 1992) and in smooth muscle of the body wall of ascidians (Kobzar and Shelkovnikov, 1985b).

Conclusions

The pioneer echinoderm muscle physiologists of the 1930s realized that the holothurian LMBW preparation would prove a valuable model for the study of the cholinergic control of smooth muscle (Welsh, 1966). It later became a system used in the study of Ca^{2+} mobilization during the process of excitation–contraction coupling (Hill, 1993). More recently, this classic echinoderm model has re-emerged as an excellent NMJ preparation in which to investigate dual GABAergic/cholinergic control of smooth muscle. The holothurian LMBW bears numerous striking similarities to the smooth muscle of the guinea pig colon in terms of neural control. First, GABA and baclofen cause dose-dependent relaxation of the holothurian LMBW, an effect also seen in the guinea pig ileum (Giotti et al., 1985; Minocha and Galligan, 1993; Ong et al., 1987). Moreover, ACh-induced contractions of both the LMBW (Devlin and Schlosser, 1999) and the smooth muscle of the guinea pig ileum (Ong et al., 1987) and bladder (Maggi et al., 1985) are blocked by GABA. Muscimol, which potentiates ACh-induced contractions in the holothurian LMBW, stimulates ACh release from neurons of the myenteric plexus innervating the intestinal smooth muscle of the guinea pig and so enhances contractility (Taniyama et al., 1988). Finally, in both the holothurian LMBW (Devlin and Schlosser, 1999) and guinea pig ileum (Kleinrok and Kilbinger, 1983) there is pharmacological evidence for distinct GABA A and GABA B receptors that appear to have opposing actions. We found that the echinoderm GABA A receptors are typically excitatory in nature, whereas the GABA B receptors are inhibitory; these are features of the GABA A and B receptors of the guinea pig myenteric plexus-smooth muscle preparation as well (Ronai et al., 1987). Because of these numerous parallels with mammalian intestinal smooth muscle, the holothurian LMBW can be viewed in a new light and studied as an evolutionary precursor to later-appearing deuterostome smooth muscle systems.

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