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NEUROMUSCULAR BLOCKING EFFECTS OF AN ALKALOIDAL EXTRACT FROM *INULA ROYLEANA:* CONTRACTILE AND ELECTRICAL STUDIES ON AMPHIBIAN SKELETAL MUSICLE *IN VITRO*

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Abstract : The neuromuscular blocking properties of an alkaloidal extract from the root of *Inula royleana* have been investigated *in vitro* using a combination of mechanical and electrophysiological approaches. Neurogenic twitches of the frog sartorius were profoundly inhibited by concentrations of the extract ≥ 20 µg/ml, being reduced to 50% of control amplitude in ~90 s at a concentration of ≥ 20 µg/ml. They were partially reversed by neostigmine (6 µg/ml), and by prolonged washout of the extract. Muscle surface action potentials, recorded with extracellular electrodes, also declined rapidly in amplitude in the presence of the extract. Direct muscle stimulation during inhibition by the extract elicited contractions and action potentials whose magnitudes were similar to control responses. Resting membrane potentials, and the intracellular input impedance of the skeletal muscle cells, were not significantly changed by the alkaloids. These results indicate that the extract has significant neuromuscular blocking activity of a partially or slowly reversible nature. The block appears to be exerted at the postjunctional end-plate nicotine receptors, thus offering promise for the identification of novel cholinergic receptor antagonist(s).

Key words : neuromuscular junction nicotinic receptor antagonist skeletal muscle *Inula royleana*

INTRODUCTION

The physiological and biochemical effects of extracts obtained from plants used in traditional Indian medicine are receiving increasing attention these days. Plants of the genus *Inula* possess a number of physiologically active constituents (1–3). An alkaloidal extract from the root of the Himalayan perennial herb *Inula royleana* was reported to have curariform activity when tested *in vivo* on the contractions of the canine gastrocnemius (4) or on other indices of curariform activity in dogs and rabbits (5). However, the studies conducted were exploratory, and detailed investigation of the characteristics of inhibition and mechanism of action have not been carried out. Since the alkaloidal extract of *Inula royleana* may hold promise for the identification of a novel

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blocker (or blockers) of the neuromuscular and other cholinergic nicotinic synapses, further elucidation of its action is warranted.

Therefore, experiments were conducted on contractile as well as electrical properties of amphibian isolated nerve-muscle preparations *in vitro* to explore more fully the actions of the extract. The contractile studies indicate that the extract possesses significant neuromuscular blocking activity, and that this activity can be explained by blockade of postjunctional cholinergic nicotinic receptors. Electrophysiological studies rule out the possibility of depolarising block and of block achieved by non-specific action of the alkaloids on muscle membrane ionic channels.

METHODS

Alkaloid extraction, tissue preparation and contractile studies

The alkaloidal extract of *Inula royleana* root was obtained using standard procedures. Briefly, 0.5 kg of the powdered root (procured from M/s United Chemicals & Allied Products, Calcutta) was dissolved in ethanol, filtered, and the filtrate evaporated. The residue was dissolved in ethyl acetate and extracted with 1N HCl. The HCl layer was basified and the liberated alkaloids were extracted with ethyl acetate, which was subsequently evaporated to provide the alkaloidal extract.

Physiological experiments were performed on twenty-one isolated sciatic nerve-sartorius muscle preparations of the leopard frog (*Rana pipiens*) at room temperature. Care was taken to dissect a sufficiently long length of nerve to provide slack length and prevent undue strain while mounting on stimulation electrodes. For contractile studies the muscle was mounted in an 80 ml capacity vertical glass organ bath and continuously superfused with aerated Ringer's solution of the following composition (mM) (6): NaCl 115.0, KCl 2.5, $CaCl_2$ 1.8, Tris HCl 5.0 to maintain the pH of the solution around 7.5. The pelvic end of the muscle, which was dissected along with a fragment of the pelvic bone, was attached to the horizontal member of an immovable glass rod placed at the lower end of the chamber. The knee end was attached to the hook of an isometric force transducer using a polypropylene suture thread passed through the tendon. The transducer output was fed to an amplifier and paper chart recorder (Polyrite, Recorders & Medicare Ltd, Chandigarh). The nerve was placed gently on a pair of Ag/AgCl wire stimulation electrodes mounted in the bath. Nerve stimulation was carried out by delivering rectangular voltage pulses (amplitude 1–9.5 V, pulse width 0.01–0.20 ms) at a frequency of 1 Hz from an electronic stimulator developed in-house. Experiments were carried out after increasing stimulation strength to about 50% more than that required to elicit just-supramaximal twitches. To introduce or wash out the extract, the bath solution was completely replaced before subsequent superfusion with the new solution.

Electrophysiology

For electrical studies the preparation was mounted, at approximately *in vivo* length, in a wax bottomed horizontal perspex organ

bath. The muscle was pinned down at its extremities and was superfused with aerated Ringer's solution at 2–3 ml/min at room temperature. The solution was fed by gravity and led off by suction using a suction pump. Ag/AgCl stimulating electrodes were used to stimulate the nerve. Change to experimental solution was effected by switching the input between two reservoirs, without complete replacement of organ bath solution. This was done to ensure that the relationship of the recording electrodes to the tissue surface (in the case of extracellular recording), which influences the strength of signal recorded, did not change during the recording and hence introduce artefactual data.

(a) Extracellular electrical recordings were obtained using bipolar Ag/AgCl wire electrodes, insulated up to 1 mm from the tips, placed on the surface of the muscle and moved using a micromanipulator (MMJ, Helmut Hund GmbH, Germany). The signals were fed to an in-house developed a.c. amplifier, displayed on an oscilloscope, and simultaneously collected on an IBM-PC compatible 80386- or 80486-based computer. Signals were band-pass filtered with-3 dB cutoff frequencies of 0.1 Hz (high-pass) and 1.2 KHz (low-pass), and digitized at 3 or 4 KHz using a PCL 208 data acquisition card. Custom-made software developed in this laboratory (7), which allowed triggering of collection by the threshold-detection method, was used for collection and display of data. Data could also be read, displayed and plotted using the software package SCAN (Synaptic Current Analysis) kindly supplied by Dr J. Dempster, University of Strathclyde, Glasgow (8).

(b) Intracellular recordings: These were made using conventional filamented capillary-glass microelectrodes filled with 3M KCl (9–10) and mounted on a micromanipulator (M33, Helmut Hund GmbH, Germany). The microelectrodes were pulled on a Brown-Flaming type horizontal puller (P-87, Sutter Instruments Corp., USA) and had tip resistances in the range 10–50 MΩ. Intracellular measurements were made with reference to an Ag/AgCl pellet in the bath. The signals were fed to the high input impedance (10^{11} Ω) probe of an intracellular electrometer (IE201, Warner Instrument Corp., USA). For resting membrane potentials (RMPs) the following criteria were used for accepting measurements: i) the impalement should be abrupt, ii) the membrane potential should not depolarize after the impalement, iii) the RMP should be stable for at least a minute before taking a reading (11). The electrometer allowed the use of an active bridge for the measurement of input impedance, such that microelectrode tip impedance could be nulled before measurement of cell input impedance (10). The same criteria were followed as for the measurement of RMPs, with the addition that data from only those cells were accepted in which microelectrode impedance did not show a change of more than 15% between the values before insertion into the cell and after withdrawal from it (10).

All data are expressed as mean ± S.E.M, (no. of observations). Student's unpaired t-test was used to assess the statistical significance of the difference between means, a P value of <0.05 being taken to indicate a statistically significant difference (12).

RESULTS

Contraction studies

In all 11 experiments on muscle force generation, the extract was found to inhibit supramaximal contractions at doses equal to or greater than 20 µg/ml. Fig. I shows the effect in a representative experiment of a 50 µg/ml solution (a) and of a 20 µg/ml solution (b) of the alkaloidal extract on supramaximal twitches. Contractions were rapidly and monotonically diminished (time of reduction to 50% of initial amplitude: ~ 40 s for 50 µg/ml, ~ 90 s for 20 µg/ml). They were ultimately abolished, in a manner similar to that reported for conventional non-depolarizing blockers, such as d-tubocurarine and gallamine (both at 1 µg/ml), investigated in pilot studies. The extract had no effect on baseline tension, indicating a non-depolarising action. In separate experiments, the extract-induced inhibition was ascertained not to be due to muscle fatigue, as the muscle could maintain supramaximal twitch force at this frequency of stimulation (1 Hz) continuously for up to 20 min., both before as well as after the action of the extract.

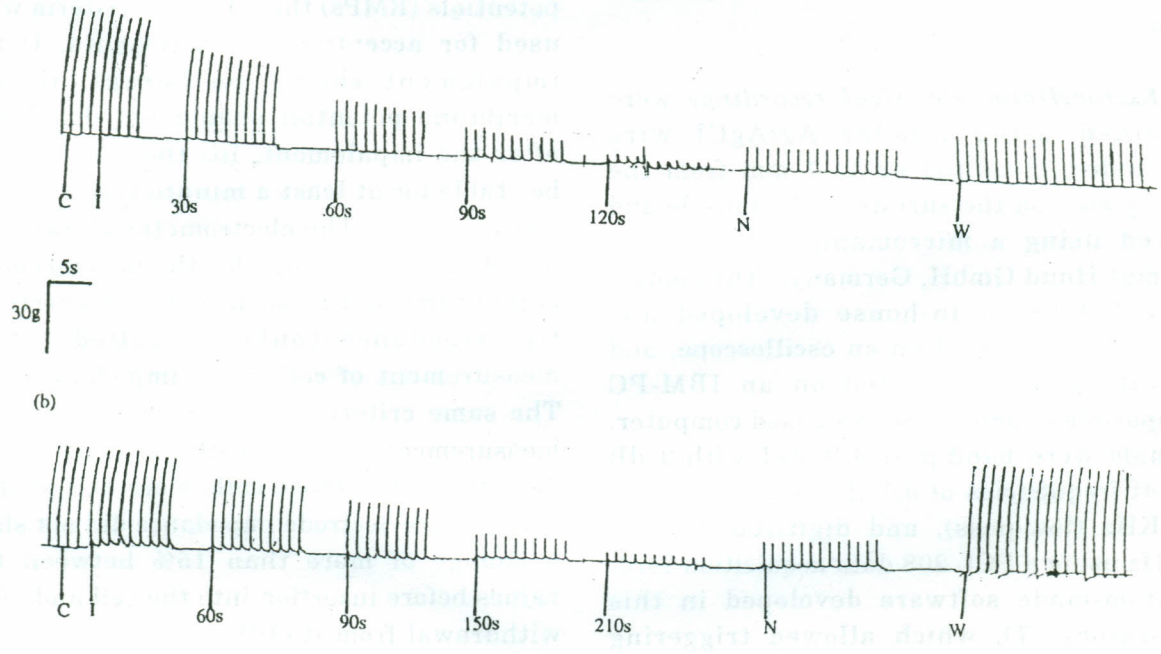

Fig. 1: Effect of the alkaloidal extract on supramaximal twitches at various time intervals after its addition (C-control twitches; I-Introduction of extract). Extract added at concentrations of 50 µg/ml (a) and 20 µg/ml (b). Note partial recovery of twitches after addition of neostigmine (N) (µg/ml) for both concentrations of extract. Also note partial restoration of magnitude of twitches on washing (40 min.) (W), after exposure to 50 µg/ml of extract, and complete recovery of twitches on washing (45 min.) after exposure to 20 µg/ml of extract.

Neostigmine at a concentration sufficient to inhibit junctional acetylcholinesterase (6 µg/ml) (6, 11) only partially restored the contractions, to 20% of control amplitude after exposure to 50 µg/ml extract (Fig. 1a, "N") and to 25% of control after exposure to 20 µg/ml extract (Fig. 1b, "N"). Prolonged washout ("W") of the extract succeeded in restoring twitches to their control amplitude after exposure to 20 µg/ml extract but not 50 µg/ml (Fig. 1). No reduction of twitch force was observed when 10 µg/ml of the extract was administered, illustrating the concentration dependence of the effect. We therefore decided to use the extract at a concentration of 20 µg/ml in most of the subsequent experiments.

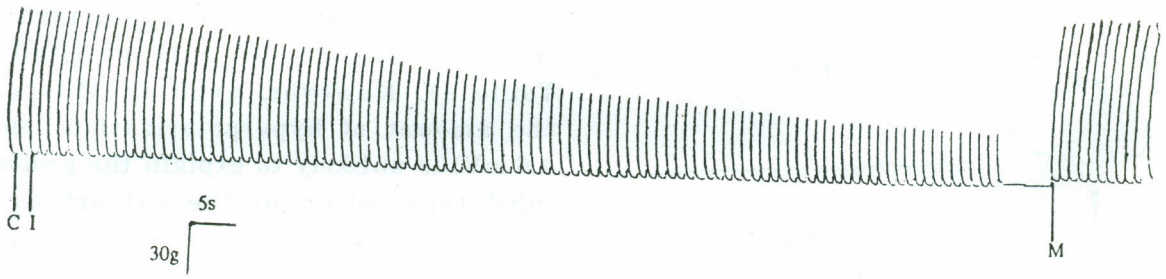

Fig. 2: Contractions evoked by direct muscle stimulation (M) following prior inhibition of neurogenic twitches by the alkaloidal extract (20 µg/ml) (C-control twitches; I-Introduction of extract).

To test whether the suppression of force was mediated by an action on non-receptor mechanism, e.g. voltage-sensitive Na^+ or K^+ channels of the muscle, stimulation electrodes were placed directly on the muscle after the extract had exerted a substantial block, depressing the twitch to about 33% of control amplitude. As shown in Fig. 2, the twitches obtained following direct muscle stimulation ("M") matched in amplitude the supramaximal twitches with nerve stimulation elicited before the extract was added.

Electrophysiology

(a) Extracellular recording: The extract was found to suppress markedly the nerve-evoked muscle electrical activity recorded extracellularly in all trials (5 experiments), in the manner expected of a non-depolarizing blocker (13). Fig. 3 shows the results from one such experiment using the extract at a concentration of 20 µg/ml. Muscle action potentials (Fig. 3a) were reduced to about 60% of control value 4 min. after the introduction of solution containing the extract (Fig. 3b), and to about 45% after 9 min. (Fig. 3c). Although the electrical activity was suppressed substantially in less than 9 minutes, this effect is not as rapid as that observed in the experiments on contractions. This may have been because of differences in the manner of introduction of experimental solution in the contractile and electrical studies (see above). However, the inhibition was qualitatively very similar to that obtained on the contractions. Direct muscle stimulation resulted in reappearance of the action potentials comparable in magnitude to control signals (Fig. 3e), again providing evidence against a non-specific effect "downstream" of cholinergic receptor antagonism.

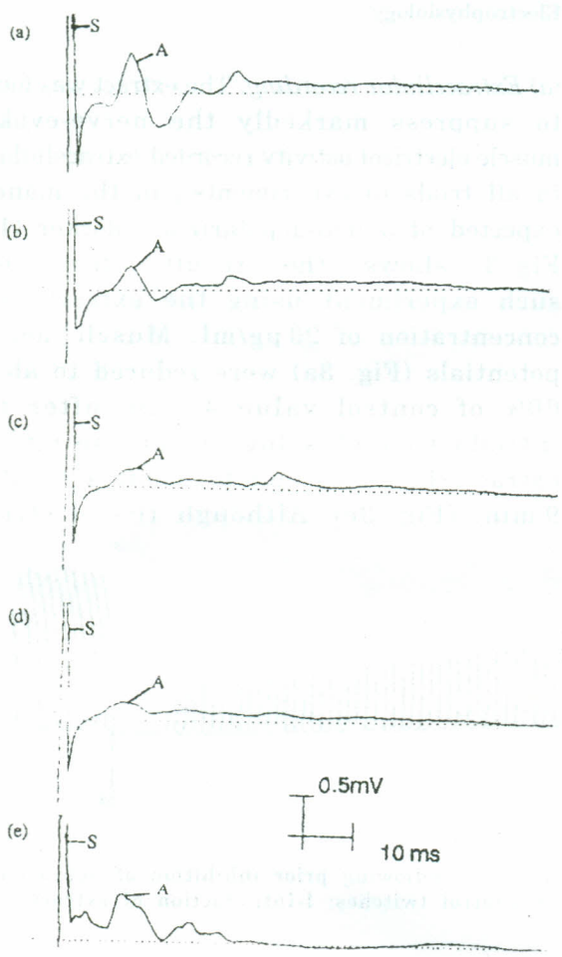

Fig. 3: Effect of 20 µg/ml solution of the alkaloidal extract on the magnitude of nerve-stimulation evoked muscle action potentials ("A" in a-d), recorded extracellularly, at various intervals of time after addition of extract. (a): Control; (b): 4 min.; (c): 9 min.; (d): 11 min. (e): Reappearance of action potentials with direct muscle stimulation during inhibition by extract. All figures are averages of 8–10 records. S indicates the stimulus artifact. The long latency, low amplitude traces in a, b, and c, observed after the action potentials marked A, are probably recordings of different action potentials arising deeper in the muscle from separate groups of muscle fibres (as suggested by their different latencies) and attenuated in amplitude.

(b) Intracellular recording: To test whether the block exerted by the extract was due to persistent depolarization, measurements of resting membrane potentials (RMP) and input impedance were made. In four experiments, at concentrations which rapidly blocked the neurogenic contractile response and the surface action potentials (20 and 40 µg/ml), the extract had no depolarizing effect on the muscle fibres (Table I). Whilst in three trials, there was no significant difference between RMPs obtained before and after the addition of the extract ($P>0.01$), in one trial, RMPs were slightly, but significantly, hyperpolarized in the presence of the extract ($P<0.05$, Table I). However, even in this case the amount of hyperpolarization (about 4.6 mV) is unlikely to explain the profound inhibitory action of the extract by an effect on resting membrane potential alone.

To see if passive membrane properties were affected independently of effects on resting membrane potential, experiments were carried out on the effect of the extract on intracellular input impedance of the skeletal muscle cells. In two trials, using extract concentrations of 20 µg/ml in one and 40 µg/ml in the other, the input impedance was observed not be altered in the presence of the extract (Table II). The fact that the block is not caused by persistent depolarization is also indicated by the studies on contractile responses, where the extract was observed to have no effect on baseline tension.

TABLE I: Values of the resting membrane potential (RMP, expressed as mean ± S.E.M.) before and after addition of the alkaloidal extract. Number of observations in brackets. Significant difference indicated by *(P<0.05).

Expt.	*Concentration of extract (µg/ml)*	*RMP (mV)*	
		Control	*After addition of the extract*
1	40	−80.64±5.04 (15)	−84.04±2.85 (15)*
2	40	−84.21±5.05 (15)	−83.8±7.58 (15)
3	40	−81.76±4.55 (11)	−79.05±6.62 (5)
4	40	−79.09±11.43 (16)	−81.06±8.76 (14)

TABLE I: Values of intracellular input impedance (mean ± S.E.M.) before and after addition of the alkaloidal extract. Number of observations in brackets. The differences were not statistically significant (P>0.1).

Expt.	*Concentration of extract (µg/ml)*	*Input impedance (MΩ)*	
		Control	*After addition of the extract*
1	20	6.19±4.16 (11)	8.73±6.68 (5)
2	20	9.68±3.66 (12)	11.02±1.70 (11)

DISCUSSION

The present observations confirm that the alkaloidal extract of *Inula royleana* possesses striking paralysant activity on skeletal muscle, most probably via antagonism of postjunctional cholinergic receptors. We discuss below the salient features of the antagonism and the likely mechanism and site of action of the active constituents of the extract.

Features of antagonism

Marked reduction in the force of contraction after superfusion with the extract-containing solution was obtained in each of the 11 experiments performed, and the inhibition was concentration-dependent. Jamwal and Anand (4) reported in their studies with the alkaloidal extract from *Inula royleana* that the curariform activity of the extract was weaker than that of the "classical" nicotinic cholinoceptor antagonist, d-tubocurarine. In their *in vivo* experiments on canine gastronemius a dose of 1.2 mg/kg of the alkaloidal extract produced quantitatively the same effect as 0.2 mg/kg d-tubocurarine (d-TC). This appears also to be the case from our *in vitro* experiments on frog sartorius: while d-TC and gallamine were effective at a concentration of 1 µg/ml, the extract was ineffective at 10 µg/ml and only effective at concentrations ≥20 µg/ml. However, a direct comparison with the potency of the established competitive antagonists is not yet feasible as the identity of the active compounds in the extract is unknown. Furthermore, the extract seems to have more than one active component (see below), and the concentration at which the individual components are effective will need

to be examined.

The reduction in the peak amplitude of the contractions by the alkaloidal extract was not accompanied by an upward shift in baseline force. Thus, the extract *per se* does not cause a "background" contraction of the muscle that would mask the peak force developed. This indicates that the extract probably does not exert depolarizing block (14), as confimed by the electrical studies (see below).

The onset of effect of the extract on the contractions is rapid and comparable with that of established neuromuscular blockers such as d-tubocurarine or gallamine, or of the narcotic analgesic morphine (15). Our data do not indicate a rapid reversibility of the block exerted by the extract, a property not examined in earlier studies (4–5). In the contractile experiments the effects of the extract were reversed only after washing for 30–45 minutes. Furthermore in the contractile studies reversal with neostigmine was only partial, and this indicates an irreversible or slowly reversible component of the antagonism. Preliminary data obtained with different fractions of the alkaloidal extract indicate the presence of three or four active constituents, one or more of which appear to exert a relatively slowly reversible block while the others appear to exert a more readily reversible block (Manchanda R., Bhat S.V., & Tiwari D., unpublished observations). In the unfractionated extract used here, the slowly reversible component of block would have necessarily masked the readily reversible one, and delayed the recovery of twitches on washout.

Mechanism and site of action

Inhibition or blockade of skeletal muscle contraction can be caused by any one of several mechanisms other than antagonism at postjunctional receptors, both prior to and following receptor activation. The blocking agent may cause interference with acetylcholine release mechanisms, or may act at a site downstream of receptor activation, such as muscle fibre action potential generation, excitation-contraction coupling, and the activation of the contractile machinery following the release of Ca^{2+} from the sarcoplasmic reticulum (16, 17). Our results would appear to exclude post-receptor events as being the site of action of the extract. At stages when the neurogenic contractile or electrical activity has been inhibited profoundly by the alkaloids, direct stimulation of the muscle successfully elicited both action potentials (Fig. 3) and contractions (Fig. 1), these responses occurring at amplitudes up to maximal neurogenic levels. The persistence of action potentials indicates that the extract does not effect the voltage-gated Na^+ and K^+ channels in the muscle membrane responsible for action potential generation and propagation. The persistence of contractile activity shows that excitation-contraction coupling and subsequent processes remained unaffected by the extract (16, 17).

Closer examination of the actions of the extract, using intracellular recording of RMPs and input impedance, also shows that the inhibition could not be attributed to non-specific electrophysiological effects. The resting potential of amphibian skeletal

muscle fibres is determined predominantly by a background K^+ permeability (18), with some contribution from a Na^+ permeability. A decrease in this permeability or an increase in Na^+ permeability due to the extract can lead to alterations in membrane electrical properties, which in turn can lead to subtle effects on the operation of voltage-gated channels. This could ultimately affect contractile activity. However, in either case the input impedance of the cell would be expected to change (10), and we have eliminated this possibility by showing that the extract has no effect on input impedance.

Taken together, these findings suggest strongly that the alkaloidal extract explored here interferes with cholinergic neurotransmission by exerting a specific blocking action on the postsynaptic nicotinic receptors rather than any site "downstram" of receptor antagonism.

In conclusion, the alkaloidal extract from *Inula royleana* appears to hold promise for the isolation of one or more fairly rapidly acting skeletal neuromuscular blocker(s). However, much remains to be done in order to characterize more thoroughly its actions. Previous chemical analysis of the chemical constituents of the *Inula* root have identified its terpenoid components (19), as well as a few alkaloidal ones (20). However, the curariform activities of individual alkaloidal constituents are unknown. Preliminary data from our laboratory indicate the presence in the extract of four principal fractions with differing spectra of neuromuscular blocking activity, and these will need to be evaluated and chemically characterised individually. Detailed studies, including those at the level of end-plate potentials and currents (21), will be required to delineate properties such as reversibility, competitiveness of block (22–23), and any presynaptic component of action, such as that suggested for d-tubocurarine (24–25). Such issues are currently being addressed in our laboratory.

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