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The Membrane Hyperpolarization of Rat Dorsolateral Septal Nucleus Neurons is Mediated by a Novel Nicotinic Receptor[1]

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ABSTRACT

The pharmacology, calcium dependence and G protein mediation of the membrane hyperpolarization of rat dorsolateral septal nucleus (DLSN) neurons in response to nicotinic agonists was examined to classify the nicotinic receptor mediating the response. Intracellular recording from DSLN neurons in a brain slice preparation was used to determine whether chlorisondamine, trimethaphan, cytisine or strychnine inhibited the membrane hyperpolarization in response to application of the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP). Chlorisondamine was found to block the response only at a high concentration (500 μM) although strychnine (100 μM) was without effect. Cytisine was neither an effective agonist nor an antagonist (500 μM). Surprisingly, trimethaphan appeared to act as an agonist, rather than an antagonist, with a potency and efficacy similar to that reported for nicotine at this receptor. The response was dependent on intracellular calcium stores because it persisted in the absence of extracellular calcium but was blocked by intracellular injection of 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA). Injection of GTPγS into the neurons blocked the nicotinic response. Apamin, iberiotoxin and charybdotoxin reduced but did not block the response at concentrations that selectively block calcium-dependent potassium channels. These results indicate that the nicotinic response in DLSN neurons may be mediated by a metabotropic nicotinic receptor coupled to a calcium-dependent potassium channel through the activation of a G-protein and release of intracellular calcium stores. The unusual pharmacology of the nicotinic receptor on DLSN neurons indicates that it may be a novel receptor which has yet to be cloned.

Nicotine is known to have both pre- and postsynaptic actions in the central nervous system (Sargent, 1993). Activation of presynaptic nicotinic receptors on nerve terminals modulates the release of neurotransmitters, usually increasing neurotransmitter release, and will be seen as either an excitatory or inhibitory response at the postsynaptic neuron depending on the specific neurotransmitter involved (Somogyi and De Groat, 1992; McMahon *et al.*, 1994; McGehee *et al.*, 1995). Direct activation of nicotinic receptors on postsynaptic cells is usually excitatory, resulting in a membrane depolarization accompanied by a decrease in the input resistance due to the net influx of cations through the open nicotinic ion channel (Sorenson and Chiappinelli, 1990; Mulle *et al.*, 1991; Alkondon *et al.*, 1992; Alkondon and Albuquerque, 1993). The postsynaptic excitatory response is sometimes followed by a membrane hyperpolarization (Housley and Ashmore, 1991; Mulle *et al.*, 1992). Both the presynaptic increase in neurotransmitter release and the postsynaptic inhibitory responses that follow the initial excitatory inward current through the nicotinic channel appear to be the result of increased intracellular calcium concentrations (Fuchs and Murrow, 1992; Mulle *et al.*, 1992; Vernino *et al.*, 1992; Vijayaraghavan *et al.*, 1992; McMahon *et al.*, 1994; Vernino *et al.*, 1994; Mollard *et al.*, 1995).

This laboratory has previously described a novel membrane hyperpolarization of DLSN neurons in response to nicotinic agonists (Wong and Gallagher, 1989, 1991). Briefly, acetylcholine in the presence of atropine, DMPP or nicotine produce a direct membrane hyperpolarization accompanied by a decrease in input resistance. A direct action of the nicotinic agonists was supported when synaptic responses were abolished by low calcium buffer but the DMPP hyperpolarization, although attenuated, persisted. Therefore the hyperpolarization in response to nicotinic agonists appears to be due to postsynaptic actions that may be calcium dependent. Additional evidence that the hyperpolarization is not due to the activation of classical nicotinic receptors was obtained by receptor inactivation studies. The brain slice was treated with the reducing agent dithiothreitol to inactivate

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ABBREVIATIONS: ABgT, α-bungarotoxin; ACSF, artificial cerebral spinal fluid; CYT, cytisine; DHβE, dihydro-β-erythroidine; DLSN, dorsolateral septal nucleus; DMPP, 1,1-dimethyl-4-phenylpiperazinium; KBgT, κ-bungarotoxin; nAChR, nicotinic acetylcholine receptor; TMP, trimethaphan camsylate; TTX, tetrodotoxin.

any excitatory nicotinic receptors (Sorenson and Gallagher, 1993b). All known α nicotinic receptor subunits contain a conserved cysteine pair at positions 192 and 193 in their amino acid sequence (Sargent, 1993). Treatment of excitatory receptors with dithiothreitol has been shown to reduce the disulfide bond formed by these cysteines at the agonist binding site, inactivating the nicotinic receptors (Kao *et al.*, 1984). Treatment of DLSN slices with dithiothreitol before applying nicotinic agonists did not block the hyperpolarizing response (Sorenson and Gallagher, 1993b). Control experiments demonstrated that the reducing treatment was effective in blocking other excitatory nicotinic responses. Therefore the response does not appear to be the result of the activation of classical excitatory nicotinic receptors on presynaptic terminals in the DLSN.

The unusual pharmacology of the hyperpolarization response supports a receptor structure distinct from classical nicotinic receptors (Wong and Gallagher, 1989, 1991). The nicotinic hyperpolarization was blocked by mecamylamine (50 μM) and KBgT (0.2 μM), both ganglionic nicotinic antagonists (Wong and Gallagher, 1991). Mecamylamine acts predominantly by blocking the nicotinic receptor ion channel (Banerjee *et al.*, 1990) whereas KBgT binds at the agonist binding site (Chiappinelli, 1983). Because DMPP, a ganglionic agonist, is a potent agonist and KBgT is an antagonist at this receptor site, the nicotinic receptor on DLSN neurons may have structural similarities to the agonist binding sites on the ganglionic class of nicotinic receptors.

We have tested the ganglionic nicotinic antagonists chlorisondamine and trimethaphan to see if they block the membrane hyperpolarization in response to DMPP. The ganglionic agonist CYT has also been tested for both agonist and antagonist properties at this receptor. CYT is reported to be a partial agonist at nicotinic receptors containing β2 subunits and an agonist at receptors containing β4 subunits or ABgT-sensitive receptors (Luetje and Patrick, 1991; Bertrand *et al.*, 1992; Amar *et al.*, 1993; Papke and Heinemann, 1994). Finally, we have examined the effects of strychnine because it has been reported to block α_7 homomers expressed in oocytes (Anand *et al.*, 1993) and the membrane hyperpolarization of cochlear hair cells in response to nicotinic agonists (Housley and Ashmore, 1991).

In addition, we have further examined the mechanism by which nicotine action is coupled to the efflux of potassium. We now confirm that the response requires the presence of intracellular calcium and demonstrate that calcium from intracellular stores appears to be sufficient to produce a response. We have also injected GTPγS to see if the hyperpolarization to nicotine is blocked by permanent activation of G proteins. The type of potassium channel involved was also further examined by comparing the effects of apamin, iberiotoxin and charybdotoxin. These results have appeared in abstract form (Sorenson and Gallagher, 1992; Sorenson and Gallagher, 1993a; Sorenson and Gallagher, 1994).

Methods

Male Sprague-Dawley rats were decapitated and 500-μm slices were obtained as previously described (Stevens *et al.*, 1984). Standard intracellular recording techniques were used to record from submerged brain slices at 32°C. The ACSF, consisting of 117 mM NaCl; 4.7 mM KCl; 1.2 mM $MgSO_4$; 1.2 mM NaH_2PO_4; 2.5 mM $CaCl_2$; 25 mM $NaHCO_3$ and 11.5 mM glucose, was superfused at a rate of 2 ml/min and bubbled continuously with 95% O_2 and 5% CO_2. Zero calcium buffer was made by substituting 2.5 mM $MgCl_2$ for the $CaCl_2$ and adding 1 mM EGTA. The final concentration of added Mg^+ in the zero calcium buffer was 3.7 mM. Microelectrodes were filled with 2 to 4 M potassium acetate and typically had resistances between 80 to 130 MΩ. Signals were amplified by an Axoclamp-2A amplifier (Axon Instruments, Inc., Axon Instruments, Foster City, CA) and continuously displayed on an analog oscilloscope (Tektronix, Tektronix, Wilsonville, OR). Data were recorded on a Gould chart recorder and a Vetter video cassette instrumentation recorder for off-line analysis. Membrane resistance was monitored by passing brief hyperpolarizing current pulses through the microelectrode. The bridge balance was monitored and adjusted during the delivery of current pulses. A DC current was passed to bring the cell to a common membrane potential, usually between -60 and -65 mV, before application of drugs, so that the resulting voltage changes could be compared among neurons. Some experiments examining the ef-

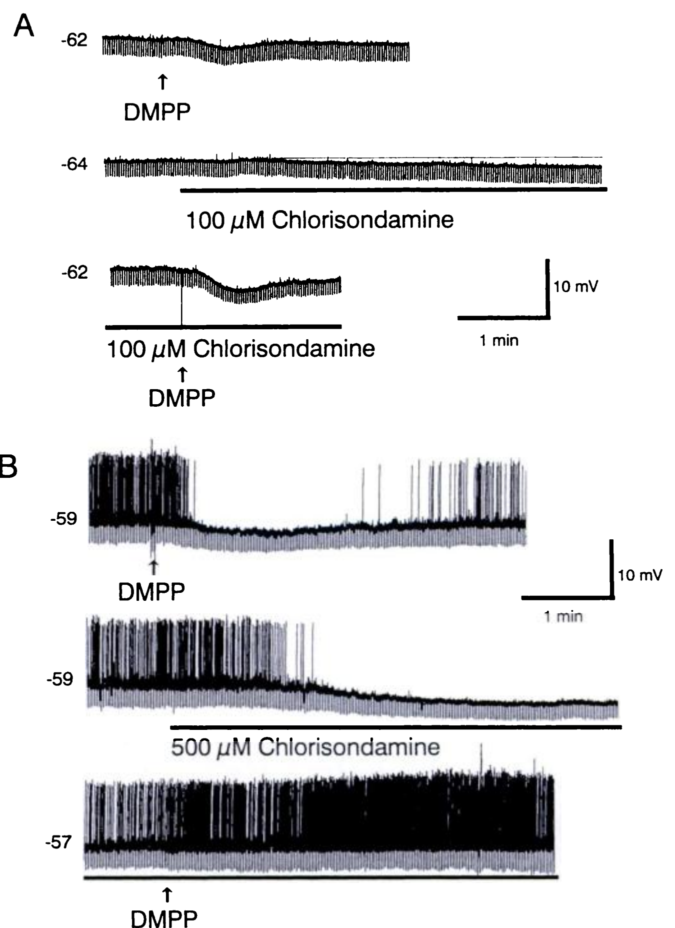

Fig. 1. A) The records in (A) were obtained from a DLSN neuron in the presence of 1 μM TTX. The top trace is a control response of a DLSN neuron to a drop of 10 mM DMPP. Superfusion with 100 μM chlorisondamine produced a membrane hyperpolarization (middle trace). After 30 min in the chlorisondamine, DMPP still produced a hyperpolarization. B) Increasing the concentration of chlorisondamine to 500 μM did block the nicotinic response in most, but not all neurons (four of five). The top trace is a control response to a drop of 10 mM DMPP. In the middle trace, it can be seen that 500 μM chlorisondamine produces a large hyperpolarization of the membrane potential. In the bottom trace, the reapplication of DMPP no longer produces a hyperpolarizing response in this neuron. The mean response of five neurons to DMPP in the presence of 500 μM chlorisondamine was 1.9 ± 0.9 mV and that is significantly lower than 4.5 ± 1.732 mV, the mean hyperpolarization in control ACSF ($P < .05$; one-tailed, paired t test).

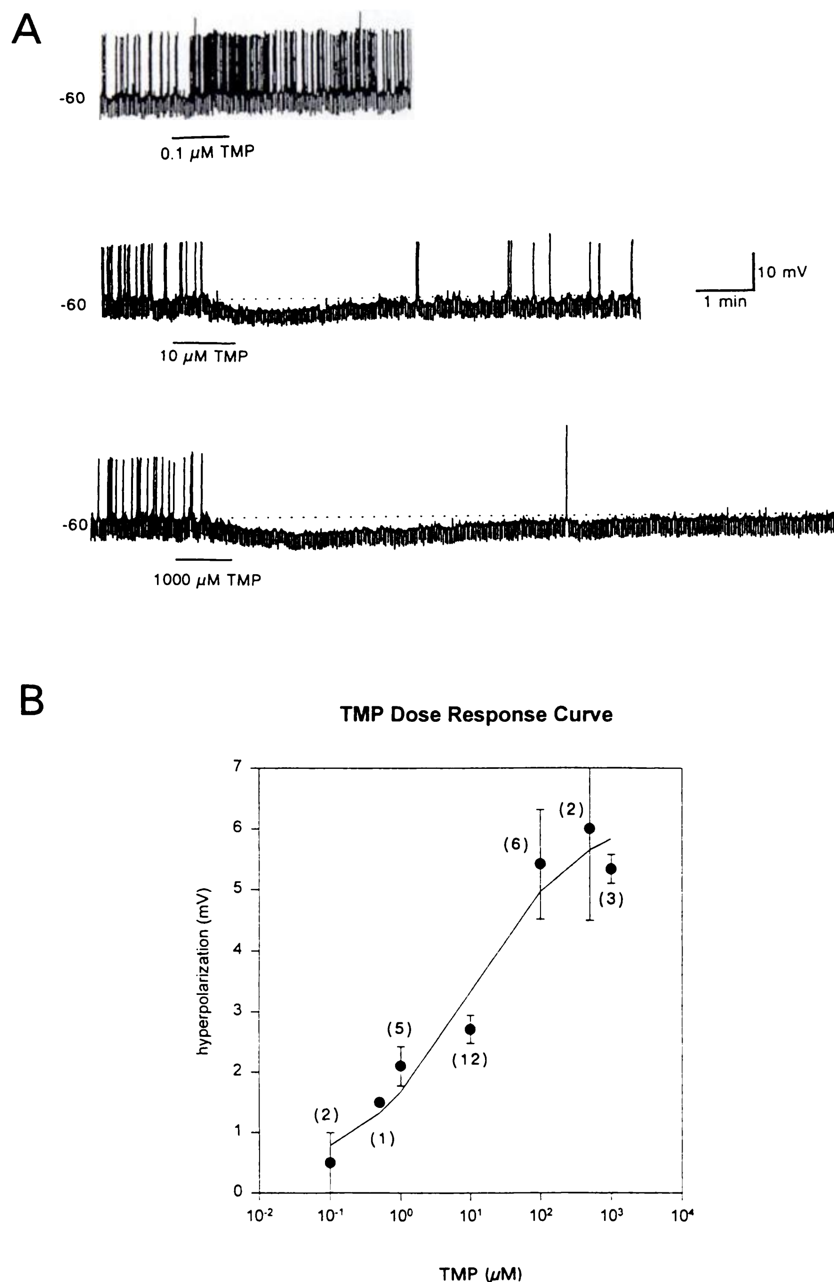

Fig. 2. A) A DLSN neuron was superfused for 1 min with different concentrations of TMP at a membrane potential of -60 mV. The TMP superfusions were at least 15 min apart to minimize receptor desensitization. The membrane hyperpolarization increased in amplitude and duration with increasing concentrations of TMP. B) A concentration response curve was plotted after determining the hyperpolarization of DLSN neurons to superfusions of 0.1, 0.5, 1, 1, 10, 100, 500 and 1000 μM TMP as described in (A). The EC_{50} from the concentration response curve was 10 μM. Twenty neurons, each from a different animal, were used in obtaining the dose response curve.

fects of potassium channel blockers were done in the single electrode voltage clamp mode. The neurons were clamped at a holding potential of -70 mV, at a sampling frequency between 5 and 7 kHz with a gain up to 8 nA/mV. The head stage was monitored continuously using a second oscilloscope.

Drugs were applied using two methods, superfusion at known concentrations and manual ejection of drug in a 10-μl drop from a pipette directly into the recording chamber upstream from the recording microelectrode. Agonists were usually applied by drop applications at least 15 min apart to minimize desensitization. In our system, a 10-μl drop of 10 mM DMPP produces an average hyperpolarization of 4 mV with individual responses ranging from 2 to 9 mV. This range of responses falls on the steep part of the dose-response curve for DLSN neurons as previously determined (Wong and Gallagher, 1991). Usually two control responses of similar magnitude were obtained before beginning experimental protocols. Antagonists were superfused over the slice for 15 min before testing agonists. Superfusion of chlorisondamine, CYT and strychnine at the higher concentrations often produced a delayed, gradual membrane hyperpolarization that we suspect is nonspecific for reasons given in the discussion. To be able to compare a neuron's response to DMPP in the presence and absence of these drugs, the membrane potential of the neurons was manually clamped back to the membrane potential during the control response. In this and previous studies, the response to nicotinic agonists was apparently unaffected by the presence of TTX. Therefore, it was not routinely included in the ACSF. When TTX is included, it is so stated in the figure legend. Superfusion solutions were switched by manually controlled valves. BAPTA and GTPγS were included in the microelectrode solution at 20 and 1.6 mM, respectively. BAPTA was injected with -0.5 nA current steps and -0.3 nA constant current. Statistics were performed using the Instat program (GraphPad, San Diego, CA) and the dose-response curve and the EC_{50} for TMP were obtained using the SigmaPlot program (Jandel Scientific, San Rafael, CA).

Apamin, iberiotoxin and charybdotoxin were obtained from Research Biochemicals International, Natick, MA. All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO) except trimethaphan camsylate and chlorisondamine that were gifts from

Hoffman-La Roche, Nutley, NJ and Ciba-Geigy, Basle, Switzerland, respectively.

Results

Chlorisondamine, a noncompetitive nicotinic antagonist, produced a membrane hyperpolarization of DLSN neurons that lasted throughout its superfusion onto the slice (fig. 1, middle traces in A and B). We tested whether DMPP was still able to induce its typical, nicotinic receptor-mediated membrane hyperpolarization in the presence of various concentrations of chlorisodamine. When the membrane potential was manually clamped back to control level, only concentrations of chlorisondamine higher than 100 μM blocked the nicotinic response. Figure 1A demonstrates that DMPP still produced a membrane hyperpolarization in the presence of 100 μM chlorisondamine (lower trace). Superfusion of 500 μM chlorisondamine onto another DLSN neuron blocked the hyperpolarization in response to DMPP (fig. 1B, lower trace). The response was blocked by 500 μM chlorisondamine in four of five neurons tested. The blockade of the DMPP hyperpolarization by chlorisondamine sometimes revealed an increase in the firing rate of the neuron in response to DMPP (fig. 1B, lower trace). This excitatory effect of nicotinic agonists was also occasionally seen in some neurons in the presence of mecamylamine (figs. 7 and 8 in Wong and Gallagher, 1991). Partial recovery from the chlorisondamine blockade was seen after approximately 30 min of washing out of the antagonist (not shown).

Unexpectedly, when the competitive ganglionic antagonist TMP was superfused onto DLSN neurons, it consistently induced a large hyperpolarization (figs. 2-5B). Responses of the same cell to a drop application equivalent to 10 μl of DMPP or TMP is shown in figure 5B. The amplitude of the hyperpolarization after TMP superfusion was concentration-dependent, such that increasing concentrations of TMP produced larger hyperpolarizations (fig. 2A). The dose response curve of the peak hyperpolarization, produced by a 1-min superfusion of known concentrations of TMP, is seen in figure 2B; the EC_{50} of TMP was 10 μM. As with the nicotinic agonists, the response to TMP persisted in the presence of

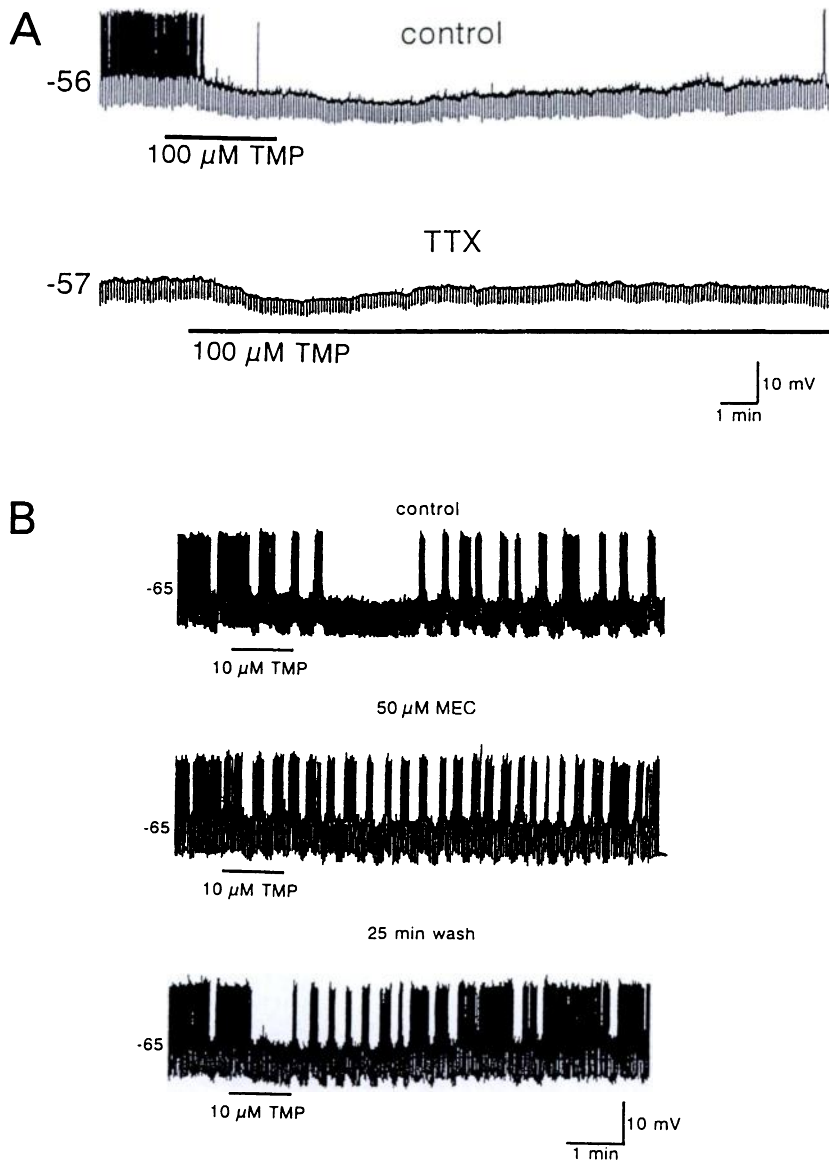

Fig. 3. A) A control response to a superfusion of 100 μM TMP was obtained (upper trace). The neuron was then superfused with 0.1 μM TTX for 15 min to block synaptic transmission. The effects of TMP were retested in the presence of TTX (lower trace). The neuron still hyperpolarized in response to TMP. Because the actions of TMP persist in the presence of TTX, it appears to be acting postsynaptically on DLSN neurons. B) A control response to a 1-min superfusion of 10 μM TMP caused a hyperpolarization and a pause in the firing of this DLSN neuron (upper trace). After a 15-min superfusion of 50 μM mecamylamine, the same dose of TMP did not cause a hyperpolarization although the firing pattern of the neuron became phasic rather than tonic (middle trace). A recovery of the TMP response could be seen after washing out the mecamylamine. Mecamylamine (50 μM) significantly reduced the hyperpolarization to 10 μM TMP ($P <$ .05, n = 4; one-tailed, paired t test).

TTX (0.1 μM, fig. 3A). The lower trace in figure 3A and the trace in figure 4B, also demonstrate that the response returns to base line in the continued presence of TMP suggesting that the receptor mediating the TMP response may be desensitized. Furthermore, the hyperpolarization in response to TMP was blocked by mecamylamine (fig. 3B). To test whether the TMP was acting at the same receptor on DLSN neurons as DMPP, 500 μM TMP was superfused onto the slice and the response was allowed to desensitize or "fade" (fig. 4). Although the cell was still being superfused with TMP, the response to DMPP was retested and found to be blocked (fig. 4B). The DMPP response was blocked in 3/3 cells tested. The response to DMPP gradually returned after washing out the TMP over a 90-min period (fig. 4C). TMP may therefore be acting as an agonist at the nicotinic receptors on DLSN neurons.

CYT, a full or partial nicotinic agonist depending on the specific receptor it is acting on, was first tested as an agonist at DLSN neurons. The response of individual neurons to the same concentration of both CYT and DMPP was examined (fig. 5A). CYT did not produce a membrane hyperpolarization in neurons that were hyperpolarized by DMPP (3/3 neurons). When superfused at 500 μM, it did produce a small hyperpolarization of DLSN neurons that did not return to base line (fig. 5C, middle trace). At receptors containing the β_2 nicotinic receptor subunit, CYT is a partial agonist (Papke and Heinemann, 1994). Therefore, after obtaining a control response, the response of DLSN neurons to DMPP was retested in the presence of 500 μM CYT (fig. 5C). Unlike the blockade of the response during continuous superfusion of trimethaphan, DLSN neurons still hyperpolarized upon DMPP application in the presence of CYT (fig. 5B, lower trace). The weak hyperpolarization of DLSN neurons after CYT application, together with the inability of CYT to desensitize the nicotinic response, suggests that CYT is not a good agonist at this receptor. CYT does not appear to be an antagonist at this site because the hyperpolarization in response to DMPP persists in the presence of 500 μM CYT.

Strychnine (100 μM) did not antagonize the membrane hyperpolarization of DLSN neurons in response to DMPP (fig. 6). This is in contrast to its blockade of the hyperpolarization of outer hair cells in response to acetylcholine (Housley and Ashmore, 1991).

From previous studies, calcium appeared to be involved in the nicotinic response (Wong and Gallagher, 1989 and 1991). The requirement for intracellular calcium was confirmed by testing the response to DMPP before and after the injection of BAPTA into DLSN neurons. Figure 7A (upper trace) shows the control response of a DLSN neuron to serotonin and DMPP. After the injection of BAPTA into the neuron, the response to serotonin remained although the response to DMPP was blocked (fig. 7A, lower trace). The serotonin response of DLSN neurons is known to be calcium independent and mediated by $5HT_{1A}$ receptors (Joëls *et al.*, 1987; Van den Hooff and Galvan, 1992). The continued presence of the serotonin response after the BAPTA injection indicates that the neuron is still healthy. The blocking of the DMPP response by intracellular BAPTA indicates that it is dependent on increased levels of intracellular calcium. The source of the calcium could be either intracellular or extracellular. To eliminate the influx of extracellular calcium, calcium was eliminated from and 1 mM EGTA was added to the superfusion buffer to chelate any extracellular calcium in the slice. Under these conditions, the hyperpolarization to DMPP was completely blocked in only one of seven neurons tested, although being reduced by more than 50% in only two other neurons. Figure 7B shows the responses of two neurons that were not blocked by elimination of extracellular calcium. When the cells were kept in zero calcium buffer for longer periods of time (>30 min), the nicotinic response would eventually be blocked (not shown). Under zero calcium conditions, the depolarization after the hyperpolarization observed in some neurons was still present (fig. 7B, cell 2).

To determine whether a G protein was involved in the nicotinic response, GTPγS was injected into DLSN neurons. Immediately after impalement, the DLSN neuron in figure 8 responded to serotonin and to DMPP. The response to drop application of DMPP does not recover back to base line, suggesting a prolonged activation of the mechanism responsible for the response. After injection of GTPγS into the

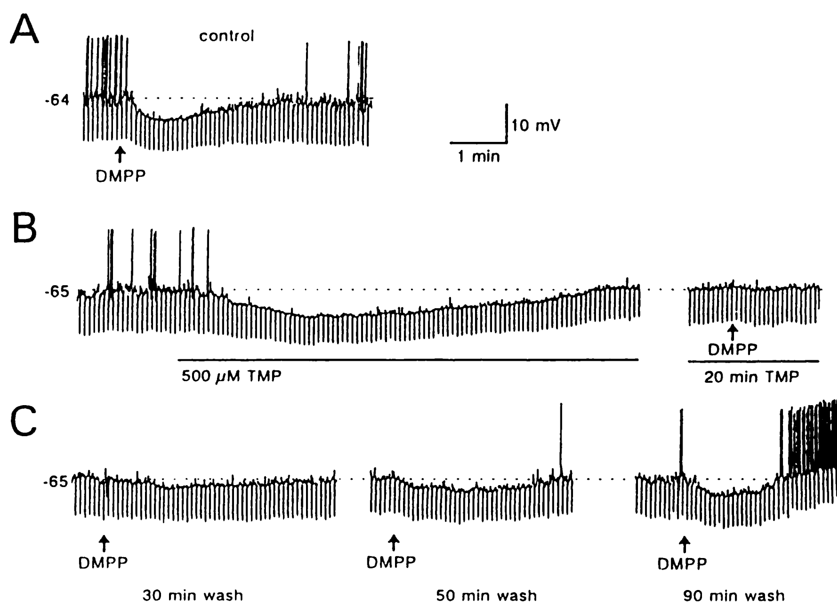

Fig. 4. A) The control response of a DLSN neuron to a microdrop of 10 mM DMPP was obtained. B) After 15 min, the neuron was superfused with 500 μM TMP that produced a large membrane hyperpolarization accompanied by a decrease in the input resistance. However, this response "faded," and the membrane potential returned to base line after 8 min in the presence of TMP. There was no response to DMPP after the membrane potential returned to baseline in the presence of TMP. The response to DMPP gradually recovered after prolonged washing.

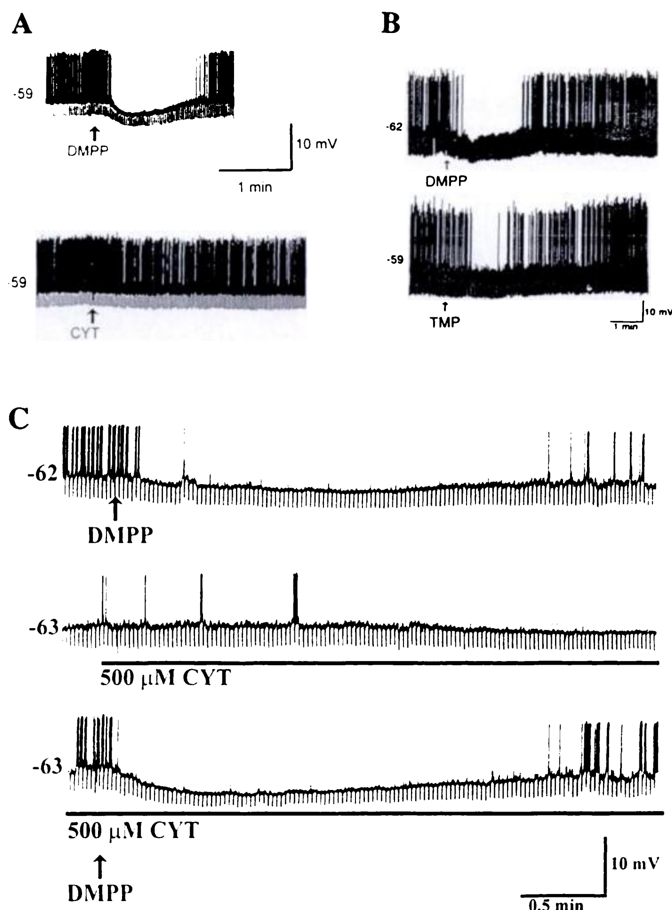

Fig. 5. A) The top trace is the response of a DLSN neuron to a 10-μl drop of 10 mM DMPP. In the same neuron, the application of an equal concentration of CYT produced little membrane hyperpolarization. The lack of response was not due to desensitization, because a reapplication of an equal concentration of DMPP immediately after the CYT still hyperpolarized the neuron (not shown). In the cells tested by drop application, CYT failed to produce a hyperpolarization in DLSN neurons that responded to an equivalent concentration of DMPP ($P > .05$, t test). The highest concentration of CYT drop applied was 50 mM. B) This DLSN neuron displays characteristic responses to 10-μl applications of DMPP or TMP both at 10 mM. The TMP was applied 20 min after the DMPP. The response to TMP was consistently smaller than the response to the same concentration of DMPP. The response to the microdrop application of TMP may be compared to the response of another neuron to the same concentration of CYT in (A). C) To test whether CYT may be acting as an antagonist, it was superfused onto a DLSN neuron after a control response to DMPP was recorded. CYT did produce a hyperpolarization at this concentration (middle trace). After bringing the membrane potential of the neuron back to that in the control response, DMPP still produced a hyperpolarization that was not significantly different from the control response ($P > .05$, $n = 4$; paired t test). CYT therefore is neither a good agonist or an antagonist of the effects of DMPP at DLSN neurons.

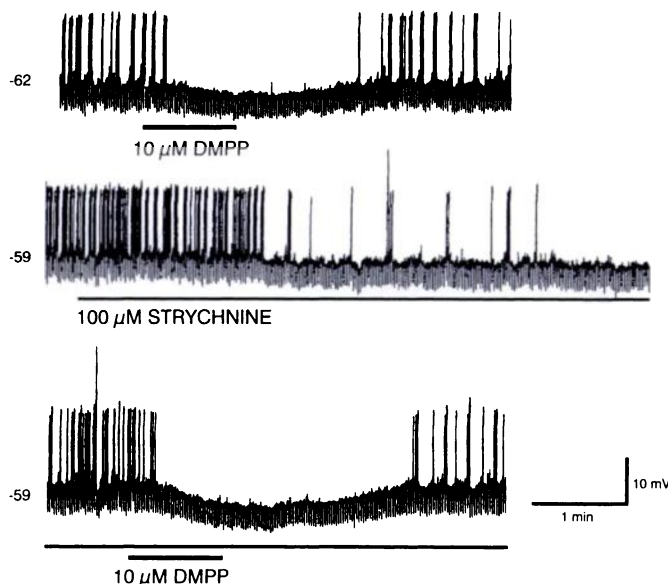

Fig. 6. The top trace is a control response to a 1-min superfusion of 10 μM DMPP. Superfusion of 100 μM strychnine alone caused the cell to hyperpolarize (middle trace). After strychnine had been superfused for 25 min, the membrane potential was brought back to control with injection of current. Then the response to 10 μM DMPP was retested in the presence of strychnine (lower trace). The cell still exhibited a strong hyperpolarization. Strychnine did not block the response at 10 to 100 μM in the three of three neurons tested.

neuron, the $5HT_{1A}$ response is blocked and so is the response to DMPP. The response to DMPP was blocked in the three of three neurons where injection of GTPγS blocked the response to serotonin.

Apamin was previously shown to block the nicotinic response, but it was tested at 10 μM, a higher concentration than reported to block specifically the SK class of potassium channels (Blatz and Magleby, 1986). Figure 9A shows the sensitivity of the response to lower concentrations of apamin. At 1 μM, apamin produces a 70% inhibition of the nicotinic response. In the three neurons tested, a significant block of the nicotinic response was not seen until concentrations of 1 μM apamin or higher were reached. In addition, charybdotoxin and iberiotoxin where tested on separate neurons. These toxins produced approximately a 30% inhibition of the response at 100 nM iberiotoxin and at 30 nM charybdotoxin (fig. 9, B and C). Iberiotoxin was tested in three neurons and charybdotoxin in two neurons. A few recordings were made in the voltage clamp mode (fig. 9C) while in the presence of the potassium channel blockers. This was done to enhance the detection of any inward currents that may be concealed by the large outward potassium current. No inward currents were detected. However, the outward currents were only partially blocked by the toxins; these residual outward currents could have still masked a small inward current.

Discussion

Neuronal nicotinic receptors can be divided into three general classes based on their pharmacology. These classes have been designated as the high affinity, the ganglionic and the ABgT-sensitive nicotinic receptors. From initial studies, the pharmacology of the nicotinic response of DLSN neurons appeared to be most like that of ganglionic receptors because DMPP was a good agonist and mecamylamine and KBgT were antagonists. These drugs all act at the ganglionic nAChR containing the α_3 subunit while ABgT does not. ABgT was also ineffective at blocking the nicotinic response of DLSN neurons. The lack of ABgT-sensitivity of the response in DLSN neurons suggested that the response was not mediated by receptors containing α_7, α_8, or α_9 nAChR subunits that are known to be ABgT-sensitive. Although mecamylamine is considered a ganglionic antagonist it is also widely used to block the central actions of nicotine where the

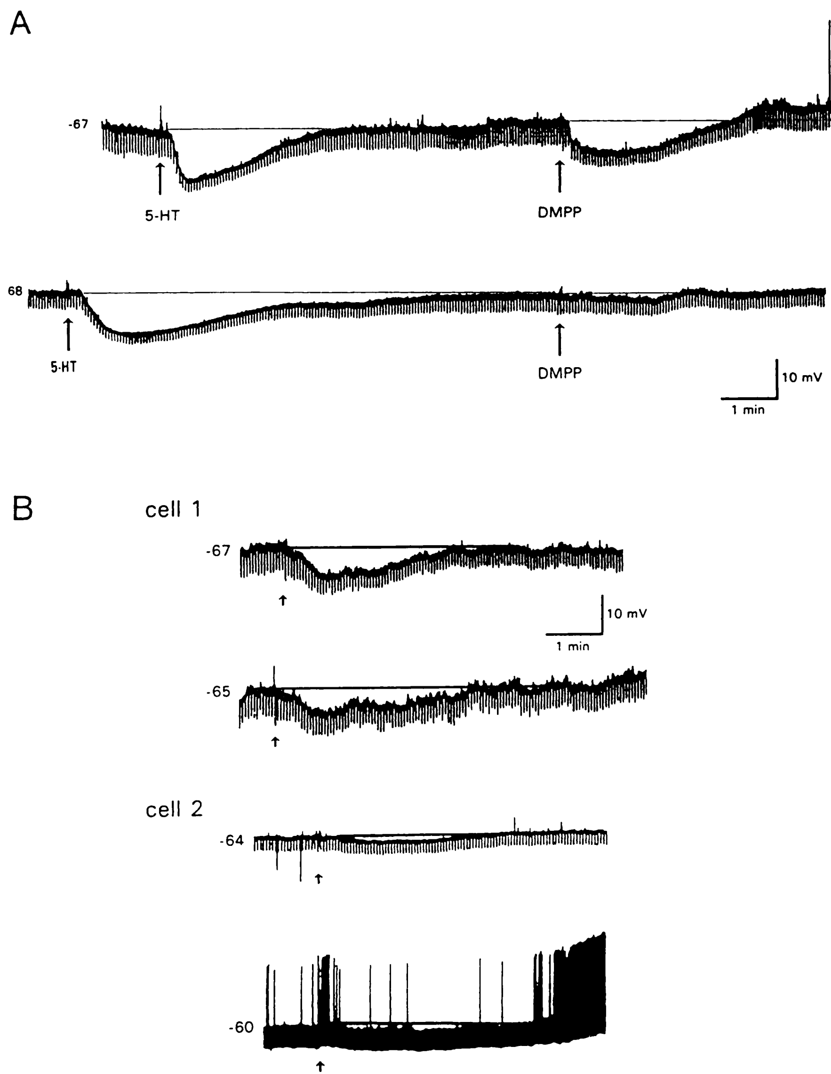

Fig. 7. A) The membrane hyperpolarization in response to DMPP requires a minimum level of intracellular calcium. The top trace in A) shows the control response to microdrops of 500 μM serotonin and 10 mM DMPP obtained upon impalement of a DLSN neuron. BAPTA was then injected into the neuron. After BAPTA injection the response to serotonin was not blocked but the DMPP response was (lower trace in A). The serotonin response in these neurons has previously been shown to be calcium-independent. The calcium-dependent after-hyperpolarizing potential of the neurons after a train of spikes was monitored to determine whether BAPTA was entering the cells. B) Entry of extracellular calcium does not appear to be required for the response to DMPP. The top traces for both cell 1 and cell 2 show the response to a microdrop application of DMPP in control ACSF. After superfusion of the slices with zero calcium buffer for 12 min the response to DMPP was retested and it persisted (lower traces, cell 1 and cell 2).

expression of the α_3 containing ganglionic class of receptors is low (London *et al.*, 1988; Clarke, 1990; Spande *et al.*, 1992). Mecamylamine acts predominantly at the nicotinic receptor ion channel and appears to block most neuronal nicotinic responses, and thus it may not be a good classification agent. The nicotinic response of DLSN neurons was not blocked by the classic nicotinic antagonists *d*-tubocurarine or dihydro-β-erythroidine both of which block all three classes of neuronal nicotinic receptors. This indicates that the structure of the nAChR on DLSN neurons may not neatly fit into the currently accepted classification of nAChR. Our studies lend further support to the conclusion that the receptor mediating the hyperpolarization of DLSN neurons in response to nicotinic agonists is not one that has been described in other systems.

The effect of the ganglionic antagonist chlorisondamine was tested on the nAChR in DLSN neurons because it has a mechanism of action similar to that of mecamylamine. Both antagonists are noncompetitive, blocking the channel of the nAChR. As with mecamylamine, chlorisondamine has been used to block the central effects of nicotine (Clarke and Kumar, 1983; Clarke *et al.*, 1994). In our study, chlorisondamine blocked the nicotinic response of DLSN neurons only at a high concentration (500 μM). Studies have indicated that the nAChR block by mecamylamine and chlorisondamine may be use-dependent as they block the open receptor ion channel. The high concentration of chlorisondamine needed to block the nicotinic response in our study did not appear to be due to a lack of receptor channel activation, since, in the neuron where the action of DMPP was not blocked by chlorisondamine, a second application of DMPP in the presence of the antagonist was also ineffective (not shown). The results may indicate that the nicotinic receptor on DLSN neurons is a ligand-gated receptor with an ion channel structurally similar to the ion channels of the other nicotinic receptors. However, mecamylamine and chlorisondamine are not very specific at high concentrations, and have been shown to block, for example, the NMDA receptor at 70 and 600 μM, respectively (Clarke *et al.*, 1994). Another possible explanation of the results is that at the high concentrations needed to block the nicotinic response of DLSN neurons, chlorisondamine is acting nonspecifically at a cation channel pore. For example, these channel blockers may be acting at the potassium channel mediating the hyperpolarization rather than at the nicotinic receptor itself.

In contrast to chlorisondamine, TMP is a competitive ganglionic antagonist (van Rossum, 1962). Unexpectedly, our results suggest that it may be acting as an agonist at the

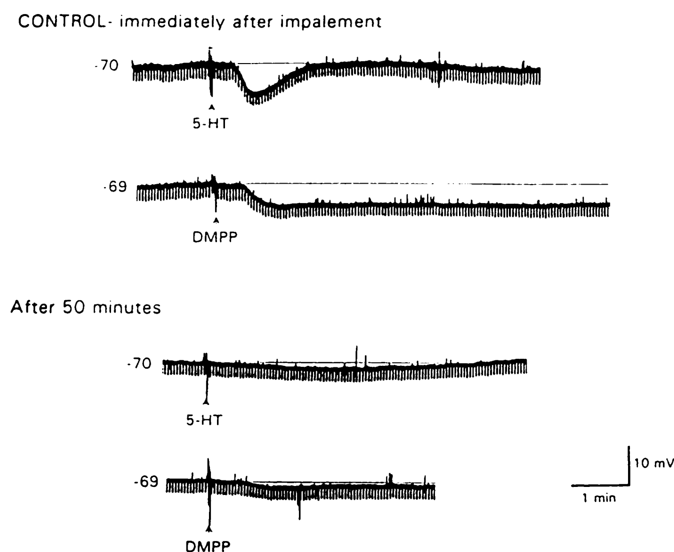

Fig. 8. A G protein appears to be involved in coupling the nicotinic receptor and the potassium channel mediating the response. Serotonin and DMPP were drop applied immediately after impaling this DLSN neuron with a GTPγS containing microelectrode (upper two traces). The response to DMPP appears to be prolonged, possibly due to the permanent activation of G proteins by GTPγS that has leaked from the electrode into the cell. GTPγS was then injected into the neuron. After 50 min the response to serotonin and DMPP were retested (lower two traces). Both the serotonin and the DMPP responses were blocked. In the three neurons in which GTPγS blocked the serotonin response, the DMPP response was also blocked. However, the two responses are not mediated by the same G protein because the serotonin response is pertussis toxin sensitive but the DMPP response is not (Charest *et al.*, 1993; Wong and Gallagher, 1991).

nAChR on DLSN neurons. The hyperpolarization of DLSN neurons in response to TMP was concentration dependent and persisted in the presence of TTX. It appeared to desensitize as does the response to nicotine and DMPP in these neurons. Inasmuch as the apparent desensitization of the nAChR on DLSN neurons by TMP prevented the action of DMPP, TMP and DMPP may be interacting with the same receptor on DLSN neurons. The EC_{50} of TMP was 10 μM, the same as found previously for nicotine at this receptor (Wong and Gallagher, 1991). As for nicotine, the maximum response to TMP is lower than that for DMPP producing an average maximal hyperpolarization near 6 mV. The lower maximal responses of TMP and nicotine may be due to desensitization of the receptor at higher drug concentrations or lower efficacies of TMP and nicotine at the receptor. It may be less surprising that TMP acts as an agonist if it is remembered that *d*-tubocurarine and dihydro-β-erythroidine are ineffective as antagonists at this receptor. The structure of the receptor seems to differ enough from those previously characterized to prevent the binding of classical antagonists or to possibly allow them to function as agonists. It has been shown that a single mutation in the amino acid sequence of the α_7 subunit produces a receptor that has a conductance in the presence of DHβE and *d*-tubocurarine (Bertrand *et al.*, 1992); the structure of the agonist binding site of the receptor on DLSN neurons may thus not have to be radically different if amino acids critical for binding or channel gating have been altered.

Antagonists could mimic the action of agonists in producing a membrane hyperpolarization if the hyperpolarization resulted from the inactivation of tonically active nAChR. The inactivation could be due to desensitization of the nAChR by agonists or block of the tonically active receptors by antagonists. These possibilities seem unlikely because the hyperpolarization, as also reported for DMPP, "fades" in the continuous presence of TMP. In the continued presence of an agonist, most of the receptors should remain desensitized while in the presence of antagonists, the response should remain blocked, both of which would not "fade."

A similar hyperpolarization in response to nicotinic agonists has been described in outer hair cells (Housley and Ashmore, 1991). Like the response in DLSN neurons, the hyperpolarization is mediated by the opening of a potassium channel. The receptor on hair cells appears to have a mixed pharmacology that is related to the α_7 class of receptors. It is blocked by ABgT and KBgT but also by 50 μM atropine and 10 μM strychnine, which also blocks α_7 receptors expressed in oocytes (Housley and Ashmore, 1991; Anand *et al.*, 1993). At concentrations of up to 100 μM, strychnine did not antagonize the response of DLSN neurons to DMPP. Together with their insensitivity to ABgT, the lack of antagonism by strychnine further suggests that the receptors on DLSN neurons do not belong to the α_7, α_8 or α_9 class of receptors.

CYT is also a good agonist at most ABgT-sensitive responses (Bertrand *et al.*, 1992; Amar *et al.*, 1993). For example, in chick ciliary ganglion cells, nicotine and CYT were both much better agonists than DMPP for the ABgT-sensitive response. When tested on DLSN neurons CYT produced no hyperpolarization until high concentrations of drug were reached. This supports the previous findings of this laboratory that CYT has little or no agonist action at these neurons (Wong and Gallagher, 1991). Although being an agonist at α_7 containing receptors, CYT is either an agonist or partial agonist at high affinity nicotine receptors depending on the structural subunits in the receptor. If β_4 subunits are present, CYT is more potent than nicotine, while if β_2 subunits are present, CYT is a partial agonist inhibiting the response to nicotine. The possibility that CYT is an antagonist at DLSN neurons was tested, and at concentrations of up to 500 μM it was found to have no blocking effect on the response to DMPP. The pharmacology of the response of DLSN neurons, therefore, remains most like that of a ganglionic nAChR.

It was noted that chlorisondamine, CYT and strychnine produce membrane hyperpolarizations when superfused onto DLSN neurons at high concentrations. This hyperpolarization does not appear to have the same time course as the DMPP response. It is slower in onset and does not return to base line in the continued presence of these drugs. Because the drugs are of different classes, a noncompetitive nicotinic antagonist, a nicotinic agonist and a glycine receptor antagonist and they all produce a similar effect, we suspect that this hyperpolarization is a nonspecific action due to the high concentration of the drugs. However, the mechanism underlying this hyperpolarization was not examined.

The experiments with potassium channel blockers suggest that, although a calcium dependent potassium channel is likely to mediate the nicotinic response, this channel is not one of the classically defined calcium-dependent potassium channels. Apamin has been reported to specifically block SK channels at nanomolar concentrations although in the DLSN micromolar concentrations are required (Blatz and Magleby,

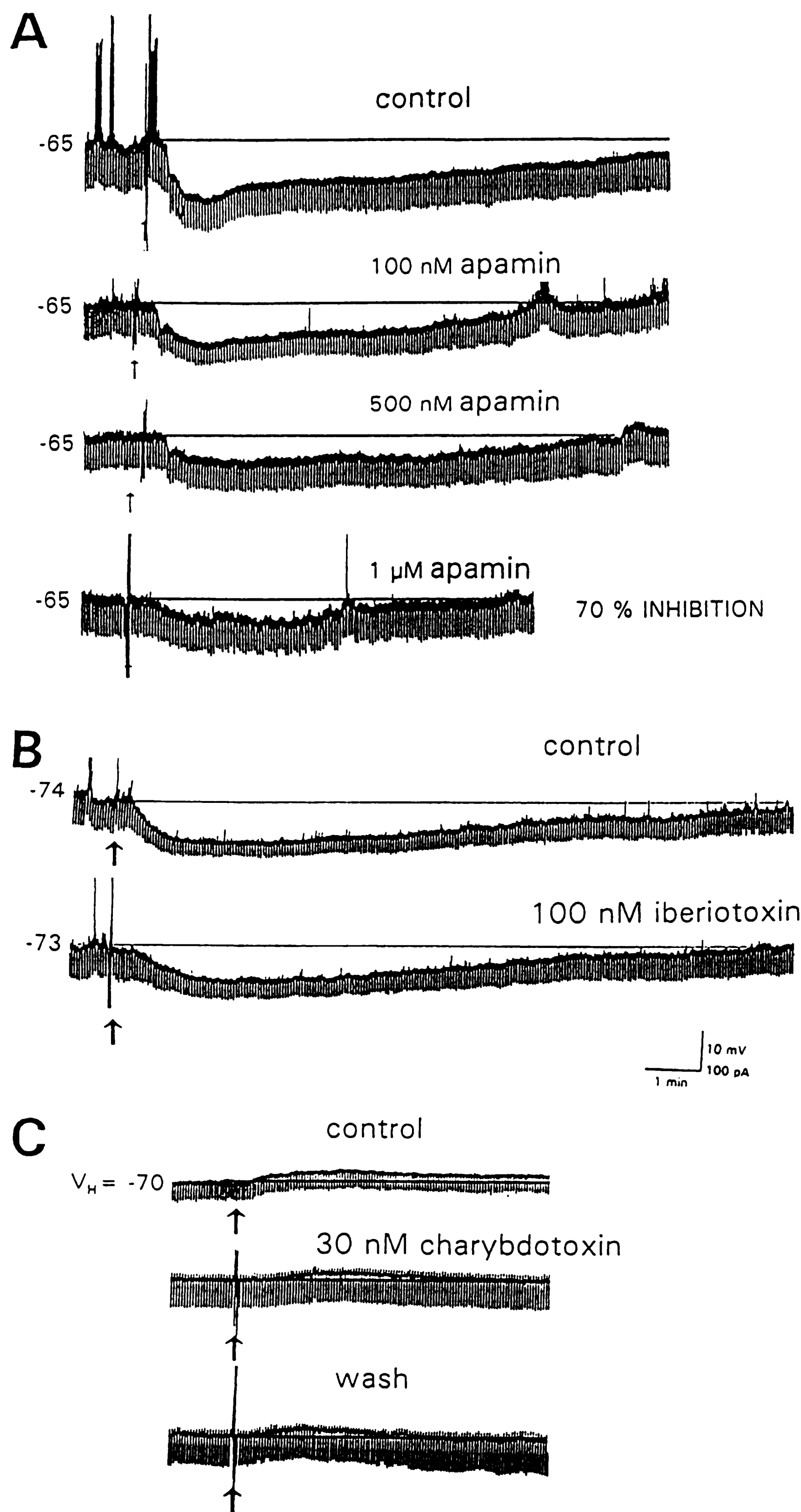

Fig. 9. The potassium channel mediating the hyperpolarization does not appear to be a classical calcium-dependent potassium channel as defined by sensitivity to specific toxins. A) Apamin blocks the membrane hyperpolarization in response to DMPP at ≥1 μM (n = 3). An almost complete blockade could be obtained with 10 μM apamin (shown previously in fig. 13 by Wong and Gallagher, 1991). SK channels are reported to be blocked by 30 nM apamin. B) Iberiotoxin blocks only 30% of the response to DMPP at 100 nM (n = 3). There was also a 30% block in one cell tested in the presence of 30 nM iberiotoxin so the block at 100 nM appears to be complete. Iberiotoxin has been shown to block BK channels at 30 nM. C) Charybdotoxin (30 nM) at a concentration reported to block IK and BK channels, also inhibits only 30% of the outward current in response to DMPP (n = 2). Only one concentration of charybdotoxin was tested. The recording in C) was done in the voltage clamp mode.

1986). Iberiotoxin has been reported to block BK channels at 2 nM and charybdotoxin should produce a significant blockade of both BK and IK channels at the concentration used (Galvez *et al.*, 1990; Vazquez *et al.*, 1990). The nicotinic response of DLSN neurons has been previously been shown to be blocked by Ba^{++} and high concentrations of TEA but not by Cs^{+}. So although the response is coupled to a potassium channel, the properties of this channel are not currently clear.

Although the nicotinic response of DLSN neurons is modulated by some ganglionic drugs, for the most part its pharmacology is different from nicotinic receptors described in the literature. It is interesting to note that expression studies have found only β_2 but not α_2, α_3 or α_4 subunit mRNA in the

DLSN and only background levels of ^{125}I-ABgT binding (Wada *et al.*, 1989; Schulz *et al.*, 1991). Inasmuch as the pharmacology of this response is unique and no α subunits appear to be expressed in these neurons, the receptor mediating the response may not be a classical ligand-gated ion channel. Several receptor models could account for the hyperpolarizing response. It could be mediated by a potassium channel that is directly activated by nicotinic agonists. This channel would also require the presence of a minimal level of intracellular calcium. Alternatively the hyperpolarization could be mediated by a metabotropic receptor with nicotinic pharmacology. This receptor would need to be coupled to the release of intracellular calcium stores through a second messenger. A metabotropic receptor could also be directly coupled by a G protein to a calcium-dependent potassium channel. The persistence of the response in the absence of extracellular calcium makes the activation of a potassium conductance by calcium entering through a calcium permeable nicotinic receptor unlikely. Both the activation of a nicotinic channel with potassium permeability and the direct activation of a potassium channel by a G protein would require that a minimum level of intracellular calcium be present. The potassium channel mediating the response would then have a dual requirement for calcium and either G protein or agonist for activation. Therefore, the simplest model that fits the experimental data is that the nicotinic receptor is a metabotropic receptor coupled to a G protein. When activated the receptor initiates the production of a second messenger, for example IP_3, with release of intracellular calcium stores. The calcium release activates a calcium dependent potassium channel, related to the apamin sensitive SK channel, resulting in hyperpolarization of the cell.

The appearance of the depolarization upon blocking of the nicotinic hyperpolarization with mecamylamine or upon return to base line of the membrane potential has been noted in this and previous studies. It is not present in all cells with DMPP responses. Since it is sometimes revealed in the presence of mecamylamine the depolarization does not appear to be due to the activation of an excitatory nicotinic receptor. For the same reason, the depolarization is unlikely to be due to the deinactivation of calcium channels upon membrane hyperpolarization in response to nicotinic agonists. DLSN neurons do express functional low threshold calcium channels but these would not be deinactivated during the block of the DMPP response with mecamylamine because there is little membrane hyperpolarization in this case. A possible explanation for this effect could be that the G protein activated by a metabotropic nicotinic receptor may have more than one action within the same neuron. If this was the case then mecamylamine could be blocking the potassium channel mediating the nicotinic response and not the nicotinic receptor itself. The depolarization of some DLSN neurons requires further study.

Only three inhibitory nicotinic responses have been reported to date in mammalian systems. The hyperpolarization of hair cells has already been discussed. An extracellular study in the cerebellum reported that an inhibitory response to nicotinic agonists was blocked by KBgT but not ABgT (de la Garza *et al.*, 1989). However it is not known whether this response is pre- or postsynaptic. Somogyi and de Groat (1992) have reported an inhibition of acetylcholine release from nerve terminals innervating the bladder by activation of presynaptic nAChR. The pharmacology of this response was not extensively tested but it was blocked by 50 μM *d*-tubocurarine; the mechanism by which neurotransmitter release was inhibited was also not investigated. In conclusion, the inhibitory nicotinic receptor on DLSN neurons appears to have a unique pharmacology from both that of nicotinic receptors expressed in oocytes and those studied *in situ* suggesting that the nicotinic receptor on DLSN neurons is as yet uncloned and may have a significantly different structure from the nicotinic receptors whose structure is already known.

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