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Muscarinic Agonists Activate an Inwardly Rectifying Potassium Conductance in Medial Pontine Reticular Formation Neurons of the Rat *in vitro*

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Intracellular recordings were obtained from neurons in pontine reticular formation slices of the rat to characterize a cholinergic-gated increase in conductance. The conductance increase was associated with a hyperpolarization of the membrane potential and with an outward current under voltage-clamp conditions. Current–voltage relations and potassium substitution experiments indicated mediation by a change in permeability, primarily to potassium. This potassium conductance exhibited inward rectification at membrane potentials negative to resting potential, a novel finding for cholinergic actions in CNS neurons. Further characterization of this inwardly rectifying potassium conductance revealed marked sensitivity to low concentrations of barium. Cholinergically evoked currents were relatively unaffected by the presence of extracellular cesium. Cholinergic effects persisted in TTX. The outward currents elicited by carbachol or methacholine were blocked only by high concentrations of pirenzepine, a selective antagonist of the M_1 muscarinic receptor. The interaction between these agents is quantitatively consistent with cholinergic action at postsynaptic muscarinic receptors of the non-M_1 subtype.

A neuronal circuit that includes the cholinergic efferents from the laterodorsal and pedunculopontine tegmental nuclei to the medial pontine reticular formation (mPRF) may be integral to the generation and maintenance of rapid eye movement (REM) sleep (Steriade and McCarley, 1990). Knowledge of the mechanisms of cholinergic action at the cellular level in the mPRF is a necessary first step in the clarification of the function of this circuit.

The predominant response to ACh acting at muscarinic receptors in the CNS is a depolarization associated with a decrease in potassium conductance (McCormick, 1989), and a similar depolarizing response to cholinergic agents has been observed in the majority of neurons in the mPRF (Greene et al., 1989). However, neurons in the parabrachial nucleus and the medial and lateral geniculate nuclei of the thalamus have been reported to hyperpolarize upon muscarinic activation as a result of an increase in potassium conductance (Egan and North, 1986; McCormick and Prince, 1986; Christie and North, 1988; McCormick and Pape, 1988). In the mPRF, approximately one-fourth of the cells hyperpolarized in response to cholinergic agonists (Greene et al., 1989). The goal of the present investigation was to characterize the conductance responsible for this hyperpolarization. At least five types of potassium conductances in the CNS are known to be enhanced by neurotransmitters, including a non-voltage-sensitive conductance (Gerber et al., 1989b), the A-conductance (Atkins et al., 1990), the M-conductance (Jacquin et al., 1988), an inwardly rectifying conductance (Williams et al., 1988b), and a voltage-insensitive calcium-dependent potassium conductance (Haas and Greene, 1984). We here present data indicating that an inwardly rectifying potassium conductance in mPRF is enhanced by activation of a cholinergic receptor, an observation not previously reported for CNS neurons. This conductance appears similar in both voltage and pharmacological sensitivity to the agonist-gated inward rectifier described in other mammalian preparations (North, 1989).

A preliminary report of some of this work has been published previously (Greene et al., 1989).

Materials and Methods

Methods of preparation and maintenance of slices of medial pontine reticular formation have been described previously (Greene et al., 1986; Gerber et al., 1989a). Coronal brainstem slices containing the medial pontine reticular formation were prepared from young Sprague–Dawley or Long–Evans rats (8–20 d) following decapitation under halothane anesthesia. Slices were fully submerged in perfusion medium containing (in mM) NaCl 124, KCl 3.75, KH_2PO_4 1.25, $NaHCO_3$ 26, glucose 10, $CaCl_2$ 2.5, $MgCl_2$ 1.4, which was kept at 30°C at a flow rate of 1.5 ml/min (dead space plus chamber volume equals 1.2 ml). Intracellular recordings were made with glass microelectrodes filled with 2 M KCl (resistance, 50–90 MΩ) for recording of membrane potential or for voltage-clamp recordings in the sample and hold single-electrode mode with an Axoclamp 2A amplifier (Axon Instruments, Burlingame, CA). Head stage output was constantly monitored to ensure adequate settling time. Drugs were applied in known concentrations to the perfusion medium or by pressure ejection through a micropipette with tip diameter of 10–50 μm filled with a 1 mM solution of carbachol dissolved in perfusate.

Duration and pressure parameters for drug ejection through puffer pipettes were chosen to evoke responses approximating those observed with 1 μM bath application of carbachol. Drugs were obtained from Sigma. All pooled data are expressed as the mean ± 1 unit standard deviation.

Results

The cholinergic agonist carbachol was applied to 96 neurons of the mPRF formation during stable intracellular recordings (resting potential, −61 ± 5 mV; input resistance, 81 ± 34 MΩ). Bath application (0.5–5 μM) or puffer ejection of carbachol depolarized 70 cells (73%), hyperpolarized 20 cells (21%), caused a biphasic hyperpolarization–depolarization response in 3 cells,

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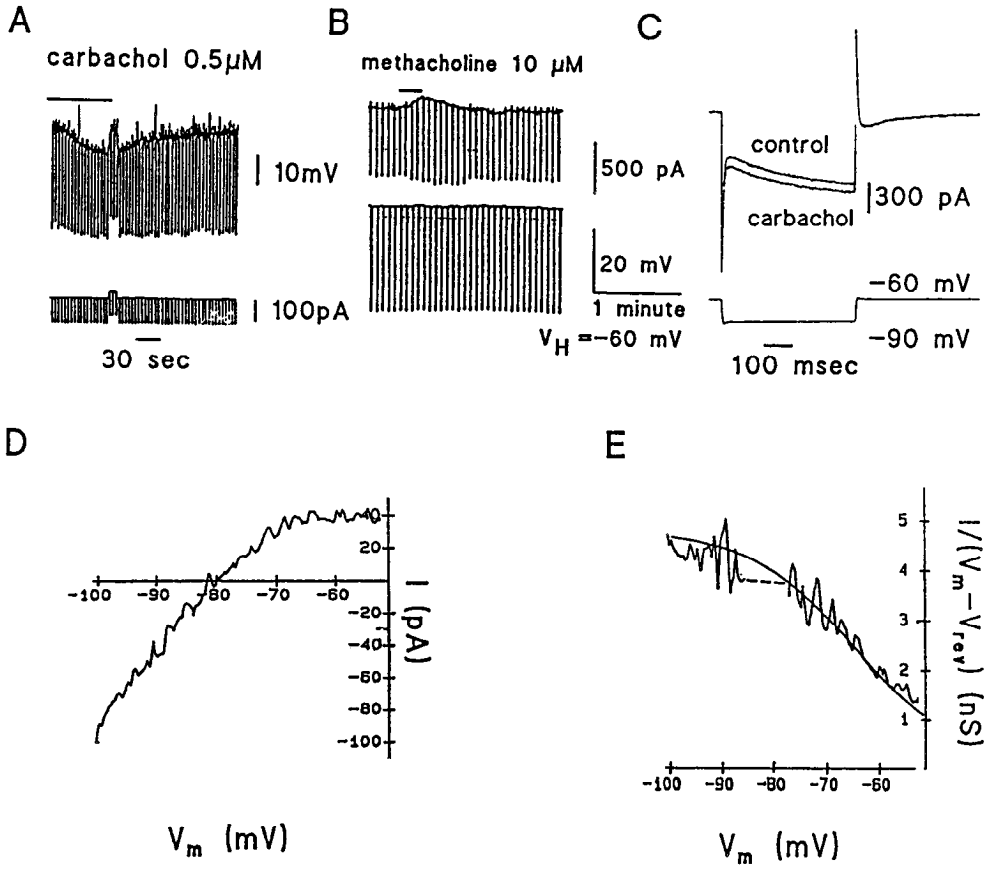

Figure 1. Muscarinic agonists increase membrane conductance. *A*, A voltage record shows that bath application of carbachol increases membrane conductance and results in hyperpolarization of membrane potential. Current pulses (400 msec duration) were injected to assess membrane conductance, and membrane potential was briefly repolarized to compensate for possible contributions from voltage-sensitive intrinsic conductances. *B*, Application of methacholine (10 μM; 0.5 μM TTX present) to a cell voltage clamped at −60 mV and stepped to −90 mV with 500 msec voltage command pulses causes an outward current associated with an increase in membrane conductance. *C*, The carbachol-sensitive conductance activates instantaneously, while there is no effect on the time-dependent inward current relaxation. A control trace is superimposed on a trace during peak carbachol effect at an expanded time base. *D*, An *I/V* plot of carbachol-evoked current shows inward rectification. Note the increased slope conductance at hyperpolarized membrane potentials. The plot was constructed by subtracting the whole-cell current responses to voltage ramp commands in control conditions from the whole-cell current obtained in the presence of carbachol. *E*, Plot of conductance versus voltage obtained by dividing the data in *D* by the driving force. Membrane conductance diminishes as membrane potential is depolarized. The data are fitted to a curve derived from the Boltzmann equation: $G_K = G_{K(max)}/\{1 + \exp[(V - V_{½})/k]\}$ (*solid line*). $G_{K(max)}$ was estimated to be 5 nS for the data in *E*. The *k* value (11.6) was determined as the slope of a least-squares fit to the conductance data points measured in *E* and graphed semilogarithmically against voltage, $V = k\{\ln(G_{max}/G) - 1\} + V_{½}$. These *k* and G_{max} values provide a good fit, accounting for 93% of the data variance.

and had no effect on 3 cells. The conductance and receptors mediating the hyperpolarizing cholinergic response are the subject of this communication.

We have previously reported that mPRF neurons can be classified into two principal types based on the presence or absence of a low-threshold calcium spike that underlies burst firing at hyperpolarized membrane potentials (Greene et al., 1986; Gerber et al., 1989a). In the present sample, 37% of the cells exhibited a low-threshold burst. Nine of the 20 neurons (45%) that were hyperpolarized by carbachol exhibited a low-threshold burst, while the remaining 11 cells were of the non-burst type. χ^2 analysis showed no statistically significant association between burst presence and muscarinic hyperpolarization ($P > 0.25$).

Potassium conductance is increased by carbachol

Figure 1 shows examples of cholinergic agonist–induced increases in neuronal membrane conductance resulting in hyperpolarization of the resting potential (*A*). Hyperpolarizations associated with treatment with carbachol (0.5–1.0 μM; $n = 11$) or methacholine (10 μM; $n = 2$) were associated with an outward current (*B*). Carbachol was applied via puffer on 9 cells and bath applied in 11 cells (0.5 or 1 μM). The average decrease in input resistance was 18 ± 6 MΩ ($n = 17$), altering resting membrane potential by −6 ± 2 mV. Figure 1*A* illustrates the measurement of the change in input resistance; carbachol was applied by bath and, for input resistance measurement, membrane potential was briefly repolarized to the control voltage (between −60 and −65 mV) to control for effects of voltage-dependent intrinsic membrane currents. Figure 1*B* illustrates, in another neuron during voltage clamp, the effects on current of bath application of methacholine (10 μM) following TTX (0.5 μM) exposure.

There was no apparent action of muscarinic agonists on time-dependent inward rectification. When the cell was held at −60 mV, carbachol elicited 80 pA of outward current. The increase in current response to the 400 msec voltage command pulses

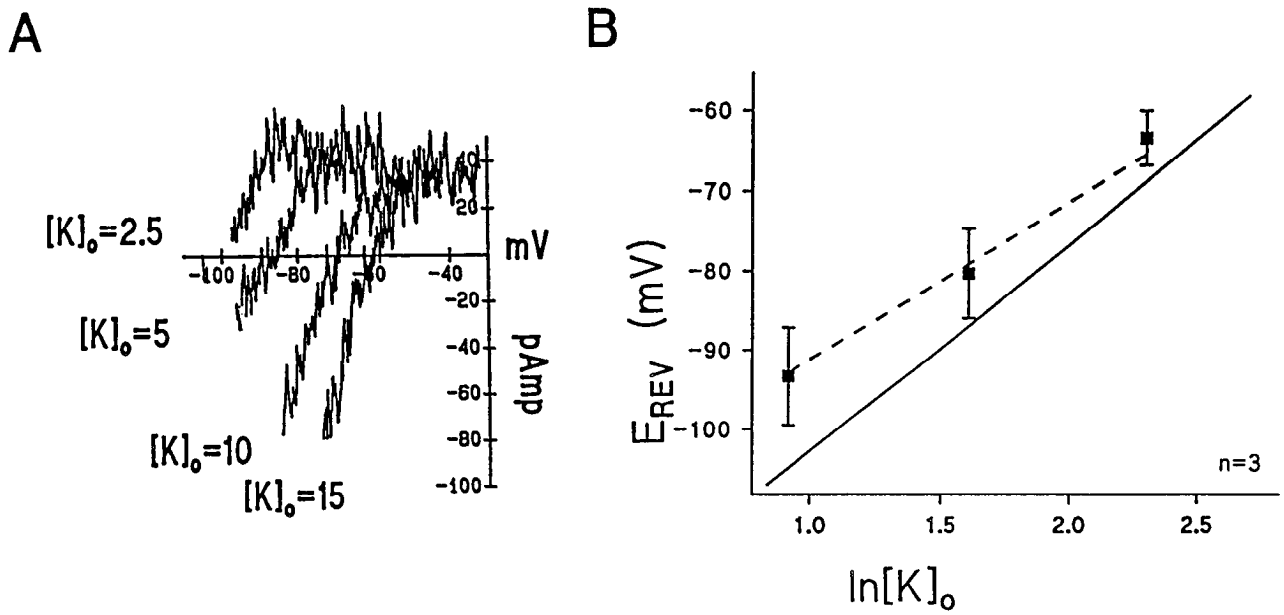

Figure 2. *A.* The current–voltage curves for carbachol-evoked current as determined by voltage ramps at different concentrations of potassium. The reversal potential as well as the inflection reflecting inward rectification is shifted in a Nernstian fashion with changes in extracellular potassium. *B,* Plot of pooled data for the reversal potential at three different potassium concentrations. The *solid line* indicates the relationship of reversal potential to $[K]_o$ for a pure potassium conductance. The slight deviation from this line may reflect a small contribution from another ion. The *broken line* indicates the reversal potential for a potassium channel with partial permeability to sodium ions (P_{Na}/P_K = 1/100). *Error bars* represent 1 unit SD.

from −60 to −90 mV during carbachol exposure was immediately observable following the capacitative transients as shown in Figure 1*C*. Superimposition of current traces in control and during maximal carbachol action by subtracting the 100 pA of carbachol-evoked outward current (measured at the holding potential) demonstrated that carbachol was without effect on a time-dependent inward current relaxation, corresponding to "Q" current or "H" current (Halliwell and Adams, 1982; Mayer and Westbrook, 1983; Bobker and Williams, 1989; Pape and McCormick, 1989).

In nine neurons, current–voltage plots were generated using a slow voltage-command ramp protocol during control conditions and in the presence of carbachol; voltage was changed at the rate of 2.5 mV/sec between −100 and −40 mV. By digitally subtracting the control from the carbachol current trace, the carbachol-sensitive current was determined (Fig. 1*D*).

The carbachol-evoked current exhibited inward rectification at potentials negative to resting membrane potential as shown in Figures 1*D*, 2*A*, and 3. The relationship between the chord conductance [computed at 50 μV increments as $I/[(V_m - V_{reversal})]$ and voltage is plotted in Figure 1*E*. Conductance rapidly decreased at membrane potentials more depolarized than resting potential. (At V_m near $V_{reversal}$ chord conductance is undefined ($\rightarrow\infty$), and consequently we have used a broken line to indicate an extrapolated conductance in this region.)

The conductance was fit by a Boltzmann expression:

$$G_K = G_{K(max)}/\{1 + \exp[(V - V_{½})/k]\},$$

where $G_{K(max)}$ is the maximum potassium conductance, $V_{½}$ is the voltage at which G_K is half-maximal, and k defines the steepness of the curve. The empirically derived values for the mPRF carbachol-activated potassium conductance at $[K]_o$ = 5 mM (n = 3 cells) were $V_{½}$ = −65.8 ± 2.4 mV and k = 9.9 ± 1.2 mV.

The inwardly rectifying properties of this conductance were maintained during changes in external potassium concentration, and $V_{½}$ varied in the same direction as the reversal potential, as reported for other inward rectifiers (Hagiwara and Takahashi, 1974) (Fig. 2*A*). The reversal potential of the response was −80 ± 4.5 mV in $[K]_o$ of 5 mM. Assuming a $[K]_i$ of 140 mM (Alger and Nicoll, 1980), the Nernst equation predicts a reversal potential of −87 mV for a response mediated by a selective increase in potassium permeability. Thus, the result obtained is consistent with a change primarily in a K^+ conductance. The reversal potential for this response varied with external potassium. There was a slight positive deviation in the data points from the theoretical line of reversal potential for a response mediated by a purely potassium-selective conductance (Fig. 2*B*).

Characterization of potassium conductance

The sensitivity of the carbachol-activated conductance to ions that specifically block inwardly rectifying potassium channels was examined. Low concentrations of barium have been shown to be selective for potassium inward rectifier currents (Standen and Stanfield, 1978; Osmanovic and Shefner, 1987; Surprenant and North, 1988; Williams et al., 1988b). Figure 3*A* shows that barium (100 μM) reversibly blocked the inwardly rectifying conductance evoked by application of carbachol (n = 3). An additional inwardly rectifying conductance prevalent in vertebrate CNS neurons is responsible for "H" or "Q" current (I_H). This current, carried by potassium and sodium ions, has been shown to be modulated by neurotransmitters (Bobker and Williams, 1989; Pape and McCormick, 1989) and is selectively blocked by external cesium but not barium (Halliwell and Adams, 1982; Mayer and Westbrook, 1983). Figure 3*B* compares the carbachol-sensitive conductance with the cesium-sensitive conductance (n = 5). The current blocked by cesium was typical for I_H in that it activated only at relatively hyperpolarized potentials, did not reverse polarity, and approached a reversal potential more depolarized than expected for a pure potassium conductance. In contrast, the carbachol-evoked current reversed

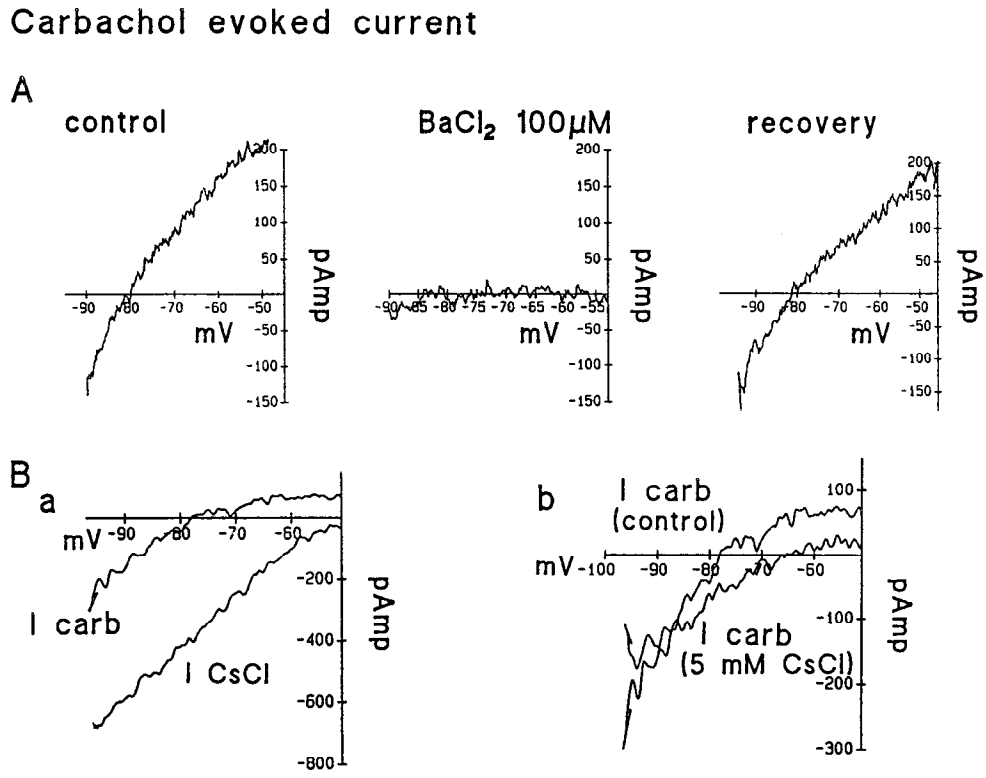

Figure 3. The sensitivity of carbachol-evoked current to barium and cesium. *A*, Barium reversibly blocks carbachol-elicited current. *Ba*, Comparison of carbachol-sensitive current and cesium-sensitive current (obtained by subtraction of the whole-cell current in the presence of cesium from the control current). Although both currents show inward rectification, they appear to result from different conductances. *Bb*, Ramp responses illustrating the effect of cesium on carbachol-evoked current.

polarity at approximately −80 mV and was not affected by 1 mM cesium at membrane potentials positive to the reversal potential. At more negative potentials, carbachol-evoked current was reduced (data not shown), similar to cesium effects on other inward rectifier potassium currents (see Discussion). In the presence of 5 mM cesium, the reversal potential was depolarized by 9.2 ± 2.1 mV (n = 3), but the slope of the I/V curve was not affected at potentials positive to −80 mV (Fig. 3*Bb*).

The potassium conductance is activated via non-M_1 muscarinic receptors

Pirenzepine has been used in a number of preparations to distinguish between muscarinic M_1 and non-M_1 binding sites (Hammer et al., 1980), and more recently, the neuronal biochemical (McKinney et al., 1989b) and electrophysiological (North et al., 1985; Egan and North, 1986; Marrion et al., 1989) effects have been correlated with these specific sites. The dissociation constant, K_i, for pirenzepine at the cortical M_1 receptor was reported as 6.99 nM and, at the brainstem non-M_1 receptor, as 219 nM (McKinney et al., 1989a). The agonist carbachol was also reported to show selectivity as follows: the dissociation constant, K_D, for cortical M_1 receptors was 0.32 μM (Potter and Ferrendelli, 1989), and for pontine reticular formation non-M_1 receptors, 1.4 μM (Cortes and Palacios, 1986). The muscarinic agonist methacholine has similar K_D values: cortical M_1 K_D was reported as 0.47 μM (Potter and Ferrendelli, 1989), and heart M_2 K_D was reported as 0.25 μM (Fuder and Jung, 1985). To provide a quantitative measure of the agonist–antagonist interaction, the ratio of the response to carbachol in the presence of pirenzepine (R_{pz}) to the response to the same dose of carbachol without pirenzepine (R_c) was experimentally determined in the mPRF. Based on the mass action law for receptor kinetics, the equation describing this ratio is

$$R_{pz}/R_c = \{K_D + [\text{Carb}]\}/\{K_D\,(1 + [\text{Pz}]/K_i) + [\text{Carb}]\},$$

where [Carb] and [Pz] are the concentration of carbachol and pirenzepine, respectively. The theoretical values (based on the K_D and K_i values mentioned above) for R_{pz}/R_c are 0.07 at the M_1 receptor and 0.48 at the non-M_1 receptor with [Carb] = 1 μM and [Pz] = 0.4 μM. We observed an average ratio of 0.49 ± 0.05 (n = 3). Expected ratios employing pirenzepine (0.4 μM) and methacholine (10 μM) are 0.28 at M_1 receptors and 0.73 at M_2 receptors. The observed ratio was 0.67 (Fig. 4).

The IC_{50} for pirenzepine, estimated from a best fit of a logistic function to a pirenzepine inhibition curve constructed from pooled data (n = 5; carbachol at 1 μM in the bath or an equivalent response to puffer application), was 670 nM, 2 orders of magnitude greater than that reported for M_1 receptors (Fig. 4).

Discussion

We have presented data demonstrating that postsynaptic muscarinic receptors mediate an increase in an inwardly rectifying potassium conductance in 21% of the neurons in the mPRF. Modulation of an inwardly rectifying potassium conductance in mammalian CNS by neurotransmitter agonists has been shown for 5-HT (Andrade and Nicoll, 1987; Colino and Halliwell, 1987; Williams et al., 1988a; Yakel et al., 1988; North and Uchimura, 1989), baclofen (Gähwiler and Brown, 1985), opioids (North et al., 1987; Williams et al., 1988b), noradrenaline (Williams et al., 1985), somatostatin (Inoue et al., 1988), adenosine (Trussel and Jackson, 1985), and substance P (Stanfield et al., 1985).

Cholinergic gating of an inwardly rectifying potassium conductance in CNS has not been previously demonstrated. Christie and North (1988) have suggested that the muscarinic receptor–

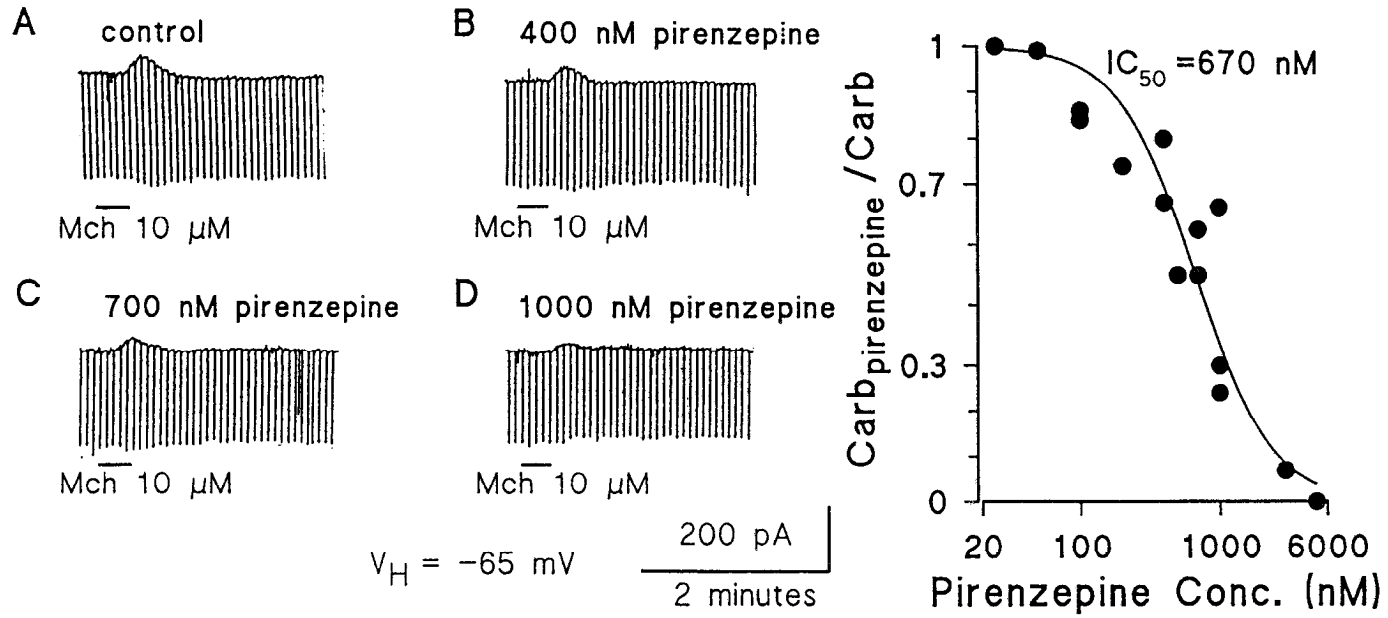

Figure 4. Effect of pirenzepine, an M_1 muscarinic antagonist, on the cholinergic response. Bath application of methacholine (10 μM) evokes outward currents in a cell voltage clamped at −60 mV. *A*, A control response to methacholine. *B–D*, Responses to identical applications of methacholine in the presence of increasing concentrations of pirenzepine. The log-concentration/response curve for pirenzepine inhibition of carbachol currents (*right panel*; results pooled from five neurons) indicates the concentration dependence of pirenzepine depression of carbachol responses. The sigmoid curve was fit using a logistic equation with a IC_{50} of 670 nM.

activated hyperpolarization in rat parabrachial neurons is mediated by an inwardly rectifying potassium conductance since it is occluded during activation of the inwardly rectifying μ-opioid response. Muscarinic agonists elicit a potassium conductance in cardiac pacemaker cells (Noma and Trautwein, 1978; Sakmann et al., 1983) that has properties similar to the inward rectifier described in the present study.

The agonist-gated inward rectifier has a $V_{½}$ near −65 mV (Stanfield et al., 1985; Williams et al., 1988b; Tatsumi et al., 1990). The close similarity of the previously described agonist-gated conductance with the muscarinic-gated conductance in the present study provides further evidence that different receptors can be coupled to the same conductance (Swann and Carpenter, 1975), with specificity occurring in the particular kind(s) of receptor found in various neuronal classes (North, 1989). The recent results reported by Selyanko et al. (1990) working on bullfrog sympathetic ganglion cells indicate that both a muscarinic and an adrenergic receptor gate the same potassium inward rectifier in C-cells.

5-HT acting at 5-HT_1 receptors and noradrenaline at α_2-receptors also increase a potassium conductance in mPRF cells (Gerber et al., 1989c; Stevens et al., 1991). The conductance activated by 5-HT was not inwardly rectifying. The adrenergically coupled potassium conductance displayed inward rectification, but further experiments will be required to determine whether muscarinic receptors gate this same conductance in mPRF.

The voltage sensitivity of the inward rectifier current can be attributed to the conductance properties of the component single channels. They pass current better in the inward direction (Sakmann and Trube, 1984; Matsuda and Stanfield, 1990) because, at least in some cases, of a blockade of the outward current by internal magnesium (Vanderberg, 1987). Other mechanisms can lead to inward rectification that do not involve inward rectifier channels. The channel population can be voltage gated, being in an active state at hyperpolarized potentials and an inactive state at depolarized potentials. This may occur in the channels mediating I_H (Halliwell and Adams, 1982; Mayer and Westbrook, 1983; Bobker and Williams, 1989; Pape and McCormick, 1989). Alternatively, there may be a simulation of inward rectification due to the agonist's activating an inward current at depolarized potentials (Freschi and Livengood, 1989) in addition to a non-voltage-sensitive potassium current, resulting in less net outward current at the depolarized potentials. The fact that the cholinergic inward rectification shifted in a manner parallel with potassium reversal potential is most consistent with inwardly rectifying channels. Further evidence identifying this potassium conductance as a true inward rectifier is provided by the barium blockade experiment. The low concentration of barium employed here has been shown to block an inwardly rectifying potassium conductance in other neurons (Osmanovic and Shefner, 1987; Surprenant and North, 1988; Williams et al., 1988a,b). Finally, as opposed to the time-dependent inward current due to the "H" conductance that is not affected by carbachol in mPRF, the conductance increase due to carbachol exhibited rapid kinetics, typical of the potassium inward rectifier (Sakmann and Trube, 1984; Inoue et al., 1988).

The effects of cesium appear more complex. At lower concentrations, the most prominent effect is the antagonism of the inward current as previously reported (Hagiwara et al., 1976; Gay and Standen, 1977; Mihara et al., 1987; Inoue et al., 1988; Williams et al., 1988a,b). At higher concentrations, an additional effect, a shift in the reversal potential, was observed. However, as with the lower concentration, slope conductance positive to the reversal potential was unaffected. This may be consistent with a low partial permeability to cesium resulting

in an additional inward current in the presence of higher external cesium ion concentrations.

Based on the experiments with the muscarinic antagonist pirenzepine and the agonists carbachol and methacholine, the receptors mediating the increase in potassium conductance are of the non-M_1 subtype. It has not been possible to obtain sufficient data to graph Schild plots in order to determine the antagonist dissociation constant, K_B, for the receptor. However, given the relative selectivity of the agents employed, determination of the electrophysiologically relevant receptor as a non-M_1 subtype appears reasonable. Furthermore, our findings are in agreement with autoradiographic data (Mash and Potter, 1986), binding studies (Lazareno and Roberts, 1989), *in situ* hybridization experiments that localized mRNA for the M_2 and M_3 gene product in the pons (Buckley et al., 1988), and behavioral results (Velazquez-Moctezuma et al., 1989), all of which are consistent with the presence of predominantly non-M_1 receptors in this brainstem region.

At the cellular level, the functional implication of an increased inwardly rectifying potassium conductance is that excitability is depressed in a nonlinear manner. For a given amount of cholinergic activation, the increase in conductance will be relatively greater at more hyperpolarized levels and thus exert a relatively greater depressant effect on excitatory input. Similarly, excitatory input will have an increased tendency toward an all-or-nothing effect.

A cholinergically induced depolarization of the pool of mPRF neurons appears to be crucial in the initiation and maintenance of the REM sleep behavioral state (Steriade and McCarley, 1990). The best pharmacological model for REM sleep is achieved with the microinjection of muscarinic (cholinergic) agonists into the mPRF of behaving animals (Baghdoyan et al., 1985). Most mPRF neurons are excited during natural and chemically induced REM. However, it is not unreasonable to expect a subpopulation of mPRF cells, perhaps the descending reticulospinal neurons important in motor activity, to be inhibited during REM sleep. Further work characterizing anatomical projection patterns will be useful in defining the behaviorally related function of the subset of neurons that exhibit muscarinic hyperpolarization.

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