Muscarinic Receptors Modulate N-, P-, and L-type Ca^{2+} Currents in Rat Striatal Neurons through Parallel Pathways

Angela R. Howe and D. James Surmeier

Department of Anatomy and Neurobiology, College of Medicine, University of Tennessee, Memphis, Tennessee 38163

Muscarinic modulation of calcium currents was studied in acutely isolated striatal neurons from the adult rat using the whole-cell configuration of the patch-clamp technique. Muscarinic agonists reduced calcium currents through two distinct signaling pathways. One pathway depended upon PTX-sensitive G-proteins and targeted N- and P-type currents. The other pathway depended upon PTX-insensitive G-proteins and was rendered inactive by high intracellular concentrations of BAPTA and targeted L-type currents. The modulation of N- and P-type currents was relieved by strong depolarizing prepulses, whereas the modulation of L-type currents was not. These findings support the proposition that parallel signaling pathways exist between muscarinic receptors and calcium channels.

[Key words: muscarine, acetylcholine, neuromodulation, dihydropyridine, ω -agatoxin, ω -conotoxin, patch clamp, acute dissociation]

Manipulation of intrastriatal cholinergic signaling has been a long-standing strategy for treating basal ganglia disorders (Calne, 1980). The intrastriatal cholinergic network arises exclusively from large interneurons with widely branching axonal arbors (Bolam et al., 1984; Phelps et al., 1985; DiFiglia, 1987; Wilson et al., 1990). These interneurons richly innervate neurons that project from the striatum, resulting in levels of acetylcholine and its synthetic enzymes that are among the highest of any brain region (Vilario et al., 1991). In spite of its functional importance, the impact of acetylcholine release on the excitability of striatal neurons is not well understood.

Within the striatum, the postsynaptic effects of acetylcholine are mediated primarily by muscarinic receptors. Five muscarinic receptors have been identified through molecular cloning and *in situ* hybridization (Kubo et al., 1986; Bonner et al., 1987, 1988; Bonner, 1989; Laio et al., 1989). These subtypes can be grouped into two broad categories based upon their preferential coupling to second-messenger systems. The m2 and m4 receptor subtypes inhibit adenylate cyclase (for review, see Hulme et al., 1990; Nathanson, 1990). On the other hand, the m1, m3, and

m5 receptor subtypes activate phospholipase C (PLC) which releases inositol 1,3,5 triphosphate (IP_3) and diacylglycerol (DAG) from membrane phospholipid (Hokin and Hokin, 1954; Berridge and Irvine, 1989). The principal neuron of the striatum—the medium-spiny neuron—expresses high levels of two of these five receptors—the m1 and m4 subtypes (Andersen and McKinney, 1988; Bernard et al., 1992), suggesting that acetylcholine may be able to act through at least two signaling pathways to affect excitability.

One peripheral neuron expressing both m1 and m4 receptor subtypes that has been extensively studied is the rat superior cervical ganglion (SCG) neuron (Beech et al., 1991, 1992; Bernheim et al., 1991, 1992; Mathie et al., 1992). In these cells, muscarinic agonists inhibit N- and L-type calcium current through fast and slow signaling pathways. The fast pathway is mediated primarily by the m4 receptor, is membrane delimited, and dependent upon the activation of a pertussis toxin (PTX) sensitive G-protein. This pathway targets only N-type currents. The slow pathway is mediated primarily by the m1 receptor, is dependent upon the activation of a PTX insensitive G-protein, and involves a BAPTA-sensitive, diffusible second messenger. This pathway targets both N- and L-type currents.

We have attempted to test whether this model of muscarinic modulation can be generalized to brain neurons and striatal neurons, in particular. To do so, we have used whole-cell voltage-clamp recording techniques with acutely isolated adult neurons. As shown below, activation of muscarinic receptors in striatal neurons triggers two parallel signaling cascades that modulate N-, P-, and L-type currents. Although the two signaling cascades are similar in many respects to those described in SCG neurons, the PTX-insensitive pathway in striatal neurons only targets L-type currents. This difference suggests that muscarinic signaling mechanisms, particularly those of the BAPTA-sensitive pathway, may not be identical in peripheral and central neurons.

Materials and Methods

Acute-dissociation. Neostriatal neurons from adult (>25 d postnatal) male rats were acutely isolated using procedures similar to those previously reported by our group (Surmeier et al., 1991, 1992). In brief, rats were decapitated under metofane anesthesia. Brains were then quickly removed, iced, and blocked prior to slicing. Slices (400 μm) were cut using a Vibroslice (Campden Instr., London, England) and transferred to a low calcium (100 μM), HEPES-buffered saline solution (in mM: 140 Na isethionate, 2 KCl, 4 MgCl_2 , 0.1 CaCl_2 , 23 glucose, 15 HEPES, pH 7.4, 300–305 mOsm/liter). Slices were then incubated for 1–6 hr in a NaHCO_3 -buffered saline bubbled with 95% O_2 , 5% CO_2 (in mM: 126 NaCl, 2.5 KCl, 2 MgCl_2 , 1 CaCl_2 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , 10 glucose, 1 pyruvic acid, pH = 7.4, 300–305 mOsm/liter). Slices were then removed into the low calcium buffer, and with the aid of a dissecting microscope, regions of the dorsal precommissural striatum were re-

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Correspondence should be addressed to D. James Surmeier, Ph.D., Department of Anatomy and Neurobiology College of Medicine, University of Tennessee, 855 Monroe Avenue, Memphis, TN 38163.

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moved and placed in a Cell Stir (Wheaton, Millville, NJ) containing protease (type XIV, 1.5 mg/ml, Sigma Chemical Co., St. Louis, MO) in HEPES buffered Hank's balanced salt solution (HBSS, Sigma Chemical Co.) at 35°C. Other enzymes, such as trypsin and papain, were also used with comparable results; however, protease treatment provided the highest yield of viable cells. After 20–30 min of the enzyme treatment, the tissue was rinsed several times in the low calcium buffer and mechanically dissociated using a series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux petri dish mounted on the stage of an inverted microscope containing 1 ml of HEPES-buffered HBSS saline. After allowing the cells to settle (about 5 min), the solution bathing the cells was changed to our normal external recording solution.

Whole-cell recordings. Whole-cell recordings employed standard techniques (Hamill et al., 1981; Surmeier et al., 1992). Electrodes were pulled from Corning 7052 or LA16 (Dagan Instr., Inc., Minneapolis, MN) glass and fire polished prior to use. The internal recording solution consisted of (in mM) 180 *N*-methyl-D-glucamine (NMG), 40 HEPES, 4 MgCl₂, 0–10 EGTA, or 0.1–20 BAPTA, 12 phosphocreatine, 2 Na₂ATP, 0.2 Na₂GTP, 0.1 leupeptin, pH = 7.2 with H₃PO₄, 270–275 mOsm/liter. The external recording solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 MgCl₂, 10 HEPES, 0.001 TTX, 5 BaCl₂, pH = 7.5 with NaOH, 300–305 mOsm/liter. The nicotinic receptor antagonist, mecamylamine (10 μ M, RBI, Inc., Natick, MA) was added to all solutions to block nonmuscarinic receptors. Nifedipine and (–)Bay K 8644 (RBI, Inc., Natick, MA) were made up as concentrated stocks in 95% ethanol and then diluted immediately prior to use. These solutions were protected from ambient light. Final ethanol concentrations never exceeded 0.05%. Although previous experiments had shown that this concentration has no effect on currents, ethanol controls were run for every cell. ω -Conotoxin GVIA (ω -CgTx, Peninsula Lab., Belmont, CA; RBI, Inc.) and ω -agatoxin IVA (ω -AgTx, Peptides International, Louisville, KY) were prepared as concentrated stocks in water, then frozen in aliquots that were thawed and diluted immediately prior to use. When using ω -AgTx, cytochrome *c* (Calbiochem, San Diego, CA) was added to all solutions (0.01%) to prevent nonspecific binding to plastics (see Mintz et al., 1992). Carbachol, muscarine, and atropine (RBI Inc.) were prepared as fresh concentrated stocks in water immediately prior to use. GTP γ S or GDP- β -S (Calbiochem) were substituted for GTP in some experiments at a concentration of 600 μ M and 1–5 mM, respectively. To determine the PTX sensitivity of the signaling pathway, we utilized the catalytic domain of the toxin (PTX a protomer, List Laboratories, Campbell, CA) at a concentration of 10 ng/ml. The protomer was added to the solution used to backfill the recording pipette. Dialysis of the protomer into the cell appeared to be complete within 10 min (Pusch and Neher, 1988).

Electrode resistances were typically 3–6 M Ω in the bath. Recordings were obtained using a Dagan 3900 Integrating patch clamp equipped with the 3911A Whole-cell Expander module (Dagan Instr., Minneapolis, MN) or an Axon Instruments 200 patch clamp. Recordings were monitored and controlled with a PC 486 clone using pCLAMP (v. 5.0–5.5) with a 125 kHz interface (Axon Instr., Inc., Foster City, CA). After seal rupture, series resistance compensation (70–80%) was routinely employed; given the amplitudes of our currents, series resistance errors should never have exceeded 2–3 mV. Recordings were made only from medium-sized neurons (somal diameter 5–10 μ m) that had at most one or two short processes. The adequacy of voltage control was assessed after compensation by examining the tail currents generated by strong depolarizations. Cells in which tail currents did not decay rapidly and smoothly at subthreshold potentials were eliminated from the study. Potentials were not corrected for the liquid junction potential with the NMG internal solution, which was estimated to be about 7 mV.

Drugs were applied using a gravity-fed “sewer pipe” system. The array of application capillaries (~150 μ m i.d.) was positioned a few hundred micrometers away from the cell under study. Solution changes were made by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport-Klinger, Inc., Irvine, CA). Complete solution changes were achieved within less than 1 sec, as judged by changes in reversal potential. As a further check, the time course of channel block by Cd²⁺ (200 μ M) was also determined. Cd²⁺ block of whole-cell Ca²⁺ current developed with a time constant less than 500 msec in all cells tested (*n* = 5).

Data were gathered using a combination of voltage step and ramp protocols. In most cases, those currents not blocked by Cd²⁺ (200 μ M) were subtracted from control records (in nearly all cells, there was little or no evidence for Cd²⁺-insensitive currents at membrane potentials

below 0 mV; above this potential, small tail currents that were sensitive to Cl[−] replacement were observed). Peak current–voltage plots derived from Ba²⁺ currents evoked with short (10–30 msec) step protocols and from slow voltage ramps (0.25–0.5 mV/msec) were very similar in most cells in which they were compared (*n* = 10/11). Faster ramps yielded current–voltage curves that were shifted toward more depolarized potentials (see Bargas et al., in press).

Data analysis. Dose–response curves were fit with a Langmuir isotherm of the form: $I_{\text{peak}} = I_{\text{sensitive}} \cdot (1 + [M]/EC_{50})^{-n} + I_{\text{resistant}}$, where I_{peak} was the maximum ramp current, $I_{\text{sensitive}}$ was the blockable current, $[M]$ was the agonist concentration, EC_{50} was the concentration of agonist producing 50% block, $I_{\text{resistant}}$ was the portion of the current resistant to modulation, and *n* was usually set to one (varying *n* between 0.5 and 1.5 did not significantly enhance the fits for the data described here). Fitting was done using a least squares criterion with a commercially available statistical package (SYSTAT, Inc., Evanston, IL) running on a Macintosh computer. Statistical analyses also used this program. Statistics on large samples (*n* > 20) are presented as means (μ) $\pm$ SEM. For smaller samples where means do not necessarily give a good measure of central tendency, medians and ranges are given. When the sample sizes allowed, box plots of the data were drawn. In the box plot, the median is represented as the central bar of the box, the edges of the box are the interquartiles (technically fourths), the ‘whiskers’ are lines drawn to the most extreme points in the sample that are not outliers (defined as points beyond interquartile $\pm$ 1.5 · interquartile range); outliers are shown as circles or asterisks (Tukey, 1977).

Results

Muscarinic agonists reversibly inhibit Ca²⁺ current through a G-protein signaling pathway

The muscarinic agonists, muscarine and carbachol, reversibly inhibited whole-cell Ca²⁺ current in rat striatal neurons (Fig. 1). Muscarine dose–response plots were well fit with a single isotherm having an EC₅₀ in the low nanomolar range. Shown in Figure 1C is a representative dose–response plot. The EC₅₀ in this neuron was 1.0 nM; similar results were obtained in three other cells (EC₅₀ = 6.5 $\pm$ 1.4 nM). Saturating concentrations of muscarine (1 μ M) reduced the peak whole-cell Ca²⁺ current an average of 46.0 $\pm$ 6.4% (*n* = 11). But, as shown in the box plot in Figure 3C, the relative reduction in current amplitude at saturating concentrations varied somewhat from cell to cell. Carbachol dose–response plots were well fit with a single isotherm having EC₅₀s in the low micromolar range. A representative dose–response plot from a single neuron is shown in Figure 1D. The EC₅₀ in this neuron was 2.7 μ M; similar results were seen in 11 other neurons (EC₅₀ = 3.25 $\pm$ 0.89 μ M). On average, saturating concentrations of carbachol (10 μ M) were not as effective in reducing peak currents as muscarine (mean reduction = 32.3 $\pm$ 1.2%, *n* = 106). However, as shown in the box plot shown in Figure 1D, there was variability in the magnitude of the modulation from cell to cell. As shown in Figure 2A, the modulation by muscarine was blocked by the muscarinic receptor antagonist atropine (1 μ M) (*n* = 5); similar results were obtained with carbachol (*n* = 10), arguing that the effects of both agonists were mediated by muscarinic receptors.

To test for the involvement of a GTP-binding protein (G-protein) in the modulation, we utilized the nonhydrolyzable GTP analog, GTP γ S. Once bound, GTP γ S cannot be metabolized, resulting in the persistent activation of the G-protein alpha subunit (for review, see Breitwieser and Szabo, 1988). Dialysis for 4–5 min with GTP γ S (600 μ M, 0 GTP) prevented the complete reversal of the reduction in currents produced by carbachol (Fig. 2B)—consistent with the involvement of a G-protein in the signaling pathway. Shown in the inset of Figure 2B is a box plot summarizing the percent recovery of the carbachol modulation 10–15 min after seal rupture in control and

Figure 1. Muscarinic agonists reduce Ca^{2+} currents in a reversible, dose-dependent fashion. Whole-cell calcium current was elicited by a ramp voltage protocol (0.5 mV/sec). Data points represent peak I_{Ca} evoked by the ramp. **A**, Plot of peak current as a function of time during the application of carbachol (10 μM). The timing of carbachol application is coded by the bar above the plot and the shading of the symbols. **B**, Representative ramp currents taken from points labeled 1 and 2 in **A**. **C**, A representative dose-response curve for muscarine was generated using increasing concentrations of agonist. The data points were well fit with a single isotherm having an EC_{50} of 1.0 nM. The inset is a box plot of the percent reduction in the presence of muscarine in 11 cells. **D**, A representative dose-response curve for carbachol was generated as in **C**. The data points were fit with a single isotherm having an EC_{50} of 2.7 μM . The inset is a box plot of the percent reduction in the presence of carbachol in 106 cells. Recordings were made in the absence of extrinsic calcium chelators.

GTP γ S dialyzed cells. Dialysis with 1–5 mM GDP- β -S did not eliminate the modulation produced by carbachol. Lowering the ATP concentration in the internal solution (1 mM) did not significantly enhance the effects of GDP- β -S. Similar negative results with GDP- β -S have been reported for modulations in other neurons with known G-protein involvement (Ikeda and Schofield, 1989).

N-, L-, and P-type components of the whole cell calcium current are modulated

Whole-cell calcium currents in adult, medium-spiny striatal neurons are of the high-voltage-activated (HVA) type. Four classes of Ca^{2+} current contribute to whole-cell currents in these cells: N-, L-, and P-type currents and a fourth, pharmacologically uncharacterized current (Bargas et al., in press). We utilized the calcium channel blockers ω -conotoxin GVIA (ω -CgTx), ω -agatoxin IVA (ω -AgTx) and nifedipine to determine which components of the whole-cell calcium current are modulated by the muscarinic agonist carbachol.

To simplify the task of determining whether P-type currents were modulated, N- and L-type currents were first eliminated with saturating concentrations of the N-channel blocker ω -CgTx (2 μM) and the L-channel blocker nifedipine (5 μM). In the presence of these antagonists, carbachol inhibited a significant component of the remaining current. A plot of the peak current evoked by a voltage ramp as a function of time and drug treatment in a typical neuron is shown in Figure 3A. In this cell, the subsequent addition of the P-channel blocker ω -AgTx (100 nM) reduced the effects of carbachol, demonstrating that P-type currents were modulated (Figure 3A). Washing led to a partial recovery that was largely attributable to reversal of the nifedipine block of L-type currents (Hoehn et al., 1993; Bargas et al., in press). A statistical summary is shown in the inset; as can be seen from the box plot, the addition of ω -AgTx (in the presence of N- and L-type blockers) reduced the modulation produced by carbachol by more than 80% in most neurons ($n = 7$). It is unclear whether the small residual modulation seen in the presence of all three channel antagonists represents modulation of

Figure 2. A muscarinic receptor and GTP-binding protein (G-protein) are involved in the signaling pathway. *A*, The modulation was eliminated in the presence of the muscarinic antagonist atropine ($1 \mu\text{M}$) indicating the involvement of the muscarinic receptor in the modulatory pathway. I_{Ca} was elicited by a 20 msec step to 0 mV from a holding potential of -90 mV. *B*, Substitution of GTP in the internal solution with the nonhydrolyzable GTP analog, GTP γ S ($600 \mu\text{M}$) resulted in an irreversible suppression of I_{Ca} in response to receptor activation by $10 \mu\text{M}$ carbachol. The inset shows the average recovery from muscarinic suppression for cells with GTP versus GTP γ S in the internal solution.

unblocked L-, N-, or P-type currents or modulation of the resistant current. Nevertheless, the impact of ω -AgTx on the modulation clearly demonstrates that P-type currents were modulated.

A similar paradigm was used to determine whether N-type currents were modulated by activating muscarinic receptors. In the presence of L- and P-type channel antagonists, carbachol reduced a significant component of the remaining current. A plot of the peak current evoked by a voltage ramp as a function of time and drug application in an exemplary neuron is shown in Figure 3*B*. The subsequent addition of the N-channel blocker ω -CgTx reduced the modulation, demonstrating that N-type currents were modulated. Washing led to a partial recovery that was largely attributable to reversal of the nifedipine block. Shown in the inset is a box plot summary of the percentage reduction in the carbachol modulation produced by ω -CgTx in the presence of nifedipine and ω -AgTx ($n = 4$).

Although its modulation has been reported infrequently (Bernheim et al., 1992; Mathie et al., 1992; Sayer et al., 1992; for

Figure 3. N- and P-type currents are modulated by muscarinic agonists. *A*, The modulation of P-type current was studied in the presence of the L-channel blocker nifedipine ($5 \mu\text{M}$) and the N-channel blocker ω -CgTx ($1 \mu\text{M}$). Carbachol ($10 \mu\text{M}$) inhibited a significant component of the remaining current. Subsequent addition of the P-channel blocker ω -AgTx (100 nM) eliminated the modulation demonstrating the involvement of P-type currents. The inset shows a statistical summary of the percentage modulation eliminated by the addition of ω -AgTx. *B*, The modulation of N-type current was studied in the presence of the L-channel blocker nifedipine ($5 \mu\text{M}$) and the P-channel blocker ω -AgTx (100 nM). Carbachol ($10 \mu\text{M}$) inhibited a significant component of the remaining current. Subsequent addition of the N-channel blocker ω -CgTx ($1 \mu\text{M}$) eliminated the modulation demonstrating the involvement of N-type currents. The inset shows a statistical summary of the percentage modulation eliminated by the addition of ω -CgTx.

review, see Tsien et al., 1988), L-type current was also modulated by carbachol in striatal neurons. An approach similar to that described for N- and P-type currents consistently revealed an occlusion of the carbachol modulation by treatment with the L-type channel antagonist nifedipine (5 – $10 \mu\text{M}$) ($n = 4$, data not shown). Another way in which the involvement of L-type channels can be examined is with the use of the dihydropyridine agonist (–)Bay K 8644. Bay K 8644 selectively enhances the current through L-type channels (Kokubun and Reuter, 1984; Plummer, 1989; Jones and Jacobs, 1990), shifting the peak of the I - V curve to more negative potentials (Fig. 4*A,B*; Fox et al., 1987; Tsien et al., 1988; Tsien, 1993) and slowing the deactivation tail currents (Fig. 4*B*; Fox et al., 1987; Tsien et al., 1988; Tsien, 1993). The slow component of the deactivation tail in the presence of Bay K 8644 is carried exclusively through L-channels (Plummer et al., 1989; Jones and Jacobs, 1990). Alterations in this slow tail current were used as a measure of muscarine's effect on L-type currents. As shown in Figure 4*C*, the application of carbachol in the presence of Bay K 8644 reduced peak currents. In addition, carbachol reduced the slow component of the tail current as judged by the difference currents shown in Figure 4*D*. This modulation readily reversed with washing. The statistical summary of the carbachol-induced re-

Figure 4. Bay K 8644-enhanced tail currents are modulated by carbachol in the absence of intracellular calcium chelators. *A*, Addition of the L-channel agonist Bay K 8644 (1 μM) selectively enhances current through L-type channels, shifting the peak of the I-V curve to more negative potentials and slowing the deactivation tail current. *B*, An expanded view of the tail currents shown in *A*. *C*, In the absence of intracellular calcium chelators, 10 μM carbachol reduced peak currents and the slow tail current. *D*, An expanded view of the tail currents in control and in the presence of carbachol. Also plotted is the difference current showing that the carbachol-induced reduction was of the slow component of the tail current.

duction in tail amplitudes (measured 12 msec into the tail, see the arrow shown in Fig. 4*B*) for a subset of neurons ($n = 15$) is shown in the inset of Figure 4*D*. In this sample, the median reduction in tail amplitude was approximately 30%. This result confirms the inference that L-type currents were reduced by carbachol.

The modulation of L-type current appears to involve a pathway distinct from that of the other types of Ca^{2+} current in striatal neurons

In SCG cells, the modulation of L-type currents is abolished by high intracellular concentrations of the calcium chelator BAPTA (Beech et al., 1991; Bernheim et al., 1991), whereas the modulation of N-type currents is not. In striatal neurons, the modulation of N- and P-currents appeared to be unaffected by high concentrations of intracellular BAPTA (20 mM), indicating that the signaling pathway targeting these channels is not dependent upon intracellular calcium ($n = 5$, data not shown). In contrast, modulation of L-type currents was blocked by dialysis with 20 mM BAPTA, as judged from failure of carbachol to reduce Bay K 8644-enhanced tail currents (Fig. 5*A,B*). Shown in the inset of Figure 5*B* is a summary of the change in the Bay K 8644-

enhanced tail current produced by carbachol in recordings using internal solutions containing 20 mM BAPTA and no added Ca^{2+} ($n = 10$). The free concentration of Ca^{2+} in the dialysate, rather than BAPTA per se, appeared to be the crucial factor in these experiments. In most cells (92%, $n = 39$), raising the estimated free Ca^{2+} concentration to 150 nM in the presence of BAPTA (20 mM) restored the ability of muscarinic agonists to reduce Bay K 8644-enhanced tail currents. An example of the effect of raising intracellular Ca^{2+} is shown in Figure 5*C* and *D*. Both the peak and slow component of the tail current were modulated by carbachol in this cell. A statistical comparison of the reduction in the slow tail measured with a low BAPTA internal (0–0.1 mM BAPTA) to that seen using an internal solution containing 20 mM BAPTA with 10 mM Ca^{2+} added is shown in the inset. Although the reductions in tail amplitude in these two situations were both significantly greater than those produced in the presence of 20 mM BAPTA without added Ca^{2+} (low BAPTA: $p < 0.001$, Kruskal–Wallis ANOVA; high BAPTA: $p < 0.001$, Kruskal–Wallis ANOVA), they were not significantly different from each other ($p > 0.05$, Kruskal–Wallis ANOVA).

A second feature distinguishing the muscarinic pathways in SCG neurons is the PTX sensitivity of the G-protein involved

Figure 5. Chelation of intracellular Ca^{2+} eliminates the reduction of the Bay K 8644-enhanced tail current but restoration of intracellular free Ca^{2+} to near 150 nM reenables the modulation. *A*, The application of carbachol (10 μM) following dialysis of the cell with a solution containing 20 mM BAPTA did not abolish the reduction in peak current but did abolish the reduction in the slow tail current. *B*, An expanded view of the tail current showing that the slow component was unaffected by carbachol. The difference current shows that only a rapidly deactivating current was reduced. Shown in the inset is a summary of the change in the Bay K 8644-enhanced tail current produced by carbachol under these conditions. *C*, When carbachol was applied to cells containing the same concentration of BAPTA but with 10 mM Ca^{2+} (to bring the free Ca^{2+} concentration to near 150 nM), the modulation of the peak current was enhanced and the reduction of the slow tail current was restored. *D*, An expanded view of the tail currents in control solution and after application of carbachol; also shown are the difference currents. Shown in the inset is a statistical comparison of the reduction in the slow tail measured with a low BAPTA internal to that seen using an internal solution containing 20 mM BAPTA with 10 mM added Ca^{2+} .

(Beech et al., 1992). In these cells, the rapid modulation of N-type current is sensitive to PTX; the slow modulation of N- and L-type currents is not. The PTX-sensitivity of the G-protein involved in muscarinic modulation of calcium currents in striatal neurons was determined by dialyzing cells with the catalytic domain of PTX, the PTX α -protomer (Haydon et al., 1991; Elmslie, 1992). As shown in the plot of peak currents in Figure 6, carbachol reduced whole-cell currents. To determine carbachol's effect on N- and P-type currents, carbachol was applied in the presence of the L-type current antagonist nifedipine. As shown at the first downward arrow, carbachol was initially capable of modulating these currents. Washing out nifedipine restored the peak currents to their previous values. After several minutes of PTX, a protomer dialysis, nifedipine was reapplied and carbachol's effects measured. Now, carbachol had no effect on the whole-cell currents (second arrow), suggesting that the signaling pathway affecting N- and P-type currents was blocked.

After washing out the nifedipine, carbachol was reapplied, revealing that the modulation of the L-type currents was still intact, and little changed from its initial value (as judged by the difference in the first and second modulations). Similar results were observed in four cells.

The loss of carbachol's effect on N- and P-type currents in these experiments was not likely to have been a consequence of rundown, as the modulation of these currents was normally very robust. In a sample of 10 cells where the modulation of N- and P-type currents was isolated, the median modulation 10–15 min after seal rupture was close to 90% of that measured 5 min after initiation of whole-cell recording (see box plot inset of Fig. 6). Desensitization also was not a factor, as repeated application of carbachol in control cells led to very little attenuation in the response magnitude (see inset Fig. 6). In six cells tested with repeated applications (like those of the inset), the median modulation after 10 min was 89% of the initial value. Taken together,

Figure 6. The modulation of N- and P-type currents is blocked by dialysis with the a-protomer of PTX. Peak ramp currents are plotted as a function of time following seal rupture. The internal solution contained the PTX a-protomer (10 ng/ml). Carbachol (10 μM) was applied periodically (shown by the bars at the top and the shading of the symbols). Initially, the L-type current antagonist nifedipine did not occlude the carbachol reduction. However, after a few minutes of dialysis, nifedipine was able to completely occlude the modulation, suggesting that the muscarinic receptors were no longer able to affect N- and P-type currents. The time course was corrected for a 20% rundown in the currents by subtraction of an exponential fit to the currents during control periods. The box plot is a statistical summary of the percentage change in the magnitude of the modulation sensitive to carbachol at 5 and 10 min into the recording showing that the observed effect is not due to rundown of the modulation. Nor is it a result of desensitization, as repeated applications of carbachol led to very little attenuation of the response as shown in the inset.

these results suggest that a PTX-sensitive G-protein is involved in the modulation of N- and P-type currents but not of L-type currents.

A third feature distinguishing muscarinic pathways in SCG cells is the voltage dependence of the modulation (Beech et al., 1992). The voltage dependence of the muscarinic effects in striatal neurons was studied by comparing the modulation of currents evoked by a step to -20 mV with and without strong ($+100$ mV) depolarizing conditioning steps (30 msec). In all neurons, strong depolarizing prepulses enhanced the kinetics and amplitude of currents evoked by the test step (Fig. 7A, inset), as described for a number of other neurons (Bean, 1989; Boland and Bean, 1993). When intracellular Ca^{2+} was not extrinsically buffered, the current reductions produced by carbachol were attenuated in most cells (3/5) by depolarizing prepulses. However, the magnitude of the attenuation, if present, was highly variable (11–60%). One possible explanation for the variation is that when Ca^{2+} is unbuffered it may be low or high, effectively enabling or disabling the Ca^{2+} -dependent pathway that is thought to be voltage independent (see below). To test this possibility, intracellular free Ca^{2+} was buffered into the low nanomolar range by dialyzing with a solution containing 20 mM BAPTA and no Ca^{2+} . As shown above, this maneuver limits the muscarinic modulation to N- and P-type currents. Under these circumstances, the reductions produced by carbachol were

more consistently attenuated by depolarizing prepulses (Fig. 7A,B) (median modulation after the prepulse was 63% of control, see inset Fig. 7B).

This result suggests that some of the variation in voltage dependence seen without Ca^{2+} buffers could have been a consequence of a voltage-independent modulation of L-type currents (Beech et al., 1992; Mathie et al., 1992). To test whether the modulation of L-type currents was voltage dependent, (–)Bay K 8644-enhanced tail currents were examined in the same protocol with free Ca^{2+} buffered to near 150 nM. Under these conditions (see above), the slow tail is reduced by carbachol (Fig. 7C). Strong depolarizing prepulses did not attenuate the carbachol-induced reduction in these slow tail currents (Fig. 7D). In fact, the median modulation was larger following a strong conditioning depolarization (median modulation = 119%, see inset Fig. 7D). Although the samples were small, statistical analysis of the change in modulation in these two conditions suggested the differences were significant ($p < 0.05$, Kruskal–Wallis ANOVA).

A final feature distinguishing the muscarinic pathways in SCG cells is onset kinetics (Bernheim et al., 1991). One pathway that targets N-type channels is fast ($\tau < 1$ sec), whereas the second pathway that targets N- and L-type currents is slower ($\tau \sim 3$ –35 sec, depending on agonist concentration). To determine whether fast and slow components could be resolved in striatal neurons, test steps to 0 mV were delivered once a second during drug application. Rapidly moving the perfusion barrels effected an agonist concentration jump that was complete in less than a second (see inset, Fig. 8A). At saturating concentrations of agonist (10 μM carbachol), plots of peak current as a function of time were well fit with a single exponential in all cases ($n = 10$). An example is shown in Figure 8A; in this neuron, the onset of the modulation developed as a single exponential with a time constant of 3.86 sec; other cells had similar kinetics (median = 3.01 sec, $n = 10$; see inset, Fig. 8B). The kinetics of the reduction in the slow, Bay K 8644-enhanced tail current subsequent to carbachol application were similar ($2.5 < \tau < 6.5$ sec, $n = 13$, data not shown). To determine whether a fast component was present but merely masked by large L-type current modulation, intracellular Ca^{2+} was chelated to low levels with 20 mM BAPTA. Under these conditions, the onset of the modulation was not significantly faster. An example is shown in Figure 8B. Here, the change in current amplitude was well fit, with an exponential having a single time constant of 3.25 sec; other neurons were similar (median = 2.8 sec, $n = 9$, see inset Figure 8B). The differences in the time constants in the two buffering conditions were not significant ($p > 0.05$, Kruskal–Wallis ANOVA).

Discussion

Muscarinic receptors modulate HVA Ca^{2+} currents

In rat striatal neurons, activation of muscarinic receptors triggers two parallel signaling cascades that result in the modulation of N-, P-, and L-type HVA Ca^{2+} currents. The muscarinic reductions in Ca^{2+} currents were dose dependent and reversible. This finding is consistent with previous reports in striatal neurons (Misgeld et al., 1986) and in other cell types (Adams et al., 1982; Brown and Selyanko, 1985; Heschler et al., 1986, 1987; Gahwiler and Brown, 1987; Wanke et al., 1987; Brown et al., 1989; Toselli and Lux, 1989; Fisher and Johnston, 1990; Beech et al., 1991; Bernheim et al., 1992; Mathie et al., 1992) showing muscarinic reductions in Ca^{2+} currents. As in rat SCG neurons (Bernheim et al., 1992), it is highly likely that these striatal

Figure 7. The modulation of N- and P-type currents was attenuated by depolarizing prepulses, but the reduction of L-type currents was not. *A*, Whole-cell calcium currents were elicited by a step to -20 mV from a holding potential of -80 mV in a cell dialyzed with an internal solution containing 20 mM BAPTA, 0 Ca^{2+} . The application of carbachol ($10 \mu\text{M}$) reduced the currents evoked by the step. Conditioning with a step to $+100$ mV (30 msec) enhanced the currents evoked by the step to -20 mV, as shown in the inset. *B*, Conditioning steps to $+100$ mV (30 msec) attenuated the modulation. The bar shows the amplitude of the modulation without the conditioning step in *A*. The inset is a statistical summary showing the percentage control modulation remaining after the conditioning step. *C*, When the internal Ca^{2+} was elevated to near 150 nM (5 mM BAPTA, 2.8 mM Ca^{2+}), carbachol ($5 \mu\text{M}$) was able to modulate the slow Bay K 8644 tail current (as shown above). *D*, Conditioning steps to $+100$ mV in these recording conditions did not attenuate the reduction in the slow tail currents produced by carbachol. The inset is a statistical summary showing the percentage control modulation remaining after the conditioning step under conditions of high and low intracellular Ca^{2+} .

pathways depend upon m1 and m4 class receptors in striatal neurons. The principal neuron of the striatum—the medium-spiny projection neuron—is known to colocalize m1 and m4 receptors (Anderson and McKinney, 1988; Bernard et al., 1992). As these neurons constitute more than 95% of all striatal neurons, the vast majority of our recordings were undoubtedly from this cell type.

N- and P-type currents are modulated through a PTX-sensitive G-protein

In spite of our inability to definitively isolate m1 and m4 pathways at the receptor level, two signaling pathways with features known to be associated with each receptor were identified by manipulation of G-proteins and intracellular Ca^{2+} . In a variety of cell types, m4 receptors couple with PTX-sensitive G_i / G_o class proteins, whereas m1 receptors do not (Nathanson, 1987; Peralta et al., 1988; Hulme et al., 1990; Bernheim et al., 1992). Dialysis with the α -protomer of PTX eliminated the muscarinic modulation of N- and P-type currents but did not noticeably affect modulation of L-type currents. This result is consistent

with studies in peripheral neurons showing that activation of the PTX-sensitive pathway reduces N-type currents (Marchetti et al., 1986; Bernheim et al., 1991; Beech et al., 1992). Recent work with central neurons (Mintz and Bean, 1993) has revealed reductions in P-currents subsequent to activation of receptors (GABA_B) that have been shown to couple to PTX-sensitive G-proteins (Alford and Grillner, 1991; Knott et al., 1993). In these reports, the modulation of N- and P-currents remained when intracellular Ca^{2+} was buffered to nominally low nanomolar levels and was relieved by strongly depolarizing prepulses (i.e., voltage dependent). The modulation of N- and P-type currents by muscarinic agonists in striatal neurons possessed both of these features, indicating that the striatal PTX-sensitive signaling pathway is similar to that found in other central and peripheral neurons.

Only the onset kinetics of the modulation of N- and P-type currents differed from previous descriptions. In SCG neurons, the rapid ($\tau < 1$ sec), PTX-sensitive reduction in N-type currents is associated with a membrane delimited, G-protein-mediated modulation. Rather than having a time constant of less than a

Figure 8. The kinetics of the muscarinic reduction were similar with and without Ca^{2+} chelators. **A**, Calcium current was elicited by a 10 msec step to 0 mV from a holding potential of -80 mV. The test step was repeated once every second. The onset of the modulation was well fit with a single exponential curve ($I = I_{\text{sen}} \exp(-(t - t_0)/\tau) + \text{constant}$, where I_{sen} was the reducible part of the current, t_0 was the time of drug application, and the constant was the nonreducible part of the current). τ in this cell was 3.86 sec. The inset is the single exponential fit of Cd^{2+} blockade of the current having a τ of 400 msec, indicating the speed with which complete solution changes are achieved with our perfusion system. **B**, The same experiment was performed in the presence of a high concentration of intracellular calcium chelator (20 mM BAPTA). A single exponential curve having a τ of 3.25 sec was fit to the onset of the muscarinic suppression. The inset is a box plot showing the distribution of time constants in neurons dialyzed without buffer and those dialyzed with 20 mM BAPTA. The distributions were not statistically different ($p > 0.05$, Kolmogorov-Smirnov, $df = 8$; $p > 0.05$, Kruskal-Wallis analysis of variance).

second, the onset of the striatal modulation of N- and P-type currents at carbachol concentrations 3–4 times the IC_{50} usually had time constants in the 2–3 sec range (see Fig. 8B). It is difficult, however, to assign much significance to this difference. In other cell types, onset kinetics very similar to ours have been reported for modulations thought to be mediated by direct

G-protein effects on Ca^{2+} channels (Jones, 1991; Boland and Bean, 1993). Hence, kinetics alone do not appear to be a reliable means of distinguishing direct and indirect signaling pathways. One reason for this experimental variability may be that in small cells (e.g., striatal neurons) patching (or dissociation) disrupts the cytoskeleton in such a way that the spatial constraints upon

signaling elements (receptors, G-proteins, and targets) are lost. Constraints upon these signaling elements appear to be crucial to the rapid kinetics of membrane delimited pathways (Brown, 1993). The loss of these constraints lead to kinetic predictions very similar to kinetics we observed (Brown, 1993).

L-Type currents are modulated through a BAPTA-sensitive pathway involving a PTX-insensitive G-protein

In the absence of extrinsic Ca^{2+} chelator, muscarinic agonists reduced L-type currents in striatal neurons. The involvement of L-type currents was deduced not only from the occlusive effects of the dihydropyridine antagonist nifedipine but also from the ability of muscarinic agonists to reduce the slow component of Bay K 8644-enhanced tail currents (Plummer et al., 1989; Jones and Jacobs, 1990). Although initially L-type currents in neurons were not thought to be neuromodulatory targets, recent work has revealed several types of receptor negatively coupled to this class of channel (Bley and Tsien, 1990; Beech et al., 1991, 1992; Bernheim et al., 1991, 1992; Mathie et al., 1992; Sayer et al., 1993).

The insensitivity of the L-type current modulation to dialysis with the PTX catalytic subunit (a-protomer) is consistent with the hypothesis that this modulation is mediated by m1 class receptors (Wanke et al., 1987). A similar linkage has been established in rat SCG neurons, where m1 receptors modulate L-type currents through a PTX-insensitive G-protein and a soluble second messenger (Bernheim et al., 1992). In these neurons, as in striatal neurons, the modulation is relatively slow ($\tau > 2$ sec), is not attenuated by depolarizing conditioning steps, but is eliminated by high concentrations of Ca^{2+} chelator (Beech et al., 1992). In spite of the BAPTA sensitivity, the SCG signaling pathway does not appear to involve Ca^{2+} other than in a permissive way. There are reasons to believe that fluctuations in intracellular Ca^{2+} may play a more direct role in the modulation of L-type currents in striatal neurons. In these neurons, m1 receptors are coupled to phospholipase C and the production of diacylglycerol (DAG) and inositol triphosphate (IP_3) (Peralta et al., 1988). In a variety of cell types, IP_3 triggers the release of Ca^{2+} from intracellular stores. Preliminary photometry experiments (Surmeier, Harrington, and Hille, unpublished observations) have confirmed this inference by showing that muscarinic agonists are capable of inducing transient elevations in cytosolic Ca^{2+} levels in striatal neurons, in contrast to SCG neurons (Bernheim et al., 1991). Furthermore, caffeine, a potent liberator of Ca^{2+} from ryanodine-sensitive Ca^{2+} stores (Endo, 1977; Leitjen and Van Breeman, 1984), also mimics the ability of muscarinic agonists to reduce L-type currents in striatal neurons (Howe and Surmeier, 1993). Given these facts, it is plausible to assume that Ca^{2+} per se is causally linked to the reduction in striatal L-type currents by muscarinic agonists. A schematic depicting our working hypotheses about this signaling pathway and the pathway targeting N- and P-type channels is shown in Figure 9.

What is less clear is why elevating free Ca^{2+} in the presence of 20 mM BAPTA also enables modulation of L-current. In this circumstance, Ca^{2+} should still be well buffered. There are two possible explanations. One possibility is that elevation of cytosolic Ca^{2+} above 100 nM by muscarinic agonists is necessary but not sufficient to bring about the modulation of L-currents. Although a number of cellular signaling molecules are Ca^{2+} dependent, the one directly linked to m1 receptors (protein kinase C) has been reported to enhance L-type currents (Yang and

Figure 9. A schematic summarizing the elements involved in the muscarinic modulation of HVA currents in neostriatal neurons. Muscarinic receptors couple to PTX-sensitive and -insensitive G-proteins. The PTX-sensitive pathway reduces N- and P-type currents through a mechanism that is not affected by chelating Ca^{2+} to low nanomolar levels. The PTX-insensitive pathway reduces L-type currents through signaling elements that are blocked by Ca^{2+} chelation.

Tsien, 1993). Another possibility is that dialyzed BAPTA cannot penetrate all cellular compartments equally well and, as a consequence, does not buffer Ca^{2+} efficiently throughout the cell. Dialysis with high millimolar concentrations of BAPTA in the absence of added Ca^{2+} may block the modulation by depleting releasable Ca^{2+} stores—a consequence not brought about by dialysis with a Ca^{2+} supplemented solution. We are testing these possibilities by combining perfused pipette whole-cell patch clamp measurements with coincident photometric measurements of free Ca^{2+} .

In spite of these caveats, the hypothesis that Ca^{2+} per se is the muscarinic signal is consistent with the well-described Ca^{2+} -dependent inactivation of L-type currents (Eckert and Chad, 1984; Kalman et al., 1988; Armstrong, 1989). In a variety of cell types, elevation of intracellular Ca^{2+} leads to a reduction in L-type current. There are a number of enzymes that are dependent upon calmodulin and intracellular Ca^{2+} that could be involved in this process (Stryer, 1988; Hille, 1992). One likely candidate is the calcium/calmodulin-dependent phosphatase, calcineurin (Armstrong, 1989). In muscle cells, calcineurin dephosphorylates protein kinase A (PKA) sites on dihydropyridine-sensitive, L-type channels and, in so doing, reduces transmembrane current. If a similar mechanism is important in striatal neurons, PKA activation should enhance L-type currents. This appears to be the case in a subset of medium-sized striatal neurons (Surmeier et al., unpublished observations). Assuming that there is constitutive activity of PKA (or a kinase with similar substrate specificity), dephosphorylation of L-type channels by calcineurin could produce the pattern of effects we have observed. This interaction—between D1 dopamine receptor-mediated activation of PKA and muscarinic receptor activation of calcineurin—could be a cellular counterpart of the long-standing clinical observation that dopaminergic and cholinergic ag-

onists oppose one another in the control of basal ganglia-dependent movements (Barbeau, 1962).

Functional implications

By reducing the entry of Ca^{2+} through voltage-dependent channels, acetylcholine will influence a variety of cellular functions. The integrative properties of striatal neurons, for example, should be altered in at least two ways. First, by minimizing the contribution of Ca^{2+} -dependent regenerative events in the dendrites (Lev-Ram et al., 1992; Amitai et al., 1993), acetylcholine should reduce the conduction of postsynaptic charge to the soma/initial segment. In agreement with this inference, activation of striatal muscarinic receptors has been shown to reduce the amplitude of synaptic potentials evoked in response to cortical stimulation (Calabresi et al., 1991). Second, the reduction of HVA Ca^{2+} entry during spiking should attenuate Ca^{2+} -dependent potassium currents that slow discharge in a variety of neurons (Lancaster et al., 1990; McCobb and Beam, 1991), including striatal neurons (Pineda et al., 1992). By acting on a portion of the dendritic tree and the soma of a neuron, cholinergic interneurons could functionally shrink targeted dendrites and enhance the somatic response to sustained excitatory inputs arising elsewhere.

Muscarinic receptors may also affect presynaptic transmitter release and junctional plasticity through Ca^{2+} channels. Acetylcholine is known to reduce depolarization-induced GABA release (Hashimoto et al., 1986; Augustine et al., 1987; Marchi et al., 1990; Raiteri et al., 1990). Muscarinic reductions in N- and P-type currents, which control transmitter release in the striatum (Turner et al., 1993), provides a cellular mechanism for this inhibition. Alterations in Ca^{2+} entry into the soma and dendrites can also be expected to modulate second-messenger pathways that are Ca^{2+} /calmodulin dependent. Changes of this sort are of importance in synaptic plasticity (see Kotsyuk, 1992, for review) and structural changes contingent upon immediate early gene induction (Kley et al., 1987; Murphy et al., 1991).

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