Regulation of M Current by Intracellular Calcium in Bullfrog Sympathetic Ganglion Neurons

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Regulation of M current (I_M) by intracellular free calcium was studied in dissociated bullfrog sympathetic ganglion B cells using whole-cell recording, intracellular perfusion, and confocal calcium imaging. BAPTA (20 mM) and appropriate amounts of calcium were added to pipette solutions to clamp calcium at different levels. A high concentration of BAPTA itself mildly inhibited I_M . Intracellular perfusion effectively controlled cellular free calcium; this was confirmed by confocal imaging with the calcium indicator fluo-3. In a calcium-free environment (no calcium added to either side of the cell membrane), average I_M was 166 pA. Raising intracellular free calcium to 60 nM or higher reversibly enhanced I_M by more than 100%. The maximum M conductance doubled upon raising calcium from 0 to 120 nM, and was accompanied by a -11 mV shift of the half-activation voltage. The kinetics of the closing and reopening relaxations of I_M were also altered by raising calcium. Enhancement of I_M by calcium required ATP in the pipette. TEA (5 mM) and *d*-tubocurarine (*d*-TC; 100 μ M) did not alter the calcium effect, indicating that it was the M current being modulated and not other K^+ currents. High calcium (450 nM) reduced I_M . The up- and downregulation of I_M paralleled the increases and decreases of fluorescence intensity observed via calcium imaging. Changing extracellular calcium had no significant effect on I_M or cellular fluorescence.

The role of calcium in muscarinic and peptidergic modulation of I_M was also explored. Muscarine (1 or 10 μ M) inhibited I_M less at zero calcium than at higher calcium. Nearly complete suppression occurred with 120 nM calcium in the presence of 20 mM BAPTA. I_M overrecovered upon washout of muscarine at 120 nM calcium, while little overrecovery of I_M developed at zero calcium. Similar effects were observed at zero and 120 nM calcium when using the peptide LHRH to inhibit I_M . We conclude that the absolute level of free calcium determines the size of I_M , and that a minimum sustained level of calcium is required both for optimal suppression of I_M by muscarine and for overrecovery. While our data

suggest that resting calcium levels play a permissive role in muscarinic suppression, an additional role for agonist-induced calcium increases cannot be ruled out.

The M current (I_M), a voltage- and time-dependent potassium current, was identified in bullfrog sympathetic neurons (Brown and Adams, 1980; Adams et al., 1982a) and has been demonstrated in central and peripheral neurons of a variety of other vertebrate species (reviewed in Brown, 1988). Activation of I_M drives the membrane potential back toward E_K , profoundly diminishing cell excitability (Adams and Brown, 1982; Adams et al., 1982b). Muscarine, luteinizing hormone-releasing hormone (LHRH), and other neurotransmitters modulate I_M in two ways: first, they suppress the current; second, after washout of the agonist, I_M is transiently enhanced. This second response is called "overrecovery" (Pfaffinger, 1988). Very high concentrations of agonist also cause desensitization of muscarinic ACh receptors (Bosma et al., 1990); this was not investigated here.

I_M was initially characterized as being " Ca^{2+} independent" in terms of not requiring Ca^{2+} influx for its activation (Adams et al., 1982b). I_M in both bullfrog (Adams et al., 1982b) and rat (Marrion et al., 1989) sympathetic neurons persists when Ca^{2+} entry is prevented either by removing Ca^{2+} from the external medium or by using selective Ca^{2+} channel blockers. In bullfrog sympathetic neurons, I_M was not changed by intracellular iontophoresis of Ca^{2+} (Adams et al., 1982b). However, a transient $[Ca^{2+}]_i$ increase is observed during I_M suppression by muscarine or by LHRH (Pfaffinger et al., 1988; Marrion et al., 1991), raising the possibility of a relationship between Ca^{2+} and I_M .

In bullfrog sympathetic neurons, cobalt only slightly suppressed I_M in the presence of 2 mM Ca^{2+} (Adams et al., 1982b). However, I_M was reduced by 90% when Ca^{2+} was substituted by cobalt or other divalent cations (Tokimasa and Akasu, 1990a). The maximum M conductance was reduced by 60% when Ca^{2+} was removed from the superfusate. It was therefore suggested that extracellular Ca^{2+} is required for M channel function (Tokimasa and Akasu, 1990a).

Some investigators have suggested that Ca^{2+} may be a second messenger regulating I_M . Stimulating Ca^{2+} release from intracellular stores by applying caffeine to bullfrog sympathetic neurons evokes a component of K^+ current whose voltage and ACh sensitivity resembles that of I_M (Koketsu et al., 1982). On the other hand, caffeine was also reported to block I_M in these cells (Pfaffinger et al., 1988; Akaike and Sadoshima, 1989). When attempts were made to increase intracellular Ca^{2+} by membrane depolarization, no obvious effects on I_M were noticed (Pfaffinger et al., 1988). However, the increase in intracellular Ca^{2+} pro-

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duced by the massive entry of Ca^{2+} during a calcium spike inhibited I_M (Tokimasa, 1985).

Inositol trisphosphate (IP_3) was injected intracellularly to stimulate Ca^{2+} release from internal stores, but produced no obvious effect on I_M in frog sympathetic ganglion cells (Brown and Adams, 1987) and NG108-15 cells (Higashida and Brown, 1986; Brown and Higashida, 1988). Nevertheless, a high concentration of IP_3 (20–60 mM in the intracellular recording electrode) blocked I_M in hippocampal CA1 cells (Dutar and Nicoll, 1988). Previous work from our laboratory (Marrion et al., 1991) showed that small increases in $[\text{Ca}^{2+}]_i$ produced by photolysis of the “caged” Ca^{2+} chelator nitr-5 or by evoking action potentials augmented I_M , while larger increases produced by repeated illumination inhibited I_M .

Evidence concerning the involvement of Ca^{2+} in muscarinic regulation of I_M is equally contradictory. Suppression of I_M by muscarine can be reduced by dialyzing the cell with concentrations of BAPTA high enough to reduce resting free calcium. One interpretation was that a certain minimum concentration of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) is required for the coupling of muscarinic receptors to M channels (Beech et al., 1991). Alternatively, a transient rise in $[\text{Ca}^{2+}]_i$ is associated with muscarinic suppression (Kirkwood et al., 1991). Muscarine and other agonists that suppress I_M elicit phosphatidylinositol (PI) turnover and produce Ca^{2+} transients. Kirkwood et al. therefore proposed that it is this rise in calcium that mediates agonist-induced suppression of I_M . In contrast, other investigators have reported that elevating $[\text{Ca}^{2+}]_i$ by membrane depolarization or manipulating $[\text{Ca}^{2+}]_i$ by adding BAPTA did not affect the actions of agonists on I_M , and it was therefore argued that $[\text{Ca}^{2+}]_i$ was not relevant to suppression of I_M (Pfaffinger et al., 1988; Marrion et al., 1991). The mechanisms by which I_M is modulated by muscarine and other agonists are still obscure. Study of these mechanisms and their regulation is important for understanding the control of neuronal excitability.

Previous studies of the relationship between calcium and I_M did not set $[\text{Ca}^{2+}]_i$ at well-defined levels. In the present study, whole-cell recordings were carried out in a configuration that allowed successive perfusions of individual cells with different internal solutions. The free Ca^{2+} in the pipette solutions and hence the cytoplasmic free Ca^{2+} could therefore be clamped at different levels. This allowed a direct assessment of the relevance of $[\text{Ca}^{2+}]_i$ for I_M modulation. Confocal imaging was used to verify the effectiveness of the manipulation of intracellular free calcium. A close correlation between $[\text{Ca}^{2+}]_i$ and I_M was revealed in this study. The muscarinic responses (suppression and over-recovery) of I_M were also affected by $[\text{Ca}^{2+}]_i$.

A portion of this work has been published previously in preliminary form (Yu et al., 1991; Yu and Adams, 1992).

Materials and Methods

Experimental preparation. Experiments were done on B cells from the lumbar VIIIth, IXth, and Xth sympathetic ganglia of the bullfrog, *Rana catesbeiana*. The isolation and dissociation of the sympathetic ganglia were described previously (Kuffler and Sejnowski, 1983). Briefly, the ganglia were dissected and cut into small pieces that were then incubated for 30 min at 37°C in Ringer solution containing 1 mg/ml collagenase (Worthington, Freehold, NJ) and 5 mg/ml dispase (Boehringer Mannheim, Germany). The ganglia were then mechanically disrupted by repeatedly passing the tissue through the tip of a fire-polished Pasteur pipette. Complete dissociation of the ganglion cells was achieved after a second digestion for 30 min at 37°C in enzyme solution with 5 mg/ml dispase, followed by further mechanical disruption. After washing the cells three times in Ringer solution, the isolated cells were plated in

plastic Petri dishes, and stored at 8°C in L-15 medium containing 10% fetal calf serum, 0.2% glucose, and 1 mM CaCl_2 (GIBCO, Gaithersburg, MD). Usually the dissociated cells attached to the bottom of the dish, which was used as the experimental chamber. The dissociated cells were roughly spherical and devoid of visible processes. The cells were used within the next 10 d; there was no detectable deterioration.

Whole-cell recording. Membrane currents were recorded using the whole-cell configuration of the patch clamp (Hamill et al., 1981). The tip resistance of the fire-polished electrode was 1–2 M Ω . Whole-cell voltage clamp was performed using an Axoclamp 2A amplifier (Axon Instruments) in the discontinuous mode. For Na^+ current recording the sampling rate on the amplifier was 20 kHz. For recording K^+ currents, sampling rates of 7–15 kHz were used. Voltage and current were displayed on a storage oscilloscope (Tektronix Inc.) and a computer monitor. They were simultaneously recorded by a data acquisition/analysis program written in ASYST language (ASYST 1.53, Macmillan Software Company, NY) on an IBM-compatible computer (Compaq Deskpro 286) and a chart recorder (Gould RS 3400). A second oscilloscope was used to monitor the headstage voltage transient decay continuously to ensure sufficient capacity compensation.

Dissociated neurons were visualized using inverted phase-contrast microscopy (Zeiss, Germany). B-type spherical neurons with diameters of 40–60 μm were chosen for recordings. After gigaseal formation (10–40 G Ω seal resistance) and establishment of the whole-cell configuration, the cells were first clamped at –60 mV to check the membrane input resistance. Neurons were used only if they had input resistances over 80 M Ω ; usually the input resistance was more than 100 M Ω .

To record Na^+ currents, electrodes were filled with the following solution (in mM): CsCl, 110 or 10; NaCl, 5 or 105; HEPES, 5; EGTA, 1; TEA, 3. The external solution for Na^+ current recording was (in mM) NaCl, 115; TEA, 5; MnCl_2 , and Tris 2.5. To record M current, a modified Ringer solution contained (in mM) NaCl, 115; KCl, 2.5; MnCl_2 , 2.0; HEPES, 10.0; EGTA [ethylene glycol bis-(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 0.5; D-glucose 5.0; and no added Ca^{2+} . Mn^{2+} was used as a substitute divalent cation to maintain membrane surface potentials and to prevent any possible Ca^{2+} influx. Patch-clamp electrodes used for normal I_M recording were filled with the following solution (in mM): K-aspartate, 60; KCl, 50; MgCl_2 , 1.5; HEPES, 10; BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid], 20; $\text{Na}_2\text{-ATP}$, 1.5; and CaCl_2 , 8.47. This solution was calculated to contain 120 nM free Ca^{2+} (see below), a value in the range of physiological Ca^{2+} levels found in bullfrog ganglion cells (Pfaffinger et al., 1988; Marrion et al., 1991). The pH of intra- or extracellular solutions was 7.2, adjusted by Tris base or HCl. Recordings were made at room temperature (21–23°C). Extracellular solutions routinely flowed at the rate of 1 ml/min. The dish contained 1.5 ml of solution, which could be replaced completely in less than 20 sec by speeding up the flow rate. Data stored on computer or chart paper were later measured by the ASYST 1.53 program or by ruler. The Student's *t* test was used for statistical tests. Mean values are given with SEM.

The K_D for BAPTA originally published by Tsien (1980), 110 nM, had been determined for a 200 mOsm solution. This value was corrected to account for the increased osmolarity of bullfrog Ringer (about 270 mOsm) based on the study by Harrison and Bers (1987). The corrected value, 160 nM, provided the best fit between calculated values of free calcium (using an iterative program written in the ASYST program language by Alvaro Villaroel and based on the methods of Fabiato (1988) and experimentally measured fluorescence levels of fluo-3 in an intracellular solution. The ASYST program takes into account binding of multiple metals (e.g., Mg^{2+} and Mn^{2+}) and multiple buffers (e.g., ATP, fluo-3). Alternatively, use of Harrison and Bers' K_D for BAPTA, 212 nM, under our control conditions, would predict a free calcium level of 160 nM rather than 120 nM, but use of this K_D did not fit the experimental calibration curve as well as the corrected value of Tsien.

Intracellular perfusion. Control of intracellular ions was achieved by an intrapipette perfusion procedure and cellular dialysis. It is referred to here as intracellular perfusion (Lopez, 1992). Several technical modifications were made as follows. A modified electrode holder (A058-C, E. W. Wright) with two outlets was used. An internal perfusion tube of about 200 μm in external diameter was placed close to the tip down the inside of the recording electrode. Electrodes were pulled from Microcaps micropipettes (Drummond Scientific Company, Broomall, PA) on a Flaming Brown Micropipette puller (P-80 PC, Sutter Instrument Co.) Five or six pull stages were programmed to control more accurately the shape of the electrode tip. The electrode shank was made as short as

Figure 1. Effect of BAPTA on I_M . *A*, Continuous recording in 10 mM and 20 mM BAPTA. The $[Ca^{2+}]_i$ concentration was kept constant at 120 nM throughout the experiment by adjusting the total added Ca^{2+} ; intracellular perfusion with 20 mM BAPTA decreased the current by about 20%. *B*, The current-voltage (I - V) relationships in 10 mM ($\blacktriangledown$) and 20 mM ($\bullet$) BAPTA; the inset shows a representative trace. The instantaneous shift is part "leak" current and part M current and is referred to here as I_{inst} (instantaneous). The amplitude of the current relaxation upon hyperpolarization provides a measure of the deactivation of I_M (Brown and Adams, 1980). In order to maximize control of free calcium, BAPTA is present at a concentration of 20 mM throughout this report, unless stated otherwise.

possible (2–3 mm) so that the distal end of the internal tube could be placed close ($\sim 500 \mu m$) to the electrode tip to facilitate diffusion and dialysis. The other end of the internal tube led into the perfusion solution in a separate reservoir through a sealed outlet. Perfusion was initiated when negative pressure (4–5 psi) was applied to the inside of the electrode through a second sealed outlet. The flow could rapidly (~ 1 sec) change the solution in the distal part of the electrode. The perfusion usually was carried on for 3 min, during which time about 0.5 ml of solution flowed through. The internal perfusion flow was then shut off. The procedure could be repeated in the same cell several times using different internal solutions without detectable deterioration of the cell. Because dialysis of the cell can be affected by clogging of the electrode tip after seal formation, the input resistance of the cell was routinely checked during the experiment. Data were used only from cells where the input resistance was not significantly increased.

When $[Ca^{2+}]_i$ was increased by adding $CaCl_2$, a junction potential shift occurred. For example, a +6 mV junction potential was detected by raising free Ca^{2+} from zero to 120 nM. This junction potential was corrected by referring to the reversal potential or applying an equal opposite voltage by adjusting the input offset on the amplifier after intracellular perfusion. In later experiments calcium acetate was used instead of $CaCl_2$ and no obvious shift of junction potential was detected.

Measurement of M current. Most M channels are closed at membrane potentials negative to -60 mV (Brown and Adams, 1980). When the membrane potential is clamped at -30 mV, a substantial fraction of the available M channels are open (Brown and Adams, 1980; Adams et al., 1982a). A hyperpolarizing voltage step, which closes M channels, results in an instantaneous ohmic current jump ($I_{instantaneous}$) followed by a slow M channel closing relaxation. When the membrane potential is commanded back to -30 mV 1 sec later, a relaxation associated with M channel reopening is seen (see inset, Fig. 1*B*). We generally measured

the size of I_M as the closing relaxation induced by a 20 mV hyperpolarizing step (from -30 to -50 mV). The steady-state current I_{ss} ($I_M + I_{instantaneous}$) upon the step was measured for constructing I - V curves. The use of closing relaxations, rather than opening relaxations, minimized contamination of the measured I_M by other K^+ currents.

Inhibitory effect of BAPTA on I_M . BAPTA is a useful Ca^{2+} buffer because of its high selectivity for Ca^{2+} over Mg^{2+} . BAPTA is also much less affected by pH than is EGTA and can bind and release Ca^{2+} more quickly than EGTA (Tsien, 1980). Most experiments used BAPTA to control intracellular Ca^{2+} . While BAPTA may exert direct actions on Ca^{2+} channels in rat sympathetic neurons (Beech et al., 1991), there is as yet no evidence that BAPTA directly binds to K^+ channels or inhibits K^+ currents. However, because a high concentration of BAPTA (20 mM) was used to clamp free calcium levels, the effects of BAPTA on I_M were evaluated by intracellular perfusion.

Experiments were initiated with an internal solution containing 120 nM free Ca^{2+} and 10 mM BAPTA; I_M was 640 ± 20 pA ($n = 4$). The concentration of BAPTA was then raised to 20 mM by intracellular perfusion, while free calcium was kept constant by a compensatory increase in total calcium. The higher concentration of BAPTA appeared to reduce current relaxations and steady-state current ($I_{ss} + I_{instantaneous}$; Fig. 1*A,B*). I - V curves showed, however, that only the portion of outward rectification at potentials positive to -60 mV was reduced (Fig. 1*B*). This outward current is generated by the progressive opening of M channels over this voltage range (Adams et al., 1982a). In 20 mM BAPTA, I_M was reduced to 405 ± 78 pA, that is, 64% of the I_M in 10 mM BAPTA ($p < 0.05$, $n = 4$). This effect was reversed by perfusing back to the lower BAPTA solution. Another set of experiments was done in the reverse order, beginning with 20 mM BAPTA and then reducing BAPTA to 10 mM. I_M was 495 ± 120 pA in 20 mM BAPTA and increased to 694 ± 110 pA in 10 mM BAPTA ($p < 0.05$, $n = 3$). These observations suggest that BAPTA selectively inhibits I_M .

While free calcium was held constant in these experiments, the increase in BAPTA from 10 to 20 mM did produce other changes, according to the ASYST program: free magnesium decreased from 252 μM to 222 μM , while free ATP increased from 337 μM to 371 μM . These changes are small relative to the twofold increase in BAPTA, making it likely that BAPTA contributes the most to the alteration in I_M . Use of Harrison and Bers' K_p for BAPTA does not affect the outcome of this experiment. Free calcium would be 160 nM at both levels of BAPTA tested and similar changes in free ATP and magnesium would result. Unless otherwise specified, the BAPTA concentration used throughout this article was 20 mM. The I_M values obtained would therefore be somewhat lower than I_M values obtained in the absence of BAPTA.

Imaging with fluo-3. Changes in $[Ca^{2+}]_i$ were visualized using the fluorescent indicator fluo-3 (Molecular Probes). Fluo-3 was the indicator of choice because of its compatibility with BAPTA. The more commonly used ratiometric indicators, fura-2 and indo-1, function at UV wavelengths. This limits their usefulness when using BAPTA, which is also sensitive to UV light. While EGTA could be used with the ratiometric indicators, binding of calcium to EGTA releases protons and would be disruptive in the types of experiments performed here. The magnitude of fluo-3's responsiveness has been determined in cultured sympathetic neurons using intracellular perfusion (O'Malley et al., 1993), and therefore the magnitude of the calcium changes can be estimated using this indicator.

The patch-clamp electrode solution was as described above and additionally contained 100 μM fluo-3 (pentapotassium salt). The cells were imaged using a Bio-Rad MRC 600 laser-scanning confocal imaging system using 488 nm excitation light and a Zeiss IM35 inverted microscope with a $50\times$, 1.0 NA water-immersion objective (Leitz). For imaging experiments, the sympathetic ganglion cells were plated on a glass coverslip that was glued to a small Petri dish. A drop of water is placed on the microscope objective, which is then raised until the drop contacts the bottom of the coverslip. The objective is raised further until the cell comes into focus. The argon laserbeam was attenuated with a 2.0 log neutral density filter and the aperture was set approximately one-third open. The specimens were slow scanned using Kalman averaging of five successive scans. Images were routinely taken at 2 or 3 min intervals. Mn^{2+} was omitted from the extracellular Ringer and replaced with Mg^{2+} to prevent quenching of the fluo-3 fluorescence (Kao et al., 1989).

Chemicals. BAPTA was purchased from Sigma or Biochemical (BDH Chemicals Ltd., Poole, England). EGTA, TEA, and *d*-tubocurarine (*d*-TC) were from Sigma. Muscarine chloride was from Sigma or RBI (Research Biochemicals Incorporated, Natick, MA). IP_3 was from RBI.

Results

Manipulation of free calcium by intracellular perfusion

As a first check in verifying the efficacy of the intracellular perfusion technique, cells were perfused with different concentrations of Na^+ while measuring Na^+ current (I_{Na} ; Jones, 1987). Raising intracellular Na^+ from 8 to 115 mM, by perfusing the patch pipette with high Na^+ solution, reversed the original inward I_{Na} to an outward current, consistent with the change in the Na^+ equilibrium potential. The change was essentially completed in 5.0 ± 0.1 min ($n = 36$). The alteration in Na^+ current was reversed by switching the internal perfusate back to the low Na^+ solution (see also Lopez, 1992).

While sodium appears to be well controlled by this technique, manipulation of intracellular calcium is potentially more problematic because of intracellular calcium binding sites, buffers, and storage organelles and because of calcium extrusion mechanisms such as the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the Ca^{2+} ATPase. To verify further the efficacy of intracellular perfusion, a laser-scanning confocal fluorescence imaging system was used to visualize changes in intracellular Ca^{2+} during perfusion with different levels of free calcium. Patch-clamp recordings were made using electrodes filled with 100 μM fluo-3, 20 mM BAPTA, and no added Ca^{2+} . Because of the complexity of combining intracellular perfusion with laser-scanning microscopy, it was necessary to use slightly higher resistance electrodes (3–4 M Ω) for these particular experiments.

During whole-cell recording, fluo-3 and BAPTA gradually diffused into the cell, as evidenced by a gradual fluorescence increase (Fig. 2A). The $[\text{Ca}^{2+}]_i$ is expected to be in the very low nanomolar range due to the high concentration of BAPTA. After the image intensity approached a plateau, internal perfusion was initiated with a solution that contained 120 nM free Ca^{2+} , but was otherwise identical to the zero calcium solution (Fig. 2A, row II). As the perfusion proceeded, the cellular fluorescence became progressively brighter, approaching a plateau in about 12 min (the range was 5–15 min in 15 cells). Although the nucleus is brighter than the cytosol, due to a greater content of fluo-3 (O'Malley et al., 1993), the relative fluorescence increase was similar in the nucleus and cytoplasm. When the cell was subsequently perfused with a zero Ca^{2+} solution to reduce $[\text{Ca}^{2+}]_i$ (row III), the cellular fluorescence decreased back toward the level initially present in zero calcium solution, showing that the change in intracellular calcium was reversible (Fig. 2A).

Relationship between free calcium and I_{M}

The effect of Ca^{2+} on I_{M} was tested by perfusing the pipette with solutions containing different concentrations of free calcium. Most experiments were started in a nominally Ca^{2+} -free environment, that is, with no Ca^{2+} added to either the inside or outside of the cells and with calcium chelator present on both sides (see Materials and Methods). In this condition (referred to as zero Ca^{2+} ; applies to the above imaging experiments as well), we consistently observed smaller I_{M} than in normal Ca^{2+} , even immediately after breaking through the patch membrane. There were usually some slight increases or decreases of the current during the first few minutes. Experimental data were collected after 5–10 min, when the size of the current stabilized. I_{M} in 0 nM Ca^{2+} was 166 ± 10 pA ($n = 82$). Upon raising $[\text{Ca}^{2+}]_i$, I_{M} gradually increased. In some cells, I_{M} was monitored simultaneously with Ca^{2+} imaging (Fig. 2A–C) to visualize the relation between $[\text{Ca}^{2+}]_i$ and I_{M} . As shown in Figure 2C, the

current was enhanced in parallel with the increase of fluorescence intensity as $[\text{Ca}^{2+}]_i$ increased. Both fluorescence and current declined when the cell was again perfused with zero $[\text{Ca}^{2+}]_i$. The parallel changes in image intensity and I_{M} demonstrates a correlation between $[\text{Ca}^{2+}]_i$ and I_{M} .

Once the enhanced I_{M} reached its peak, it remained at that level for as long as the recording lasted (10–30 min) (Fig. 3A). Low-resistance electrodes (1–2 M Ω) were used in the nonimaging experiments throughout this study; note that the time course by which changes in calcium modulated M current (Fig. 3A) was similar to the time course of changes in sodium current described above and elsewhere (Lopez, 1992). The I - V curves in Figure 3B show that the outwardly rectifying M current was selectively enhanced by increasing free calcium from 0 nM to 120 nM Ca^{2+} via intracellular perfusion. The outward rectification moved to a lower level after returning to zero Ca^{2+} , showing that the enhancement of I_{M} by Ca^{2+} was reversible. Changes of I_{M} could be reproduced in a single cell by successive internal perfusions; most of the data points, however, reflect experiments where calcium was raised, by a single perfusion change, from 0 nM to one of the higher values illustrated. The reversal potential (about -85 mV) remained the same after changing Ca^{2+} . In order to rule out artifactual influences of the perfusion procedure on I_{M} , some cells were perfused with the same internal perfusate, that is, from 0 nM Ca^{2+} to 0 nM Ca^{2+} or from 120 nM Ca^{2+} to 120 nM Ca^{2+} ; no obvious alterations of I_{M} occurred.

The dose-response curve in Figure 3C illustrates that the most substantial changes in I_{M} occurred between 0 and 60 nM Ca^{2+} . Increasing calcium from 0 nM to 60 nM enhanced I_{M} by about twofold (ratio of post- to preperfusion, 2.08 ± 0.25 ; $n = 12$; $p < 0.05$). Calcium levels higher than 60 nM, reached either by starting from 0 nM Ca^{2+} or by successive perfusions in a single cell, produced only limited further increases of I_{M} (Fig. 3C). For example, in cells initially recorded at 120 nM Ca^{2+} , I_{M} was 351 ± 61 pA. $[\text{Ca}^{2+}]_i$ was then increased to 240 nM (a "net" 120 nM increase in free Ca^{2+}). I_{M} recorded after the perfusion was 374 ± 79 pA ($n = 3$), a ratio of 1.06 relative to the I_{M} before the perfusion. Raising Ca^{2+} beyond 400 nM tended to reduce I_{M} as compared to 60 or 240 nM Ca^{2+} . The amplitude of I_{M} was 278 ± 58 pA ($n = 8$) when Ca^{2+} was raised to 450 nM, although this value was still larger than when the current was measured at zero Ca^{2+} (Fig. 3C).

Since some authors suggested that a Ca^{2+} binding site on the outside of the membrane might modulate the opening of M channels (Tokimasa and Akasu, 1990a), we also examined whether changing extracellular calcium levels would modulate I_{M} . The experiments were started in the Ca^{2+} -free environment described above. I_{M} was 184 ± 83 pA ($n = 14$). The extracellular Ca^{2+} -free Ringer was then switched to a Ringer containing 2 mM CaCl_2 (in place of 2 mM MnCl_2), and I_{M} increased to 203 ± 111 pA. This change was insignificant at a 10% level (1.11 ± 0.47 , $n = 14$; $p > 0.05$). Calcium imaging studies showed only minuscule changes in cellular fluorescence when switching between these Ringer solutions.

In Ca^{2+} -free solution, there will be more free BAPTA available than in the 120 nM Ca^{2+} solution, since total BAPTA is held constant at 20 mM. Because BAPTA had an inhibitory effect on I_{M} , it was possible that the enhancement of I_{M} in 120 nM free calcium resulted from the lowering of free BAPTA (a greater proportion of the BAPTA now being complexed with calcium). To explore this possibility, the free BAPTA was main-

Figure 2. Manipulation of free calcium by intracellular perfusion. Fluo-3 (100 μM) and BAPTA (20 mM) were included in all internal solutions. Images were acquired via confocal laser-scanning microscopy (Bio-Rad MRC 600). Whole-cell currents were simultaneously recorded in the same neurons. **A**, Images were acquired at about 3 min intervals; representative images acquired during cell filling and successive perfusions are shown. **B**, The current traces correspond to the last image in each of the three rows of **A**: *I*, fluo-3 and BAPTA were loaded into the cell with zero Ca^{2+} ; *II*, during perfusion with 120 nM Ca^{2+} ; *III*, the cell was perfused back to zero Ca^{2+} . **C**, Parallel changes in fluorescence intensity (arbitrary units) or free calcium and I_M occur during the successive intracellular perfusions. Fluorescence intensity was taken as the average of the measurements from the cytosol and the nucleus. For imaging experiments, electrodes of higher resistance were used (3–4 M Ω ; 3 M Ω in this instance). Thus, the time course of reaching a plateau, for example, about 12 min in row *II*, is longer than in the nonimaging experiments in this report, where lower resistance electrodes were used.

Figure 3. Effect of free calcium on I_M . *A*, Continuous recording in 0 nM and 120 nM Ca^{2+} . *B*, I - V relationships before (●) and during (■) perfusion with 120 nM Ca^{2+} , followed by perfusion back to 0 nM Ca^{2+} (▼). The inset shows the superimposed current traces before (left) and during (right) the 120 nM Ca^{2+} perfusion. *C*, Dose-response curve obtained from perfused (●) and nonperfused (○) cells. Data for the perfused cells were principally acquired in experiments where calcium was raised from 0 nM to one of the higher levels of calcium shown. The two representative examples of M current shown were obtained in 0 nM and 120 nM Ca^{2+} . V_{hold} , -30 mV; V_{step} , -20 mV for 1 sec.

tained constant while Ca^{2+} was varied. For the Ca^{2+} -free solution, 11.4 mM total BAPTA was used, while 20 mM BAPTA was used in the 120 nM Ca^{2+} solution. The amount of free BAPTA was calculated to be the same in both solutions. Under these conditions, I_M was 382 ± 71 pA in 0 Ca^{2+} and augmented to 825 ± 96 pA ($n = 3$) after 120 nM Ca^{2+} perfusion. Thus, I_M was again about doubled in 120 nM Ca^{2+} (2.16-fold increase), while free BAPTA was held constant, eliminating changes in free BAPTA as a cause of the augmentation of I_M .

An alternative Ca^{2+} chelator EGTA (20 mM) was substituted for BAPTA in some experiments. I_M was 83 ± 70 pA at 0 nM Ca^{2+} and rose to 275 ± 34 pA ($n = 14$) after 80 nM Ca^{2+} perfusion, a 3.3-fold increase. This further indicates that alterations of I_M after raising $[\text{Ca}^{2+}]_i$ are not due to direct effects of BAPTA.

Figure 4. Calcium affects the activation of I_M . Activation curves were obtained before (○) and after (●) raising $[\text{Ca}^{2+}]_i$ from 0 nM to 120 nM. The curves were fitted by the Boltzmann equation $G = G_{\text{max}} \{ [1 + \exp((V_{\text{half}} - V)/A)]^{-1} \}$, where V is membrane potential; V_{half} , the potential for half activation; and A , the slope factor (Adams et al., 1982a). The channels were mostly closed at voltages negative to -60 mV. Raising $[\text{Ca}^{2+}]_i$ enhanced the conductance and shifted its voltage sensitivity. The experiment was done with 5 mM TEA, 1 mM 4-aminopyridine, 1 mM cesium, 3 μM TTX in the external Ringer. Cesium was used to block the slow sodium-potassium inward H current (Tokimasa and Akasu, 1990b), which may be activated by the hyperpolarization jumps used to close the M channels.

Electrophysiological controls

Switching between internal solutions can create liquid junction potentials that could change the true size of the holding potential and subsequently affect the size of voltage-dependent currents. Although the junction potential was carefully checked and corrected (see Materials and Methods), some nonperfusion experiments were carried out to avoid this possible artifact. Patch-clamp electrodes were initially filled with solutions containing different concentrations of free Ca^{2+} . The electrode "input" offset was compensated to zero at the beginning of each experiment and checked at the end. Thus, these nonperfusion experiments eliminated potential errors arising from the intracellular perfusion procedure, particularly any errors that might have resulted from the switching of solutions. I_M was measured in cells initially dialyzed with 0, 15, 60, 120, and 240 nM free Ca^{2+} (open circles, Fig. 3C). The amplitude of I_M varied systematically at each Ca^{2+} concentration. I_M values obtained from perfusion and nonperfusion experiments coincided quite well. I_M at 60 nM or higher Ca^{2+} was always more than two times larger than I_M at 0 nM Ca^{2+} .

In some experiments, the membrane potential was held at -60 mV during the 0–120 nM Ca^{2+} perfusion. After 5–10 min of perfusion with 120 nM calcium, the holding potential was stepped to -30 mV, and the size of I_M was immediately measured. The increase in I_M was found to be the same as in experiments where the cell was held at -30 mV during the switch. I_M increased 2.13-fold ($n = 3$) after the perfusion. This indicates that the enhancement of I_M by calcium occurs independently of voltage; thus, the effects of calcium occurred regardless of whether the channels were mostly open (-30 mV) or largely closed (-60 mV).

Intracellular perfusion with raised Ca^{2+} also enhanced mem-

Figure 5. ATP is required for the calcium-dependent enhancement of I_M . *A*, The I - V relationship of voltage and M current; ■, the basal M current in a Ca^{2+} -free environment. Perfusion with 120 nM Ca^{2+} without ATP (●) failed to increase the current significantly. Addition of 1.5 mM ATP to the perfusion solution, in the continued presence of 120 nM Ca^{2+} (▲), considerably enhanced the current at voltages positive to -60 mV. *B*, The three current traces correspond to the respective I - V curves in *A*. V_{hold} , -30 mV; V_{step} , -20 mV for 1 sec.

brane conductance. The membrane chord conductance (G_{CH}) increased 7.93 nS from 0 nM Ca^{2+} (11.83 ± 1.48 nS) to 120 nM Ca^{2+} [19.76 ± 1.87 nS; $n = 23$, $p < 0.05$; $G_{\text{CH}} = I_{\text{instantaneous}}(V)/(V - V_H)$, $V = -50$ mV, $V_H = -30$ mV]. Figure 4 shows the activation curves of I_M before and after 120 nM Ca^{2+} perfusion. At 0 nM Ca^{2+} , M channels were mostly closed at potentials negative to -50 mV. The maximum conductance (G_{max}) was 8.5 nS with a half-activation potential (V_{half}) of -26 mV. Raising Ca^{2+} shifted the curve upward, increasing G_{max} to 20.1 nS. In addition, the voltage dependence of M channels moved toward more negative potentials. V_{half} became -41 mV in this instance (Fig. 4). The average shift of V_{half} was -11 ± 6 mV in four analyzed cells.

ATP is required for maintaining I_M (Pfaffinger, 1988). We observed that when ATP was omitted from the electrode solution I_M gradually declined (the "rundown" of I_M) even when normal Ca^{2+} was present. To test whether ATP was necessary for I_M enhancement, $[\text{Ca}^{2+}]_i$ was raised without ATP in the internal solution. Intracellular perfusions were initiated to raise $[\text{Ca}^{2+}]_i$ in the absence of exogenous ATP, about 5 min after establishing the whole-cell recording configuration. I_M did not increase when $[\text{Ca}^{2+}]_i$ was raised to 120 nM (ratio of 1.03 ± 0.11 ; $n = 5$). Figure 5 shows that raising $[\text{Ca}^{2+}]_i$ to 120 nM, with no ATP in the perfusate, produced little effect on I_M or the I - V relationship. Upon addition of 1.5 mM ATP to the perfusate, however, the outward rectification of the I - V curve was enhanced, due to the progressive opening of M channels.

Bullfrog sympathetic ganglion cells exhibit several potassium currents in addition to I_M , some of which are Ca^{2+} dependent (I_C , I_{AHP} ; Adams et al., 1982a; Pennefather et al., 1985). To confirm that the current enhanced by Ca^{2+} was indeed I_M , the following experiments were conducted. First, the current was identified as a K^+ current by changing the external K^+ concentration. After raising $[\text{Ca}^{2+}]_i$ from 0 to 120 nM, the current showed a reversal potential of -90 mV in normal Ringer (2.5 mM KCl). Upon increasing external potassium to 10 mM, the

Figure 6. Muscarinic inhibition of I_M is affected by free calcium. Cells were divided into four groups; each group contained five cells ($n = 5$). Each point in the graph represents the data from one group. Muscarine (1 and 10 μM) was tested at 0 nM (○) and 120 nM (●) Ca^{2+} , respectively. Both concentrations of muscarine produced larger reductions in current at 120 nM calcium than at 0 nM calcium.

reversal potential shifted to about -60 mV. Both reversal potentials were similar to the values predicted by the Nernst equation. This shows that the current increased by Ca^{2+} was principally carried by K^+ .

TEA (5 mM) was applied in the bathing medium to block I_K and I_C (Adams et al., 1982b), and d -tubocurarine (d-TC; 100 μM) was used to block I_{AHP} (Pennefather et al., 1985; Goh and Pennefather, 1987). Neither of these agents prevented the increase in K^+ current normally produced by raising $[\text{Ca}^{2+}]_i$. I_M was enhanced 2.54 ± 0.21 -fold ($n = 4$) and 2.80 ± 0.86 -fold ($n = 3$) in the presence of TEA and d-TC, respectively. To construct the activation curves (Fig. 4), the external Ringer contained TEA (5 mM), 4-aminopyridine (1 mM), cesium (1 mM), and TTX (3 μM). In the presence of this group of compounds, the M conductance was still enhanced by raising $[\text{Ca}^{2+}]_i$. These results rule out substantial contributions from other K^+ currents.

The M channel is tonically active at the holding potential of -30 mV; hence, the standing outward holding current at -30 mV reflects the size of I_M (Bosma et al., 1990). It was thus expected that the holding current should be smaller in 0 Ca^{2+} than in higher Ca^{2+} . This prediction was confirmed. The holding current at -30 mV was 245 ± 61 pA ($n = 26$) at 0 Ca^{2+} and increased to 651 ± 84 pA after perfusion with 120 nM Ca^{2+} . The holding current was increased 2.65-fold by raising $[\text{Ca}^{2+}]_i$ ($p < 0.05$), similar to the I_M increase measured from the closing relaxation (a ratio of 2.11).

Muscarinic modulation of I_M : effects of calcium

The effects of muscarine were measured at different levels of free calcium to examine the role of Ca^{2+} in I_M suppression and overrecovery. To avoid desensitization of muscarinic receptors, muscarine was usually applied only once to each cell, typically for about 20 or 30 sec, which allowed muscarine to attain its maximal effect. When two muscarine tests were conducted in a single cell, the cell was extensively washed by drug-free Ringer and there was at least 10 min between applications of muscarine.

Figure 7. Calcium plays a role in sustained suppression of I_M . A decrease in $[Ca^{2+}]_i$ by intracellular perfusion resulted in a partial recovery of I_M that had been blocked by $10 \mu M$ muscarine. The cell was first recorded at $120 nM$ Ca^{2+} and I_M was totally suppressed by muscarine. In the continued presence of $10 \mu M$ muscarine in the bathing solution, a reduction in $[Ca^{2+}]_i$ by intracellular perfusion of zero Ca^{2+} caused a substantial increase in I_M .

At $0 nM$ Ca^{2+} , $1 \mu M$ muscarine produced about a 30% (0.29 ± 0.11 , $n = 8$) reduction of I_M , while 70% (0.69 ± 0.09 , $n = 6$) of I_M was blocked at $120 nM$ Ca^{2+} (Fig. 6). Increasing the concentration of muscarine to $10 \mu M$ slightly increased inhibition of I_M at both levels of free calcium, but muscarine was still much more effective at $120 nM$ calcium. At $0 nM$ Ca^{2+} , 40% of I_M was blocked (0.38 ± 0.14 , $n = 4$), while the blockage was about 80% (0.77 ± 0.08 , $n = 4$) at $120 nM$ Ca^{2+} (Fig. 6). Similar results were obtained for LHRH. At $0 nM$ Ca^{2+} , $1 \mu M$ LHRH blocked $59 \pm 17\%$ ($n = 4$) I_M , while $85 \pm 6\%$ ($n = 11$) of the current was blocked at $120 nM$ Ca^{2+} .

The following experiments were designed to test whether reducing $[Ca^{2+}]_i$ during exposure to agonist could influence the suppression of I_M . Starting with $120 nM$ $[Ca^{2+}]_i$, $10 \mu M$ muscarine essentially eliminated I_M . In the continued presence of muscarine, intracellular perfusion was started to reduce $[Ca^{2+}]_i$. Earlier, it was shown that zero calcium effectively reduced I_M . However, under this condition, the $0 nM$ Ca^{2+} perfusion resulted in a partial recovery of the current suppressed by muscarine ($n = 3$; Fig. 7). This implies that a minimal amount of Ca^{2+} is required for the sustained suppression of I_M by muscarine. This is not an artifact of the perfusion procedure, since reducing calcium normally decreases I_M rather than increasing it. The lessening of the muscarinic suppression does not appear to be a result of receptor desensitization. In two control experiments, when muscarine ($10 \mu M$) was maintained in the bathing medium for the same period of time (5–6 min) without changing $[Ca^{2+}]_i$, I_M suppression was faithfully maintained. Also, the time course of the increase in I_M parallels the change in calcium level. A very different time course would be expected for desensitization of muscarinic receptors.

The level of free calcium also has dramatic effects on the overrecovery of M current that occurs after removal of muscarine (Fig. 8). With $120 nM$ Ca^{2+} present, upon washout of $10 \mu M$ muscarine, I_M transiently overrecovered by 50% ($150 \pm 10\%$ recovery; $n = 9$), compared with its control level before muscarine. When muscarine was tested at $0 nM$ Ca^{2+} , although the suppressed current eventually returned to its original size after removing muscarine there was little overrecovery. The recovery of I_M averaged $106 \pm 5\%$ ($n = 19$) of the control current recorded before muscarine. A similar effect was found with LHRH. At zero Ca^{2+} , there was only a $113 \pm 11\%$ ($n = 4$) recovery, while

Figure 8. Effects of free calcium on overrecovery. *A*, Continuous recording in $120 nM$ $[Ca^{2+}]_i$ shows that normal muscarinic suppression of I_M and overrecovery of I_M are observed in the presence of $20 mM$ BAPTA. I_M was mostly blocked by $2 \mu M$ muscarine and overrecovered upon washout of muscarine. *B*, The effects of muscarine ($10 \mu M$) are shown in a representative cell recorded at $0 nM$ and $120 nM$ $[Ca^{2+}]_i$. The following recordings were made at each calcium level: *I*, control recording; *II*, 30 sec after bath application of $10 \mu M$ muscarine; and *III*, 1 min after washout of muscarine with drug-free Ringer. I_M was noticeably smaller in $0 Ca^{2+}$, and inhibited less by muscarine (51% block) than it was in $120 nM$ Ca^{2+} (96% block). I_M only partially recovered (75% recovery) after 1 min of washout of muscarine at $0 nM$ Ca^{2+} , while it overrecovered (150% recovery) following the same procedure at $120 nM$ Ca^{2+} . Notice that the recovery of I_M after the second application of muscarine was greater than the first one; this was never observed when $[Ca^{2+}]_i$ was kept constant. The presence of $20 mM$ BAPTA in these experiments should prevent the occurrence of calcium transients that would normally result from application of muscarine (Marrion et al., 1991; O'Malley et al., 1993).

an 88% “overshoot” of I_M ($188 \pm 25\%$ recovery; $n = 10$) appeared in $120 nM$ Ca^{2+} after removal of $1 \mu M$ LHRH.

Overrecovery normally diminishes following repeated application of agonists. Under consistent conditions, the second application of muscarine always produces less overrecovery than the previous one. Muscarine responses were examined in single cells before and after raising $[Ca^{2+}]_i$. The initial $10 \mu M$ muscarine applied at $0 nM$ Ca^{2+} was followed by a $110 \pm 5\%$ recovery. After $[Ca^{2+}]_i$ was increased to $120 nM$, the second application of muscarine produced $127 \pm 5\%$ recovery of I_M ($p < 0.05$ from paired t test; $n = 11$). An example is shown in Figure 8.

Alteration of I_M kinetics by calcium

Increasing calcium slowed the deactivation of I_M , while accelerating reactivation of the current (Marrion et al., 1991). The kinetics of I_M were obviously altered after raising $[Ca^{2+}]_i$ (Fig. 9). An initial analysis was made by fitting the relaxations to single exponentials. The closing relaxation was slower at $120 nM$ Ca^{2+} than at $0 nM$ Ca^{2+} . The time constant (τ) was prolonged from 109.3 ± 14.5 msec at $0 Ca^{2+}$ to 139.2 ± 12.1 msec at $120 nM$ Ca^{2+} ($n = 8$; Table 1). On the other hand, the τ of the opening relaxation became faster at $120 nM$ Ca^{2+} than at $0 nM$ Ca^{2+} , going from 216.1 ± 18.2 msec at $0 Ca^{2+}$ to 149.1 ± 13.9 msec at $120 nM$ Ca^{2+} (Table 1, Fig. 9*A,B*). The kinetic changes pro-

Figure 9. Changes in I_M kinetics at different levels of free calcium. *A*, Superimposed traces of closing relaxation (deactivation) recorded at 0 nM Ca^{2+} (*a*) and 120 nM Ca^{2+} (*b*). Calibration: 200 msec; 122 pA (*a*), 210 pA (*b*). *B*, The superimposed traces of reopening relaxation (reactivation) at 0 nM Ca^{2+} (*a*) and 120 nM Ca^{2+} (*b*). Calibration: 160 msec; 168 pA (*a*), 330 pA (*b*). The traces were fitted by two exponentials. For closing relaxations τ_1 and τ_2 were 61.7 msec and 242.8 msec, respectively, at 0 nM Ca^{2+} ; after raising $[\text{Ca}^{2+}]_i$ to 120 nM, τ_1 and τ_2 became 81.0 msec and 379.5 msec. For reopening relaxation at 0 nM Ca^{2+} , τ_1 and τ_2 were 198.0 msec and 1272.9 msec, and the τ values were reduced to 38.6 msec and 150.2 msec, respectively, at 120 nM $[\text{Ca}^{2+}]_i$.

duced by lowering Ca^{2+} are consistent with a reduction in M channel open time or opening frequency, which would cause the channels to spend more time in the closed state.

As can be seen from the superimposed traces of magnified tail currents in Figure 9, a slow component appeared at the later phase of the relaxations. The relaxations hence appear to consist of two exponential components. This might reflect multiple kinetic states or different subpopulations of channels (Owen et al., 1990; Marrion et al., 1992) or perhaps nonuniform Ca^{2+} concentrations. We further analyzed the kinetic changes by introducing a second τ in the computer curve fitting. The fit was noticeably improved in six out of eight cells analyzed. According to the two- τ curve fitting, both fast and slow components for closing relaxation were longer at 120 nM Ca^{2+} than at 0 Ca^{2+} . The two τ s for the opening relaxation both became faster in higher Ca^{2+} (Table 1). There was no sign that raising Ca^{2+} brought out or eliminated either of the components.

At 120 nM Ca^{2+} , the time constant for the fast closing component (τ_1 ; Table 1) was 69.4 msec, consistent with a previous separate study (62.5 msec; Marrion et al., 1992). The τ_2 for the slow component (337.2 msec) was smaller than in the previous study (573 msec). The difference might be due to the shorter voltage command (1 sec vs 3 sec) used in this study.

These kinetic changes might be caused by activation of Ca^{2+} -dependent K^+ currents, such as I_C or I_{AHP} , by the rise in $[\text{Ca}^{2+}]_i$. Some experiments were thus performed with TEA (5 mM) or d-TC (100 μM) in the external superfusate to block these two

currents. After perfusion with 120 nM Ca^{2+} , similar kinetic changes were observed (two cells for each blocker, data not shown).

At high $[\text{Ca}^{2+}]_i$ (450 nM), τ values for the closing relaxation obtained from either one- or two- τ fitting (Table 1) became faster than at the lower Ca^{2+} . The τ values obtained from two exponential fittings were even smaller than the values at zero Ca^{2+} . This reduction in time constant was consistent with the lowered amplitude of I_M at this Ca^{2+} level (see Fig. 3C). Furthermore, the τ values for the opening relaxation became progressively faster as $[\text{Ca}^{2+}]_i$ increased (Table 1).

Discussion

While it is clear that M current is modulated by changes in cytosolic calcium, the mechanisms subserving this modulation remain controversial. Two of the main conclusions reached here are (1) that I_M is activated when free calcium is raised into the physiological range and (2) that I_M is inactivated when calcium is raised well above resting calcium levels. The electrophysiological aspects of this modulation are discussed, as are technical issues concerning the control and measurement of calcium levels. A third conclusion reached is that calcium transients do not appear to be necessary for the suppression of I_M by muscarine under our experimental conditions. There is substantial disagreement concerning this third issue (Pfaffinger et al., 1988; Beech et al., 1991; Kirkwood et al., 1991; Marrion et al., 1991). The mechanisms by which I_M is regulated are discussed in this context.

Establishment of calcium levels

Intracellular perfusion is a powerful technique for modifying the internal milieu of patch-clamped nerve cells (Soejima and Noma, 1984; Lopez, 1992). The rapid change in sodium current (reversal within 5 min) illustrates the effectiveness of internal perfusion. Our electrophysiological evidence indicates that free calcium levels can be modulated on the same time scale as sodium levels (Fig. 3). However, because free calcium is regulated in the nanomolar range (vs millimolar levels of free sodium), its regulation may be more powerful. To confirm the effectiveness of internal perfusion in manipulating calcium, free calcium levels were measured using the calcium indicator fluo-3.

As shown in Figure 2, large, reversible changes in fluorescence were produced by perfusing cells first with 120 nM free calcium, then with zero calcium solution. Based on intracellular calibration curves obtained in these cells (O'Malley et al., 1993), it is estimated that the greater than twofold increase in fluorescence corresponds to an increase in cytosolic free calcium of about 80–90 nM. Upon returning to zero calcium solution, the fluorescence returned to its basal level in the nucleus and to nearly

Table 1. Kinetic changes of I_M relaxations at different $[\text{Ca}^{2+}]_i$ levels

	Closing relaxation			Opening relaxation			<i>n</i>
	One- τ fitting	Two- τ fitting		One- τ fitting	Two- τ fitting		
	τ (msec)	τ_1 (msec)	τ_2 (msec)	τ (msec)	τ_1 (msec)	τ_2 (msec)	
0 nM Ca ²⁺	109.3 $\pm$ 14.5	56.6 $\pm$ 4.9	284.0 $\pm$ 44.7	216.0 $\pm$ 18.2	118.0 $\pm$ 39.0	330.7 $\pm$ 105	8
120 nM Ca ²⁺	139.0 $\pm$ 12.0	69.4 $\pm$ 5.5	337.2 $\pm$ 36.9	149.0 $\pm$ 13.9	87.6 $\pm$ 14.4	229.9 $\pm$ 31.4	8
240 nM Ca ²⁺	206.7 $\pm$ 22.1	143.2 $\pm$ 37.7	393.9 $\pm$ 35.9	183.5 $\pm$ 25.5	98.8 $\pm$ 24.9	233.1 $\pm$ 27.3	5
450 nM Ca ²⁺	167.7 $\pm$ 14.7	49.6 $\pm$ 8.7	215.0 $\pm$ 40.7	116.4 $\pm$ 16.8	58.6 $\pm$ 10.7	439.1 $\pm$ 136.2	5

its basal level in the cytoplasm. These results indicate that internal perfusion with different levels of calcium produces, at a minimum, most of the desired degree of change in free calcium. The failure of the cytoplasmic signal to fully recover is thought to be due to slow internalization of fluo-3 into organelles (O'Malley et al., 1993).

One possible reason why fluo-3 fluorescence changes in the cell were somewhat smaller than in calibration solutions is that calcium dyes behave differently *in situ* as compared to in droplets. Such deviations in behavior are common with calcium indicators. Alternatively, cellular mechanisms, such as extrusion of calcium from the cytosol by ATPases and sodium/calcium exchangers, may have compensated against our dialysis. Thus, free calcium could have been a little greater than 0 nM, and perhaps a little less than 120 nM during the separate stages of a typical perfusion. The key issue, however, is that fluorescence increases were large enough to indicate that most of the desired control of free calcium was observed and that I_M behaved differently in very low versus physiological/resting calcium levels.

Fluo-3 was the calcium indicator of choice because it is the most compatible with BAPTA, which is a faster buffer than the standard calcium chelator, EGTA. BAPTA also does not give off protons, as EGTA does, upon binding calcium. BAPTA is not normally used in conjunction with the ratiometric indicators fura-2 and indo-1 because BAPTA is itself fluorescent within the excitation range of these dyes, making calibration problematic (N. V. Marrion, personal communication). With free calcium levels set by high concentrations of BAPTA, fluo-3 provides useful signals for monitoring changes in free calcium (O'Malley et al., 1993).

Additional controls

Ion channel behavior is known to be influenced by surface charge (Perozo and Bezanilla, 1990), so simple charge effects on I_M must be considered. Because the intracellular solution contained 1.5 mM magnesium, and because BAPTA has a high selectivity for Ca^{2+} over Mg^{2+} ($>10^5$; Tsien, 1980), the free Mg^{2+} concentration was orders of magnitude higher than the free calcium concentration. Free magnesium was calculated to be 222 μM in zero calcium, and 252 μM in 120 nM free calcium solutions. The change in free Ca^{2+} was thus only a tiny fraction ($\sim 0.03\%$) of free Mg^{2+} ; calcium's effect on surface charge would therefore be negligible. The 15% increase of free Mg^{2+} (30 μM) produced when free calcium was increased is also unlikely to cause the large changes of I_M observed.

The changes in I_M are not due to alterations of liquid junction potential, driving force, or contamination by other currents such as I_{Ca} or I_{C} . The measurement of M current relaxation under our voltage protocol ensures that other potassium conductances do not significantly contribute to the measured I_M (Adams et al., 1982a). The use of TEA and d-TC to block potentially problematic K^+ currents further strengthens this identification. Effects of liquid junction potential also cannot account for the large changes observed in I_M , because these potentials were carefully corrected for and any residual, uncorrected potential would not account for the substantial changes in I_M .

A small inhibitory effect of BAPTA on I_M , which was observed when $[\text{Ca}^{2+}]_i$ was held constant (Fig. 1), could be due to either calcium-free or calcium-bound BAPTA. In 10 mM and 20 mM BAPTA solutions, with free calcium set at 120 nM, there would be 5.6 mM and 11.3 mM free BAPTA, respectively, while cal-

cium-bound BAPTA would be 4.4 and 8.7 mM, respectively. These effects of BAPTA were tested at 120 nM $[\text{Ca}^{2+}]_i$, where small fluctuations in free calcium would not greatly affect I_M (Fig. 3C). Efforts to carefully control free calcium levels, for example, use of a single batch (i.e., purity) of BAPTA, should have minimized any deviations in free calcium. It should be noted that these effects of BAPTA were quite small (20% inhibition) in relation to the much larger effects occurring when free calcium was changed (up to 80% inhibition), so the effects observed upon perfusion of different levels of free calcium are not due to changes in BAPTA. This was confirmed in the experiments where free BAPTA was held constant and calcium was increased from 0 to 120 nM: the typical increases in I_M were observed.

Good dialysis of the cell was necessary to manipulate effectively intracellular Ca^{2+} . With a longer shank electrode and higher tip resistance ($>5 \text{ M}\Omega$), large I_M persisted even in nominally zero calcium solution. But with our typical low-resistance electrodes, in the low calcium range, changes of a few tens of nanomolars dramatically altered I_M (Fig. 3C). Since electrode geometry strongly affects the time constant of diffusion and dialysis (Oliva et al., 1988), it seems likely, with high-resistance electrodes, that calcium levels were not well controlled. This is consistent with our observation that changes in calcium were somewhat slower with the 3 $\text{M}\Omega$ electrodes used for calcium imaging.

Effects of calcium on M current

The alteration of I_M by changes in free calcium are reversible and reproducible. At the lower limit of physiological calcium levels, small increases in $[\text{Ca}^{2+}]_i$ will strongly enhance I_M , while small decreases will reduce it. When $[\text{Ca}^{2+}]_i$ is raised well above resting calcium levels, I_M is again reduced, but not to nearly the same extent. This is qualitatively consistent with the results of Marrion et al. (1991), who showed that step increases in calcium, produced by photolysis of nitr-5, a photoreleased calcium chelator, caused I_M to increase at low levels of free calcium and decrease at higher levels. As also reported here, these were sustained effects. Thus, it is the absolute level of $[\text{Ca}^{2+}]_i$, not the nanomolar increase, which determines the size of I_M . The increase of M conductance by calcium is accompanied by a negative shift of the half-activation voltage. Thus, at a given voltage, a greater fraction of the available g_M will be active following stimulation. A similar shift in V_{half} was found in gastric smooth muscle cells for modulation of I_M by a β -adrenergic agent and cAMP analogs (Sims et al., 1990). Also, calcium appears to regulate M channels whether they are open or closed. This was shown by switching from 0 nM to 120 nM calcium while holding most of the channels closed at a holding potential of -60 mV . However, at -60 mV a small fraction of M channels might still be open, so it remains possible that the channels were modulated by calcium while in the open state.

Modulation of I_M by calcium involves changes in channel kinetics. Calcium accelerates opening of M channels and shows a biphasic action on channel closing rate, depending on its concentration. The kinetic analysis suggests that the secondary inhibition of I_M in high Ca^{2+} (450 nM) is due to facilitation of the channel "off" rate rather than a decrease in open probability. This indicates that although I_M appears smaller either in very low or high calcium, the underlying mechanisms may not be the same. High calcium, for example, 450 nM, stimulates acti-

vation as well as deactivation of M channels. High calcium may push M channels to a "new" gating state in which the channels exhibit accelerated opening and closing rates (see, e.g., Marrion, 1993). Because the potassium channel blockers TEA and d-TC were not used at the high calcium levels, it is possible that other potassium currents may have contaminated the opening relaxation, but the closing relaxation would still be essentially pure M current.

Calcium is also involved in the muscarinic suppression of I_M . M current was more suppressible at resting calcium levels than in low calcium. This suggests that the muscarinic suppression pathway has calcium-dependent steps, but an alternative possibility is that there may be a fraction of M channels that are insensitive to muscarine. Thus, the greater suppression observed in 120 nM calcium, versus zero calcium, may be due simply to the possibility that in zero calcium, the muscarine-insensitive channels constituted a larger fraction of the total M current. This, however, does not explain the increase in I_M observed during muscarinic suppression when calcium is lowered to 0 nM (Fig. 7). Normally, this would suppress I_M ; the substantial increase that occurs suggests that calcium indeed plays a role in sustaining muscarinic suppression.

Calcium may also play a role in the overrecovery of I_M that occurs when muscarine is removed. A residual elevation of calcium, induced by application of muscarine, was proposed to activate M channels. This activation would then be unveiled upon removal of muscarine (Marrion et al., 1991). In our experiments, however, overrecovery occurs while calcium is apparently held close to its resting level by the 20 mM BAPTA present (see discussion below). Also, our resting level of calcium should maximally activate I_M (Fig. 3C) and so a residual increase present after muscarine removal should not further activate it. Our data do suggest that free calcium is involved in overrecovery, but in a different way. We find that overrecovery occurs at 120 nM calcium but not at zero calcium (Fig. 8). This again suggests that a sustained level of free calcium is important for the functioning of the regulatory pathway, in this case for the pathway that allows I_M to exceed the level present before application of muscarine.

Our data argue against a role for extracellular calcium in modulating I_M . Tokimasa and Akasu (1990a) reported reduction of I_M by an external Ca^{2+} -free solution that depressed the maximum M conductance. They proposed that calcium binding sites on the outside of the membrane may be affiliated with M channels. Our results show, however, that with intracellular calcium tightly controlled by BAPTA, changing extracellular calcium had minimal effects on I_M . Calcium imaging showed that changing extracellular calcium had minuscule effects on intracellular calcium when 20 mM BAPTA was present, consistent with the lack of effect on M current.

Is there a role for calcium transients in muscarinic suppression of I_M ?

The study by Kirkwood et al. (1991) provides the strongest argument that calcium transients are an element of the signaling pathway through which muscarine inhibits I_M . The essential finding of that report is that muscarine can suppress I_M in cells loaded with 0.3 mM BAPTA, but perfusing these same cells with 20 mM BAPTA strongly diminishes the muscarinic suppression. Thus, the elimination of the muscarine-induced calcium transient, by 20 mM BAPTA, is proposed to uncouple activation of the receptor from the modulatory pathway. In contrast, earlier

reports showed that when calcium transients were prevented by a similarly high level of calcium chelator, muscarinic suppression proceeded as usual (Pffaffinger et al., 1988; Beech et al., 1991; Marrion et al., 1991). Two distinct issues must be resolved to evaluate the role of calcium transients. The first issue is whether muscarine is truly capable of suppressing I_M in the presence of 20 mM BAPTA. A second, distinct, issue is whether powerful buffering by 20 mM BAPTA can truly suppress calcium transients.

Kirkwood et al. (1991) suggest that higher dosages of muscarine might account for the conflicting results. However, we observe a 70% inhibition of I_M with as little as 1 μM muscarine. A related issue is the duration of application of muscarine. A long application of muscarine might produce a longer release of calcium that could overwhelm the buffering capacity of 20 mM BAPTA. However, Figure 7 shows that muscarinic inhibition reaches a maximum in less than 15 sec, so any later release of calcium is probably irrelevant. In agreement with this, Marrion et al. (1991, Fig. 3) show that the time course of muscarinic suppression is the same in 20 mM BAPTA or in 0.1 mM EGTA. It should take substantially longer for muscarine to overwhelm the buffer in 20 mM BAPTA.

This agrees with previous studies reporting that 20 mM BAPTA is insufficient to block the muscarinic effect (Beech et al., 1991; Marrion et al., 1991). In contrast, in the Kirkwood study, the time course of inhibition is substantially slowed by 20 mM BAPTA, so the different results are not readily reconciled. By our calculations, the intracellular formula used in Kirkwood et al.'s 20 mM BAPTA solution should have set free calcium at a level of about 100 nM, a level well into the range where I_M should be highly suppressible. Kirkwood et al.'s data could be explained if for some unknown reason perfusion with 20 mM BAPTA lowered resting free calcium in their experiments.

Marrion et al. (1991) report that 10 μM muscarine induces a 50 nM increase in free calcium in cells loaded with only 0.2 mM buffer (100 μM fura 2, 100 μM EGTA). We have measured calcium transients in voltage-clamp experiments, where calcium influx is controlled via voltage-gated calcium currents (O'Malley et al., 1993; D. M. O'Malley, unpublished observations). In those experiments, highly localized, brief transients were routinely measured in low concentrations of calcium buffer (100 μM fluo-3). Inclusion of 10 mM BAPTA, however, completely prevented the appearance of transients during an extremely rapid influx of calcium (50 msec depolarizations) that would have raised calcium to micromolar levels near the membrane. Given then that muscarine produces only a small, relatively slow increase in low buffer, it seems likely that 20 mM BAPTA strongly inhibited the size and spread of muscarine-induced calcium transients. This agrees with the results of Beech et al. (1991), who showed that the normally large increase in free calcium induced by 10 mM caffeine was completely buffered by 20 mM BAPTA (also see Marrion and Adams, 1992).

This type of experiment cannot rule out, however, highly localized actions of calcium occurring between calcium release channels and calcium binding molecules (e.g., protein kinase C) that might be situated adjacent to one another and to the M channels. Thus, a role for calcium transients in the muscarinic suppression pathway is still an open issue. An alternative explanation, one that we favor, is that muscarine induces prolonged production of a second messenger other than calcium and that removal of muscarine allows this messenger to decline quickly.

Mechanism of regulation of I_M by calcium

Low levels of free calcium clearly reduce I_M . This observation could result from a direct action of calcium on the M channel or on any affiliated molecules that affect channel gating; resting calcium levels would therefore be necessary for normal functioning. A more complex form of inhibition is suggested, however, by the observation that ATP is required to restore M current to normal levels when calcium is raised from 0 nM to 120 nM (Fig. 5). Since most of the restoration depended on ATP (see also Tokimasa and Akasu, 1990b), this suggests that a phosphorylation event is involved in the regulation of I_M by absolute calcium levels. A simple explanation is that phosphorylation of the M channel at resting calcium levels, by a calcium-dependent enzyme, maintains a resting level of I_M activation. In low calcium, the channel then becomes dephosphorylated, and cannot be reactivated in the absence of ATP. One candidate for a role in reactivation of I_M is calmodulin (reviewed by Walaas and Greengard, 1991). Increasing calcium from 0 nM to 120 nM could potentially stimulate calmodulin and activate calmodulin-dependent protein kinases. Preliminary experiments showed that a calmodulin antagonist W-7 (20 μ M; $n = 3$) failed to influence the enhancement of I_M by calcium (data not shown), but firm conclusions should not be drawn from this.

At high calcium levels, I_M is again inhibited. Because this modulation is kinetically different than the modulation by low calcium, a different mechanism is likely to be involved. The observation that high levels of calcium (450 nM) do not suppress M current to nearly the same extent as zero calcium further suggests an independent mechanism. The reduction of I_M at high calcium could be due to a direct action of calcium on the channel, since the effects of ATP were not evaluated at this calcium level. These high calcium levels are likely to occur during physiological stimulation (Llinas et al., 1992). It is less certain whether or not the low calcium levels required for reduction of I_M occur naturally, although some groups report resting levels of 30–50 nM (Nohmi et al., 1988; Pfaffinger et al., 1988; Bosma et al., 1990). In summary, calcium increases may either up- or downregulate I_M , depending on resting levels of free calcium.

Another candidate system considered for the modulation of M current is protein kinase C (PKC). Activation of protein kinase C by phorbol esters partially suppresses I_M (Brown and Adams, 1987; Bosma and Hille, 1989). The activation of PKC is Ca^{2+} dependent (reviewed by Huang, 1989) which is consistent with the inhibition of I_M at high levels of calcium (Fig. 3; Marrion et al., 1991). PKC was also considered to be a candidate for mediating the suppression of I_M by muscarine and LHRH. While evidence is accumulating against this possibility (Bosma and Hille, 1989; Tokimasa and Akasu, 1990b; Marrion, personal communication), the involvement of other protein kinases in muscarinic suppression remains an open issue.

The role of calcium in muscarinic suppression of I_M appears both complex and controversial. While our experiments do not indicate a direct role for calcium transients in muscarinic suppression, single-channel recording experiments do suggest that suppression is mediated by an unknown cytoplasmic signal (Marrion, 1993). One explanation is that there are calcium-dependent steps in this regulatory pathway. At resting calcium, muscarinic suppression proceeds swiftly. But in zero calcium, this pathway cannot proceed efficiently, and the muscarinic suppression of I_M is incomplete (Fig. 7). Because suppression of I_M by low calcium and by muscarine are distinct from each other,

there may be multiple sites on the M channel or associated structures that are independently regulated. An intriguing possibility, based on the observation that the M channels exists in distinct gating modes, is that only one mode is sensitive to muscarine (Marrion, 1993).

One candidate that appears to play a role in muscarinic modulation of I_M is arachidonic acid (AA), which together with its metabolites in hippocampal CA1 pyramidal neurons may mediate somatostatin augmentation of I_M (Schweitzer et al., 1990). Arachidonic acid liberation by phospholipase A_2 is known to be calcium dependent (Ballou and Cheung, 1985; Clark et al., 1991). Also, purified phospholipase A_2 from rat kidney is activated at physiological calcium concentrations (Gronich et al., 1990). The activation of several metabolic enzymes in the AA pathway requires both calcium and ATP (Vonakis and Vanderhoek, 1989; Dragan and Ellis, 1990; Wong et al., 1991). In bullfrog sympathetic neurons, AA increased I_M (Bosma et al., 1990). Work in our lab shows that AA and specific metabolites are involved in the modulation of I_M (Yu and Adams, 1991, 1992). Recent work suggests that distinct branches of the AA metabolic pathway subserve the modulation of this widespread channel (S. P. Yu and P. R. Adams, unpublished observations).

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