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Modulation of M-Current by Intracellular Ca^{2+}

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Summary

I_M is a voltage- and time-dependent K^+ current that is suppressed by muscarinic receptor activation. I_M augmentation following agonist washout was blocked by heavily buffering $[Ca^{2+}]_i$ using BAPTA. Although I_M is not primarily Ca^{2+} dependent, small increases in $[Ca^{2+}]_i$ by photolysis of the "caged" Ca^{2+} chelator nitr-5 or by evoking action potentials augmented, while larger increases inhibited, I_M. Raising $[Ca^{2+}]_i$ for prolonged periods, by nitr-5 photolysis, reduced its sensitivity to agonist, leaving a poorly reversible response. These results suggest that I_M can be regulated by physiologically relevant changes in $[Ca^{2+}]_i$, placing I_M in a unique position to modulate cell excitability.

Introduction

The M-current (I_M) is a time- and voltage-dependent K^+ current found in bullfrog sympathetic neurons (Brown and Adams, 1980) and a variety of other vertebrate neurons (Brown, 1988). It is suppressed during slow synaptic transmission (Adams and Brown, 1982), leading to increased input resistance, depolarization, and increased excitability (Adams et al., 1982a).

The evidence concerning Ca^{2+} involvement in I_M regulation is conflicting. It has been shown that I_M is not "Ca^{2+}-dependent," in the sense of requiring priming Ca^{2+} current for its activation (Adams et al., 1982a), unlike certain other K^+ currents in these cells, such as I_C (Adams et al., 1982b) and I_{AHP} (Pennefather et al., 1985). I_M in both bullfrog (Adams et al., 1982a) and rat (Marrion et al., 1989) sympathetic neurons persists when Ca^{2+} entry is prevented, either by removing Ca^{2+} from the perfusing medium or by using divalent Ca^{2+} channel blockers. Attempts to increase intracellular Ca^{2+} have yielded an array of effects. Raising intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in bullfrog sympathetic neurons by injecting Ca^{2+} ions had no obvious effect upon I_M (Adams et al., 1982a). Application of caffeine to bullfrog sympathetic neurons (presumably resulting in the release of intracellular Ca^{2+}) (Kuba, 1980; Pfaffinger et al., 1988; N. V. M. and P. R. A., submitted) evoked a component of K^+ current whose voltage and acetylcholine-sensitivity resembled that of I_M (Koketsu et al., 1982). But caffeine can also block I_M in these cells (Pfaffinger et al., 1988; Akaike and Sadoshima, 1989; Marrion et al., 1989, Soc. Neurosci., abstract). It has also been reported that Ca^{2+} influx can be followed by a long lasting inward current that was attributed to suppression of I_M (Tokimasa, 1985).

Agonists that suppress I_M in frog sympathetic neurons, such as muscarine and luteinizing hormone releasing hormone (LHRH), simultaneously increase $[Ca^{2+}]_i$ (Pfaffinger et al., 1988). Possible effects of Ca^{2+} on I_M are of particular interest because it was argued that this Ca^{2+} increase is irrelevant to I_M changes because the former, but not the latter, is prevented by BAPTA (Pfaffinger et al., 1988). We have used a combination of techniques to attempt to resolve whether I_M itself, or its modulation by muscarine, is affected by changes in $[Ca^{2+}]_i$. Intracellular Ca^{2+} levels were monitored using image analysis of fura 2-loaded (Grynkiewicz et al., 1985) whole-cell voltage-clamped bullfrog sympathetic neurons. Imaging allows resolution of Ca^{2+} signals near the membrane, which may be of particular relevance to electrophysiological effects. Elevation of Ca^{2+} was achieved either by photolysis of the caged Ca^{2+} chelator nitr-5 (Adams et al., 1988) or by evoking action potentials. Changes in $[Ca^{2+}]_i$ were prevented by dialyzing neurons with 20 mM BAPTA (Tsien, 1980).

Results

I_M is tonically active at potentials positive to −70 mV (Brown and Adams, 1980). Thus, when these neurons are voltage clamped to a depolarized level (−38 mV) I_M greatly contributes to the outward holding current. Hyperpolarizations to −58 mV then reveal I_M as inward current relaxations (Figure 1). I_M reactivates on restoring the potential to −38 mV, producing a slow increase in outward current. Bath application of muscarine (10 μM) reversibly suppressed I_M (Figure 1). Inhibition of I_M results in a net inward current and a loss of the time-dependent current relaxations induced by the hyperpolarizing voltage pulse. As illustrated in Figure 1, muscarine also increased $[Ca^{2+}]_i$ simultaneously with suppression of I_M. Measured at the peak of I_M suppression, muscarine (10 μM) evoked a mean $[Ca^{2+}]_i$ increase of 52 ± 2 nM (n = 23, mean ± SEM). Removal of muscarine caused a net outward current that was associated with I_M relaxations, which were larger than before muscarine application. This transiently enhanced I_M was first reported by Pfaffinger (1988), who termed it overrecovery. After further washing (3 min) with muscarine-free Ringer solution $[Ca^{2+}]_i$ returned to near control levels, while I_M always tended to stabilize to an amplitude smaller than seen before musca-

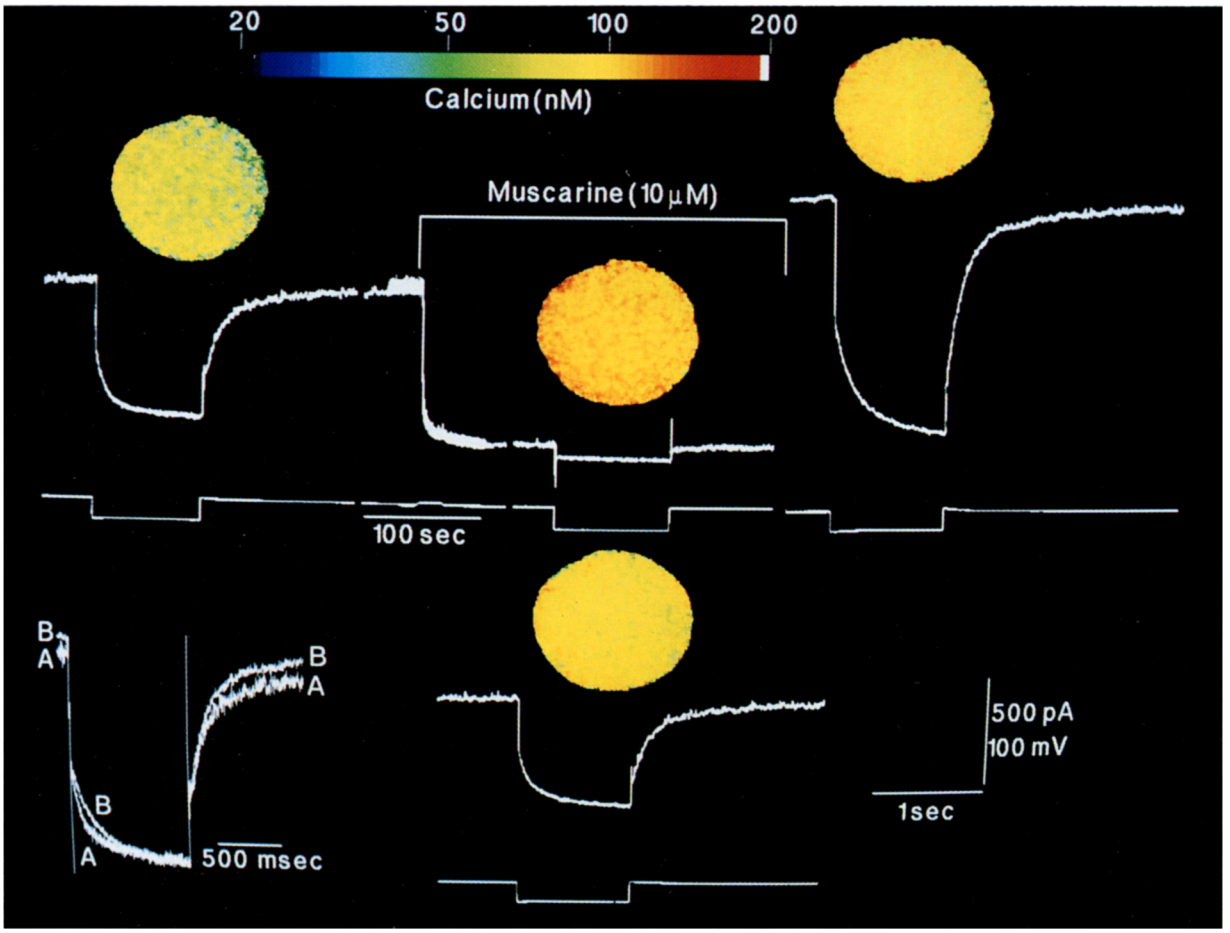


Figure 1. Muscarine Raises $[Ca^{2+}]_i$ Simultaneous with Suppression of I_M

Sustained I_M was activated by whole-cell voltage clamping a dissociated bullfrog sympathetic neuron at −38 mV. I_M was revealed a by 20 mV hyperpolarizing voltage step of 1 s duration. $[Ca^{2+}]_i$ levels, determined by image analysis of fura 2 signals, were measured before, during, and after muscarine application. The color bar at the top of the figure gives $[Ca^{2+}]_i$ in nM. Values for $[Ca^{2+}]_i$ are obtained by averaging all pixels in the cell. Application of muscarine (10 μM) suppressed the outward I_M, resulting in a net inward current (note the change in time scale) and a loss of the deactivation and reactivation current relaxations associated with M-channel closing and reopening. Simultaneous with this, muscarine raised $[Ca^{2+}]_i$ from 40 nM to 78 nM. Removal of muscarine caused I_M to transiently overrecover, while $[Ca^{2+}]_i$ declined to 60 nM. After 3 min wash (bottom trace) both membrane current and $[Ca^{2+}]_i$ (53 nM) returned to near control levels.

Inset shows superimposed currents obtained before addition of 10 μM muscarine (A) and after removal of muscarine at the peak of the overrecovery (B). Currents were scaled so the amplitudes of I_M deactivation before and after muscarine were the same. Note the slowing of the deactivation and the faster reactivation kinetics upon enhancement of I_M following removal of agonist.

rine. This increase in $[Ca^{2+}]_i$ was similar to that described in frog sympathetic neurons and was also seen with other agonists that suppress I_M, such as LHRH (Pfaffinger et al., 1988). This rise in $[Ca^{2+}]_i$ was not localized to any region of the cell (see Figure 1). Although some "hot" regions are observable in Figure 1, these differed with repeated additions of muscarine.

I_M kinetics changed during overrecovery. As illustrated in the inset of Figure 1, the deactivation current relaxation was slowed, while reactivation was accelerated. This transient change in kinetics was also seen after photolysis of nitr-5 (see below).

The transiently enhanced or overrecovered I_M observed on removal of agonist was fully sensitive to subsequent additions of either LHRH or muscarine. Figure 2 shows that if LHRH is applied at the peak of overrecovery, induced by a prior muscarine application, it completely suppresses I_M. After washout of LHRH I_M overrecovered relative to the original drug naive control response, although it did not attain the peak value seen during the first overrecovery. A second muscarine application again eliminated I_M, and after washout I_M again attained a value that was larger than the original control, but smaller than that just prior to this second muscarine application. Waning of overrecovery with repetitive agonist applications was frequently observed, and after approximately six agonist applications overrecovery was often no longer present. The $[Ca^{2+}]_i$ rise evoked by muscarine was found also to wane with repeated applications of ago-

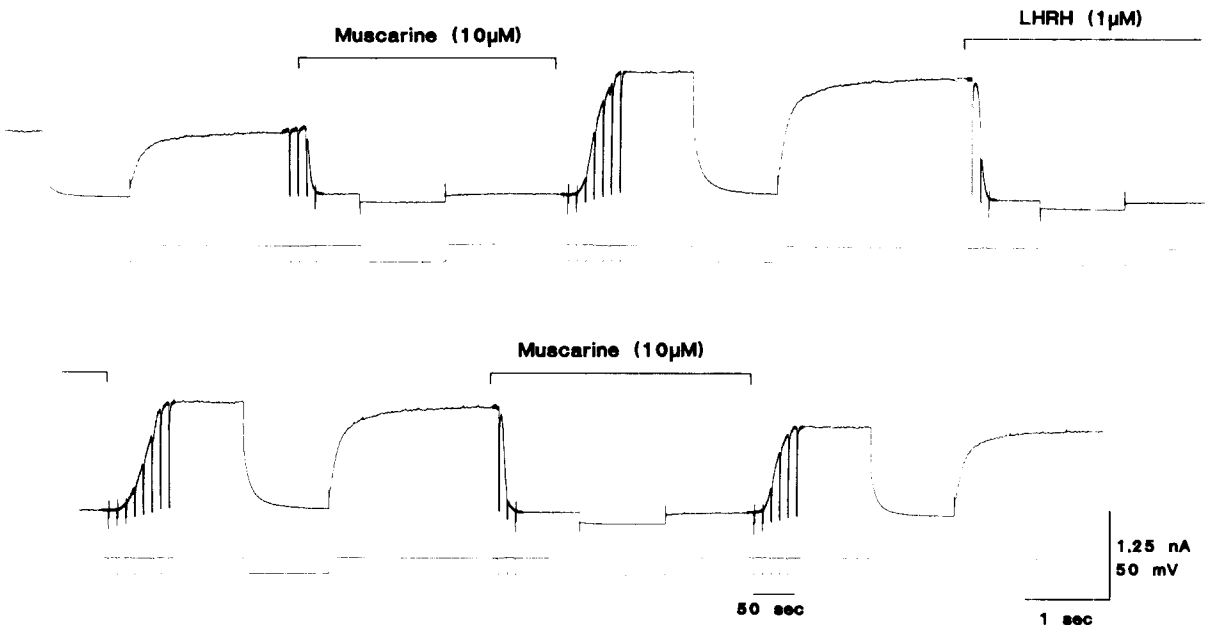


Figure 2. Agonist-Enhanced I_M Is Fully Sensitive to Second Agonist Application

I_M was activated by whole-cell voltage clamping the neuron at −38 mV with an electrode solution containing 0.1 mM EGTA. I_M was revealed by a 20 mV hyperpolarizing voltage step (1 s duration) repeated every 10 s (note the change in time scale). Application of muscarine (10 μM) suppressed I_M, and upon removing the agonist I_M rapidly recovered to an overrecovered level. Bath application of LHRH (1 μM) fully suppressed the agonist-enhanced current. Washing the cell of LHRH caused the I_M to again rapidly overrecover, but to a lower level than before. Addition of muscarine (10 μM) to this enhanced current again caused complete suppression. Removing muscarine caused the I_M to recover to an even smaller enhanced level.

nist (see also Pfaffinger et al., 1988), until after approximately six additions of muscarine the increase in $[Ca^{2+}]_i$ was no longer observed. The observation that both agonist-evoked I_M overrecovery and $[Ca^{2+}]_i$ increases waned after successive agonist additions suggests that the two may be linked. The next experiments tested whether such increases in $[Ca^{2+}]_i$ would have effects on the I_M.

Effect of Buffering $[Ca^{2+}]_i$ on Agonist Modulation of I_M

Changes in $[Ca^{2+}]_i$ can be reduced or even eliminated by dialysis with a whole-cell electrode solution containing a high concentration of the rapid Ca^{2+} chelator BAPTA (Tsien, 1980). In contrast, whole-cell recording with a low concentration of EGTA allows normal $[Ca^{2+}]_i$ changes to occur (see Pfaffinger et al., 1988; N. V. M. and P. R. A., submitted). Figure 3A shows I_M recorded in the whole-cell configuration with an electrode solution containing 0.1 mM EGTA. Under these conditions (assuming a contaminating $[Ca^{2+}]$ of less than 10 μM) the $[Ca^{2+}]$ in the electrode solution would be less than 20 nM, with very little (approximately 70 μM, accounting for Mg^{2+} binding to EGTA) free EGTA. $[Ca^{2+}]_i$ should therefore be free to change and be controlled by the cell's own regulatory mechanisms, without major influence from exogenous buffer. Application of muscarine (10 μM) to this cell caused a normal suppression of I_M. On removing muscarine I_M rapidly reversed to a net outward current as the I_M overrecovered. In over 30 cells dialyzed with this electrode solution I_M transiently overrecovered after removing the first application of muscarine or LHRH.

Whole-cell dialysis with an electrode solution containing 20 mM BAPTA (with an estimated free $[Ca^{2+}]_i$ of <10 nM) gave different results. Figure 3B shows I_M recorded after 15 min dialysis with a BAPTA (20 mM)-containing electrode solution. I_M was somewhat smaller (453 ± 219 pA, n = 8, mean ± SEM) than control (942 ± 177 pA, n = 32, mean ± SEM) (Figure 3A) and could be fully suppressed with muscarine (10 μM). The rate of suppression was comparable to that seen in control cells. On removing muscarine however, I_M remained suppressed for approximately 50 s before any recovery was obvious. In control cells washing for 50 s was sufficient to regain over 80% of I_M (see Figure 2; Figure 3A). As well as slowing recovery, inclusion of BAPTA prevented overrecovery. In eight out of ten examples, cells dialyzed with 20 mM BAPTA failed to exhibit the transiently enhanced I_M on removing muscarine. These results suggest that although suppression of I_M by muscarine was unaffected by heavily buffering $[Ca^{2+}]_i$, as reported by Pfaffinger et al. (1988), overrecovery was blocked, and recovery was slowed.

Raising $[Ca^{2+}]_i$ by Photolysis of Nitr-5

The effects of intracellular BAPTA suggested that changes in $[Ca^{2+}]_i$ may affect I_M. This was tested by raising $[Ca^{2+}]_i$ by various methods. Increasing $[Ca^{2+}]_i$ by

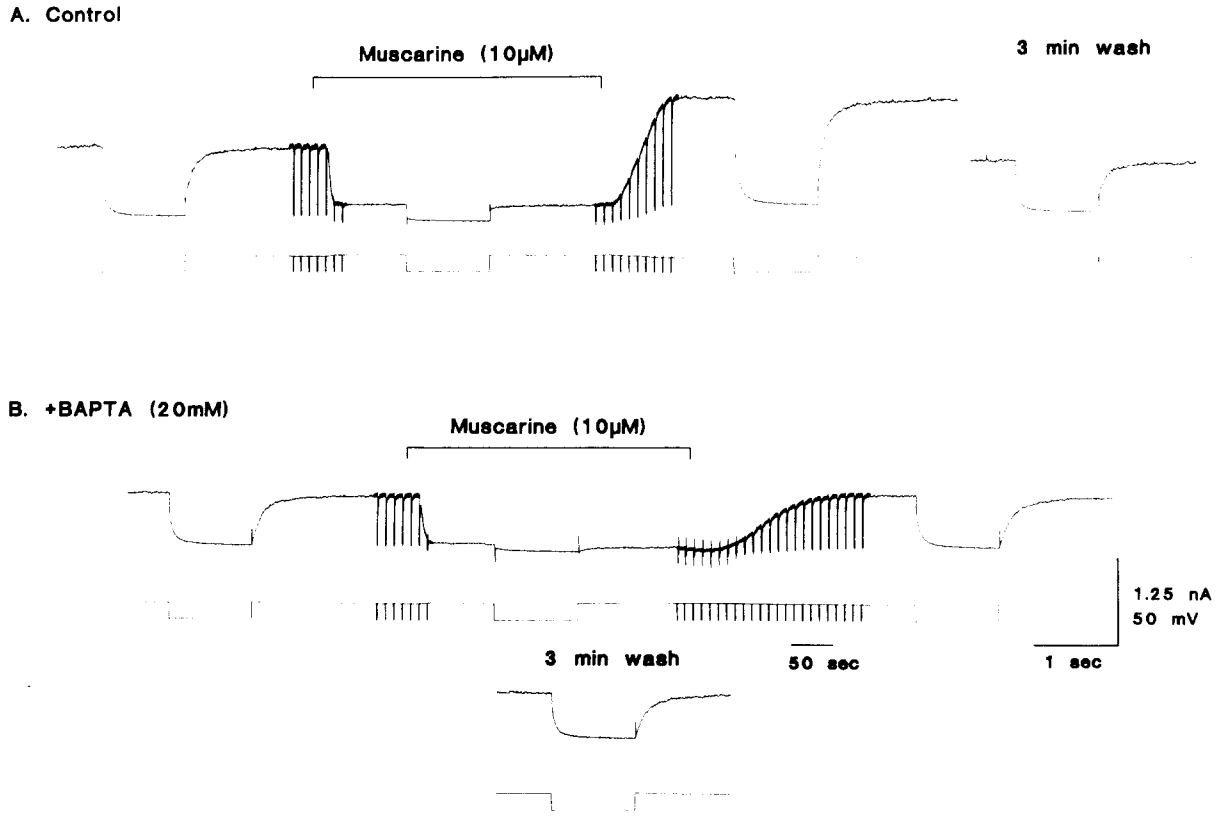


Figure 3. Heavily Buffered $[Ca^{2+}]_i$ Prevents I_M from Overrecovering

Cells were voltage clamped at −38 mV with whole-cell electrode solutions containing (A) 0.1 mM EGTA and (B) 20 mM BAPTA. I_M was revealed by a 20 mV hyperpolarizing voltage step of 1 s duration. This voltage step was repeated every 10 s (note the change in time base) while muscarine was applied. Muscarine reversibly suppressed I_M in the control cell (A) (different cell from Figure 1), its removal resulting in an enhanced I_M as it transiently overrecovered. Addition of muscarine to the cell dialyzed with 20 mM BAPTA (B) suppressed I_M as in control cells. Recovery from muscarine was retarded and did not result in overrecovery in cells in which $[Ca^{2+}]_i$ was heavily buffered.

bath application of caffeine (10 mM) produced either a suppression (also see Pfaffinger et al., 1988) or a sustained enhancement of I_M (Marrion et al., 1989; unpublished data). The Ca^{2+} ionophore A-23187 produced biphasic effects on I_M: initially an enhancement followed by a suppression of I_M as $[Ca^{2+}]_i$ rose to approximately 200 nM (data not shown). However, neither of these techniques were ideal because the amplitude and duration of $[Ca^{2+}]_i$ increases could not be controlled. To achieve this, the photolyzable Ca^{2+} chelator nitr-5 (Adams et al., 1988) was used. Based on BAPTA (Tsien, 1980), nitr-5 has a high affinity for Ca^{2+} (K_D = 145 nM) before photolysis. Upon illumination with ultraviolet light, nitr-5 is cleaved yielding a photolyzed product with a greatly diminished affinity for Ca^{2+} (K_D = 6.3 μM), resulting in a release of Ca^{2+} (Adams et al., 1988).

As discussed in the Experimental Procedures, photolysis of nitr-5 for different durations yields predictable increases in $[Ca^{2+}]_i$ (Landò and Zucker, 1989). The duration of $[Ca^{2+}]_i$ rises is dependent on regulatory mechanisms operating within the cell and replacement of the cell contents with unphotolyzed nitr-5 from the electrode. Because the light source was focused on the cell and the electrode tip, most of the nitr-5 in the electrode tip was also photolyzed, so little exchange with unphotolyzed nitr-5 should have occurred. In addition, the high concentration of nitr-5 (15 mM) used would be expected to swamp endogenous buffering mechanisms and retard the removal of $[Ca^{2+}]_i$. Potential problems in the use of nitr-5 have been previously discussed (Landò and Zucker, 1989). It has to be emphasized that the amount of high affinity buffer will change on photolysis. Illumination will decrease the amount of the high affinity nitrobenzhydrol and increase the degree of Ca^{2+} loading of the remaining high affinity buffer. This means that the amount of free nitrobenzhydrol will decrease with photolysis so that endogenous regulatory mechanisms within the cell will be free to play more of a role after sufficient photolysis.

Although the free Ca^{2+} level achieved by defined nitr-5 photolysis can be calculated, eventually cell regulatory mechanisms can remove some of the Ca^{2+} introduced with nitr-5. This makes it desirable to monitor $[Ca^{2+}]_i$ as an independent check. Long wavelength

Ca^{2+} indicators could have been used, such as fluo-3 or rhod-2 (Minta et al., 1989). Rhod-2 is, however, unsuitable for measuring small increases in Ca^{2+} (K_D = approximately 1 μM) (Minta et al., 1989). Fluo-3 is not ideal because quantitation of $[Ca^{2+}]_i$ would be approximate and may be subject to variation with different degrees of loading from cell to cell. Measurement with fura 2 has the advantage of circumventing problems associated with nonhomogeneous dye distribution or uncertain loading, by taking the ratio of data obtained at two excitation wavelengths (Grynkiewicz et al., 1985).

However, potential problems in combining nitr-5 and fura 2 techniques have to be recognized. First, the range of wavelengths used to photolyze nitr-5 (approximately 360 nm) coincides with the wavelengths used to excite fura 2 (340 and 380 nm). This may lead to some bleaching of fura 2 by the photolysis light source. If extensive, then high levels of $[Ca^{2+}]_i$ would be underestimated, because a Ca^{2+}-insensitive intermediate of fura 2 formed upon bleaching is still fluorescent over the range 340–380 nm (Becker and Fay, 1987). We have attempted to minimize this error by selecting for analysis those records that showed minimal fura 2 bleaching. Second, the photolyzed product of nitr-5, a nitrobenzophenone, has a peak absorbance at 380 nm (Adams et al., 1988). It is therefore possible that an accumulation of the nitrobenzophenone would diminish the 380 nm light used to excite fura 2 and give artifactually low values of emission. This error would then tend to overestimate values of $[Ca^{2+}]_i$. For these reasons the estimates of $[Ca^{2+}]_i$ after photolysis of nitr-5 must be considered to be approximate, although they usually agreed well with expectations from calculation (Landò and Zucker, 1989). Finally, the illumination used to excite fura 2 fluorescence can photolyze nitr-5, so that $[Ca^{2+}]_i$ might be significantly perturbed by its measurement. By taking repeated images we found that single $[Ca^{2+}]_i$ measurements typically elevated $[Ca^{2+}]_i$ by 15 nM or less.

Under normal imaging conditions, cell dialysis can be monitored by periodic illumination. When nitr-5 was included in the electrode solution this could not be done because of the possibility of photolysis of nitr-5. Thus, in most cases, dialysis was monitored by observing blockade of the Ca^{2+}-activated K^+ current I_C (Adams et al., 1982b), evoked by a 20 ms, 50 mV depolarizing voltage step.

After sufficient dialysis (between 5 and 10 min) cells were voltage clamped at −38 mV to activate sustained I_M. Figure 4A shows an example of I_M recorded after 6 min dialysis with a nitr-5-containing electrode solution. As the cell was illuminated for 6 s an outward current developed that was associated with increased amplitudes of I_M deactivation and reactivation current relaxations (Figure 4A). Coincident with the enhancement of I_M, illumination raised $[Ca^{2+}]_i$ from 52 to 132 nM due to photolysis of nitr-5. Figure 4B shows I_M recorded from a different cell after 6 min dialysis with 12 mM nitr-5, 40% bound to Ca^{2+}. Illuminating the cell for 12 s caused a net outward current associated with an enhancement of I_M together with an increase in $[Ca^{2+}]_i$ from 125 nM to 296 nM. This enhancement of I_M was not affected by the level of resting $[Ca^{2+}]_i$. This was set to different values by changing the initial percent Ca^{2+} bound to nitr-5 in the whole-cell electrode solution. Values of resting $[Ca^{2+}]_i$, measured by imaging of fura 2 signals, ranged from 15 nM (Figure 5) to 170 nM (Figure 6). In all cells tested (n = 24), regardless of the resting $[Ca^{2+}]_i$ level, the first illumination (and rise in $[Ca^{2+}]_i$) caused a sustained enhancement of I_M. In cells in which current-voltage relationships were constructed, no change in "leak" conductance (not attributable to I_M; see Experimental Procedures) was observed. Fura 2 images could not be obtained during nitr-5 photolysis because of the conflict in excitation wavelengths, so the spatial and temporal profile of the growth of the evoked $[Ca^{2+}]_i$ rise could not be studied.

If nitr-5 was omitted from the fura 2–containing electrode solution, illumination caused only a small transient membrane current and no change in $[Ca^{2+}]_i$. Unlike the sustained effect described earlier, this effect (either an inward or outward current) only occurred while the light was on and rapidly dissipated when the light was switched off. It probably resulted from light polarization of the Ag/AgCl reference electrode or electrode wire. Thus, the sustained effects seen in nitr-5 experiments are not an artifact of illumination nor do they reflect damage from ultraviolet light exposure. In some cells, photolysis of nitr-5 did not cause an increase in $[Ca^{2+}]_i$, and no sustained effect on membrane current was observed. This suggests that the photolyzed product of nitr-5 did not affect I_M and that measured $[Ca^{2+}]_i$ rises were not an artifact of an accumulation of the photolyzed nitr-5.

Little or no delay between starting the illumination and enhancement of I_M was observed. As can be seen in Figures 4A and 4B, the effect on membrane current stopped developing upon cessation of the light. In those cells in which bleaching of fura 2 was not significant, $[Ca^{2+}]_i$ levels and amplitudes of I_M were measured before and after illumination. In cells that were illuminated for 6 s, I_M increased by a mean value of 28% ± 5% (n = 5, mean ± SEM), while the mean increase in $[Ca^{2+}]_i$ was 108 ± 19 nM (n = 5, mean ± SEM). Cells that were illuminated for 12 s showed a mean increase in I_M of 39% ± 2% (n = 3, mean ± SEM), with a mean rise in $[Ca^{2+}]_i$ of 193 ± 14 nM (n = 3, mean ± SEM). Examples of longer illumination times were not analyzed due to excessive bleaching of fura 2.

I_M often showed a slowing in deactivation and an acceleration of reactivation kinetics after illumination (Figure 4). This was also observed when I_M was transiently enhanced following washout of agonist (see Figure 1 inset; Discussion). One possible explanation of this change in I_M kinetics following nitr-5 photolysis would be activation of Ca^{2+}-dependent K^+ currents by the rise in $[Ca^{2+}]_i$. Two such currents are present in bullfrog sympathetic neurons, I_C (Adams et al., 1982b)

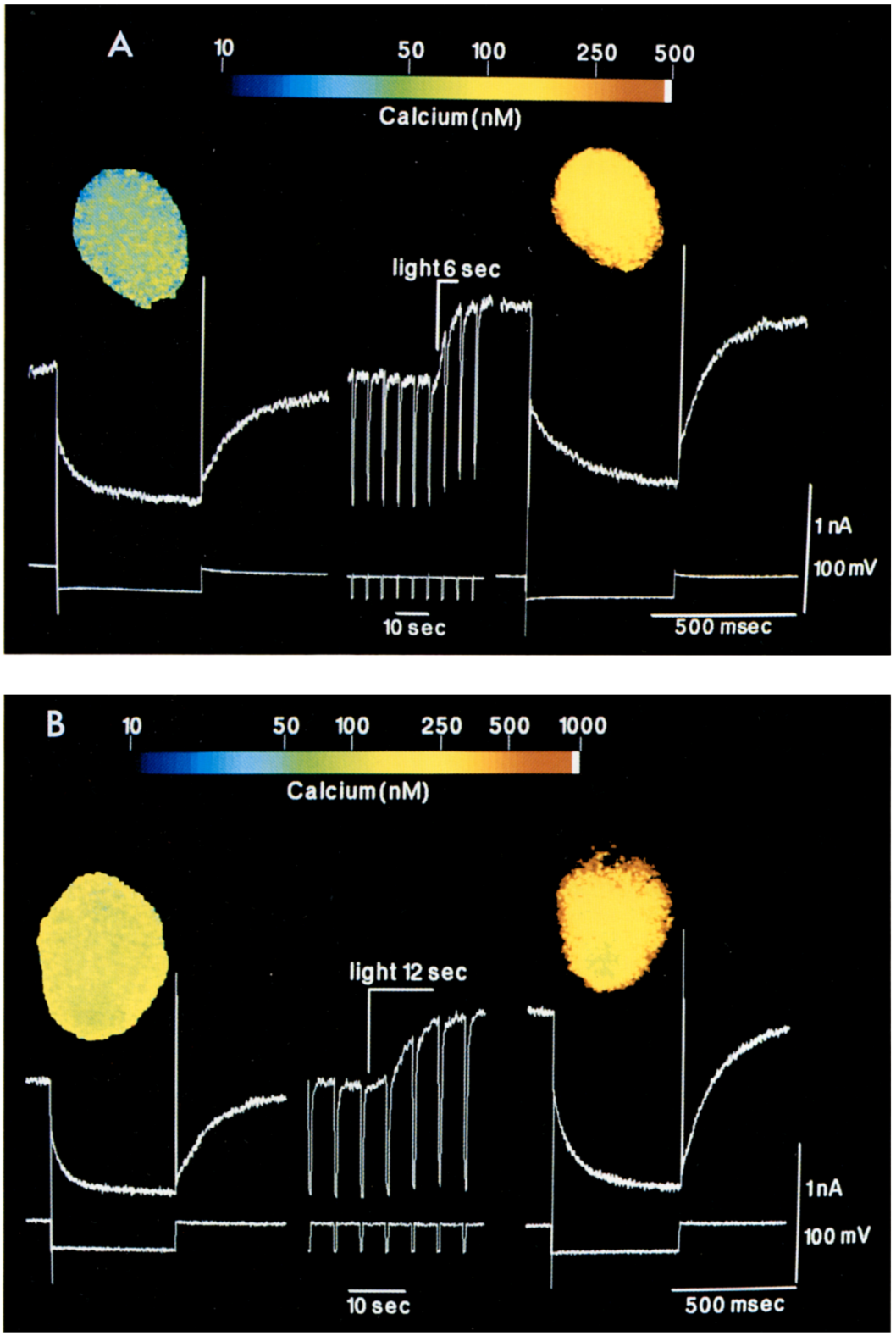


Figure 4. Photolysis of Nitr-5 Increases $[Ca^{2+}]_i$ and Augments I_M

Cells were voltage clamped at −38 mV with electrode solutions containing (A) 15 mM nitr-5, 35% bound to Ca^{2+}, and (B) 12 mM nitr-5, 40% bound to Ca^{2+}. I_M was revealed by 500 ms long, 20 mV hyperpolarizing voltage steps that were repeated every 5 s (note the change in time scales).

(A) Resting $[Ca^{2+}]_i$ was measured to be 52 nM. Illumination for 6 s evoked a net outward current associated with increased amplitudes of the current relaxations due to I_M deactivation and reactivation. Measurement of I_M deactivation current relaxation showed that I_M had been augmented by 20%. Accompanying the augmentation of I_M, $[Ca^{2+}]_i$ increased to 132 nM. (B) Resting $[Ca^{2+}]_i$ was estimated to be 125 nM. Illumination for 12 s raised $[Ca^{2+}]_i$ to 296 nM and evoked an outward current associated with an enhanced I_M of approximately 35%.

Note that in both cases, raising $[Ca^{2+}]_i$ changed I_M deactivation and reactivation kinetics similarly to what was seen during the transient overrecovery on removal of muscarine (Figure 1).

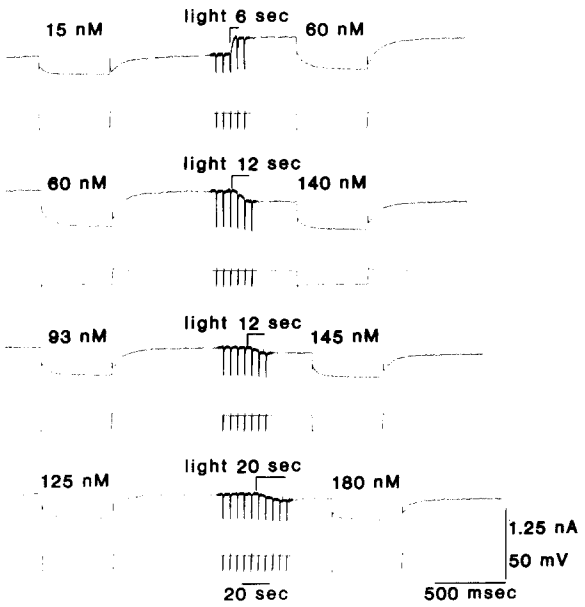


Figure 5. Higher $[Ca^{2+}]_i$ Loads Inhibit I_M

After whole-cell voltage clamping the cell at −38 mV with an electrode solution containing 15 mM nitr-5 (35% bound to Ca^{2+}), I_M was revealed by a 20 mV hyperpolarizing voltage step of 500 ms duration. This voltage step was repeated every 5 s (note the change in time base). Illumination for 6 s raised $[Ca^{2+}]_i$ from 15 to 60 nM and augmented I_M by 50% (measured from the deactivation current relaxation). After a further 120 s, during which the I_M remained enhanced, a 12 s light was applied to the cell. $[Ca^{2+}]_i$ rose from 60 to 140 nM, and I_M was inhibited by 33%. During the next 120 s I_M partially recovered (by about 10%), while $[Ca^{2+}]_i$ fell to 93 nM. A third light application (of 12 s duration) further suppressed I_M (by 22%), while increasing $[Ca^{2+}]_i$ to 145 nM. During a 120 s respite I_M again partly recovered (by about 10%), and $[Ca^{2+}]_i$ declined to 125 nM. A final light application (of 20 s duration) reduced I_M by another 28% and increased $[Ca^{2+}]_i$ to at least 180 nM. This latter measurement, however, is likely to be an underestimate due to significant bleaching of fura 2.

and I_{AHP} (Pennefather et al., 1985). These two currents can be inhibited by tetraethylammonium or d-tubocurarine, respectively. Illumination of nitr-5-filled neurons superfused with 1 mM tetraethylammonium and 200 μM d-tubocurarine (n = 4) still evoked a sustained outward current associated with an enhancement of I_M and changed I_M kinetics (data not shown). Thus the main effect of small increases in $[Ca^{2+}]_i$ (rising from 49 ± 8 to 157 ± 17 nM by a 6 s illumination, n = 5, mean ± SEM) is to augment I_M.

The Biphasic Action of $[Ca^{2+}]_i$ on I_M

As described above, the initial effect of raising $[Ca^{2+}]_i$ by nitr-5 photolysis was to increase I_M. However, if illumination episodes were repeated (with approximately 120 s between each light application) I_M was reduced. Figure 5 shows an experiment in which an initial illumination increased both $[Ca^{2+}]_i$ and I_M. Subsequent illuminations produced further increases in $[Ca^{2+}]_i$ but reduced I_M, eventually to below control levels. The initial enhancement was seen in all cells (n = 17), while the subsequent inhibition of I_M was seen in 12 out of 17 cells. In cells in which this did not occur, successive light applications did not further increase $[Ca^{2+}]_i$ either, probably because the nitr-5 was already fully photolyzed.

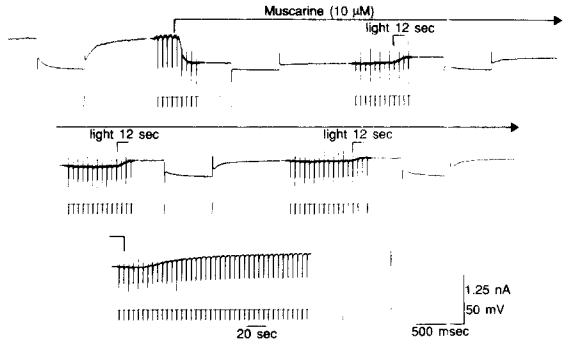


Figure 6. Raising $[Ca^{2+}]_i$ Restores Muscarine-Suppressed I_M

I_M was recorded at −38 mV and revealed by stepping the membrane voltage to −58 mV for 500 ms every 5 s (note the change in time scales). After muscarine (10 μM) suppressed I_M, the cell was illuminated and $[Ca^{2+}]_i$ rose from 170 nM to 230 nM, causing I_M to partially recover. Successive light applications increased the amplitude of the muscarine-suppressed I_M. Removal of muscarine allowed I_M to recover to near control levels.

Action of Raising $[Ca^{2+}]_i$ on I_M Suppression by Muscarine

The size of I_M is influenced both by the presence of muscarine and by increases in $[Ca^{2+}]_i$. Possible interactions between these factors were assessed in two types of experiments. In the first (Figure 6), illumina-

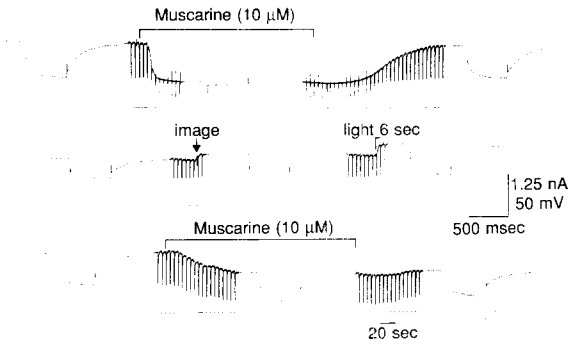
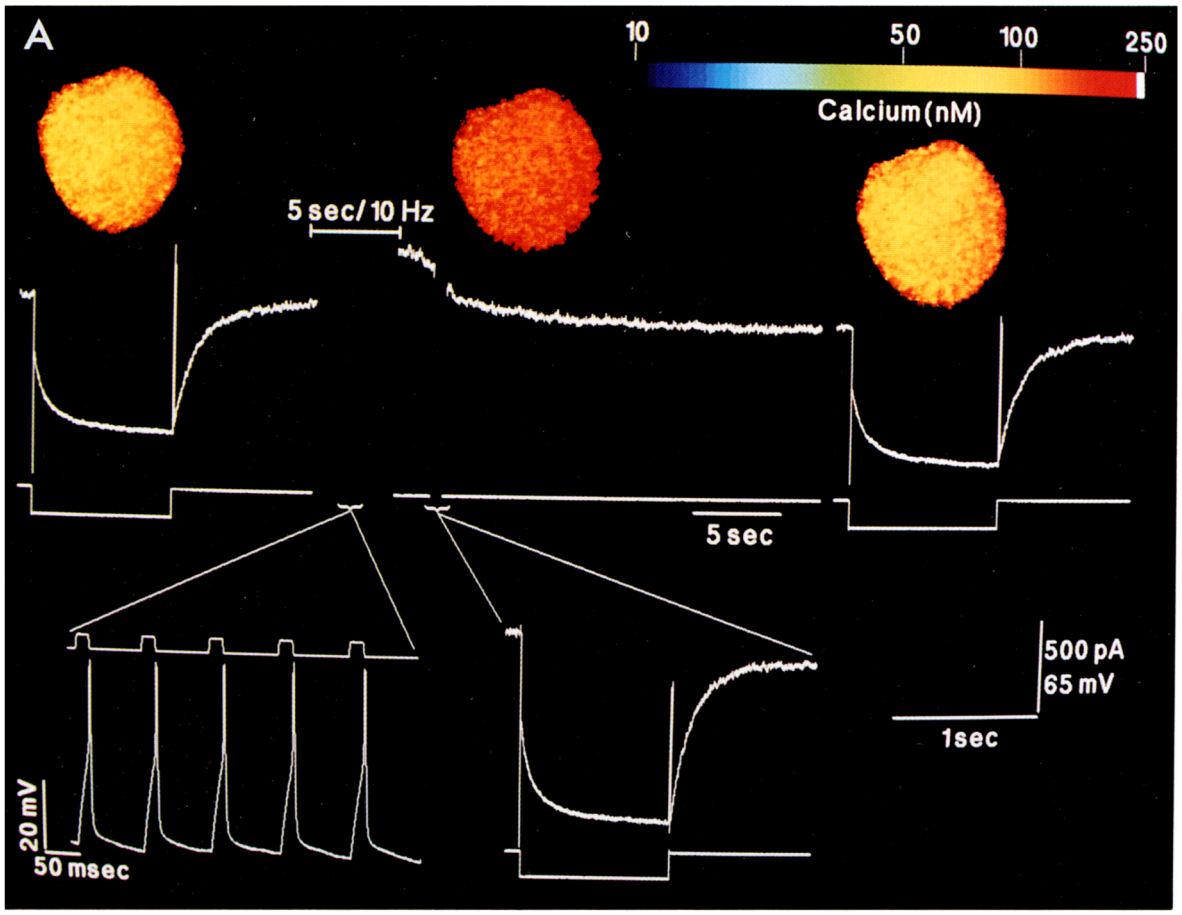
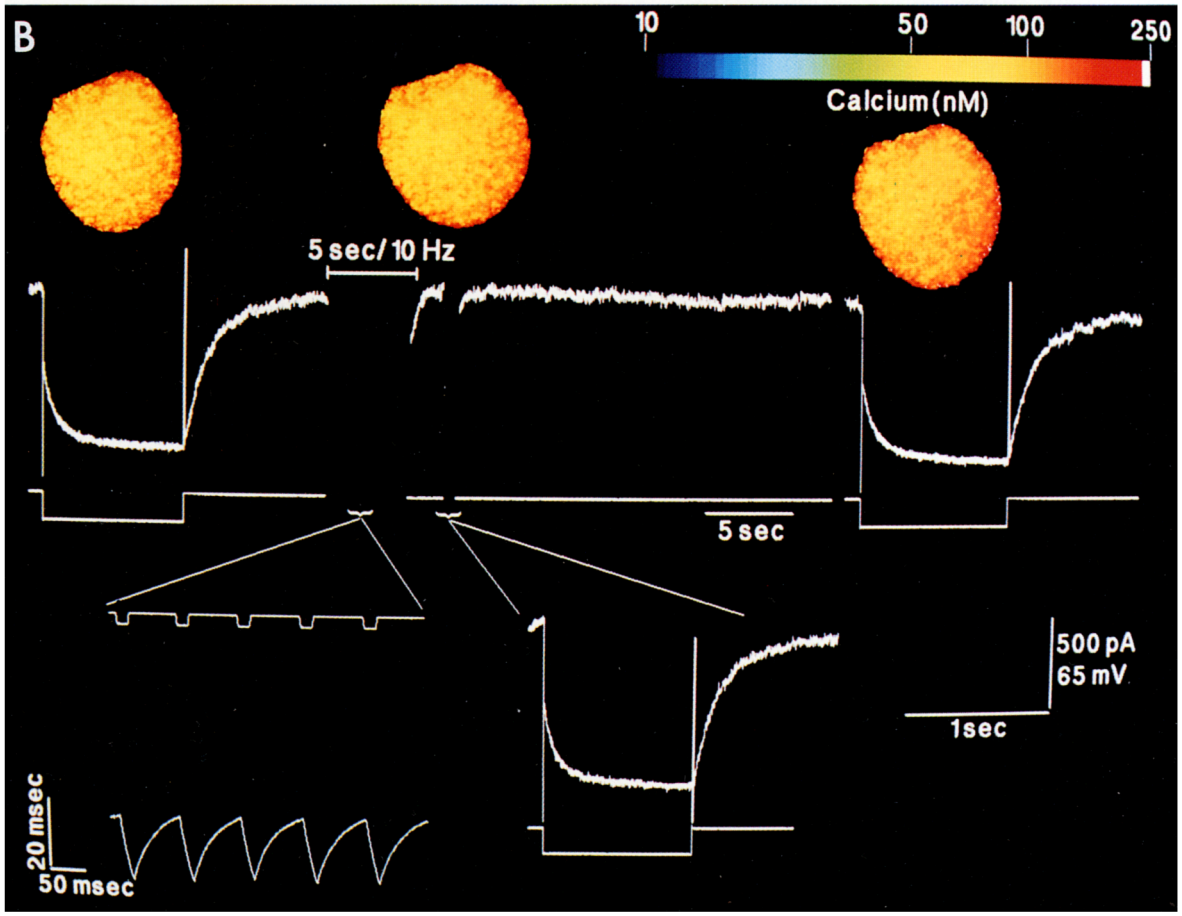


Figure 7. The Muscarine Response Can Be Blocked by High $[Ca^{2+}]_i$

The cell was whole-cell voltage clamped at −38 mV with an electrode solution containing 15 mM nitr-5, 35% bound to Ca^{2+}. After I_M was revealed by repeated 20 mV hyperpolarizing voltage steps (500 ms duration, every 5 s), muscarine (10 μM) was applied (note the change in time base). Muscarine rapidly suppressed I_M, after which the recovery was retarded (see text). $[Ca^{2+}]_i$ was then measured (note the augmented I_M upon gathering the image). Illumination for 6 s raised $[Ca^{2+}]_i$ from 82 nM to 220 nM, augmenting I_M by 30%. After a subsequent 12 s light application, raising $[Ca^{2+}]_i$ to 458 nM, muscarine (10 μM) was reapplied. Muscarine now only partially suppressed I_M (by 20%) and evoked an increase in leak. Removing muscarine reversed the leak increase, but I_M did not recover.


A
10
50
100
250
Calcium (nM)
5 sec/10 Hz
5 sec
500 pA
65 mV
1sec
20 mV
50 msec


B
10
50
100
250
Calcium (nM)
5 sec/10 Hz
5 sec
500 pA
65 mV
1sec
20 msec
50 msec

tion was applied during continuous exposure to muscarine. Each light application led to some recovery of I_M despite the continued presence of muscarine. Such recovery was not seen during similar muscarine exposure when light was not applied.

In the second type of experiment the effect of illumination was assessed before and after muscarine applications (Figure 7). As expected, given the Ca^{2+}-buffering properties of unphotolyzed nitr-5, overrecovery was not seen following termination of the first muscarine application (see Figure 3). Two light exposures were then applied, the first augmenting I_M and the second slightly reducing I_M. These lights raised $[Ca^{2+}]_i$ from 82 to 460 nM. Reapplication of muscarine now evoked a slow inward current, reflecting mainly an increase in leak conductance with only a small decrease in I_M (20%). Washout of muscarine reversed the leak increase but not the small decrease in I_M. This effect of raised $[Ca^{2+}]_i$ was seen in eight out of eight cells, in two of which the response to muscarine was completely blocked by high $[Ca^{2+}]_i$. This observation suggests that high $[Ca^{2+}]_i$ for long periods may inhibit coupling of the muscarinic receptor to the channel.

A Train of Action Potentials Can Augment I_M

These results show that small increases in $[Ca^{2+}]_i$ enhance I_M, while larger increases inhibit I_M. A physiologically relevant source of Ca^{2+} is entry through voltage-gated Ca^{2+} channels activated during an action potential. Figure 8A shows a hybrid clamp experiment in which $[Ca^{2+}]_i$ and I_M were increased by a train of action potentials. This cell was bathed in a superfusate containing 1 mM tetraethylammonium and 200 μM d-tubocurarine to block both I_C and I_{AHP}. After voltage clamping the neuron at −38 mV and observing I_M, the clamp was released and the cell returned to its resting membrane potential (−70 mV). A train of action potentials were evoked by injecting 20 ms, 300 pA current pulses at 10 Hz for 5 s. At the end of the induced train the voltage clamp was reapplied and $[Ca^{2+}]_i$ was measured. $[Ca^{2+}]_i$ increased from 88 to 144 nM, and a decaying outward current was evoked. Superimposition of a 20 mV hyperpolarizing voltage step onto this outward current showed that the I_M was enhanced (by 38%). After the outward current had fully decayed, both I_M and $[Ca^{2+}]_i$ (to 82 nM) returned to control levels. Reversing the polarity of the train of current pulses, to evoke only passive electrotonic potentials had no effect on $[Ca^{2+}]_i$ or I_M (Figure 8B). Thus, I_M can be enhanced by small $[Ca^{2+}]_i$ increases resulting from a train of action potentials.

Discussion

We have used several procedures to determine whether $[Ca^{2+}]_i$ levels could modulate I_M and/or its suppression by muscarine. $[Ca^{2+}]_i$ rises were prevented by dialyzing the neuron interior with BAPTA (20 mM). Controlled rises in $[Ca^{2+}]_i$ were achieved by photolysis of the "caged" Ca^{2+} chelator nitr-5 and monitored using fura 2. Increases in $[Ca^{2+}]_i$ were also produced by evoking a train of action potentials.

Suppression of I_M by muscarine was accompanied by a small increase in $[Ca^{2+}]_i$ of 52 ± 2 nM (n = 23, mean ± SEM). When muscarine was removed a transiently enhanced I_M, or overrecovery, was observed (Figure 1). Inclusion of BAPTA in the electrode solution retarded recovery and inhibited the development of the enhanced I_M, or overrecovery, evoked on removing agonist (Figure 3). Photolysis of nitr-5, inducing small increases in $[Ca^{2+}]_i$ (50–140 nM), enhanced I_M. This enhancement was accompanied by a change in I_M kinetics (Figures 4A and 4B; see below). Larger and more prolonged rises in $[Ca^{2+}]_i$ suppressed I_M (Figure 5).

$[Ca^{2+}]_i$ increases produced by muscarine, action potentials, and particularly by nitr-5 photolysis were not completely uniform across the cell, increases near the cell periphery being particularly pronounced (Figures 4A and 4B; Figure 8A). One would expect that diffusion would "iron out" such gradients within a few hundred milliseconds (Hernández-Cruz et al., 1990), yet they persisted for many seconds. Such long lasting gradients have been seen in images from sympathetic neurons (Lipscombe et al., 1988) and other cell types such as smooth muscle (Williams et al., 1985), neuroblastoma cells (Silver et al., 1989), adrenal chromaffin cells (O'Sullivan et al., 1989), CA1 hippocampal pyramidal neurons (Connor et al., 1988), and cerebellar Purkinje cells (Hockberger et al., 1989). They may represent residual dye distribution nonuniformities not eliminated by taking the ratio or genuine Ca^{2+} compartmentalization. It seems unlikely that they are machine artifacts, because they are not seen when imaging solution droplets.

Increases in $[Ca^{2+}]_i$ also had an effect on muscarine

Figure 8. I_M Can Be Augmented by a Train of Action Potentials

(A) After treating this cell with tetraethylammonium (1 mM) and d-tubocurarine (200 μM) to block I_C and I_{AHP}, respectively, I_M was revealed by voltage clamping at −38 mV and stepping the voltage to −58 mV for 1 s. Then the time base was slowed, and the clamp was released, allowing the cell to return to its resting membrane potential (−70 mV). A train of action potentials were evoked by injecting 300 pA current pulses (20 ms duration) at 10 Hz for 5 s. On resetting the voltage clamp (and the time base) a decaying outward current was evoked together with a $[Ca^{2+}]_i$ increase from 88 to 180 nM. Superimposition of a 20 mV hyperpolarizing voltage step on the initial decay of the outward current showed it was associated with a 38% augmentation of I_M. Once the outward current had fully decayed both $[Ca^{2+}]_i$ and I_M had recovered to control levels.

(B) Same protocol to that described in (A) except for reversing the polarity of the current pulses to evoke passive electrotonic potentials. On resetting the voltage clamp both membrane current and $[Ca^{2+}]_i$ were unaffected.

suppression of I_M. During suppression by muscarine, I_M could be "step-wise" restored upon each photolysis of nitr-5 (Figure 6). This effect may reflect inhibition of receptor-channel coupling or else insensitivity of the muscarine-closed M-channel to high $[Ca^{2+}]_i$ levels. The former explanation is more likely because raising $[Ca^{2+}]_i$ can inhibit muscarinic suppression of a previously muscarine-sensitive I_M (Figure 7). This effect is unlikely to be due to the enhanced I_M being insensitive to agonist, because the overrecovery (induced by agonist removal) is equally sensitive to agonist (Figure 2). It will be important to determine whether the various effects of Ca^{2+} and muscarine occur at independent or interacting sites.

I_M showed changed kinetics following agonist washout or raising $[Ca^{2+}]_i$ by nitr-5 photolysis. The deactivation current relaxation slowed and became more "monoexponential," while the reactivation of I_M became more rapid and more "biexponential." Recent analysis of macroscopic I_M has revealed that the M-channel may exist in at least four kinetic states (Gruner et al., 1989, Soc. Neurosci., abstract; unpublished data). Changes in I_M kinetics upon raising $[Ca^{2+}]_i$ could reflect a change in these single-channel properties.

Because overrecovery was blocked by heavily buffering $[Ca^{2+}]_i$ and mimicked by raising $[Ca^{2+}]_i$, it seems likely that I_M enhancement following removal of agonist results from the agonist-evoked increase in $[Ca^{2+}]_i$ during I_M suppression (Figure 1). Images obtained during the cell's response to muscarine showed that $[Ca^{2+}]_i$ had declined toward control levels at the peak of overrecovery. This consistent observation implies that the effect of raised $[Ca^{2+}]_i$ on I_M is delayed. It is possible that the M-channels are modulated as a result of the raised $[Ca^{2+}]_i$, even while they are closed by muscarine. Relief of suppression on removing muscarine would then allow the enhanced current to become apparent. The finding that this overrecovery wanes with successive agonist applications (Figure 2) is in keeping with a role for Ca^{2+}, because the agonist-induced increase in $[Ca^{2+}]_i$ also declines upon repeated applications of agonist (see also Pfaffinger et al., 1988). However, it is possible, though less likely, that the block of overrecovery seen with 20 mM BAPTA reflects an absolute lowering of $[Ca^{2+}]_i$ (from below 20 nM to below 10 nM), rather than just the increased buffer capacity. Against this are observations that in experiments with electrode solutions containing 1 mM EGTA and no added Ca^{2+} (which should also lower Ca^{2+}, though not quite as far), overrecovery was seen (data not shown).

Agonist-induced enhancements of I_M have also been observed in other preparations. Isoprenaline augments the I_M in toad smooth muscle (Sims et al., 1988), and somatostatin enhances I_M in both hippocampal (Moore et al., 1988) and solitary tract neurons (Jacquin et al., 1988). Although $[Ca^{2+}]_i$ was not measured in the above experiments, the isoprenaline-induced enhancement was replicated by 8-bromo cyclic AMP or by forskolin, implying it was generated by activation of adenylate cyclase and presumably cyclic AMP-dependent phosphorylation (Sims et al., 1988). Although no such effect of isoprenaline, cyclic AMP, or forskolin has been detected on neuronal I_M (Adams et al., 1982a; Brown and Adams, 1987; Brown et al., 1989), it remains possible that the effect of nitr-5 photolysis is akin to this action. For example, cardiac Ca^{2+} currents are enhanced by isoprenaline (see Tsien et al., 1986) acting through a cyclic AMP-dependent phosphorylation (Reuter, 1983). These currents are also augmented by raising $[Ca^{2+}]_i$ using flash photolysis of nitr-5 (Gurney et al., 1989). This enhancement could be blocked if the Ca^{2+} current was previously enhanced by isoprenaline, suggesting that a Ca^{2+}-dependent process, presumably phosphorylation, can modulate Ca^{2+} channels in the same way as isoprenaline (Gurney et al., 1989). It seems possible, therefore, that neuronal M-channels may share this phenomenon, allowing them to be modulated by a Ca^{2+}-dependent phosphorylation. This putative phosphorylation may increase the probability of M-channels being open and/or actually increase the number of functional channels (as for cardiac Ca^{2+} channels, see Tsien et al., 1986).

Recently, enhancement of hippocampal I_M by somatostatin was reported to be mimicked by arachidonic acid and its leukotriene metabolites (Schweitzer et al., 1990). Arachidonic acid liberation by phospholipase A_2 is Ca^{2+} dependent, raising the possibility that the augmentation of I_M in this study could result from elevated $[Ca^{2+}]_i$ levels activating phospholipase A_2 (Gronich et al., 1990; see van Scharrenburg et al., 1985).

The secondary inhibition of I_M by higher $[Ca^{2+}]_i$ levels may be a more nonselective effect. It could reflect the activation of an enzyme(s) causing a modification in the M-channel making it unable to open. The most likely candidates for this are a Ca^{2+}-calmodulin-dependent protein kinase or protein kinase C (PKC). The latter is more likely because inhibitors of Ca^{2+}-calmodulin-dependent phosphorylation, for example W-7, have no effect on the profile of muscarine's response (N. V. M., S. J. M., and P. R. A., unpublished data), whereas I_M can be inhibited by activators of PKC, for example phorbol esters (Brown and Adams, 1987; Brown et al., 1989), and PKC can be activated by raising $[Ca^{2+}]_i$ (see Huang, 1989).

The third effect of raised $[Ca^{2+}]_i$ was a partial inhibition of muscarine's suppression of I_M. This reduction in muscarine sensitivity is similar to that following phorbol ester application to these cells (Brown and Adams, 1987). In both cases, the residual effect of muscarine is slow to develop, resulting in a partial suppression of I_M and an increase in leak, with only the leak increase reversing after removing the agonist (Figure 7). Possibly raised $[Ca^{2+}]_i$ activates PKC, which then inhibits the pathway for muscarine responses.

The inhibition of agonist suppression of I_M by raised $[Ca^{2+}]_i$ suggests possible mechanisms of how muscarine closes M-channels. Muscarine may suppress I_M through two convergent pathways, the first a rapid

and easily reversible pathway (as yet unknown) and a second slower and less reversible route. The existence of two pathways is also suggested by the observation that the time course of I_M suppression by muscarine may comprise at least two components (an early, rapid suppression followed by a slowly developing phase; see Figure 1; Figure 7). Muscarinic receptor activation stimulates the formation of inositol phosphates in frog sympathetic ganglia (Pfaffinger et al., 1988), suggesting that the second messengers (1,4,5)IP_3 and diacylglycerol should be formed in response to muscarine. It is known that PKC activation by phorbol esters can inhibit I_M. This inhibition is slow to plateau, irreversible, and affects only a part of I_M. The response to muscarine seen with high $[Ca^{2+}]_i$ levels was also incomplete, slow to plateau, and irreversible (Figure 7). The high $[Ca^{2+}]_i$ levels obtained in these experiments may inhibit the first pathway, leaving the second pathway, possibly operating via PKC activation, intact.

Enhancement of I_M by Ca^{2+} may be physiologically important, since increasing $[Ca^{2+}]_i$ by making the cell fire a train of action potentials transiently augmented I_M (Figure 8). It is possible, therefore, that a component of spike after-hyperpolarization could be contributed by enhanced I_M (see Storm, 1989). This finding places the I_M in a unique position to modulate cell excitability. During cholinergic synaptic activation I_M is suppressed by receptor-mediated inhibition (Adams and Brown, 1982), making the cell more excitable. If other excitatory inputs were to initiate a train of action potentials then $[Ca^{2+}]_i$ would rise, augmenting I_M, thereby hyperpolarizing the cell and inhibiting cell firing.

Experimental Procedures

Preparation of Cells

Lumbar VIIIth, IXth, and Xth sympathetic ganglia were isolated from adult bullfrogs (Rana catesbiana) and dissociated as described previously (Kuffler and Sejnowski, 1983). Briefly, ganglia were chopped and incubated with collagenase (1 mg/ml; Worthington) and dispase (5 mg/ml; Boehringer Mannheim) for 30 min at 37°C. After mechanical disruption another dispase treatment (5 mg/ml, 30 min at 37°C) preceded final mechanical trituration. Dispersed cells were plated on polylysine-coated no.1 coverslips within a Lucite ring cemented into place with Sylgard (Dow Corning Corp.). When imaging was not required cells were plated into 35 mm plastic petri dishes. Isolated neurons were stored at 8°C in a supplemented L-15 medium (Gibco) and were used after 1–7 days in culture.

Membrane Current Recording and Fura 2 Imaging of $[Ca^{2+}]_i$

Coverslips or petri dishes were mounted in a Lucite block and superfused at 15 ml/min (bath volumes 0.2 and 1 ml, respectively) with a modified Ringer solution (115 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, and 10 mM D-glucose [pH 7.2]) at room temperature (20–22°C). Dissociated neurons were visualized using inverted phase-contrast optics (40× Nikon diaphot, 0.85 N.A.) and voltage clamped with a discontinuous amplifier (Axoclamp 2A, sampling rate 5–8 kHz; see Jones, 1987) using the whole-cell method (Hamill et al., 1981). Fire-polished whole-cell electrodes (3–5 MΩ resistance, 2–3 μm tip diameter) contained the following: 90 mM potassium aspartate, 20 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM Na_2ATP, 5 mM HEPES, and 3 mM NaOH. For imaging cytoplasmic Ca^{2+} levels electrodes also contained the following: 0.1 mM EGTA, 0.1 mM fura 2, and 73 μM $CaCl_2$ (assuming a contaminating $[Ca^{2+}]$ of 10 μM), giving an initial $[Ca^{2+}]_i$ of 80 nM (Fabiato and Fabiato, 1979). When $[Ca^{2+}]_i$ changes were prevented electrodes instead contained 20 mM BAPTA with no added Ca^{2+}. In experiments in which $[Ca^{2+}]_i$ was raised by photolysis of nitr-5, electrodes now also contained 0.1 mM fura 2 and 15 mM nitr-5 (normally 35% loaded with Ca^{2+}; see text). At an ionic strength of about 100 mM, this solution should buffer free Ca^{2+} to approximately 75 nM (Adams et al., 1988). Electrodes were manufactured from filamented borosilicate glass. When using nitr-5-containing electrode solution electrodes were backfilled by placing a 0.5 μl droplet onto the filament so that the tip could be rapidly filled with this small volume. Electrodes were painted with M-coat (Measurements Group, Inc.) to within approximately 200 μm of the tip to prevent light polarizing the Ag/AgCl wire and generating a junction potential while nitr-5 photolysis was occurring. A junction potential of +8 mV was recorded between the Ringer and electrode solutions used in this study. This junction potential was subtracted from all recorded potentials. I_M was measured as the amplitude of the reactivation current relaxation evoked at the cessation of the hyperpolarizing voltage pulse. This current (extrapolated to time zero) was measured before and after changes in conditions, and the percent change was calculated. Leak currents were measured as the instantaneous current response to hyperpolarizing voltage pulses from a holding potential of −70 mV.

Ca^{2+} was elevated by release from the nitr-5 photolyzed by ultraviolet light, previously described in detail (Landò and Zucker, 1989). We used a 150 W xenon arc lamp fitted with a shutter that had been found to photolyze 35% of a dilute solution of fully Ca^{2+}-loaded nitr-5 in 2.0 s at the same source-to-target distance as we used in our experiments. The expected average rise in free $[Ca^{2+}]$ upon exposure to light source was calculated as described in Landò and Zucker (1989). Briefly, from the cell diameter, absorbencies of cytoplasm, each species of nitr-5 (free and Ca^{2+}-bound, unphotolyzed and photolyzed), and the initial concentrations of each species in cells, we could estimate the average light intensity, rate of photolysis, and evolution of free Ca^{2+}. Considering equilibration of all buffer species we could calculate free $[Ca^{2+}]$ after a light exposure of any duration. For example, a 6 s exposure should raise Ca^{2+} to near 120 nM. A subsequent 6 s exposure should elevate $[Ca^{2+}]$ to nearly 200 nM. A cumulative exposure of 24 s might raise $[Ca^{2+}]$ to 700 nM, but as the high affinity buffer is eliminated, Ca^{2+} extrusion and sequestration mechanisms are more effective in removing Ca^{2+} from the remaining buffer, and these calculations will overestimate the level of free Ca^{2+} achieved. For this reason, we did not rely entirely on calculation to determine free $[Ca^{2+}]$, but rather measured intracellular Ca^{2+} with fura 2.

Imaging $[Ca^{2+}]_i$ was achieved by illuminating dialyzed neurons with 340 and 380 nm light, alternating at 10 Hz and filtering the emission with a 480 nm long-pass barrier filter. Light was collected with a low-light level camera (Dage MTI), through an intensified photocathode (Videoscope, KS-1380), and digitized to 8-bit resolution (Tracor Northern). Pixel images (512 × 480) were collected for 1 s (5 frames at each wavelength) and Kalman averaged. For each cell, prior to going "whole cell," background images were taken. After subtraction of the background images, final images were obtained by constructing a binary template from the brightest image and superimposing it on the ratio taken of the image, thereby removing extraneous background noise. $[Ca^{2+}]$ was calculated according to a calibration curve using a K_D of 224 nM (Grynkiewicz et al., 1985). R_{min}, R_{max}, and intermediate $[Ca^{2+}]$ were determined (with 340 and 380 nm wavelengths alternating at 10 Hz) by whole-cell dialysis of bullfrog neurons with known Ca^{2+}–EGTA/EGTA mixtures (10 mM EGTA) (Fabiato and Fabiato, 1979) included in the electrode solution. We obtained a ratio of R_{max}/R_{min} = 12.5, somewhat less than in vitro estimates (Grynkiewicz et al., 1985). This may reflect some "spillover" between the two wavelength signals, which alternate every 100 ms, about twice the settling time of our camera. Figures showing membrane currents (digitized at 1000 Hz and filtered at 300 Hz) and ratios taken of images were composed on a Sun workstation,

and a 64-color log(10) calibration bar was superimposed. Stated values of intracellular Ca^{2+} were obtained by averaging all pixels in the cell. Upon a rise in $[Ca^{2+}]_i$, fura 2 emissions (from 340 and 380 nm excitation wavelengths) change. The signal from 340 nm excitation increases, while that with 380 nm excitation decreases (Grynkiewicz et al., 1985). Measurement of $[Ca^{2+}]_i$ rises evoked by nitr-5 photolysis have the complication of accumulation of the photolyzed product of nitr-5. This nitrobenzophenone absorbs at the UV wavelengths used to excite fura 2 with a peak absorbance at 380 nm. Thus, nitr-5 photolysis alone, without Ca^{2+} release, could theoretically increase the 340/380 signal ratio, leading to an artifactual Ca^{2+} signal. There are three arguments against such a possibility. First, in most of the experiments there was an absolute increase in the signal with 340 nm excitation, not just that relative to the 380 nm signal. For example, in Figure 4A, the elevation of $[Ca^{2+}]_i$ was indicated by an 18% rise in the 340 nm signal and a 28% drop in the 380 nm signal. Second, the observed Ca^{2+} increases were close to those calculated as described in Landò and Zucker (1989). Third, in some cells illumination failed to increase Ca^{2+} as measured by fura 2 fluorescence and failed to produce physiological effects, even though the nitrobenzophenone was presumably generated. These were probably cells in which pumps were able to remove most of the Ca^{2+} injected with nitr-5. All chemicals were obtained from Sigma except HEPES (Calbiochem) and fura 2 (pentapotassium salt, Molecular Probes). Nitr-5 was a gift from Drs. R. Y. Tsien and S. R. Adams or was obtained from Calbiochem.

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Note Added in Proof

We have recently discovered that nitr-5 has fluorescence properties that can interfere with the fura 2 measurement of $[Ca^{2+}]_i$. Calibration with fura 2/nitr-5-containing solutions has shown that R_{max} was reduced by 67%, while R_{min} declined by 22%. Under these conditions, in the range of $[Ca^{2+}]_i$ found in this study, measurements of $[Ca^{2+}]_i$ will be underestimated by 10%–20%.