

Muscarinic Stimulation of $\alpha 1E$ Ca Channels Is Selectively Blocked by the Effector Antagonist Function of RGS2 and Phospholipase C- $\beta 1$

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Neuronal $\alpha 1E$ Ca channel subunits are widely expressed in mammalian brain, where they are thought to form R-type Ca channels. Recent studies have demonstrated that R-type channels contribute to neurosecretion and dendritic Ca influx, but little is known concerning their modulation. Here we show that $\alpha 1E$ channels are strongly stimulated, and only weakly inhibited, through M1 muscarinic acetylcholine receptors. Both forms of channel modulation are mediated by pertussis toxin-insensitive G-proteins. Channel stimulation is blocked by regulator of G-protein signaling 2 (RGS2) or the C-terminal region of phospholipase C- $\beta 1$ (PLC $\beta 1$ ct), which have been previously shown to function as GTPase-activating proteins for G αq . In contrast, RGS2 and PLC $\beta 1$ ct do not block inhibition of $\alpha 1E$ through M1 receptors. Inhibition is prevented, however, by the C-terminal region of β -adrenergic receptor kinase 1, which sequesters G $\beta\gamma$ dimers. Thus, stimulation of $\alpha 1E$ is mediated by a pertussis toxin-

insensitive G α subunit (e.g., G αq), whereas inhibition is mediated by G $\beta\gamma$. The ability of RGS2 and PLC $\beta 1$ ct to selectively block stimulation indicates these proteins functioned primarily as effector antagonists. In support of this interpretation, RGS2 prevented stimulation of $\alpha 1E$ with non-hydrolyzable guanosine 5'-0-(3-thiotriphosphate). We also report strong muscarinic stimulation of rbE-II, a variant $\alpha 1E$ Ca channel that is insensitive to voltage-dependent inhibition. Our results predict that G αq -coupled receptors predominantly stimulate native R-type Ca channels. Receptor-mediated enhancement of R-type Ca currents may have important consequences for neurosecretion, dendritic excitability, gene expression, or other neuronal functions.

Key words: Ca_v2.3; R-type calcium channel; $\alpha 1E$; RGS protein; phospholipase C- $\beta 1$; GAP; effector antagonist

Native R-type Ca channels have been defined by their resistance to selective antagonists of L-, N-, and P/Q-type Ca channels (Randall and Tsien, 1995). Only recently has a selective antagonist of R-type channels been reported (Newcomb et al., 1998). Antisense depletion experiments suggest that neuronal R-type Ca channels are formed by $\alpha 1E$ subunits (Piedras-Rentería and Tsien, 1998; Totene et al., 2000). $\alpha 1E$ subunits are widely expressed in mammalian brain (Niidome et al., 1992; Soong et al., 1993; Wakamori et al., 1994; Williams et al., 1994; Yokoyama et al., 1995), and several splice variants have been described (cf. Pereverzev et al., 1998). Although the physiological functions of R-type Ca channels are incompletely known, available evidence indicates that they contribute to dendritic Ca influx (Kavalali et al., 1997) and neurosecretion by some presynaptic terminals (Turner et al., 1995; Wu et al., 1998, 1999; Allen, 1999; Wang et al., 1999).

The G-protein-dependent modulation of N- and P/Q-type Ca channels has been extensively studied (for review, see Hille, 1994; Jones and Elmslie, 1997; Zamponi and Snutch, 1998; Ikeda and Dunlap, 1999; Bean, 2000). In contrast, much less is known concerning the modulation of R-type Ca channels. Previously, we reported that R-type channels formed by rabbit $\alpha 1E$ subunits are both inhibited and stimulated through M2 muscarinic acetylcholine receptors (Meza et al., 1999). Our experiments demonstrated that inhibition and stimulation of $\alpha 1E$ are separate processes that occur through distinct signaling pathways, both of which couple to M2 receptors. Inhibition occurs through a fast, pertussis toxin (PTX)-sensitive pathway, whereas stimulation occurs through a slower,

PTX-insensitive pathway (Meza et al., 1999). It is intriguing that R-type channels can be stimulated through muscarinic receptors, because the closely-related N- and P/Q-type channels are typically inhibited through G-protein-coupled receptors. Receptor-mediated enhancement of R-type Ca currents may have important consequences for dendritic Ca signaling, neurosecretion, or other neuronal functions.

In the present study, we have further examined the muscarinic stimulation of recombinant R-type Ca channels. $\alpha 1E$ subunits were expressed in HEK293 cells with M1 muscarinic acetylcholine receptors, which preferentially couple to heterotrimeric G-proteins of the G αq subfamily (Felder, 1995). We find that M1 receptors predominantly stimulate, rather than inhibit, $\alpha 1E$ channels. Additionally, we find that stimulation of $\alpha 1E$ is selectively blocked by regulator of G-protein signaling 2 (RGS2) and the C-terminal region of phospholipase C- $\beta 1$ (PLC $\beta 1$ ct), two proteins known to function as GTPase-activating proteins (GAPs) for G αq . Interestingly, the effects of RGS2 and PLC $\beta 1$ ct in our experiments can only be explained if these proteins functioned primarily as effector antagonists. Altogether, our data suggest that $\alpha 1E$ is stimulated through a G αq -coupled signaling pathway. These observations predict that G αq -coupled receptors stimulate native R-type Ca channels *in vivo*. Our results also provide new insight into mechanisms by which RGS proteins can influence the receptor-mediated modulation of voltage-gated Ca channels.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney (HEK293) cells were obtained from the American Type Culture Collection (Manassas, VA) and propagated in culture medium containing 90% DMEM, 10% fetal bovine serum, and 50 μ g/ml gentamycin. The cells were trypsinized weekly and replated onto 60 mm culture dishes at 20% confluence. CaPO₄ precipitation was used to transfect these cells within 3–5 d of plating. The transfection mixture contained expression plasmids encoding $\alpha 1E$, $\alpha 2$ - δ , and $\beta 3$ Ca channel subunits at 1.25 μ g of each cDNA per dish, plus an expression plasmid encoding the M1 muscarinic acetylcholine receptor at 0.25 μ g of cDNA per dish. In selected experiments, the transfection

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mixture also included expression plasmids encoding RGS2, RGS8, or the C-terminal region (Thr⁹⁰³-Leu¹²¹⁶) of PLC $\beta 1$ at 0.625 μ g of cDNA per dish. RGS2, RGS8, and PLC $\beta 1$ ct were expressed as fusions to the C terminus of enhanced green fluorescence protein (EGFP). In specific experiments, an expression plasmid encoding Gly⁴⁹⁵-Leu⁶⁸⁹ of β -adrenergic receptor kinase 1 (BARK1; denoted β ARK1ct) was transfected at 1.25 μ g of cDNA per dish. For the experiments illustrated in Figure 7, cells were transfected at 1.25 μ g/dish with an expression plasmid encoding a variant $\alpha 1E$ subunit (denoted rE-II) cloned from rat hippocampus by Soong et al. (1993). These cells were cotransfected with the M1 receptor as described above or alternatively with a plasmid encoding the M2 muscarinic acetylcholine receptor at 0.0625 μ g/dish. For all transfections that did not include EGFP fusion proteins, a separate plasmid encoding EGFP was included at 0.125 μ g/dish. The day after transfection, cells were briefly trypsinized and replated onto 12 mm round glass coverslips. Electrophysiological experiments were performed 16–24 hr later. Successfully transfected cells were visually identified by their green fluorescence under ultraviolet illumination; only green cells were used for experiments.

Expression plasmids. cDNA encoding rabbit $\alpha 1E$ (GenBank accession number X67856) was in pcDNA3.1+ (Invitrogen, San Diego, CA). rE-II (accession number L15453) and rat $\alpha 2\text{-}\delta$ (M86621) were in pMT2 (Genetics Institute, Cambridge, MA). Rabbit $\beta 3$ (X64300) was in pcDNA3 (Invitrogen). Human M1 muscarinic acetylcholine receptor (X52068) was in pCD. Human M2 muscarinic receptor (X15264) was in pRK5 (Genentech, South San Francisco, CA). Jellyfish enhanced green fluorescent protein (U55763) was in pEGFP (Clontech, Cambridge, UK). Human RGS2 (L13463) and rat RGS8 (AB006013) were in pCI (Promega, Madison, WI). EGFP-RGS2 and EGFP-RGS8 were in pEGFP-C2 and pEGFP-C1 (Clontech), respectively. A deletion mutant of RGS8 (denoted Δ RGS8) was in pEGFP-C1; this construct encodes EGFP fused to an RGS8 protein lacking Phe⁵⁷-Pro¹⁶⁰. cDNA encoding Thr⁹⁰³-Leu¹²¹⁶ of rat PLC $\beta 1$ (M20636) was in pEGFP-C1 (Clontech). cDNA encoding Gly⁴⁹⁵-Leu⁶⁸⁹ of β ARK1 (M34019) was in pRK5 (Koch et al., 1994).

Patch-clamp recordings. Large-bore patch pipettes were pulled from 100 μ l borosilicate glass micropipettes (VWR 53432-921) and filled with a solution containing (in mM): 155 CsCl, 10 Cs₂-EGTA, 4 Mg-ATP, 0.32 Li-GTP, and 10 HEPES, pH 7.4, with CsOH. For the experiments illustrated in Figure 6, equimolar guanosine 5'-0-(3-thiotriphosphate) (GTP- γ S) was substituted for GTP in the pipette solution. Aliquots of the pipette solution were stored at -80°C , kept on ice after thawing, and filtered at 0.22 μ m immediately before use. Pipette tips were coated with paraffin to reduce capacitance and then fire-polished. Filled patch pipettes had DC resistances of 1.0–1.5 M Ω . The bath solution contained (in mM): 145 NaCl, 40 CaCl₂, 2 KCl, and 10 HEPES, pH 7.4, with NaOH. Ca currents were recorded in the whole-cell configuration. After forming a gigaohm seal in the cell-attached configuration, residual pipette capacitance was compensated using the negative capacitance circuit of the amplifier. The DC resistance of the whole-cell configuration was routinely >1 G Ω . The steady holding potential was -90 mV. No corrections were made for liquid junction potentials. Depolarizations to potentials near the peak of the current-voltage relationship ($+30$ mV) were delivered every 1–10 sec; the stimulation rate was adjusted for each cell to maximize sampling resolution and to minimize cumulative inactivation. Currents were filtered at 2–10 kHz using the built-in Bessel filter (four-pole low-pass) of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and sampled at 10–50 kHz using a Digidata 1200 analog-to-digital board installed in a Gateway Pentium computer. The pCLAMP 7.0 software programs Clampex and Clampfit were used for data acquisition and analysis, respectively. Figures were made using the software program Origin (version 6.0).

Linear cell capacitance (C) was determined by integrating the area under the whole-cell capacity transient, which was evoked by a voltage-clamp step from -90 to -80 mV; the whole-cell capacitance compensation circuit of the amplifier was turned off during this measurement. The average value of C was 18 ± 1 pF (mean \pm SEM; $n = 160$ cells). To minimize voltage errors, the time constant for decay of the whole-cell capacity transient (t) was reduced as much as possible using the analog series resistance compensation circuit of the amplifier. Series resistance (R_s) was calculated as $t \times (1/C)$, where t was the time constant for decay of the whole-cell capacity transient. The average values of t and R_s , measured before electronic compensation, were 54 ± 2 μ sec and 3.2 ± 0.1 M Ω , respectively ($n = 160$). Maximal Ca current amplitude was 1790 ± 140 pA ($n = 160$; test potential, $+30$ mV). After electronic compensation of t and R_s , the average maximum voltage error was 3.9 ± 0.3 mV ($n = 160$).

All currents were corrected for linear capacitance, and leakage currents using $-P/6$ or $-P/4$ subtraction. Ca current amplitudes were measured at the time of peak inward current. Comparisons were by ANOVA or by unpaired, two-tailed t tests, with $p < 0.05$ considered significant. Application of carbachol (CCh) was by bath exchange or local superfusion through macropipette positioning close to the cell under study. CCh was dissolved directly in the bath solution. Temperature ($20\text{--}24^{\circ}\text{C}$) was continuously monitored using a miniature thermocouple placed in the recording chamber.

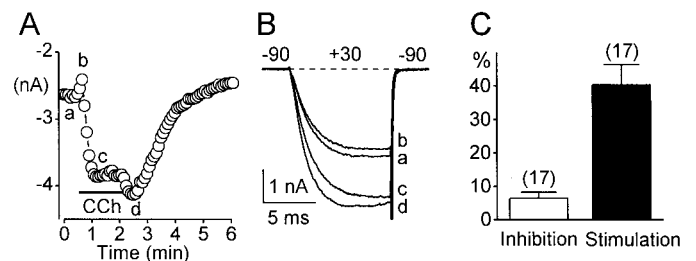


Figure 1. $\alpha 1E$ Ca channels are predominantly stimulated through M1 muscarinic acetylcholine receptors. *A*, Whole-cell Ca currents were evoked every 5 sec by step depolarizations from -90 to $+30$ mV. Ca current amplitudes are plotted as a function of time during a representative experiment. Application of CCh (1 mM) is indicated by a horizontal bar. Linear cell capacitance (C) = 13 pF; series resistance (R_s) = 2.1 M Ω . *B*, Selected Ca currents recorded at the times indicated in *A*. *C*, Summary data for inhibition and stimulation of $\alpha 1E$ Ca currents through coexpressed M1 receptors. For each cell, inhibition was measured as the difference between the amplitudes of currents *a* and *b*, normalized with respect to the amplitude of current *a*. Stimulation was measured as the difference between the amplitudes of currents *b* and *c*, normalized with respect to the amplitude of current *a*. Error bars represent \pm SEM.

RESULTS

$\alpha 1E$ Ca channels are predominantly stimulated through M1 muscarinic acetylcholine receptors

Figure 1 illustrates Ca currents recorded from an HEK293 cell coexpressing rabbit $\alpha 1E$ subunits and human M1 muscarinic acetylcholine receptors. As seen in the plot of current amplitudes versus time (Fig. 1*A*), application of CCh initially produced a small, rapid decrease (inhibition) of $\alpha 1E$ current amplitude (point *b*). This initial inhibition was soon followed (point *c*) by a substantial increase in the current amplitude (stimulation). After CCh washout, current amplitude again transiently increased (point *d*) by an amount comparable with that of the initial inhibition. This secondary increase at washout apparently corresponds to relief of the initial inhibition (see below). The observed modulation of $\alpha 1E$ Ca channels was attributable to coexpressed M1 receptors, because it was absent from cells not cotransfected with muscarinic receptors and was completely blocked by atropine (Meza et al., 1999).

$\alpha 1E$ current amplitudes were inhibited by only $6 \pm 2\%$ ($n = 17$) through M1 receptors. In contrast, the magnitude of stimulation was substantially larger ($40 \pm 6\%$; $n = 17$). Thus, M1 receptors predominantly stimulate $\alpha 1E$ Ca channels (Fig. 1*C*). Previously, we found that $\alpha 1E$ currents were inhibited by $\sim 40\%$ and stimulated by $\sim 20\%$ through the M2 subtype of muscarinic acetylcholine receptor (Meza et al., 1999). This comparison indicates that M1 receptors produce smaller inhibition and larger stimulation of $\alpha 1E$ Ca channels than M2 receptors.

We next examined the dose dependency for stimulation of $\alpha 1E$ Ca channels through M1 receptors (Fig. 2). Application of 10 μ M CCh produced approximately half-maximal stimulation, and 100 μ M CCh generated maximal stimulation. The concentration of CCh used throughout the remainder of this study (1 mM) was therefore clearly saturating. These dose-response data are in general agreement with previously reported agonist binding affinities of cloned M1 receptors (Peralta et al., 1987).

In our previous study (Meza et al., 1999), we found that the M2 receptor-mediated stimulation of $\alpha 1E$ was prevented by staurosporine, a broad-spectrum inhibitor of serine-threonine kinases. As shown in Figure 2, staurosporine also prevented stimulation of $\alpha 1E$ through M1 receptors, suggesting that stimulation results from a pathway that couples to both receptor subtypes. Because M1 receptors produce larger stimulation than M2 receptors, the responsible signaling pathway apparently couples more efficiently to M1 receptors. The effect of staurosporine is consistent with a previous report that $\alpha 1E$ Ca channels are stimulated through a protein kinase C-dependent pathway in *Xenopus* oocytes (Stea et al., 1995).

As shown in Figure 2, the average magnitude of inhibition was

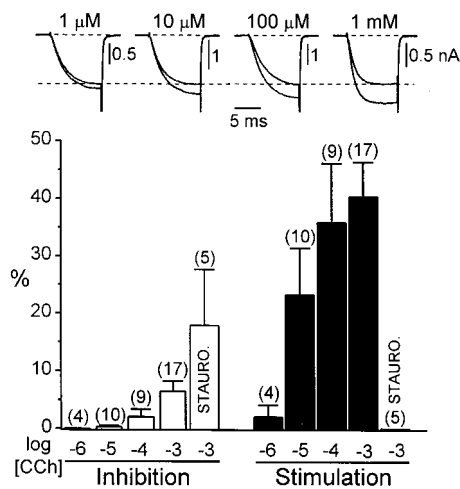


Figure 2. Dose–response data for modulation of $\alpha 1E$ Ca channels through M1 receptors. *Top*, Representative Ca currents recorded before the application of CCh and during maximal stimulation with various concentrations of CCh. The control currents are aligned with the horizontal dotted line. *Bottom*, Average inhibition and stimulation of $\alpha 1E$ currents by various CCh concentrations. Inhibition and stimulation are expressed relative to the control Ca current in each cell, recorded immediately before CCh application. Each cell was exposed once to a single concentration of CCh. The voltage protocol is as in Figure 1. In selected experiments, cells were exposed to 100 nM staurosporine before, during, and after the application of CCh. Staurosporine (STAURO.) was dissolved in DMSO to make a stock solution of 1 mM. The final concentration of DMSO in the bath was 0.01%, which alone had no effects on $\alpha 1E$ currents.

somewhat larger in staurosporine-treated cells ($18 \pm 10\%$) than in control cells ($6 \pm 2\%$), although this difference was not statistically significant ($p > 0.05$). Our impression is that stimulation causes a slight underestimation in the measurement of inhibition; however, this underestimation does not affect the conclusions of this study (see below).

Stimulation of $\alpha 1E$ is mediated by a PTX-insensitive $G\alpha$ subunit

PTX catalyzes the ADP ribosylation of $G\alpha$ subfamily proteins at a cysteine residue near the C terminus, thereby decoupling these $G\alpha$ subunits from receptors (West et al., 1985; Avigan et al., 1992). We used PTX to investigate which G-proteins are responsible for modulation of $\alpha 1E$ through M1 receptors. Figure 3B shows currents recorded from a cell exposed to PTX (500 ng/ml) overnight, and Figure 3A illustrates currents recorded from an untreated control cell. It was clear that PTX had no appreciable effects on inhibition or stimulation of $\alpha 1E$. Averaged results are presented in Figure 5. These data suggest that, in the case of M1 receptors, inhibition and stimulation of $\alpha 1E$ are both mediated by PTX-insensitive G-proteins.

To determine which subunits ($G\alpha$ or $G\beta\gamma$) of the PTX-insensitive G-protein are responsible for modulation of $\alpha 1E$, we expressed $\beta ARK1ct$. This region (Gly⁴⁹⁵-Leu⁶⁸⁹) of $\beta ARK1$ sequesters $G\beta\gamma$ dimers (Koch et al., 1994), including $G\beta\gamma$ released through activation of $G\alpha q$ -coupled M3 receptors (Stehno-Bittel et al., 1995). As shown in Figure 3C, coexpression of $\beta ARK1ct$ blocked the initial inhibition of $\alpha 1E$ as well as the secondary increase in current amplitude after CCh washout. Overall, $\alpha 1E$ currents were inhibited by only $0.7 \pm 0.7\%$ ($n = 8$) in $\beta ARK1ct$ -expressing cells, compared with $6.4 \pm 1.8\%$ ($n = 17$) inhibition in control cells ($p < 0.05$; see Fig. 5). These results with $\beta ARK1ct$ suggest that inhibition of $\alpha 1E$ is mediated by $G\beta\gamma$ dimers released as a consequence of M1 receptor activation.

In contrast, stimulation of $\alpha 1E$ was unaffected by $\beta ARK1ct$ (see Fig. 5). Thus, $\alpha 1E$ currents were stimulated by $40 \pm 6\%$ in control cells ($n = 17$) and by $43 \pm 7\%$ in $\beta ARK1ct$ -expressing cells ($n = 7$). Altogether, these data indicate that stimulation involves signaling by a PTX-insensitive $G\alpha$ subunit.

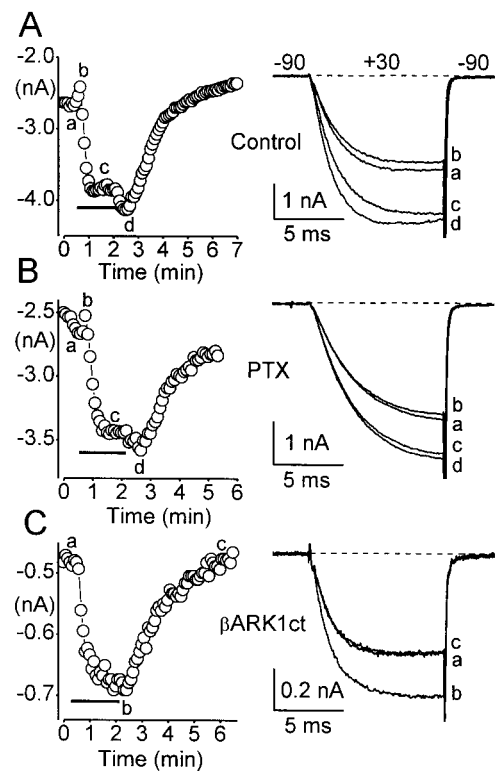


Figure 3. Stimulation of $\alpha 1E$ is mediated by a PTX-insensitive $G\alpha$ subunit. *Left*, $\alpha 1E$ current amplitudes are plotted as a function of time during representative experiments. *Right*, Whole-cell Ca currents recorded at times indicated in the corresponding plots. *A*, Modulation of $\alpha 1E$ currents by M1 receptors in a control cell (same cell as in Fig. 1A). $C = 13$ pF; $R_s = 2.1$ M Ω . *B*, PTX does not affect inhibition or stimulation of $\alpha 1E$ through M1 receptors. Cells were preincubated with PTX; 200–500 ng/ml for at least 20 hr. $C = 9$ pF; $R_s = 3.6$ M Ω . *C*, Coexpression of $\beta ARK1ct$ selectively blocks inhibition of $\alpha 1E$ through M1 receptors. $C = 11$ pF; $R_s = 3.2$ M Ω . Other details are as in Figure 1.

PLC $\beta 1ct$, RGS2, and RGS8 selectively block stimulation of $\alpha 1E$

M1 receptors preferentially couple to G-proteins of the $G\alpha q$ subfamily (Felder, 1995), suggesting that $G\alpha q$ mediates stimulation of $\alpha 1E$. To test this hypothesis, we took advantage of recent work showing that PLC $\beta 1$ and RGS2 can modify interactions of $G\alpha q$ with its downstream effectors. PLC $\beta 1$ is the principal effector enzyme of $G\alpha q$; interestingly, this phospholipase also functions as a powerful GAP for $G\alpha q$ (Berstein et al., 1992; Biddlecome et al., 1996). For our purposes, we chose to express only the C terminus of PLC $\beta 1$ (Thr⁹⁰³-Leu¹²¹⁶; denoted PLC $\beta 1ct$), because this portion of the protein contains intrinsic GAP activity (Paulssen et al., 1996), but it completely lacks phospholipase activity (Wu et al., 1993).

As illustrated in Figure 4B, coexpression of PLC $\beta 1ct$ greatly reduced stimulation of $\alpha 1E$. Thus, stimulation was only $\sim 4\%$ in cells expressing PLC $\beta 1ct$, compared with $\sim 40\%$ stimulation in control cells (Fig. 5). Surprisingly, PLC $\beta 1ct$ did not also reduce inhibition of $\alpha 1E$ through M1 receptors. Overall, $\alpha 1E$ currents were inhibited by $10 \pm 2\%$ ($n = 10$) in PLC $\beta 1ct$ -expressing cells, compared with $6 \pm 2\%$ ($n = 17$) inhibition in control cells ($p > 0.1$). The lack of effect on inhibition was unexpected, given the previously demonstrated ability of PLC $\beta 1$ to act as a powerful GAP for $G\alpha q$ (Berstein et al., 1992).

To further test the hypothesis that $G\alpha q$ mediates stimulation of $\alpha 1E$, we used RGS2, because previous studies have found that RGS2 preferentially interacts with $G\alpha q$ *in vitro* (Chen et al., 1997; Heximer et al., 1997; but see Ingi et al., 1998). Stimulation of $\alpha 1E$ was also significantly reduced by coexpression of RGS2 (Fig. 4C). Altogether, stimulation was $7 \pm 4\%$ in RGS2-expressing cells ($n =$

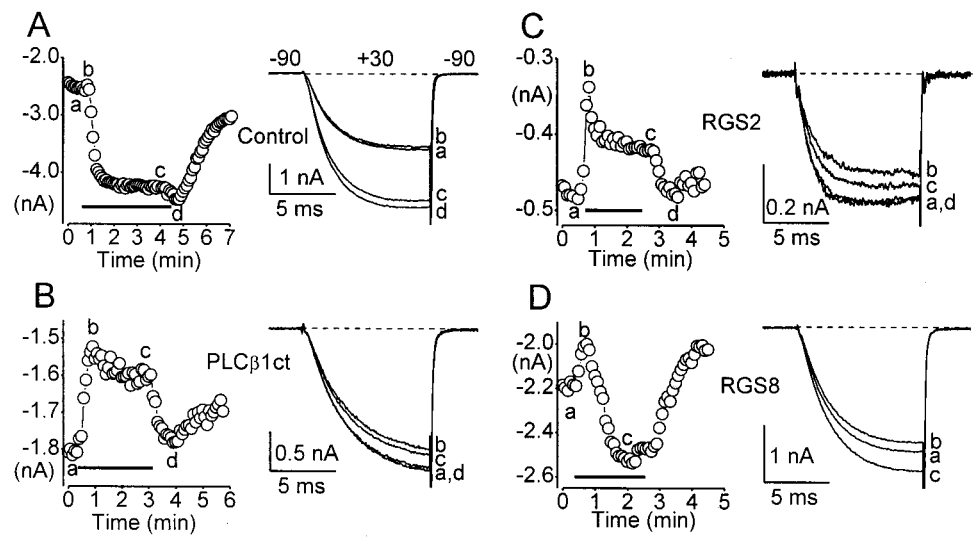


Figure 4. Stimulation of $\alpha 1E$ is selectively blocked by PLC $\beta 1ct$, RGS2, and RGS8. *A*, Modulation of $\alpha 1E$ by M1 receptors in a control cell. $C = 16$ pF; $R_S = 2.8$ M Ω . *B*, Modulation of $\alpha 1E$ by M1 receptors in a cell coexpressing PLC $\beta 1ct$. $C = 24$ pF; $R_S = 1.3$ M Ω . *C*, Modulation of $\alpha 1E$ by M1 receptors in a cell coexpressing RGS2. $C = 11$ pF; $R_S = 5.1$ M Ω . *D*, Modulation of $\alpha 1E$ by M1 receptors in a cell coexpressing RGS8. $C = 13$ pF; $R_S = 2.9$ M Ω . Other details are as in Figure 1.

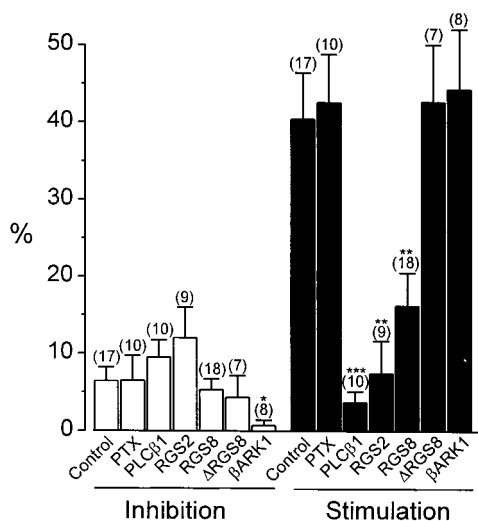


Figure 5. Modulation of $\alpha 1E$ Ca currents through M1 receptors. Inhibition and stimulation are expressed relative to the control Ca current in each cell, recorded immediately before CCh application. Each cell was exposed once to a single concentration of CCh. Means were compared using one-way ANOVA or by an unpaired, two-tailed *t* test. Asterisks indicate significant differences from the control mean (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The average Ca current densities (measured in response to a test pulse to +30 mV) in each group were 106 ± 17 pA/pF ($n = 17$) in control, 104 ± 23 pA/pF ($n = 10$) in PTX-treated, 80 ± 21 pA/pF ($n = 10$) in PLC $\beta 1ct$ -expressing, 196 ± 88 pA/pF ($n = 9$) in RGS2-expressing, 68 ± 10 pA/pF ($n = 8$) in $\beta ARK1ct$ -expressing, 124 ± 19 pA/pF ($n = 18$) in RGS8-expressing, and 149 ± 38 pA/pF ($n = 7$) in $\Delta RGS8$ -expressing cells.

9), compared with $40 \pm 6\%$ ($n = 17$) stimulation in control cells ($p < 0.01$). In contrast, inhibition of $\alpha 1E$ was unaffected by RGS2 (Fig. 5). Thus, the effects of RGS2 were basically identical to those of PLC $\beta 1ct$; that is, both proteins strongly reduced stimulation of $\alpha 1E$ without also reducing inhibition. Because PLC $\beta 1ct$ and RGS2 have been previously demonstrated to interact with G αq , these findings support the hypothesis that stimulation is mediated through a G αq -coupled signaling pathway.

For comparison with PLC $\beta 1ct$ and RGS2, we tested RGS8, which has been previously shown to act as a GAP for G αi subfamily proteins (Saitoh et al., 1997; Melliti et al., 1999). Coexpression of RGS8 significantly reduced stimulation of $\alpha 1E$ (Fig. 4D) but to a lesser extent than either PLC $\beta 1ct$ or RGS2 (Fig. 5). Inhibition of $\alpha 1E$ was unaffected by RGS8 (Fig. 5). The ability of RGS8 to reduce stimulation suggests that it can interact with G αq , although less effectively than either PLC $\beta 1ct$ or RGS2.

As a control for expression of PLC $\beta 1ct$, RGS2, and RGS8, we

used the deletion mutant $\Delta RGS8$ (Melliti et al., 1999). This mutant lacks amino acids Phe⁵⁷-Pro¹⁶⁰, which constitute the major portion of the conserved RGS core domain (Berman and Gilman, 1998). The RGS core domain mediates binding of RGS proteins to the switch regions of G α (Tesmer et al., 1997). We have previously shown that $\Delta RGS8$ does not act as a GAP to attenuate N-type Ca channel inhibition by G αi subfamily proteins (Melliti et al., 1999). As summarized in Figure 5, coexpression of $\Delta RGS8$ had no effect on the modulation of $\alpha 1E$ through M1 receptors. Thus, the RGS core domain is apparently necessary for the observed effects of RGS2 and RGS8 in our experiments.

In summary, only stimulation of $\alpha 1E$ was attenuated by PLC $\beta 1ct$, RGS2, and RGS8. Notably, these proteins failed to reduce inhibition of $\alpha 1E$ through M1 receptors (Fig. 5). The selective block of stimulation by PLC $\beta 1ct$, RGS2, and RGS8 cannot be explained by the GAP activity of these proteins, because simply accelerating GTP hydrolysis should have attenuated signaling by both G α and G $\beta \gamma$ and should have reduced both stimulation and inhibition of $\alpha 1E$. However, the selective block of stimulation is consistent with the interpretation that PLC $\beta 1ct$, RGS2, and RGS8 functioned primarily as effector antagonists in our experiments.

RGS2 prevents GTP- γ -S-mediated stimulation of $\alpha 1E$

Previous studies have found that RGS2 and RGS4 reduce signaling by G αq activated with GTP- γ -S (Hepler et al., 1997; Heximer et al., 1997). GTP- γ -S is nonhydrolyzable; thus, these effects of RGS2 and RGS4 cannot be attributed to their GAP activities. Rather, it is thought that RGS2 and RGS4 can function as effector antagonists by binding to the switch regions of G αq , thereby blocking its interactions with downstream effectors. To further examine whether PLC $\beta 1ct$, RGS2, and RGS8 could have functioned as effector antagonists in our experiments, we used GTP- γ -S to produce stimulation of $\alpha 1E$. These experiments were performed on cells coexpressing $\alpha 1E$ Ca channels and M1 receptors and incubated with PTX (200–500 ng/ml) overnight to inactivate G $\alpha i/o$ proteins. Equimolar GTP- γ -S was substituted for GTP in the pipette solution, and $\alpha 1E$ current amplitudes were monitored as a function of time after establishing the whole-cell configuration (i.e., break-in). As shown in Figure 6, intracellular dialysis with GTP- γ -S significantly increased $\alpha 1E$ currents in control cells. The magnitude of this increase was comparable (~40%) with that produced by coexpressed M1 receptors (compare Fig. 1). By contrast, GTP- γ -S failed to stimulate $\alpha 1E$ currents in cells coexpressing RGS2 (Fig. 6). Apparently, GTP- γ -S activated the stimulatory pathway in control cells but not in cells coexpressing RGS2. Because GTP- γ -S is nonhydrolyzable, RGS2 cannot have blocked the stimulatory pathway by functioning as a GAP; it must have functioned as an effector antagonist. These results support our hypoth-

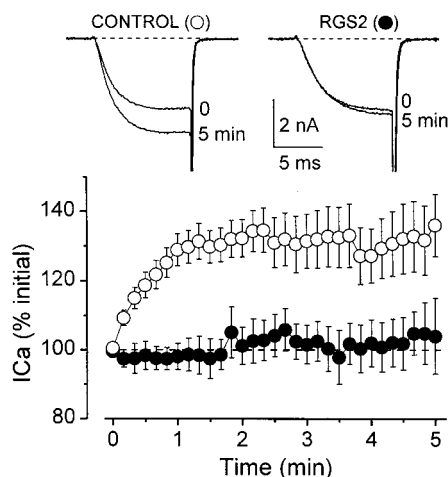


Figure 6. RGS2 prevents stimulation of $\alpha 1E$ by intracellular GTP- γ -S. Whole-cell Ca currents were recorded from HEK293 cells expressing rabbit $\alpha 1E$ Ca channels and human M1 receptors; some cells also expressed RGS2. The pipette solution contained 0.32 mM GTP- γ -S in place of GTP; aliquots of this solution were thawed from -80°C , kept on ice during experiments, and discarded within 3 hr of thawing. Recordings from control and RGS2-expressing cells were alternated. The cells were incubated with PTX (200–500 ng/ml) overnight before experiments. Immediately after establishment of the whole-cell configuration (break-in), Ca currents were evoked by 10 msec depolarizations to +30 mV, delivered every 10 sec from a steady holding potential of -90 mV. *Top*, Representative whole-cell Ca currents recorded at break-in and 5 min later from a control (*left*) and an RGS2-expressing (*right*) cell. $R_s = 2.5$ M Ω (control) and 2.4 M Ω (RGS2). *Bottom*, Average \pm SEM Ca current amplitudes at various times after break-in. Current amplitudes are expressed relative to the initial Ca current amplitude recorded in each cell at break-in. For times between 0 and 2 min, *symbols* represent data from 15 control and 10 RGS2-expressing cells; beyond 2 min, *symbols* represent data from 8 control and 7 RGS2-coexpressing cells. Initial Ca current densities were 99 ± 17 pA/pF (control) and 124 ± 22 pA/pF (RGS2).

esis that PLC $\beta 1$ ct, RGS2, and RGS8 selectively blocked stimulation of $\alpha 1E$ by acting as effector antagonists.

rbE-II is stimulated through muscarinic receptors

Previous studies have demonstrated that rbE-II, an $\alpha 1E$ subunit cloned from rat hippocampus (Soong et al., 1993), is insensitive to inhibition through μ -opioid and dopamine receptors (Bourinet et al., 1996; Page et al., 1998). It was recently shown by Page et al. (1998) that an N-terminal domain within $\alpha 1B$ and $\alpha 1E$ subunits is essential for their voltage-dependent inhibition by G $\beta\gamma$ dimers. rbE-II lacks this domain, which accounts for its insensitivity to voltage-dependent inhibition (Page et al., 1998).

To determine whether rbE-II can undergo muscarinic stimulation, we coexpressed it with M1 or M2 receptors. In agreement with Bourinet et al. (1996) and Page et al. (1998), we found that rbE-II displayed no appreciable voltage-dependent inhibition through either muscarinic receptor. However, rbE-II was prominently stimulated through both receptors. In response to M2 receptors, rbE-II exhibited stimulation that simply reversed after CCh washout (Fig. 7A). However, with M1 receptors rbE-II currents often exhibited a secondary increase after CCh washout (Fig. 7B). The mechanism of this secondary increase is presently unclear; further work is being done.

Results obtained with rbE-II Ca channels are summarized in Figure 7C. Altogether, rbE-II currents were stimulated by $35 \pm 11\%$ ($n = 10$) through M2 receptors and by $55 \pm 8\%$ ($n = 13$) through M1 receptors (only the primary phase of stimulation is included in the latter measurement). The larger stimulation of rbE-II through the M1 receptor is consistent with the hypothesis that stimulation is mediated by G α_q , because M1 receptors couple preferentially to this G α subunit (Felder, 1995). Stimulation of rbE-II was statistically indistinguishable ($p > 0.1$) from stimulation of the rabbit $\alpha 1E$ Ca channel [$19 \pm 3\%$ through M2 receptors ($n = 11$) and $40 \pm 6\%$ through M1 receptors ($n = 17$)]. In summary,

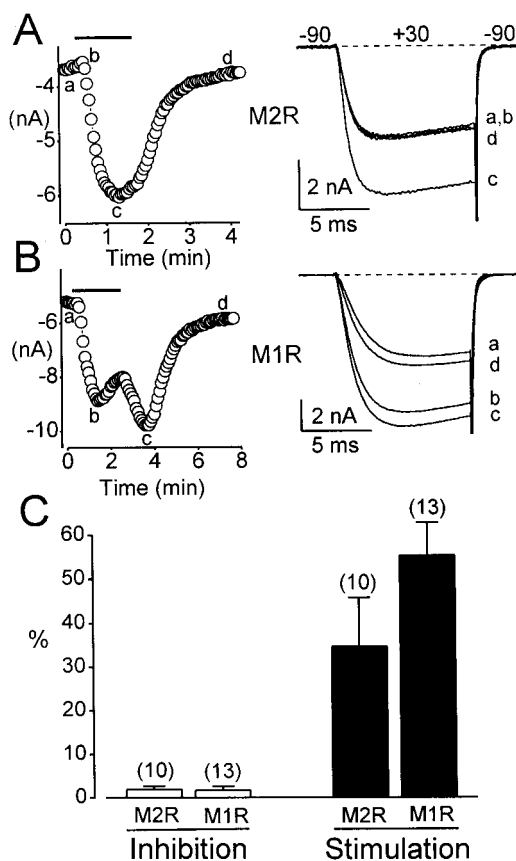


Figure 7. rbE-II Ca channels are strongly stimulated through muscarinic receptors. *Left*, rbE-II current amplitudes are plotted as a function of time during representative experiments. *Right*, Whole-cell Ca currents recorded at times indicated in the corresponding plot. *A*, Stimulation of rbE-II current through M2 receptors (M2R). Ca currents were evoked every 3 sec by step depolarizations from -90 to +30 mV. $C = 50$ pF; $R_s = 1.1$ M Ω . The application of CCh (50 μM) is indicated by a horizontal bar; this CCh concentration produces maximal activation of M2 receptors (Melliti et al., 1999; Meza et al., 1999). *B*, Stimulation of rbE-II current through M1 receptors (M1R). Ca currents were evoked every 5 sec by step depolarizations from -90 to +30 mV. $C = 28$ pF; $R_s = 1.5$ M Ω . The concentration of applied CCh was 1 mM. *C*, Average stimulation of rbE-II currents through M1 and M2 receptors.

these experiments demonstrate that rbE-II Ca channels are significantly stimulated through M1 and M2 muscarinic receptors. Because rbE-II lacks the N-terminal domain essential for voltage-dependent, G $\beta\gamma$ -mediated inhibition (Page et al., 1998), this domain apparently is not involved in the muscarinic stimulation of $\alpha 1E$ Ca channels.

DISCUSSION

We have shown that $\alpha 1E$ Ca channels are strongly stimulated and only weakly inhibited through M1 muscarinic acetylcholine receptors. Both forms of channel modulation are insensitive to PTX. Stimulation of $\alpha 1E$ is blocked by PLC $\beta 1$ ct and RGS2, two proteins previously demonstrated to function as GAPs for G α_q (Berstein et al., 1992; Paulssen et al., 1996; Heximer et al., 1997). In contrast, stimulation is unaffected by β ARK1ct, which sequesters G $\beta\gamma$ subunits and which blocks inhibition of $\alpha 1E$ through M1 receptors (Figs. 4C, 5). Together these results indicate that stimulation of $\alpha 1E$ involves signaling by a PTX-insensitive G α subunit. Because M1 receptors preferentially couple to heterotrimeric G-proteins of the G α_q subfamily (Felder, 1995), we hypothesize that stimulation is mediated through G α_q . Our results predict that native R-type Ca channels are predominantly stimulated through endogenous muscarinic and possibly other G α_q -coupled receptors. This possibility is supported by the strong muscarinic stimulation of rbE-II Ca channels (Fig. 7).

The finding that $\beta ARK1ct$ blocks inhibition of $\alpha 1E$ suggests that inhibition is mediated by $G\beta\gamma$ dimers (Figs. 3C, 5). Notably, M1 receptors produce relatively weak inhibition of $\alpha 1E$ (compare Figs. 1, 5). Thus, even in staurosporine-treated cells where stimulation was prevented and inhibition was consequently not underestimated, $\alpha 1E$ currents were inhibited by only $\sim 18\%$ (Fig. 2). In comparison, the M2 subtype of muscarinic receptor produces $\sim 40\%$ inhibition of $\alpha 1E$ Ca currents under identical experimental conditions (Meza et al., 1999). The weak inhibition of $\alpha 1E$ through M1 receptors may reflect the type of $G\beta\gamma$ dimer involved. Previously, Fletcher et al. (1998) found that $G\beta 5$ preferentially associates with $G\alpha q$, suggesting that $G\alpha q$ -coupled receptors such as the M1 receptor will liberate $G\beta\gamma$ dimers containing $G\beta 5$ (assuming that $G\beta 5$ is present in HEK293 cells). Because $G\beta 5$ produces relatively weak voltage-dependent inhibition of native N-type Ca channels (García et al., 1998; Ruiz-Velasco and Ikeda, 2000), it may also produce relatively weak inhibition of R-type Ca channels formed by $\alpha 1E$.

PLC $\beta 1ct$, RGS2, and RGS8 functioned primarily as effector antagonists

PLC $\beta 1ct$, RGS2, and RGS8 blocked stimulation of $\alpha 1E$ without also reducing its inhibition through M1 receptors (Fig. 5). The selective block of stimulation is surprising, given that PLC $\beta 1$ and RGS2 have been previously shown to act as powerful GAPs for $G\alpha q$ *in vitro* (Berstein et al., 1992; Biddlecome et al., 1996; Ingi et al., 1998). If PLC $\beta 1ct$ and RGS2 had behaved mainly as GAPs, they would have accelerated conversion of $G\alpha$ -GTP into $G\alpha$ -GDP, thereby promoting reassociation of $G\alpha$ with its $G\beta\gamma$ dimer (Berman and Gilman, 1998). In this event, both inhibition and stimulation of $\alpha 1E$ would have been attenuated. That only stimulation was reduced argues that PLC $\beta 1ct$, RGS2, and RGS8 functioned primarily as effector antagonists in our experiments. This interpretation is supported by results obtained using GTP- γ -S. We found that GTP- γ -S produced stimulation of $\alpha 1E$ in control cells but not in cells coexpressing RGS2 (Fig. 6). Because GTP- γ -S cannot be hydrolyzed by $G\alpha$ subunits, RGS2 must have functioned exclusively as an effector antagonist to prevent stimulation of $\alpha 1E$ in these experiments (Fig. 6). However, these experiments with GTP- γ -S do not preclude the possibility that RGS2, RGS8, and PLC $\beta 1ct$ also functioned as GAPs, in addition to functioning as effector antagonists, in experiments in which M1 receptors and hydrolyzable GTP produced stimulation of $\alpha 1E$ (e.g., Fig. 4).

Previous studies have demonstrated that RGS2 and RGS4 can act as effector antagonists under certain conditions (Hepler et al., 1997; Heximer et al., 1997; Yan et al., 1997). In contrast, effector antagonism by PLC $\beta 1$ or RGS8 has not been previously reported. However, Kammermeier and Ikeda (1999) found that PLC $\beta 1ct$ blocked the voltage-independent, $G\alpha q$ -mediated component of N-type Ca channel inhibition in superior cervical ganglion neurons but left the voltage-dependent, $G\beta\gamma$ -mediated component of inhibition intact. Their experiments suggest that PLC $\beta 1ct$ interfered with signaling by $G\alpha q$ but not with signaling by its $G\beta\gamma$ dimer. These results of Kammermeier and Ikeda (1999) are also consistent with the idea that PLC $\beta 1ct$ can function as an effector antagonist for $G\alpha q$.

Physical interactions between $G\alpha$ subunits and RGS proteins take place at the switch regions of $G\alpha$, which are also involved in binding $G\beta\gamma$ dimers and downstream effectors such as PLC $\beta 1$ (Wall et al., 1995; Lambright et al., 1996; Tesmer et al., 1997; Berman and Gilman, 1998). It is therefore unlikely that $G\alpha$ can associate with $G\beta\gamma$ while an RGS protein (or effector) is bound. Prolonged association between $G\alpha$ and an RGS protein would be expected to delay heterotrimer formation and would be predicted to block signaling by $G\alpha$ but to allow continued signaling by its $G\beta\gamma$ dimer. This mechanism was recently proposed by Bünemann and Hosey (1998) to explain the apparently increased availability of $G\beta\gamma$ dimers in cells overexpressing RGS4. Prolonged association between $G\alpha$ and an RGS protein might result if GTP hydrolysis was relatively slow or if the RGS protein dissociated slowly from $G\alpha$ after GTP hydrolysis (Berman and Gilman, 1998). Alternately,

an RGS protein might remain associated with $G\alpha$ over multiple GTPase cycles, as proposed for PLC $\beta 1$ and $G\alpha q$ (Biddlecome et al., 1996). In this latter example, an RGS protein (or PLC $\beta 1$) could function simultaneously, or alternately, as an effector antagonist and as a GAP.

Previously, we demonstrated that RGS proteins shift the dose-response curve for Ca channel inhibition to higher agonist concentrations (Melliti et al., 1999). RGS proteins have also been shown to accelerate recovery of Ca channels from inhibition after agonist washout (Jeong and Ikeda, 1998; Melliti et al., 1999). These effects of RGS proteins can be adequately explained by their GAP activity. In these two previous studies, Ca channels were inhibited by PTX-sensitive G-proteins belonging to the $G\alpha i$ subfamily (Jeong and Ikeda, 1998; Melliti et al., 1999). In the present study, channel modulation was mediated by PTX-insensitive G-proteins (probably $G\alpha q$), and the coexpressed RGS proteins (and PLC $\beta 1ct$) appeared to function primarily as effector antagonists. Thus, whether $G\alpha$ -interacting proteins such as RGS and PLC $\beta 1$ behave mainly as GAPs or as effector antagonists might depend on the $G\alpha$ subunit involved. Consistent with this idea, most previous studies have used $G\alpha q$ to reveal the effector antagonist function of RGS proteins (Hepler et al., 1997; Heximer et al., 1997; Yan et al., 1997; Kammermeier and Ikeda, 1999).

Our present results are the first demonstration that RGS proteins (and PLC $\beta 1ct$) can influence receptor-mediated stimulation, as opposed to inhibition, of voltage-gated Ca channels. Our findings contribute to a growing body of evidence that RGS and other $G\alpha$ -interacting proteins play important roles in ion channel modulation (cf. Doupnik et al., 1997; Saitoh et al., 1997; Bünemann and Hosey, 1998; Jeong and Ikeda, 1998; Herlitze et al., 1999; Kammermeier and Ikeda, 1999; Melliti et al., 1999).

Physiological significance of $\alpha 1E$ Ca channel stimulation

Muscarinic receptors are widely expressed in mammalian brain and are prevalent in hippocampus, dentate gyrus, amygdala, and cortex (Buckley et al., 1988). These same regions of the brain also express $\alpha 1E$ subunits (Niidome et al., 1992; Soong et al., 1993; Wakamori et al., 1994; Williams et al., 1994; Yokoyama et al., 1995). Additionally, muscarinic receptors and $\alpha 1E$ subunits are both found on neuronal somata and dendrites (Hersch et al., 1994; Yokoyama et al., 1995; Westenbroek et al., 1998). Thus, we speculate that native R-type Ca channels are modulated through muscarinic receptors in central neurons.

Only a few studies have examined receptor-mediated modulation of native R-type Ca channels. Jeong and Wurster (1997) observed muscarinic inhibition of R-type currents in intracardiac neurons, and Overholt and Prabhakar (1999) found adrenergic inhibition of R-type currents in carotid body glomus cells. To our knowledge, receptor-mediated stimulation of native R-type Ca channels has not been reported. However, our present results demonstrate that $\alpha 1E$ Ca channels are strongly stimulated through $G\alpha q$ -coupled M1 receptors. It seems likely that native R-type Ca channels are also stimulated through muscarinic and perhaps other $G\alpha q$ -coupled receptors *in vivo*. Stimulation of native R-type currents may have important consequences for dendritic Ca signaling, neurosecretion, gene expression, or other neuronal functions.

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