Spike Patterning by Ca\(^{2+}\)-Dependent Regulation of a Muscarinic Cation Current in Entorhinal Cortex Layer-II Neurons

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**Running head:** Ca\(^{2+}\)-dependent modulation of muscarinic cation current

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In entorhinal cortex layer-II neurons, muscarinic receptor activation promotes depolarization via activation of a non-specific cation current ($I_{NCM}$). Under muscarinic influence, these neurons also develop changes in excitability that result in activity-dependent induction of delayed firing and bursting activity. In order to identify the membrane processes underlying these phenomena, we examined whether $I_{NCM}$ may undergo activity-dependent regulation. Our voltage-clamp experiments revealed that appropriate depolarizing protocols increased the basal level of inward current activated during muscarinic stimulation, and suggested that this effect was due to $I_{NCM}$ up-regulation. In the presence of low buffering for intracellular Ca$^{2+}$, this up-regulation was transient and its decay could be followed by a phase of $I_{NCM}$ down-regulation. Both up- and down-regulation were elicited by depolarizing stimuli able to activate voltage-gated Ca$^{2+}$ channels (VGCC); both were sensitive to increasing concentrations of intracellular Ca$^{2+}$-chelating agents, with down-regulation being abolished at lower Ca$^{2+}$-buffering capacities; both were reduced or suppressed by VGCC block or in the absence of extracellular Ca$^{2+}$. These data indicate that relatively small increases in [Ca$^{2+}$], driven by firing activity can induce up-regulation of a basal muscarinic depolarizing-current level, whereas more pronounced [Ca$^{2+}$] elevations can result in $I_{NCM}$ down-regulation. We propose that the interaction of activity-dependent positive and negative feedback mechanisms on $I_{NCM}$ allows entorhinal cortex layer-II neurons to exhibit emergent properties, such as delayed firing and enhanced or suppressed responses to repeated stimuli, that may be of importance in the memory functions of the temporal lobe and in the pathophysiology of epilepsy.

**Keywords**: cholinergic, plateau-potential, TRP, plasticity, memory.
INTRODUCTION

The entorhinal cortex (EC) in the parahippocampal region plays an important role in memory processes as shown by converging evidence from clinico-psychological (Leonard et al. 1995; Manns et al. 2003b; Scoville and Milner 1957) and brain imaging (Fernandez et al. 1999; Owen et al. 1996) studies in humans, as well as neurophysiological studies in humans (Fried et al. 1997) and animals (Suzuki et al. 1997; Young et al. 1997). Significantly, it has been shown that the level of sustained entorhinal neural activity correlates positively with “declarative” memory performance (Fernandez et al. 1999), and during working memory tasks neurons in the EC may display sustained activity during the delay period and enhanced or suppressed responses to matching stimuli (Hasselmo et al. 2000; Suzuki et al. 1997; Young et al. 1997).

The cholinergic innervation of the EC by the basal forebrain (Mesulam et al. 1983) is very robust and cholinergic fibers profusely innervate EC layer-II neurons (Alonso and Amaral 1995). EC layer II funnels polysensory cortical signals into the hippocampus (Insausti et al. 1987) thereby occupying a critical position in the neocortical-hippocampal-neocortical memory circuit. It is well established that the cholinergic system modulates the level and patterns of cortical activation (Celesia and Jasper 1966) and that cholinergic influences contribute to cortical plasticity (Bear and Singer 1986; Dykes 1997; Winkler et al. 1995), state-dependent learning (Shulz et al. 2000) and declarative memory formation (Aigner and Mishkin 1986; Chang and Gold 2003; Hasselmo et al. 1996; Tang et al. 1997). The contribution of the basal forebrain cholinergic system to memory processes is highlighted by the degeneration of cholinergic neurons in Alzheimer’s disease (Dunnett and Fibiger 1993), and cholinergic modulation of EC neuronal activity might be critical in the normal memory function of this structure (Hasselmo et al. 2000). Indeed, cholinergic activation of EC promotes the emergence of oscillatory neuronal dynamics (Buzsáki 1996) which are implicated in learning and memory processes (Hasselmo 1999b; Huerta and Lisman 1993; Larson and Lynch 1986).

Principal neurons from layer II of the EC belong to the broad category of regular spiking neurons (Alonso and Klink 1993; Connors and Gutnick 1990) and respond to step depolarizations with slowly-adapting trains of action potentials followed by a typical, slow afterhyperpolarization (Alonso and Klink 1993; and Fig. 1A I). Previous current-clamp investigations carried out in EC slices with intracellular sharp electrodes have shown that cholinergic stimulation of these neurons through muscarinic receptors affects their electrophysiological state and properties in at least two ways: on the
one hand, it produces a basal, sustained depolarization; on the other hand, it drastically modifies their firing behavior by inducing Ca$^{2+}$-dependent plateau potentials and bursting activity (Klink and Alonso 1997). In addition, in a recent voltage-clamp analysis based on whole-cell, patch-clamp experiments we showed that the muscarinic-receptor-dependent depolarization of EC layer-II neurons largely relies on the activation of a non-specific cation inward current that we referred to as $I_{NCM}$, and reported preliminary evidence suggesting that $I_{NCM}$ could be bi-directionally regulated by Ca$^{2+}$ influx (Shalinsky et al. 2002). A Ca$^{2+}$- and activity-dependent regulation of $I_{NCM}$ could introduce plasticity in the firing behavior of the cells so as to lead to afterdischarges and make the cellular responses dependent on the previous firing history. The goal of the here-presented patch-clamp study was therefore two-fold. First, we needed to re-examine, under whole-cell recording conditions, the firing repertoire of EC layer-II cells undergoing muscarinic stimulation, and test the hypothesis that the occurrence of plateau-potential-driven activity is influenced by the previous firing history. Second, by carrying out a voltage-clamp analysis and using intracellular Ca$^{2+}$ chelators we aimed to verify the hypothesis that an activity-(Ca$^{2+}$-) dependent regulation of $I_{NCM}$ is implicated in patterning spike discharge in EC layer-II cells during cholinergic modulation. Our results show that, indeed, $I_{NCM}$ appears to be subject to both Ca$^{2+}$-dependent up- and down-regulation, and are consistent with an activity-dependent regulation of this current playing an important role in patterning the firing discharge of EC layer-II cells during muscarinic modulation. Some of these data has been previously presented in preliminary form (Magistretti et al. 2001).
MATERIALS AND METHODS

Slice preparation. Brain slices were prepared from male Long-Evans rats (100-250 g, i.e., 30 to 60 days of age) as previously described (Alonso and Klink 1993). Briefly, animals were quickly decapitated, and the brain was rapidly removed from the cranium and placed in a cold (4 °C) Ringer’s solution containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH2PO4, 2 CaCl2, 2 MgSO4, 26 NaHCO3, 10 glucose (pH 7.4 by saturation with 95% O2, 5% CO2). Horizontal slices of the retrohippocampal region were cut at 350-400 μm on a vibratome (Pelco Series 1000, Redding, CA), and then transferred to an incubation chamber in which they were kept submerged for at least a one-hour period at room temperature (~22 °C).

Patch-clamp, whole-cell recordings. The recording chamber was mounted on the stage of an upright microscope (see below). Slices were transferred, one at a time, to the chamber and perfused with one of the extracellular solutions described in Table 1, according to the specific experimental purpose. In current-clamp experiments, extra- and intracellular solutions were always E1 and I1. Patch pipettes were fabricated from thick-wall borosilicate glass capillaries by means of a Sutter P-97 horizontal puller. The solutions used to fill the patch pipettes are also described in Table 1. When filled with one of these solutions, the patch pipettes had a resistance of 3-5 MΩ. Slices were observed with an Axioskop microscope (Zeiss, Oberkochen, FRG) equipped with a ×40 water-immersion objective lens and differential-contrast optics. A near-infrared charge-coupled device (CCD) camera (Sony XC-75) was also connected to the microscope, and used to improve cell visualization for identification of neuron types and during the approaching and patching procedures. With this equipment, the principal cells of EC layer II were easily distinguished based on their somato-dendritic shape, size, and position (Dickson et al. 2000). Patch pipettes were brought in close proximity to the selected neurons while manually applying positive pressure inside the pipette. Tight seals (> 10 GΩ) and the whole-cell configuration were obtained by suction. Series resistance ($R_s$) was estimated on-line by canceling the fast component of whole-cell capacitive transients evoked by −10-mV voltage steps with the amplifier compensation section (with the low-pass filter set at 10 kHz) and reading out the corresponding value, and was on average ~16-18 MΩ. $R_s$ was always compensated by about 40% with the amplifier’s built-in compensation section. Current- and voltage-clamp recordings were performed at room temperature.
(~22 °C) using an Axopatch 1D amplifier or an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). All current-clamp experiments were carried out using the Axopatch 200B amplifier in the “I-Clamp fast” mode, which, in the presence of \( R_s \) values like those typically obtained in our conditions, allows for reliable recording of membrane-voltage signals (Magistretti et al. 1998; Magistretti et al. 1996). The low-pass filter (~3 dB) was set at 5 kHz. In voltage-clamp recordings, the general holding potential was –60 mV unless otherwise indicated.

**Drugs.** Carbamyl choline (carbachol, CCh) was delivered by bath superfusion at 5-30 µmol/l, during synaptic transmission block with kynurenic acid (1 mM) and picrotoxin (100 µM). Stock solutions of nifedipine (20 µM) were prepared using dimethylsulfoxide (DMSO) as the solvent, and stored at –20 °C in the dark. Nifedipine aliquots were then re-dissolved in recording solution and applied through a light-shielded perfusion channel. All chemicals and reagents, including those listed in Table 1 and CCh, were purchased from Sigma (St. Louis, MO), except tetrodotoxin (TTx), which was purchased from Alomone labs (Jerusalem, Israel).

**Data acquisition.** All recordings were stored by PCL coding on VHS tape (Neurocorder, Neurodata, New York). In voltage-clamp experiments, voltage protocols were commanded and current signals were acquired with a Pentium PC interfaced to an Axon DigiData 1200 interface, using the Clampex program of the pClamp software (V8.0, Axon Instruments). Ramp voltage protocols consisted in 1-s linear depolarizations from –100 to –50 mV (depolarization rate = 50 mV/s), always preceded by a 500-ms fixed step at –100 mV. Data stored on VHS tape was digitized and plotted off-line by sampling at 20 kHz using the Axoscope software (V1.1, Axon Instruments).

**Data analysis.** Whole-cell recordings were analyzed by means of the Clampfit program of the pClamp software (Axon Instruments). Linear regressions were performed using Origin 6.0 (MicroCal Software, Northampton, MA, USA). Average values were expressed as mean ± SEM. Statistical significance was evaluated, when not otherwise explicitly stated, by means of the two-tail Student’s \( t \) test for unpaired data, or, in other cases, by applying the one-way ANOVA and/or the Bonferroni multiple comparison test. In current-clamp recordings, neurons were electrophysiologically categorized as stellate cells if they displayed robust time-dependent inward rectification (sag% > 40%; Dickson et al. 2000).
RESULTS

Self-terminating plateau potentials and dependence of activity on firing history

Under current-clamp conditions, we found that in all neurons examined \((n = 43)\) triggering a short train of action potentials during bath application of carbachol (CCh, 5-20 \(\mu\)M) elicited a depolarizing afterpotential (DAP) that could sustain delayed spiking activity. Two exemplary cases are illustrated in Fig. 1A2 and B. Afterdischarges displayed spike-frequency patterns qualitatively similar in all neurons examined, independently of whether the cells were electrophysiologically categorized as stellate or pyramidal-like neurons (Alonso and Klink 1993; Dickson et al. 2000). However, in response to comparable stimuli (0.1-nA, 1-s current steps, resting potential \(\sim -60\) mV under superfusion with 5-\(\mu\)M CCh) pyramidal-like cells typically displayed afterdischarges with a more pronounced accelerating-decelerating spike-frequency pattern than stellate cells. This behavior was characterized by a significantly higher peak firing frequency during the afterdischarge in pyramidal cells (4.03 ± 0.69 Hz; \(n = 5\)) than in stellate cells (2.29 ± 0.26 Hz; \(n = 8\)) \((p < 0.05)\), and a shorter (though not statistically significant) afterdischarge duration in pyramidal cells (15.24 ± 0.97 s) than in stellate cells (29.52 ± 7.57 s).

Importantly, in all cases tested \((n = 13)\), we also noted that when a given suprathreshold stimulus was repeatedly applied, the DAPs that developed following each subsequent stimulus were first enhanced and then suppressed back to the initial level (Fig. 2A). This biphasic enhancement-suppression pattern always took place, whether (Fig. 2A1) or not (Fig. 2A2) an afterdischarge developed. However, when one of the stimuli did give rise to an afterdischarge, the responses to subsequent stimuli were always suppressed (Fig. 2A1). The suppression period following afterdischarges is better illustrated by the example shown in Fig. 2B. Note in Fig. 2B1 that a brief depolarizing stimulus led to an afterdischarge that lasted for \(\sim 20\) s, and that immediately after this event the same stimulus triggered a spike train with minimal DAP. However, following a recovery period of \(\sim 1\) min the stimulus was again capable of triggering an afterdischarge. Fig. 2B2 illustrates another example of the phenomenon in the same neuron: in this case the stimulus was re-applied at increasing intervals during the suppression period. Note that a stimulus interval of 27 s was required to
elicited a subsequent afterdischarge. Although we did not systematically analyze the duration of the suppression period, the elicitation of two consecutive afterdischarges of equivalent duration required a minimal recovery period of about 20 s ($n = 5$).

The above observations indicate that the firing pattern of EC layer-II neurons during muscarinic modulation is always dependent on its previous short-term firing history, because spiking activity leads to a cycle consisting of initially positive feedback, then no or negative feedback.

**Voltage-clamp analysis of $I_{NCM}$ responses to activating stimuli**

Having established that during muscarinic modulation the activity pattern of EC layer-II neurons is dependent on the previous firing history, we then tested the hypothesis that a bi-directional activity-(Ca$^{2+}$-) dependent regulation of $I_{NCM}$ (Shalinsky et al. 2002) might, at least partially, underlie the observed firing phenomena. With this goal in mind, we first performed a series of voltage-clamp experiments to analyze the behavior of $I_{NCM}$ in response to brief step depolarizations to voltage levels expected to trigger Ca$^{2+}$ influx; 50- to 500-ms depolarizing voltage steps to 0 mV were applied prior to and during bath application of CCh. To maximize the isolation of $I_{NCM}$ and improve clamp conditions, these experiments were initially carried out with Cs$^+$ as the main intracellular cation (in order to completely block K$^+$ conductances), and with 0.5-mM intra-pipette EGTA (intracellular soln. I2) (Shalinsky et al. 2002). Results from a representative cell are illustrated in Fig. 3. Whereas 100-ms voltage steps to 0 mV caused no modifications in the holding current under control conditions, during the CCh response they triggered prominent, inward tail-like currents that decayed in seconds (Fig. 3B) and were followed by a transient depression of $I_{NCM}$ (Fig. 3A). Qualitatively (see below and Fig. 4 for quantification), the same result was observed in all cells tested in this way ($n = 12$).

For simplicity, the slow, inward tail-like currents that followed depolarizing voltage steps will be referred to as $I_{tail}$. It could be argued that $I_{tail}$ may actually represent tail currents resulting from some voltage-dependent conductance activated upon depolarization. Since in our experiments voltage-gated Na$^+$ currents were blocked with TTx, voltage-dependent Ca$^{2+}$ currents (VDCC) are the obvious candidate; however, several observations make this possibility unlikely: 1) tail Ca$^{2+}$ currents are several orders of magnitude faster than $I_{tail}$; 2) $I_{tail}$ are accompanied by an increase in current noise (Figs. 3B, 4 and 5; see also Shalinsky et al., 2002) which is unlikely to be produced by voltage-gated Ca$^{2+}$ channels due their very low conductance; 3) in a number of neuronal systems, muscarinic-receptor activation has been shown to produce negative (instead of positive) modulation of high-voltage-
activated Ca\textsuperscript{2+} currents with a G-protein-dependent mechanism (e.g. Wanke et al. 1994), whereas low-voltage-activated, T-type Ca\textsuperscript{2+} channels, which can be enhanced by muscarinic receptor activation (Fisher and Johnston, 1990), are expected to be largely inactivated in the present experimental conditions (holding potential was \(-60\) mV). This notwithstanding, we directly addressed the above issue by comparing CCh-dependent \(I_{\text{tail}}\) induction with CCh effects on VDCC. Depolarizing voltage steps that triggered prominent \(I_{\text{tail}}\) during CCh delivery also evoked inward voltage-dependent Ca\textsuperscript{2+} currents (identified by their sensitivity to 200-\(\mu\)M Cd\textsuperscript{2+}: not shown) that in all cases (\(n = 5\)) were markedly depressed by CCh (by \(~33\%\) on average) (Fig. 3C). These results clearly demonstrate that \(I_{\text{tail}}\) cannot coincide with tail Ca\textsuperscript{2+} currents resulting from CCh potentiation of VDCC.

**Up- and down-regulation of CCh-activated inward current are Ca\textsuperscript{2+} dependent**

Since both \(I_{\text{tail}}\) and \(I_{\text{NCM}}\) down-regulation were triggered by step depolarizations able to activate voltage-dependent Ca\textsuperscript{2+} currents, we next examined whether these phenomena depend on changes in intracellular Ca\textsuperscript{2+} concentration induced by voltage-dependent Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels. A series of experiments was performed in which different concentrations of Ca\textsuperscript{2+} chelating agents were used in the intra-pipette solution, in the presence of Cs\textsuperscript{+} as the main intracellular cation (intracellular solns. I2-I5) (Fig. 4).

\(I_{\text{tail}}\) induction and \(I_{\text{NCM}}\) down-regulation both proved to be markedly sensitive to intracellular Ca\textsuperscript{2+}-buffering conditions. We first examined the current modifications triggered by 500-ms voltage pulses to 0 mV which were delivered as the first depolarizing stimuli after the full development of a steady \(I_{\text{NCM}}\). In the presence of 0.5-mM intra-pipette EGTA (Fig. 4A1), \(I_{\text{tail}}\) evoked by these stimuli were always prominent and fast-developing. At 400 ms from the end of the depolarizing step (a time long enough to allow passive transients to fully subside), \(I_{\text{tail}}\) amplitude exceeded by 524.7 \(\pm\) 107.7\% (\(n = 7\)) that of the preceding basal \(I_{\text{NCM}}\). Moreover, \(I_{\text{tail}}\) were always followed by a transient \(I_{\text{NCM}}\) down-regulation. At the peak of \(I_{\text{NCM}}\) down-regulation, which was reached after 11.4 \(\pm\) 1.9 s from the end of the depolarizing pulse, current amplitude was decreased on average by 41.0 \(\pm\) 8.3\% (\(n = 7\)). With increasing concentrations of intracellular Ca\textsuperscript{2+} chelators (5-mM EGTA, 5-30-mM BAPTA), \(I_{\text{tail}}\) peak amplitude became progressively smaller (Fig. 4A2-A4), and their onset more slowly developing (Fig. 4A2, A3, insets), such that in these cases \(I_{\text{tail}}\) peak amplitude could be unequivocally determined.
Average data on basal $I_{\text{NCM}}$ and $I_{\text{tail}}$ peak amplitude for various intracellular-Ca$^{2+}$ buffering conditions are illustrated in Fig. 4B. First of all, it is worth to note that the basal $I_{\text{NCM}}$ response was not abolished even at the highest Ca$^{2+}$-buffering capacity tested (30-mM BAPTA), neither was its peak amplitude significantly reduced by increasing concentrations of the Ca$^{2+}$ chelators ($p > 0.05$ for the comparisons between 0.5-mM EGTA and all the other conditions; Bonferroni multiple comparison test), indicating that muscarinic-receptor-dependent induction of basal $I_{\text{NCM}}$ does not have an absolute dependence on intracellular Ca$^{2+}$. Second, in the presence of 30-mM intra-pipette BAPTA, $I_{\text{tails}}$ were almost completely abolished (Fig. 4A4 and B), indicating that this form of muscarinic-receptor-dependent inward-current potentiation depends on intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) increases elicited by the depolarizing pulses applied. Additional information was provided by the intermediate Ca$^{2+}$-chelator concentrations used. In the presence of 5-mM intracellular EGTA, the $I_{\text{tails}}$ evoked by the depolarizing pulses had a peak current-amplitude over the basal $I_{\text{NCM}}$ level ($\Delta I$) that averaged ~900 pA, with an average, percent increase ($\% \Delta I$) of ~700% (Fig. 4C). In the presence of the same concentration of BAPTA, $I_{\text{tails}}$, although still prominent, where significantly smaller, with an average, peak $\% \Delta I$ of ~300% (Fig. 4C). (The different behaviors observed when using the same concentration of EGTA and BAPTA, which have similar $K_D$s for Ca$^{2+}$, are likely a consequence of the slower kinetics of EGTA in binding Ca$^{2+}$ as compared to BAPTA: after abrupt triggering of Ca$^{2+}$ influx to the cytoplasm, higher [Ca$^{2+}$], levels are expected to be transiently reached in the presence of EGTA than in the presence of BAPTA.) 10-mM BAPTA caused a further decrease of $\% \Delta I$ at $I_{\text{tail}}$ peak (although not statistically significant with respect to 5-mM BAPTA; Fig. 4C). As illustrated in Fig. 4D, the increase in $I_{\text{tail}}$ inhibition due to more efficient or stronger Ca$^{2+}$ buffering was paralleled by a progressive decrease in the speed of $I_{\text{tail}}$ onset, as quantified by the measurement of $I_{\text{tail}}$ time-to-peak.

Down-regulation of $I_{\text{NCM}}$ following a depolarizing pulse proved to be even more sensitive than $I_{\text{tails}}$ to increases in buffering capacity for intracellular Ca$^{2+}$, as it was already abolished by 5-mM intra-pipette EGTA (Fig. 4A2). Remarkably, in the presence of 5-mM EGTA and 5- and 10-mM BAPTA, $I_{\text{NCM}}$ down-regulation was replaced by a prominent inward-current plateau (Fig. 4A2, A3), and by an increase in current noise level, indicative of increased channel activity. Plateau amplitude exceeded by $58.7 \pm 21.6\%$ that of the preceding basal $I_{\text{NCM}}$ in the case of 5-mM EGTA ($n = 5$), by $39.0 \pm 15.0\%$ in the case of 5-mM BAPTA ($n = 4$), and by $60.7 \pm 13.8\%$ in the case of 10-mM BAPTA ($n = 15$).
Plateau amplitude was measured 60 to 120 s after $I_{\text{tail}}$ peak, depending on the time course of $I_{\text{tail}}$ development.

The above results demonstrate that $I_{\text{tail}}$, plateau-current induction, and $I_{\text{NCM}}$ down-regulation all depend on $[\text{Ca}^{2+}]_i$ elevations triggered by membrane depolarization, and strongly suggest that these three distinct phenomena are caused by different $[\text{Ca}^{2+}]_i$ dynamics (i.e., different degrees of $[\text{Ca}^{2+}]_i$ increase and/or different spatio-temporal distribution of $[\text{Ca}^{2+}]_i$) secondarily to voltage-dependent Ca$^{2+}$ entry. In particular, $I_{\text{NCM}}$ down-regulation would require higher levels of free intracellular-Ca$^{2+}$ than those resulting in induction of $I_{\text{tail}}$ and plateau currents, since $I_{\text{NCM}}$ down-regulation was abolished at lower $[\text{Ca}^{2+}]_i$ chelating levels. This interpretation was also supported by experiments in which 50-, 100-, and 500-ms step depolarizations at 0 mV were delivered in sequence during an $I_{\text{NCM}}$ response in the presence of 5-mM EGTA or 10-mM BAPTA in the recording pipette (Fig. 5). In the former condition, a 50-ms depolarizing pulse typically induced a small $I_{\text{tail}}$, whereas a 100-ms pulse elicited a more pronounced $I_{\text{tail}}$ followed by an evident plateau; a 500-ms pulse, delivered on the plateau phase, evoked an even bigger $I_{\text{tail}}$ which was then followed by a sharp, profound $I_{\text{NCM}}$ down-regulation phase (Fig. 5A1). In the presence of 10-mM intra-pipette BAPTA, 50-, 100-, and 500-ms depolarizing pulses induced $I_{\text{tail}}$ of increasing amplitude, with the 500-ms pulse only being followed by a prominent plateau (Fig. 5A2). No $I_{\text{NCM}}$ down-regulation was ever observed in this condition. In the presence of 30-mM intra-pipette BAPTA, none of the above depolarizing stimuli caused significant modifications of the basal $I_{\text{NCM}}$ amplitude level (Fig. 5A3). Average data on these effects are illustrated in Fig. 5B1 and B2 (peak $I_{\text{tail}}$ amplitude) and Fig. 5C (plateau potentiation or down-regulation). These results not only indicate that $I_{\text{tail}}$, plateaus, and $I_{\text{NCM}}$ down-regulation are all Ca$^{2+}$-dependent processes, but also strongly suggest that the induction, in sequence, of the three phenomena depends on increasing degrees of $[\text{Ca}^{2+}]_i$ elevations (either in terms of absolute levels at one location or in terms of spatio-temporal distribution) that can be obtained with cumulative voltage-dependent Ca$^{2+}$ entry.

**Potentiation and down-regulation of CCh-activated inward current require voltage-dependent Ca$^{2+}$ entry**

The intimate relation between voltage-dependent Ca$^{2+}$ entry and induction of both $I_{\text{tail}}$ and plateaus as well as $I_{\text{NCM}}$ down-regulation during muscarinic stimulation was further confirmed by experiments carried out in the presence of partial block of voltage-gated Ca$^{2+}$ channels. In EC layer-II neurons, a
current fraction equal to ~45% of total VDCCs is sensitive to the L-type current blocker, nifedipine (Castelli and Magistretti, unpublished results). Therefore, experiments were carried out in the presence of 20-μM nifedipine in the bath and 0.5-mM intra-pipette EGTA (recording solutions: E2, I2). In other control experiments the extracellular solution was added with the same amount of vehicle (DMSO) used to dissolve nifedipine aliquots (final concentration 0.1% v/v). In the presence of nifedipine, CCh application evoked the usual $I_{NCM}$, but the $I_{tail}$ responses that followed the application of 500-ms voltage pulses at 0 mV were significantly reduced in amplitude as compared to those obtained in control conditions (Fig. 6A). The percent increase in peak post-depolarization current was 151.3 ± 135.0% ($n = 4$) in the presence of nifedipine vs. 605.1 ± 84.7% ($n = 3$) in control conditions ($p < 0.05$). $I_{NCM}$ down-regulation was also markedly reduced (percent decrease in peak current of 10.7 ± 8.4% vs. 40.0 ± 3.4%; $p < 0.05$).

Similar experiments were also performed in 0 nominal Ca$^{2+}$ plus 0.5-mM EGTA in the extracellular solution. In these conditions, no significant up- or down-regulation of the CCh-evoked inward-current response was ever observed following the application of 500-ms voltage pulses at 0 mV (Fig. 6B; $n = 4$) in the presence of 0.5-mM intra-pipette EGTA (recording solutions: E4, I2). The above results provide conclusive demonstration that the depolarization-triggered modifications of CCh-induced inward current are caused by voltage-dependent Ca$^{2+}$ entry through VGCCs.

**Potentiated currents require the maintenance of the muscarinic stimulus and display the same sensitivity to Ca$^{2+}$ and current-voltage relationship as $I_{NCM}$**

As discussed above, induction of potentiated, plateau inward currents could be obtained in the presence of 5-mM intra-pipette EGTA or 10-mM intra-pipette BAPTA using appropriate depolarization patterns. In the continuous presence of CCh stimulation, the plateau currents, after an initial, transient decay phase, reached a steady level that showed little or no tendency to decrease further for at least 5 min ($n = 10$), and up to ~15 min ($n = 2$; Fig. 7A1). By contrast, if the continuous application of CCh with the perfusing solution was interrupted after the development of a plateau, the current decayed to the baseline within 4-5 min (Fig. 7A2; $n = 3$). These results show that plateau currents, like both the basal $I_{NCM}$ and $I_{tail}$s, are strictly muscarinic-receptor-dependent, and that they can persist for prolonged
periods, provided the muscarinic stimulus is continuously present.

We then analyzed the sensitivity of both basal $I_{NCM}$ and plateau currents to Co$^{2+}$ (recording solutions: $E2, I4$), since it had been previously shown that Co$^{2+}$ blocks both CCh-induced depolarizations and plateau potentials in EC layer-II neurons (Klink and Alonso 1997). Consistent with our previous observations, $I_{NCM}$ responses could not be evoked in the presence of 2-mM Co$^{2+}$ in the perfusing medium (Fig. 7B1; $n = 4$), suggesting that the underlying ion channels are sensitive to Co$^{2+}$ block. Remarkably, the application of 2-mM Co$^{2+}$ after the development of a plateau current evoked in control conditions resulted in a fast inhibition of the same current and a decay of the total CCh-induced inward current to the baseline (Fig. 7B2; $n = 2$). Hence, both $I_{NCM}$ and potentiated currents show similar sensitivity to Co$^{2+}$.

Finally, the current-voltage relationship of both the basal $I_{NCM}$ and depolarization-induced potentiated currents was studied using relatively short depolarizing voltage ramps (1 s, 50 mV/s; Fig. 8A and B) (recording solutions: $E2, I4$). The voltage range explored by these ramps was limited to a window between –100 and –60/–50 mV, to prevent further current up-regulation resulting from voltage-dependent Ca$^{2+}$ entry at more positive voltages. The currents recorded during ramp application in control conditions, at the peak of the $I_{NCM}$ response, and after the development of a $I_{tail}$ (evoked by a 500-ms pulse at 0 mV) in a representative cell are shown in Fig. 8B. The control ramp current was then subtracted from the $I_{NCM}$ ramp current, and the $I_{NCM}$ ramp current was subtracted from the $I_{tail}$ ramp current, to obtain the voltage dependence of the basal $I_{NCM}$ and that of the pulse-evoked $I_{tail}$, respectively. The current-voltage relationships of the subtracted currents are illustrated in Fig. 8C. Both the basal $I_{NCM}$ and the up-regulated current showed a linear behavior from –100 to –60 mV in all cases studied ($n = 9$). Moreover, the average, extrapolated reversal potential determined by linear regression of subtracted currents was not significantly different in $I_{NCM}$ ramp currents ($+13.8 \pm 7.6$ mV) and $I_{tail}$ ramp currents ($+21.8 \pm 4.2$ mV; $p = 0.3$, $t$ test for paired data). The parallel sensitivity to muscarinic receptor stimulation and block by Co$^{2+}$, together with the similarity in the current-voltage behavior suggest that $I_{NCM}$, $I_{tails}$, and plateau currents may represent different modulatory states of the same current.

*Repetitive depolarizations can induce phasic or sustained inward-current up-regulation depending on the stimulation pattern*
The data presented above have shown that \( I_{\text{tail}} \), plateau currents, and \( I_{\text{NCM}} \) down-regulation can be sequentially induced in a voltage- and Ca\(^{2+}\)-dependent manner with increasing degrees of intracellular-Ca\(^{2+}\) accumulation. In an attempt to further clarify how the activity- (Ca\(^{2+}\)-) dependent regulation of \( I_{\text{NCM}} \) may influence the generation of distinct firing patterns during an afterdischarge (i.e., sustained delayed firing or “bursty” discharge with a pronounced accelerating-decelerating pattern), we carried out further experiments involving trains of step depolarizations. To facilitate comparison with the current-clamp observations (Figs. 1 and 2), this last series of experiments was also performed using K\(^+\) as the main intracellular cation and 0.5-mM intra-pipette EGTA (intra-pipette solution II). However, the extracellular solution contained Cs\(^+\) (5 mM) and Ba\(^{2+}\) (1 mM) (extracellular solution E3), a cocktail that blocks Ca\(^{2+}\)-dependent slow afterhyperpolarization (sAHP) (not shown) and isolates \( I_{\text{NCM}} \) from K\(^+\) currents modulated by CCh in a subthreshold voltage range (Shalinsky et al., 2002).

In these conditions, single 500-ms step depolarizations to 0 mV in the continuous presence of CCh (30 \( \mu \)M) always triggered a large \( I_{\text{tail}} \) that slowly decayed towards the pre-step current level (Fig. 9A, main panel and inset) \( n = 10 \). However, the application of a long train of identical depolarizing steps was followed by a transient decay of \( I_{\text{NCM}} \) to a level lower than that preceding the train. A slow, transient depression of \( I_{\text{NCM}} \) was invariably observed with trains of 200-500 ms step depolarizations to 0 mV delivered at 0.3-1 Hz \( n = 6 \). Hence, in the presence of intracellular K\(^+\), potentiation can be dissociated from \( I_{\text{NCM}} \) down-regulation, with down-regulation requiring repetitive depolarizing stimuli to be induced. The observation that, using 0.5-mM intra-pipette EGTA, \( I_{\text{tail}} \) elicited by equal stimuli are followed by \( I_{\text{NCM}} \) down-regulation in the presence of Cs\(^+\), but not K\(^+\), as the main intracellular cation can be easily explained on the basis of our conclusion that down-regulation requires higher levels of [Ca\(^{2+}\)]\(_{i}\) increase than potentiation: indeed, higher [Ca\(^{2+}\)]\(_{i}\) rises, able to induce \( I_{\text{NCM}} \) down-regulation, are more likely to occur in an in situ neuron during poorly clamped dendritic Ca\(^{2+}\) events, that are expected to be greatly facilitated by Cs\(^+\) block of K\(^+\) conductances. Alternatively, VDCC could by differently affected by intracellular K\(^+\) vs. Cs\(^+\) (see Brette et al., 2003).

Further, we analyzed the effects of repetitive stimulation on current-potentiation expression and time course. Fig. 9B shows a typical recording in a different neuron, in which, after the full development of a CCh-induced \( I_{\text{NCM}} \) response, five trains of depolarizing voltage steps of variable amplitude and frequency were delivered. Trains 1 to 3 consisted of pulses of equal large amplitude (to nominal +20 mV), but the pulse frequency was increased from train to train (train 1: 0.167 Hz; train 2: 0.33 Hz; and train 3: 0.5 Hz). We applied this protocol since it is expected to lead to larger and faster
[Ca$^{2+}$]$_i$ rises as the pulse frequency is increased. If so, and muscarinic-receptor-dependent inward-current induction is bidirectionally regulated in an activity-dependent manner, one would expect as well larger $I_{\text{tail}}$s (inward-current up-regulation) and more pronounced $I_{\text{NCM}}$ down-regulation as the pulse frequency is increased. Note that this was indeed the case. In each train from 1 to 3 the pulses triggered $I_{\text{tail}}$s that summated to each other so that the peak current level of successive $I_{\text{tail}}$s was initially increasingly negative; the higher the step frequency, the larger the peak amplitude of the initial $I_{\text{tail}}$ summation. Note, however, that the fast initial rise in $I_{\text{tail}}$ amplitude was followed by a progressive decrease that became more pronounced as the pulse frequency was increased from train 1 to train 3. In trains 4 and 5, the pulse frequency was left equal to that of train 3 (0.5 Hz) but the pulse amplitude was progressively decreased from train to train (train 3: nominal +20 mV; train 4: nominal –20 mV; train 5: nominal –30 mV). The decrease in pulse amplitude was aimed to lead to a lower Ca$^{2+}$ influx and, if our interpretation regarding the Ca$^{2+}$-dependent regulation of muscarinic inward current(s) is correct, to less pronounced $I_{\text{tails}}$ and $I_{\text{NCM}}$ down-regulation. Note that this was again the case, and that for train 5 an almost sustained “plateau” $I_{\text{NCM}}$ level was reached during the train. The same changes in $I_{\text{tail}}$ behavior during pulse trains of variable step-depolarization amplitude or frequency were observed in all neurons tested ($n = 3$). These data show that “phasic” and “plateau” inward-current potentiation can be differentially induced depending on the amplitude of repetitive depolarizing stimuli and, therefore, are likely dependent on the levels of Ca$^{2+}$ influx triggered by the repetitive activating stimuli (step depolarizations in voltage clamp, spikes in current clamp).
DISCUSSION

Previous experimental work has shown that a main component of the muscarinic-induced depolarization in EC layer-II principal neurons is represented by the activation of a non-specific cation current that we referred to as $I_{NCM}$ (Shalinsky et al. 2002). In the present study, we provide evidence that $I_{NCM}$ is Ca$^{2+}$-sensitive and, most importantly, that it can be substantially modified in amplitude in opposite directions by voltage-dependent Ca$^{2+}$ influx, thus providing a simple mechanism by which cholinergic input can modulate the firing behavior of these cortical neurons in an activity-dependent manner.

First, we found that, during $I_{NCM}$ induction, step depolarizations able to activate VGCC were followed by transient, slow tail-like inward currents that substantially potentiated the effect of muscarinic stimulation in terms of induction of depolarizing current. Post-depolarization tail-like currents ($I_{tails}$) were sensitive to increasing concentrations of intracellular Ca$^{2+}$-chelating agents, although their complete abolishment required very strong intracellular-Ca$^{2+}$ buffering (30-mM BAPTA). $I_{tails}$ were reduced or suppressed by VGCC block or in the absence of extracellular Ca$^{2+}$, indicating that they depend on [Ca$^{2+}$]$_i$ elevations secondary to voltage-dependent Ca$^{2+}$ entry.

Second, under particular conditions, $I_{tails}$ were followed by a marked depression of pre-depolarization $I_{NCM}$ amplitude. Similarly to $I_{tail}$ induction, $I_{NCM}$ down-regulation depended on [Ca$^{2+}$]$_i$ elevations caused by voltage-dependent Ca$^{2+}$ entry. However, $I_{NCM}$ down-regulation showed higher sensitivity to increases in intracellular-Ca$^{2+}$ buffering than $I_{tail}$ induction, being readily abolished by 5-mM intracellular EGTA. This suggests that $I_{NCM}$ down-regulation requires larger [Ca$^{2+}$]$_i$ rises (either in absolute terms or in terms of spatio-temporal distribution) than those sufficient to trigger $I_{tails}$. Consistent with this interpretation, during an $I_{NCM}$ response evoked in intracellular K$^+$, trains of step depolarizations expected to cause smaller degrees of Ca$^{2+}$ influx resulted in sustained “plateau-like” increases the inward-current amplitude, whereas trains of step depolarizations expected to cause larger Ca$^{2+}$ influx produced a more pronounced, transient current potentiation, and were followed by the apparent development of $I_{NCM}$ depression. Thus [Ca$^{2+}$]$_i$ elevations can result in either potentiation or depotentiation of the muscarinic induction of inward current, depending on the pattern of depolarization.

Third, recordings carried out in “intermediate” conditions of intracellular-Ca$^{2+}$ buffering revealed that $I_{tails}$ may be followed by inward-current plateaus. These plateaus were always
accompanied by an increase in the current noise level, indicating an increased channel activity, and were strictly dependent on the maintenance of the muscarinic stimulus. Our results indicate that the three phenomena of $I_{\text{tail}}$, current plateaus, and $I_{\text{NCM}}$ down-regulation may be induced in sequence with increasing degrees of intracellular-$\text{Ca}^{2+}$ accumulation.

Our data did not allow us to definitely establish whether the basal $I_{\text{NCM}}$ and depolarization-induced potentiated currents (which included $I_{\text{tail}}$ and plateau currents) are based on the same channel population. However, $I_{\text{NCM}}$ and potentiated currents shared several common features: 1) strict dependence on muscarinic receptor activation; 2) suppression by 2-mM $\text{Co}^{2+}$; 3) linear current-voltage dependence between $-100$ and $-60/-50$ mV; 4) down-regulation in a voltage- and $\text{Ca}^{2+}$-dependent manner (see, for instance, Fig. 9B); and 5) association to an increase in current noise, indicative of increased activity of a population of channels characterized by relatively high conductance. We have previously estimated that the channel activity underlying $I_{\text{NCM}}$ has a single-channel conductance of $\sim 14$ pS (Shalinsky et al. 2002). The above observations are consistent with a homogeneous population of channels being responsible for both the basal $I_{\text{NCM}}$ and $\text{Ca}^{2+}$-potentiated currents, although they do not exclude the possibility that distinct channels sharing some common features generate $I_{\text{NCM}}$, $I_{\text{tail}}$, and plateau currents.

In addition to EC neurons, muscarinic-dependent plateau potentials have been shown in a variety of cortical neuronal populations including hippocampal pyramidal cells (Caeser et al. 1993; Fraser and MacVicar 1996; Young et al. 2003) and interneurons (McQuiston and Madison 1999), and neocortical pyramidal cells (Andrade 1991; Haj-Dahmane and Andrade 1998; Schwindt et al. 1988), and typically suggested to reflect the activation of a $\text{Ca}^{2+}$-dependent non-specific cation current (Caeser et al. 1993; Haj-Dahmane and Andrade 1998; Schwindt et al. 1988). A noteworthy case is that of prefrontal cortex pyramidal cells in which, similarly to EC neurons, the mechanism of sustained muscarinic depolarization has also been shown to rely on the activation of a non-specific cation current (Haj-Dahmane and Andrade 1996), and in which it was hypothesized that both the basal muscarinic-receptor-induced, sustained inward current and the current generating the depolarizing afterpotential reflect a common underlying mechanism (Haj-Dahmane and Andrade 1998). In fact, in smooth muscle, the muscarinic-receptor-activated cation current, $I_{\text{CAT}}$, displays a very prominent, transient $\text{Ca}^{2+}$-dependent up-regulation (Inoue and Isenberg 1990; Kim et al. 1998). As discussed above, our data suggest that in EC layer-II neurons a $\text{Ca}^{2+}$-dependent up-regulation of the same channel activity responsible for $I_{\text{NCM}}$ may be the mechanism underlying the generation of potentiated currents.
To our knowledge, no previous study carried out in vertebrate neurons has shown a \( \text{Ca}^{2+} \)-dependent down-regulation of a neurotransmitter-activated inward current operated by metabotropic-type receptors. Nevertheless, \( \text{Ca}^{2+} \)-dependent inactivation is a phenomenon well known to occur in a variety of cationic channels, including voltage-activated \( \text{Ca}^{2+} \) channels (Gutnick et al. 1989), NMDA receptors (Legendre et al. 1993), cyclic nucleotide-activated cation channels (Zufall et al. 1991), and the light-dependent channels (\textit{trp} and \textit{trpl}) in \textit{Drosophila} photoreceptors (Hardie and Minke 1994; Ranganathan et al. 1991). It seems noteworthy that in the invertebrate visual cascade, \( \text{Ca}^{2+} \)-influx mediates both an early facilitation followed by a somewhat slower down-regulation of the light-induced current which displays transient inactivation (Hardie and Minke 1995; Scott et al. 1997). Overall, similar properties also characterize the \( I_{\text{NCM}} \) current expressed by EC neurons and, as discussed by Shalinsky et al. 2002, suggest that the channels underlying \( I_{\text{NCM}} \) may belong to the TRP family (Clapham 2003; Harteneck et al. 2000; Voets and Nilius 2003). Indeed, there has been increasing evidence indicating that TRP channels may mediate many metabotropic responses in CNS neurons (Chuang et al. 2002; Clapham et al. 2001; Gee et al. 2003; Strubing et al. 2001), and a recent report has shown that in cerebellar Purkinje cells a metabotropic slow EPSC is mediated by the TRPC1 cation channel (Kim et al. 2003). Also, a recent report has shown that heterologous expression of human TRPM5 in HEK-293 cells generates a non-selective cation channel that carries \( \text{Na}^{+} \), \( \text{K}^{+} \) and \( \text{Cs}^{+} \) equally well and is regulated by \( [\text{Ca}^{2+}]_{i} \), being activated at low and inhibited at high \( [\text{Ca}^{2+}]_{i} \) levels, thus resulting in a bell-shaped dose-response curve for \( [\text{Ca}^{2+}]_{i} \) (Prawitt et al. 2003). A similar \( \text{Ca}^{2+} \)-dependent regulation is also manifested by TRPM4 channels which are good candidates as mediators of plateau potentials (Nilius et al. 2003).

Interactions between physiological processes involving positive and negative feedback mechanisms allow physiological signals to exhibit emergent properties, most notably plateau potentials and oscillation. The \( \text{Ca}^{2+} \)-dependent up- and down-regulation of muscarinic-receptor-dependent inward current(s) are likely to participate in the emergence of plateau potentials and oscillatory trains of activity in EC layer-II cells. Indeed, some key features of such firing behaviors showed clear similarities with the modulatory characteristics of muscarinic inward current(s). In particular: 1) in current clamp (CC), afterpotentials of variable amplitude could be evoked by trains of action potentials; likewise, in voltage clamp (VC), \( I_{\text{uni}} \) followed depolarizing protocols; 2) in CC, successive afterpotentials could sum up to reach firing threshold, which could result in sustained low-frequency firing; likewise, in VC, repetitive depolarizations could produce summation of \( I_{\text{uni}} \) and plateau
currents; and 3) in CC, afterdischarges superimposed to afterpotentials were self-terminating and followed by a period of suppression; likewise, in VC, high-frequency trains of depolarizing pulses were followed by $I_{\text{NCM}}$ down-regulation and transient depression.

We also noted that some neurons (preferentially the stellate cells) during cholinergic modulation could produce prolonged delayed responses of regular, low-frequency firing, whereas others (preferentially pyramidal-like cells) generated more pronounced self-terminating bursts of activity (Klink and Alonso 1997). The Ca$^{2+}$-dependent modulatory properties of $I_{\text{NCM}}$ are consistent with an important role of this current in the generation of these self-terminating responses. On one hand, the ability of elevated $[\text{Ca}^{2+}]_i$ to produce current potentiation can lead to the generation of activity-dependent, prolonged afterdepolarizations. On the other hand, when $[\text{Ca}^{2+}]_i$ rises further secondarily to intense spike discharge, then a slower, transient process of $I_{\text{NCM}}$ inhibition takes place and is followed by refractoriness. The latter properties would necessarily confer hysteresis on the $[\text{Ca}^{2+}]_i$ dependence of inward-current amplitude which, in turn, may lead to oscillation.

Metabotropic activation of non-specific cation conductances by neurotransmitters and peptides has been shown in many types of mammalian brain neurons (Chakfe and Bourque 2000; Congar et al. 1997; Li et al. 1999; Shen and North 1992). In many instances, neuromodulatory actions lead to the development of emergent properties such as bursting oscillations and bistable behavior (Andrade 1991). Ca$^{2+}$-dependent up- and down-regulation of a neurotransmitter-operated cation current may be a widespread mechanism to switch the firing modalities of different brain neuronal populations and the dynamics of the networks in which they are embedded (Marder and Calabrese 1996). In the case of the entorhinal network, cholinergically-induced changes in neuronal dynamics by $I_{\text{NCM}}$ may have profound implications in normal memory functioning (Fransen et al. 2002; Hasselmo et al. 2000; Lisman and Idiart 1995). In addition, given the apparent involvement of muscarinic receptors in the generation of epileptic seizures (Turski et al. 1989), $I_{\text{NCM}}$ may also be a factor contributing to the hyperexcitability of the EC and related temporal lobe structures.

Finally, further cues on the possible role of plasticity in cholinergic-dependent firing patterns are provided by the observation that cholinergic stimuli differentially regulate the intrinsic activity of neurons in superficial and deep layers of the EC. During cholinergic stimulation, EC layer-II principal neurons respond to a short stimulus with a delayed, self-terminating response, which can be suppressed by repetitive stimulus application. In contrast, we have recently shown that pyramidal neurons of EC layer V respond to an input with persistent activity which can be stepped up by repetitive application of
the same input (Egorov et al. 2002). At the network level, EC layers II and V represent the input and output interface, respectively, of the EC-hippocampal system. Whereas sensory input from multiple cortical areas converges upon layer-II cells, which then send associative information to the hippocampus via the perforant path, layer-V neurons receive the hippocampal output and project back to neocortex (Amaral and Witter 1995). Significantly, both EC layer II and layer V receive a dense cholinergic innervation (Alonso and Amaral 1995). Both the importance of the EC-hippocampal system in the “declarative” memory processes (Eichenbaum 2000; Scoville and Milner 1957; Squire 1998), and the particular role of acetylcholine in multiple aspects of plasticity (Hasselmo 1999a; Whitehouse et al. 1982) are well established. We suggest that the behavior of EC layer-II neurons is more appropriate for the formation of short-term associations of on-going sensory information, and that the activity-dependent up- and down-regulation of $I_{NCM}$ may represent a cellular mechanism partly underlying the enhanced and suppressed responses that are frequently observed in parahippocampal neurons during delayed matching tasks (Brown and Aggleton 2001; Suzuki et al. 1997; Young et al. 1997). This firing behavior could be perhaps utilized for the detection of novelty and recency of sensory events (Brown and Aggleton 2001; Fransen et al. 2002) and contribute to recognition memory (Manns et al. 2003a). On the other hand, the graded persistent firing more characteristic of layer-V neurons could constitute the basis of a neural integrator of hippocampal outputs capable of sequentially organizing the distinct elements of a memory trace or, in other words, forming the basis of a tracking device for episodic memories.
ACKNOWLEDGMENTS

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GRANTS

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Table 1. *Solutions*

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<td>2</td>
<td>-</td>
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<th>Cs⁺ MeS</th>
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<th>HEPES</th>
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**TABLE 1 LEGEND**

The composition of the extracellular recording solutions (*Extra soln.*) and intra-pipette solutions (*Intra soln.*) employed in the patch-clamp experiments of the present study is specified. All concentrations are indicated in mmol·l⁻¹. In voltage-clamp experiments, all extracellular solutions were also added with the synaptic blockers, kynurenic acid (1 mM) and picrotoxin (100 μM), the nicotinic-receptor antagonists, mecamylamine (10 μM) and α-bungarotoxin (100 nM), and 300-nM tetrodotoxin. The pH of extracellular solutions was maintained at 7.4 by continuous bubbling with 95% O₂, 5% CO₂. All intracellular solutions were added with 2-mM adenosine 5’-triphosphate (ATP) and 0.4-mM guanosine 5’-triphosphate (GTP). The pH of intracellular solutions was adjusted at 7.2 with KOH (*I1, I2*) or CsOH (*I3-I5*). Abbreviations: Cs⁺ MeS, Cs⁺ methanesulphonate; HEPES, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid]; EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N,N’,N’-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.
FIGURE LEGENDS

FIG. 1. Cholinergic stimulation induces depolarizing afterpotentials (DAPs) and afterdischarges in EC layer-II neurons. A, current-clamp recordings in a representative neuron in the absence (A1) and in the presence (A2) of 5-µM CCh in the perfusing solution. In each subpanel, the upper trace is the voltage recording and the lower trace is the current command. Note that, in the presence of CCh stimulation, suprathreshold depolarizing pulses were followed by a prolonged afterdischarge (A2) that was never observed in control conditions (A1). B, current-clamp recording in another representative neuron in the presence of 5-µM CCh in the perfusing solution. Note that a suprathreshold depolarizing pulse was followed by a pronounced DAP (arrow) on which an afterdischarge superimposed. The cells in A and B were electrophysiologically identified as stellate and pyramidal-like, respectively (see the text for details). Calibration bars in A2 and B: same values as in A1. Recording solutions were E1 and I1.

FIG. 2. CCh-induced DAPs and afterdischarges show summation, activity-dependent suppression, and refractoriness. A, Effects of repetitively-applied suprathreshold depolarizing pulses in a representative neuron in the presence of 20-µM CCh in the perfusing solution. In each subpanel, the upper trace is the voltage recording and the lower trace is the current command. Note that the repetitive application of +50-pA current pulses resulted in DAPs that initially summated, reaching (A1) or not (A2) the threshold for triggering an afterdischarge, and then were suppressed. Calibration bars in A2: same values as in A1. B, Repetitively-applied suprathreshold depolarizing pulses in another representative neuron reveals refractoriness and time-dependent recovery of DAPs and afterdischarges. The recordings were carried out in the presence of 20-µM CCh in the perfusing solution. Calibration bars in B2: same values as in B1.

FIG. 3. Single step depolarizations promote potentiation and down-regulation of CCh-induced inward current in intracellular Cs⁺, while revealing CCh-dependent inhibition of voltage-gated Ca²⁺ currents. A, Current response to CCh application in a representative neuron in the presence of 0.5-mM intrapipette EGTA (recording solutions: E2, I2). Traces in A1 and A2 represent the current recording and the voltage command, respectively. CCh (30 µM) was applied by continuous bath superfusion (during the
time span marked by the horizontal bar). The voltage protocol applied included a holding potential at –50 mV and 100-ms depolarizing steps at 0 mV commanded both before and during the CCh response. 

B, The post-step currents following depolarizing steps 1 and 2 in A (boxes) are shown over an expanded time scale. Note the prominent, post-step potentiation of CCh-induced inward current, which then decayed to levels lower than the pre-step amplitude. C, The currents recorded during depolarizing steps 1 and 2 in A. The 100-ms steps at 0 mV (lower trace) evoked inward currents (upper traces) that were abolished by 200-μM Cd²⁺ (not shown), and were therefore identifiable as voltage-gated Ca²⁺ currents. Note the marked Ca²⁺-current inhibition under CCh.

**FIG. 4.** Post-depolarization potentiation and down-regulation of CCh-induced inward current are sensitive to intracellular-Ca²⁺ buffering. A, Effects of single 500-ms voltage pulses at 0 mV on the current response induced by continuous bath superfusion of CCh (30 μM) in four different neurons, in the presence of 0.5-mM EGTA (A1), 5-mM EGTA (A2), 10-mM BAPTA (A3), or 30-mM BAPTA (A4) in the patch pipette (recording solutions: E2; I2-I5). In each sub-panel, the horizontal bar indicates the period of CCh perfusion, the arrow the time point at which the 500-ms voltage pulse was applied. In the *insets*, the post-step currents following the depolarizing steps (dotted-line boxes) are shown over an expanded time scale (x-axis window: 20 s; y-axis window: 1.05 nA). The horizontal, dotted line in each inset marks the pre-step current level. B, Bar diagram of average peak $I_{NCM}$ amplitude (empty columns) and peak $I_{tail}$ amplitude (hatched columns) observed in the presence of 0.5-mM and 5-mM EGTA, and 5-, 10-, and 30-mM BAPTA in the patch pipette, using the same experimental procedure illustrated for the previous panel. Numbers of observations are specified over each column pair; n.a., not available. C, Bar diagram of the average, percent inward-current increase ($\% \Delta I$) at the peak of $I_{tail}$ following 500-ms voltage steps at 0 mV, in the same experimental conditions of the previous panels. $\% \Delta I$ was calculated as $[I_{tail(peak)} - I_{NCM(pre)}] / I_{NCM(pre)} \times 100$, where $I_{tail(peak)}$ is current amplitude at the peak of $I_{tail}$ and $I_{NCM(pre)}$ is $I_{NCM}$ amplitude just before the application the depolarizing pulse. Numbers of observations are the same as in panel B. One-way ANOVA revealed that the differences between the means of the various groups were statistically highly significant ($p < 10^{-4}$). *** and **, $p < 0.001$ and $p < 0.01$, respectively, with respect to 5-mM EGTA (Bonferroni multiple comparison test). According to the same test, there was also statistical significance in the differences between 5-mM and 30-mM BAPTA, and between 10-mM and 30-mM BAPTA ($p < 10^{-2}$ in both cases). D, Bar diagram of average
$I_{\text{tail}}$ time-to-peak (same experimental conditions and numbers of observation as in the previous panels; n.a., not available). Means were statistically different at the 95.0% level of significance (one-way ANOVA). *, $p < 0.05$ with respect to 5-mM EGTA (Bonferroni multiple comparison test).

**FIG. 5.** Sequentially-applied voltage steps of increasing duration induce $I_{\text{tail}}$s of increasing amplitude and differentially promote inward-current plateaus or down-regulation depending on intracellular-Ca$^{2+}$ buffering. **A**, Effects of three 50-, 100-, and 500-ms voltage pulses at 0 mV on the current response induced by continuous bath superfusion of CCh (30 μM) in three different neurons, in the presence of 5-mM EGTA ($A1$), 10-mM BAPTA ($A2$), or 30-mM BAPTA ($A3$) in the patch pipette (recording solutions: $E2$; $I3-I5$). In each sub-panel, the horizontal bar indicates the period of CCh perfusion, the numbers the time points of application of the 50-, 100-, and 500-ms voltage pulse. In the insets, the post-step currents following the 500-ms depolarizing steps (dotted-line boxes) are shown over an expanded time scale (x-axis window: 20 s; y-axis window: 1.05 nA). The horizontal, dotted line in each inset marks the pre-step current level. **B**, Average, absolute ($B1$) and percent ($B2$) peak inward-current increase [$\Delta I$(peak)] following voltage steps at 0 mV, as a function of step duration. The two quantities were calculated as $\Delta I = I_{\text{tail(peak)}} - I_{(\text{pre})}$, and $\% \Delta I = \Delta I / I_{(\text{pre})} \times 100$, where $I_{\text{tail(peak)}}$ is current amplitude at the peak of $I_{\text{tail}}$ and $I_{(\text{pre})}$ is the current level just before the application each depolarizing pulse. The stimulation protocol applied was the same as illustrated in A. Filled circles: 5-mM EGTA; empty squares: 10-mM BAPTA; filled triangles: 30-mM BAPTA. $n = 3$ (5-mM EGTA), 4 (10-mM BAPTA), and 3 (30-mM BAPTA). ** and * indicate that the means at each pulse duration were statistically different at the 99.0% and 95.0% level of significance, respectively (one-way ANOVA). **C**, Bar diagram of the percent modifications in plateau inward-current level [$\Delta I$(plateau)] following 50-, 100-, and 500-ms voltage steps at 0 mV (same stimulation protocol as in the previous panels). $\% \Delta I$(plateau) was calculated as $[I_{(\text{pl})} - I_{(\text{pre})}] / I_{(\text{pre})} \times 100$, where $I_{(\text{pl})}$ is the plateau current level observed after each depolarizing pulse and $I_{(\text{pre})}$ is the current level just before the same depolarizing pulse. Filled columns: 5-mM EGTA; empty columns: 10-mM BAPTA. Numbers of observations are the same as in panel B. *** and *, $p < 0.001$ and $p < 0.05$, respectively (two-tail $t$ test for unpaired values).
FIG. 6. Voltage-gated Ca\(^{2+}\)-channel blockers and the absence of extracellular Ca\(^{2+}\) reduce or prevent post-depolarization up- and down-regulation of CCh-induced inward current. A, Effects 20-µM nifedipine in the bath. Panel A1 illustrates the effects of a single 500-ms voltage pulse at 0 mV (arrow) on the current response to continuous bath superfusion of CCh (30 µM, horizontal bar) in a representative neuron recorded in the presence of 20-µM nifedipine in the bath. The inset is a detail of the trace stretch delimited by the dotted-line box in the main panel (x-axis window: 20 ms; y-axis window: 660 pA). The horizontal, dotted line in the inset marks the pre-ramp current level. Panel A2 shows the average amplitude of \(I_{\text{NCM}}\) (open bars) and \(I_{\text{tail}}\) (hatched bars) in control conditions (DMSO; \(n = 3\)) and in the presence of nifedipine (Nif.; \(n = 4\)). B, Effects of extracellular-Ca\(^{2+}\) removal. Panel B1 illustrates the effects of a single 500-ms voltage pulse at 0 mV (arrow) on the current response to continuous bath superfusion of CCh (30 µM, horizontal bar) in a representative neuron in 0 extracellular Ca\(^{2+}\) plus 0.5-mM extracellular EGTA. Panel B2 shows the average peak amplitude of \(I_{\text{NCM}}\) (open bars) and \(I_{\text{tail}}\) (hatched bars) in control conditions (\(n = 7\)) and in the absence of extracellular Ca\(^{2+}\) (0 Ca; \(n = 4\)). In all cases illustrated in this figure, 0.5-mM EGTA was present in the patch pipette (recording solutions were \(E_2\) and \(I_2\) in A; \(E_4\) and \(I_2\) in B). Current amplitudes were always measured immediately before the onset (\(I_{\text{NCM}}\)) and 400 ms after the end (\(I_{\text{tail}}\)) of 500-ms voltage pulses at 0 mV.

FIG. 7. Potentiated currents and basal \(I_{\text{NCM}}\) both require the maintenance of the muscarinic stimulus (A) and display the same sensitivity to Co\(^{2+}\) (B). A1, Effects of three 50-, 100-, and 500-ms voltage pulses at 0 mV on the current response induced by long-lasting bath superfusion of CCh (30 µM, horizontal bar) in a representative neuron. A2, Effect of washout of CCh superfusion on the plateau current evoked by a single 500-ms voltage pulse at 0 mV in another representative neuron. In both cases, 10-mM BAPTA was present in the patch pipette (recording solutions: \(E_2\), \(I_4\)). B1, Current recording from a representative neuron in which 30-µM CCh was applied by continuous bath superfusion (empty horizontal bar) in the presence of 2-mM extracellular CoCl\(_2\) (hatched horizontal bar). No \(I_{\text{NCM}}\) response was observed in these conditions. B2, Effect of application of 2-mM extracellular CoCl\(_2\) (hatched horizontal bar) on the plateau current evoked by a 500-ms voltage pulse at 0 mV during an \(I_{\text{NCM}}\) response induced in another representative neuron by continuous bath superfusion of CCh (30 µM;
empty horizontal bar). In both cases, 10-mM BAPTA was present in the patch pipette (recording solutions: \textit{E2, I4}). The arrows indicate the time points at which 500-ms voltage pulses at 0 mV were applied.

**FIG. 8.** Current-voltage relation of basal \( I_{\text{NCM}} \) and potentiated currents. \( A \), In a representative neuron an \( I_{\text{NCM}} \) response was evoked by continuous bath superfusion of CCh (30 \( \mu \)M; horizontal bar), then an \( I_{\text{tail}} \) and a plateau current were elicited by a 500-ms voltage pulse at 0 mV. 10-mM BAPTA was present in the patch pipette (recording solutions: \textit{E2, I4}). To determine the voltage dependence of CCh-induced currents, 1-s voltage ramps (50 mV/s) were commanded in control conditions, at the peak of the \( I_{\text{NCM}} \) response, and during the post-depolarization current potentiation. Traces in \( A1 \) and \( A2 \) represent the current recording and the voltage command, respectively. \( B \), The currents recorded in response to the ramp protocols labeled 1, 2, and 3 in \( A \). \( C \), The currents obtained by subtracting the control ramp current (labeled 1 in \( B \)) from the \( I_{\text{NCM}} \) ramp current (labeled 2 in \( B \)), and the \( I_{\text{NCM}} \) ramp current from the \( I_{\text{tail}} \) ramp current (labeled 3 in \( B \)), as a function of ramp voltage. The straight lines are linear regressions to experimental data, which returned slope conductance values of 1.49 nS (2 – 1) and 2.88 nS (3 – 2), and \( x \)-axis intercept values of –2.2 mV (2 – 1) and +12.8 mV (3 – 2).

**FIG. 9.** Different protocols of repetitive stimulation can result in CCh-induced inward-current potentiation and/or down-regulation. \( A \), Single and repetitive step depolarizations promote potentiation and down-regulation, respectively, of CCh-induced inward-current in intracellular K\(^+\). The main panel shows the current response to CCh application recorded in a representative neuron in the presence of 0.5-mM intra-pipette EGTA (recording solutions: \textit{E3, I1}). The upper and lower traces represent the current recording and the voltage command, respectively. CCh (30 \( \mu \)M) was applied by continuous bath superfusion (during the time span marked by the horizontal bar). The voltage protocol applied included a holding potential at –50 mV and 500-ms depolarizing steps at +20 mV commanded both before and during the CCh response (1, 2), plus a 38.5-s pulse train (3) consisting of 39 of the same steps delivered at 1 Hz. In the inset, the post-step currents following depolarizing steps labeled 1 and 2 in the main panel are shown over an expanded time scale. Note the prominent, post-step inward-current potentiation under CCh. Note also, in the main panel, the prominent depression of CCh-induced inward current in the post-train period. \( B \), Trains of depolarizing voltage steps can promote transient and/or
steady potentiation of CCh-induced inward current depending on the stimulation pattern. The main panel shows the effects of the application of five pulse trains of variable frequencies consisting of 400-ms depolarizing steps at various voltage levels during a CCh-evoked $I_{NCM}$ response in a different neuron. Each train consisted of 9-18 pulses at +20 mV (trains 1-3), –20 mV (train 4), and –30 mV (train 5). The upper and lower traces in the main panel represent the current recording and the voltage command, respectively. In the insets, the currents recorded during the five trains are shown over an expanded time scale (calibration bars: 20 pA, 5 s). The experiment was performed in the presence of K$^+$ as the main intracellular cation and 0.5-mM intra-pipette EGTA (recording solutions: $E3$, $I1$).
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Magistretti et al.,
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Magistretti et al.,
Fig. 4
Magistretti et al.,
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Magistretti et al.,
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Magistretti et al.,
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Magistretti et al.,
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Magistretti et al.,