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Muscarinic and β-adrenergic depression of the slow Ca^{2+}-activated potassium conductance in hippocampal CA3 pyramidal cells is not mediated by a reduction of depolarization-induced cytosolic Ca^{2+} transients

(afterhyperpolarization/fura-2/voltage clamp)

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ABSTRACT **Combined intracellular and microfluorometric recording techniques were used to evaluate whether the inhibition by cholinergic or adrenergic transmitters of the Ca^{2+}-activated potassium current (I_{AHP}) in hippocampal CA3 pyramidal cells was mediated by an alteration of depolarization-induced change in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Low concentrations of isoproterenol (1–10 μM) and muscarine (0.25–1 μM) reversibly abolished I_{AHP} without affecting concomitant Ca^{2+} transients or the steady-state $[Ca^{2+}]_i$. Only after application of higher concentrations of muscarine, $[Ca^{2+}]_i$ increased; in the presence of potassium channel blockers, muscarine depressed Ca^{2+} currents and concomitant Ca^{2+} transients. These observations provide direct evidence that the inhibition of I_{AHP} by isoproterenol and muscarine are not mediated by an alteration of Ca^{2+} dynamics.**

Hippocampal pyramidal cells show a prolonged, Ca^{2+}-dependent K^+ afterhyperpolarization (AHP) (1–4) and aftercurrent (I_{AHP}; ref. 5) after single or repetitive action potentials. This AHP is blocked by removing external Ca^{2+} or by adding a divalent Ca^{2+}-channel blocker, or by intracellular injection of Ca^{2+} chelators and, hence, has been attributed to an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) after entry of Ca^{2+} through voltage-gated Ca^{2+} channels.

I_{AHP} can be readily inhibited by several neurotransmitters, including norepinephrine (6, 7) and acetylcholine (8–12). The action of norepinephrine is mediated by a β-adrenergic receptor and can be replicated by isoproterenol (13). Its effect probably results from the activation of adenylate cyclase and consequent formation of cAMP, since it can also be replicated by cAMP derivatives or by forskolin and the action of norepinephrine is potentiated by phosphodiesterase inhibitors and blocked by an adenylate cyclase inhibitor (14). The effect of acetylcholine is mediated by muscarinic receptors, probably of the M_1 subtype (15). Its action might involve the activation of protein kinase C by diacylglycerols formed from the hydrolysis of membrane phospholipids by phospholipase C (16, 17), since I_{AHP} is inhibited by exogenous activators of protein kinase C such as phorbol esters (18, 19). An alternative messenger for acetylcholine could be cGMP since cholinergic agonists increase cGMP levels in rat hippocampus (20) and 8-bromo-cGMP has been reported to reduce the Ca^{2+}-activated AHP in hippocampal pyramidal cells (21).

The mechanism of I_{AHP} inhibition by norepinephrine and acetylcholine is unknown. Norepinephrine does not appear to reduce the Ca^{2+} current; on the contrary, it may be enhanced (13, 22). Norepinephrine might therefore modify either the cytoplasmic Ca^{2+} change produced by the transmembrane Ca^{2+} current or the response of the K^+ channels to the presumed increment in cytosolic Ca^{2+}. The effect of acetylcholine is even less clear. Initial experiments suggested that acetylcholine, like norepinephrine, did not inhibit the Ca^{2+} current (as measured from the Ca^{2+} spike recorded in tetrodotoxin/tetraethylammonium solution; ref. 21) but some subsequent observations have shown that Ca^{2+} currents in hippocampal pyramidal neurons recorded under voltage clamp can be inhibited both by muscarinic agonists (23, 24) and by the compounds previously used to test for possible second messengers, 8-bromo-cGMP and phorbol esters (25). Hence, acetylcholine might modify Ca^{2+} entry, cytosolic Ca^{2+} concentrations, or the K^+ current itself.

To test some of these possibilities, we have measured the cytosolic Ca^{2+} transients accompanying I_{AHP} in single voltage clamped hippocampal CA3 neurons by using the Ca^{2+} indicator fura-2 (26). We find that neither norepinephrine nor acetylcholine reduced these transients in concentrations that eliminate I_{AHP} and hence conclude that their primary effect is to inhibit the K^+ current *per se*.

MATERIALS AND METHODS

Hippocampal Slice Cultures. Slices of hippocampus (400 μm thick) were prepared from 5- to 7-day-old rat pups and were cultured by means of the roller tube technique as described (27–29).

Electrophysiological and Microfluorometric Recordings. For electrophysiological studies, cultures were transferred to a perfused temperature-controlled (32°C) chamber, which was mounted on the stage of an inverted microscope. The composition of the perfusate was as follows: 156 mM Na^+, 2.7 mM K^+, 159 mM Cl^-, 3.8 mM Ca^{2+}, 2.5 mM Mg^{2+}, 11.6 mM HCO_3^-, 0.4 mM $H_2PO_4^-$, 5.6 mM D-glucose. CA3 pyramidal cells were impaled with thin-walled microelectrodes, the very tip of which was filled with a solution containing 1 mM fura-2 and 150 mM potassium acetate, and the shank was back-filled with 2 M potassium methylsulfate (pH 7.4). After injection of the dye, the electrode resistance dropped from an initial value of 150–200 MΩ to ≈50 MΩ (the normal resistance with $KMeSO_4$ alone), thus allowing switched current- and voltage-clamp recordings to be made with an Axoclamp-2 amplifier (Axon Instruments, Burlingame, CA). Voltage clamp was achieved by using switching frequencies from 2–3 kHz and a current gain of 2.5–5 nA/mV. For fura-2 epifluorescence measurements, a mercury arc lamp driven by a stabilized DC power supply was used. Excitation was ac-

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Abbreviations: AHP, afterhyperpolarization; I_{AHP}, Ca^{2+}-dependent K^+ aftercurrent; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration.

complished by means of a shutter, excitation filters (either an interference filter centered around 360 nm or a broad-band filter centered around 380 nm), heat filter, and a ×40 Zeiss Neofluar objective. Fluorescence from a 35 × 35 μm area centered around the soma of the injected cell was projected through a 470-nm long-pass filter on one of a 10 × 10 array of photodiodes. Lack of dye filling of neighboring cells was ascertained visually. A very low noise current/voltage converter for the output of this photodiode was positioned directly behind the array. The output of this circuit was fed into a high-gain amplifier. Fluorescence and microelectrode signals were recorded on a chart recorder and fed into a data acquisition system for off-line analysis and reproduction.

Calculation of Ca^{2+} Signals. The calculation of $[Ca^{2+}]_i$ from fura-2 fluorescence recordings takes advantage of the difference in the excitation spectra of fura-2 in the free and Ca^{2+}-bound form. The ratio R of fura-2 fluorescence obtained at two different excitation wavelengths is independent of the fura-2 concentration and allows the Ca^{2+} concentration to be calculated according to the following equation (26):

$$[Ca^{2+}]_i = k^*(R - R_{min})/(R_{max} - R). \quad [1]$$

The values of the constant k^* as well as R_{min} and R_{max} (the limiting values of the fluorescence ratios at low and high Ca^{2+}) depend on the apparatus used. These calibration constants were evaluated by measuring the fura-2 fluorescence ratios from calibration solutions, which were either suspended in paraffin oil as neuron-sized droplets or filled into glass tubes. The calibration solution contained 110 mM K^+, 10 mM Na^+, 1 mM Mg^{2+}, 5 mM Hepes, 10 μM fura-2, 5 mM Ca^{2+} buffer 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,-*N*′-tetraacetic acid (BAPTA), and various concentrations of free Ca^{2+} (free $[Ca^{2+}]$ was calculated by using a K_d of 107 nM for BAPTA). For calculation of $[Ca^{2+}]_i$ transients, we chose an approach that takes advantage of the linear relation of the fluorescence intensities ($F1$, $F2$) obtained at two wavelengths ($f1$, $f2$):

$$F2 = mF1 + q. \quad [2]$$

The constant m is independent of the fura-2 concentration and was determined from Ca^{2+} transients repeatedly monitored at $f1$ and $f2$. Before and after each pharmacological experiment, the fluorescence ratio $F1°/F2°$—corresponding to resting Ca^{2+}—was measured, from which q was calculated. The time course of fura-2 fluorescence was recorded at wavelength $f1$ and Ca^{2+} transients were calculated from Eqs. 1 and 2:

$$[Ca^{2+}]_i = k^* \frac{F1 - R_{min}[m(F1 - F1°) + F2°]}{R_{max}[m(F1 - F1°) + F2°] - F1}. \quad [3]$$

RESULTS

Depolarizations Induce $[Ca^{2+}]_i$ Transients and Slow AHPs. Fig. 1 shows combined electrophysiological and optical recordings from a CA3 pyramidal cell injected with fura-2. Action potentials induced by depolarizing current injections were followed by a slow AHP of similar duration and amplitude to those previously reported (see Introduction). This was accompanied by an increase in cytosolic Ca^{2+}, which began during the action potential train and lasted for the duration of the AHP. Hence, the amounts of fura-2 injected were sufficiently low so as not to buffer the increase in Ca^{2+} and thus inhibit the AHP (cf. refs. 2, 3, and 30).

For subsequent work with transmitters, propagated action potentials were suppressed with tetrodotoxin (to preclude indirect effects) and the AHP current was generated and recorded under voltage clamp by applying a short (40–100 ms) depolarizing voltage step (Fig. 1*B*; cf. ref. 5). Under these conditions, I_{AHP} is seen as a slow outward aftercurrent, of similar time course to the AHP following an action potential train. I_{AHP} could be reversed at the expected K^+ equilibrium potential under our conditions (data not shown; cf. ref. 5), demonstrating that I_{AHP} is generated by a K^+ conductance. The induction of I_{AHP} was accompanied by an increase in cytosolic Ca^{2+}, peaking slightly earlier and lasting somewhat longer than the outward current. Parallel changes in Ca^{2+} transients and I_{AHP} occurred on varying the Ca^{2+} load either by varying the voltage command or by changing extracellular Ca^{2+} (Fig. 1*B*).

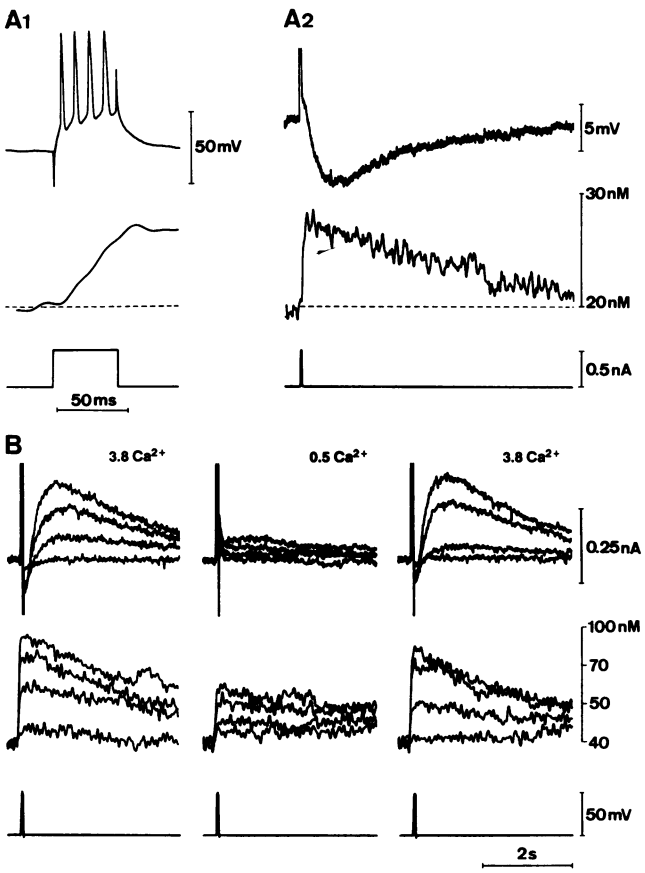

FIG. 1. Combined intracellular recordings and microfluorometric measurements of cytosolic free Ca^{2+} in a hippocampal CA3 pyramidal cell. (*A*) Action potentials evoked by injection of a current pulse were followed by a slowly decaying AHP and induced a transient increase in $[Ca^{2+}]_i$. Reproduction at fast (*A1*) and slow (*A2*) time scale of membrane potential (upper trace), $[Ca^{2+}]_i$ (middle trace), and injected current (lower trace). (*B*) In the presence of 1 μM tetrodotoxin a voltage-clamped CA3 pyramidal cell (holding potential, −53 mV) was subjected to 100-ms voltage steps to −33, −23, −13, and −3 mV. Voltage steps to −23 mV or more positive values induced an outward aftercurrent (upper recordings) and a transient increase in $[Ca^{2+}]_i$ (middle recordings). Reducing the Ca^{2+} concentration in the recording solution from 3.8 mM (*Left*) to 0.5 mM (*Center*) reversibly (*Right*) depressed both the aftercurrent and the concomitant Ca^{2+} transients. Note nonlinear scale for $[Ca^{2+}]_i$.

Ca^{2+} Transients Are Unchanged During β-Adrenergic Depression of I_{AHP}. The effect of the selective β-adrenergic receptor agonist isoproterenol was tested on the depolarization-induced outward aftercurrents and the concomitant Ca^{2+} transients. Bath application of 1–2.5 μM isoproterenol for 1–2 min reversibly depressed the I_{AHP} without affecting the evoked calcium transient or the resting calcium concentration (Fig. 2*A*). This effect of isoproterenol was consistently observed in the eight neurons tested. Analysis of the current–voltage relationship of the cell measured at the peak of I_{AHP}

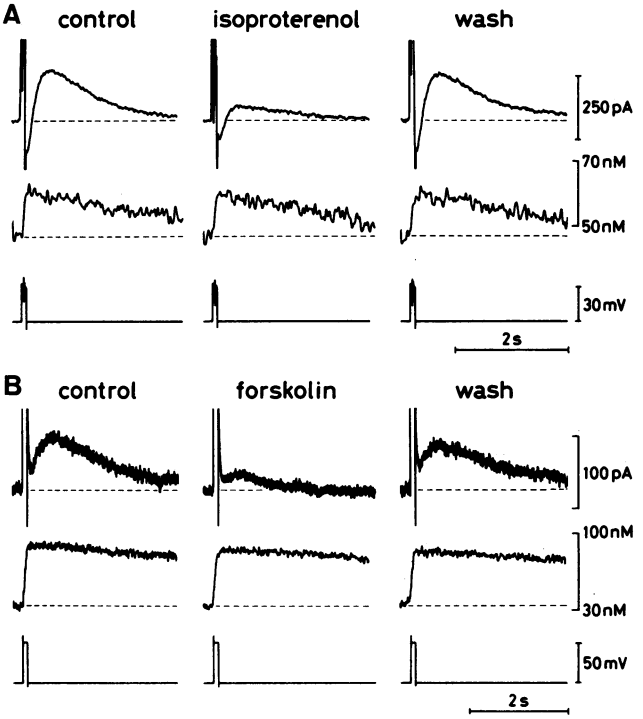

FIG. 2. Effect of isoproterenol and forskolin on depolarization-induced slow aftercurrents and on cytosolic $[Ca^{2+}]$. CA3 pyramidal cells were superfused with a solution containing 1 μM tetrodotoxin and voltage clamped at −55 mV. Voltage jumps of 100 ms duration to ≈0 mV induced slow outward aftercurrents and Ca^{2+} transients. Clamp current (upper traces), $[Ca^{2+}]_i$ (middle traces), and voltage (lower traces) before (control), during, and 20 min (*A*) or 15 min (*B*) after (wash) application of 1.25 μM isoproterenol (*A*) and 5 μM forskolin (*B*).

revealed that the effect of isoproterenol was to reduce a K^+ conductance (data not shown). Depression of the slow aftercurrent by isoproterenol was not accompanied by either a shift in steady-state holding current or a small inward current. Like isoproterenol, norepinephrine reversibly depressed the aftercurrent without affecting the dynamics of cytosolic free Ca^{2+} (n = 3).

The β-adrenergic effect appears to be mediated by an increase of cAMP (see Introduction). This hypothesis is supported by experiments with forskolin, an activator of adenylate cyclase. Like activation of β-adrenergic receptors, 5–10 μM forskolin applied for ≈1 min reversibly depressed the slow aftercurrent without affecting Ca^{2+} transients (n = 2; Fig. 2*B*).

Muscarine Can Depress the Slow AHP Without Affecting Ca^{2+} Transients. Bath application of low concentrations of muscarine (0.25–1 μM for 30–60 s) was followed by a depression of the depolarization-induced slow aftercurrent. These low doses of muscarine could fully abolish I_{AHP} without affecting the calcium transient or the resting calcium concentration (n = 5; Fig. 3). In contrast to the actions of β-adrenergic receptor activation, muscarine always induced an inward current, which was, however, small with these relatively low concentrations of muscarine (see Fig. 3). The spike AHP was preceded by an afterdepolarization (Fig. 1*A*). Under voltage clamp, this was frequently revealed as an inward current preceding I_{AHP} (see Figs. 1*B*, 2, 3, and 4*B*). In the presence of low concentrations of muscarine, this inward current was exaggerated (Fig. 3). An afterdepolarization in the presence of muscarinic agonists has previously been noted in hippocampal cells (8, 9) and also in cortical Betz cells where it has been attributed to a Ca^{2+}-activated nonspecific cation conductance (31). The present observation supports this view that it might be Ca^{2+}-activated since it coincided with the peak of the Ca^{2+} transient.

Higher Concentrations of Muscarine Induce Alterations of Cytosolic Ca^{2+}. Higher concentrations of muscarine (1–100 μM) not only depressed I_{AHP} but also induced a pronounced inward current of up to 0.5 nA. This inward current appeared immediately after the onset of the muscarine application and typically exhibited a biphasic time course consisting of a larger fast component and a smaller slow component (cf. ref. 32). During this inward current, $[Ca^{2+}]_i$ dynamics were significantly altered. A typical experiment is shown in Fig. 4. In parallel with the inward current, cytosolic Ca^{2+} increased and the Ca^{2+} transients increased in amplitude. The inward current and the Ca^{2+} signals dropped to initial values after a wash of ≈5 min. The depolarization-induced outward aftercurrents recovered, however, only after ≈15 min.

Prolonged Application of Muscarine Reduces Ca^{2+} Currents. We have previously noted that muscarine can reduce the Ca^{2+} current in these neurons (23). In the experiments described above, muscarine did not reduce the Ca^{2+} transient evoked by depolarizing steps in normal recording solution,

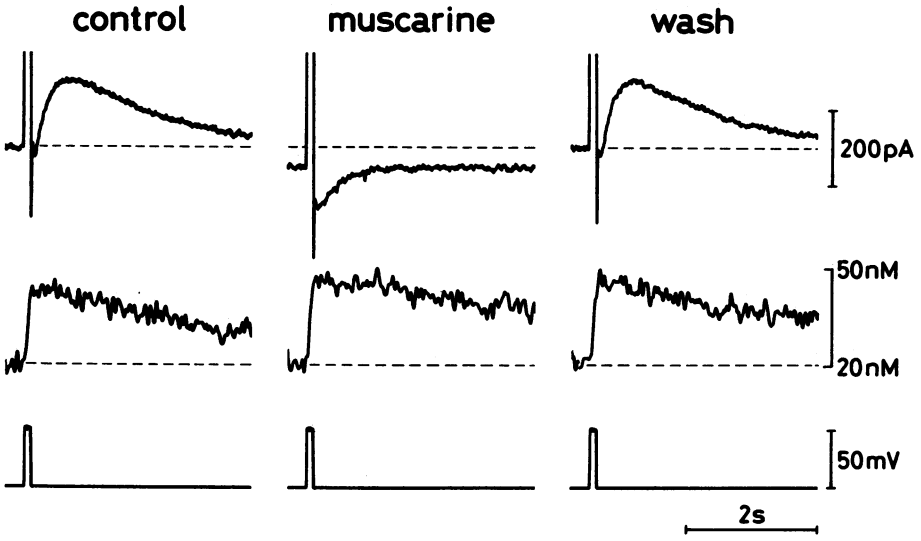

FIG. 3. Low concentration (0.25 μM) of muscarine inhibits I_{AHP} but not the Ca^{2+} transient. Voltage jumps of 100 ms duration to −5 mV induced outward tail currents in a cell voltage clamped at −55 mV. Clamp current (upper traces), $[Ca^{2+}]_i$ (middle traces), and voltage (lower traces) before (control), during, and 5 min after (wash) application of muscarine.

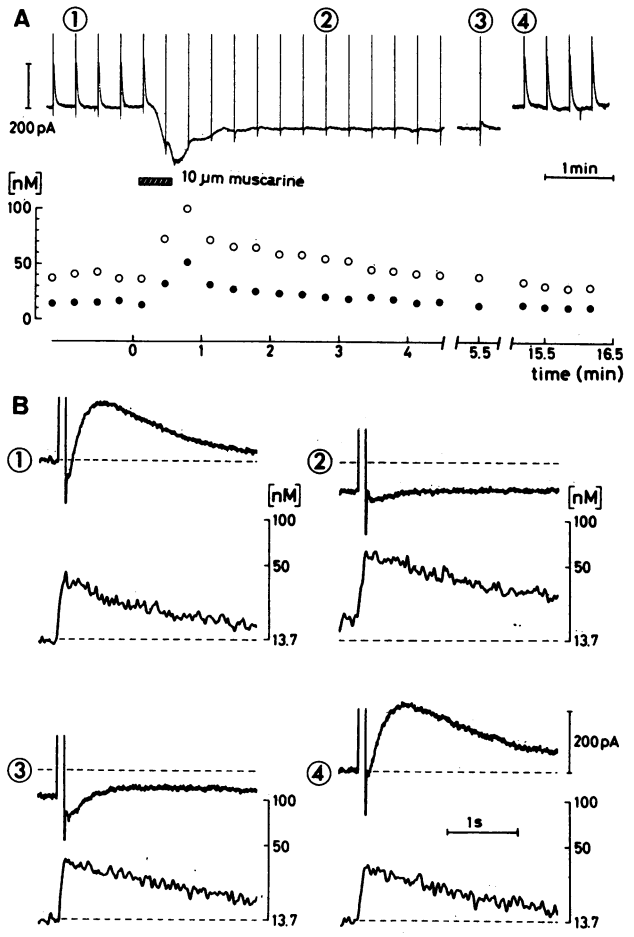

FIG. 4. Effect of higher concentrations of muscarine on $[Ca^{2+}]_i$. Outward slow tail currents were induced by 100-ms voltage jumps from a holding potential of −50 mV to 0 mV at 0.05 Hz in a CA3 pyramidal cell bathed in 1 μM tetrodotoxin. (*A*) Recording of clamp current (upper trace), time course of $[Ca^{2+}]_i$, and time course of peak amplitudes of Ca^{2+} transients (lower plots). (*B*) Records of clamp currents, cytosolic Ca^{2+} transients, and voltage obtained at times 1–4 indicated in *A*.

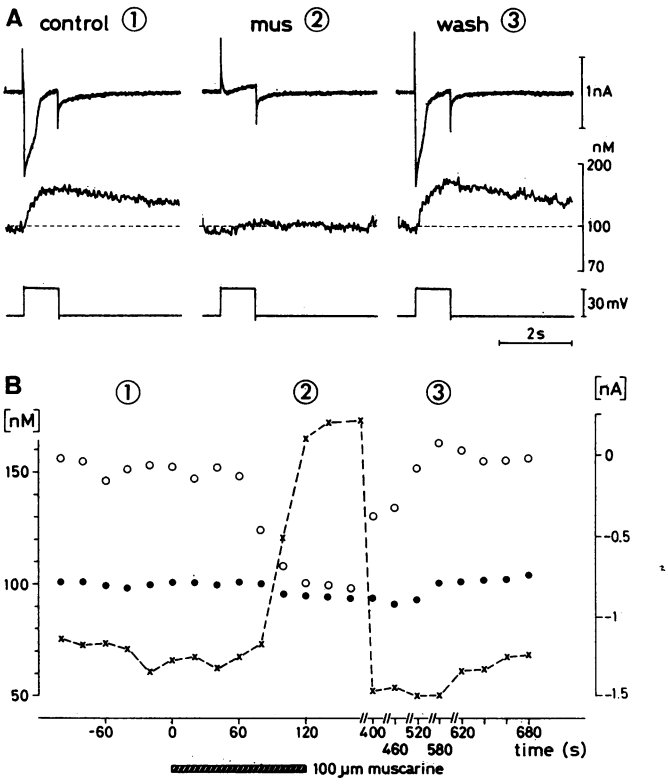

FIG. 5. Effect of higher concentrations of muscarine on Ca^{2+} currents recorded when potassium currents were depressed. A CA3 pyramidal cell was voltage clamped at −40 mV and superfused with a solution containing 1 μM tetrodotoxin, 10 mM tetraethylammonium, and 1 mM Ba^{2+}. (*A*) Currents and Ca^{2+} transients evoked by 1-s voltage commands to −10 mV at 0.05 Hz before (control, 1), during (mus, 2), and after (wash, 3) application of 100 μM muscarine (a high concentration of muscarine was necessary to overcome the partial M-receptor block by tetraethylammonium; see ref. 23). Upper records, current (inward current downward); middle records, Ca^{2+} signal; lower record, voltage. (*B*) Time course of the effects on peak inward currents (×), steady-state $[Ca^{2+}]_i$ (●), and peak amplitude of evoked Ca^{2+} transients (○). Circled numbers refer to the records shown in *A*.

although an occasional reduction occurred at a late stage of muscarine perfusion. Hence, we tested the effect of muscarine under conditions previously used to record Ca^{2+} currents—that is, after reduction of K^+ conductances with 10 mM tetraethylammonium and 1 mM Ba^{2+}. Inward currents evoked by depolarizing commands in this solution were accompanied by transient increases in cytosolic Ca^{2+} (Fig. 5), showing that they were due (in part at least) to inward Ca^{2+} currents. Furthermore, muscarine was clearly capable of reducing both the inward current and the consequent increase in $[Ca^{2+}]_i$ (Fig. 5; $n = 3$). However, three features differentiated this effect from those recorded in normal recording solution. First, the resting cytosolic Ca^{2+} concentration was appreciably higher. Second, higher concentrations of muscarine were required. Third, the effect was slower in onset (lag time, 40–120 s) than the depression of the AHP current or inward current generation (cf. Fig. 4).

DISCUSSION

The primary purpose of the present experiments was to determine whether the inhibition of the Ca^{2+}-activated AHP current produced by β-adrenergic receptor agonists or muscarinic agonists could be ascribed to a reduction of the "Ca^{2+} load." Our results suggest not: both β-agonists (isoproterenol and norepinephrine) and muscarine (at low concentrations) could reduce or suppress I_{AHP} without any clear change in either the peak amplitude or the time course of the underlying Ca^{2+} transient as measured with fura-2. Hence, we would conclude that the depression of I_{AHP} produced by these agonists results primarily from an inhibition of the K^+ current itself or of the ability of Ca^{2+} to activate the current, rather than from a change in Ca^{2+} influx or sequestration. This substantiates the initial suggestion of Madison and Nicoll (6, 13) regarding the action of β-agonists; and the comparable action of forskolin also supports their view (14) that the β-adrenergic effect results from activation of adenylate cyclase.

Notwithstanding, it is also clear that the action of muscarine is more complex than that of the β-adrenergic receptor agonists in two respects: at higher concentrations than those required to inhibit I_{AHP}, it produced an inward current and inhibited the Ca^{2+} current; and these effects were accompanied by an increase in resting cytosolic Ca^{2+} concentration and an inhibition of the Ca^{2+} transients, respectively.

The inward current produced by muscarine has been reported many times previously and has been attributed to inhibition of at least two species of K^+ current: the voltage-dependent M current (33, 34) and a voltage-independent leak current (12, 35). Since I_{AHP} was inhibited at concentrations of muscarine below those producing a substantial inward current, and since norepinephrine and isoproterenol could in-

hibit I_{AHP} without producing an inward current, it seems unlikely that I_{AHP} contributed substantially to the resting K^+ currents or that its inhibition contributed to the inward current produced by muscarine. In our experiments, the inward current appeared biphasic, with an initial peak and slow sustained phase, and the accompanying increase in cytosolic Ca^{2+} was also biphasic. Pitler *et al.* (32) have recently described a biphasic depolarization of hippocampal neurons by acetylcholine, the first phase of which was reduced when external Ca^{2+} was replaced with Mn^{2+}, and hence appeared to be "Ca^{2+} dependent." Kudo *et al.* (36) have also described a biphasic increase in cytosolic Ca^{2+} produced by acetylcholine in fura-2 ester-loaded (unclamped) hippocampal cells in culture: they suggested that the initial phase was due to activation of a Ca^{2+} entry pathway, whereas the later phase resulted from release of stored intracellular Ca^{2+}. Our experiments do not shed any light on this suggestion. Moreover, although the time course of the inward current and Ca^{2+} increase were strikingly similar, this does not mean that Ca^{2+} induces the inward current: it is equally possible that Ca^{2+} entry might be induced through an increased voltage-dependent Ca^{2+} conductance consequent upon the inward current in regions remote from the point clamp. It may be relevant that agonist-induced inhibition of I_M in sympathetic neurons can also be accompanied by an elevation of cytosolic Ca^{2+} without any direct causal relationship between the two (37).

We have confirmed previous observations (23, 24) that, when K^+ currents are blocked, muscarine can inhibit the Ca^{2+} current. Since this occurred in the absence of any increase in resting cytosolic Ca^{2+}, it cannot be due to Ca^{2+}-induced $I_{Ca^{2+}}$ inactivation; instead, it presumably reflects some effects on Ca^{2+} channel activity itself. When $I_{Ca^{2+}}$ was inhibited under these conditions, then Ca^{2+} transients were suppressed. However, this does not contribute to the inhibition of I_{AHP} since higher concentrations of muscarine were needed and the effect was much more delayed in onset. Toselli and Lux (24) have reported that the inhibition of $I_{Ca^{2+}}$ in cultured dissociated neurons by acetylcholine is blocked by preincubation with pertussis toxin. In contrast, inhibition of I_{AHP} and I_M by muscarine is resistant to pertussis toxin (15). Hence, inhibition of $I_{Ca^{2+}}$ probably involves a different receptor subtype—perhaps in low abundance (m_4; see ref. 38)—coupling to a different GTP-binding protein than that mediating inhibition of I_{AHP}.

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