Acetylcholine raises excitability by inhibiting the fast transient potassium current in cultured hippocampal neurons

(A-current/muscarinic agonists/4-aminopyridine/whole-cell recording)

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ABSTRACT The effects of acetylcholine on cultured hippocampal neurons were investigated by using the whole-cell version of the patch-clamp technique. The CA1 region of the hippocampus was excised from brain slices of young rats (12–19 day old), incubated in a papain solution, and dissociated. Neurons were plated on a glial feeder layer. The experiments were conducted mostly on neurons cultured for 2–6 days. Upon depolarization under voltage clamp, these cells exhibited a fast transient outward current (A-current), which was inhibited by 4-aminopyridine (2.5 mM). Acetylcholine (0.1 μM) also inhibited this A-current, as did the muscarinic agonists bethanechol and muscarine. As expected from their inhibition of the A-current, acetylcholine and 4-aminopyridine both increased the amplitude of the action potential and prolonged its duration. We conclude that the inhibition of the A-current constitutes a mechanism by which acetylcholine exerts its excitatory influence on hippocampal neurons.

Neuronal excitation produced by the muscarinic action of acetylcholine (AcCho) is an important cellular event for brain function, but details of its ionic mechanism are not fully understood. In 1971 Krnjević et al. (1) investigated the muscarinic action of AcCho on cortical neurons by using intracellular microelectrodes, and they concluded that AcCho probably produces its excitatory action by reducing both resting and delayed potassium currents. Recently, Brown and Adams (2) and Halliwell and Adams (3) analyzed the muscarinic action under voltage clamp and explained muscarinic excitation as being due primarily to an inhibition of a potassium current, which they named the M-current.

We have investigated the action of cholinergic agonists on cultured hippocampal neurons by using the whole-cell patch-clamp technique. Our results show that cholinergic agonists inhibit the fast transient potassium current, which was designated as the A-current by Connor and Stevens (4). This inhibition of the A-current would play a role in cholinergic excitation. The effect of AcCho resembles that of 4-aminopyridine (APD), which has been shown to be a relatively specific inhibitor of the A-current (5). Previously, it has been shown that thyrotropin releasing hormone (6), noradrenaline (7), and the intracellular level of cyclic AMP (8, 9) may modify neural activity via their effects on A-current.

MATERIALS AND METHODS

Culture. The culture method was similar to that developed for dissociated cell cultures of locus coeruleus (10) and nucleus basalis (11). Young rats (Long–Evans, postnatal, 12–19 day old; Charles River Breeding Laboratories) rather than newborn or embryonic rats were used in the main, because it is known that muscarinic receptors in the hippocampus begin to increase after the birth (12). Coronal sections (400 μm thick) of brains were obtained with a vibratome. From these brain slices, the CA1 region of hippocampus was excised under a dissecting microscope. We began the work by using trypsin dissociation (10, 11), but we soon realized that the papain treatment (13) yielded a healthier culture, particularly when carried out with cells from young rather than newborn animals. Thus, the main work was done using the papain treatment. The dissected fragments were incubated twice (15 min each) in an oxygenated L-15 culture medium (pH 7.3) containing papain (12 units/ml), dl-cysteine (0.2 mg/ml), and bovine serum albumin (0.2 mg/ml) at 37°C. Tissue fragments were then dissociated by trituration in modified Eagle's minimal essential culture medium with Earle's salt, L-glutamine (0.292 mg/ml), glucose (6 mg/ml), NaHCO3 (3.7 mg/ml), l-ascorbic acid (10 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% heat-inactivated rat serum (14). In some cultures, 10% fetal bovine serum and 10% heat-inactivated horse serum were used instead of rat serum. We used a small well at the center of a Petri dish as a culture chamber, and the dissociated neurons were plated on a feeder layer of glial cells (11). Most experiments were conducted on materials cultured for 2–6 days, but in some experiments materials cultured for up to 27 days were used.

Electrophysiology. The techniques were similar to those described (11). We used the whole-cell version of the patch-clamp method (15). During the experiments, the culture was superfused continuously with oxygenated Krebs solution containing 148 mM (or 145.5 mM) NaCl, 2.5 mM (or 5 mM) KCl, 2.4 mM CaCl2, 1.3 mM MgCl2, 5 mM Hepes NaOH buffer, 11 mM glucose (pH 7.4). The patch pipette was filled with either internal solution I (0.5 mM EGTA Ca buffer) or internal solution II (5 mM EGTA, Ca-free). Internal solution I was 120 mM potassium aspartate/40 mM NaCl/5 mM Hepes/KOH buffer/0.5 mM EGTA/KOH/0.25 mM CaCl2/3 mM MgCl2/2 mM Na2ATP, pH 7.1–7.2. Internal solution II was 115 mM potassium aspartate/40 mM NaCl/5 mM Hepes/KOH/5 mM EGTA/KOH/3 mM MgCl2/2 mM Na2ATP, pH 7.1–7.2. Drugs were applied by pressure ejection (16) from micropipettes with a tip diameter of 4–5 μm placed within 15–20 μm of the neuronal soma surface. The values of membrane potentials were corrected for a 9-mV liquid junction potential between the internal solution and the bathing solution. The bath temperature near the neuron was kept at 30°C–32°C.

RESULTS

The neurons that we used from the CA1 area of the hippocampus were ≈20 μm in diameter and were pyramidal, fusiform, or multipolar. Previously, it has been shown that hippocampal neurons in brain slices or in culture exhibit fast transient outward currents (A-currents; refs. 17–19). Our

Abbreviations: AcCho, acetylcholine; APD, 4-aminopyridine.
AcCho (0.1 μM) and APd (2.5 mM) suppress the fast transient outward currents in a cultured hippocampal neuron. The culture was superfused with Krebs solution containing tetrodotoxin (1 μM), and the cell was voltage-clamped by the whole-cell clamp technique. The pipette contained internal solution II (5 mM EGTA). The upper records are currents and lower records are potentials. The outward currents were activated repeatedly, by steps, to -35 mV from a holding potential of -90 mV. The resting potential of the cell was -78 mV. The bars indicate the time while the drug was pressure-ejected from the pipette. The drug pipettes were initially located far away from the soma. The pipette was brought near the soma (within 20 μm) immediately before the drug ejection was started. After the end of the ejection, the drug pipette was again removed from the vicinity of the cell. Note that the transient outward currents began to be suppressed just before the start of the APd ejection: this is due to a leak of APd from the approaching pipette.

cultured neurons from young rats also exhibited a fast transient outward current under the whole-cell patch clamp. In Fig. 1 the cell was depolarized repeatedly from a holding potential of -90 mV to the level of -35 mV under voltage clamp. With the slow time base used in Fig. 1, the fast transient outward currents are seen as spike-like upstrokes, which rapidly decline, followed by more slowly decaying outward currents. The application of APd (2.5 mM) or AcCho (0.1 μM) produced a substantial reduction of the transient outward current and a much smaller reduction of the delayed outward current (Fig. 1).

In Fig. 2 the outward currents evoked by three different depolarizations are displayed. In the control solution, depolarizations to -43 mV or to -25 mV evoked large outward currents, reaching a peak at 6–11 msec and then declining rapidly with a time constant of 15–18 msec (Fig. 2A2 and A3). Records in Fig. 2B and C illustrate the effects of AcCho (0.1 μM) or APd (2.5 mM) on these outward currents. At a moderate depolarization (-43 mV), the fast transient outward currents were almost completely eliminated by either AcCho or APd (Fig. 2B2 and C2), but at a higher depolarization (-25 mV), the inhibition of the transient outward currents by these drugs was not complete (Fig. 2B3 and C3). To measure AcCho- or APd-sensitive currents, digital subtraction of currents in the presence of AcCho or APd from the control currents was used (Fig. 2D and E) (cf. ref. 19 for the APd-sensitive currents). The time courses of the AcCho-sensitive and the APd-sensitive currents look very similar, except that the latter has a larger residual current at -25 mV, indicating that APd exerts a greater effect than AcCho on the delayed part of the outward currents. These results indicate that the fast transient outward currents in our cultured neurons are most probably A-currents and that AcCho has an inhibitory effect on the A-current.

The dose dependency of the inhibitory action of AcCho on the A-current has not been worked out completely. But

**Fig. 1.** AcCho (0.1 μM) and APd (2.5 mM) suppress the fast transient outward currents in a cultured hippocampal neuron. The culture was superfused with Krebs solution containing tetrodotoxin (1 μM), and the cell was voltage-clamped by the whole-cell clamp technique. The pipette contained internal solution II (5 mM EGTA). The upper records are currents and lower records are potentials. The outward currents were activated repeatedly, by steps, to -35 mV from a holding potential of -90 mV. The resting potential of the cell was -78 mV. The bars indicate the time while the drug was pressure-ejected from the pipette. The drug pipettes were initially located far away from the soma. The pipette was brought near the soma (within 20 μm) immediately before the drug ejection was started. After the end of the ejection, the drug pipette was again removed from the vicinity of the cell. Note that the transient outward currents began to be suppressed just before the start of the APd ejection: this is due to a leak of APd from the approaching pipette.

**Fig. 2.** AcCho and APd effects on outward currents in a neuron bathed in the Krebs solution containing tetrodotoxin (1 μM). The patch pipette contained internal solution II (5 mM EGTA). The membrane was depolarized, by steps, from a holding potential of -108 mV to -62 mV (A1–E1), -43 mV (A2–E2), and to -25 mV (A3–E3). (A1–A3) Averages of two control currents before and after the drug applications. While AcCho (0.1 μM) or APd (2.5 mM) was applied (for 25 sec), depolarizations of various amplitudes were applied to record the currents in B1–B3 or C1–C3. Arrows indicate the zero current levels. In D1–D3 (or E1–E3), the AcCho records (B1–B3) (or the APd records) were subtracted from the control records (A1–A3). Digital sampling rate was 5 kHz.

**Neurobiology: Nakajima et al.**


![Graph showing AcCho and APd effects on outward currents](image-url)
AcCho at high concentrations (1 μM or more) did not necessarily produce larger effects on the A-current than at 0.1 μM. Very low concentrations (<0.01 μM AcCho) or control solution (without AcCho) did not produce inhibitory effects on the A-current. Sometimes the control solution produced a slight enhancement of the A-current, but the effect was very small, and it may have a trivial explanation.

We have attempted to analyze the mechanisms of the AcCho and APd effects by studying their voltage dependencies. In Fig. 3, the open symbols show the steady-state inactivation of the A-current as a function of membrane potential. The membrane potential was held at −40 mV and then was hyperpolarized to various levels (for 700 msec) before it was depolarized to −22 mV. The peak current produced by this depolarization was plotted against the level of hyperpolarization. These data were fitted by an equation similar to the one that describes the steady-state Na inactivation (20); namely, \( I = \bar{I}[1 + \exp\left((V-V_\text{h})/k\right)]^{-1} + C \), in which \( k \) and \( V \) are the parameters for the steepness and the half-inactivated voltage, respectively, and \( C \) is the current that is not inactivated (including the leak current). Table 1 gives the average values of \( k \) and \( V \) for six cells. The data for \( V \) indicate that the inactivation curves are shifted in the depolarizing direction by 13 mV or 7 mV in the presence of AcCho or APd. The value of \( \bar{V} \) represents the magnitude of the maximum activatable A-current at −22 mV. For the six cells in Table 1, AcCho (0.1 μM) reduced \( \bar{V} \) by 55% ± 5% (mean ± SEM) and APd (2.5 mM) reduced it by 41% ± 8%, indicating that the potencies of 0.1 μM AcCho and 2.5 mM APd are comparable.

The solid symbols in Fig. 3 illustrate the relationship between potential and the activation of A-currents. The potential was held at −108 mV and various steps of depolarization were applied. The peak of the A-current was plotted against the depolarization. These data show that the A-currents are activated at about −50 to −60 mV in the control and also suggest that the curves are shifted in the depolarizing direction by AcCho or APd. We did not further analyze the activation mechanisms in this paper. Nevertheless, our APD results agree well with the recent data by Kasai (21), who found similar effects of APD on activation and inactivation by single channel analysis.

To eliminate the possibility of contamination from Ca²⁺ currents, we also performed experiments on cells bathed in a Kresbs solution containing 5 mM cobalt chloride. The A-current persisted in the presence of cobalt ions, although its activation curve tended to be shifted in the depolarizing direction, in agreement with the effect of manganese on A-currents in other mammalian neurons (22). In the cobalt solution, we observed that the A-current was still inhibited by AcCho as well as by APd.

**Table 1. Steady-state inactivation of A-current**

<table>
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<tr>
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<th>Control (0.1 μM)</th>
<th>APd (2.5 mM)</th>
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<tbody>
<tr>
<td>( V ), mV</td>
<td>−73 ± 1</td>
<td>−60 ± 2</td>
</tr>
<tr>
<td>( k ), mV</td>
<td>5.7 ± 0.3</td>
<td>6.2 ± 0.4</td>
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Values are mean ± SEM (n = 6). The inactivation curve was fitted by the equation \( I = \bar{I}[1 + \exp\left((V-V_\text{h})/k\right)]^{-1} + C \).

**DISCUSSION**

The A-current has been described in many neurons. It is an outward K current with a rapidly inactivating time course similar to the one described in this paper and also with activation and inactivation curves like those in Fig. 3 (4, 7, 17–19, 21, 22, 24–28). In addition, the A-current of mammalian neurons is inhibited by APd. In the present experiments, AcCho produced almost the same inhibition of the transient outward current, as did APD. Thus, we conclude that AcCho inhibits the A-current in our hippocampal neurons.

We propose that A-current inhibition constitutes a mechanism by which AcCho exerts its excitatory influence through muscarinic receptors. As shown in Fig. 4, under the influence of AcCho, neurons tend to produce high-frequency repetitive firing. At first sight, this repetitive firing does not
appear to be caused by the A-current inhibition, since after the end of the first spike, the membrane potential was never sufficiently negative for inactivation of the A-current to be removed. Nevertheless, according to the recent single channel data by Kasai (21), the A-current is inactivated with two time constants, 100 msec and 4 sec. Thus, it is very likely that after the end of the control spike (Fig. 4A1), a part of A-current still remains without being completely inactivated. Inhibition of this remaining A-current by AcCho would produce a substantial effect on the ease of firing of the subsequent spikes. The role of the A-current in modulating the spike repetition rate has already been well explained by Connor and Stevens (29). It should be noted, however, that some effects of AcCho or APd other than the inhibition of A-current could exist, and these effects may play a role in producing the repetitive tendency. In fact, Fig. 2 shows that less inward current is required to hold the potential at $-108$ mV in the presence of AcCho or APd, an effect that may not be attributed to an effect on the A-current. Cole and Nicoll (23) suggested that inhibition of the Ca-induced K current underlies AcCho-induced repetitive firing.

Inhibition of the A-current would allow a single spike to be evoked more easily, provided the resting potential before the spike was sufficiently negative. This situation was predicted by Connor et al. (30) and has been recently observed by Segal et al. (18) in connection with inhibition of the A-current by APd. Thus, an excitatory postsynaptic potential, which would have been below threshold, may now, under the influence of AcCho, be able to initiate a spike.

The increase in the amplitude and the duration of the spike by AcCho and APd, seen in Fig. 4 B2 and D2, would certainly be related to the inhibition of A-currents. The A-current, with its fast kinetics, is an important factor in determining the size of the action potential and its time course of repolarization; the role played by the A-current in determining the repolarization phase was emphasized by Belluzzi et al. (28). An enhancement of spike height and duration would exert a powerful excitatory action if these changes took place at presynaptic nerve terminals: there, small changes of action potential height and shape produce remarkable changes in the amount of transmitter released (31-34). In fact, a presynaptic excitatory effect mediated by muscarinic receptors has recently been suggested by Raiteri et al. (35). In summary, AcCho mimics the excitatory action of APd, a convulsant, which would exert its excitatory influence mainly through the inhibition of the A-current.

All the excitatory effects we have described in this study took place in the probable absence of M-currents. Under our experimental conditions, we could rarely observe M-currents, perhaps because of the presence of 3 mM magnesium in the patch pipette (36). Under more physiological conditions, the modification of both the M-current and the A-current could work together in producing the excitatory action of AcCho. The muscarinic effects are multifaceted phenomena (1,3,23,37-40). For a muscarinic effect to take place, the cell must have the following three components: (i) the receptor, (ii) the messenger, and (iii) the target channel. Different responses could arise not only because of the existence of a variety of target channels but also because of the existence of multiple classes of muscarinic receptors (41) or because of the possible involvement of more than one messenger. Probably our experimental conditions were favorable for one facet of these complex events to be observed.

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