Ionic Mechanism of the Slow Afterdepolarization Induced by Muscarinic Receptor Activation in Rat Prefrontal Cortex

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Haj-Dahmane, Samir and Rodrigo Andrade. Ionic mechanism produces an increase in cellular excitability that is the result of the slow afterdepolarization induced by muscarinic receptor of several specific effects, including a membrane depolarization and the appearance of a slow afterdepolarization (sADP). In the current report we examine the mechanism underlying the sADP with the use of sharp microelectrode and whole cell recording techniques in vitro brain slices. The ability of acetylcholine (ACh) and carbachol to induce the appearance of an sADP in pyramidal cells of layer V of prefrontal cortex is antagonized in a surmountable manner through which acetylcholine (ACh) regulates cortical networks to allow for normal cognitive functioning. We recently have shown that the muscarinic induced depolarization in this region is mediated by the activation of a voltage-sensitive nonselective cation current (Haj-Dahmane and Andrade 1996). However the mechanism responsible for the sADP in this region was not elucidated. sADPs, similar to those observed in prefrontal cortex, were also observed after muscarinic (Constanti and Bagetta 1991; Schwindt et al. 1988), 5-HT2 (Araneda and Andrade 1991; Spain 1994), and glutamate metabotropic receptor activation (Constanti and Libri 1992; Greene et al. 1992) in several different cortical regions. This suggested that the current underlying the sADP might be an important target for neurotransmitter regulation in cortex. In this study we examined the ionic mechanisms responsible for the generation of the sADP in prefrontal cortex.

METHODS

Preparation of brain slices

Cortical brain slices that included the medial prefrontal cortex (Krettek and Price 1977) were prepared as previously described (Andrade 1991). Male albino rats (200–250 g) were anesthetized with halothane and killed by decapitation. The brain was removed and cooled in ice-cold, oxygenated Ringer solution (composition in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1 NaH2PO4, 26.2 NaHCO3, and 11 glucose), and the anterior forebrain was isolated. The anterior pole of the brain was then affixed to a stage with cyanocrylate glue and sectioned into 400-μm-thick coronal slices with the use of a vibratome (Lancer series 1000, Ted Pella, Irvine, CA). The brain slices were then placed on filter paper saturated with oxygenated Ringer solution inside a chamber filled with a moist atmosphere of 95% O2-5% CO2 at room temperature. After 1–2 h of recovery, slices were transferred as needed to a recording chamber of standard design...
Acetylcholine (ACh) and carbachol induce a slow afterdepolarization (sADP). A: response of a pyramidal neuron recorded in current-clamp mode to a long depolarizing pulse under control conditions (left), after a brief pressure application of ACh (middle), and after recovery (right). Note the blockade of the afterhyperpolarization (AHP) and its replacement by an sADP after pressure application of ACh. Insert: spiking activity during the pulse with the use of an expanded timescale (calibration 20 mV, 2 nA, 100 ms). Resting membrane potential, −69 mV. B: bath application of carbachol (30 μM) mimics the effect of pressure-applied ACh in a different pyramidal neuron. Resting membrane potential, −75 mV. C: camera Lucida drawing of a neurobiotin-filled cell exhibiting the typical electrophysiological profile of the regular spiking cells used in this study. The response of this cell to muscarine is illustrated in Fig. 4.

(Nicoll and Alger 1981). In this chamber the slices were held submerged between two nylon nets and continuously perfused (2–4 ml/min) with normal Ringer solution bubbled to saturation with 95%O₂-5% CO₂ at 30 ± 1°C.

Electrophysiological recordings

Intracellular recording were obtained from pyramidal neurons of layer V of the prelimbic and anterior cingulate subdivisions of the medial prefrontal cortex with the use of patch clamp or conventional sharp microelectrode recording techniques. For standard intracellular recording sharp microelectrodes were pulled from 1.2-mm OD borosilicate glass with the use of a Flaming-Brown horizontal puller (Model PC80/PC, Sutter Instrumentrs, Novato, CA) to give resistance ranging from 90 to 150 MΩ when filled with 2 M potassium methylsulfate. Neurons were impaled by delivering a high-voltage pulse through the electrode or by “ringing” induced by overcompensating the capacity compensation circuit of the amplifier. Data were collected only from neurons with a stable resting membrane potential more negative than −60 mV and overshooting spikes. Whole cell recordings were performed with the use of the blind tight seal technique (Blanton et al. 1989). The recording pipettes were pulled from 1.2-mm OD borosilicate glass with the use of a Flaming-Brown horizontal puller to give resistances ranging from 4 to 6 MΩ when filled with an internal solution of the following composition (in mM): 125 KMeSO₄, 5 NaCl, 1 MgCl₂, 10 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 0.02 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′′,N′′′-tetraacetic acid (EGTA), 2 Na₂ATP, and 0.5 Na₃ guanosine 5′-triphosphate (Na₃GTP). The pH was adjusted to 7.3–7.4 with KOH. In some experiments, the calcium buffering capacity of the pipette solution was increased with 10 mM EGTA or bis-(o-aminophenoxo)-N,N,N′,N′′-tetraacetic acid (BAPTA) and added calcium to bring the free calcium concentration to ~10 nM. In some experiments, potassium methylsulfate was substituted by cesium gluconate or cesium methanesulfonate in the internal solution to examine the possible role of potassium current in the sADP. In these cases the pH of the internal solution was adjusted to 7.3–7.4 with CsOH and the osmolarity to ~5 mosM lower than the osmolarity of standard Ringer (~295 mosM). With all these solutions access resistance measured with the use of the bridge compensation circuit of the amplifier ranged from 10 to 25 MΩ.

Voltages were measured and current was injected with the use of an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Electrical signals were filtered at 5–10 kHz and recorded on-line with the use of a paper chart recorder (Model 3200, Gould Instruments, Valley View, OH). Fast events such as voltage steps and actions potentials were digitized with the use of an Intel processor-based computer equipped with a 12-bit A/D converter under the control of pClamp 5.5 software (Axon Instruments). Voltage-clamp experiments were conducted with the use of either continuous or discontinuous voltage-clamp techniques. In the case of continuous voltage-clamping, series resistance was corrected by ~70%. In the case of discontinuous voltage-clamp recordings, the headstage output was monitored on an oscilloscope, and the sampling frequency was adjusted to obtain complete discharge of the electrode between cycles. In some experiments a “hybrid” current/
FIG. 2. sADP is mediated by a slow inward aftercurrent. A1a: burst of spikes followed by an AHP under control conditions. The current underlying this AHP can be examined with a "hybrid" current/voltage-clamp protocol. A1b: after bath administration of carbachol (30 μM) this AHP is replaced by sADP. This sADP can be seen to be mediated by a slow inward aftercurrent (I_{sADP}) when recording with a hybrid current/voltage-clamp protocol. A1c: a similar I_{sADP} can be observed following 2 depolarizing constant current pulses in the presence of tetrodotoxin (TTX; 1 μM) in the same cell. Previous studies have shown that the transient depolarizations (A1c) elicited in these cells by the depolarizing pulses is mediated at least in part by the activation of low-threshold calcium currents (Sutor and Zieglgänsberger 1987). A1d: in the same cell, a depolarizing step to −20 mV under voltage clamp can also elicit I_{sADP}. 1a and 1b, insets: spiking induced by the current pulse illustrated with an expanded timescale. Inset 1c depicts a low-threshold calcium spike (calibration 100 ms, 20 mV, 2 nA). Resting membrane potential, −69 mV. B: carbachol induces I_{sADP} in a concentration-dependent manner. Cells were depolarized for 800 ms from −65 to −30 mV to allow calcium into the cell, and the peak amplitude of the resulting inward aftercurrent was plotted. This plot reflects data obtained in 4 cells tested. The raw data were fitted with an occupational model to an EC50 of 3.2 μM and the Hill coefficient of 1.6. Right traces: examples of the traces used to construct the dose-response curve. Holding potential, −65 mV; holding current at rest, 210 pA.

voltage-clamp protocol was used to record aftercurrents after a stimulus, generally a calcium spike. In these experiments the amplifier was switched from voltage clamp to current clamp 200–500 ms before the constant current stimulus and back to voltage clamp 50–250 ms after the end of the stimulus under the control of the Master 8 stimulator.

Drugs were applied by bath, dissolved in the Ringer at known concentration. In a few experiments, a patch pipette containing 100 mM ACh was placed immediately above the slice as close as possible to the cell of interest, and ACh was ejected by a brief pressure pulse. The pressure and duration used for ejecting ACh were adjusted to produce a small droplet at the tip of each pipette under visual inspection under the dissecting microscope used to visualize the slice. These parameters were subsequently optimized to produce a reliable inward current or depolarization with the ejection pipette in place. The low-sodium Ringer was prepared by substituting N-methyl-D-glucamine (NMDG) for sodium chloride, and the pH was adjusted to 7.4 with the use of HCl. In those experiments where extracellular sodium was reduced to 10 mM, the control Ringer was modified by omitting the NaH2PO4 and replacing it with 10 mM HEPES. The pH of this solution was adjusted to 7.4 with the use of HCl, and pure oxygen was bubbled to equilibrium. Osmolarity was adjusted to match that of the control solution with the use of sucrose. This solution became our control solution in these experiments. To reduce extracellular sodium to 10 mM this control solution was replaced by a low-sodium solution prepared following the same protocol except that NMDG was substituted for all but 10 mM of the sodium. The low-chloride Ringer was prepared by substituting sodium isethionate for sodium chloride. Most of the compounds used in this study were obtained from Sigma (St. Louis, MO). Tetrodotoxin (TTX) was from Calbiochem (La Jolla, CA). Data were analyzed and plotted with the use of Origin (Microcal Software, Northampton, MA). Numerical data are presented as means ± SE. Statistical
comparisons used Student’s paired or unpaired two-tailed t-test or analysis of variance; *P < 0.05 was considered to be statistically significant.

**RESULTS**

Recordings were obtained from >300 cells from layer V of the dorsal anterior cingulate and prelimbic subdivisions of the rat medial prefrontal cortex (Krettek and Price 1977). As previously described, the vast majority of neurons encountered in these recordings could be classified as regular spiking neurons based on their electrophysiological characteristics (Haj-Dahmane and Andrade 1996). We previously have shown that, as elsewhere in cortex (Connors and Gutnick 1990; Connors et al. 1982; Foehring et al. 1991; McCormick et al. 1985), these regular spiking neurons correspond to morphologically identified pyramidal cells (Fig. 1C). All experiments reported herein were conducted on this cell population.

Pyramidal neurons in medial prefrontal cortex respond to a suprathreshold constant-current-depolarizing pulse with a burst of spikes, which is followed by an AHP. As illustrated in Fig. 1A, a brief pressure administration of ACh elicits a marked reduction in this AHP and its replacement by sADP (n = 10 cells, Fig. 1A). This effect can be elicited repeatedly on the same cell with little evidence of desensitization or rundown for ≥30 min after breaking into the cell. Essentially identical results are obtained after bath administration of the poorly hydrolyzable ACh analogue carbachol (3–100 µM, Fig. 1B) (n >100 cells). Concurrent with the appearance of this sADP, pressure application of ACh or bath administration of carbachol also elicits a membrane depolarization that is mediated through a nonselective cation current (not shown) (Haj-Dahmane and Andrade 1996). When examining the sADP in current clamp, this depolarization was neutralized by injection of hyperpolarizing current to maintain a constant membrane potential.

**FIG. 3.** Ability of carbachol to induce sADP is blocked by atropine. Effect of carbachol under control conditions (top traces) and in the presence of 100 nM of the selective muscarinic antagonist atropine (bottom traces). Bath administration of atropine blocks the ability of carbachol to induce sADP in a surmountable manner. Cell resting membrane potential, −72 mV.

**FIG. 4.** Selective muscarinic cholinergic agonists oxotremorine and muscarine also induce sADP. A: depolarizing pulses capable of triggering a burst of action potential were delivered under control condition (left trace), in the presence of oxotremorine (30 µM) (middle trace), and after bath application of atropine (1 µM) (right trace). Note that oxotremorine induces sADP and that this effect is completely reversed after administration of atropine. Resting membrane potential, −73 mV. B: in a different cell, a depolarizing pulse delivered in the presence of muscarine induces a strong sADP, which reached threshold and generated sustained firing activity (middle trace). This effect is completely reversed by atropine (right trace). Resting membrane potential, −68 mV.
With the use of a hybrid current/voltage-clamp protocol, in which the cell is voltage clamped immediately after a depolarizing stimulus, it is possible to examine directly the current underlying the sADP. Fig. 2A illustrates one such experiment. Under control conditions a burst of spikes is followed by a slow outward aftercurrent that corresponds to the current responsible for the AHP. Carbachol administration reduces this outward aftercurrent and induces the appearance of a slow inward aftercurrent. This inward aftercurrent is responsible for the sADP observed in current-clamp conditions and will be referred to as $I_{sADP}$. Action potentials are not required for triggering this $I_{sADP}$ because this current can also be triggered by depolarizing pulses capable of opening low-threshold calcium channels (Fig. 2A1c) or, when recording entirely in voltage clamp, by depolarizing steps to potentials positive to -40 to -50 mV (Fig. 2A1d).

**Pharmacology of the receptors mediating the sADP**

The concentration-response relationship for the carbachol-induced $I_{sADP}$ was determined by examining the effect of increasing concentrations of carbachol on the amplitude of the sADP after a constant depolarizing step. As illustrated in Fig. 2B, under control conditions a depolarizing step is followed by an outward aftercurrent that decays completely within 1–2 s from the end of the depolarizing step. Addition of low micromolar carbachol to the bath reduces this outward aftercurrent. Concomitant with this reduction carbachol induces the appearance of a slower inward aftercurrent ($I_{sADP}$). This inward aftercurrent decays slowly and generally incompletely over several seconds and thus exhibits a slower time course than the outward aftercurrent. Increasing the carbachol concentration results in a concentration-dependent increase in the amplitude of $I_{sADP}$ (Fig. 2B) that reaches a maximum at concentrations $>30 \mu M$. The EC$_{50}$ for carbachol in these experiments is $3.2 \mu M$ ($n = 4$ cells). For a given carbachol concentration it is generally possible to increase the amplitude of the sADP by increasing the intensity of the stimulus to allow more calcium to enter the cell, but this effect shows saturation. No obvious changes in the kinetics of the sADP could be detected over the range of stimulus strengths examined in this study.

As illustrated in Fig. 3, atropine at a concentration as low as 100 nM completely blocks the ability of carbachol (10 $\mu M$) to elicit an sADP (Fig. 3, $n = 5$ cells tested), and this antagonism can be surmounted by increasing the carbachol concentration. These results indicate that carbachol induces sADP by stimulating muscarinic receptors. Consistent with these findings, the selective muscarinic cholinergic agonists muscarine (30 $\mu M$, $n = 6$ cells tested, Fig. 4A) and oxotremorine (30 $\mu M$, $n = 4$ cells tested, Fig. 4B) are also capable of eliciting the appearance of sADP. Recovery from these agonists is slower than with carbachol but can be easily obtained by the addition of atropine to the bath (Fig. 4). Because carbachol washes out faster than the other muscarinic agonists tested, it was used to induce the sADP in the rest of the study.

**Properties of the muscarinic-induced sADP**

As indicated above, action potentials are not necessary for generating the sADP. A depolarizing pulse capable of triggering low-threshold calcium spikes or depolarizing voltage steps positive to -40 to -50 mV capable of opening low-threshold calcium channels (Sutor and Ziegglänsberger 1987) is as effective as a burst of action potentials in inducing an $I_{sADP}$ (Fig. 2A). These results, combined with the previous observation that the carbachol-induced sADP is reduced by lowering extracellular calcium and inhibited by calcium channel blockers (Andrade 1991), suggested that this afterpotential depends on calcium influx into the cell.

Calcium-dependent ADPs similar to those studied here were previously reported to be mediated by either calcium (Hounsgaard and Kiehn 1989) or calcium-activated (or inactivated) currents (Kramer and Levitan 1988; Partridge and Swandulla 1988; Yoshimura et al. 1987). To distinguish between these possibilities recordings were obtained with the use of intracellular solutions with differing calcium-buffering capacities. When recordings were conducted with the use of an intracellular solution of low calcium-buffering capacity (20 $\mu M$ EGTA), low-threshold calcium spikes reli-
ably produced sADP in the presence of carbachol (30 μM) (Fig. 5A). In contrast, when the calcium-buffering capacity of the intracellular solution was increased by 10 mM EGTA or 10 mM BAPTA, the sADP triggered with the use of the same stimulation protocol was greatly reduced or completely suppressed (Fig. 5B). In this experiment the amplitude of the sADP was reduced from 16.3 ± 4.0 mV (n = 11 cells) in 20 μM EGTA to 3.0 ± 0.47 mV in 10 mM EGTA (n = 11 cells, P = 0.008) and 0.4 ± 0.24 mV in 10 mM BAPTA (n = 6 cells, P = 0.003). In contrast to these results buffering intracellular calcium had no effect on the carbachol-induced depolarization (Fig. 5B) (Haj-Dahmane and Andrade 1996), indicating that the inhibition of the sADP was not simply secondary to a loss of muscarinic receptor functioning. These results indicated that the carbachol-induced sADP most likely resulted from a calcium-activated (or -inactivated) current.

Ionic mechanism underlying the sADP

Previous studies have shown that muscarinic agonists depress the AHP in cortex (McCormick and Prince 1986; Schwindt et al. 1988) and hippocampus (Benardo and Prince 1982; Nicoll et al. 1990). Thus muscarinic agonists could induce sADP simply by unmasking a constitutively present inward aftercurrent. Alternatively, muscarinic receptor stimulation could activate a previously silent current whose expression depends on an influx of calcium into the cell. To distinguish between these possibilities recordings were obtained with the use of a cesium-based intracellular solution. As illustrated in Fig. 6A1, under control conditions depolarizing steps to potentials above −50 mV are followed by a slow outward aftercurrent mediated by a calcium-activated potassium current (Schwindt et al. 1988). Substitution of potassium by cesium in the intracellular solution completely blocks the outward aftercurrents (Fig. 6, A2 and A3, n = 5 cells tested) but fails to unmask an sADP (Fig. 6A4). This failure of cesium to unmask sADP does not reflect merely an inabilty of the cesium-loaded cell to express the sADP because an sADP comparable with that seen under control conditions is observed after administration of carbachol (30 μM, Fig. 6B, n > 50 cells). Depolarizing steps to potentials positive to −30 to −40 mV in contrast generally resulted in the appearance of a faster ADP. However this early ADP...
E \text{inward aftercurrent} \((\text{only a very small inward aftercurrent is detected at this time. However, after bath application of carbachol, a large, slow})\)

\text{the appearance of} \(I_{\text{sADP}}\) \((\text{inset})\) \((\text{Approximately } 250 \text{ ms after the end of the depolarizing voltage-clamp protocol,}}\) \text{the amplifier was switched to voltage-clamp mode} \((a)\) \text{to measure any slow aftercurrents after the calcium spike. Under control conditions only a very small inward aftercurrent is detected at this time. However, after bath application of carbachol, a large, slow inward aftercurrent} \((I_{\text{sADP}})\) \text{becomes evident. Discontinuous voltage clamp, holding potential, } -71 \text{ mV; holding current at rest, none.} \)

\text{FIG. 7. Effect of carbachol on a cell recorded with a cesium gluconate-based intracellular solution in the presence of TTX (1 \(\mu M\)). Under these conditions a brief depolarizing pulse delivered with a hybrid current/voltage-clamp protocol results in an all-or-none calcium spike} \((\text{inset})\). \text{Approximately } 250 \text{ ms after the end of the depolarizing voltage-clamp protocol, the amplifier was switched to voltage-clamp mode} \((a)\) \text{to measure any slow aftercurrents after the calcium spike. Under control conditions only a very small inward aftercurrent is detected at this time. However, after bath application of carbachol, a large, slow inward aftercurrent} \((I_{\text{sADP}})\) \text{becomes evident. Discontinuous voltage clamp, holding potential, } -71 \text{ mV; holding current at rest, none.} \)

decayed within 100–200 ms and was clearly temporarily distinct from the sADP reported \((\text{Haj-Dahmane and Andrade 1997})\).

Essentially identical results on the effects of cesium on the sADP were obtained when the experiments were repeated in current clamp. In the presence of TTX \((1 \mu M)\), cesium-loaded cells respond to a suprathreshold-depolarizing pulse with an all-or-none calcium action potential. This calcium spike was followed by a fast ADP lasting 200–300 ms \((\text{Fig. 7, inset})\), but there was no evidence for sADP \((\text{or } I_{\text{sADP}}, n = 40 \text{ cells, Fig. 7})\). As previously seen in voltage clamp, the subsequent administration of carbachol \((10–30 \mu M)\) elicited the appearance of \(I_{\text{sADP}}\) in most of the cells tested \((29/40 \text{ cells, Fig. 7})\).

The results above suggest that muscarinic receptor activation induces the appearance of a calcium-dependent current, which in turn is responsible for the sADP. The subsequent experiments were designed to characterize its underlying ionic mechanisms.

Several distinct ionic mechanisms could underlie sADP. Previous studies in olfactory cortex identified a muscarinic induced ADP similar to that seen in prefrontal cortex \((\text{Constanti and Bagetta 1991; Constanti et al. 1993})\) and concluded that it is mediated by the closure of potassium channels. Therefore we examined the possibility that a similar mechanism could account for the muscarinic induced sADP in prefrontal cortex. If the sADP were mediated through a calcium-inactivated potassium conductance, the amplitude of this afterpotential should diminish with hyperpolarization and should reverse polarity near \(E_K\) \((\text{approximately } -104 \text{ mV under our experimental conditions})\). To test this prediction, we examined the voltage dependence of \(I_{\text{sADP}}\) from \(-50 \text{ to } -120 \text{ mV}\). Voltages positive to \(-50 \text{ mV} \text{ could not be examined because the resulting calcium influx by itself activates } I_{\text{sADP}}\). As illustrated in Fig. 8, \(I_{\text{sADP}}\) diminished with hyperpolarization but remained inward even at \(-120 \text{ mV}\) \((n = 5 \text{ cells})\). In none of the cells tested did the peak \(I_{\text{sADP}}\) reverse below \(E_K\). In three of the five cells tested, the very late portion of \(I_{\text{sADP}}\), but not the peak, appeared to reverse 5–10 mV below \(E_K\).

There are multiple reasons that could account for our failure to observe reversal of \(I_{\text{sADP}}\) at \(E_K\) in control extracellular potassium. Therefore we reexamined this issue with the use of elevated extracellular potassium, a condition that should favor observing the reversal of a current mediated by a decrease in potassium conductance. In this experiment we raised extracellular potassium from 2.5 to 10 mM to shift \(E_K\) to near \(-65 \text{ mV}\). As expected, this resulted in the suppression of the outward potassium current responsible for the AHP in these cells \((\text{Fig. 9A})\). However, even under these conditions, bath application of carbachol still induces the appearance of \(I_{\text{sADP}}\) \((\text{Fig. 9B, } n = 6 \text{ cells tested})\). In three of these cells it was possible to examine \(I_{\text{sADP}}\) in the \(-60 \text{ to } -90 \text{ mV range} \((\text{i.e., below } E_K \text{ under these conditions})\). In all of these cells \(I_{\text{sADP}}\) remained inward over this voltage range. Moreover, no evidence of a reversing late component of \(I_{\text{sADP}}\) could be detected. From these observations and the persistence of \(I_{\text{sADP}}\) after intracellular cesium loading we conclude that this current must be mediated largely by a mechanism other than a decrease in potassium conductance.

sADP such as that studied here could in principle be mediated by changes in chloride conductance. Indeed, previous studies in other neuronal cell types identified ADPs mediated by calcium-activated chloride currents \((\text{Mayer 1985; Owen et al. 1984})\). In our study the muscarinic-induced sADP can be recorded at potentials very close to \(E_{Cl}\) estimated from the chloride concentration in the intracellular solution. Thus the carbachol-induced sADP is unlikely to be mediated by a calcium-dependent chloride current. Nevertheless, because a chloride gradient could conceivably be present at sites distal from the recording electrode, we tested the effect of reducing the extracellular chloride concentration on the amplitude of the carbachol-induced sADP. As illustrated in Fig. 10, lowering the extracellular concentration of chloride from 126.5 to 57.5 mM, which should shift \(E_{Cl}\) by \(-20 \text{ mV} \text{ in the depolarizing direction} \text{ and effectively reverse a chloride-}
mediated potential at -65 to -70 mV, had no effect on the amplitude of the carbachol-induced sADP (control = 4 ± 0.4 mV, low chloride = 4.75 ± 0.47 mV, n = 4 cells tested). These results indicate that the carbachol-induced sADP is not mediated by a change in chloride permeability.

An alternative mechanism for the sADP could involve a calcium-activated nonselective cation current. Such currents were shown to mediate ADPs in a variety of preparations (Caeser et al. 1993; Partridge and Swandulla 1988). To test the possible involvement of nonselective cation currents we examined the effect of lowering the extracellular concentration of sodium from 126 to 46 mM on the amplitude of the carbachol-induced sADP. This reduction of extracellular concentration should shift $E_{Na}$ in the negative direction by ~25 mV. As illustrated in Fig. 11, substitution of 60% of extracellular sodium with NMDG reversibly reduced the amplitude of the carbachol-induced sADP. Overall, in a group of five cells tested with the use of this protocol, 60% sodium substitution with the use of NMDG reduced the sADP from 5.7 ± 0.58 mV to 2.8 ± 0.43 mV (n = 5 cells, $P < 0.005$). The reduction in the amplitude of the sADP appeared to be most prominent during the early part of the sADP. This is most evident on superimposition of the traces obtained under control condition and in low sodium. No change in the input resistance was observed during the perfusion of low sodium.

The ion substitution employed above should have reduced a pure sodium current in direct proportion to the reduction in driving force, in this case ~20%. However, we observed a reduction of 50%. This could be explained if the sADP were mediated by a nonselective cation current. However, other explanations cannot be readily ruled out from this experiment. Therefore we reexamined the sodium dependence of $I_{ADP}$ after cesium loading, a condition that would partially isolate this current and minimize space-clamp artifacts. As illustrated in Fig. 12, lowering extracellular sodium reduced the amplitude of $I_{ADP}$ recorded in cesium-loaded cells, and this inhibition depended on the fraction of extracellular sodium replaced with NMDG ($n = 5$ cells for 50% replacement and $n = 4$ cells for 93% replacement, Fig. 12B). These effects were again somewhat larger than expected for sodium-selective channels and argued for a nonselective cation current.

sADP mediated by a nonselective cation current should be associated with an increase in chord conductance. Surprisingly, when we looked for changes in the apparent membrane resistance during $I_{ADP}$ with the use of brief depolarizing steps, we observed the opposite, an apparent decrease in membrane conductance (Fig. 13). This effect was even more prominent when we repeated the experiment with the use of depolarizing steps. This asymmetry suggested that the voltage steps were not simply measuring membrane conductance, but rather that the current was rapidly activating (and deactivating) during the voltage steps. To test directly whether $I_{ADP}$ was indeed voltage dependent, we reexamined this current in -50 to -110 mV under conditions that would isolate this current from other currents also expressed in the cell. Under these conditions, $I_{ADP}$ was found to exhibit marked voltage sensitivity (Fig. 14), deactivating quickly with hyperpolarization in the subthreshold range (where our conductance measurements were conducted). This voltage dependence would predict the observed reduction in the amplitude of the conductance pulse observed during $I_{ADP}$.

**DISCUSSION**

One of the most striking effects of cholinergic stimulation of pyramidal cells in the rat prefrontal cortex is the appearance of an sADP. In the current study this sADP could be induced by brief applications of ACh or by bath administration of carbachol, muscarine, or oxotremorine. Because all of these effects could be blocked by low concentrations of atropine, these results indicate that the sADP is elicited by...
In this study we investigated the ionic basis for the sADP. Previous studies have shown that the muscarinic receptor-induced sADP seen in prefrontal (Andrade 1991), sensorimotor (Schwindt et al. 1988), and olfactory (Constanti et al. 1993) cortices as well as hippocampus (Caeser et al. 1993; Fraser and MacVicar 1996) depended on extracellular calcium influx into the cell. This could indicate that the sADP is triggered by a rise in intracellular calcium or that calcium itself carries the sADP current (Hounsgaard and Kiehn 1989). Buffering intracellular calcium with EGTA or BAPTA greatly reduced or blocked the sADP. Because this inhibition was observed in the absence of a reduction of the muscarinic depolarization also present in these cells, this inhibition was unlikely to reflect a nonspecific effect of the calcium buffers on muscarinic receptor functioning. These results, together with earlier results indicating a critical role for calcium influx in the generation of the sADP (Andrade 1991), indicate that the sADP seen in prefrontal cortex is triggered by a rise in intracellular calcium. The observation that calcium chelation inhibits the sADP is consistent with similar results in hippocampus (Fraser and MacVicar 1996) but disagrees with the report that injections of EGTA or BAPTA by sharp microelectrodes fail to reduce the ADP in olfactory cortex (Constanti et al. 1993).

A rise in intracellular calcium could produce the sADP through several distinct mechanisms, including changes in sodium, potassium, or chloride permeabilities, the activation of an electrogenic calcium pump, or a combination of these. From these experiments we conclude that the mechanism of the activation of muscarinic receptors. The potency of carbachol for eliciting this response was comparable with that previously reported for eliciting a membrane depolarization in these same cells (Haj-Dahmane and Andrade 1996) and for reducing \( I_{\text{m}} \) in CA1 pyramidal neurons (Madison et al. 1987). However, it was considerably higher than that required to reduce the AHP in hippocampus (Madison et al. 1987).

Muscarinic receptor activation could elicit the appearance of the sADP directly by inducing its expression or indirectly by inhibiting the AHP and unmasking a constitutive sADP. Because inhibition of the AHP with the use of intracellular cesium failed to unmask sADP, we conclude that muscarinic receptors induce the de novo expression of an sADP in these cells. A similar conclusion was reached for the muscarinic receptor-induced ADP in sensorimotor and olfactory cortices based on the reversal potential of the AHP (Schwindt et al. 1988) and the inability of cAMP, which inhibits the AHP, to induce an ADP (Constanti et al. 1993).

In this study we investigated the ionic basis for the sADP. Previous studies have shown that the muscarinic receptor-induced sADP seen in prefrontal (Andrade 1991), sensorimotor (Schwindt et al. 1988), and olfactory (Constanti et al. 1993) cortices as well as hippocampus (Caeser et al. 1993; Fraser and MacVicar 1996) depended on extracellular calcium influx into the cell. This could indicate that the sADP is triggered by a rise in intracellular calcium or that calcium itself carries the sADP current (Hounsgaard and Kiehn 1989). Buffering intracellular calcium with EGTA or BAPTA greatly reduced or blocked the sADP. Because this inhibition was observed in the absence of a reduction of the muscarinic depolarization also present in these cells, this inhibition was unlikely to reflect a nonspecific effect of the calcium buffers on muscarinic receptor functioning. These results, together with earlier results indicating a critical role for calcium influx in the generation of the sADP (Andrade 1991), indicate that the sADP seen in prefrontal cortex is triggered by a rise in intracellular calcium. The observation that calcium chelation inhibits the sADP is consistent with similar results in hippocampus (Fraser and MacVicar 1996) but disagrees with the report that injections of EGTA or BAPTA by sharp microelectrodes fail to reduce the ADP in olfactory cortex (Constanti et al. 1993).

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Two lines of evidence support this conclusion. The first is based on the exclusion of possible mechanisms. Previous studies in olfactory cortex suggested that the sADP observed in that region in response to muscarinic receptor activation is mediated by a calcium-inactivated potassium conductance (Constanti and Bagetta 1991; Constanti et al. 1993). Two major lines of evidence lead us to reject the possibility that this mechanism was responsible for the sADP seen in prefrontal cortex. First, a robust $I_{\text{sADP}}$ could be observed in the presence of intracellular cesium, a manipulation that should block most potassium channels. Although it is possible that cesium might not block, or block very weakly, this particular potassium channel, cesium is a fairly nonselective potassium channel blocker. Thus its failure to reduce $I_{\text{sADP}}$ suggests that potassium channel closure is unlikely to be the predominant mechanism underlying the sADP. Second, and most importantly, a robust $I_{\text{sADP}}$ persists as an inward current at and below $E_K$. Together these two lines of evidence indicate that a calcium-inactivated potassium current is unlikely to be primarily responsible for the generation of the sADP. However, they cannot completely exclude the possibility that a calcium-inactivated potassium current could make a small contribution to the generation of this afterpotential.

The sADP could also be mediated by a change in chloride permeability or an electrogenic pump. A change in chloride permeability could be ruled out as the fundamental mechanism responsible for the sADP because this afterpotential was not affected by extracellular chloride substitution or, as previously shown (Andrade 1991), by elevation of intracellular chloride. Similarly, a sodium/calcium electrogenic pump also did not appear to be responsible for the sADP because $I_{\text{sADP}}$ was voltage dependent and diminished, rather than increased, on depolarization. Equally important, the amplitude of the sADP increased in amplitude until saturation but did not increase in duration with increasing stimulus strength. This is contrary to what was reported previously for afterpotentials mediated by electrogenic pumps (Rang and Ritchie 1968).

In contrast to the negative findings outlined above, the sADP (or $I_{\text{sADP}}$) is reduced by replacing extracellular sodium with NMDG, as expected for a current whose main charge carrier is sodium. Moreover, the amplitude of this reduction depends on the fraction of sodium replaced. This indicated that $I_{\text{sADP}}$ is carried by sodium influx into the cell. It is noteworthy that this dependence on extracellular sodium is more pronounced than expected for a pure sodium current. We interpret these observations to suggest that the sADP is not mediated by a pure sodium current but rather by a nonselective cation current. Technical limitations intrinsic to slice preparations make it difficult to conduct a precise determination of the contribution of different cations to this current. However, a permeability to potassium and/or cesium could explain the observed deviations.

Although the results outlined above lead us to conclude that $I_{\text{sADP}}$ corresponds to a nonselective cation current, we were surprised to find that $I_{\text{sADP}}$ was associated with an apparent decrease in membrane conductance when assessed by brief hyperpolarizing steps. Similar observations were made by others for the muscarinic induced sADP in olfactory cortex (Constanti and Bagetta 1991; Constanti et al. 1993), and the metabotropic glutamate receptor elicited sADP in sensorimotor cortex (Greene et al. 1994). In the first of these preparations, the apparent decrease in conductance was attributed to a reduction in potassium conductance. However, as outlined above, this explanation could not account for the sADP in prefrontal cortex.

Alternatively, the sADP in sensorimotor cortex was attributed to a mixed ionic mechanism involving both calcium-activated nonselective cation and calcium-inactivated potassium conductances. In this scenario the apparent decrease in conductance could be explained by a predominantly somatic localization of the potassium channels. Such a mechanism would be similar to that proposed to mediate slow depolar-
Lowering extracellular sodium reduces $I_{sADP}$ in proportion to the degree to which sodium is replaced by N-methyl-D-glucamine (NMDG). A: hybrid current/voltage-clamp protocol was used to record $I_{sADP}$. Recordings were obtained with a cesium-based intracellular solution, and calcium spikes were used to trigger $I_{sADP}$ under control conditions and in low extracellular sodium. Lowering extracellular sodium concentration to 10 mM resulted in a large reduction in $I_{sADP}$. Discontinuous voltage-clamp recording; holding potential, −70 mV; holding current at rest, −50 pA. B: superimposition of traces depicting $I_{sADP}$ under control conditions and in low extracellular sodium. C: summary plot illustrating the relationship between the amplitude of $I_{sADP}$ elicited in the presence of 30 μM carbachol and the percent substitution of extracellular sodium by NMDG; $n = 5$ cells; error bars correspond to SE.

An alternative explanation for the apparent decrease in conductance during the sADP could be that $I_{sADP}$ itself is voltage dependent. Rapid voltage-dependent relaxations during the steps could give the appearance of decreases in conductance. In support of this possibility, the apparent decrease in conductance during $I_{sADP}$ was found to be highly dependent on the polarity of the voltage step used. This asymmetrical behavior can be explained if depolarizing steps resulted in an increase and hyperpolarizing steps resulted in a decrease in the amplitude of $I_{sADP}$. To test this possibility we directly examined the voltage dependence of $I_{sADP}$. Consistent with the idea that $I_{sADP}$ is voltage dependent, the amplitude of $I_{sADP}$ recorded in isolation after cesium loading of the cells was found to diminish steeply with hyperpolarization. Of course, a variety of voltage-clamp artifacts, resulting from the difficulty of clamping neurons with intact dendritic arbors, could have conferred the appearance of voltage dependence to an otherwise voltage-independent cation current. However, in these same cells and with techniques identical to those used in the current study, it is possible to demonstrate the presence of a well-behaved, voltage-insensitive, calcium-activated nonselective cation current (Haj-Dahmame and Andrade 1997). Thus we conclude that $I_{sADP}$ corresponds to a current carried most likely by voltage-dependent nonselective cation channels. We cannot exclude the possibility that a small component of $I_{sADP}$ could involve potassium or chloride channels. However, the involvement of such channels is not required to explain the current observations.

sADPs similar to those studied here can be induced not only by muscarinic stimulation elsewhere in cortex (Constanti and Bagetta 1991; Schwindt et al. 1988) but also by serotonin (Spain 1994) acting on 5-HT$_2$ receptors (Araneda and Andrade 1991), norepinephrine acting on α$_1$ receptors (Araneda and Andrade 1991), and metabotropic glutamate...
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muscarinic activation also induces a calcium- and voltage-
ated by a voltage-dependent nonselective cation current. In-
membrane conductance was assessed with depolarizing steps. Note that in both cases the largest
change in apparent membrane conductance occurred during the peak of the
increase in a voltage-dependent cation conductance. Further
increase in membrane resistance. A pyramidal neuron was voltage clamped
at $-71$ mV, and membrane conductance was assessed with hyperpolarizing
or depolarizing voltage steps (10 mV) before and after a jump to $-20$ mV
designed to allow calcium influx into the cell. A: under control conditions the
depolarizing jump is followed by an outward aftercurrent. As expected
for a current associated with an increase in potassium conductance, this
outward aftercurrent is associated with a conductance increase. B: after
carbachol administration, the outward aftercurrent is replaced by I_{sADP}. B_1:
when membrane conductance was assessed with hyperpolarizing steps there
was an apparent decrease in membrane conductance during I_{sADP}. B_2; this
apparent decrease in membrane conductance was even more pronounced
when assessed with depolarizing steps. FIG. 13. The I_{sADP} elicited by carbachol is accompanied with an apparent
increase in membrane resistance. A pyramidal neuron was voltage clamped
receptors ( Constanti and Libri 1992; Greene et al. 1992;
unpublished observations ). This suggests that the appear-
ance of sADP is a widespread response to activation of a
variety of metabotropic neurotransmitters receptors. Indeed,
we previously suggested that sADP might be one of the
common effects signaled by neurotransmitter receptors in
cerebral cortex coupled to G proteins of the G_s family ( Ar-
andada and Andrade 1991 ). If this were the case, it could be expected that all of these sADPs should exhibit a common
underlying mechanism ( Araneda and Andrade 1991 ). How-
ever, as outlined above, different groups studying the sADP
in cortex reached different conclusions regarding their ori-
gin. Thus, in olfactory cortex, the sADP was reported to be
mediated by a decrease in potassium conductance ( Constanti
and Bagetta 1991; Constanti et al. 1993 ), whereas in sensori-
motor cortex ( Greene et al. 1994; Schwindt et al. 1988 ), it
was attributed to a mixed ionic mechanism. In this study we
conclude that in prefrontal cortex the muscarinic-induced
sADP is mediated predominantly, if not exclusively, by an
increase in a voltage-dependent cation conductance. Further
studies will be required to determine whether different ionic
mechanisms underlie the ADP in different regions of cortex.
Interestingly, muscarinic or glutamate metabotropic recep-
tor activation of CA3 neurons also results in the appear-
ance of an ADP in hippocampal slice cultures ( Caesar et al.
1993 ). Like the sADP in prefrontal cortex, this ADP was
reported to be mediated most likely by a calcium-activated
cation current. However, it differs from the sADP seen in
prefrontal cortex in being $\sim 10$ times faster. In this regard,
the hippocampal ADP more closely resembles fast ADP ( fADP ) also seen in prefrontal cortex ( Haj-Dahmane and
Andrade 1997 ).
Muscarinic stimulation of pyramidal neurons of the pre-
frontal cortex results not only in the appearance of sADP
but also in the generation of a slow membrane depolariz-
(Haj-Dahmane and Andrade 1996 ). We previously have
shown that this depolarization, like the sADP, is also medi-
atmed by a voltage-dependent nonselective cation current. In-
interestingly, in smooth muscle cells of the guinea pig ileum,
muscarinic activation also induces a calcium- and voltage-
dependent nonselective cation current ( Inoue and Isenberg
1990a,b; Zholos and Bolton 1994, 1995 ) that mediates a
membrane depolarization and an inward aftercurrent not unlike
those seen in prefrontal cortex. It is tempting to speculate
that in prefrontal cortex, like in guinea pig ileum, the musca-
rinic depolarization and sADP might also reflect different
manifestations of a unitary underlying ionic mechanism: the
depolarization corresponding to the activation of the current
at a basal intracellular calcium level and the sADP corre-
spending to the transient, calcium-induced enhancement of
this same current. Further studies will be needed to validate
or disprove this hypothesis. A similar current was also seen
in Aplysia bag cells, where it makes possible a long-lasting
afterdischarge ( Wilson et al. 1996 ). The widespread distrib-
ution of this nonselective cation current(s) suggests that it
may play an ubiquitous role in regulating membrane excit-
ability.
What could be the role for this sADP in prefrontal cortex? Al-
though ADPs are often associated with bursting activity,
in prefrontal cortex activation of I_{sADP} fails to induce burst-
ing. Instead, activation of I_{sADP} leads to a long-lasting ADP
that, if large enough, produces self-sustained spiking activity
that greatly outlasts the original stimulus ( Andrade 1991 ).
This behavior is reminiscent of that seen for some prefrontal
cortex cells during memory tests, where a cell continues to
fire long past presentation of a relevant stimulus ( Goldman-
Rakic 1990 ). This sustained firing has been interpreted as
representing the neuronal substrate of working memory.
Given the well-established role of ACh on memory function
(Winkler et al. 1995 ), it is tempting to speculate on a possible
relationship between these two phenomena. However,
careful experiments in vivo will be required to examine this
possibility. On a more general level, it is possible that the
dependence of sADP on concerted muscarinic receptor activation and calcium influx into the cell could be an important element in determining how a widespread cholinergic input to cortex might be capable of flexibly regulating membrane excitability of specific cortical cell populations.

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REFERENCES


Hounsfield, J. and Kiehn, O. Serotonin-induced bistability of turtle mo...


