Role of Phosphoinositide Metabolites in the Prolongation of Afterhyperpolarizations by α_1 -Adrenoceptors in Rat Dorsal Raphe Neurons

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Afterhyperpolarizations and outward tail currents of rat dorsal raphe neurons were measured by intracellular recording and single-electrode voltage clamping in the brain slice preparation. The α_1 -agonist phenylephrine, and (in the presence of propranolol) norepinephrine, elicited an increase in the duration, but not of the initial amplitude, of afterhyperpolarizations and associated outward tail currents which followed depolarizing pulses. These effects were antagonized by prazosin, indicating that they were mediated by α_1 -adrenoceptors. The outward tail currents were sensitive to apamin, a blocker of certain Ca^{2+} -activated K^+ currents. A prolongation of afterhyperpolarizations would offset the major excitatory α_1 effects, which are associated with suppression of resting K^+ currents and of the A-current.

Since polyphosphoinositide metabolites have been reported to be second messengers for Ca^{2+} -dependent receptor actions, we compared their effects with those of α_1 -receptor stimulation on these cells. Intracellular ejection of the putative second messenger *myo*-inositol-1,4,5-trisphosphate from the recording electrode transiently mimicked the actions of α_1 -agonists on the afterhyperpolarization. Superfusion with 1 mM LiCl, simulating therapeutic levels of lithium, had no effect on the rate of recovery from inositol trisphosphate ejection.

Superfusion with water-soluble phorbol esters (which mimic actions of another phosphoinositide metabolite, 1,2-diacylglycerol) suppressed rather than mimicked the activation of raphe cell firing by phenylephrine; this occurred with a rank-order potency consistent with activation of protein kinase C and was associated with suppression of a slow inward current and of the outward tail current. Our results suggest that phosphoinositide turnover is more likely to mediate modulatory or negative-feedback effects of α_1 -adrenoceptors than to mediate the major excitatory effects.

The serotonergic neurons of the rat dorsal raphe nucleus display excitatory responses mediated by α_1 -adrenoceptors (Baraban and Aghajanian, 1980). This excitation is associated with suppression of resting K^+ currents and of the early transient outward

current or A-current (Aghajanian, 1985). Rat brain α_1 -receptors are known to stimulate phosphatidylinositol (PI) turnover (Brown et al., 1984; Janowsky et al., 1984; Minneman and Johnson, 1984; Schoepp et al., 1984; Gonzales and Crews, 1985; Kemp and Downes, 1986). Polyphosphoinositide metabolism, in turn, generates the putative second messengers *myo*-inositol-1,4,5-trisphosphate (IP_3), which mediates elevation of intracellular free Ca^{2+} , and 1,2-diacylglycerol (DAG), which activates the Ca^{2+} - and phospholipid-dependent protein kinase C (Berridge, 1984; Nishizuka, 1984).

We now report that, in addition to their major excitatory effects, dorsal raphe α_1 -adrenoceptors mediate a prolongation of the afterspike afterhyperpolarization (AHP) and associated outward currents; this action would tend to modulate or offset the major excitatory effects. The AHP seen in dorsal raphe neurons is in large part mediated by a Ca^{2+} -activated K^+ current (Aghajanian, 1985). Because of the association of IP_3 and DAG with Ca^{2+} -dependent effects, we attempted to determine if there was any resemblance between their actions and those of α_1 -agonists on these cells. We report that intracellular ejection of IP_3 mimics the effects of α_1 -agonists in prolonging the AHP and that phorbol esters, agents which potentially mimic DAG (Nishizuka, 1984; Ashendel, 1985), suppress α_1 -agonist-induced cell firing. Our results suggest that PI metabolism is more likely to mediate modulatory effects than the major excitatory effects of α_1 -adrenoceptors in these cells.

Materials and Methods

Drugs. Tetrodotoxin (TTX), apamin, *l*-phenylephrine hydrochloride, *d,l*-propranolol hydrochloride, *l*-isoproterenol bitartrate, *myo*-inositol trisphosphate (lot number 85F-84402, 4.7 mol K^+ /mol), and all phorbol derivatives were from Sigma Chemical Company. Prazosin hydrochloride was from Pfizer, Inc. *l*-Norepinephrine bitartrate was from Regis Chemical Company. *d*-Propranolol hydrochloride was from Ayerst Laboratories. Ascorbic acid was from Calbiochem. Inorganic salts were of analytic reagent grade.

Methods. Recordings were obtained from serotonergic neurons of the dorsal raphe nucleus in the brain slice preparation by minor modifications of methods that have been described in detail previously (Aghajanian, 1985). Briefly, 450–500 μm coronal sections were prepared at 0–4°C from chloral hydrate-anesthetized male albino rats, 125–175 gm (Charles River) using a Vibroslice 752/M vibrotome (WP Instruments). Slices were placed directly in a slice chamber at a fluid–gas interface at $33 \pm 0.5^\circ\text{C}$. The chamber was continuously circulated with humidified 95% O_2 /5% CO_2 , and superfused with artificial cerebrospinal fluid (ACSF) at 1.0–1.2 ml/min. ACSF consisted of (in mM): NaCl, 126; KCl, 5; CaCl_2 , 2.5; MgSO_4 , 2; NaHCO_3 , 28; NaH_2PO_4 , 1.25; and *d*-glucose, 10, and was pre-equilibrated with 95% O_2 /5% CO_2 to a pH of 7.36–7.42. Recordings were typically obtained 4–10 hr after preparing the slice.

Drug solutions in gassed ACSF were introduced to the chamber through

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Figure 1. Reversal potential of the outward tail current of dorsal raphe neurons. Single-electrode voltage clamping was performed in the presence of $1 \mu\text{M}$ TTX. The cell was step depolarized from -60 to -30 mV, then returned to potentials between -60 and -120 mV, in 10 mV increments. Traces are superimposed. Note that the tail currents following termination of the step depolarizations are outward at < -90 mV and inward > -90 mV.

a stopcock arrangement; latency was less than 1 min. To minimize oxidation, norepinephrine and isoproterenol were prepared within 30 min of use and were administered in the presence of 0.1 mM ascorbic acid, which was without effect on these cells in control experiments (Aghajanian, 1985). Prolonged superfusions with LiCl were begun not less than 1 hr after preparing the slice, to avoid losses of cell viability that were observed when LiCl was applied immediately after preparing the slice. Phorbol derivatives were diluted from 0.1 mM stock solutions, which were prepared without organic solvents by sonication in ACSF.

Microelectrodes for intracellular recording were pulled from 1.5 mm Microstar tubing (Radnoti Glass) on a Brown-Flaming puller (Sutter Instrument Co.). They were filled with 2 M KCl and had resistances of 10 – 20 M Ω . Single-electrode voltage clamping was performed with an Axoclamp-2 (Axon Instruments) as previously described (Aghajanian, 1985); data were photographed on line from a storage oscilloscope. For current-clamp studies of the AHP, spike trains were elicited at 30 sec intervals, with sufficient constant current passed through the electrode between spike trains so that membrane potential would reach a standard value of -65 mV before the next spike train. The $t_{1/2}$ of the AHP was defined as the time elapsed from the end of the depolarizing pulse until the amplitude of the AHP had decayed to 50% of its maximum amplitude. Hyperpolarizing pulses preceding the depolarizing pulses were used to estimate apparent input resistance. Cells deemed suitable for inclusion in this study had input resistances >150 M Ω (typically 200 – 300 M Ω) and spike amplitudes >75 mV (typically 85 – 95 mV); criteria for recognizing serotonergic neurons were as described previously (Aghajanian, 1985). Comparisons of tail current durations and of AHP $t_{1/2}$ values were made using a Student's t test.

For intracellular ejection of IP_3 , electrodes were filled with 20 mM IP_3 , dissolved fresh daily in 1 M KCl, 20 mM HCl (final pH near 7). To eject IP_3 (an anion), sufficient negative current (typically -0.10 to -0.25 nA) was passed to hyperpolarize the cell to -100 mV for 2 min, and the AHP was observed at various times after returning the cell to -65 mV.

Extracellular recordings were performed in the presence of $2 \mu\text{M}$ phenylephrine to induce cell firing (VanderMaelen and Aghajanian, 1983). At this concentration, cells fire at rates comparable to those seen *in vivo*, where the cells receive a tonic noradrenergic input (Baraban and Aghajanian, 1980). Single-barrel electrodes were filled with 2 M NaCl and had impedances of 4 – 6 M Ω , measured in saline at 60 Hz. Cell firing rates were recorded on a Gould chart recorder.

Results

Prolongation of outward tail currents and AHPs by α_1 -adrenoceptors

Step depolarization of dorsal raphe neurons from -60 to -35 mV in the presence of $1 \mu\text{M}$ TTX elicited an initial inward current, followed by a slow outward current and tail current. The outward current had a reversal potential of approximately

-90 mV (Fig. 1), which was previously shown to be the K^+ reversal potential of these cells in the presence of 5 mM KCl (Aghajanian and Lakoski, 1984). This current was suppressed 95 – 100% by agents that blocked Ca^{2+} currents (see figure 4B of Aghajanian, 1985). Apamin, a neurotoxic peptide from bee venom that is known to block some types of Ca^{2+} -activated K^+ currents ($I_{\text{K(Ca)}}$) (Hugues et al., 1982; Pennefather et al., 1985), also suppressed this current (see below).

Application of the α_1 -agonist phenylephrine ($25 \mu\text{M}$) elicited an increase in the duration of the outward tail current (Fig. 2, *A* and *B*; $n = 14$ cells). This increase in time course was not accompanied by any significant increase in the initial amplitude of the current. The total duration of the tail current was 0.32 ± 0.04 sec (mean $\pm$ SD) before application of phenylephrine, and increased to 1.3 ± 0.2 sec after phenylephrine, an increase representing 306% of control ($p < 0.001$; $n = 14$ cells). The peak amplitude of the tail current in the presence of phenylephrine was suppressed 80 – 90% by 100 – 200 nM apamin (Fig. 2, *C* and *D*; $n = 4$ cells); increasing the concentration of apamin to 400 nM produced no further effect. Apamin suppressed the tail current in the absence of phenylephrine to an equivalent extent ($n = 4$ cells, not shown).

Figures 3 and 4 show that in current-clamp mode, phenylephrine elicited a comparable prolongation of the AHP, and that this prolongation was reversed by $5 \mu\text{M}$ prazosin ($n = 6$ cells). The high degree of α_1 -selectivity of prazosin (Menkes et al., 1981) indicates that this effect of phenylephrine was mediated by α_1 -adrenoceptors on these cells. The $t_{1/2}$ of the AHP (see Materials and Methods) was 0.19 ± 0.04 sec (mean $\pm$ SD) before application of phenylephrine and increased to 1.0 ± 0.2 sec after application of phenylephrine, an increase representing 426% of control ($p < 0.001$; $n = 6$ cells).

The endogenous neurotransmitter norepinephrine ($25 \mu\text{M}$) failed, when applied alone, to exert the same effect on the AHP as did phenylephrine (Fig. 5, *A*, *B*; $n = 4$ cells). In voltage clamp, norepinephrine showed little or no prolongation of the outward tail current but caused a partial decrease in the amplitude of the current (Fig. 5, *C*, *D*; $n = 4$ cells). However, when norepinephrine was applied in the presence of $10 \mu\text{M}$ of the β -antagonist propranolol (Madison and Nicoll, 1986a), it was as effective as phenylephrine in prolonging the AHP, and the prolongation was antagonized by prazosin (Fig. 6; $n = 5$ cells). *d*-Propranolol, a

Figure 2. Prolongation by an α_1 -agonist of the outward tail current and its suppression by apamin. *A*, Control voltage-clamp record in the presence of $1 \mu\text{M}$ TTX. *B*, Prolongation of the tail current 10 min after addition of $25 \mu\text{M}$ phenylephrine (PE) plus TTX. *C* and *D*, Records 5 and 10 min, respectively, after addition of 200 nM apamin (AP). Note that in the control sweep (*A*) the tail current returns to baseline within 0.5 sec , whereas after PE (*B*) the tail current is still elevated even at the end of the sweep (arrow). The cell was clamped at -60 mV and depolarized to -35 mV to elicit the outward tail current (200 msec step). Prior to the addition of PE the holding current at -60 mV was $+0.2 \text{ nA}$; PE induced an inward current of 0.14 nA . Note that apamin did not change the holding current despite reduction in outward tail current.

stereoisomer lacking serotonergic antagonist activity (Sprouse and Aghajanian, 1986), was as active as the racemic mixture. In the presence of propranolol, the $t_{1/2}$ of the AHP was $0.19 \pm 0.02 \text{ sec}$ before norepinephrine and increased to $1.2 \pm 0.3 \text{ sec}$ after norepinephrine, a 532% increase ($p < 0.001$; $n = 5$ cells). The β -agonist isoproterenol ($25 \mu\text{M}$) failed to diminish the AHP or to prevent phenylephrine from prolonging the AHP ($n = 4$ cells, data not shown).

Prolongation of AHPs by IP_3

Cells impaled with electrodes containing 20 mM IP_3 initially showed no apparent difference from cells impaled with control electrodes. Within 5 sec after passing negative current through the electrode to eject IP_3 , the AHP was prolonged much as after application of α_1 -agonists (Fig. 7, *A*, *B*). Of 9 cells tested with IP_3 -containing electrodes, 8 responded as in Figure 7 and 1 did

not respond. At 5 sec after ejection, the increase in duration of the AHP was $305 \pm 110\%$ for the responsive cells ($p < 0.01$; Fig. 7*D*). When the same protocol of passing negative current was performed with cells impaled with control electrodes, no change in the AHP was observed (Fig. 8; $n = 4$ cells). The prolongation of the AHP was rapidly reversible, with the AHP time course returning to control values in less than 1 min (Fig. 7, *C*, *D*).

The antimanic drug lithium has been reported to inhibit inositol-monophosphate phosphatase and, to a lesser extent, other enzymes involved in the breakdown of IP_3 to *myo*-inositol (Berridge et al., 1982; Thomas et al., 1984). On this basis, it is possible that lithium might prolong the actions of IP_3 by interfering with its breakdown. To investigate whether concentrations of lithium corresponding to tissue levels attained at therapeutic dosages would have effects on raphe cell AHPs, slices

Figure 3. Prolongation by an α_1 -agonist of the afterhyperpolarization. *A*, Control current-clamp record before addition of drug. *B*, Prolongation of the afterhyperpolarization (arrow) 15 min after addition of $25 \mu\text{M}$ phenylephrine (PE). The initial resting potential of this cell was -65 mV (as seen in *A*). In *B*, to maintain the cell at -65 mV , negative current was injected (-0.16 nA) to compensate for the depolarizing effect of PE.

Figure 4. α_1 -Adrenoceptor-mediated prolongation of the afterhyperpolarization, at a faster sweep speed than in Figure 3. *A*, Control current-clamp record before addition of drug. *B*, Prolongation of afterhyperpolarization (arrow) 15 min after addition of 25 μ M phenylephrine (PE). *C*, Reversal of prolongation 15 min after addition of 5 μ M prazosin (PRAZ).

were superfused with 1 mM LiCl for 4–6 hr. In these experiments, cell AHPs showed the same response to IP_3 injection, and the response wore off at the same rate, as in cells not exposed to lithium (Fig 7D; $n = 4$ cells). Application of LiCl at 2 mM or above—concentrations associated clinically with toxic side effects (Byck, 1975)—rapidly caused a variety of effects, includ-

ing a decrease in inward currents and a general decrease in cell viability ($n = 5$ cells, data not shown).

Effects of protein kinase C activators

We found in preliminary experiments that organic solvents such as dimethyl sulfoxide and dimethyl formamide, which would normally have to be used to introduce DAG analogs or long-chain phorbol esters into our interface slice chamber, exerted toxic effects by themselves on raphe slices. We therefore tested a series of short-chain phorbol diesters that could be dissolved directly in ACSF. One of these was 4- β -phorbol-12,13-diacetate (PDA). At a concentration of 500 nM, which is at the midpoint of the dose-response relationship for protein kinase C binding and skin irritation by PDA (Dunn and Blumberg, 1983), and is near or below PDA concentrations reported to be effective in hippocampal pyramidal cells (Baraban et al., 1985), PDA not only failed to prolong the AHP, but actually partially decreased its amplitude (Fig. 9A; $n = 10$ cells). In voltage clamp, 500 nM PDA similarly suppressed the outward tail current (Fig. 9, B–D; $n = 4$ cells). This was accompanied by suppression of the inward current which preceded the outward current (Fig. 9, B–D). The effects of PDA on these currents were essentially indistinguishable from the effects of the Ca^{2+} channel blocker $CdCl_2$ (1 mM) (see figure 4A, B, of Aghajanian, 1985). PDA also reversed the net steady inward current elicited by phenylephrine (Fig. 9, B–D) and blocked the depolarizing response to phenylephrine seen in current clamp (not shown). PDA also elicited further steady outward currents in 2 of 4 cells tested in voltage clamp, and further hyperpolarized 4 of 10 cells tested in current clamp; in no case did it elicit net inward current or depolarization.

Because there was no obvious similarity between the above effects of PDA and the effects of α_1 -agonists, we tested a series of soluble phorbol esters extracellularly, in order to detect other

Figure 5. Effects of norepinephrine on the afterhyperpolarization and on the outward current. *A* and *B*, Current-clamp records before and after addition of 25 μ M norepinephrine (NE). *C* and *D*, Voltage-clamp records, in the presence of 1 μ M TTX, before and after addition of 25 μ M norepinephrine.

Figure 6. Prolongation of the afterhyperpolarization by norepinephrine in the presence of propranolol. *A*, Control current-clamp record before addition of drug. *B*, Record 10 min after addition of 10 μ M propranolol (PROP). *C*, Prolongation of afterhyperpolarization (arrow) 10 min after addition of 25 μ M norepinephrine (NE). *D*, Reversal of prolongation 15 min after addition of 5 μ M prazosin (PRAZ). The decrease in input resistance in *D* was not typical of responses to prazosin.

possible effects and to establish a rank-order potency. The compounds tested were PDA, 4- β -phorbol-12,13-dibutyrate (PDB), and 4- β -phorbol-12,13,20-triacetate (PTA). In extracellular recordings, all 3 compounds suppressed phenylephrine-induced cell firing (Fig. 10). As shown, when added at the minimum concentration sufficient to suppress firing, phorbol esters were slow to exert their effects, and their actions were slowly reversible. In the presence of 2 μ M phenylephrine, the concentrations of phorbol esters that suppressed firing by 50% (in nM, mean $\pm$

SD) were: PDB, 41 ± 21 ($n = 11$ cells); PDA, 190 ± 46 ($n = 11$ cells); and PTA, 630 ± 220 ($n = 6$ cells). The parent alcohol, 4- β -phorbol, was without effect at a concentration of 5 μ M (Fig. 10D; $n = 4$ cells). These potencies correlate closely with published values for protein kinase C activation and for skin irritation by phorbol esters (Dunn and Blumberg, 1983). Since cell firing rates increased with the concentration of α_1 -agonists (Fig. 10A, and VanderMaelen and Aghajanian, 1983), phorbol esters and α_1 -agonists had opposite effects on raphe cell firing rates.

Figure 7. Prolongation of the afterhyperpolarization by inositol trisphosphate (IP₃). *A*, Current-clamp record of a cell impaled with an electrode containing 20 mM IP₃. *B*, Prolongation of the afterhyperpolarization (arrow) 5 sec after ejecting IP₃ as described in Materials and Methods. *C*, Partial reversal of the prolongation 20 sec after ejecting IP₃. *D*, Time course of IP₃ reversal. The $t_{1/2}$ of the afterhyperpolarization was measured as described in Materials and Methods. The percentage increase in $t_{1/2}$ after IP₃ ejection, relative to the $t_{1/2}$ before ejection, is plotted at various times after ejection. Data are means $\pm$ SD for 8 cells superfused with control medium (circles) and 4 cells superfused for 4–6 hr with medium containing 1 mM LiCl (triangles).

Figure 8. Control experiment showing lack of effect on the afterhyperpolarization of a sham intracellular ejection. The experiment was performed exactly as in Figure 7, *A* and *B*, except that inositol trisphosphate was omitted from the electrode.

Discussion

The major findings of this study are that the α_1 -adrenoceptors of rat dorsal raphe neurons mediate a prolongation of the AHP, and that IP_3 mimics this effect, fulfilling one necessary criterion of a second messenger candidate. Fulfillment of other second messenger criteria by IP_3 remains to be established for these particular cells. Indeed, one of the most striking features of this study is the extent to which the effects of IP_3 and of activation of protein kinase C seemed not to resemble the prominent excitatory effects of α_1 -agonists, whose responses they have been hypothesized to mediate.

We have used single-electrode voltage clamping to describe a postactivation outward current of these cells with a reversal potential consistent with K^+ being a major carrier ion. Previous studies (Aghajanian, 1985) showed this current to be sensitive

to Ca^{2+} channel blockers. We report now that about 80–90% of this current was sensitive to apamin and therefore conclude that 80–90% of the peak outward tail current was an apamin-sensitive $I_{K(Ca)}$. There are several possibilities as to the identity of the current or currents comprising the 10–20% that was apamin-resistant. These include an apamin-insensitive subtype of $I_{K(Ca)}$ (Pennefather et al., 1985) and the interruption of a persistent inward Ca^{2+} current (Kramer and Zucker, 1985).

The tail current resulting from the decay of these outward currents was consistently prolonged by the α_1 -agonist phenylephrine. Although the additional current at late time points was small (<0.1 nA), such small currents can have significant effects on membrane potential in raphe cells, which have high input resistances (typically 200–300 M Ω). It is not clear to what extent this prolongation of the total tail current reflected a prolongation of the apamin-sensitive $I_{K(Ca)}$, as opposed to prolongation of

Figure 9. Current- and voltage-clamp studies of the effects of a phorbol ester. *A*, Current-clamp records of the diminution of the afterhyperpolarization (arrow) 20 min after application of 500 nM phorbol diacetate (PDA). The current pulses were -0.08 and $+0.30$ nA. *B*, Voltage-clamp record of a different cell in the presence of $1 \mu M$ TTX. Single arrow denotes the inward current; double arrows denote the outward tail current. I_h is the holding current needed to clamp the cell at -60 mV. *C*, Record 10 min after addition of $2 \mu M$ phenylephrine (PE). At this concentration, the tail current was not consistently prolonged. *D*, Record 20 min after addition of 500 nM PDA.

Figure 10. Suppression of agonist-induced cell firing by phorbol esters. Cell firing rates were measured extracellularly in the presence of 2 μ M phenylephrine. *A*, Increase in cell firing rate in response to raising the concentration of phenylephrine (*PE*) from 2 to 25 μ M, followed by a decrease in firing in response to 400 nM phorbol diacetate (*PDA*), which was very slowly reversible. *B*, Suppression of firing by 50 nM phorbol dibutyrate (*PDB*). *C*, Suppression of firing by 1 μ M phorbol triacetate (*PTA*). *D*, Lack of direct effect of 5 μ M 4- β -phorbol (*PHOR*), followed by normal response to 400 nM *PDA*.

other outward currents or the induction of currents not expressed in the absence of phenylephrine.

We wished to introduce IP_3 into raphe cells via the recording electrode. However, we found that IP_3 -containing microelectrodes conducted less well than those containing only KCl, rendering them unsuitable for single-microelectrode voltage clamping, although current-clamp recording was possible. If the effect of phenylephrine on the tail current had been primarily an increase in initial amplitude, it would have been unreliable to attempt to infer analogous effects in current clamp, due to the voltage dependence of the currents. However, voltage-clamp data indicated that the primary effect of phenylephrine was an increase in time course rather than in initial amplitude, and time course can be validly measured in current clamp. We found that both IP_3 and α_1 -agonists prolonged the AHP in current clamp. The lack of increase in tail current peak amplitude makes it unlikely that the prolongation was secondary to an increase in inward Ca^{2+} currents.

Because the effect of phenylephrine on the AHP was antagonized by prazosin, it is likely to have been mediated by α_1 -adrenoceptors (Menkes et al., 1981). The endogenous neurotransmitter norepinephrine also elicited this effect in a prazosin-sensitive manner, but only after prior exposure of the cells to propranolol. Since β -adrenoceptors in hippocampal pyramidal cells decrease the amplitude of the AHP through a cyclic AMP-mediated mechanism (Madison and Nicoll, 1986a, b), it is tempting to speculate that β -receptors on dorsal raphe cells exerted effects opposed to the α_1 effects and that propranolol unmasked the α_1 actions of norepinephrine. However, the β -agonist isoproterenol was apparently inactive in our experiments, indicating that further experiments will be needed to elucidate why propranolol had this effect. Conceivably norepinephrine, but not isoproterenol, could act at additional sites to augment the activation of adenylate cyclase by β -receptors, as

has been reported in some brain regions (Duman et al., 1986; Stone and Herrera, 1986).

Because the major actions of IP_3 are the mobilization of intracellular Ca^{2+} (Berridge, 1984) and possibly the slowing of Ca^{2+} reequilibration after Ca^{2+} entry (Popescu et al., 1986), IP_3 deserves consideration as a candidate second messenger for the effects of α_1 -receptors on Ca^{2+} -dependent conductances. Rat brain α_1 -adrenoceptors are known to be positively coupled to PI turnover (Brown et al., 1984; Janowsky et al., 1984; Minneman and Johnson, 1984; Schoepp et al., 1984; Gonzales and Crews, 1985; Kemp and Downes, 1986); however, this has not been demonstrated specifically in the dorsal raphe. Passing negative current through IP_3 -containing electrodes appeared to mimic the effects of α_1 -agonists on the AHP. Since passing negative current through electrodes not containing IP_3 did not have this effect, ruling out fluctuations in membrane potential as a cause of the prolongation, this action must have been caused in our experiments by either: (1) IP_3 itself; (2) a compound generated by metabolism of IP_3 , such as *myo*-inositol-1,3,4,5-tetrakisphosphate (Hansen et al., 1986); or (3) a substance present as a contaminant in our samples of IP_3 . The rapid onset and reversal of this effect seems consistent with rapid metabolic turnover of the active substance and provides indirect evidence against exogenous IP_3 being metabolized to a more active compound. However, in the absence of voltage-clamp measurements of isolated currents, we cannot rule out the possibility that IP_3 and α_1 -agonists influenced different currents underlying the AHP. Thus, while by far the simplest interpretation of our data is that IP_3 (or a metabolite) fulfills one necessary criterion of a second messenger by appearing to mimic the actions of agonists on the AHP, IP_3 has yet to be proven to be a second messenger in dorsal raphe neurons. IP_3 has previously been shown to enhance $I_{K(Ca)}$ in the NG108-15 cell line (Higashida and Brown, 1986).

The antimanic drug lithium is known to inhibit some of the

enzymes involved in the breakdown of IP_3 to *myo*-inositol (Berridge et al., 1982; Thomas et al., 1984). Raphe serotonergic cells display enhanced synaptic effects in response to therapeutic levels of lithium (Blier and deMontigny, 1985). In assessing whether lithium could exert its antimanic actions through an effect on brain PI turnover, it is crucial to consider the concentration of lithium used. LiCl at 1 mM corresponds to about the maximum CSF concentration attained in humans at clinically therapeutic dosages, whereas concentrations above 1 mM are associated with toxic side effects (Byck, 1975). We found that exposure of raphe slices to 1 mM LiCl for up to 6 hr had no effect on the rate at which the effects of exogenous IP_3 wore off. Thus our negative results failed to provide evidence that lithium slowed the breakdown of IP_3 , but of course we cannot rule out effects under other experimental conditions, such as with more long-term exposure to 1 mM LiCl. It is worth noting that in intact cells external lithium concentrations of 5 mM or more are often needed to substantially affect IP_3 levels, as opposed to levels of inositol or inositol monophosphate (Thomas et al., 1984). Menkes et al. (1986) have used a somewhat different protocol to describe a dampening effect of LiCl concentrations at or just above 1 mM on reversal of receptor-mediated contractions in tracheal smooth muscle.

To mimic the other putative second messenger associated with PI turnover, DAG, we have utilized a series of water-soluble phorbol esters, thereby avoiding organic solvent effects. Rather than mimicking α_1 -agonists, these phorbol esters had effects diametrically opposite that of agonists on the rate of raphe cell firing and on the outward tail current and AHP. The principal action of phorbol esters is known to be DAG-like activation of protein kinase C, although the degree of activation with phorbol esters is much more massive than with DAG itself (Nishizuka, 1984; Ashendel, 1985). However, phorbol esters may have some additional actions as well (Kreutter et al., 1985; Yamamoto et al., 1985; Cochet et al., 1986; Gschwendt et al., 1986; Vaartjes et al., 1986). To assess the validity of our effects, we tested extracellularly a series of phorbol ester analogs as well as the biologically inactive parent alcohol, determining the minimum effective concentration for each analog. This rank-order potency agreed extremely well with published values for binding to C-kinase (Dunn and Blumberg, 1983); as in hippocampal pyramidal cells, potencies correlated with the skin-irritant rather than tumor-promoting subtype (Baraban et al., 1985). Indeed, our results are not surprising in light of a number of recent reports that phorbol esters and α_1 -agonists have opposing effects in other cell types (Corvera and Garcia-Sainz, 1984; Labarca et al., 1984; Cooper et al., 1985; Lynch et al., 1985; McMillan et al., 1986). Leeb-Lundberg et al. (1985) have shown that C-kinase phosphorylates α_1 -receptors, uncoupling receptor from response, and have proposed that this could be a negative-feedback mechanism of the receptor, which seems consistent with our results.

Another likely mechanism, not exclusive of the one above, by which phorbol esters could have suppressed cell firing would be through inhibition of a transient low-threshold Ca^{2+} conductance, which has recently been observed in these cells (Burlhis and Aghajanian, 1986). In the present study, we used voltage clamping in the presence of TTX to describe a high-threshold slow inward current. Since this current was inhibited by Ca^{2+} channel blockers (Aghajanian, 1985), there is a good probability that it was, at least in part, a high-threshold I_{Ca} . The amplitude of this current was diminished by phorbol esters. Madison et

al. (1986) have described a chloride current suppressed by phorbol esters in hippocampal pyramidal cells, but this seems distinct from the currents that we have described in that it was activated by step hyperpolarization. Although it is possible that phorbol esters could have appeared to suppress the inward current by actually stimulating competing outward currents, the simplest interpretation of our results is that the primary action of phorbol esters was a suppression of I_{Ca} , with suppression of Ca^{2+} -dependent outward currents and thus of the AHP occurring secondarily to decreased Ca^{2+} entry. Suppression of Ca^{2+} conductance associated with C-kinase activation has also been described in chick dorsal root ganglion cells (Werz and MacDonald, 1985; Rane and Dunlap, 1986), the PC12 and RINm5F cell lines (DiVirgilio et al., 1986), the AtT-20 cell line (Weight and Lewis, 1986), and *Helix aspersa* neuron D2 (Hammond et al., 1986). However, this is not true in all cell types, since C-kinase activation stimulates Ca^{2+} currents in *Aplysia* bag cell neurons (DeRiemer et al., 1985), and *Hermisenda* photoreceptors (Farley and Auerbach, 1986). Phorbol esters also suppress $I_{K(Ca)}$ in hippocampal pyramidal neurons (Baraban et al., 1985; Malenka et al., 1986) but do so independently of any change in the amplitude of Ca^{2+} spikes (Malenka et al., 1986).

α_1 -Adrenoceptors excite dorsal raphe neurons by suppressing 2 classes of outward K^+ current: resting K^+ currents and the A-current (Aghajanian, 1985). Our data indicate that, paradoxically, these receptors have an *enhancing* effect on a third type of outward current, that underlying the AHP. We suggest that prolongation of the AHP serves to modulate or offset the major excitatory effects, resulting in a moderate, rather than massive, excitation of the cells. Our data also indicate that IP_3 , or a metabolite, meets one of the criteria for a second messenger mediating this prolongation of the AHP. It is unclear precisely how DAG would relate to the known effects of α_1 -agonists on these cells, since phorbol esters and α_1 -agonists appeared to have opposing effects. Since under certain conditions in other cell types, DAG can be produced independently of IP_3 by receptor-activated hydrolysis of lipids other than PI-4,5-bisphosphate (Griendling et al., 1986; Saltiel et al., 1987), DAG and C-kinase may sometimes serve as a negative-feedback mechanism in raphe neurons. This could result from both a small decrease in I_{Ca} (smaller than seen with phorbol esters), which could modulate, in turn, the putative effects of IP_3 on the AHP, and from the uncoupling effects of receptor phosphorylation.

Could an increase in PI turnover mediate the major α_1 excitatory effects? On the whole, our results suggest that the answer may be no. The major excitatory effects are not Ca^{2+} -sensitive in these cells (Aghajanian, 1985), and so it would be hard to fit them with the known actions of IP_3 , but of course we cannot rule out a novel, Ca^{2+} -independent action of IP_3 . Phorbol esters not only failed to mimic the excitatory effects, but actually blocked them. Of course, we cannot rule out other actions of phorbol esters, masking excitatory effects, but there is no evidence for this having occurred. How then are the major excitatory effects mediated? Possibilities include direct coupling of receptor to ion channel, coupling via some other second messenger system, or coupling of the receptor via a guanyl nucleotide binding protein directly to ion channels without a second messenger, as has been shown to occur for cardiac muscarinic cholinergic receptors (Pfaffinger et al., 1985; Cohen-Armon and Sokolovsky, 1986). Thus, these α_1 -adrenoceptors exert multiple electrophysiologic effects, only some of which may be mediated by a given second messenger.

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