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Serotonin Inhibits the Peptide FMRFamide Response Through a Cyclic AMP–Independent Pathway in *Aplysia*

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SUMMARY AND CONCLUSIONS

1. The S-K^+ conductance was isolated by voltage-clamping near the resting potential pleural mechanosensory neurons of *Aplysia* in culture. This background conductance is modulated in opposite directions by two distinct, transmitter-controlled second-messenger cascades: it is enhanced by the peptide FMRFamide through the 12-lipoxygenase pathway of arachidonic acid, and it is decreased by serotonin (5-HT) through adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent phosphorylation.

2. The dose-dependent activating effect of FMRFamide (0.01–500 μM) on the S-K^+ conductance was measured in the presence and the absence either of 1–100 μM 8-bromo-cAMP (8b-cAMP, a membrane-permeable and hydrolysis-resistant analogue of cAMP), or of 0.01–0.1 μM 5-HT.

3. When 8b-cAMP was applied, it produced a slow inward current response due to closure of the S-K^+ conductance. This response was antagonized by FMRFamide in a dose-dependent mode. Application of 100 μM FMRFamide, in the presence of 1–10 μM 8b-cAMP, produced an outward current response *larger* than the control FMRFa response and equal to the sum of the responses to FMRFamide alone and to 8b-cAMP alone. Similarly, at 500 μM, FMRFamide completely antagonized the closing action of maximal 8b-cAMP levels (100 μM). This observation confirms previous work that indicated that FMRFamide can reopen S-K^+ channels closed by FMRFamide.

4. In contrast, in the presence of moderate concentrations of 5-HT (0.01 μM), which produce a slow inward current due to the closing of the S-K^+ conductance, FMRFamide elicited a response that only partially antagonized this 5-HT action. Under maximal 5-HT concentrations (0.1 μM), the 5-HT response was not antagonized by any FMRFamide concentration: instead, the FMRFamide response was *smaller* than the control response without 5-HT. This experiment suggests that 5-HT, with an action independent from cAMP, inhibits the effect of FMRFamide on the S-K^+ channel.

5. The dose-dependent inhibitory effect of 5-HT (0.001–10 μM) on the S-K^+ conductance was measured in the presence and the absence either of FMRFamide (1–50 μM), which stimulates the release and metabolism of arachidonic acid in *Aplysia* sensory neurons or of arachidonic acid (25 μM).

6. Under these conditions, supramaximal concentrations of 5-HT could not completely suppress the slow outward current evoked by FMRFamide or by arachidonic acid, indicating that a component of the arachidonic-mediated response to FMRFamide is resistant to actions that maximally increase the S-K^+ channel phosphorylation.

7. The dose-dependent activating effect of arachidonic acid (1–100 μM) on the S-K^+ conductance was measured in the presence and the absence of 5-HT (0.01 μM). Under this fixed dose of 5-HT, the higher concentrations of arachidonic acid evoked outward currents larger than those evoked in the absence of 5-HT. However, much like FMRFamide, arachidonic acid invariably failed to fully antagonize the 5-HT–evoked inward current. This experiment confirms that arachidonic acid metabolism also mediates the action of FMRFamide, which is antagonistic to that of cAMP (reopening).

8. This work reconciles divergent findings present in the literature and, in addition, uncovers a novel action of 5-HT. In the sensory neurons of *Aplysia*, 5-HT and FMRFamide regulate the S-K^+ channel, each using two separate mechanisms: both transmitters exert opposing control on the level of phosphorylation of the S-K^+ channels, which in turn regulates the number of channels available for the gating. In addition to this "competitive" interaction on the S-K^+ channels, FMRFamide directly increases their probability of opening, and 5-HT depresses the actions of FMRFamide through a cAMP-independent pathway.

INTRODUCTION

Receptors modulate the activity of ion channels and thus neuronal excitability through a variety of biochemical cascades (for recent reviews, see Belardetti and Siegelbaum 1988; Levitan 1988; Piomelli and Greengard 1990; Sternweis and Pang 1990; Yau and Baylor 1989). As a consequence, neurons normally integrate different second-messenger effects acting on the same ion channel (Belardetti et al. 1987; Kramer and Levitan 1990; Lotshaw and Levitan 1988; Schaad et al. 1987; Schweitzer et al. 1990; see also Hartzell and Fischmeister 1986). However, little is known about the mechanisms underlying this type of interaction. To study the interplay between two antagonistic transmitter actions, we used a well-studied system (Belardetti and Siegelbaum 1988; Kandel and Schwartz 1982), the nervous system of the mollusk *Aplysia*.

In *Aplysia* mechanosensory neurons, the background S-K^+ channel undergoes a dual antagonistic type of regulation. Serotonin (5-HT) produces long closures in the S-K^+ channel spontaneous activity, leading to a decrease in the number of active channels in the membrane. This effect is mediated by adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent phosphorylation (presumably of the S-K^+ channel itself: Klein et al. 1982; Shuster et al. 1985; Siegelbaum et al. 1982) and results in a slow decreased-conductance depolarization, thus contributing to the facilitation of transmitter release induced by 5-HT (Kandel and Schwartz 1982). In contrast, the peptide FMRFamide increases the S-K^+ channel opening, an action at the basis of a slow increased-conductance hyperpolarization, thus contributing to the presynaptic inhibition of transmitter release (Abrams et al. 1984; Belardetti et al. 1987; Brezina et al. 1987a; Cottrell et al. 1984; Price and Greenberg 1977;

Sweatt et al. 1989). This FMRFamide channel opening action has two distinct components. *1*) In basal conditions, FMRFamide increases the S-K^+ channel opening probability (Belardetti et al. 1987) through activation of the 12-lipoxygenase pathway of arachidonic acid (Piomelli et al. 1987). The active metabolite of this pathway in intact sensory cells, 12-hydroperoxyeicosatetraenoic acid (12-HPETE), needs further metabolism by a P_{450}-like enzyme (possibly to hepoxilin A_3; Piomelli et al. 1989) to increase the opening of the S-K^+ channel (Belardetti et al. 1989). This opening action does not involve a phosphorylation or a dephosphorylation reaction (Belardetti et al. 1989; Buttner et al. 1989), and it is thought to be direct. *2*) In the presence of elevated cAMP levels, FMRFamide completely antagonizes the closing action of cAMP and reopens the S-K^+ channels (Belardetti et al. 1987). There is evidence that this antagonistic action of FMRFamide is also mediated by a lipoxygenase pathway of arachidonate metabolism (Volterra and Siegelbaum 1988), which ultimately leads to dephosphorylation of the S-K^+ channel (Ichinose et al. 1990; Sweatt et al. 1989). However, the mechanism that leads to this dephosphorylation is not known. In apparent conflict with the observation of complete antagonism at single-channel level, parallel studies using macroscopic current measurements indicated that FMRFamide can only partially antagonize the S-K^+ current-reducing actions of 5-HT or cAMP (Sweatt et al. 1989). Moreover, in *Aplysia* neurons other than the mechanosensory, 5-HT or cAMP profoundly depresses the activation of the S-K^+ current by FMRFamide (Brezina et al. 1987a).

A possible difficulty when attempting to isolate the macroscopic S-K^+ channel is that multiple conductances are regulated by FMRFamide and 5-HT in *Aplysia* neurons. However, several lines of evidence indicate that, when the membrane is voltage-clamped near the resting potential, the FMRFamide response is solely mediated by the enhancement of the S-K^+ current (Brezina et al. 1987a). Superimposed on the S-K^+ current action, FMRFamide also decreases the voltage-dependent Ca^{2+} current [$I_{Ca(V)}$], but only if step commands positive to −20 mV are applied to activate this current. In addition, the Ca^{2+}-dependent K^+ current [$I_{K(Ca)}$] is also decreased by FMRFamide, but only indirectly as a result of the $I_{Ca(V)}$ decrease (Brezina et al. 1987a,b; Edmonds et al. 1990). In contrast, 5-HT enhances the $I_{Ca(V)}$ (Edmonds et al. 1990) and also modulates the delayed rectifier [$I_{K(V)}$; Baxter and Byrne 1989] after their activation by steps positive to −20 mV. At membrane potentials negative to −20 mV, similarly to FMRFamide, the 5-HT response is due to a decrease of the S-K^+ current (Baxter and Byrne 1989; Klein et al. 1982). However, a recent report suggests that a second K^+ current, a steady-state $I_{K(Ca)}$, is decreased by 5-HT at membrane voltages as negative as −35 mV (Walsh and Byrne 1989). A mechanism of this type is not easily reconciled with two sets of observations. *1*) The resting Ca^{2+} concentration is low in sensory cells, and it is not increased by 5-HT if the membrane is held at negative potentials (Blumenfeld et al. 1990; Boyle et al. 1984). This low internal Ca^{2+} is expected to keep the Ca^{2+}-dependent K^+ channels closed at resting potential, as confirmed by single-channel recordings from cell-attached patches (Belardetti et al. 1986). *2*) The response to 5-HT when the membrane is held at negative potentials (Baxter and Byrne 1989; Klein et al. 1982) shows little sensitivity to external tetraethylammonium (TEA), much like the S-K^+ channel (Shuster and Siegelbaum 1987), whereas the *Aplysia* $I_{K(Ca)}$ is highly sensitive to TEA (Hermann and Gorman 1981). One alternative explanation of the original observations (Walsh and Byrne 1989) is that the 5-HT response is metabolically Ca^{2+} dependent.

In this work, we have characterized in quantitative form the interactive effects of FMRFamide and 5-HT on the S-K^+ conductance to obtain a better understanding of the underlying mechanisms and to clarify the controversy about the efficacy of FMRFamide antagonism. To this purpose, we have restricted our analysis to voltages near the resting potential as a convenient way to isolate the S-K^+ conductance from other conductances modulated by FMRFamide and 5-HT.

Some of these results have already been presented in abstract form (Belardetti and Shi 1991; Shi and Belardetti 1990).

METHODS

Cell culture

Aplysia californica were purchased from the *Aplysia* Research Facility at the University of Miami (Miami, FL), kept in a refrigerated (15°C) aquarium, and fed with fresh red gracyllaria. The animals were anesthetized with isotonic $MgCl_2$ solution for 20 min, and all the ganglia were then dissected out. Tail mechanosensory neurons from the pleural cluster (Walters et al. 1983) were cultured as previously described (Schacher and Proshansky 1983).

Solutions

The cells were superfused with artificial sea water (ASW), containing (in mM) 460 NaCl, 10 KCl, 55 $MgCl_2$, 11 $CaCl_2$, and 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), brought to pH 7.6 with NaOH. All the solutions were filtered through a 0.2-μm filter before use. TEA containing sea water was obtained by replacing the appropriate amount of NaCl with either TEA-Cl or TEA-Br (Fluka, Buchs, Switzerland).

Chemicals

The components for the ASW and the cAMP analogues were purchased from Sigma Chemical (St. Louis, MO), FMRFamide from Peninsula Laboratories (Belmont, CA), 5-HT from Calbiochem (San Diego, CA), and arachidonic acid from Nu Chek Prep (Elysian, MN).

Drug application

The cAMP analogues and the transmitters were stored dry at −25°C. Arachidonic acid was stored in toluene under argon and at −70°C. Stock solutions of the cAMP analogues and the transmitters in ASW were prepared at the beginning of each day and were kept in ice. An aliquot from the arachidonic acid solution was dried under gentle argon flux in a glass tube immediately before use; then 200 μl of ASW were added, and the mixture was sonicated for 15 s before the application. cAMP or 5-HT were manually pipetted into the experimental bath as concentrated aliquots from the stock solution. Their concentrations, as given in the text and figures, are their final bath concentrations. FMRFamide and arachidonic acid were pressure-applied from a wide-mouthed pi-

pette positioned near the neuronal cell body; the concentrations of these compounds refer to the pipette concentrations. During a typical experiment, a given concentration of FMRFamide (or arachidonic acid) in the absence of 5-HT or 8-bromo-cAMP (8b-cAMP) was applied twice (see Fig. 1*A*, B_1 and D_1) to control for a possible decline of the response, and the mean between these two values was used for the comparison with the response to FMRFamide (or arachidonic acid) in the presence of 5-HT or 8b-cAMP (Fig. 1*A*, C_1).

Voltage-clamp recordings

Intracellular microelectrodes were pulled from WPI (New Haven, CT) thin-wall glass capillaries and were filled with 2.5 KCl to resistances of 20–25 MΩ. Only sensory cells with a steady resting potential of at least −40 mV were used. The microelectrode was connected to an Axoclamp 2-A amplifier (Axon Instruments, Burlingame, CA) with a Ag-AgCl wire, and the bath was connected to the ground via an agar-salt bridge. The amplifier was used in the single-microelectrode, discontinuous voltage-clamp mode. The switching frequency was set between 2 and 4 kHz. The current record was filtered at 100 Hz, digitized at 22 kHz with a Sony PCM 501 audio adapter (Dagan, Minneapolis, MN), and stored in a conventional videotape recorder.

A simple voltage-clamp protocol was used: the membrane voltage was held at a fixed value of −35 mV. The voltage was stepped every 5 s for 0.5 s at −40 mV to measure the membrane slope conductance.

Data analysis

Current records were played back on chart paper. We measured the peak current responses to the drug applications in the outward and in the inward directions (Fig. 1), using a holding current of zero for all our measurements. This analysis is based on two assumptions: *1*) that the $S\text{-}K^+$ already opens and closes spontaneously at the resting potential, in the absence of transmitter stimulation, and *2*) that 5-HT and cAMP close the $S\text{-}K^+$ channel, whereas FMRFamide and arachidonic acid open the same channel. The average number of channels open cannot be estimated in absolute terms with the use of macroscopic current recordings. Thus, to compare the $S\text{-}K^+$ channel-closing and -opening actions of various treatments within a cell and among cells, we normalized within each cell the currents produced by these treatments to the current produced by a fixed dose of FMRFamide.

When the FMRFamide dose-response relationship was studied (Fig. 1*A*), all the responses within a given cell were normalized to the value of the maximal response to 100 μM FMRFamide (A_2). In the absence of 5-HT (or 8b-cAMP), the normalized mean response to a given concentration of FMRFamide $[(B_2 + D_2)/2]$ was plotted with zero as baseline. In the same cell, in the presence (E_1) of 5-HT (or 8b-cAMP), the normalized response to FMRFamide (C_2) was plotted with the normalized and averaged value of the 5-HT (or 8b-cAMP) response as baseline. The responses to a given dose of 5-HT (or of 8b-cAMP) in the absence of FMRFa were separately normalized within each cell to the maximal response to FMRFa in that cell. This is a legitimate operation, because all these responses are the results of the closing and opening of the same channel. However, for 5-HT (and 8b-cAMP), the minus sign was used. These normalized values of the 5-HT or 8b-cAMP responses were then averaged to give the normalized and averaged value of the 5-HT (or 8b-cAMP) response for any given cell. Finally, the mean of all cells was obtained and plotted in E_2. Similarly, when the dose-dependent actions of 5-HT were studied (Fig. 1*B*), all the responses within a given cell were normalized to the value of the maximal response to FMRFamide, $(B_2 + D_2)/2$. The dose-dependent action of 5-HT on the S current, in the absence of FMRFamide or arachidonic acid, was

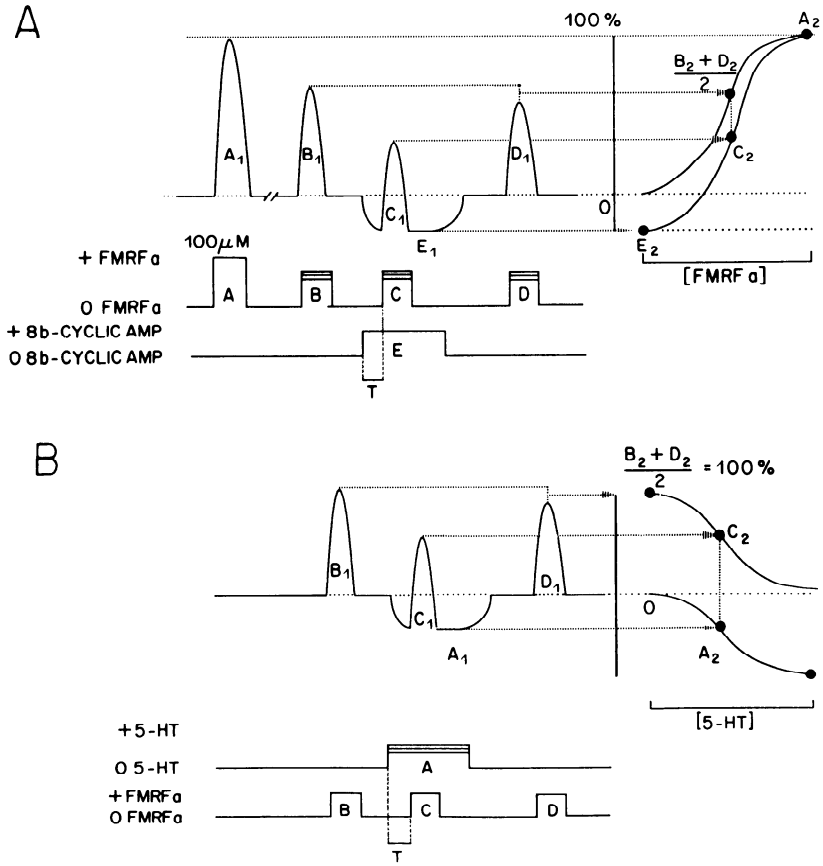

FIG. 1. Drawings showing the method used to measure, normalize, and plot the dose-response relationship of FMRFamide (or arachidonic acid, *A*) and 5-HT (or 8b-cAMP, *B*). See METHODS for explanations.

obtained by plotting the normalized responses to each concentration (A_2), which were measured before the application of FMRFamide or arachidonic acid. The dose-dependent action of 5-HT on the FMRFamide (or arachidonic) response was similarly plotted (C_2) as percentages of the FMRFamide response in the absence of 5-HT.

Data are expressed as means ± SE throughout the text and the figures.

RESULTS

S-K$^+$ conductance is the dominant target of regulation at the resting potential level

Three K^+ currents are modulated by FMRFamide and 5-HT in *Aplysia* mechanosensory neurons: the S-K^+ current, $I_{K(Ca)}$, and $I_{K(V)}$. We exploited their different sensitivities to TEA to confirm that, under our experimental conditions, the S-K^+ conductance is the dominant target of the regulation by FMRFamide and 5-HT. The macroscopic S-K^+ current response to serotonin at −35 mV is insensitive to external TEA up to 100 mM (Fig. 2*A*; $n = 7$). The macroscopic current is the product of $N \cdot P_o \cdot i$. At the single-channel level, external TEA reduces the elementary S-K^+ channel current, i (K_d = 90 mM), but also increases its probability of opening, P_o (Shuster and Siegelbaum 1987). Because 5-HT decreases N, the number of active channels (Siegelbaum et al. 1982), the microscopic mechanism of action of TEA explains its lack of effect at macroscopic level, as proposed by Shuster and Siegelbaum (1987). Whatever the interpretation, this experiment indicates that, under our experimental conditions, the $I_{K(Ca)}$ and the $I_{K(V)}$ do not significantly contribute to the 5-HT response, because they are highly sensitive to external TEA (K_d = 0.4 and 6.0 mM, respectively; Hermann and Gorman 1981). The S-K^+ current response to FMRFamide at −35 mV is modestly reduced under 50 mM TEA (Fig. 2, B_1 and B_3, $n = 4$). This effect is presumably due to occlusion, because both substances increase P_o. In the presence of 5-HT (Fig. 2, B_2 and B_4), the response to FMRFamide displays the same degree of sensitivity to TEA (B_4 vs. B_2) that is displayed by the response to FMRFamide in the absence of 5-HT (B_3 vs. B_1). This experiment ($n = 4$) suggests that the same type of K^+ current is activated by FMRFamide in the presence and in the absence of 5-HT. Taken together, the experiments with TEA indicate that modulation of the S-K^+ conductance plays a dominant, if not exclusive, role in the current response generated by the combined application of 5-HT and FMRFamide under our experimental conditions.

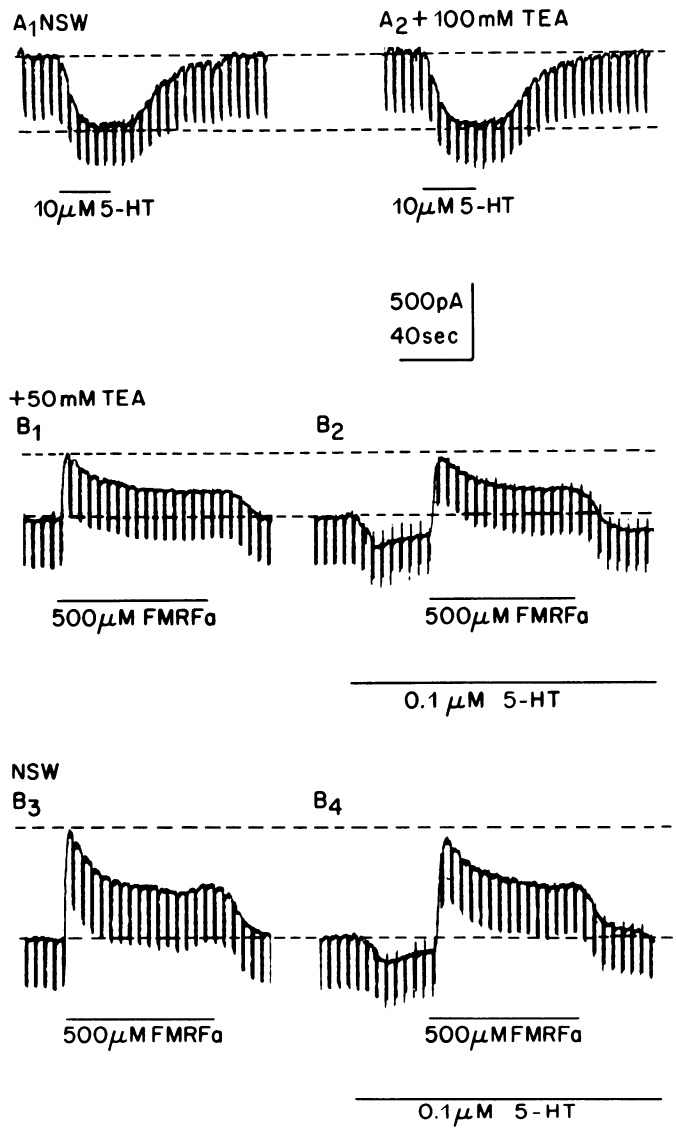

FIG. 2. Effect of TEA on responses to 5-HT and FMRFamide and on their interaction. Macroscopic current recordings from 2 cells (*A* and *B*, respectively). Horizontal bars underlying records mark drug applications. A_1 and A_2: inward current in response to 10 μM 5-HT in the absence and in the presence, respectively, of 100 mM TEA. B_1–B_4: outward current responses to 500 μM FMRFamide in the presence and in the absence of TEA and 5-HT. Records in B_1 and B_3 are in the absence and B_2 and B_4 in the presence of 0.1 μM 5-HT. Records in B_1 and B_2 are in the presence and B_3 and B_4 in the absence of 50 mM TEA. Records B_1–B_4 are in chronological order. A modest decline of the peak response to 5-HT with repeated applications was often observed (compare the peak inward current in B_2 and B_4) and was unrelated to TEA application. Holding potential, −35 mV. Step voltage commands to −40 mV applied every 5 s.

FMRFa overrides the cAMP-induced closure of the S-K$^+$ conductance

We initially asked whether FMRFamide fully antagonizes the action of cAMP on the macroscopic S-K^+ current, as reported with the use of single S-K^+ channel recordings (Belardetti et al. 1987). To determine this, we applied a broad range of concentrations of FMRFamide in the absence and in the presence of a membrane-permeable and hydrolysis-resistant cAMP analogue (Figs. 3 and 4). In some experiments, we used 8-(4-chlorophenylthio)-cAMP (8cpt-cAMP), and in the remaining experiments we used 8b-cAMP. No obvious differences were observed between the two analogues. Selected examples of this interaction are shown in Fig. 3. Pressure applications of FMRFamide (1–500 μM, Fig. 3, A_1, B_1, and B_3) produced an outward current accompanied by increased slope conductance, which reflects the increased opening of the S-K^+ channels induced by the peptide. Bath application of either cAMP analogue (Fig. 3, A_2, B_2, and B_4) produced an inward current with decreased slope conductance, generated by the closure of the same channel that is opened by FMRFamide. If, in the presence of 1.4 μM 8cpt-cAMP, 1 μM FMRFamide is applied (A_2), an outward current equal to the sum

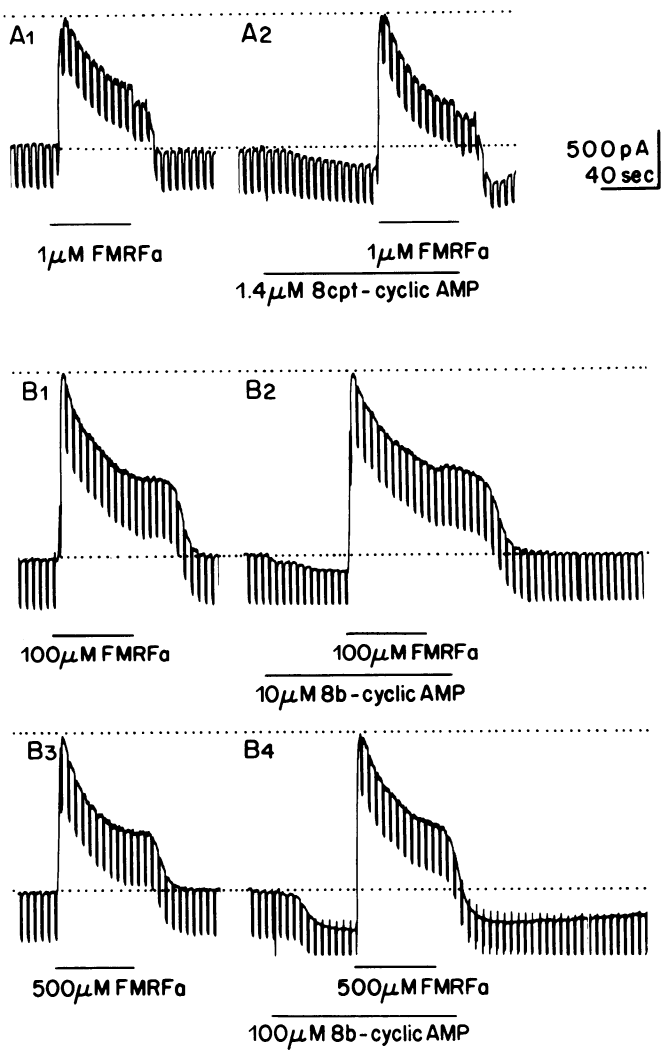

FIG. 3. Peptide FMRFamide action on the S-K^+ conductance in the presence of 2 cAMP analogues. Macroscopic current recordings from 2 cells (A and B, respectively). Horizontal bars underlying records mark drug applications. Current responses to increasing concentrations of FMRFamide are shown in the absence (A_1, B_1, and B_3) and in the presence of increasing concentrations of cAMP analogue (A_2, B_2, and B_4). In the experiment shown in A_2, 8cpt-cAMP was used, whereas in B_2 and B_4 8b-cAMP was applied. Holding potential, −35 mV. Step voltage commands to −40 mV applied every 5 s.

of the currents produced by the same concentration of FMRFamide alone (A_1) and 8cpt-cAMP alone (A_2) resulted, so that, with the combined application, the peak current flowing out of the cell was equal to the peak current flowing under FMRFamide alone. Thus, under these conditions, FMRFamide can completely antagonize the S-K^+ closing action of the nucleotide. Can a larger dose of FMRFamide fully antagonize the closing action of high levels of the cAMP analogue? This is shown in the remaining part of Fig. 3, with the use of a different cell: application of 100 μM FMRFamide fully antagonizes (B_2) the action of 10 μM 8b-cAMP. Finally, at maximal concentrations of 8b-cAMP (100 μM), 500 μM FMRFamide were required to completely antagonize the nucleotide action (Fig. $3B_4$).

The results from a total of 13 cells are plotted in Fig. 4. The *left* curve (filled circles) shows the control dose response of FMRFamide alone. Maximal S-K^+ current is evoked at 100 μM. In the presence of 8b-cAMP, the dose response for FMRFamide starts from more inward levels (half-filled and empty circles). In the presence of moderate 8b-cAMP concentrations (1–10 μM, half-filled circles), FMRFamide produces a response larger (as measured from the baseline) than the control response at all the concentrations tested, with complete antagonism of the 8b-cAMP effect at 100 μM FMRFamide. However, in the presence of maximal 8b-cAMP concentrations (100 μM, empty circles), the response to low concentrations (0.01–1 μM) of FMRFamide is depressed compared with the control response in the absence of the nucleotide; but, at concentrations of 10 μM or higher, FMRFamide again produces a response larger than the control, with full antagonism at 500 μM. The full antagonism of the effect of 1–10 μM 8b-cAMP by FMRFamide was present in 6 of 7 cells; with 100 μM 8b-cAMP, in 2 of 3 cells.

This group of experiments fully confirms the conclusion from the previous work at single-channel level (Belardetti et al. 1987) that FMRFamide can fully antagonize the S-K^+ channel–closing action of cAMP. It is also in agreement with the observation that FMRFamide dephosphorylates neuronal proteins phosphorylated by cAMP (Sweatt et al. 1989).

cAMP-independent action of serotonin

Having shown that FMRFamide fully antagonizes the cAMP-induced closure of the S-K^+ conductance, we next investigated (Figs. 5 and 6) whether FMRFamide could

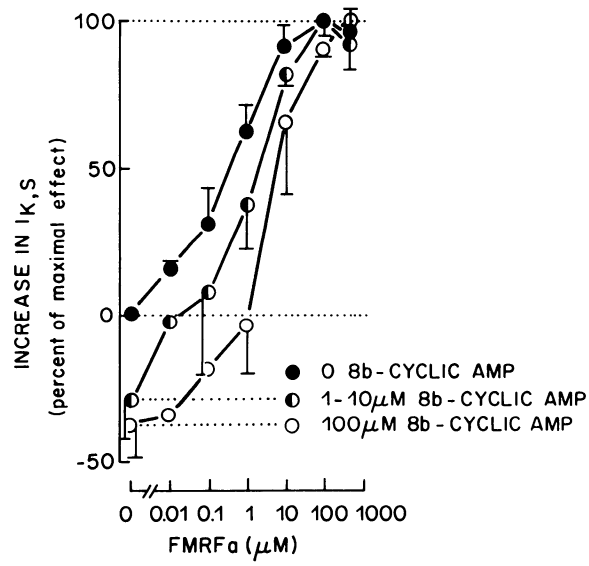

FIG. 4. Dose-response relationships of FMRFamide in the absence and in the presence of 8b-cAMP. Curve with filled symbols plot mean normalized responses to FMRFamide as a function of various concentrations of FMRFamide (0.01–500 μM). Half-filled symbols plot mean normalized responses to FMRFamide as a function of same concentrations of FMRFamide, but in the presence of a moderate concentration of 8b-cAMP (1–10 μM). Empty symbols plot mean normalized responses to FMRFamide in the presence of maximal levels of 8b-cAMP (100 μM). Baselines are shifted in the inward direction by the presence of 8b-cAMP. Thirteen cells were used, 7 with low–8b-cAMP and 6 with high–8b-cAMP experiments. For each cell, responses were normalized to the response to 100 μM FMRFamide.

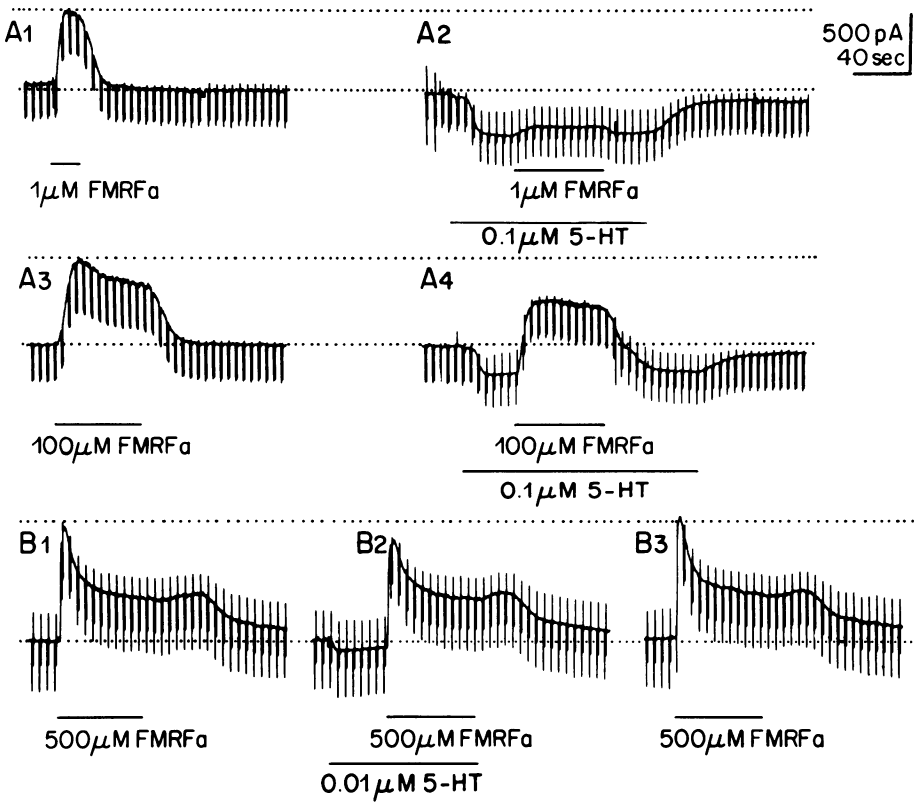

FIG. 5. FMRFamide action on the S-K^+ conductance in the presence of 5-HT. Current recordings are shown from 2 cells (A and B, respectively). Horizontal bars mark drug applications. Current responses to increasing concentrations of FMRFamide are shown before (A_1, A_3, and B_1), in the presence of various concentrations of 5-HT (A_2, A_4, and B_2), and after washing 5-HT out of the bath (B_3). Holding potential, −35 mV. Step voltage commands to −40 mV applied every 5 s.

also antagonize the 5-HT–induced closure of the same conductance, which, as shown by several lines of evidence, is mediated by cAMP (Klein et al. 1982; Ocorr and Byrne 1985; Shuster et al. 1985; Siegelbaum et al. 1982; Sweatt et al. 1989). Application of FMRFamide produced an outward S-K^+ current (Fig. 5, A_1, A_3, and B_1). In the presence of maximal 5-HT concentration (0.1 μM; see Fig. 7 B_2), the response to a moderate concentration of FMRFamide (1 μM) was substantially smaller (Fig. 5 A_2) than the control response to the same concentration of FMRFamide in the absence of 5-HT (Fig. 5 A_1), despite the fact that FMRFamide was applied for a longer time. In the same cell, a maximal concentration of FMRFamide (100 μM) did not overcome the effect of a maximal concentration of 5-HT (0.1 μM; Fig. 5, A_3 and A_4). Finally, in a different cell, at a moderate 5-HT concentration (0.01 μM), a supramaximal FMRFamide application (500 μM) still did not overcome the 5-HT action (Fig. 5, B_1 and B_2). This depression of the FMRFamide responses under 5-HT was not the result of desensitization, because the FMRFamide response returned to the control level after washout of 5-HT (Fig. 5 B_3). This indicates that the interactions of cAMP and 5-HT with FMRFamide differ in a qualitative manner.

The results from a total of 16 cells are plotted in Fig. 6. The filled circles show the dose response to FMRFamide. In the presence of 5-HT (half-filled and empty circles), the FMRFamide dose response starts from a more inward level, as in the cAMP experiment (Fig. 4). However, in contrast with that group of experiments, in the presence of a moderate dose of 5-HT (0.01 μM, half-filled symbols) FMRFamide produced responses that were, on the average, approximately equal (as measured from the new baseline in 5-HT) to the control responses, indicating lack of net antagonism of the 5-HT response. At supramaximal concentrations of FMRFamide (500 μM), the 5-HT response was still not antagonized. Finally, in the presence of maximal concentrations of 5-HT (0.1 μM, empty symbols), the full range of FMRFamide concentrations tested produced re-

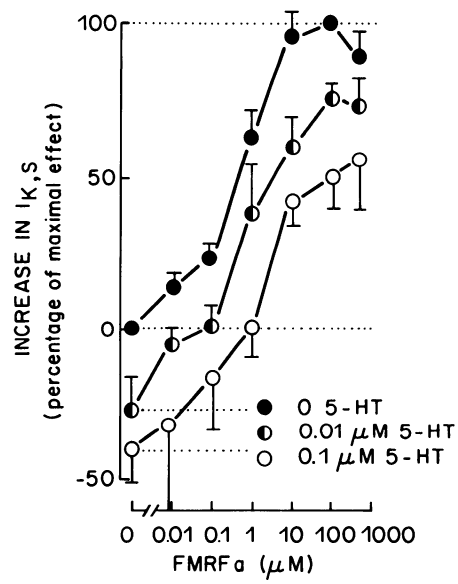

FIG. 6. Dose-response relationships of FMRFamide in the absence and in the presence of 5-HT. Filled symbols plot mean normalized current responses to FMRFamide as a function of various concentrations of FMRFamide (0.01–500 μM). Half-filled symbols plot of mean normalized responses to FMRFamide as a function of the same concentrations of FMRFamide, but in the presence of a moderate concentration of 5-HT (0.01 μM). Empty symbols show the plot of the mean normalized response to FMRFamide in the presence of maximal levels of 5-HT (0.1 μM). Much as in Fig. 4, the baselines are shifted in the inward direction by the presence of 5-HT. Sixteen cells were used, 10 for low–5-HT and 6 for high–5-HT experiments. For each cell, responses were normalized to the response to 100 μM FMRFamide.

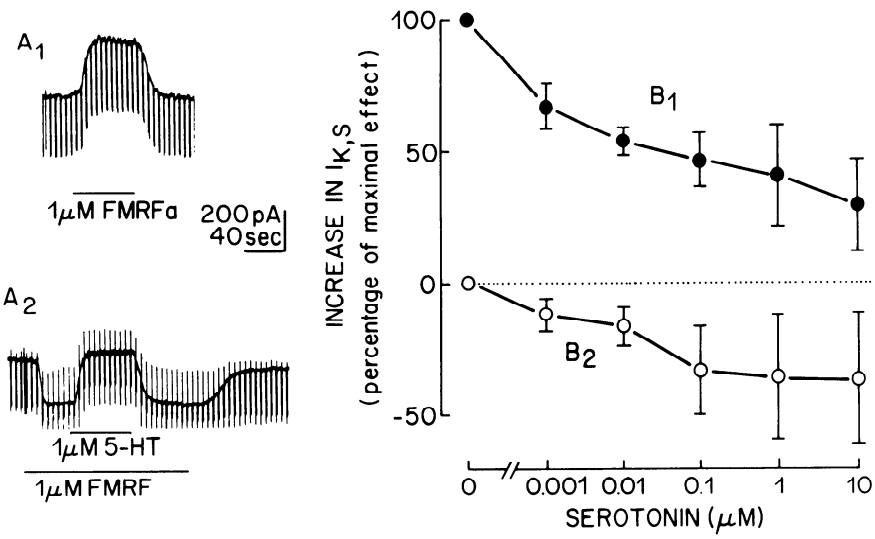

FIG. 7. Inhibitory actions of 5-HT on the FMRFamide response. *A*: macroscopic current recordings from 1 cell. Horizontal bars mark drug applications. FMRFamide (1 μM) before (A_1) and during application of 5-HT (A_2). Holding potential, −35 mV. Step voltage commands to −40 mV applied every 5 s. B_2: mean normalized responses to 5-HT as a function of various concentrations of 5-HT (0.001–10 μM). B_1: mean normalized responses to the application of a fixed concentration of FMRFamide (1–50 μM) in the presence of various 5-HT concentrations, as a function of the concentration of 5-HT. Six cells were used. For each cell, responses were normalized to the response to FMRFamide in the absence of 5-HT.

sponses that were almost invariably smaller than the control responses to the same concentration of FMRFamide. This depression of the FMRFamide responses under 5-HT was present in all the experiments.

We also observed that the degree of inhibition induced by 5-HT on the FMRFamide response became larger as the interval between the application of 5-HT and the application of FMRFamide with 5-HT became longer (Fig. 1, letter T), whereas the inhibition by 8b-cAMP seemed independent from that same interval. Although we did not investigate this observation in detail, we kept the T interval constant within each experiment.

In the data presented so far, we have shown that FMRFamide can reopen S channels closed by cAMP, whereas almost no net reopening can be observed if the channels are closed by 5-HT. Because it is well established that cAMP mediates the known 5-HT effects on the S channel, it must be concluded that additional, previously unknown actions are produced by the specific involvement of the 5-HT receptor. We call these additional effects "cAMP independent," because they cannot be reproduced by any concentration of cAMP analogue used.

Dual action of FMRFa confirmed by high doses of 5-HT

The data presented so far indicate that 5-HT has complex antagonistic effects on the FMRFamide-induced opening of the S-K^+ conductance, involving at least two parallel mechanisms: one through a cAMP-independent action, and the other through a cAMP-dependent action.

Previous experiments have indicated that FMRFa also has a dual mechanism of action on the S-K^+ conductance. The first mechanism leads to increase of channel P_o (Belardetti et al. 1987), and it is mediated by a direct action on the channel of 12-lipoxygenase metabolites (Belardetti et al. 1989; Buttner et al. 1989). The second mechanism reopens the channels closed by cAMP (Belardetti et al. 1987), and it probably acts through protein dephosphorylation (Sweatt et al. 1989). These two actions cannot be directly separated with macroscopic current recording. However, information about their properties can still be obtained with this technique. The experiments in Figs. 3 and 4, for example, show the dose dependency of the second mechanism of action of FMRFamide (the reopening). In the group of experiments presented here, to study the actions of FMRFamide, we applied a broad range of 5-HT concentrations in the presence and the absence of a fixed concentration of FMRFamide (Fig. 7). Control applications of FMRFamide at 1 μM produced a slow outward current (Fig. 7A_1). Application of increasingly higher concentrations of 5-HT progressively inhibited the response to FMRFamide. However, maximal levels of 5-HT (1 μM) could not suppress the FMRFamide response (A_2). This inhibition by 5-HT was reversible, because the FMRFamide response returned to the initial levels when FMRFamide was applied in the absence of 5-HT. The data from five experiments of this type are summarized in B_1 and B_2.

We next examined, in another group of experiments (n = 5; Fig. 8), the dose dependent actions of 5-HT (0.001–10 μM) on the response to a fixed concentration of arachidonic acid (25 μM), which mediates the FMRFamide response. Under these conditions, the arachidonic acid response was progressively reduced by increasing the concentration of 5-HT, but not abolished even by supramaximal concentrations of 5-HT (10 μM), much the same as in the experiments with FMRFamide. Finally, we investigated the dose-dependent action of 8b-cAMP (1–1,000 μM) on a fixed concentration of FMRFamide (1–10 μM, n = 3; not shown). When 8b-cAMP was applied alone, maximal responses were observed at 100 μM. When FMRFamide was applied in the presence of the nucleotide, its response was depressed but not abolished by supramaximal 8b-cAMP, similar to the effect of 5-HT.

We interpret these observations as follows. cAMP-dependent phosphorylation reduces the number of S channels available for gating (Siegelbaum et al. 1982). Maximal stimulation of phosphorylation keeps a fraction of channels closed, unavailable for opening by FMRFa, thus explaining the depression of the FMRFamide and arachidonic acid responses observed in this group of experiments. However, a component of the FMRFamide action, mediated by arachidonic acid, is resistant to maximal stimulation of phosphorylation by 5-HT or cAMP. This conclusion supports the view that the primary action of FMRFamide is not mediated by a dephosphorylation step (Belardetti et al. 1989;

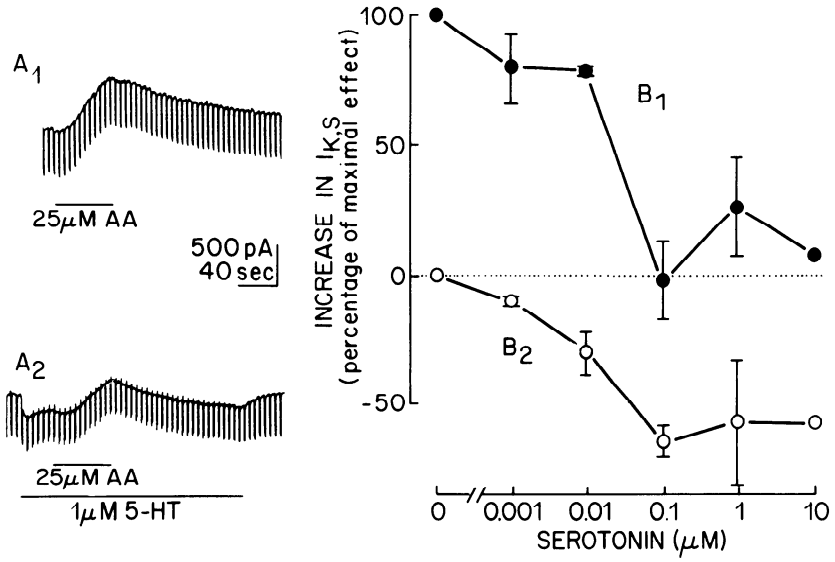

FIG. 8. Inhibitory actions of 5-HT on the response to a fixed concentration of arachidonic acid (25 μM). Same symbols and explanations as in Fig. 7. Five cells were used. For each cell, the responses were normalized to the response to arachidonic acid in the absence of 5-HT.

Buttner et al. 1989). Under opposite conditions, high FMRFamide doses can completely overcome the channel-closing action of cAMP-dependent phosphorylation, as shown in Figs. 3 and 4. This second action probably is mechanistically distinct because it requires dephosphorylation of the channel (Ichinose et al. 1990; Sweatt et al. 1989).

Arachidonic acid metabolism probably mediates both actions of FMRFamide

We have presented evidence that the inhibitory action of 5-HT on the FMRFamide S-K^+ conductance-opening response has two components. The first is cAMP mediated and antagonized by FMRFamide; the second is cAMP independent and not antagonized by FMRFamide. The purpose of the last group of experiments was twofold. First, we investigated whether the cAMP-independent component of the 5-HT action depresses the FMRFamide pathway at the level of the release of arachidonic acid or downstream. Second, we wanted to collect direct evidence of the involvement of arachidonic acid in the antagonistic action of FMRFamide. We measured the dose dependent activating effect of arachidonic acid on the S-K^+ conductance in the absence and in the presence of a moderate level of 5-HT

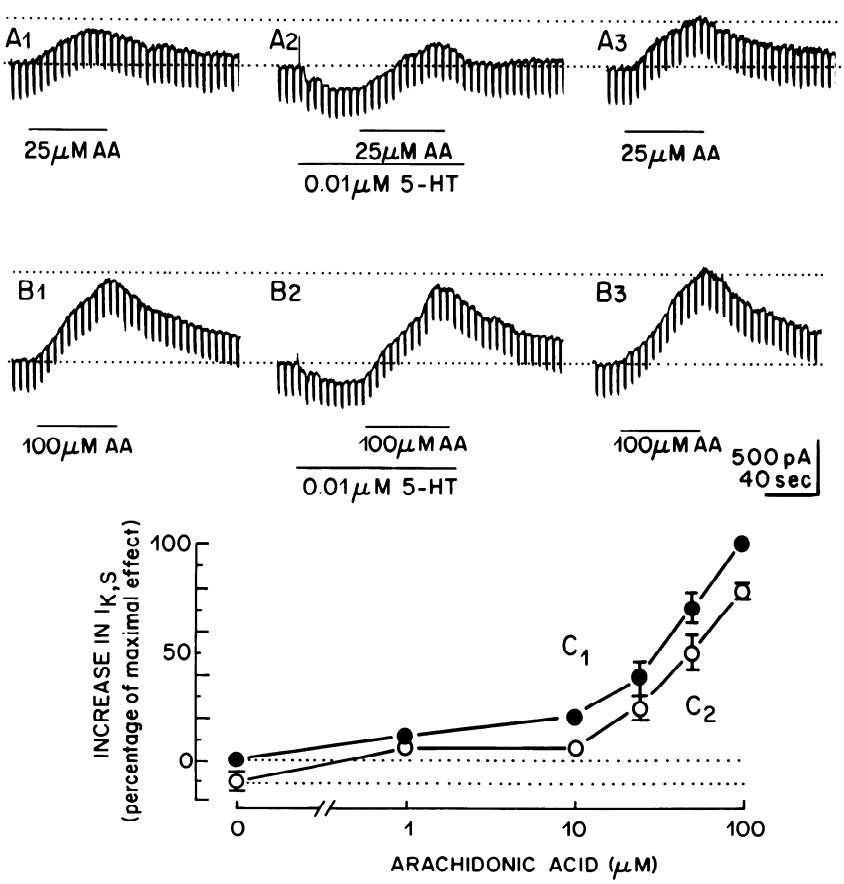

FIG. 9. Dose-response relationship of arachidonic acid in the presence and in the absence of 5-HT. Outward currents generated by external application of 2 different concentrations of arachidonic acid (A, 25 μM; B, 100 μM) before (A_1 and B_1), during (A_2 and B_2), and after (A_3 and B_3) application of 0.01 μM 5-HT. Horizontal bars mark drug applications. Holding potential, −35 mV. Step voltage commands to −40 mV applied every 5 s. C_1: mean normalized responses to arachidonic acid as a function of various concentrations of arachidonic acid (1–100 μM). C_2: in the presence of a moderate concentration of 5-HT (0.01 μM). Baseline is shifted inward by the presence of 5-HT. Four cells were used. For each cell, responses were normalized to the response to 100 μM arachidonic acid.

(0.01 μM, Fig. 9). We reasoned that if 5-HT inhibits the release of arachidonic acid, then when arachidonic acid is exogenously applied, the site of the cAMP-independent inhibition by 5-HT would be bypassed. In this case, high concentrations of arachidonic acid, by stimulating the dephosphorylation mechanism (Sweatt et al. 1989), would fully antagonize the S-K^+ conductance closure induced by 5-HT. We used a moderate dose of 5-HT to maximize the likelihood of seeing the full antagonism of the 5-HT action by arachidonic acid. The result from such an experiment is shown in Fig. 9. This experiment was limited by two factors: first, we could not reliably apply extracellular concentrations of arachidonic acid higher than 100 μM, thus completing the dose-response curve, because of irreversible damage to the cell membrane at these high concentrations. Second, during the application of arachidonic acid, the peak of the response is not reached. This apparent failure to reach an equilibrium in the response might be the result of the presence of a diffusion barrier between the site of application of arachidonic acid and its site of conversion to an active metabolite. Application of arachidonic acid produces an outward current because of the opening of the S-K^+ conductance [Fig. 9, A_1 and A_3 (25 μM) and B_1 and B_3 (100 μM); see also Piomelli et al. 1987]. Under 5-HT [A_2 and B_2 (0.01 μM)], the response to arachidonic acid is larger than the control response, but it fails to fully antagonize the 5-HT effect at both concentrations. The plot summarizing the data from four cells is shown in Fig. 9, C_1 and C_2. Under our experimental conditions, at all the concentrations tested, arachidonic acid under 5-HT was invariably unable to fully antagonize the 5-HT action. Within the limits of this experiment, these data indicate that the cAMP-independent mechanism acts downstream from the release of arachidonic acid, a necessary step in mediating both actions of FMRFamide on the S-K^+ conductance.

DISCUSSION

Antagonism of the cAMP action by FMRFamide

Although we now have a great deal of information concerning the second-messenger system that mediates the FMRFamide basal opening action on the S-K^+ conductance (Belardetti et al. 1987, 1989; Piomelli et al. 1987), less is known about the cascade responsible for the antagonism to cAMP. Our data, by showing that arachidonic acid application in the presence of 5-HT can produce responses larger than in the absence of 5-HT (Fig. 9), support the idea that arachidonic acid metabolism might also mediate the antagonistic actions of FMRFamide. A similar conclusion was reached when an inhibitor of lipoxygenase metabolism was used (Volterra and Siegelbaum 1988).

Previous work (Ichinose et al. 1990; Sweatt et al. 1989) indicated that FMRFamide antagonizes 5-HT action by ultimately producing dephosphorylation of the S-K^+ channel: for example, okadaic acid, a specific inhibitor of certain protein phosphatases, mimics the 5-HT–induced inward current and also blocks the S-K^+ current activation by FMRFamide. However, the inhibition by okadaic acid of the protein phosphatase activity, by not allowing the escape of the S-K^+ conductance from the closed state, is expected to prevent its reopening by FMRFamide, no matter which step of the 5-HT cascade is regulated by FMRFamide. This type of approach, therefore, does not indicate which site of the 5-HT cascade is primarily regulated by FMRFamide. Our data, by showing that the modulatory actions of two nonhydrolyzable cAMP analogues can be fully antagonized by FMRFamide, indicate that activation of a cAMP-dependent phosphodiesterase is not the primary site for this action of FMRFamide and point to a step downstream from cAMP as the regulated site for this action. Three mechanisms can be proposed: *1*) a protein phosphatase could be activated (Fig. 10*C*); *2*) the cAMP-dependent kinase could be inhibited by FMRFamide (Fig. 10*C*); or *3*) the binding of the 12-lipoxygenase/P_{450} metabolite to the channel, in addition to increasing its opening, could alter the substrate, therefore making the phosphatase more effective or the kinase less effective (Fig. 10*A*). Our experiments with 8b-cAMP show a competitive type of antagonism by FMRFamide. This is compatible with the idea that FMRFamide antagonizes the phosphorylating action of cAMP by activating a protein phosphatase, but to support this idea more direct evidence is needed at the biochemical level.

cAMP-independent inhibitory action of 5-HT

The more novel finding from the present study is the clear evidence that, in the presence of 5-HT (the action of which on the S-K^+ channel is mediated by cAMP), the FMRFamide response is smaller than would be expected in the presence of an equivalent dose of cAMP and that high doses of FMRFamide cannot antagonize this 5-HT action. The simplest explanation is that 5-HT exerts a cAMP-independent inhibitory action either on the FMRFamide pathway or on the S-K^+ channel itself. This effect is resistant to FMRFamide and is parallel and distinct from the inhibition exerted by 5-HT through cAMP-dependent phosphorylation, which is fully antagonized by FMRFamide.

An alternative explanation for this 5-HT–specific effect —an FMRFamide-insensitive inward current activated by 5-HT superimposed on that evoked by cAMP—is ruled out by three observations. First, the maximal 5-HT– and 8b-cAMP–evoked responses are of roughly similar size (compare Fig. 4 with Fig. 6; also, by pooling all our experiments, we obtained on the average 154 ± 19 pA/cell with 0.1 μM 5-HT, n = 19; 182 ± 57 pA/cell with 100 μM 8b-cAMP, n = 14). Second, if a second inward current were activated by 5-HT, it would be expected to shift inwardly a response to FMRFamide that remains larger (because it includes the antagonism to the cAMP-mediated component of the 5-HT response) than the control without 5-HT, whereas, on the opposite under 5-HT, the response to FMRFamide becomes smaller than the control. Third, TEA does not eliminate this 5-HT–specific effect (Fig. 2), ruling out the inhibition of a residual delayed rectifier current at −35 mV (Baxter and Byrne 1989).

We do not know which biochemical pathway mediates this additional cAMP-independent 5-HT action; possible candidates include activation of kinase C (Braha et al. 1990; Sacktor and Schwartz 1990), release of arachidonic metabolites (Piomelli et al. 1987), or direct G protein action (Sternweis and Pang 1990). We also do not know what stage of the FMRFamide-activated cascade is inhibited by

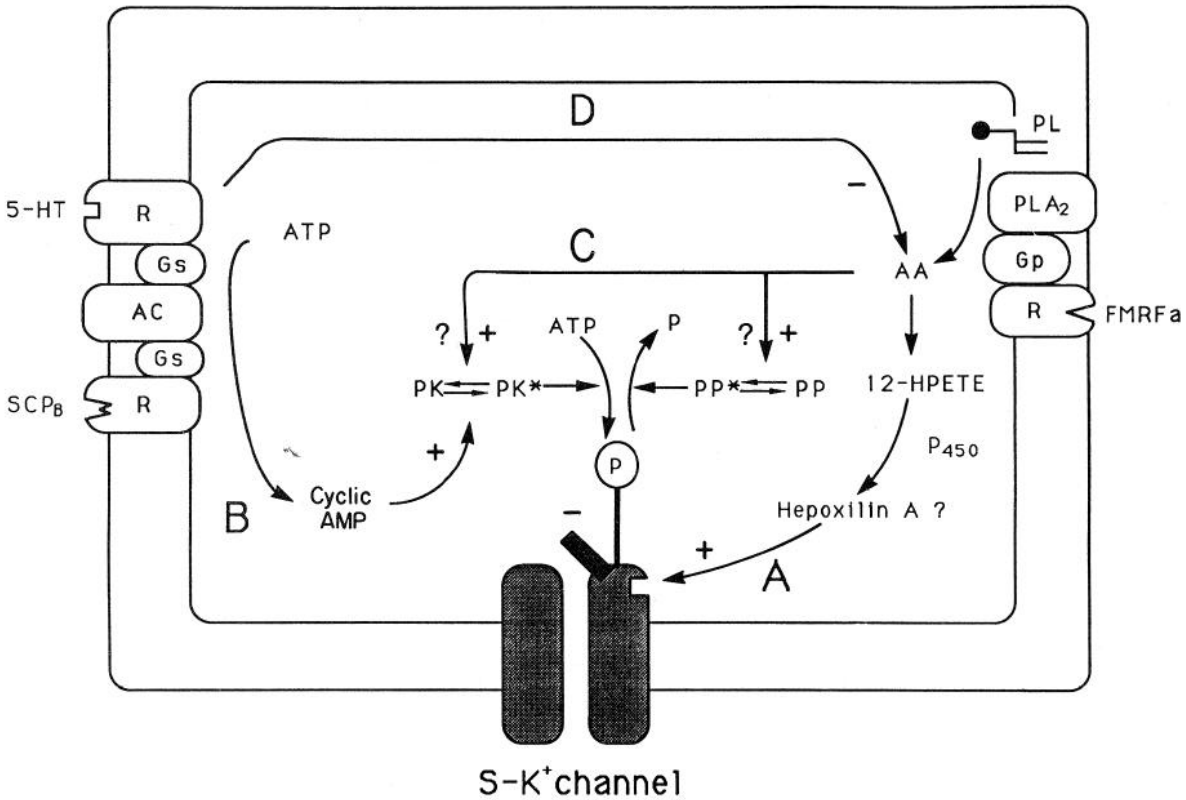

FIG. 10. Model for mechanisms of interaction of FMRFamide and 5-HT cascades. SCP_B, small cardioactive peptide; R, receptor; AC, adenylyl cyclase; PLA_2, phospholipase A_2; G_s and G_p, G proteins activating cyclase and phospholipase, respectively; PL, phospholipids; PK and PK*, inactive and active forms of cAMP-dependent protein kinase; PP and PP*, inactive and active forms of a hypothetical protein phosphatase regulated by a metabolite of arachidonic acid; P, phosphate group. Question marks indicate hypothetical components of the model. For other symbols and explanations, see the DISCUSSION.

this action of 5-HT. In the experiment shown in Fig. 9, we could use only a limited range of concentrations of arachidonic acid. Thus this experiment provides only preliminary evidence that 5-HT acts downstream from arachidonic acid release. One interesting possibility is that the cAMP-independent action of 5-HT selectively blocks the reopening action of FMRFamide (compare Fig. 6 with Fig. 4).

Model for the 5-HT and FMRFamide interacting effects on the S-K$^+$ channel conductance

In summary (Fig. 10), these experiments place in a coherent framework previous findings on the interaction between FMRFamide and 5-HT in *Aplysia* sensory neurons and, in addition, indicate a novel action of 5-HT on the FMRFamide response. In basal conditions, FMRFamide increases the opening of the available S-K$^+$ channels by direct action of a lipoxygenase metabolite of arachidonic acid (*A*). By increasing the cAMP levels, 5-HT closes the S-K$^+$ channels and reduces the number of channels available for gating (*B*). When the number of active S-K$^+$ channels is reduced by cAMP, then FMRFamide, in a competitive mode, can antagonize this action (*C*) by somehow stimulating the channels' dephosphorylation. The effect of FMRFamide is inhibited in the presence of 5-HT as a result of a second action of 5-HT (*D*). As a result of this reciprocal mechanism, FMRFa cannot antagonize the presynaptic facilitation and behavioral sensitization produced by 5-HT. However, the level of cAMP in sensory cells can be elevated not only by 5-HT but also by other transmitters, such as the small cardioactive peptide (SCP_B). Perhaps the ability of FMRFa to fully antagonize sensitization is contingent on the sensitization being produced by transmitters other than 5-HT, such as SCP_B (Fig. 10; Abrams et al. 1984; Baxter and Byrne 1989; Ocorr and Byrne 1985).

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