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Serotonin receptors expressed in *Xenopus* oocytes by mRNA from brain mediate a closing of K^+ membrane channels

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Membrane currents evoked by serotonin (5-HT) were studied in *Xenopus* oocytes injected with rat brain mRNA. Intracellular EGTA was used to abolish the Ca^{2+}-dependent oscillatory Cl^- current to 5-HT, revealing an underlying smooth inward current. This was associated with a *decreased* membrane conductance, was antagonized by Ba^{2+} and Zn^{2+} (but not TEA), and probably arises through a closing of K^+ channels. Half-maximal responses were obtained with 30 nM 5-HT, while 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT) was ineffective. Furthermore, methysergide, mianserin and lysergic acid antagonized the K^+-closing response to 5-HT, consistent with it being mediated through 5-HT_{1C} receptors. The largest K^+-closing responses were induced by a size fraction of mRNA which also induced a large K^+ conductance, suggesting that the response requires expression of both receptors and K^+ channels. The K^+-closing response induced in the oocyte resembles the M- and S-type currents described in, respectively, mammalian and invertebrate neurons.

INTRODUCTION

Oocytes of *Xenopus laevis* usually show no membrane current responses to the neurotransmitter serotonin (5-HT)[17]. However, we had previously shown that sensitivity to 5-HT can be consistently induced by injecting oocytes with messenger RNA (mRNA) from brain[9,11]. The most prominent response is an oscillatory chloride current[9], which arises because exogenous 5-HT receptors expressed by the brain mRNA cause the activation of an endogenous phosphoinositide–calcium signalling system in the oocyte[7,21,24–27,33]. Subsequently, it has been found that receptors of the 5-HT_{1C} sub-type mediate an oscillatory response[19], and the gene encoding this receptor has been cloned[15,19], and functionally expressed in the oocyte and in fibroblasts[15].

However, we had already briefly reported that 5-HT receptors expressed by brain mRNA mediate other types of ionic current response in addition to the oscillatory current; specifically, a smooth chloride current[26], and a current which arises from a closing of membrane channels[12]. The present paper is a more detailed description of the latter response.

MATERIALS AND METHODS

Experiments were done on oocytes of *Xenopus laevis*, which were treated with collagenase to remove follicular and other enveloping cells[21,23]. These were injected a few days before recording with either whole, or size fractionated, poly(A)$^+$ mRNA from the cerebral cortex or other areas of the rat brain, to induce the appearance of 5-HT receptors and ion channels. Procedures for the extraction of mRNA, injection into oocytes and electrophysiological recording were as described previously[20,22]. Size fractionation of mRNAs was done by sucrose density centrifugation[32], and lower fraction numbers correspond to larger mRNA sizes. In a parallel gradient, the 28S and 18S ribosomal mRNAs sedimented, respectively, in fraction numbers 10 and 17. Membrane currents were recorded from voltage-clamped oocytes, in response to drugs applied via continuous bath perfusion of Ringer solution at room temperature (21–23 °C). Intracellular loading of oocytes with EGTA was accomplished by pneumatic pressure injections through a pipette filled with 100–400 mM EGTA[26]. Each oocyte was usually loaded with ca. 10 pmol EGTA, and the ability of the EGTA to chelate intracellular calcium was monitored by the abolition of the calcium-dependent transient outward current evoked by depolarization of the oocyte to 0 mV[20,21].

RESULTS

Oscillatory and smooth currents evoked by 5-HT

Bath application of 5-HT to oocytes injected with rat brain mRNA evoked large oscillatory currents, which were inward at a clamp potential of –60 mV, and are due to a flux of chloride ions[9]. Occasionally, however, the oscillatory currents could be seen to be preceded by a smaller, smooth inward current (Fig. 1A), suggesting that 5-HT might activate other ionic currents in addition to

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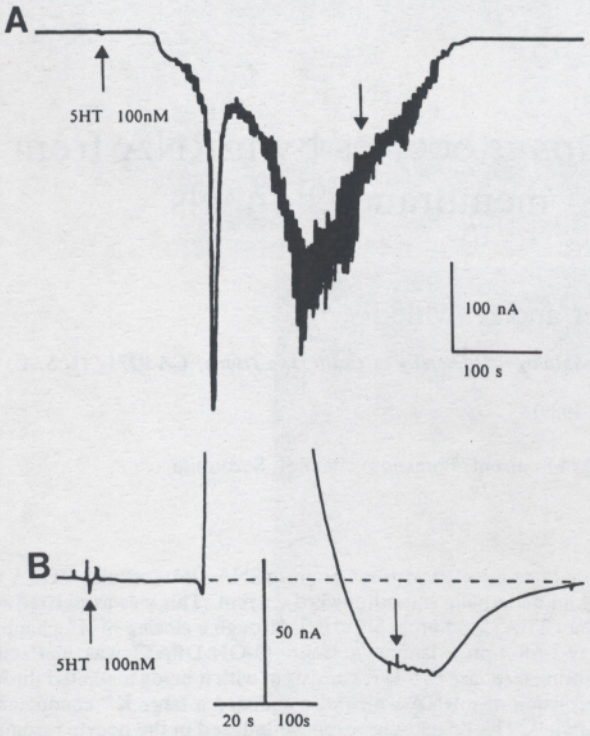

Fig. 1. Membrane currents evoked in mRNA-injected oocytes by 5-HT show more than one component. A: membrane current response to bath application of 5-HT, recorded at a clamp potential of −60 mV. Perfusion with 5-HT (100 nM) began and ended as marked by the arrows. In this, and other figures, downward deflections correspond to inward membrane currents. The oocyte was injected with mRNA from rat cerebral cortex. B: record from a different oocyte, demonstrating inward and outward membrane currents evoked by 5-HT (100 nM) at a clamp potential of −10 mV. Note that the recording speed was slowed at the time marked by the vertical calibration bar. The dashed line indicates the current baseline.

the oscillatory chloride response. To study these components, we employed a combination of two techniques.

The first was to clamp oocytes at potentials slightly (10–20 mV) more positive than the chloride equilibrium potential in the oocyte (ca. −20 mV; ref. 17). Thus, chloride currents were inverted and appeared outwardly directed, while components which remained inward at this potential could be easily distinguished. Fig. 1B illustrates a record obtained in this way. Application of 5-HT evoked an initial inward current, which was overridden after about 1 s by a much larger outward chloride current. In the oocyte illustrated the chloride current attained a peak value of over 1 μA but it subsequently desensitized to reveal an underlying inward current of about 40 nA. This inward current was maintained whilst 5-HT remained present, and returned to the baseline on washing.

Although recordings at clamp potentials around −10 mV revealed the presence of a 5-HT-evoked current other than that carried by chloride ions, the records were still greatly obscured by the large oscillatory chloride

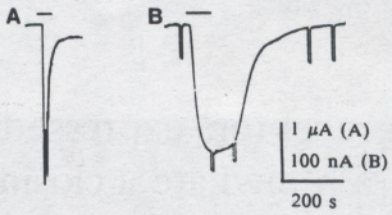

Fig. 2. Intracellular loading with EGTA abolishes the oscillatory response to 5-HT, and reveals a smooth inward current. A: control record at a clamp potential of −60 mV of a response to 5-HT (10 μM) applied as indicated by the bar. B: the oocyte was loaded with EGTA, clamped at −16 mV, and the 5-HT application was repeated. Note the higher recording gain compared to A. Occasional steps in the current level result from brief changes in clamp potential to −21 mV, which were applied to monitor the membrane conductance.

response. Indeed, the inward current could only be discerned in those oocytes where the chloride current desensitized rapidly. For other experiments described here we therefore injected oocytes with EGTA, so as to chelate intracellular calcium and block activation of the calcium-dependent chloride membrane channels which mediate the oscillatory response. As described previously[12,26], this procedure abolishes the oscillatory response to 5-HT, leaving a smooth current, which appears to be comprised of two components. One of these is the apparent inward current described above, while the other is due to an increased conductance for chloride ions[26], and has recently been suggested to arise through a class of chloride channels which are activated by low levels of free calcium[2]. The object of the present paper was to study the inward current. Recordings were therefore usually made at potentials slightly positive to the chloride equilibrium potential, so that any remaining chloride current would be small, and would be apparent as an outwardly directed current. Furthermore, this enhanced the inward current, which increases as the membrane is depolarized (see later).

5-HT closes membrane channels

As illustrated in Fig. 2, 5-HT evoked a large oscillatory response in a mRNA-injected oocyte with its membrane potential clamped at −60 mV. The oocyte was then loaded with EGTA, and 5-HT was re-applied while clamping at −16 mV. Serotonin now evoked a much smaller, smooth inward current of about 200 nA (Fig. 2B). This response showed no detectable oscillations, and was more maintained than the transient oscillatory response seen before loading the oocyte with EGTA.

An important characteristic of the smooth response was that it was associated with a *decreased* membrane conductance, as demonstrated by the reduced amplitude of the currents evoked by test voltage pulses applied during 5-HT action in Fig. 2B. Thus, the apparently inward directed current evoked by 5-HT arises not

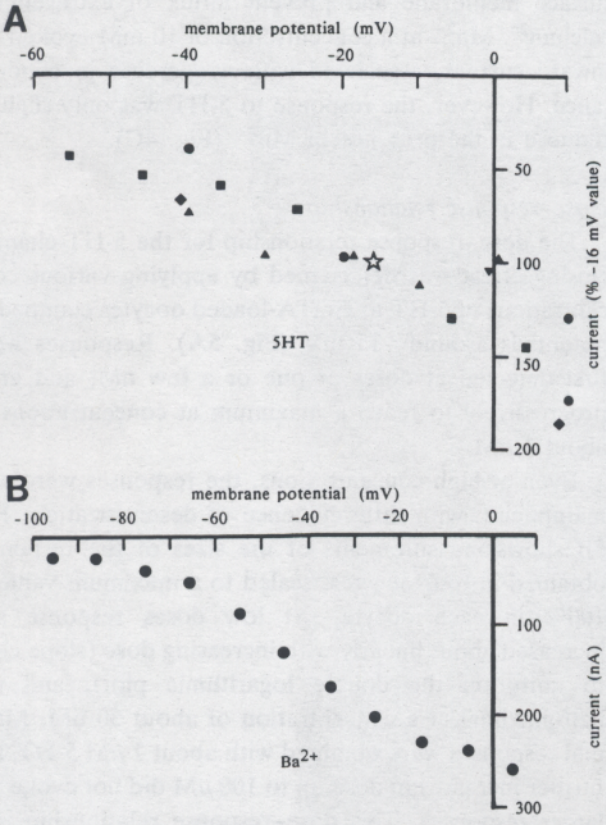

Fig. 3. Voltage-dependence of the underlying currents which are reduced by 5-HT (A) and by Ba^{2+} ions (B). In both cases, measurements were made by briefly stepping the oocytes to different voltage levels from a holding potential of –16 mV, during application of serotonin (10 μM) or Ba^{2+} (10 mM), and are plotted after subtraction of 'passive' currents evoked by the same voltage steps in normal Ringer solution. A: data are from four oocytes (different symbols), which were loaded with EGTA and had been injected with whole or fractionated poly(A)$^+$ mRNA from rat cerebral cortex. Measurements in each oocyte are scaled as a percentage of the current at –16 mV. B: data from a single oocyte, injected with rat cerebral cortex mRNA fraction 9, and loaded with EGTA.

through an opening of membrane channels but, instead, because of a closing of channels which are normally open and which carry an outward current.

mRNAs from several sources induce the 5-HT response

Control oocytes, which were not injected with mRNA or were injected with ineffective mRNA fractions, showed neither oscillatory nor smooth current responses to 5-HT (10 oocytes examined). In contrast, oscillatory and smooth 'closing' responses were consistently observed in oocytes which were injected with mRNA derived from several neuronal sources, and the results presented here are based on recordings in over 40 injected oocytes. Most experiments were done on oocytes injected with total, or size fractionated mRNA from rat cerebral cortex. However, 5-HT closing responses were also induced by mRNAs from the following tissues obtained from rats; hippocampus, spinal cord, and cultured cortical neurons.

Ionic basis of the current modulated by 5-HT

To determine the ionic basis of the current which is closed by 5-HT, we determined its current–voltage relationship by stepping the clamp potential to different values before and during application of 5-HT to EGTA-loaded oocytes. Data from 4 oocytes are shown in Fig. 3A where the points represent the 5-HT-sensitive current, after subtraction of passive membrane currents. The voltage range where measurements could be obtained was restricted between about –50 and +10 mV. At more negative potentials another current (probably the smooth chloride current activated by 5-HT) became appreciable, while voltage-gated conductances interfered with measurements at more positive potentials. Nevertheless, the observation that the underlying current which is closed by 5-HT was outwardly directed over this voltage range, already indicates that it must be carried predominantly by K^+ ions. The K^+ equilibrium potential in the oocyte is about –90 mV[17], so that K^+ currents would be outward at all potentials positive to this. On the other hand, sodium and calcium currents would be inward over the voltage range –50 to +10 mV, while chloride currents would be inward at potentials negative to –20 mV, and outward at more positive potentials.

Further evidence implicating K^+ ions in the 5-HT closing response comes from the action of Ba^{2+} ions, which have been found to block K^+ currents in a variety of cells (for review see ref. 13). Application of Ba^{2+} (10 mM) to mRNA-injected oocytes evoked an apparently inward directed current, which was maintained for as long as Ba^{2+} was present, and returned to the baseline after washing (Fig. 4A). This response was also associated with a decreased conductance, indicating that it arises from the reduction of a pre-existing outward current. The current/voltage relationship for the Ba^{2+} response was obtained in the same way as described above for the 5-HT response, and Fig. 3B shows measurements from one oocyte. At potentials between about –95 and +5 mV the response to Ba^{2+} appeared as an inward current, suggesting that it arises because of a reduction of an outward K^+ current. A complication with this interpretation is that, if Ba^{2+} ions were acting only on K^+ channels, the current is expected to reduce to zero at a potential of about –90 mV. A complete abolition of the current was not observed, but this may be explained because divalent cations are able to block several conductances in the oocyte in addition to those to K^+ (R. Miledi, I. Parker and R.M. Woodward, unpublished data). When 5-HT was applied in the presence of 10 mM Ba^{2+}, the size of the 5-HT response was reduced to less

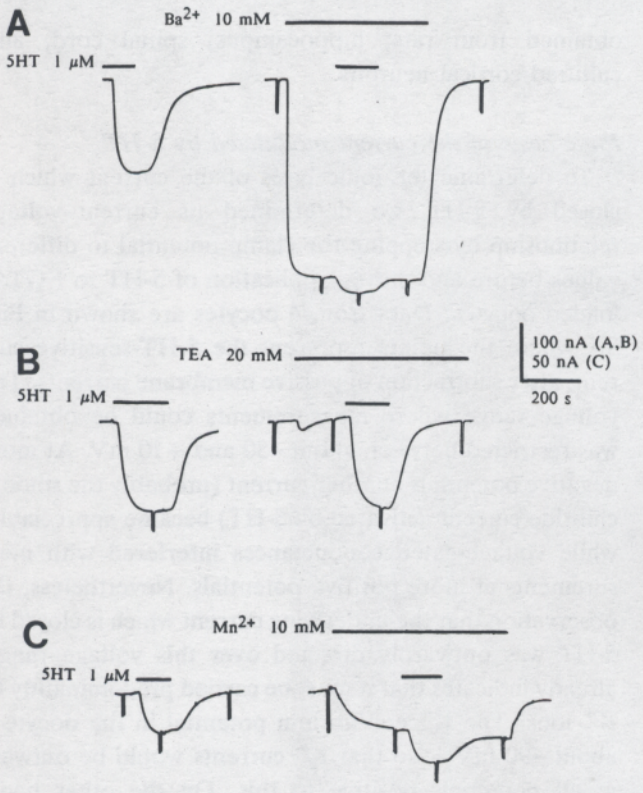

Fig. 4. The response due to 5-HT closing K^+ channels is greatly reduced by Ba^{2+} (A), but not by TEA (B) or Mn^{2+} (C). Records are from two oocytes, injected with rat cerebral cortex mRNA fraction 9 and loaded with EGTA. Currents were monitored at a clamp potential of –16 mV, with occasional brief steps to –21 mV to monitor conductance. The traces on the left show control responses to 5-HT (1 μM) applied in normal Ringer, while those on the right show responses to the same dose of 5-HT given during application of 10 mM Ba^{2+} (A), 20 mM TEA (B) and 10 mM M^{2+} (C).

than one fifth of the control value (Fig. 4A), consistent with the idea that Ba^{2+} and 5-HT both act to reduce currents through the same population of K^+ channels.

In contrast to the large closing effect produced by Ba^{2+}, tetraethylammonium (TEA), a K^+ channel blocker with different specifity[13,31], was almost ineffective. Application of 20 mM TEA gave only a small, transient inward current, with no detectable change in membrane conductance. Furthermore, the response to 5-HT was almost unchanged in the presence of TEA (Fig. 4B).

The 5-HT-closing response does not involve calcium

Unlike the intracellular calcium-dependent oscillatory response to 5-HT, it was already clear that the response associated with the conductance decrease did not depend upon intracellular calcium, because these responses were still present in EGTA-loaded oocytes. To examine further the role of calcium, we added Mn^{2+} ions to the bathing solution so as to block calcium channels in the surface membrane and prevent influx of extracellular calcium[20]. Mn^{2+} at a concentration of 10 mM evoked an inward current, associated with a decrease in conductance. However, the response to 5-HT was only slightly reduced in the presence of Mn^{2+} (Fig. 4C).

Dose–response relationship

The dose–response relationship for the 5-HT channel closing effect was determined by applying various concentrations of 5-HT to EGTA-loaded oocytes clamped at potentials around –15 mV (Fig. 5A). Responses were first detected at doses of one or a few nM, and grew progressively to reach a maximum at concentrations of about 1 μM.

Even at high concentrations, the responses were well maintained, with little evidence of desensitization. Fig. 5B shows measurements of the sizes of the responses obtained in four oocytes, scaled to a maximum value of 100% in each oocyte. At low doses response size increased about linearly with increasing dose (slope close to unity on the double logarithmic plot), and was half-maximal at a concentration of about 30 nM. Maximal responses were obtained with about 1 μM 5-HT, and further increases in dose up to 100 μM did not evoke any larger responses. This dose–response relationship is in marked contrast to that of the oscillatory response to 5-HT, which increases very non-linearly with dose and shows an all-or-nothing characteristic near threshold[9,27].

Pharmacology of the 5-HT-induced conductance decrease

The reduction in K^+ conductance produced by 5-HT was strongly antagonized by methysergide. For example, at a concentration of 100 nM, methysergide abolished the response to an equal concentration of 5-HT. Mianserin also acted as a potent antagonist and, as illustrated in Fig. 6A, gave an almost complete block of the response to 300 nM 5-HT when applied at a concentration of 100 nM. This blocking effect was, however, time dependent. In a different oocyte, 50 nM mianserin initially had little effect on the response to 100 nM 5-HT, but repeated applications of 5-HT in the continued presence of mianserin evoked progressively smaller currents, which reduced to about one third after forty minutes. Lysergic acid (LSD) evoked, by itself, small inward currents, and substantially reduced the response to an equi-molar concentration of 5-HT (Fig. 6B). Thus, LSD may act as both an agonist and antagonist on the K^+-closing response, similar to its ability to evoke and inhibit the oscillatory response to 5-HT[9]. The 5-HT_{1A}-selective agonist 8-hydroxy-2(di-*n*-propyl-amino)tetraline (8-OH-DPAT) evoked no detectable response when applied at concentrations as high as 1 μM to an oocyte which gave a large response to 5-HT. Apamin, a specific blocker of

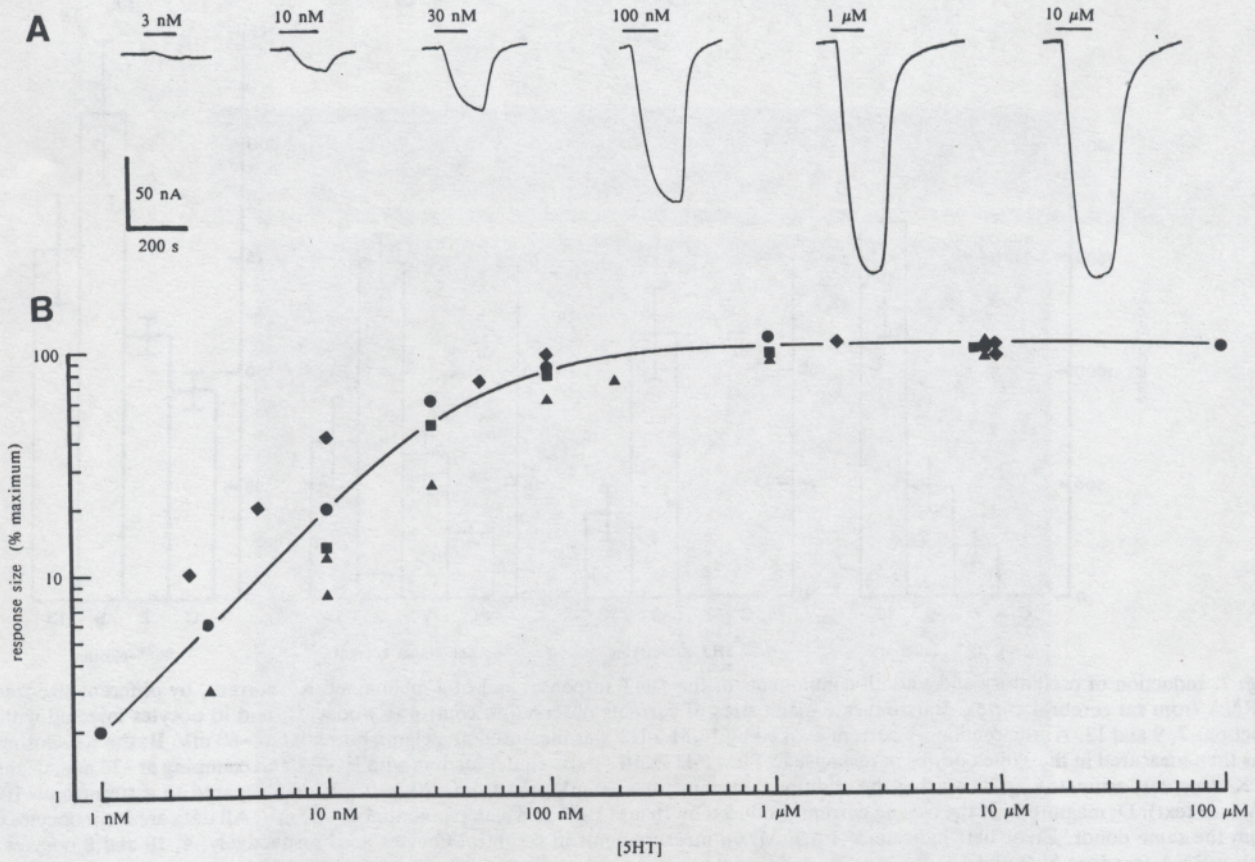

Fig. 5. Dose-dependence of the 5-HT-closing effect. A: sample traces from a single oocyte showing responses evoked by bath application of 5-HT at the molar concentrations indicated. B: double-logarithmic plot showing the dose–response relationship measured in four oocytes (different symbols) from traces like those in A. The data are scaled as a percentage of the mean maximal response in each oocyte. All measurements are from oocytes injected with fractions of mRNA from rat cerebral cortex. The oocytes were loaded with EGTA, and clamped at potentials between –16 and –20 mV.

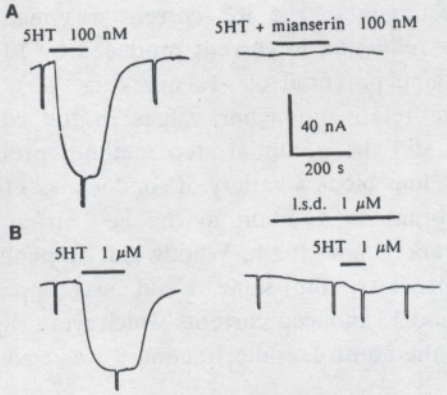

Fig. 6. Blocking of the 5-HT closing response by mianserin (A) and lysergic acid (B). The records in each row are from a single oocyte, and show a control response to 5-HT (left) and a subsequent test response to 5-HT applied in the presence of the blocking drug (right). The clamp potential was –16 mV, with brief steps to –21 mV. Both oocytes were injected with fraction 9 mRNA, and were loaded with EGTA. In A, mianserin (100 nM) was applied to the oocyte beginning a few minutes before the start of the right-hand trace. In B, when lysergic acid (1 μM) was applied is indicated by the bar.

calcium-activated K^+ channels failed to evoke any current at a concentration of 10 μM, and did not alter the response to 1 μM 5-HT.

Serotonin receptors and K^+ channels may be expressed by different mRNAs

It is clear from the above results that the binding of 5-HT to a receptor can lead to the closing of a K^+ channel. We were interested, therefore, to determine whether the functional response required the expression of both channels and receptors and, if so, whether the mRNAs encoding these two proteins could be distinguished. To answer this point, oocytes were injected with size fractionated mRNA from rat cerebral cortex[32].

Measurements were first made of the oscillatory chloride current evoked by 5-HT in these oocytes at a clamp potential of –60 mV. The oocytes were then loaded with EGTA, and the K^+-closing current was measured at –10 mV. The potencies of three mRNA fractions to induce oscillatory and K^+-closing responses are shown in Fig. 7A,B. An important result was that the sizes of the oscillatory and K^+-closing responses were not correlated.

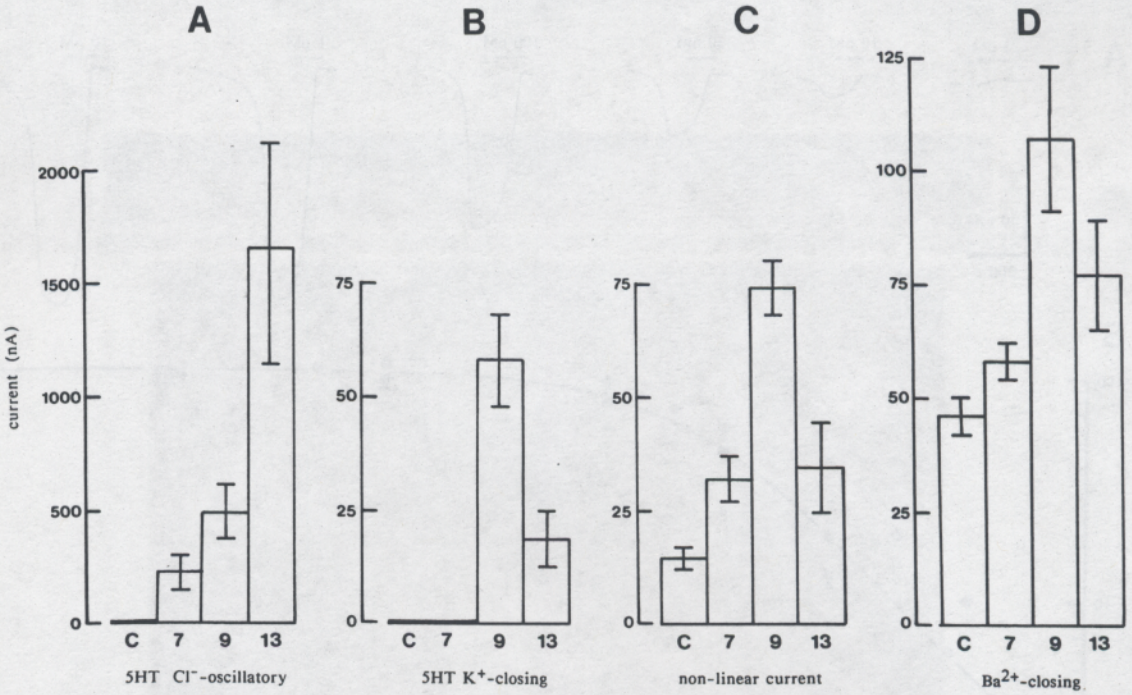

Fig. 7. Induction of oscillatory and smooth components of the 5-HT response, and of a maintained K^+ current, by different size-fractions of mRNA from rat cerebral cortex. Bars indicate mean sizes of currents observed in control (C) oocytes, and in oocytes injected with mRNA fractions 7, 9 and 13. A: the oscillatory current evoked by 1 μM 5-HT was measured at a clamp potential of –60 mV. B: the K^+-closing current was then measured in the same oocytes in response to 1 μM 5-HT, after intracellular loading with EGTA and clamping at –20 mV. C: magnitude of K^+ current, estimated by measuring the additional current for a 20 mV step from –20 to 0 mV as compared to a step from –100 to –80 mV (see text). D: magnitude of the closing current produced by 10 mM Ba^{2+} at a clamp potential of –20 mV. All data are from oocytes obtained from the same donor. Error bars indicate ± 1 S.E.M. on measurements in 6 control oocytes and, respectively, 4, 10 and 8 oocytes injected with mRNA fractions 7, 9 and 13.

For example, mRNA fraction 9 gave the largest K^+-closing response, but a relatively small oscillatory current; while fraction 13 gave the largest oscillatory response, but a small K^+ response. Non-injected oocytes failed to give any responses, and those injected with fraction 7 mRNA showed small oscillatory and no K^+ responses.

This separation of the two types of responses to 5-HT might arise if they are mediated through distinct receptors, encoded by mRNAs which were separated by the fractionation. Alternatively, both responses might be mediated by identical receptors (or receptors encoded by mRNAs of similar sizes), but the closing response may require the co-expression of mRNA encoding the K^+ channel. We had previously observed that rat brain mRNA induced the appearance of a maintained, as well as a transient K^+ current[10]. The ability of different mRNA fractions to induce the maintained K^+ current was therefore examined.

Two methods were used to estimate the size of the K^+ current. Firstly, we recorded the membrane currents evoked by steps in potential from –100 to –80 mV and from –20 to 0 mV, and calculated the difference in current between these pairs of potential steps. Since the K^+ current is activated more strongly at less negative potentials, the extra (non-linear) current needed for the –20 to 0 mV step should give an indication of the size of the underlying K^+ current. All of the mRNA fractions induced non-linear currents which were larger than that in control oocytes, but fraction 9 (the fraction which gave the largest 5-HT-closing response) was clearly the most potent (Fig. 7C).

Another estimate of the K^+ current was made by recording the reduction in current produced by 10 mM Ba^{2+} at a clamp potential of –16 mV (Fig. 7D). This measure gave relatively higher values in the control oocytes than did the potential step method, probably because Ba^{2+} ions block a variety of conductances in the oocyte membrane in addition to the K^+ current (R. Miledi, I. Parker and R.M. Woodward, unpublished data). Nevertheless, the same trend was apparent. Fractions 7 and 13 induced currents which were slightly greater than the control, while fraction 9 was again the most potent.

DISCUSSION

The results demonstrate that 5-HT receptors expressed in *Xenopus* oocytes by brain mRNA modulate a membrane current by reducing a resting ionic conductance.

This response is usually obscured by a much larger increase in chloride conductance produced by serotonergic activation of a phosphoinositide signalling pathway, but it can be studied in relative isolation by loading oocytes with EGTA and clamping them at depolarized potentials. The membrane channels which are closed by 5-HT appear to be permeable primarily to K^+ ions, since the underlying current is outwardly directed at potentials between −50 and +10 mV, while currents due to other ions are expected to be inward over at least part of this potential range. Furthermore, the effect of 5-HT was greatly reduced by Ba^{2+}, which is widely used as a blocker of K^+ channels[13]. Calcium ions are involved in the generation of the oscillatory chloride response to 5-HT, but do not appear to be required for the action on the potassium channels. In particular, the K^+-closing response remained after chelating intracellular calcium by EGTA and after blocking external calcium influx with Mn^{2+} ions, and it was unchanged by apamin, a drug which blocks calcium-dependent K^+ channels[14].

At low doses of 5-HT, the magnitude of the K^+-closing current increased about linearly with concentration, suggesting that the binding of a single molecule of 5-HT is sufficient to cause the closing of a channel. Half-maximal response was obtained at a concentration of about 30 nM 5-HT, and concentrations above about 100 nM gave close to maximal responses. The simplest interpretation of these data is that they reflect the affinity of the receptor for 5-HT, but we cannot exclude the possibility that the maximal response represents a maximal closing of K^+ channels, rather than a maximal occupancy of receptor sites.

The pharmacological properties of the receptor mediating the K^+-closing response are most consistent with it being of the 5-HT_{1C} subtype[28,29]. 5-HT_2 and 5-HT_3 receptors can be ruled out, because they display a low (micromolar) affinity for 5-HT, and because methysergide is ineffective as an antagonist at 5-HT_3 receptors. Further, no responses were observed to the 5-HT_{1A} selective agonist 8-OH-DPAT, but responses to 5-HT were blocked by sub-micromolar levels of mianserin, which is consistent with a 5-HT_{1C}, but not 5-HT_{1B} or 5-HT_{1D} receptors.

Receptors of the 5-HT_{1C} subtype are also able to mediate an oscillatory chloride response in the oocyte[19], so that a single receptor type may give rise to two different ionic responses. Expression of the gene encoding a 5-HT_{1C} receptor in oocytes has so far revealed only the oscillatory chloride response[15], but this may be because functional K^+-closing responses require the presence of appropriate K^+ channels in the membrane. It already appears that the receptor molecules mediating the oscillatory response do not possess an integral K^+ channel sensitive to 5-HT, since a size fraction of mRNA which induced large oscillatory responses was relatively ineffective in inducing the K^+-closing response. Moreover, native (non mRNA-injected) oocytes do not usually display a sufficiently large K^+ conductance to account for the 5-HT-mediated closing current. Thus, the response probably does not arise because exogenous receptors are able to couple to endogenous channels in the oocyte membrane, unlike the chloride current to 5-HT, which is activated through exogenous receptors coupling to a native messenger/channel system. Instead, it is likely that those mRNA preparations which induce the appearance of the K^+-closing response include messengers coding for both receptors and K^+ channels. It will be possible to settle this point more clearly when cloned mRNAs encoding the K^+ channel become available.

The nature of the coupling between 5-HT receptors and the K^+ channels is as yet unclear. 5-HT_{1C} receptors are known to couple through G-proteins to stimulate the metabolism of inositol phospholipids, leading to the formation of the second messengers inositol triphosphate (IP_3) and diacylglycerol[1,6]. Activation of the chloride current in the oocyte arises because IP_3 causes a rise in intracellular free calcium which, in turn, opens calcium-dependent chloride channels[21,33]. If 5-HT_{1C} receptors also modulate the K^+ current, it is clear that this does not occur via intracellular calcium. Instead the channels might be linked to other stages in the signalling pathway, including a direct coupling to the receptor, coupling through a G-protein, or calcium-independent actions of IP_3 or diacylglycerol.

The 5-HT-sensitive K^+ current expressed in the oocyte closely resembles the M-current described in many mammalian neurons[3,4,34]. This is a voltage-dependent K^+ current, which is under the control of several synaptic receptors, including those to acetylcholine and 5-HT[5]. A related current is also seen in invertebrate neurons, in which 5-HT decreases a K^+ conductance via activation of a cyclic AMP messenger pathway[8,16,30]. However, unlike the serotonin K^+-closing current in the oocyte, this S-current is not blocked by Ba^{2+} (ref. 16). We do not yet know whether the K^+ response expressed in the oocyte is identical to the neuronal M-current, but a further similarity is that muscarinic receptors expressed in the oocyte are also able to mediate a closing of a K^+ current (I. Parker and R. Miledi, unpublished data). Whatever the case, it seems that the utility of the oocyte expression sytem for the study of neurotransmitter receptors may now be extended to include those receptors which cause a closing, rather than opening, of ionic channels.

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