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Neurotensin and substance P receptors expressed in *Xenopus* oocytes by messenger RNA from rat brain

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Xenopus oocytes were induced to acquire sensitivity to neurotensin and substance P, by injecting them with a fraction of poly(A)$^+$ mRNA from rat brain. Non-injected oocytes, and oocytes injected with other brain mRNAs, failed to show responses, suggesting that receptors to these peptides were expressed by specific brain mRNAs. Responses to substance P and neurotensin comprised an oscillatory chloride current, and a smooth current having different ionic basis. These currents resembled those seen during activation of muscarinic and serotonergic receptors, but were not blocked by the corresponding antagonists atropine and methysergide. The responses to substance P, and to a lesser extent to neurotensin, showed a long-lasting desensitization. Similarities between the oscillatory currents evoked by the peptides acetylcholine and serotonin suggest that all these receptors may 'link in' to a common intracellular messenger pathway.

Introduction

Signal transmission between nerve cells in the brain takes place via a bewildering variety of neurotransmitter substances, which include acetylcholine(ACh), serotonin, catecholamines, amino acids and peptides. The receptor proteins that respond to these transmitters are encoded by messenger RNAs (mRNAs), and by injecting *Xenopus* oocytes with mRNAs derived from brain we have previously been able to express functional receptors to all of the above types of transmitters, with the exception of neuropeptides (Miledi *et al.* 1982; Gundersen *et al.* 1983, 1984*a*, *b*; Sumikawa *et al.* 1984*b*). Some native (non mRNA-injected) oocytes give an inward current (at −60 mV) in response to substance P, but this response was too small and infrequent for convenient study. Our earlier attempts to induce functional expression of peptide receptors also gave only small responses of a few nanoamps. However, those experiments were done with the use of oocytes injected with total poly(A)$^+$ mRNA from brain. In the present experiments, we used instead fractions of mRNA obtained by sucrose density gradient centrifugation, because appropriate fractions are more effective in inducing receptors than the whole mRNA from which the fractions derive (Sumikawa *et al.* 1984*a*).

Methods

Oocytes of *Xenopus laevis* were injected with fractions of mRNA obtained by sucrose density gradient centrifugation of poly(A)$^+$ mRNA derived from brains of adult Wistar rats. Procedures for extraction and fractionation of the mRNA were as described before (Miledi & Sumikawa 1982; Sumikawa *et al.* 1984*a*), but the present fraction numbers do not correspond exactly to those previously described. Electrophysiological recordings from oocytes were made 3–7 days after injection, by using techniques as before (Miledi 1982; Miledi & Sumikawa 1982). During recording, oocytes were continuously superfused with normal frog Ringer solution at room temperature (*ca.* 24 °C), and drugs were added to this bathing solution. Neuropeptides were obtained from Sigma Chemical Co., and were prepared as stock solutions of 100 μM in water.

Results

Induction of neuropeptide responses

Oocytes previously injected with rat brain mRNA fraction 10 responded to bath application of neurotensin and substance P (both 10^{-6} M) by giving slow membrane currents, which were inward at a clamp potential of −60 mV and showed characteristic oscillations (figure 1). In contrast to this sensitivity, control (non mRNA-injected) oocytes from the same donors failed to show appreciable responses. Thus the responses to the neuropeptides did not arise from 'native' receptors already present in the oocyte membrane, but instead were induced by particular mRNAs from rat brain.

Several other neuropeptides, including somatostatin, luteinizing hormone releasing hormone, and leucine and methionine enkephalins were tested on oocytes injected with rat brain fraction 10 mRNA. So far we have failed to detect responses of more than a few nanoamps to any of these substances.

The sensitivity to neurotensin and substance P might have arisen because of expression of receptors in the oocyte membrane proper, or because receptors were expressed in the surrounding follicular and epithelial cells. To discriminate between these possibilities, oocytes that had previously been injected with rat brain mRNA fraction 10 were defolliculated by treatment with collagenase (Miledi & Parker 1984). When examined a few days after collagenase treatment, these oocytes showed responses to neurotensin and substance P, indicating that sensitivity to the neuropeptides did not depend upon the presence of the follicular and epithelial cells. However, oocytes examined only one day after treatment gave very small responses, or none, even though they still gave a large response to serotonin. Possibly this occurred because initially the collagenase destroyed the substance P and neurotensin receptors already expressed in the oocyte membrane, and the peptide sensitivity returned later as new receptors were inserted in the membrane.

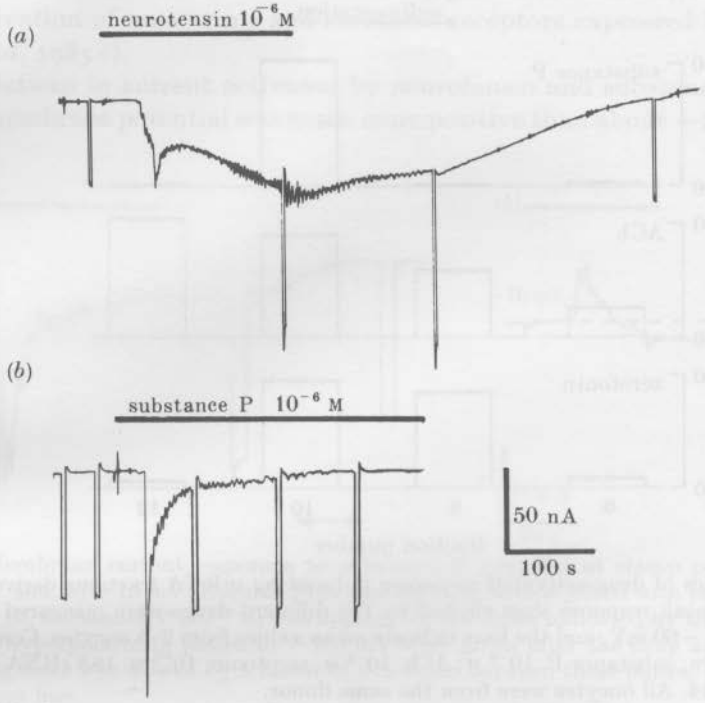

FIGURE 1. Membrane currents elicited by (*a*) neurotensin and (*b*) substance P in oocytes injected with rat brain mRNA fraction 10. The oocytes were voltage-clamped at −60 mV, with occasional brief steps to −120 mV. In this and other figures, downward deflexions correspond to inward membrane currents. Neurotensin and substance P, both at concentrations of 10^{-6} M, were applied for the times indicated by the bars.

Sensitivity to substance P resides in a particular fraction of mRNA

The sizes of responses to substance P and some other neurotransmitters were assayed in oocytes injected with different fractions of rat brain mRNA, obtained by sucrose density gradient centrifugation. Only fraction 10 was found to induce any significant sensitivity to substance P (figure 2). The peak sensitivity for muscarinic and serotonergic activation also occurred at about the same position in the gradient, but unlike with substance P, the induction of sensitivity to these agonists extended over a wider range of fractions (figure 2).

Desensitization

A striking property of the responses to substance P was their long-lasting desensitization. When an oocyte had once responded to substance P (10^{-6} M), a second application usually failed to elicit any detectable response (smooth or oscillatory), even after washing for many minutes. This property was a considerable handicap during experiments, as it was usually practicable to record only a single response in each oocyte examined. One oocyte was left to wash for 2 h after an exposure to substance P (10^{-6} M), and a subsequent application then gave a response of about one half of the initial size. The responses to neurotensin also

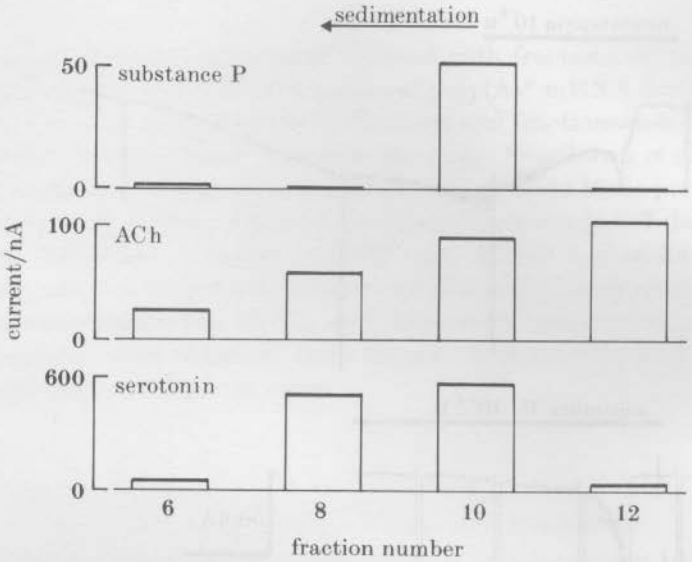

FIGURE 2. Profiles of drug-activated responses induced by mRNA fractions derived from rat brain. The peak response sizes elicited by the different drugs were measured at a clamp potential of −60 mV, and the bars indicate mean values from 2–5 oocytes. Concentrations of drugs were: substance P, 10^{-6} M; ACh, 10^{-4} M; serotonin, 10^{-5} M. 18*S* rRNA sedimented in fraction 14. All oocytes were from the same donor.

showed a slow recovery from desensitization, but this was less marked than with substance P. An interval of about 30 min between applications was sufficient for nearly complete recovery of the response.

We did not see any large 'cross-desensitization' between the responses to substance P and neurotensin: that is, a response to neurotensin was still obtained when tested shortly after application of substance P, and vice versa. Responses to serotonin and acetylcholine were also obtained shortly after exposure to substance P, even though the response to substance P was itself completely abolished at this time.

Properties of neuropeptide responses

Responses to neurotensin (10^{-6} M) were well maintained throughout drug applications lasting a few minutes, and consisted of a steady current with superimposed oscillations (figure 1*a*). After washing, the oscillations died away, leaving a smooth current that declined slowly over several minutes. Responses to substance P were less well maintained, usually showing an initial transient 'spike', followed by a series of oscillations that soon declined, even in the continued presence of agonist (figure 1*b*). The oscillations were usually superimposed upon a steady current, which persisted while substance P was present, and declined slowly after washing (see figure 3*a* for an example). Hyperpolarizing voltage steps applied during responses to neurotensin and substance P evoked larger clamp currents than at rest (figures 1*a* and 3*a*), indicating that the responses were associated with an increase in membrane conductance. In addition, hyperpolariz-

ation evoked small transient inward currents (figures 1*a*,*b* and 3*a*), like those seen during activation of muscarinic and serotonin receptors expressed in the oocyte (Parker *et al.* 1985*a*).

The ocillations in current activated by neurotensin and substance P inverted when the membrane potential was made more positive than about −20 mV (figure

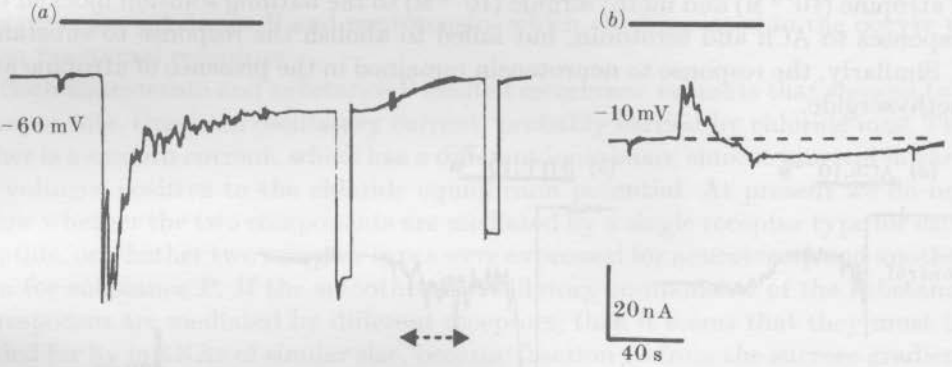

FIGURE 3. Membrane current responses to substance P recorded at clamp potentials of (*a*) −60 mV and (*b*) −10 mV. Records from two oocytes, both injected with rat brain mRNA fraction 10. Substance P (10^{-6} M) was applied for the times indicated by the solid bars. In (*a*), two hyperpolarizing pulses to −100 mV were given after the drug application. The recording trace was slowed by a factor of five times between these pulses, as indicated by the broken line.

3*b*), which corresponds to the chloride equilibrium potential in the oocyte (Kusano *et al.* 1982; Barish 1983). In one oocyte the oscillatory responses to neurotensin and substance P were almost completely suppressed at −18 mV, as was the oscillatory response to serotonin, which has been shown to be due to a chloride current (Gundersen *et al.* 1983). However, when oocytes were clamped at potentials around −10 mV, both neurotensin and substance P gave biphasic responses. The oscillatory currents inverted to become outward currents, but the smooth components of the responses remained as inward currents (see figure 3*b*).

It has been shown that an influx of calcium into the oocyte transiently opens chloride channels (Miledi 1982). However, responses to neurotensin and substance P were still obtained in oocytes perfused with Ringer solution including cobalt (4 mM) or manganese (5 mM), beginning a few minutes before the drug application. These agents block calcium channels in the oocyte membrane (Miledi 1982; Parker *et al.* 1985*a*), and the results suggest that the peptide responses do not depend upon an influx of external calcium.

Peptide receptors are different to those for ACh and serotonin

The fraction of rat brain mRNA used in these experiments induced sensitivity to serotonin and ACh, as well as to neurotensin and substance P. The currents evoked by all of these agonists show several similarities, raising the possibility that the responses to the peptides arose because of activation of muscarinic or serotonin receptors. It already seemed unlikely that this was the case, because native

oocytes can possess muscarinic receptors and still show practically no response to substance P. Moreover, the different sedimentation profiles of the mRNAs and the lack of cross-desensitization also suggest the presence of distinct receptor types. To obtain more conclusive evidence, we used atropine and methysergide as selective antagonists against muscarinic and serotonergic receptors respectively (Kusano *et al.* 1982; Gundersen *et al.* 1984*a*). As illustrated in figure 4, addition of atropine (10^{-6} M) and methysergide (10^{-6} M) to the bathing solution blocked the responses to ACh and serotonin, but failed to abolish the response to substance P. Similarly, the response to neurotensin remained in the presence of atropine and methysergide.

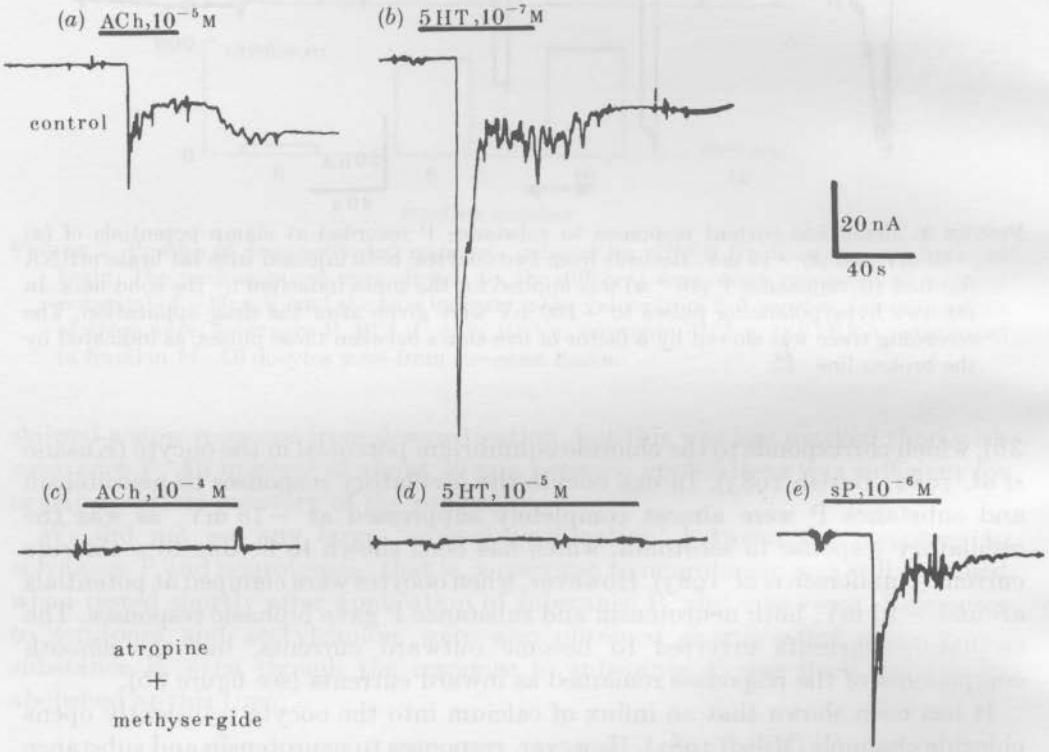

FIGURE 4. Blocking of responses to ACh and serotonin, but not to substance P, by atropine and methysergide. Upper traces (*a*, *b*) show control responses to ACh and serotonin in the absence of blocking drugs. The oocyte was then perfused continuously with solution including atropine (10^{-6} M) and methysergide (10^{-6} M), beginning about 5 min before obtaining the lower traces (*c–e*). All records are from one oocyte, which was injected with rat brain mRNA fraction 10.

DISCUSSION

The results show that an appropriate fraction of poly(A)$^+$ mRNA from rat brain is able to induce *Xenopus* oocytes to acquire sensitivity to neurotensin and substance P. The membrane currents activated by these peptides were very similar to responses activated by muscarinic and serotonergic receptors, which

were also expressed by the same mRNA fraction. However, it seems that the peptide responses are mediated via specific receptors because (i) they were not blocked by muscarinic and serotonergic antagonists, (ii) we did not observe cross-desensitization between the different agonists, and (iii) the profile of sensitivity to substance P induced by different fractions of mRNA differed from that for ACh or serotonin. Thus the adult rat brain contains mRNAs coding for specific receptors for substance P and neurotensin, which are translated in the oocyte to form functional receptors.

Both neurotensin and substance P elicited membrane currents that showed two components. One is an oscillatory current, probably carried by chloride ions. The other is a smooth current, which has a different ionic basis, since it was still inward at voltages positive to the chloride equilibrium potential. At present we do not know whether the two components are mediated by a single receptor type for each peptide, or whether two receptor types were expressed for neurotensin and another two for substance P. If the smooth and oscillatory components of the substance P responses are mediated by different receptors, then it seems that they must be coded for by mRNAs of similar size, because fraction 10 from the sucrose gradient gave both components of the response, whereas fractions 8 and 12 gave practically no smooth or oscillatory responses. From their positions in the gradient, the mRNAs coding for substance P receptors appear to be slightly larger than the mRNAs coding for muscarinic receptors and slightly smaller than those for serotonin receptors.

The oscillatory currents activated by substance P and neurotensin resemble in their time course and ionic basis those elicited by activation of serotonin and muscarinic receptors in the oocyte (Gundersen *et al.* 1983; Kusano *et al.* 1982; Sumikawa *et al.* 1984*a*). Further similarities include (i) the resistance of the peptide responses to cobalt and manganese in the bathing medium, suggesting that, like the muscarinic and serotonin responses, they do not depend on an influx of extracellular calcium (Parker *et al.* 1985*b*; Dascal *et al.* 1985), and (ii) the development of a transient inward current activated by hyperpolarization during responses to the peptides, like that seen during muscarinic and serotonin activation (Parker *et al.* 1985*a*). The oscillatory responses to all these agonists, including the development of the transient inward current, can be mimicked by intracellular injection of inositol 1,4,5-trisphosphate into the oocyte (Oron *et al.* 1985; Miledi *et al.* 1986; Parker & Miledi 1986). It therefore seems likely that these different neurotransmitter receptors, including those to substance P and neurotensin, may link in to a common intracellular messenger system in the oocyte. In this scheme, receptor activation causes the production of inositol trisphosphate from membrane inositol phospholipids. This in turn liberates calcium from intracellular stores (cf. Berridge & Irvine 1984), leading finally to the activation of calcium-sensitive chloride channels in the oocyte membrane (Miledi 1982; Miledi & Parker 1984). Substance P and neurotensin have been found to stimulate the breakdown of inositol phospholipids in several neuronal tissues (Downes 1983), suggesting that they may exert some of their actions in nerve cells through a similar internal messenger pathway to that which causes the oscillatory currents in the oocyte. However, the resulting changes in membrane excitability might differ from those

in the oocyte, depending for example on the types of calcium-sensitive channels present in the neuronal membrane.

A striking feature of the responses to substance P, and to a lesser extent to neurotensin, is their long-lasting desensitization. However, responses could still be obtained to serotonin and acetylcholine when the peptide responses were desensitized, suggesting that this did not arise because of 'exhaustion' of the mechanism giving the oscillatory currents. Instead, the desensitization probably arises at the level of the specific receptors.

Substance P is widely considered as a neurotransmitter candidate in the central nervous system (see, for example, Otsuka & Takahashi 1977; Krieger *et al.* 1983). In mouse spinal cord neurons, excitatory effects of substance P have been described (Otsuka & Yanagisawa 1980), resulting both from an increase in membrane conductance (Vincent & Barker 1979) and from a decrease in potassium conductance (Nowak & Macdonald 1982). The relations between these responses, and the different components of the response to substance P induced in the oocyte, remain to be further investigated.

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