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A transient inward current elicited by hyperpolarization during serotonin activation in *Xenopus* oocytes

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Activation of serotonin, glutamate or muscarinic receptors, incorporated into the membrane of *Xenopus* oocytes following injection of messenger RNA from rat brain, caused the development of a transient inward (T_{in}) current when the membrane was hyperpolarized. A detailed study was made of the T_{in} current induced during serotonin activation. The current is due principally to efflux of chloride ions, and is presumably activated by an influx of calcium ions, because it was blocked by removal of calcium from the bathing medium, by addition of manganese, cobalt or lanthanum, or by intracellular injection of EGTA. During application of serotonin, the amplitude of the T_{in} current increased slowly, and after washing it persisted for longer than the direct serotonin-induced current. The amplitude of the T_{in} current was sensitive to temperature and pH, and was abolished at pH 6.5 or by cooling to 12 °C. The T_{in} current may be of importance in regulating the excitability of neurons in the central nervous system.

Introduction

It has been noted previously that membranes of fully grown, immature oocytes react fairly passively to electrical hyperpolarization (Kusano *et al.* 1977, 1982; Baud *et al.* 1982). However, occasionally some oocytes were found to have a transient inward current which was activated by hyperpolarization of the membrane (R. Miledi, unpublished data), and a similar current has been described in ovulated oocytes (Peres & Bernadini 1983). In this paper we report that this membrane current is very prominent during activation of serotonin, glutamate or muscarinic receptors, in oocytes that had been induced to acquire these receptors by injection of messenger RNA isolated from the brain of adult rats (Gundersen *et al.* 1983, 1984*a*). We also describe some of the properties of this current elicited during serotonin activation.

Methods

Experiments were made on oocytes of *Xenopus laevis*, by using techniques described previously (Kusano *et al.* 1982; Miledi 1982). With the exception of the oocyte illustrated in figure 1, all experiments were made on oocytes which had been

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injected, a few days before use, with poly(A)$^+$ messenger RNA (mRNA) obtained from rat brains, to induce the formation of drug receptor-channel complexes in the oocyte membrane (Gundersen *et al.* 1983, 1984*a–c*). Oocytes were voltage-clamped by using a conventional two-electrode system, and were continuously perfused with Ringer solution. Unless otherwise stated, the temperature was 20–25 °C. Normal Ringer solution contained: NaCl, 115 mmol l^{-1}; KCl, 2 mmol l^{-1}; $CaCl_2$, 1.8 mmol l^{-1}; HEPES, 5 mmol l^{-1}; at pH 7.2. Sodium-free Ringer was made by substituting tetraethylammonium bromide or chloride for sodium chloride. Zero calcium Ringer solution contained no added calcium, and included additionally 5 mM $MgCl_2$ and 1 or 2 mM EGTA. Low chloride Ringer was made by substituting sodium methylsulphate for sodium chloride. In some experiments, a third micropipette filled with 200 mM ethylene glycol-bis (β-amino-ethyl ether)*N*,*N*-tetraacetic acid (EGTA) was used for intracellular injection. EGTA was injected iontophoretically by passing pipette negative current pulses of 50–80 nA and 0.5 s duration at a repetition rate of 1 Hz.

Results

Transient inward current in non-injected oocytes

Currents recorded from normal oocytes (not injected with mRNA) usually showed comparatively passive, 'ohmic', increases in clamp current in response to hyperpolarizing voltage steps. However, in a small fraction of oocytes a transient inward (T_{in}) current was elicited, which had a duration of a few seconds, and

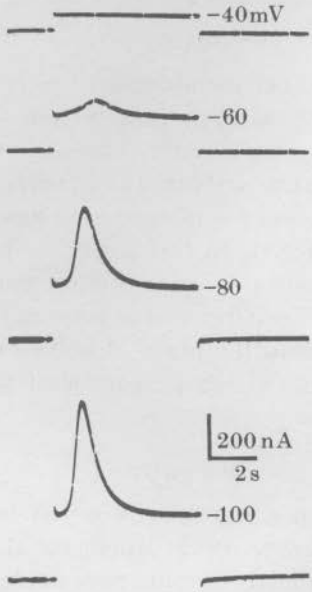

Figure 1. Transient inward current recorded from a non-injected oocyte bathed in normal Ringer solution. The oocyte was voltage-clamped at a potential of −20 mV, and hyperpolarized by pulses of 6 s duration to potentials of (from top to bottom) −40, −60, −80 and −100 mV. In this, and all other figures, an upward deflection corresponds to an inward membrane current.

increased in amplitude with increasing hyperpolarization (figure 1). The response was often labile, and oocytes which showed a T_{in} current soon after insertion of the microelectrodes could fail to respond when tested again a few minutes later. A possible explanation for this is that the T_{in} current may have been dependent upon an influx of calcium ions at the site of impalement (see later), which disappeared as the membrane sealed around the microelectrodes. The native T_{in} current was found both in oocytes that had low (*ca.* −20 mV), or high (−40 to −80 mV) resting potentials and input resistances.

Transient inward current during serotonin activation

Injection of exogenous mRNA into oocytes causes the incorporation of various functional drug receptors into the oocyte membrane (Barnard *et al.* 1982; Miledi & Sumikawa 1982; Miledi *et al.* 1982*a*, *b*; Gundersen *et al.* 1983, 1984*a*–*c*). Application of serotonin to oocytes injected with rat brain mRNA activates an oscillatory chloride current (Gundersen *et al.* 1983); in addition, it creates a state in the membrane such that hyperpolarization elicits a T_{in} current, even in oocytes which showed no such response when their membrane was hyperpolarized before applying serotonin.

Collagenase-treated oocytes, in which the follicular and other enveloping cells had been removed (Kusano *et al.* 1982; Miledi & Parker 1984), still showed T_{in} currents during serotonin activation. This indicates that the current arises across the oocyte membrane, and does not depend on the presence of the enveloping cells. Moreover, application of serotonin to non-injected oocytes from the same donors did not elicit any membrane current responses (cf. Gundersen *et al.* 1983*a*), nor did serotonin cause the appearance of T_{in} currents during hyperpolarization in these cells.

The development and decay of T_{in} currents elicited during bath application of serotonin to an oocyte previously injected with rat brain mRNA is illustrated in figure 2. Before application of serotonin, hyperpolarizing the membrane from −60 mV to −120 mV produced only a small, passive increase in membrane current (trace 1, figure 2*b*). However, soon after beginning perfusion with serotonin, a T_{in} current activated by hyperpolarizing pulses appeared, and increased progressively throughout the drug application (traces 2–4, figure 2*b*). The peak T_{in} current often grew to be as large as several microamperes, even in oocytes where the current directly elicited by the serotonin was small, as in the oocyte from which figure 2 was taken. Furthermore, some oocytes developed a T_{in} current even during application of a very low concentration of serotonin (*ca.* 10^{-9} M), which by itself failed to evoke any membrane current.

The development of the T_{in} current response to hyperpolarization was always much slower than that of the direct current response induced by serotonin. Also, the T_{in} current continued to increase with time, even when the serotonin-elicited current declined during the period of drug application. Often, the largest T_{in} current was recorded shortly after washing out serotonin from the bath, suggesting that serotonin itself may inhibit or block the membrane channels underlying the response. The fall of the T_{in} current after removal of serotonin lagged behind that of the direct serotonin-induced current, although the time course was very variable

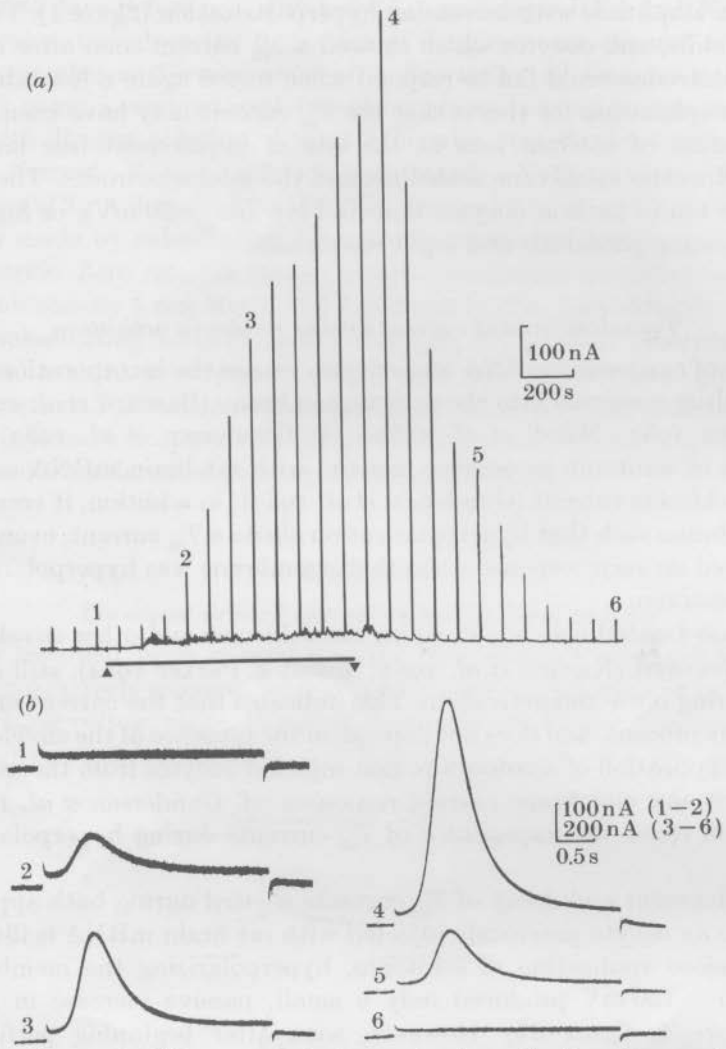

FIGURE 2. Development of T_{in} current during serotonin application. An oocyte, which had previously been injected with rat brain mRNA, was voltage-clamped at a potential of −60 mV and hyperpolarized to −120 mV by pulses of 3 s duration at intervals of 90 s. (*a*) Record of clamp current at a slow speed. (*b*) Selected responses from (*a*) at a faster sweep. Note that the recording gain was reduced after the first two traces in (*b*). Serotonin (10^{-5} M) was applied for the duration indicated by the bar in (*a*).

between different oocytes. In some oocytes the T_{in} current disappeared much more slowly than in figure 2, and persisted for many minutes after the serotonin-induced current had ceased.

Injection of mRNA derived from human foetal cerebral cortex causes oocytes to develop sensitivity to serotonin (Gundersen *et al.* 1984*b*), in a similar way to that seen with rat brain mRNA. Activation of these serotonin receptors also induced the appearance of a T_{in} current, with maximal amplitudes similar to those

in oocytes injected with rat brain mRNA. Evidence presented later indicates that the T_{in} current is carried by chloride ions, and is dependent upon an influx of calcium ions. In this context it is interesting that the calcium-dependent chloride current, activated by depolarization (Miledi 1982), was enhanced in oocytes injected with rat brain mRNA, while oocytes injected with human cortex mRNA showed currents no larger than those in native oocytes (Gundersen *et al.* 1984*b*).

Oscillatory currents following hyperpolarizing pulses

Many oocytes showed a series of oscillations of decaying amplitude following hyperpolarizing pulses applied during perfusion with serotonin (figure 3). This behaviour was most prominent in cells that showed marked spontaneous oscillations

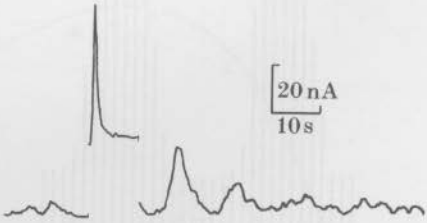

FIGURE 3. Oscillatory currents following a hyperpolarizing pulse given during serotonin application. The oocyte was perfused with serotonin (2×10^{-8} M) for several minutes before the start of the record, and the potential was stepped from −74 to −154 mV during a 10 s pulse.

in the serotonin-induced current, and it appeared that the potential step might act to 'synchronize' the drug-induced fluctuations. The reversal potential of the current oscillations, measured by stepping the potential to different levels following a hyperpolarizing pulse, was close to the chloride equilibrium potential (*ca.* −25 mV).

A surprising, and still unexplained, change in the response to serotonin occurred when the flow of perfusion fluid was altered, and in some oocytes changes in flow rate appeared to synchronize the current oscillations. Here, the current declined when the flow was stopped, but showed a large transient increase upon re-starting the perfusion, followed by a decaying series of oscillations.

Transient inward current during activation of other drug receptors

Injection of mRNA from rat brain induces the oocyte to acquire responses to several drugs in addition to serotonin. These include oscillatory chloride currents activated by glutamate (Gundersen *et al.* 1984*a*), steady chloride currents activated by γ-aminobutyric acid (GABA) (Miledi *et al.* 1982*b*) and glycine (Gundersen *et al.* 1984*c*), and a steady sodium–potassium current activated by kainate (Gundersen *et al.* 1984*a*, *b*). Activation of glutamate receptors caused the development of a T_{in} current, similar to that seen with serotonin (figure 4). In contrast, the T_{in} current did not develop during the activation of the steady membrane currents elicited by kainate, GABA and glycine.

Activation of 'native' muscarinic receptors present in the membrane of non-injected oocytes (Kusano *et al.* 1982) also caused the development of a T_{in} current. However, oocytes from many donors showed only small, or no responses at all to acetylcholine. In these cases, injection of rat brain mRNA induced the formation of exogenous muscarinic receptors (cf. Gundersen *et al.* 1984*a*), and a T_{in} current developed during acetylcholine application (figure 4).

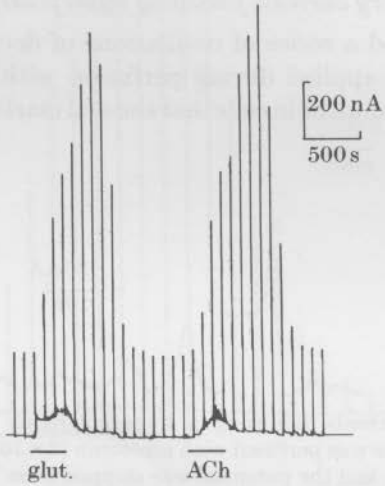

FIGURE 4. Development of T_{in} current during application of glutamate and ACh to a rat brain mRNA injected oocyte. The membrane potential was held at −60 mV, and stepped to −130 mV during pulses of 3 s duration at intervals of 90 s. Glutamate (10^{-3} M) and ACh (10^{-4} M) were applied as indicated by the bars.

Voltage and temperature dependence of the T_{in} current

The peak size of the T_{in} current elicited during serotonin application increased progressively with hyperpolarization up to about −120 mV, and the rates of onset and decay of the current became faster (figure 5). In some oocytes polarization to more negative potentials elicited larger responses (figure 5*a*), while in others the current reached a maximal value, or even declined (figure 5*b*). The size of the T_{in} current elicited by a fixed hyperpolarizing step was larger when the initial holding potential was made more positive. For example, one oocyte gave a T_{in} current of over 1 μA when the potential was stepped from 0 mV to −70 mV, but a step from −100 to −170 mV gave a response of less than 100 nA.

The amplitude and time course of the T_{in} current elicited by polarization from −60 to −130 mV during continued serotonin application were strongly temperature-dependent. As the temperature was lowered the peak amplitude declined, and the time course of rise and decline became slower. At 16 °C the response amplitude was about one half of that at 23 °C, and at 12 °C it was undetectable.

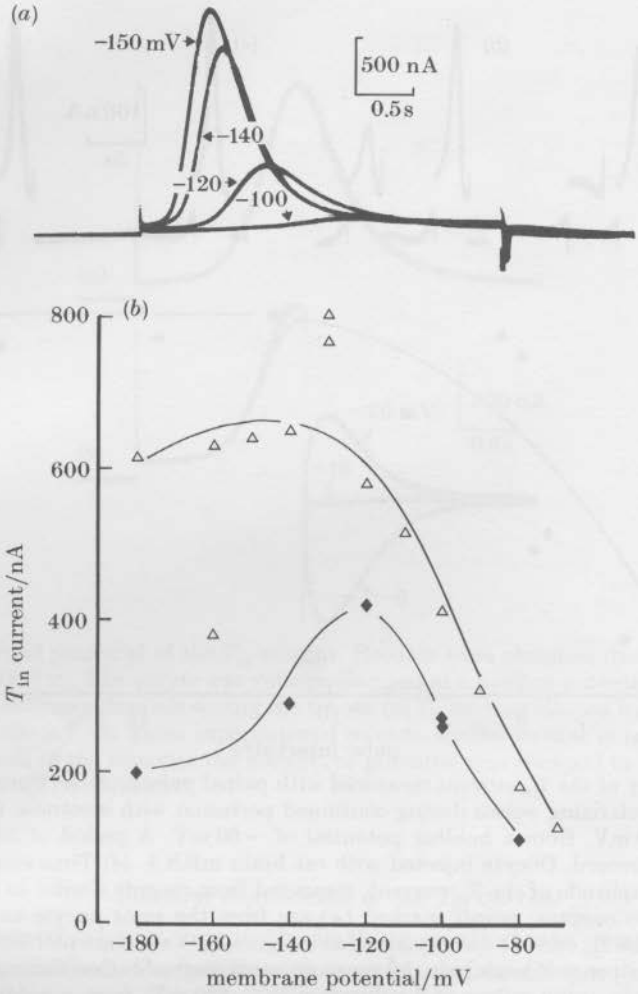

FIGURE 5. Voltage dependence of T_{in} current elicited during serotonin application. (*a*) Four superimposed records obtained about 25 min after beginning perfusion with serotonin (10^{-7} M). At this time the T_{in} current elicited by test hyperpolarizations to −120 mV had reached a stable maximal level. The oocyte was voltage clamped at a holding potential of −60 mV, and was hyperpolarized during 3 s pulses to the potentials indicated (in millivolts). Oocyte injected with rat brain mRNA. (*b*) Voltage-dependence of the T_{in} current measured from two other oocytes (different symbols). In both cases, records were obtained about 20 min after beginning perfusion with serotonin (10^{-7} M), and the holding potential was −60 mV. T_{in} currents were measured as the difference between the 'passive' current just after the beginning of the pulse, and the peak of the response.

Recovery from inactivation

The T_{in} current declined during a 3 s pulse to −130 mV, and a second pulse applied about 1 s after returning the potential to −60 mV elicited only small responses (figure 6*a*). However, when the pulse interval was lengthened, the size of the T_{in} current elicited by a second pulse gradually increased, recovering to the original size at intervals longer than about 10 s (figure 6*b*, *c*). The time course of

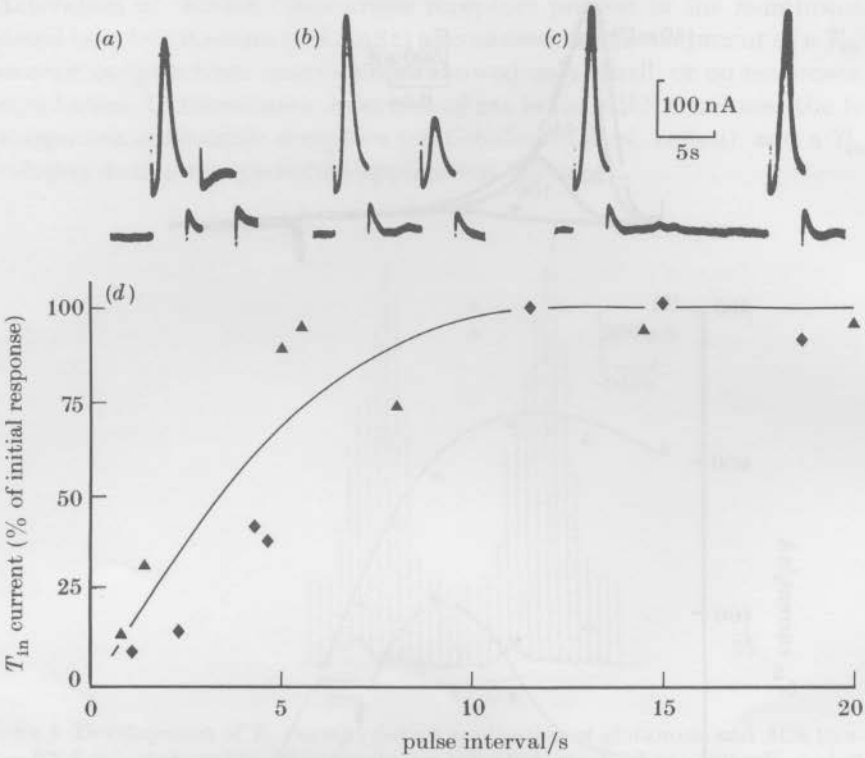

FIGURE 6. Recovery of the T_{in} current measured with paired pulses. (*a*)–(*c*) Currents elicited by paired hyperpolarizing pulses during continued perfusion with serotonin (10^{-7} M). Pulses were to −130 mV, from a holding potential of −60 mV. A period of 30 s was allowed between each record. Oocyte injected with rat brain mRNA. (*d*) Time course of recovery of the peak amplitude of the T_{in} current, measured from records similar to those in (*a*)–(*c*). Data from two oocytes; points marked (◆) are from the same oocyte as in (*a*)–(*c*). The amplitude of the T_{in} current was measured as in figure 5*b*. Values are plotted as a percentage of the initial response in each pair. All measurements were obtained during perfusion with 10^{-7} M serotonin, using pulses of 3 s duration to −130 mV, from a holding potential of −60 mV. Temperature 23 °C. Curve is drawn by eye.

recovery, measured in this way, is plotted in figure 6*d*. For these oocytes it took about 5 s for half recovery.

The T_{in} current is carried by chloride ions

Measurements of the equilibrium potential of the T_{in} current elicited during serotonin application were made by stepping the membrane potential to different levels just after the peak of the current (figure 7*a*, *b*). Tail currents recorded in this way decreased in size as the membrane was depolarized, and inverted direction at a potential of about −20 mV. This potential corresponds to the chloride equilibrium potential in *Xenopus* oocytes (Kusano *et al.* 1982). Further evidence suggesting that the T_{in} current is mainly carried by chloride ions was provided by the observations that the size of the T_{in} current (at −130 mV) was almost unchanged in sodium-free Ringer solution, but was enhanced in low chloride (methylsulphate) Ringer.

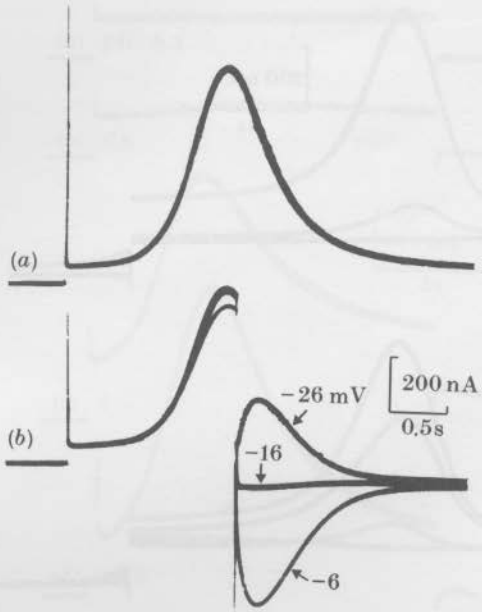

FIGURE 7. Reversal potential of the T_{in} current. Records were obtained during perfusion with serotonin (10^{-7} M). The oocyte was voltage-clamped at a holding potential of −60 mV, and stepped to various potentials during the traces. (*a*) T_{in} current elicited by a hyperpolarizing pulse to −120 mV. (*b*) Three superimposed records, similar to that in (*a*), except that just after the peak of the response the membrane potential was clamped to potentials of −26, −16 or −6 mV, as indicated.

Calcium dependence of the T_{in} current

The presence of calcium ions in the bathing solution was necessary for the T_{in} current to be elicited. Perfusion with a calcium-free Ringer (containing 1 mM EGTA and 5 mM magnesium) during serotonin application rapidly and reversibly abolished the T_{in} current (figure 8*b*). The T_{in} current was reversibly blocked also by addition of manganese (5 mM) to normal Ringer (figure 8*a*), even though this had little effect on the serotonin-activated current. Addition of cobalt (10 mM) and lanthanum (100 μM) also blocked the T_{in} current, while magnesium (5 mM) caused a small (*ca.* 25 %) reduction in amplitude. An increase in the calcium concentration four-fold to a total of 7 mM gave a slight (*ca.* 10 %) increase in amplitude of the T_{in} current.

These experiments indicated that external calcium was necessary for the generation of the T_{in} current, and suggested that the current might be dependent upon a rise in intracellular calcium subsequent to a calcium influx. To test this idea further, we injected the calcium chelating agent EGTA into oocytes so as to buffer any rise in intracellular free calcium concentration. After loading oocytes with EGTA for 30–60 min, application of serotonin elicited maintained membrane currents, without any obvious oscillations. Hyperpolarizing pulses given during serotonin application caused a slow rise in this maintained current, but did not elicit any obvious T_{in} current (figure 9).

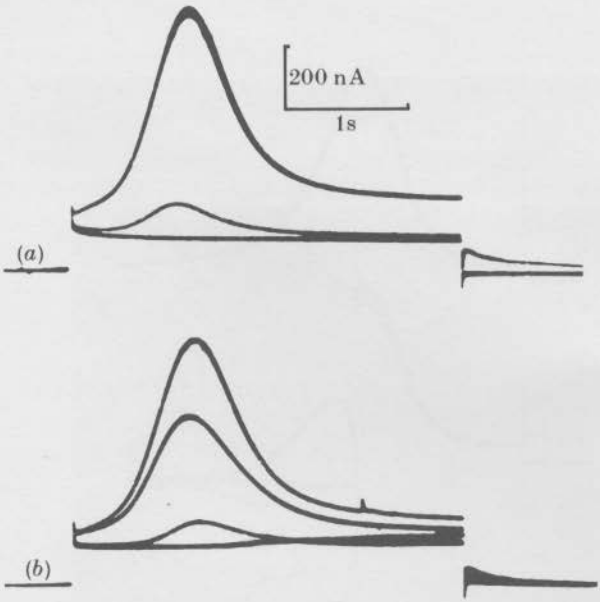

FIGURE 8. Blocking of the T_{in} current by manganese (*a*) and zero calcium solution (*b*). The oocyte was exposed to serotonin (10^{-8} M) for about 20 min before obtaining records. Superimposed traces show currents elicited by pulses from −60 to −130 mV, given at 20 s intervals. (*a*) The first trace (large T_{in} current) was recorded in normal Ringer, and 5 mM manganese was then added. The subsequent pulse elicited a smaller current, and the T_{in} current was virtually abolished during the third pulse. After changing back to normal Ringer, the T_{in} current recovered to its original amplitude within a few minutes (not shown). (*b*) Records obtained after those in (*a*), showing the effect of perfusion with calcium-free Ringer.

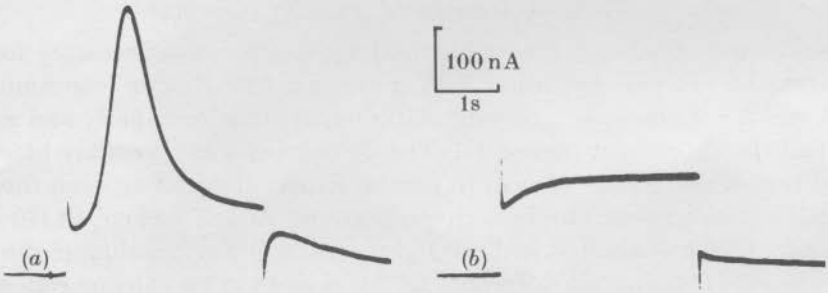

FIGURE 9. Blocking of the T_{in} current by intracellular injection of EGTA. Both traces show currents elicited by pulses from −60 to −130 mV, applied about 20 min after starting perfusion with serotonin (10^{-8} M). (*a*) Control record; (*b*) response after loading the oocyte with EGTA for 50 min.

Effect of pH on the T_{in} current

Small changes in the pH of the perfusing solution produced large changes in both the T_{in} current (figure 10*a–e*), and in the direct current induced during serotonin application. At pH values more acid than about 6.5 the T_{in} current was abolished, while it was enhanced when the pH was increased to about 8 from the normal value of 7.2. These changes were reversible, and the T_{in} current and the serotonin-induced

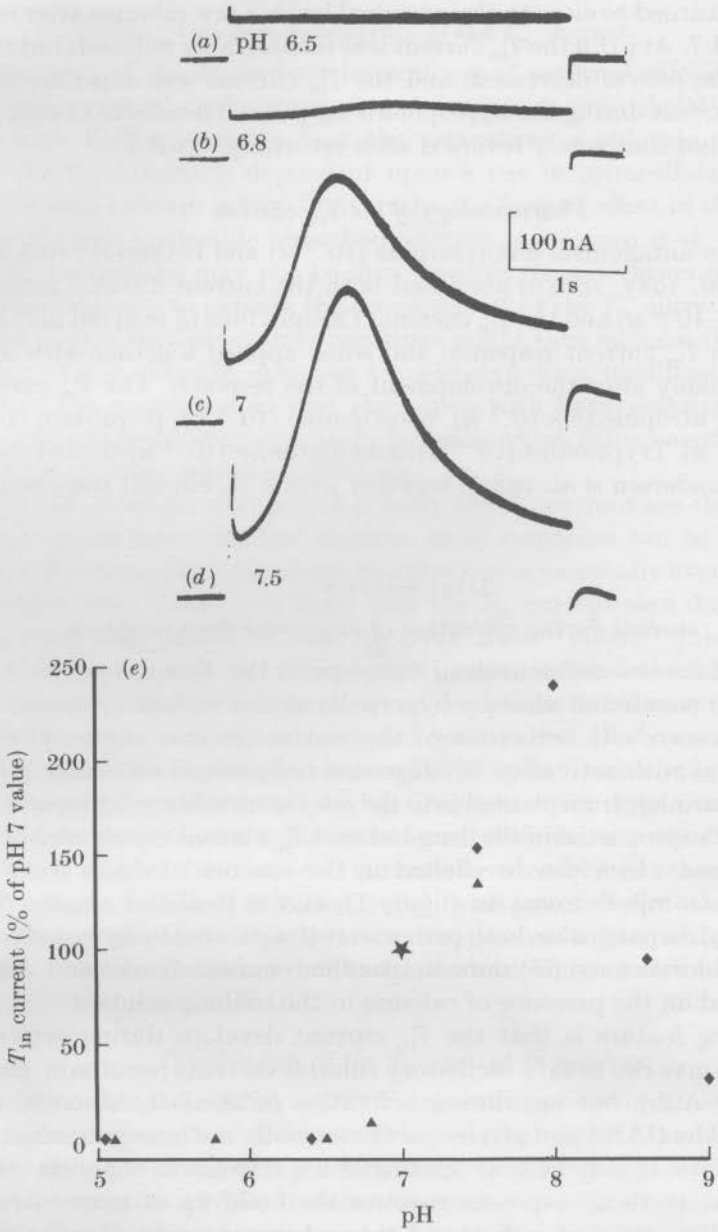

FIGURE 10. (*a*)–(*d*) T_{in} currents recorded at different pH values. The oocyte was perfused with serotonin (10^{-7} M) in normal Ringer at pH 7 for about 20 min, and the pH of this solution was then altered. The oocyte was returned to pH 7 solution after each change, and these control responses remained approximately constant. In all records, the oocyte was polarized to −130 mV from a holding potential of −60 mV. Ringer solution included 5 mM HEPES buffer; the pH was adjusted with HCl or NaOH. (*e*) Dependence of T_{in} current on pH, measured from records similar to those in (*a*)–(*d*). Results from two oocytes, injected with rat brain mRNA. Points marked (▲) were from the same oocyte as (*a*)–(*d*). Peak currents are plotted as a percentage of the control value at pH 7 obtained before each pH change. Serotonin concentration 10^{-7} M. Solutions contained HEPES buffer, except for pH 9 solution, which was made with Tris buffer (5 mM).

current both returned to close to their original levels a few minutes after returning to Ringer at pH 7. At pH 9 the T_{in} current was considerably reduced; but the input resistance of the oocyte decreased, and the T_{in} current was superimposed on a slowly rising current during the hyperpolarizing pulse. The effects of very alkaline solutions were not completely reversed after returning to pH 7.

Pharmacology of the T_{in} current

The serotonin antagonists methysergide (10^{-6} M) and ketanserin (0.5 mg ml^{-1}) (Gundersen *et al.* 1983, 1984*a*) abolished both the current directly activated by serotonin (10^{-8}–10^{-6} M) and the T_{in} current. Theophylline (5 mM) did not, by itself, give rise to the T_{in} current response, and when applied together with serotonin did not appreciably alter the development of the response. The T_{in} current was not blocked by atropine (5×10^{-7} M), neostigmine (10^{-4} M), picrotoxin (1 mM), or morphine (10^{-4} M). Tryptamine (10^{-6} M) and bufotenine (10^{-4} M) elicited oscillatory currents (cf. Gundersen *et al.* 1983), together with a T_{in} current response.

Discussion

T_{in} current during activation of exogenous drug receptors

Activation of several different drug receptors in the *Xenopus* oocyte creates a condition in the membrane whereby hyperpolarization evokes a transient inward current. This is seen with activation of the 'native' muscarinic receptors in the oocyte, as well as with activation of exogenous receptors to serotonin, glutamate and ACh (muscarinic), transplanted into the oocyte membrane by injection of rat brain mRNA. The properties of the drug-induced T_{in} current closely resemble those of the T_{in} current which can be elicited in the absence of drugs from a small proportion of non-injected oocytes (figure 1), and in ovulated oocytes (Peres & Bernardini 1983). In particular, both currents are (i) activated by hyperpolarization; (ii) carried by chloride ions; (iii) show similar time courses of onset and decay; and (iv) both depend on the presence of calcium in the bathing solution.

An interesting feature is that the T_{in} current develops during activation of receptors which give rise to slow oscillatory chloride currents (serotonin, glutamate and muscarinic ACh), but not during activation of the fast, 'smooth' chloride currents elicited by GABA and glycine, or the 'smooth' sodium–potassium current elicited by kainate. It may be that opening of the membrane channels mediating the slow oscillatory drug responses requires the build up of some intracellular messenger (cf. Kusano *et al.* 1982), and this substance might also facilitate the activation of the channels responsible for the T_{in} current. Such a mechanism would be consistent with the very slow increase and decline of the T_{in} current response during and after drug application. The pronounced latency to onset of the current following a hyperpolarizing step, and its strong temperature dependence, also suggest that activation of the response involves a multi-step process.

Calcium dependence of the T_{in} current

The abolition of the T_{in} current by removal of external calcium, by blocking calcium influx with cobalt, lanthanum, and manganese, or by chelating intracellular calcium with EGTA, suggests that the activation of chloride channels which mediate the T_{in} current is dependent upon a rise in intracellular free calcium, subsequent to a calcium influx. Conversely, the lack of effect of theophylline, an inhibitor of cyclic nucleotide phosphodiesterase (Appleman *et al.* 1973), suggests that cyclic nucleotides may not be important in the activation of the response. Possible mechanisms to explain the development of the T_{in} current as a result of activation of slow oscillatory drug responses might thus include; (i) an increase in sensitivity of the chloride channels to calcium; (ii) a modification of calcium channels in the membrane so that they open with hyperpolarization; or (iii) a calcium influx through drug-operated channels, which is increased by the greater driving force at more negative potentials.

The chloride selective channels that carry the T_{in} current are clearly present in the membrane of some 'native' oocytes, since responses can be elicited during activation of endogenous muscarinic receptors, or occasionally even in the absence of any added drug. Thus, it is likely that the T_{in} current seen during activation of exogenous drug receptors also involves these 'native' chloride channels. However, it may be that the foreign mRNA also induced the formation of new chloride channels, in addition to the drug receptors. Calcium-activated chloride currents are seen in non-injected oocytes both at rest (Robinson 1979; Kusano *et al.* 1982) and following depolarization (Miledi 1982; Barish 1983). The fact that large T_{in} currents can be elicited during drug activation from oocytes that show only small calcium-activated transient outward chloride currents on depolarization might seem to suggest that these two currents involve different channels. However, recent experiments (Miledi & Parker 1984), with intracellular calcium injections, indicate that native (non-injected) oocytes possess large numbers of calcium-activatable chloride channels, even though the transient outward current activated by depolarization may be small.

Possible role of the T_{in} current in neurons

We presume that the mRNA coding for serotonin receptors arose from neuronal cells in the brain tissue, but at present this is not certain (cf. Gundersen *et al.* 1983) and we are trying to clarify this point by isolating mRNA separately from neurons and glia. Irrespective of this, it seems likely that a conductance similar to that underlying the T_{in} current may exist in neuronal cells, since it is seen also with activation of glutamate and muscarinic receptors. Such a current could play an important role in regulating the excitability of neurons, and would show some interesting and unusual features.

The chloride equilibrium potential is more negative in neurons than in the oocyte, and the T_{in} current would thus tend to hyperpolarize the cell, perhaps giving rise to a regenerative hyperpolarizing 'action potential'. Prolonged activation of a serotonergic pathway might directly induce only a small inhibitory current, or no current at all, while the neuron was at rest or in a depolarized state.

However, when the cell began to repolarize (for example, during an inhibitory synaptic potential), activation of the T_{in} current could trigger a regenerative response, tending to pull the membrane potential rapidly towards the chloride equilibrium potential.

The properties of the T_{in} current would also make it important in the long-term regulation of cell excitability. For example, the response might still be triggered by hyperpolarization many minutes after serotonin (or ACh or glutamate) had disappeared from the synaptic cleft. Furthermore, the oscillatory nature of the currents elicited by serotonin, ACh or glutamate, together with the synchronized oscillations following a hyperpolarizing pulse, could play important roles in generating rhythmical activity in neurons. A final point is that if the responses in neurons are similar to those in the oocyte, then very small changes in pH (cf. figure 10) would have large effects on the behaviour of the neurons.

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