Journal of Physiology (1989), **408**, *pp.* 223–232
With 4 text-figures
Printed in Great Britain

RAT BRAIN GLUTAMATE RECEPTORS ACTIVATE CHLORIDE CHANNELS IN *XENOPUS* OOCYTES COUPLED BY INOSITOL TRISPHOSPHATE AND Ca^{2+}

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(*Received 2 March 1988*)

SUMMARY

1. Ionic currents were studied in *Xenopus laevis* oocytes using the patch-clamp and the whole-cell voltage-clamp techniques.

2. Single-channel currents were recorded from the cell-attached patches in oocytes injected with rat brain mRNA when glutamate was applied locally outside the patch. The single-channel conductance was 3·66 pS, and the extrapolated equilibrium potential was −23·0 mV, indicating that the channels were chloride selective.

3. Single-channel currents with similar characteristics were observed in cell-attached patches in native oocytes in response to injection of inositol 1,4,5-trisphosphate (IP_3) or Ca^{2+}.

4. Whole-cell currents were evoked by glutamate in oocytes injected with rat brain mRNA. They usually showed an oscillatory component, and reversed direction at about the chloride equilibrium potential. Injection of IP_3 or Ca^{2+} into a native oocyte evoked a transient whole-cell current. The reversal potential was near the chloride equilibrium potential, and it changed from negative to positive in low-chloride solution.

5. The results suggest that the glutamate receptors are not directly coupled with the endogenous chloride channels but indirectly activate these via the messenger system IP_3–Ca^{2+}.

INTRODUCTION

The *Xenopus laevis* oocyte is a convenient model system for the investigation of neurotransmitter-induced membrane electrical responses induced by injections of foreign mRNA. A glutamate receptor can be induced in the oocyte by injection of rat brain mRNA, and glutamate-activated currents were observed, which reversed direction at about the chloride equilibrium potential (Gundersen, Miledi & Parker, 1984; Houamed, Bilbe, Smart, Constanti, Brown, Barnard & Richards, 1984; Sugiyama, Ito & Hirono, 1987). Similar membrane current responses were also observed in the oocytes on application of serotonin (Gundersen, Miledi & Parker, 1983; Takahashi, Neher & Sakmann, 1987). These membrane currents are activated by intracellular Ca^{2+} (Dascal, Gillo & Lass, 1985; Sugiyama *et al.* 1987), and can be mimicked by intracellular injection of inositol 1,4,5-trisphosphate (IP_3) (Oron,

Dascal, Nadler & Lupu, 1985). IP_3 has been identified as a second messenger that can mobilize Ca^{2+} from the endoplasmic reticulum (Berridge & Irvine, 1984). Injection of IP_3 into *Xenopus* oocytes caused an increase in intracellular free Ca^{2+} released from intracellular stores (Busa, Ferguson, Joseph, Williamson & Nuccitelli, 1985; Parker & Miledi, 1986) and furthermore, injection of Ca^{2+} into oocytes elicited a chloride current (Miledi & Parker, 1984; Dascal *et al.* 1985). These observations suggest that the membrane responses to neurotransmitters are activated through an internal messenger system involving IP_3 and Ca^{2+}. In this scheme, activation of newly induced glutamate receptors leads to the production of IP_3, which then causes mobilization of Ca^{2+} from internal stores, and activates the endogenous membrane chloride channels. Therefore this glutamate receptor is different from the glutamate receptor channel which is activated directly by glutamate (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Cull-Candy & Ogden, 1985). However, no direct evidence has been given that glutamate is received by a mRNA-induced receptor which is not directly coupled with but is located distant from the chloride channel. By using the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), we have examined the hypothesis that the glutamate receptors from rat brain mRNA couple to intracellular messengers and activate endogenous chloride channels. In cell-attached-patch experiments, glutamate applied outside the patch area activated chloride channels in the patch membrane. Injection of IP_3 or Ca^{2+} into the oocytes also activated endogenous channels in the patch membrane. For this glutamate activation process the intracellular messenger system is indispensable. Thus we report here that the activation of rat brain glutamate receptors activates endogenous chloride channels utilizing IP_3 and mobilized Ca^{2+} in the cell.

METHODS

Injection of mRNA into oocytes

Total cellular RNA was extracted from adult rat brains by the guanidinium–hot phenol method (Maniatis, Fritsch & Sambrook, 1982). The poly(A)$^+$ mRNA purified by oligo(dT) affinity chromatography (Maniatis *et al.* 1982), and size-fractionated by sucrose gradient centrifugation (Hirono, Yamagishi, Ohara, Hisanaga, Nakayama & Sugiyama, 1985), was kindly provided by Dr H. Sugiyama. Fractions corresponding to 20–40 S mRNA were used. Female frogs (*Xenopus laevis*) more than 2 years old were obtained from Hamamatsu Seibutsu Kyozai Co. Ltd (Shizuoka, Japan). Small pieces of ovaries were removed from *Xenopus laevis* as described by Hirono *et al.* (1985). In order to disperse oocytes and remove follicular and epithelial cells, they were treated with collagenase (1·5 mg/ml) in a Ca^{2+}-free modified Barth's solution (Ca^{2+}-free MBS) for 1 h at 25 °C with gentle agitation. Defolliculated oocytes were placed in the injection chamber filled with Ca^{2+}-free MBS, then 60–90 nl of mRNA dissolved in H_2O (1 mg/ml) was injected into an oocyte by means of an N_2 gas pressure pulse (240 kPa, 50–150 ms). The tip diameter of the glass micropipette used for microinjection was 5–10 μm. Each micropipette was calibrated for ejection volume by measuring the distance of displacement of the meniscus caused by several successive pressure pulses. Oocytes injected with mRNA were cultured at 20 °C for 3–4 days in MBS containing penicillin (10 μg/ml) and streptomycin sulphate (10 μg/ml).

Microinjection of drugs

The intracellular injection of inositol 1,4,5-trisphosphate (IP_3, dissolved to 1·0 mM in H_2O) and $Ca(NO_3)_2$ (0·5 mM) was done by means of a gas pressure pulse (N_2, 240 kPa). The tip diameter of the glass micropipette was 8–15 μm. The volume of injection (10–50 nl) was calibrated as described above.

Glutamate application

Glutamate was applied by pressure from a capillary tube filled with 10 mM-sodium glutamate located with its opening close to the oocyte and opposite to the area from which patch currents were recorded.

Single-channel current measurements

For patch-clamp experiments, oocytes were skinned of their vitelline membranes (Methfessel, Witzemann, Takahashi, Mishina, Numa & Sakmann, 1986). Immediately before a patch-clamp experiment, the oocytes were placed for 20 min at 18 °C in a 'stripping' solution which was hypertonic with respect to MBS by adding 200 mM-sucrose to MBS. Within about 20 min after transfer of the oocyte into this solution the vitelline membrane became detached from the plasma membrane. When the vitelline membrane had become detached from the plasma membrane the oocyte was placed into a fresh tissue-culture dish containing normal Ringer solution. The vitelline membrane was removed with a pair of forceps. Without the vitelline membrane, the oocytes are mechanically fragile. Within several minutes, they adhere tightly to the dish.

TABLE 1. Compositions of bath solutions (in mM)

No.	Solution	NaCl	KCl	$CaCl_2$	Sodium isethionate
1	Normal Ringer	90	2	2	—
2	Ca^{2+}-free Ringer (no added Ca^{2+})	90	2	—	—
3	Na^+-free Ringer	—	90	2	—
4	Low-Cl^- Ringer	—	2	2	90

Glass patch pipettes were made from borosilicate glass by a standard two-step pulling procedure, subsequently coated with Sylgard and then fire-polished (Hamill *et al.* 1981). Single-channel currents were measured with an L/M-EPC-7 patch-clamp amplifier (List Electronics) and recorded on magnetic tape. Experiments were performed on cell-attached membrane patches at the animal hemisphere in normal Ringer solution. Patch pipettes also contained normal Ringer solution. In recording from cell-attached patches the resting membrane potential of the oocyte was measured by using an intracellular microelectrode filled with 3 M-KCl. The transmembrane voltage across the membrane patch was the sum of the oocyte resting potential and the negative value of the voltage applied to the patch pipette. Voltage was recorded on magnetic tape as above. Current traces were low-pass filtered (200 or 100 Hz) and digitized at 2 kHz.

Whole-cell current measurements

Oocytes were placed in a tissue-culture dish (diameter 35 mm). The membrane potential of the oocyte was voltage clamped with two microelectrodes. One (tip diameter 1 μm) was filled with 3 M-KCl and used for voltage recording. The other (tip diameter 1–2 μm) was filled with 3 M-potassium acetate and used for current supply. Voltage and current were recorded on magnetic tape. Current–voltage (I–V) relations were obtained by applying ramp-command voltages with slopes of about 200 mV/s. The current records stored on magnetic tape were digitized and stored in a personal computer. The I–V curves were obtained by subtraction of the control current (i.e. current before glutamate application or drug injection) from the current obtained during glutamate or drug injection responses.

Solutions

MBS (modified Barth's solution): 88 mM-NaCl, 1·0 mM-KCl, 0·41 mM-$CaCl_2$, 0·33 mM-$Ca(NO_3)_2$, 0·82 mM-$MgSO_4$, 2·4 mM-$NaHCO_3$ and 7·5 mM-Tris-Cl (pH 7·6). Ca^{2+}-free MBS: MBS minus $CaCl_2$ and $Ca(NO_3)_2$. Other solutions are listed in Table 1. All solutions listed in Table 1 contained 10 mM-Tris-Cl (pH 7·6). The experiments were done at room temperature (23–26 °C).

RESULTS

Glutamate activates single chloride channels in brain-mRNA-injected oocytes

In mRNA-injected oocytes, patch-clamp experiments were performed according to the cell-attached version (Hamill *et al.* 1981). Figure 1*A* shows examples of

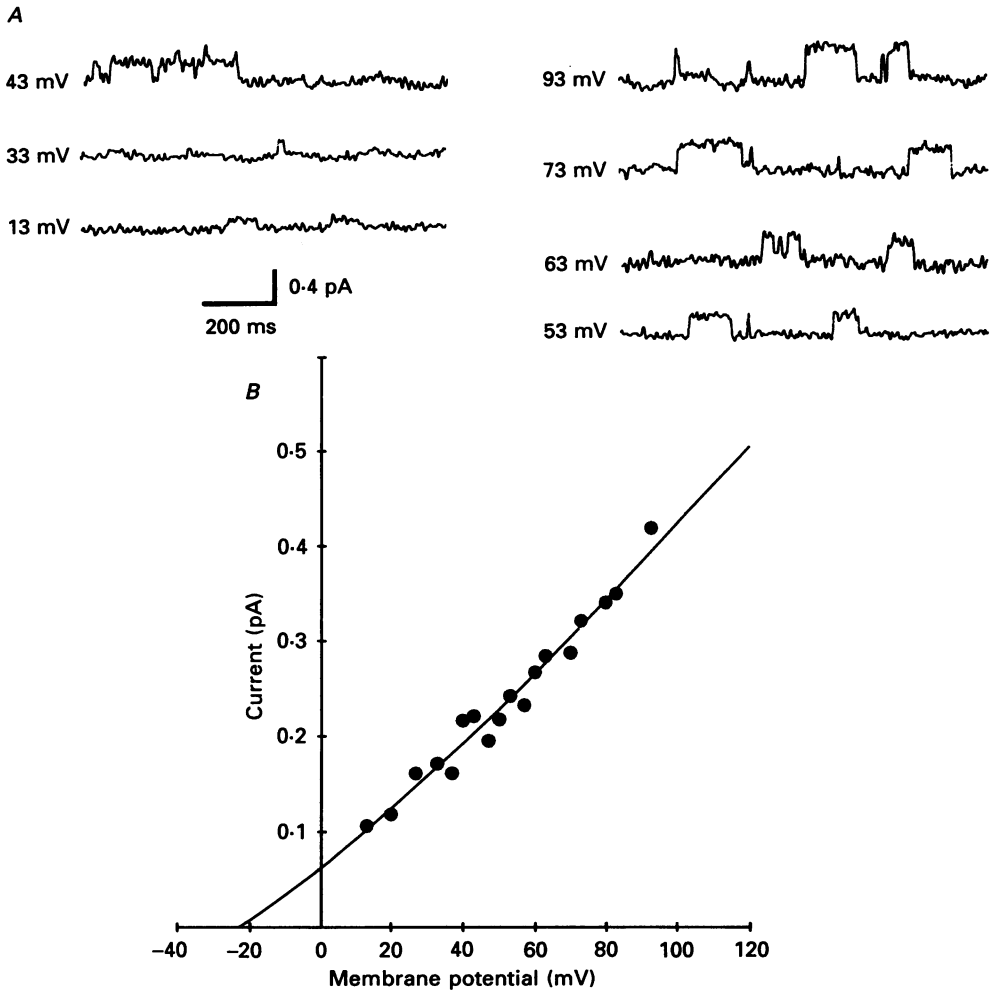

Fig. 1. *A*, single-channel currents activated by glutamate in a mRNA-injected oocyte at various membrane potentials as indicated on each trace. Cell-attached patches. Bath solution and patch-pipette solution were normal Ringer solution. Glutamate (10 mM) was applied to the membrane from a capillary placed opposite the patch pipette. In this, and all other figures, upward deflections denote outward membrane currents. Low-pass filtering at 200 Hz (−3 dB). *B*, single-channel current–voltage relation of glutamate-activated currents (four oocytes). The continuous line was drawn according to eqn (1).

glutamate-activated single-channel currents at various positive membrane potentials. They are outward currents at positive membrane potentials and increase in size with more positive membrane potentials. Glutamate was applied to the oocyte at a point opposite to the region where the patch pipette was placed. The tight seal

between the edge of the glass pipette and the membrane would presumably prevent access of applied glutamate to glutamate receptors within the membrane patch. This implies that applied glutamate could activate only glutamate receptors located in the membrane outside of the patch, and that the currents shown in Fig. 1*A* were stimulated by intracellular messengers released by glutamate receptors which were located in the membrane outside of the patch. The *I–V* relation of single-channel currents could be calculated with the constant-field assumption by using the Goldman–Hodgkin–Katz equation:

$$I = \frac{Pz^2F^2E}{RT} \frac{(c_e - c_i \exp(zFE/RT))}{(1 - \exp(zFE/RT))}, \quad (1)$$

where F, R and T are the usual thermodynamic quantities, z is the valency of the ion and P is the permeability. c_e and c_i are the external and internal ion concentrations,

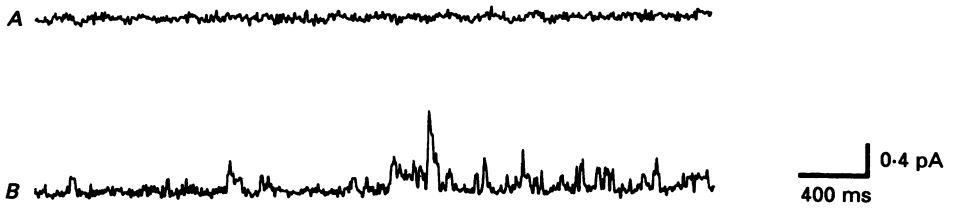

Fig. 2. Single-channel currents activated by IP_3 injection, before injection (*A*) and after injection (*B*). Cell-attached patches. Membrane potential was +40 mV. The intracellular injection was done by means of a gas pressure pulse into the oocyte from the opposite side to the patch pipette. The oocyte was injected with 25 nl of 1 mM-IP_3, yielding a total of 25 pmol/oocyte. Low-pass filtering at 100 Hz (−3 dB).

respectively. E and I are the membrane potential and current, respectively. From the results of whole-cell current experiments described later, we consider that the major ion which permeated through the channel was chloride. Thus, we assigned the values of z and c_e as −1 amd 96 mM, respectively. Figure 1*B* shows the single-channel *I–V* relation obtained from four oocytes. A continuous line was drawn according to eqn (1). The best-fit parameters indicate that: (1) slope conductance was 3·66 pS at +50 mV; (2) the extrapolated equilibrium potential was −23·0 mV; (3) internal chloride concentration was 38·3 mM. These results support the idea that glutamate-activated single-channel currents are carried predominantly by chloride. The mean open time of this channel at +53 mV was 41·6 ms.

Injection of IP_3 or Ca^{2+} activates single chloride channels in native oocytes

The above experiment employing the cell-attached system strongly suggests that the glutamate receptor requires an intracellular messenger system for the generation of current response. Therefore, we tried direct intracellular injection of IP_3 or Ca^{2+} as candidate second messengers into native oocytes after forming a cell-attached patch. Figure 2 shows single-channel currents in cell-attached patches activated by IP_3 injection into the oocytes (*A*, before IP_3 injection; *B*, after IP_3 injection).

Single-channel currents activated by IP_3 are outward at positive membrane

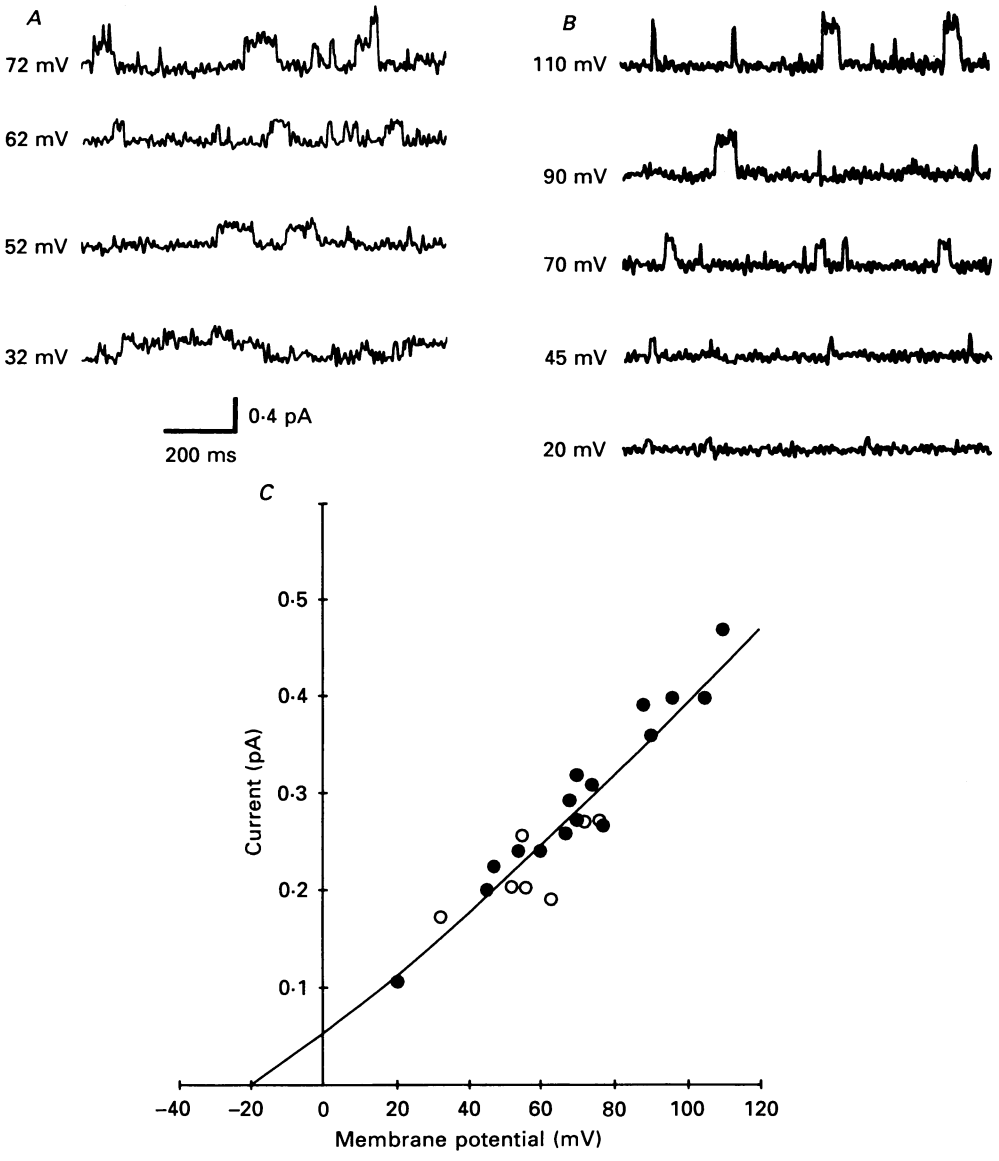

Fig. 3. Single-channel currents activated by IP_3 injection (*A*) and Ca^{2+} injection (*B*). Cell-attached patches. Membrane potentials are indicated on each trace. The oocyte was injected with 25 nl of 1 mM-IP_3, yielding a total of 25 pmol/oocyte (*A*) or with 25 nl of 0·5 mM-$Ca(NO_3)_2$, yielding a total of 12·5 pmol/oocyte (*B*). Low-pass filtering at 200 Hz (−3 dB). *C*, single-channel current–voltage relation of IP_3-activated currents (four oocytes, ○) and Ca^{2+}-activated currents (eight oocytes, ●). The continuous line was drawn according to eqn (1).

potentials, and increase in size with more positive membrane potentials as shown in Fig. 3*A*. Injection of Ca^{2+} into oocytes also elicited single-channel membrane currents that were outward when the membrane potential was clamped at positive potentials in normal Ringer solution (Fig. 3*B*). Figure 3*C* shows the single-channel *I–V* relation of both IP_3 and Ca^{2+}-activated currents. The continuous line was

drawn according to eqn (1) as in Fig. 1*B*. The result indicates that: (1) the slope conductance was 3·44 pS at +50 mV; (2) the extrapolated equilibrium potential was −20·0 mV; (3) the internal chloride concentration was 43·2 mM. These results also suggest that single-channel currents activated by injection of IP_3 or Ca^{2+} are carried predominantly by chloride. The mean open time of the IP_3-activated channel was 27·8 ms at +55 mV and that of the Ca^{2+}-activated channel was 30·4 ms at +67 mV.

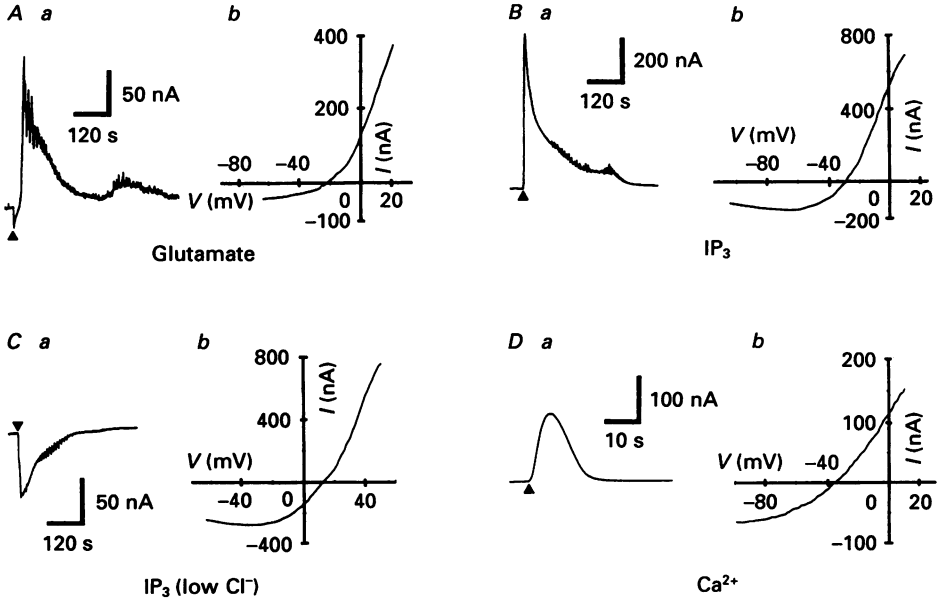

Fig. 4. *A*, glutamate activation of whole-cell currents in *Xenopus* oocytes injected with rat brain poly(A)$^+$ mRNA. Whole-cell current response to application of glutamate (▲) at 0 mV in normal Ringer solution. Current–voltage relation of glutamate-activated current as measured during rampwise shifts of command potential. The current before glutamate application was subtracted. *B* and *C*, whole-cell current response to IP_3 injection (▲) into native oocytes (no mRNA injected) at 0 mV in normal Ringer solution (*B*) and in low-Cl^- solution (*C*) and current–voltage relations of IP_3-activated current. The oocyte was injected with 25 nl of 1 mM-IP_3, yielding a total of 25 pmol/oocyte (*B*) and with 12·5 pmol-IP_3 (*C*). *D*, whole-cell current response to Ca^{2+} injection (▲) at 0 mV in normal Ringer solution (*D a*) and current–voltage relation of Ca^{2+}-activated current in normal Ringer solution (*D b*). The oocyte was injected with 25 nl of 0·5 mM-$Ca(NO_3)_2$, yielding a total of 12·5 pmol/oocyte.

Properties of whole-cell currents

In connection with the above cell-attached-patch experiment, we repeat here briefly the corresponding results on the whole-cell current.

Glutamate activates chloride currents in brain-mRNA-injected oocytes. Whole-cell current responses were observed by glutamate application to *Xenopus* oocytes injected with rat brain mRNA (Fig. 4*A a*). A brief inward current after application of glutamate might be the smooth component of the glutamate response (Gundersen *et al.* 1984). This inward current sometimes appeared, but the values of the reversal

potential were similar whether the inward current appeared or not. Figure 4*Ab* shows the whole-cell current–voltage (*I–V*) relation of the glutamate-activated current. The mean reversal potential was -19 ± 2 mV ($\pm$S.E.M., eight oocytes). In native oocytes which were not injected with rat brain mRNA, no response was observed to glutamate application.

Intracellular injection of IP_3 or Ca^{2+} evokes a chloride conductance increase in native oocytes. Injection of IP_3 into native oocytes generated outward membrane currents in normal Ringer solution (Fig. 4*B*). The mean reversal potential was -26 ± 1 mV ($\pm$S.E.M., ten oocytes). In Ca^{2+}-free solution (solution 2, Table 1) and in Na^+-free solution (solution 3), the reversal potential was -30 ± 1 mV (mean $\pm$ S.E.M., four oocytes) and -36 ± 2 mV (four oocytes), respectively. The reversal potential and the shape of the *I–V* relation showed no significant difference compared with those in normal Ringer solution. Replacement of NaCl with sodium isethionate caused a significant change in the reversal potential. In low-chloride solution (solution 4) the membrane currents turned inward at 0 mV, opposite to the current in normal Ringer solution (Fig. 4*C*). The reversal potential was $+15 \pm 1$ mV (four oocytes). These results suggest that chloride is the major ion carrying the IP_3-injected current.

Pressure injection of Ca^{2+} into oocytes elicited membrane currents which rose and decayed slowly (Fig. 4*D*). The reversal potential was -33 ± 5 mV (seven oocytes). Replacement of NaCl with sodium isethionate caused a significant change in the reversal potential towards more positive voltages. These results suggest that Ca^{2+}-activated currents were predominantly chloride currents.

DISCUSSION

Our patch-clamp and whole-cell experiments showed that rat brain glutamate receptors were induced in the *Xenopus* oocyte membrane, and that application of glutamate to the receptors activated chloride-selective channels. The patch-clamp method gave direct evidence that the glutamate receptor has no direct connection with the chloride channels but has influences on the channels via the second messenger system. The chloride channel activation was induced through intracellular second messengers (IP_3 and Ca^{2+}). Similar chloride-selective channels were activated in native oocytes by injection of IP_3 or Ca^{2+}.

Single-channel currents were recorded from the cell-attached patches in oocytes injected with rat brain mRNA when glutamate was applied locally outside the patch. The tight seal between the glass pipette and the membrane should have prevented access of applied glutamate to glutamate receptors within the membrane patch. This implies that single-channel currents activated by glutamate must be stimulated through the action of intracellular messengers. Single-channel conductance and the extrapolated equilibrium potential were 3·66 pS at +50 mV and −23·0 mV, respectively, which indicated that the channels were chloride selective. The mean open time of the channel at +53 mV was 41·6 ms. Considering that IP_3 and Ca^{2+} are candidates as intracellular messengers of this chloride-selective channels, we injected IP_3 or Ca^{2+} into oocytes after forming cell-attached patches. Single-channel currents with similar characteristics were observed in cell-attached patches in response to injection of IP_3 or Ca^{2+} in native oocytes.

The application of glutamate to oocytes injected with rat brain mRNA induced whole-cell membrane currents that reversed direction at about the chloride equilibrium potential (Fig. 4*A* in this paper; Gundersen *et al.* 1984). The glutamate-induced currents were inhibited by injection of the Ca^{2+}-chelating agent EGTA into oocytes, indicating that the glutamate-induced currents depend on intracellular Ca^{2+} (Parker, Gundersen & Miledi, 1985; Sugiyama *et al.* 1987). Injection of IP_3 into oocytes mimicked this membrane current and the reversal potential was at about the chloride equilibrium potential (Fig. 4*B*; Oron *et al.* 1985). Injection of IP_3 into oocytes caused an increase in intracellular free Ca^{2+}, not depending upon extracellular Ca^{2+} (Parker & Miledi, 1986). Injection of Ca^{2+} into the oocytes evoked currents that reversed direction at about the chloride equilibrium potential (Fig. 4*D*; Miledi & Parker, 1984; Dascal *et al.* 1985).

In conclusion, our results suggest: (1) glutamate receptors induced by rat brain mRNA are activated by glutamate, leading to the formation of IP_3; (2) IP_3 induces Ca^{2+} release from intracellular stores; (3) the increase of intracellular Ca^{2+} concentration activates endogenous Ca^{2+}-dependent chloride channels.

Recently it was reported that rat brain serotonin receptors were incorporated into *Xenopus* oocytes, and thus single-channel currents were observed from cell-attached patches when serotonin was applied locally outside the patch (Takahashi *et al.* 1987). This channel had a small conductance (about 3 pS) and was chloride selective. A similar channel was observed in inside-out patches in response to elevated Ca^{2+} concentrations on the cytoplasmic side. This channel had similar characteristics (single-channel conductance and extrapolated equilibrium potential) to the channel that we have observed in response to glutamate application, injection of IP_3 and injection of Ca^{2+}. It was reported that exposure of mammalian brain cells to glutamate or its analogues caused enhanced hydrolysis of inositol phospholipids (Sladeczek, Pin, Récasens, Bockaert & Weiss, 1985; Nicoletti, Meek, Iadarola, Chuang, Roth & Costa, 1986). Glutamate increased the intracellular Ca^{2+} concentration in isolated hippocampal neurones (Kudo & Ogura, 1986). Our results clearly suggest the existence of glutamate receptors which couple to intracellular messengers (IP_3 and Ca^{2+}) in the brain. However the final activated channel of this receptor system in the brain is not necessarily the chloride channel observed in *Xenopus* oocytes. Further investigation of the operation of this glutamate receptor in the brain is needed.

We thank Dr H. Sugiyama for the generous gift of poly(A)$^+$ mRNA. We also thank Dr K. Furuya and Dr C. Hirono for helpful discussions, and Mr T. Sazi for his technical assistance.

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