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Rat brain serotonin receptors in *Xenopus* oocytes are coupled by intracellular calcium to endogenous channels

[brain poly(A)$^+$ mRNA/calcium release/second messenger/chloride channel]

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ABSTRACT **Serotonin activates chloride currents in *Xenopus* oocytes injected with a subfraction of rat brain poly(A)$^+$ mRNA. Patch-clamp recordings from cell-attached patches showed that serotonin, applied locally outside the patch, caused the opening of channels of ≈3 pS conductance and an average lifetime of ≈100 msec. The extrapolated reversal potential indicated that the channels are chloride-selective. Single-channel currents with similar characteristics were observed in inside-out patches from native oocytes in response to elevated calcium concentrations on the cytoplasmic side. Measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by fura-2 fluorescence showed ≈10-fold increases in $[Ca^{2+}]_i$ in response to serotonin application in both normal and calcium-free Ringer solution in mRNA-injected oocytes. Little or no response to serotonin was observed in native oocytes. These results suggest that serotonin activation of receptors that are inserted into the oocyte membrane following injection of rat brain poly(A)$^+$ mRNA can induce calcium release from intracellular stores. The increase in $[Ca^{2+}]_i$ subsequently activates calcium-dependent chloride channels. Because calcium-dependent chloride channels and a receptor-controlled mechanism of internal calcium release have been shown to exist in native oocytes, we conclude that the newly inserted serotonin receptors utilized the endogenous second-messenger-mediated calcium release to activate endogenous calcium-dependent chloride channels.**

Serotonin (5-hydroxytryptamine) is an important transmitter and modulator in the mammalian brain. Biochemical studies show that serotonin receptors can stimulate phosphatidylinositol turnover as well as enhance adenylate cyclase activity (1, 2). Serotonin has been found to have a variety of effects on the electrical properties of molluscan neurons, including changes in sodium and chloride conductances (3) as well as inhibition of a serotonin-sensitive potassium channel (4). Less is known about the mechanism of serotonin action in the mammalian brain because of the heterogenous actions of serotonin receptors, although recent work has demonstrated changes in potassium conductance (5).

To study the properties of the serotonin receptors present in the vertebrate central nervous system, we have used serotonin receptors implanted into the plasma membrane of *Xenopus laevis* oocytes following injection of mRNA extracted from mammalian brain (6–9). In mRNA-injected oocytes, serotonin activated a current whose reversal potential and dependence on the extracellular ion concentration suggest that it is mainly carried by chloride ions and is dependent on calcium released from intracellular stores (6, 7, 10). This response resembles an effect produced by another neurotransmitter, acetylcholine, in native oocytes (11). Acetylcholine-induced increase in chloride conductance can be mimicked by injection of inositol trisphosphate (InsP_3) and is believed to reflect the activation of endogenous calcium-sensitive chloride channels by calcium released from InsP_3-sensitive intracellular stores (12, 13). The similarities between the two effects suggest that serotonin receptors inserted after mRNA injection activate an endogenous oocyte second-messenger system that controls oocyte channels, the end effect being determined not only by the properties of the foreign receptor but also by its interactions with elements specific for the host cell.

Our present results support this view by showing that (*i*) single-channel currents induced by serotonin agree in all their parameters with single-channel currents elicited by an increase in intracellular calcium concentration ($[Ca^{2+}]_i$); (*ii*) serotonin applied outside the patch area induces channels in the pipette-enclosed patch, indicating that its action is mediated by intracellular second messengers; and (*iii*) serotonin applied to oocytes that have been injected with brain mRNA leads to a transient increase in $[Ca^{2+}]_i$.

METHODS

Injection of mRNA into *X. laevis* Oocytes. A sucrose density-gradient fraction of poly(A)$^+$ mRNA extracted from adult Wistar rat brain by the guanidinium/cesium chloride method (23) and oligo(dT)-cellulose chromatography (24) was kindly supplied by K. Nakayama and S. Nakanishi (Kyoto University). The fraction with sedimentation coefficient 20–26 S, corresponding to an estimated size of 2.4–4.0 kilobases, was used for injection. *X. laevis* oocytes were pressure-injected with 20 nl (≈50 ng) of poly(A)$^+$ mRNA and kept at 19°C in Barth's medium (14) containing gentamycin (50 μg/ml).

Current Measurements. Oocytes injected 3–5 days previously with mRNA were placed in a recording chamber (volume ≈ 0.4 ml) and continuously perfused with frog Ringer solution (see below). The membrane potential of the oocyte was voltage-clamped with two microelectrodes filled with 4 M potassium acetate. Current–voltage (*I–V*) relations were obtained by applying ramp-command voltages with slopes of ≈150 mV/sec. The current records were stored on magnetic tape and replayed to electronically subtract control current (i.e., current before serotonin application) from the *I–V* curves obtained during serotonin responses. Serotonin (0.1–4 μM) was applied to the bath by switching the perfusion line.

For patch-clamp experiments, oocytes were "skinned" of their follicular and vitelline membranes (14). Experiments were made at 20 ± 1°C in normal frog Ringer solution (115 mM NaCl/2.5 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes, pH 7.20) or in a modified extracellular solution where Ca^{2+} was replaced by 5 mM Mg^{2+}. Patch-clamp pipettes contained

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; InsP_3, inositol trisphosphate.

divalent-cation-free extracellular solution to which 10 mM EGTA was added. The membrane potential of oocytes during patch-clamp experiments was measured by an intracellular microelectrode connected to a standard electrometer (Fig. 2*a*). The potential across the membrane under the patch pipette (V_m) was determined from the intracellular potential (V_i) and the pipette holding potential (V_p). Serotonin was applied from a capillary tube filled with 1 μM serotonin and located with its opening close ($\approx$100 μm) to the oocyte and opposite to the area from which patch currents were recorded. Inside-out patches were isolated from native oocytes bathed in 115 mM KCl/10 mM EGTA.

Calcium Measurement by Fura-2 Fluorescence. $[Ca^{2+}]_i$ was measured by fura-2 fluorescence (15), using a dual-wavelength microphotometer as described (16, 17). Briefly, a Zeiss IM-35 inverted microscope equipped with epifluorescence optics, a Xenon lamp, and a photomultiplier was modified to allow alternating fluorescence excitation at 350 nm and 390 nM. Fluorescence was measured at 500 ± 20 nm through a pinhole diaphragm, which limited the region from which light was collected to a circular spot of 40 μm diameter (or, in some cases, 16 μm diameter). The calibration constants for the fura-2 measurement were determined by injecting oocytes with 50 nl each of 2.5 mM fura-2 solutions having one of the following calcium concentrations: (*i*) Ca^{2+}-free (75 mM EGTA), (*ii*) high Ca^{2+} (75 mM $CaCl_2$), (*iii*) 0.2 μM Ca^{2+} (75 mM EGTA + 50 mM $CaCl_2$). Values of fluorescence ratio were taken 10–15 min after injection, when fluorescence reached a more or less steady-state level at the measurement site. At the same time the fluorescence ratio went through a maximum (following $CaCl_2$ injection). After 10–15 min, $[Ca^{2+}]_i$ was back in the range 1–10 μM, which points towards very efficient calcium homeostasis in oocytes.

We used a ×40, 0.65-n.a. objective and placed oocytes, with their animal pole directed upwards, to fill the whole field of view. Both absolute fluorescence intensities and fluorescence ratios depended on the focal position of the microscope. When moving the focal plane from below the chamber bottom upwards, fluorescence intensity went through a maximum. In both calibration and test measurements, we adjusted the focus at a position slightly below the maximum, so that fluorescence intensity was $\approx$80% of the maximum. This position coincided within $\approx$10 μm with the bottom of the chamber.

For experiments, oocytes were injected with 50 nl of 100 mM KCl containing fura-2 at a concentration of 10 mM, 2.5 mM, or 1 mM. Assuming that fura-2 is uniformly distributed inside a spherical oocyte having a diameter of 1.25 mm, the final fura-2 concentration was estimated to be 0.5 mM, 0.12 mM, or 0.05 mM, respectively.

RESULTS

Activation of Chloride Channels by Serotonin in Brain-mRNA-Injected Oocytes. Bath application of micromolar concentrations of serotonin to *X. laevis* oocytes previously injected with rat brain poly(A)$^+$ mRNA caused large (μA) whole-cell current responses (Fig. 1*a*, lower trace). Serotonin responses in native, uninjected oocytes were rarely observed (3 out of 60 oocytes) and when present were of minimal amplitude (20–100 nA), suggesting that most of the serotonin effect following mRNA injection is due to newly expressed and inserted receptors (see also refs. 6 and 7). The serotonin response was most prominently observed as outward current when the membrane potential was clamped at positive potentials. The current response started with a variable delay after serotonin application and showed characteristic fluctuations. For command voltage pulses of fixed amplitude (Fig. 1*a*, upper trace), the corresponding ohmic currents were markedly increased in amplitude during the response (Fig. 1*a*, lower trace), indicating an increased input conductance of the oocyte. Fig. 1*b* shows the whole-cell current–voltage (*I–V*) relation of the serotonin-activated current. It reversed in polarity at −24 ± 2 mV (mean ± SD, 15 oocytes), and the *I–V* curve was characterized by a marked rectification at negative potentials similar to that of the chloride current activated by intracellular calcium injection (18). The serotonin-activated current was also observed in calcium-free Ringer solution (10) with no significant change in reversal potential or shape of the *I–V* relation. Replacement of two-thirds of the external chloride with isethionate (2-hydroxyethanesulfonic acid) caused an $\approx$20-mV shift in the reversal potential toward more positive voltages, whereas replacement of half of the external NaCl with KCl did not measurably change the reversal potential. When two-thirds of the NaCl in the Ringer solution was replaced by sucrose, the reversal potential was essentially the same as that when two-thirds of the chloride was replaced with isethionate. These results suggest that chloride is the major ion carrying the serotonin-activated current.

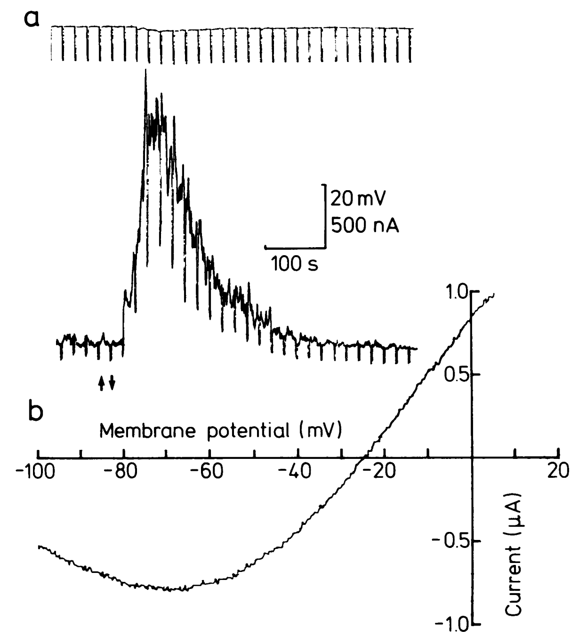

FIG. 1. Serotonin activation of whole-cell currents in *Xenopus* oocytes injected with rat brain poly(A)$^+$ mRNA. (*a*) Whole-cell current response (lower trace) to application of 1 μM serotonin (between arrows). The oocyte membrane potential was voltage-clamped to 0 mV. Hyperpolarizing command pulses were applied at 0.05 Hz (upper trace) to monitor the oocyte input conductance. (Normal frog Ringer bath solution.) (*b*) Current–voltage relation of serotonin-activated current as measured during rampwise shifts of command potential. The current before serotonin application was subtracted.

Serotonin Receptors Activate Chloride Channels via Intracellular Messengers. Following a delay of tens of seconds after application of serotonin, the intracellular membrane potential of unclamped oocytes depolarized from resting values between −60 mV and −80 mV to a steady level between −21 to −32 mV (−29 ± 6 mV, 7 oocytes). Coincident with the membrane depolarization, elementary currents were observed in cell-attached patch-clamp recordings (Fig. 2 *a* and *b*). The frequency of occurrence of these elementary currents slowly increased during continuous serotonin application and resulted in fluctuations of the current between different unitary levels. Later, bursts were observed where two or more channels were active for periods of several hundred milliseconds (Fig. 2*c*). During the intervals between bursts,

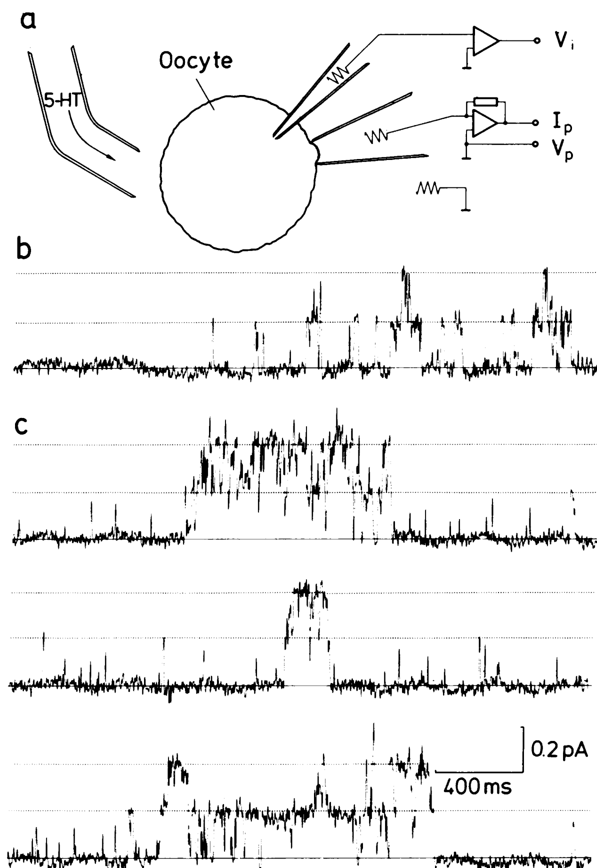

FIG. 2. Serotonin activation of single-channel currents in mRNA-injected oocyte. (*a*) Schematic representation of cell-attached patch current (I_p) recording. V_i is intracellular potential. The potential across the membrane patch under the patch pipette was clamped to the desired value by changing the pipette potential (V_p). Serotonin (5-HT, 1 μM) was applied to the oocyte from a capillary tubing placed opposite to the patch pipette. (*b*) Single-channel currents activated by serotonin (cell-attached patch). Bath solution was frog Ringer solution; patch pipette contained calcium-free extracellular solution. Intracellular potential was −29 mV. Membrane potential across the patch in the pipette was 61 mV. Current trace shows onset of the response ≈30 sec after the beginning of serotonin application and after the intracellular potential had depolarized from −58 mV to −29 mV. Low-pass filtering at 150 Hz [−3 decibels (dB), Bessel characteristic]. Upward deflection represents outward current. Depolarization of the membrane patch to 60 mV prior to serotonin application did not activate outward currents. (*c*) Three examples of single-channel current bursts, recorded between 40 sec and 2 min after the beginning of serotonin application. Same experiment as in *b*.

outward currents of similar amplitude but of much shorter duration were recorded.

Serotonin was applied to the oocyte membrane at a point opposite to the region where the patch pipette was placed (Fig. 2*a*). The tight seal between the glass and the membrane prevented access of applied serotonin to serotonin receptors within the membrane patch from which the current was recorded. This implies that the currents shown in Fig. 2 *b* and *c* were stimulated by intracellular messengers released upon occupation of serotonin receptors that were located in the oocyte membrane outside the tip of the pipette.

Conductance and Gating of Serotonin-Activated Channels. Fig. 3*a* shows examples of serotonin-activated currents at various membrane potentials. They are in the outward direction at 0 mV and increase in size with more positive membrane potentials. The single-channel *I–V* relation in the range between 0-mV and +60-mV membrane potential (Fig. 3*b*) indicates a slope conductance of ≈3 pS for the serotonin-activated channel and an extrapolated reversal potential of −29 mV. When sodium in the pipette solution was replaced by potassium, the slope of the *I–V* relation as well as the extrapolated reversal potential were not appreciably changed. These results suggest that serotonin-activated single-channel currents are carried predominantly by chloride, as observed for serotonin-activated whole-cell currents.

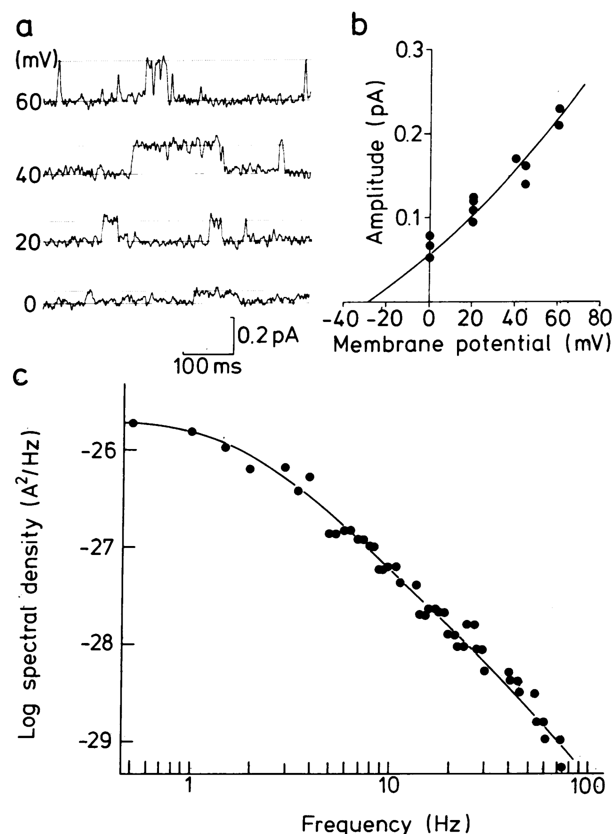

FIG. 3. Conductance and gating of serotonin-activated channels in mRNA-injected oocyte. (*a*) Single-channel currents recorded at various membrane potentials as indicated on each trace. Low-pass filtering at 150 Hz (−3 dB, Bessel). (*b*) Single-channel current–voltage relation of serotonin-activated currents (pooled data from 5 different oocytes). The continuous curve represents an eye-fit by the constant-field equation assuming selective chloride permeability as 0.8×10^{-14} cm^3·sec^{-1}, derived from the result of inside-out patch experiments (Fig. 5). External chloride concentration was 115 mM. Slope conductance at 50 mV membrane potential was 3.2 pS. (*c*) Power-density spectrum of serotonin-activated current. The continuous line represents a fit by a single Lorentzian component with corner frequency 1.4 Hz. Current records were filtered at 200 Hz (−3 dB) and sampled at 2-msec intervals. The difference spectrum is based on the average of 24 individual spectra of 1024 points each.

The power-density spectrum of a current recording from a patch where several channels were activated (Fig. 3*c*) could be fitted by a single Lorentzian component. The average duration of the serotonin-activated elementary currents determined from such power-density spectra was 116 ± 68 msec (n = 7). In 4 out of 14 patches an additional class of currents with larger amplitude and shorter mean duration was observed. The *I–V* relation of these currents indicated that serotonin may activate an additional class of channels of 22-pS conductance and −3-mV reversal potential.

Endogenous Oocyte Channels Activated by Intracellular Calcium in Native Oocytes. Comparison of the *I–V* relations of serotonin-activated currents in mRNA injected oocytes and those elicited by intracellular calcium injection in native oocytes (18, 19) suggested that the serotonin-activated channels are similar to these endogenous oocyte channels. In two

of six recordings from native oocytes, spontaneously occurring outward currents were observed that had a size and an extrapolated reversal potential similar to those observed in mRNA-injected oocytes stimulated with serotonin. However, the currents had a much shorter average duration (10 msec and 14 msec at 40 mV, two recordings) and occurred at a very low frequency (<1 sec^{-1}).

The activation of endogenous chloride channels in native oocytes by calcium acting on the cytoplasmic membrane face was also observed in current recordings made from inside-out patches. The top trace in Fig. 4 shows a record from a patch that was initially exposed on its cytoplasmic side to a bathing solution containing <10 nM Ca^{2+}. Subsequently, the calcium concentration on the cytoplasmic face was raised to about 10 μM, causing the appearance of outward currents (second and third trace). When the calcium concentration was decreased to <10 nM, the outward currents disappeared again (bottom trace). The conductance of the calcium-activated channels was ≈3 pS (Fig. 5 *a* and *b*), and the average duration of the elementary currents as determined from power-density spectra (Fig. 5*c*) were 73 msec and 98 msec in two patches. Both conductance and average lifetime agree well with the values of serotonin-activated channels in mRNA-injected oocytes and are comparable to those reported for calcium-activated chloride channels in exocrine gland cells (20).

Stimulation by Serotonin Increases $[Ca^{2+}]_i$. The experiments described above showed that calcium-dependent chloride channels are present in both native and mRNA-injected oocytes and are activated by serotonin only in mRNA-injected oocytes. This suggests that newly inserted serotonin receptors cause an increase in $[Ca^{2+}]_i$, a hypothesis that was tested by directly measuring $[Ca^{2+}]_i$ during serotonin application.

In four oocytes injected with 1 mM fura-2 (final concentration ≈ 50 μM), the "resting" free $[Ca^{2+}]_i$ (measured while the oocyte was incubated in normal Ringer solution) was 0.17 ± 0.06 μM. In an oocyte injected with 2.5 mM fura-2, the $[Ca^{2+}]_i$ was 0.08 μM; in one injected with 10 mM fura-2, it was 0.06 μM. The lower $[Ca^{2+}]_i$ values at higher fura-2 concentrations probably reflect the chelating effect of fura-2.

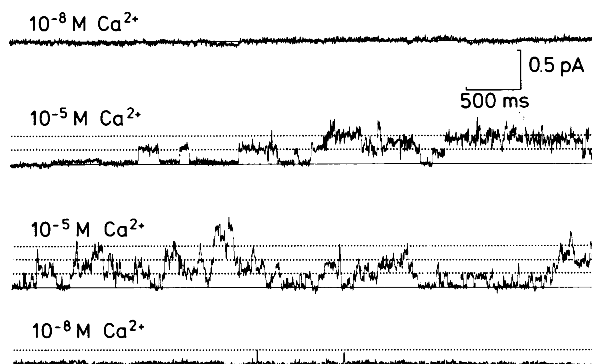

FIG. 4. Calcium activation of single-channel currents in native oocyte membrane. Elementary currents were recorded from an inside-out patch at 60-mV transmembrane potential. The pipette contained calcium-free extracellular solution. Initially the bath solution, facing the cytoplasmic side of the inside-out patch, contained a low concentration of calcium (115 mM KCl/10 mM EGTA/10 mM Hepes, pH 7.2) (top trace). Then, the calcium concentration on the cytoplasmic side was raised by changing to a solution that contained calcium (115 mM KCl/10 μM $CaCl_2$/10 mM Hepes, pH 7.2). Second trace shows onset of response to increased calcium. Third trace shows current after equilibration. Finally, the bath solution was changed back to the initial low-calcium solution (bottom trace). Low-pass filtering at 150 Hz (−3 dB, Bessel). Outward current is represented by upward deflection.

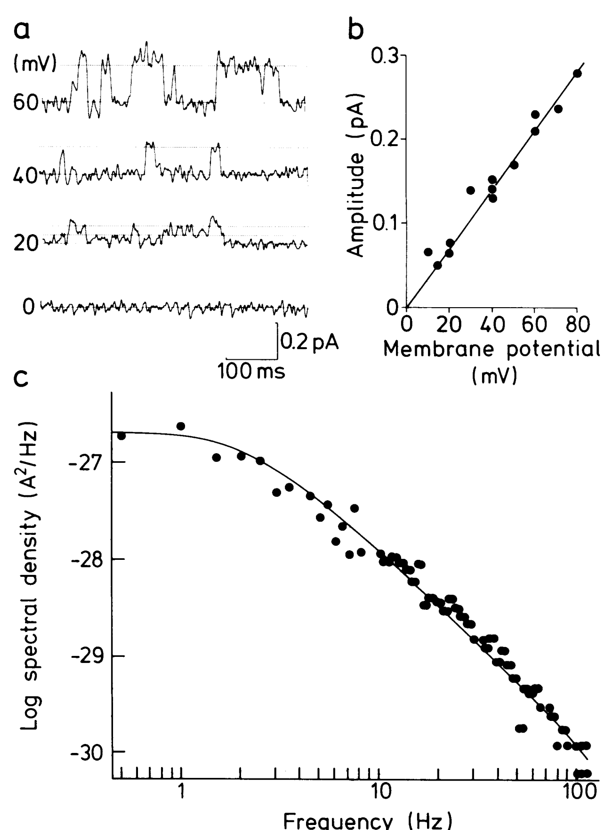

FIG. 5. Conductance and gating of calcium-activated channels in inside-out patches from native oocytes. The pipette contained calcium-free extracellular solution. Bath solution was KCl/Hepes solution with 10 μM Ca^{2+} as in Fig. 4 (second and third trace). (*a*) Single-channel currents recorded at various membrane potentials as indicated for each trace. Low-pass filtering at 150 Hz (−3 dB, Bessel). (*b*) Single-channel current–voltage relation; pooled data from three patches. Linear regression yielded a single-channel conductance of 3.6 pS. Constant-field assumption of chloride-selective channel yields permeability of 0.8×10^{-14} $cm^3 \cdot sec^{-1}$. (*c*) Power-density spectrum of calcium-activated current. The continuous line represents a fit by a single Lorentzian component with corner frequency 2.3 Hz.

Upon addition of 5 μl of 100 μM serotonin solution to the bath (volume ≈ 1 ml), there was a transient increase in $[Ca^{2+}]_i$ after a variable delay. The delay was most likely due to diffusion of serotonin in the chamber, since in some cases, when serotonin was released exactly above the oocyte, the transient increase started within the time required for the addition. $[Ca^{2+}]_i$ typically rose within 5–20 sec to a peak in the range 1–2 μM (Fig. 6*a*). The noise of the $[Ca^{2+}]_i$ trace increased markedly during the transient. In seven measurements on oocytes injected with either 1 mM or 2.5 mM fura-2, the peak value was 1.24 ± 0.72 μM. The mean of the 1 mM fura-2 values (1.28 μM) was slightly, but not significantly, higher than that of the 2.5 mM values (1.16 μM). In contrast, two serotonin applications to an oocyte injected with 10 mM fura-2 gave peak $[Ca^{2+}]_i$ values of only 0.29 and 0.43 μM. Also, the rising phases of the transients were prolonged markedly in these cases (Fig. 6*b*), probably a consequence of the calcium-buffering action of fura-2 at high concentrations.

Two oocytes that had not been injected with brain mRNA but that otherwise were treated identically to the mRNA-injected oocytes showed either no calcium response to serotonin application or only a marginal elevation of $[Ca^{2+}]_i$ and a slight increase in noise (Fig. 6*c*).

The serotonin-induced $[Ca^{2+}]_i$ transients were not dependent on the presence of extracellular calcium. When Ringer

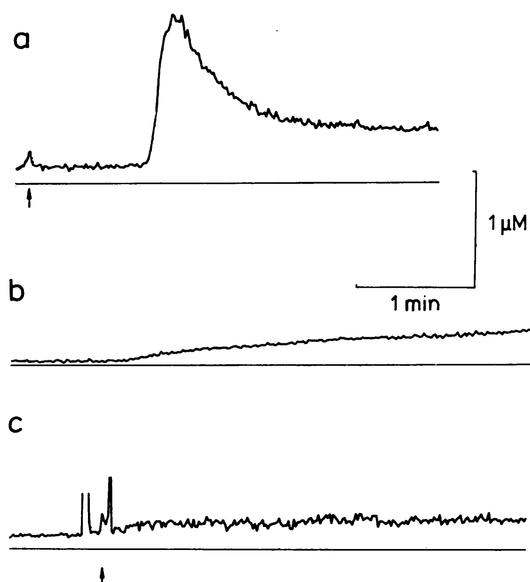

FIG. 6. Changes in $[Ca^{2+}]_i$ following addition of 5 μl of 100 μM serotonin to the bath (arrows). In *b*, the addition was shortly before the start of the record. Total bath volume was 500 μl. (*a*) mRNA-injected oocyte injected with 2.5 mM fura-2 in 100 mM KCl (≈120 μM final fura-2 concentration). (*b*) mRNA-injected oocyte injected with 10 mM fura-2 in 100 mM KCl. (*c*) Oocyte, not injected with mRNA, injected with 1 mM fura-2 in 100 mM KCl. The artifacts in parts *a* and *c* (truncated) are due to erratic background light during serotonin addition. Calibration bar represents $[Ca^{2+}]_i$ for *a*–*c*.

solution in the bath was replaced by Ca^{2+}-free saline with 5 mM Mg^{2+} a few minutes before serotonin application, peak $[Ca^{2+}]_i$ values (for 1 and 2.5 mM fura-2 injections) were 1.29 ± 0.84 μM (5 oocytes), which is indistinguishable from the value obtained in normal Ringer solution (1.24 μM, see above).

CONCLUSIONS

Our experiments show that activation of mammalian central nervous system serotonin receptors, expressed in the *Xenopus* oocyte plasma membrane, causes an ≈10-fold increase in $[Ca^{2+}]_i$, to 1–2 μM. This is presumably caused by the rise in the intracellular InsP_3 concentration being triggered by the serotonin–receptor interaction. It has been reported that in *Xenopus* oocytes, endogenous acetylcholine–receptor interaction can cause an increase in the intracellular InsP_3 content (12) and injection of InsP_3 caused release of calcium from internal stores (13, 21). The increased $[Ca^{2+}]_i$ acts as a second messenger and increases the opening probability of chloride-selective channels that are endogenous oocyte channels. They are comparable in their properties to chloride-selective channels in the rat lacrimal gland, in that they are activated by micromolar concentrations of cytoplasmic calcium, have a small conductance (≈3 pS), and a long open time (≈100 msec). The implanted serotonin receptor thus gates oocyte-specific channels so that the response to serotonin in the oocyte does not necessarily reflect the mode of action of serotonin when it acts on receptors that are in their native neuronal membrane. This behavior is different from that of implanted nicotinic receptors, which carry their own channels and whose response is similar in the oocyte and in their native muscle membrane (22). The diverse responses to serotonin in different vertebrate neurons may therefore be due in part to the serotonin-receptor-linked second-messenger system controlling different cell-specific ion channels.

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