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SINGLE 5-HT$_3$ RECEPTOR-GATED ION CHANNEL EVENTS RESOLVED IN N1E-115 MOUSE NEUROBLASTOMA CELLS

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In order to resolve single channel events underlying the 5-HT$_3$ receptor-gated ion current in N1E-115 neuroblastoma cells patch clamp experiments have been performed on excised outside-out membrane patches under optimized experimental conditions. When the driving force for Na^+ through the 5-HT$_3$ receptor-gated ion channel is enhanced by raising the external Na^+ concentration to 180 mM and by substituting internal K^+ with the relatively impermeable cation N-methyl-D-glucamine, a single conductance level is observed in the presence of 5-HT. Single channel activity is observed only in the presence of the agonist and is blocked by 50 nM of the selective 5-HT$_3$ receptor antagonist MDL 72222 in a reversible manner. At membrane potentials more negative than -60 mV discrete single channel events can be resolved with a conductance of 5.6 ± 1.2 pS.

Clonal neuroblastoma and hybrid cell lines express 5-HT$_3$ receptors. In whole-cell voltage clamped mouse neuroblastoma cells of the clone N1E-115, 5-HT causes a large transient inward current, which is blocked by nanomolar concentrations of the selective 5-HT$_3$ receptor antagonists tropisetron and MDL 72222 (1,2). The 5-HT$_3$ receptor-gated ion current is mediated by non-selective cation channels (3-5).

In excised outside-out patches of N1E-115 cells, 5-HT merely evokes a compound response, which cannot be resolved into discrete single channel events (3). From fluctuation analysis unitary conductances of 5-HT$_3$ receptor-gated ion channels have been estimated to be 310 fS in N1E-115 (3), 575 fS in N18 (4) and 4 pS in differentiated NG108-15 cells (6). Unitary events with a conductance of 12 pS have been detected in undifferentiated NG108-15 cells, but the nature of these ion channels has not been

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confirmed pharmacologically (6). Distinct unitary conductances of 9 - 19 pS reported for dissociated guinea pig submucous plexus, rabbit nodose ganglion and rat superior cervical ganglion neurons may reflect species differences in 5-HT_3 receptor-gated ion channels (7-10).

Since detailed knowledge is available from neuroblastoma cells on 5-HT_3 receptor pharmacology (11,12) and structure (13,14), the possibility to record single channel events would greatly facilitate the investigation of the basic mechanisms involved in the operation of 5-HT_3 receptor-gated ion channels. Here we describe optimized experimental conditions that allow to record discrete unitary events underlying the 5-HT_3 receptor-gated ion current in N1E-115 neuroblastoma cells.

MATERIALS AND METHODS

Cell culture. Mouse neuroblastoma cells of the clone N1E-115 (15) were grown under conditions identical to those described before (16). Differentiation of subcultures of passage numbers 32-42 was initiated on day 2 in subculture by the addition of 1 mM dibutyryl-cAMP and 1 mM 3-isobutyl-1-methylxanthine to the culture medium. This medium was refreshed about every other day. Cells were used for experiments on day 6-10 after subculture.

Patch clamp recordings. Experiments were performed on outside-out membrane patches (17) with an EPC-7 patch clamp circuit (List-Electronic, Darmstadt, Germany). Glass pipettes (Clark, GC150 borosilicate glass) with a resistance of 10-30 MΩ were used. The composition of the pipette and external solutions is listed in Table 1. Unless noted otherwise, patches were voltage clamped at -100 mV and signals were low-pass filtered at a corner frequency of 600 Hz using an 8-pole Bessel filter. Signals were digitized (8 bits; 1024 points per record) and stored on magnetic disc for off-line analysis. Experiments were performed at room temperature (20-24°C).

Table 1A. The ionic composition of the pipette solutions (in mM)

	NaCl	KCl	$MgCl_2$	HEPES	Na-HEPES	NMDG[a]	sucrose
P1	10	150	1	10	-	-	-
P2	-	-	-	-	20	100	120

Pipette solutions were buffered at pH=7.25.
[a]N-methyl-D-glucamine.

Table 1B. The ionic composition of the external solutions (in mM)

	NaCl	KCl	NH_4Cl	$CaCl_2$	$MgCl_2$	HEPES	Na-EDTA	glucose	sucrose
E1	125	5.5	-	1.8	0.8	20	-	24	36.5
E2	-	-	130	0.5[a]	0.8[a]	20	1	-	40
E3	180	-	-	1.8	0.8	10	-	-	-

External solutions were buffered at pH=7.3.
[a]Calculated free Ca^{2+} and Mg^{2+} concentrations amount to 2 μM and 300 μM, respectively.

Application of drugs. Membrane patches were continuously superfused with external solution and ion currents were evoked by changing to agonist- and/or antagonist-containing solution using a servomotor-operated valve (16). Patches were exposed to the agonist at intervals of at least 100 s in order to allow complete recovery from desensitization. Frozen stock solutions of 1 mM 5-hydroxytryptamine creatine sulphate (5-HT) and 20 μM 1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate (MDL 72222) were freshly thawn and diluted in external solution prior to the experiments.

RESULTS

The application of 10 μM 5-HT to excised outside-out membrane patches in external (E1) and pipette (P1) solution, which are similar to the solutions that have been used before to evoke large whole-cell 5-HT_3 receptor-gated ion currents, did evoke a compound response (Fig. 1A). These compound responses could be evoked repeatedly in the same patch. Figure 1B shows a compound 5-HT response recorded at high temporal resolution. No clear single channel events could be resolved, suggesting that the compound response is composed of multiple unitary events that cannot be resolved individually.

In order to augment the amplitude of the 5-HT_3 receptor-gated single channels external Na^+ and K^+ were substituted with highly permeable NH_4^+ (4) and external Ca^{2+} and Mg^{2+} were reduced (18). Figure 1C shows that under these conditions (solutions

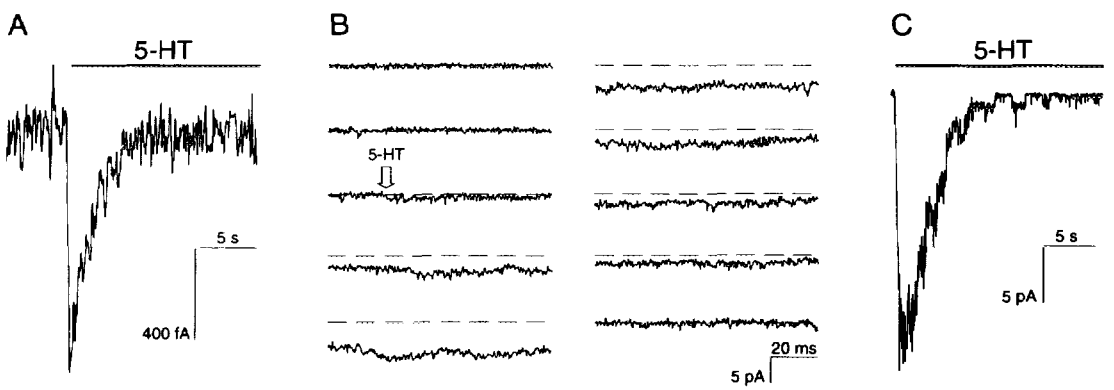

Figure 1. A. Compound response evoked by 10 μM 5-HT (solid bar) in an outside-out N1E-115 patch bathed in standard external (E1) and pipette (P1) solutions. No clear unitary events can be resolved. **B.** Compound response evoked by superfusion of 10 μM 5-HT, recorded at high temporal resolution. Depicted are 100 ms current traces recorded at regular intervals over a total period of 15 s. The approximate arrival of 5-HT at the membrane is indicated by the arrow. In the continued presence of 5-HT a transient inward current is evoked, but discrete single channel events cannot be distinguished. Dashed lines indicate the zero current level. Traces were low-pass filtered at a corner frequency of 1 kHz. **C.** Compound response evoked by 10 μM 5-HT (solid bar) in an outside-out patch in NH_4^+ external solution (E2) and standard pipette solution (P1). In the desensitized phase of the compound response, discrete single channel events are observed.

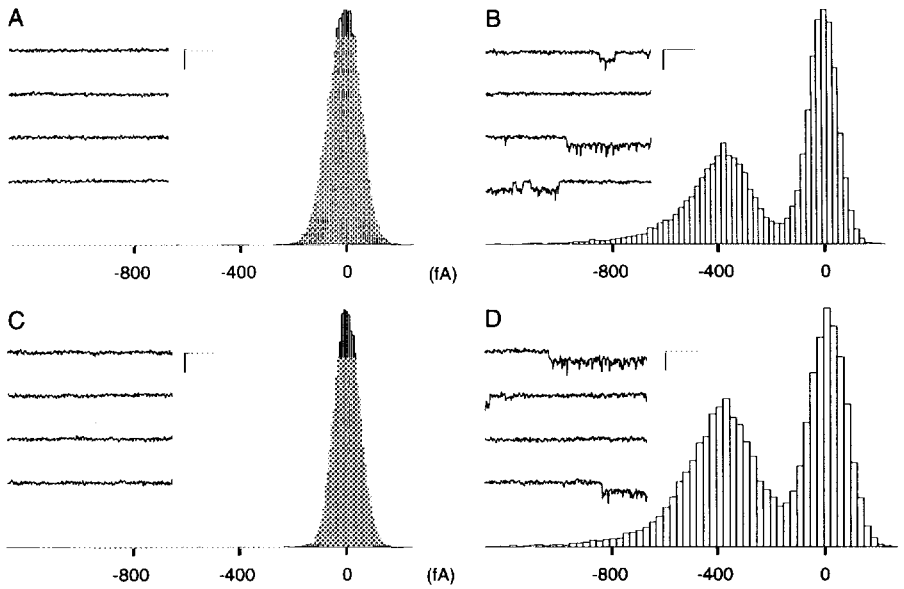

Figure 2. 5-HT_3 receptor-gated single channels in an outside-out N1E-115 patch in high Na^+ external (E3) and NMDG pipette (P2) solutions. **A.** Control traces show that in the absence of the agonist no single channel activity is present. The amplitude histogram shows a single Gaussian distribution centered around zero current. **B.** In the presence of 5 μM 5-HT discrete single channel openings are resolved. Apart from the peak at zero current, the amplitude histogram shows an additional peak at -380 fA. **C.** After washout of the agonist, the patch was superfused with 50 nM MDL 72222 for 4 min. Subsequent superfusion with 5 μM 5-HT in the continuous presence of the antagonist no longer evoked single channel activity. **D.** Reversal of the blocking effect of MDL 72222 after 20 min of superfusion with external solution (E3). Traces in A-D are from the same patch and recording started after approximately 10 s of superfusion with 5-HT. All histograms were obtained from a total recording period of 50 s. The histograms in B and D are constructed from traces with channel openings only. Similar results were obtained in two other patches. Scale bars: abscissa 100 ms, ordinate 800 fA.

E2/P1) discrete single channel events were detected during the desensitized phase of the compound response. However, in patches superfused with solution E2 single channel activity was also observed in the absence of agonist (not shown) and the reduced external Ca^{2+} and Mg^{2+} tended to destabilize the membrane patches.

As an alternative approach the driving force for Na^+ was enhanced by raising the external Na^+ concentration to 180 mM (solution E3) and by substituting K^+ in the pipette solution with N-methyl-D-glucamine ions (solution P2), which are relatively impermeable (4). Under these conditions channel activity was not observed in the absence of the agonist (Fig. 2A) and in the presence of 5 μM 5-HT discrete unitary events could be distinguished in the desensitized phase of the compound response (Fig. 2B). These single channels were blocked by 50 nM of the selective 5-HT_3 antagonist MDL 72222 (Fig. 2C) and block was gradually reversed within 20 min of washing with the external solution

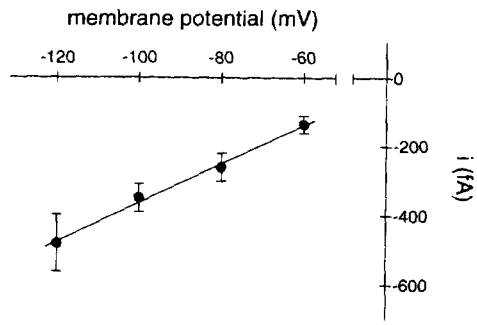

Figure 3. I-V relationship of the 5-HT_3 receptor-gated single channel events. Patches were voltage clamped at -120 mV, -100 mV, -80 mV and -60 mV in random order and the single channel amplitudes were determined from amplitude histograms. The mean values of the single amplitude (± SD bars) are depicted and the straight line drawn was fitted to the combined data obtained from 3 patches. The mean of the slope conductances, determined by linear regression of the data of the individual patches, amounts to 5.6 ± 1.2 pS (n=3).

(Fig. 2D). From the slope of the I-V relationship (Fig. 3) the unitary conductance of 5-HT_3 receptor-gated ion channels in the range of -120 mV to -60 mV was estimated to be 5.6 ± 1.2 pS (mean ± SD; n=3). Reversal of the single channel current was not observed, even when the membrane potential was changed to +80 mV (not shown).

DISCUSSION

The results demonstrate that, under conditions of enhanced driving force for external Na^+, discrete 5-HT_3 receptor-gated single channel events can be resolved in outside-out membrane patches of N1E-115 neuroblastoma cells. The observations that the single channel events exclusively occur in the presence of 5-HT (Fig. 2A,B) and are sensitive to block by a low concentration of the selective 5-HT_3 receptor antagonist MDL 72222 (Fig. 2C,D) unequivocally confirm the nature of the unitary events. The absence of discrete single channel events in physiological saline (Fig. 1A,B) confirms the previous conclusion that 5-HT_3 receptor-gated single channels are too small to be detected in N1E-115 cells under standard ionic conditions (3).

Initially, the use of NH_4^+ was preferred to augment the amplitude of unitary events. The amplitude of ion channels observed in NH_4^+ solution containing 5-HT was 0.8 - 2.4 pA at the holding potential of -100 mV (19). These values are much larger than the presently obtained value of 380 fA (Fig. 2B,D), consistent with the observation that the 5-HT_3 receptor-gated ion channel is much more permeable to NH_4^+ than to Na^+ (4). However, the presence of single channel activity in the absence of 5-HT complicates the conclusion with respect to the nature of the ion channels involved and the analysis of

results. Recently, NH_4Cl has been reported to induce inward current in whole-cell voltage clamped chick sensory neurons (20), indicating that NH_4^+ ions activate a separate membrane conductance.

From the slope of the I-V relationship the unitary conductance of the 5-HT_3 receptor-gated single channels is estimated to be 5.6 pS. Since the amplitude of the single channels at membrane potentials more positive than -60 mV was too small to be detected, it is impossible to determine the reversal potential. The data of Figure 3, together with the fact that outward currents were not observed at membrane potentials up to +80 mV indicate that substantial rectification occurs under the given experimental conditions. From fluctuation analysis the unitary conductance has been estimated to be 300 - 500 fS (3,4). The difference with the present value of 5.6 pS may be accounted for by the enhanced external cation concentration, the reduced permeability of internal cations and by the fact that in the fluctuation analysis possible rectification has been neglected.

The small value of the conductance of the 5-HT_3 receptor-gated ion channel in neuroblastoma cells is unexpected for a non-selective cation channel with an effective pore diameter of 0.76 nm, as estimated from ion substitution experiments in N18 cells (4). This is slightly larger than the pore size of 0.65 nm of endplate type nicotinic acetylcholine receptor-gated ion channels (21), which have a unitary conductance of 30 pS under normal ionic conditions (22). In order to explain this apparent discrepancy further patch clamp investigations are required.

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