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Ca^{2+} Permeability of Cloned and Native 5-Hydroxytryptamine Type 3 Receptors

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SUMMARY

We have used single-cell imaging of fura-2-loaded cells to examine the Ca^{2+} signals evoked by activation of 5-hydroxytryptamine type 3 (5-HT_3) receptors in undifferentiated N1E-115 neuroblastoma cells and in human embryonic kidney (HEK) 293 cells transfected with either of the two cloned 5-HT_3 receptor subunits. The selective 5-HT_3 receptor agonist 1-(*m*-chlorophenyl)-biguanide (mCPBG) caused a concentration-dependent increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in N1E-115 cells and in HEK 293 cells transfected with either the 5-HT_3 A subunit or the 5-HT_3 As subunit. In each case, the $[Ca^{2+}]_i$ rise was steeply dependent on the mCPBG concentration (n_H = 2–4) and abolished by removal of extracellular Ca^{2+} or addition of ondansetron. Pretreatment of N1E-115 cells with thapsigargin, caffeine, and ryanodine to deplete intracellular Ca^{2+} stores had no effect on the mCPBG-evoked Ca^{2+} signals, indicating that they result entirely from stimulated Ca^{2+} entry. The steep concentration-effect curves therefore are not a consequence of amplification of Ca^{2+} influx by Ca^{2+}-induced Ca^{2+} release from intracellular stores and probably reflect cooperative activation of 5-HT_3 receptors by mCPBG. Depolarization of transfected HEK 293 cells with medium containing increased K^+ concentrations invariably failed to evoke an increase in $[Ca^{2+}]_i$, confirming the absence of voltage-gated Ca^{2+} channels and indicating that the mCPBG-evoked rise in $[Ca^{2+}]_i$ results from Ca^{2+} permeation of 5-HT_3 receptors. However, in N1E-115 cells and transfected HEK 293 cells, both extracellular Na^+ and K^+ substantially inhibited the Ca^{2+} influx evoked by activation of 5-HT_3 receptors, possibly by inhibition of agonist binding or by competition with Ca^{2+} for permeation of the channel. We conclude that 5-HT_3 receptors are Ca^{2+} permeant, that the Ca^{2+} influx is sufficient to generate a significant rise in $[Ca^{2+}]_i$, and that, because the A and As subunits behave similarly, conflicting electrophysiological analyses of Ca^{2+} currents cannot be explained by differences between these two subunits.

The 5-HT_3 receptor shares many structural and functional features with other members of the family of ligand-gated ion channels (1). It has substantial sequence similarity with other family members (2) and its predicted membrane topology is similar; it is pentameric (3). It responds rapidly and cooperatively to its agonists by opening an ion channel that forms an intrinsic part of the receptor (4, 5), and it desensitizes during prolonged exposure to 5-HT (6–8). Further interest in 5-HT_3 receptors has been generated by the therapeutic potential of 5-HT_3 receptor antagonists as neuroleptic and anxiolytic agents (9) and most notably in the treatment of chemotherapy- or radiation-induced emesis (10).

Electrophysiological recordings from neuroblastoma cell lines and neurons have established that the 5-HT_3 receptor is a cation-selective channel with similar permeability to Na^+ and K^+, although its conductance differs between tissues (11). The 5-HT_3 receptor is substantially permeable to Ca^{2+} in both mouse N18 neuroblastoma cells (12) and rat superior cervical ganglion neurons (8), although the relative permeability of the latter is only half that of the neuroblastoma cells. This contrasts with mouse N1E-115 cells, where changes in either extracellular Ca^{2+} or Mg^{2+} concentrations do not affect the reversal potential of the response evoked by activation of 5-HT_3 receptors, suggesting that neither divalent cation significantly contributes to the current through the open channel (13). These electrophysiological analyses of voltage-clamped cells are useful means of establishing the ion selectivity and conductance of the 5-HT_3 receptor and they clearly suggest differences between the ion permeation properties and conductances of the receptors in different tissues, but they do not

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; HBM, HEPES-buffered medium; HEK, human embryonic kidney; HEK/5-HT_3A cells, human embryonic kidney 293 cells transfected with the A form of the 5-hydroxytryptamine type 3 receptor subunit; HEK/5-HT_3As cells, human embryonic kidney 293 cells transfected with the As form of the 5-hydroxytryptamine type 3 receptor subunit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[K^+]_o$, extracellular K^+ concentration; $[Na^+]_o$, extracellular Na^+ concentration; mCPBG, 1-(*m*-chlorophenyl)biguanide; NMDG, *N*-methyl-D-glucamine; CICR, Ca^{2+}-induced Ca^{2+} release; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

establish the extent to which activation of 5-HT_3 receptors evokes a physiologically significant increase in $[Ca^{2+}]_i$. The problem is exacerbated by the observation that extracellular Ca^{2+} both blocks 5-HT_3 receptors in several tissues and increases the rate of receptor desensitization (2, 13, 14). $[Ca^{2+}]_i$ can be more directly measured using fura-2 but, although a previous study of fura-2-loaded NG108 neuroblastoma/glioma cells demonstrated that activation of 5-HT_3 receptors evoked an increase in $[Ca^{2+}]_i$ (15), neither it nor a subsequent study (16) established whether the Ca^{2+} signal was a direct result of Ca^{2+} passing directly through the channel of the receptor or a consequence of the opening of voltage-gated Ca^{2+} channels.

The electrophysiological evidence in favor of 5-HT_3 receptor heterogeneity is supported by pharmacological studies, which suggest the existence of receptor subtypes, in different species, that differ in their affinities for antagonists (11, 17, 18) and in the efficacy of their interactions with mCPBG (19) and 2-methyl-5-HT (20).[1] Molecular cloning has so far identified only two forms of the 5-HT_3 receptor subunit, the 5-HT_3 receptor A subunit (2) and a splice variant, the 5-HT_3 receptor As subunit (21), that lacks six amino acids present in the large cytoplasmic loop of the 5-HT_3 receptor A subunit. These modest differences between the cloned receptor structures are not, however, sufficient to explain the functional and pharmacological differences between the 5-HT_3 receptors found in different species (20), suggesting that additional 5-HT_3 receptor subunits have yet to be identified.

Mouse N1E-115 neuroblastoma cells were the first cell line in which 5-HT_3 receptors were identified (22–24), and they have subsequently been extensively used for electrophysiological (6, 13), radioligand binding (25, 26), receptor purification (27), and cloning (21) studies. In the present study, we have used single-cell video-imaging of fura-2-loaded cells to examine the Ca^{2+} signals evoked by activation of 5-HT_3 receptors in undifferentiated N1E-115 cells and in HEK 293 cells transfected with either of the two 5-HT_3 receptor subunits. The extent to which the signals reflect Ca^{2+} permeation of the 5-HT_3 receptor, their modulation by extracellular Na^+ and K^+, and the mechanisms underlying the positively cooperative responses of 5-HT_3 receptors to their agonists were investigated.

Materials and Methods

Reagents. N1E-115 neuroblastoma cells and HEK 293 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). All cell culture reagents were obtained from GIBCO-BRL (Paisley, UK) except for fetal calf serum, which was from Advanced Protein Products (West Midlands, UK). Fura-2/acetoxymethyl ester and fura-2 pentapotassium salt were from Molecular Probes (Eugene, OR). Thapsigargin and ryanodine were from Calbiochem. mCPBG was from Cookson Chemicals (Southampton, UK). Ondansetron was a gift from Glaxo Group Research (Ware, UK). [^{3}H]Granisetron (61 Ci/mmol) was from DuPont. All other reagents, including 5-HT, were obtained from Sigma (Poole, UK).

HBM contained 115 mM NaCl, 10 mM KCl, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25 mM HEPES, and 15 mM glucose, pH 7.4 at 20°. Ca^{2+}-free HBM was supplemented with 2 mM EGTA and contained no added $CaCl_2$, and low-Na^+ HBM was prepared by replacement of the NaCl with 115 mM NMDG. Media containing increased K^+ concentrations were prepared by substitution of the appropriate K^+ concentrations either for NMDG in low-Na^+ HBM or for Na^+ in HBM; details are given in the Results section and in Figure legends.

Cell culture and transfection. N1E-115 neuroblastoma cells (passages 23–33) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 4 mM L-glutamine. Cells were maintained at 37° in a humidified atmosphere of 5% CO_2. Culture medium was changed 4 days after subculture and every 2 days thereafter. For measurements of $[Ca^{2+}]_i$, cells were plated into 35-mm Petri dishes containing a round coverslip (22-mm diameter, size 1.5). The cells were used within 2–4 days, when they were subconfluent and showed little neurite formation.

HEK 293 cells were grown either in 90-mm Petri dishes or on round glass coverslips (22-mm diameter, size 1.5), in a mixture of 50% F-12 medium/50% Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, in a humidified atmosphere of 7% CO_2. When the cells reached 50–80% confluence, they were transfected with the eukaryotic expression vector pRc/CMV (Invitrogen) containing the complete coding sequence for either the 5-HT_3 receptor A subunit (2) or the 5-HT_3 receptor As subunit from N1E-115 cells,[2] using the calcium phosphate precipitation method (28). The transfected cells were incubated for an additional 48 hr in an atmosphere of 3% CO_2 before being loaded with fura-2.

Measurement of $[Ca^{2+}]_i$. Cells were washed once with HBM and then incubated at room temperature (19–22°) for 30 min in the same medium supplemented with fura-2/acetoxymethyl ester (2 μM) and bovine serum albumin (1 mg/ml). After this initial loading, the cells were washed and incubated in HBM for at least an additional 30 min, to allow hydrolysis of the dye. From the decrease in cellular fluorescence (excitation wavelength, 360 nm) after treatment of cells either with saponin (50 μg/ml for 5 min) to selectively permeabilize the plasma membrane or with mCPBG (400 nM) in Na^+-free HBM containing Mn^{2+} instead of Ca^{2+} to allow Mn^{2+} to reach the cytosol via active 5-HT_3 receptors, >95% of the cellular fura-2 was estimated to be both cytosolic and hydrolyzed to the Ca^{2+}-sensitive fura-2 free acid.

Fura-2-loaded cells on a coverslip were placed in a perfusion chamber at room temperature (19–22°) on the stage of a Nikon Diaphot inverted epifluorescence microscope with 20× Fluor objective. Cells were excited with light from a 100-W xenon arc lamp, which was passed alternately through narrow-band interference filters of 340- and 380-nm wavelengths. The emitted light was passed through a 400-nm dichroic mirror and then a 480-nm barrier filter, and the images were collected with an extended ISIS-M CCD camera (Photonic Science). In most experiments, a 340/380-nm image pair was collected at 1.4-sec intervals, although this was extended to 2.1 sec for longer time courses. Digitized images were stored and processed using a Magiscan cell-imaging system, utilizing TARDIS software (Applied Imaging, Gateshead, UK). Background fluorescence was recorded at both excitation wavelengths from a cell-free area of the slide and was substracted from all images. Fluorescence ratios (340/380 nm) were formed by dividing adjacent pairs of images, and they were then converted to $[Ca^{2+}]_i$ by reference to a table created by measuring the 340/380-nm ratios of solutions of Ca^{2+} and EGTA containing 1 μM fura-2 pentapotassium salt (Ca^{2+} calibration buffer kit with 1 mM Mg^{2+}; Molecular Probes). The traces shown were produced by calculating the average $[Ca^{2+}]_i$ within the perimeter of individual cells.

Media were perfused over the slide chamber (volume, 1 ml) via a gravity-fed perfusion system (5–6 ml/min); from measurements using solutions of fura-2 pentapotasssium salt, media exchanged with a half-time of about 4 sec and exchange was essentially complete within 12 sec.

The fluorescence spectrum of 5-HT hydrochloride (Sigma or Research Biochemicals) overlaps with that of fura-2 and, although the 5-HT creatinine sulfate complex (Sigma) is less fluorescent, we have used the selective 5-HT_3 receptor agonist mCPBG, which is not fluorescent at the relevant wavelengths, to avoid potential problems.

[1] S. C. R. Lummis and M.-I. Sepúlveda, Splice variants of the mouse 5-hydroxytryptamine$_3$ receptor reveal different characteristics, submitted for publication.

[2] S. C. R. Lummis, unpublished observations.

Analysis of results. Concentration-response relationships were fitted by nonlinear least-squares regression analysis (using Kaleidagraph; Abelbeck Software) to a logistic equation, response = $E_{max}[A]^n/(EC_{50}^n + [A]^n)$, where E_{max} is the maximal response, [A] is the concentration of agonist, and n is the Hill coefficient (n_H elsewhere in the text).

In about 70% of recordings it was impossible to reliably fit a logistic equation to the concentration-response results from a single cell. To avoid any potential bias from selective sampling of only 30% of the population, the combined responses of a field of four to eight cells were used to establish the concentration-response relationships. There was no noticeable difference between the characteristics of the concentration-response relationships calculated in this way and those that could be calculated from individual cells (Figs. 2a, 5c, and 6b). Results are presented as the means ± standard errors of the parameters (EC_{50}, maximal response, and n_H) derived from at least three fields of cells on separate slides in independent experiments.

Results

The basal $[Ca^{2+}]_i$ in undifferentiated N1E-115 cells was 30 ± 2 nM (three experiments). Addition of a maximal concentration of mCPBG (400 nM) to N1E-115 cells caused $[Ca^{2+}]_i$ to increase to a maximum within 50 sec of addition, followed by a slower return to basal $[Ca^{2+}]_i$ over approximately 200 sec (Fig. 1a). The first application of mCPBG (400 nM) to naive cells increased $[Ca^{2+}]_i$ by 58 ± 3 nM (seven experiments),[3] but all subsequent applications of the same concentration of mCPBG caused significantly smaller (36 ± 4 nM), although very reproducible, increases in $[Ca^{2+}]_i$. By briefly (60 sec) pretreating cells with 400 nM mCPBG and then excluding that initial response from subsequent analysis, reproducible responses to both maximal and submaximal concentrations of mCPBG, irrespective of their order of addition, were obtained (Fig. 1b). Addition of mCPBG caused a concentration-dependent increase in $[Ca^{2+}]_i$ (EC_{50} = 131 ± 21 nM, n_H = 4.3 ± 0.3, three experiments) (Fig. 2a). The threshold for the response occurred at 100 nM mCPBG, and the maximal response, an increase in $[Ca^{2+}]_i$ of 36 ± 4 nM, occurred with 300–400 nM mCPBG. There was no significant difference between the fraction of cells that responded to a threshold concentration of mCPBG (100 nM) (84 ± 4%, three experiments) and the fraction that responded to a maximal concentration (400 nM) (92 ± 3%, three experiments) (Fig. 2a). These observations suggest that most cells are similarly sensitive to mCPBG. The response to mCPBG was completely inhibited by pretreatment of the cells for 5 min with a selective antagonist of 5-HT_3 receptors, ondansetron (50 nM) (Fig. 2b).

Pretreatment of cells for 2 min in Ca^{2+}-free medium completely inhibited the mCPBG-evoked rise in $[Ca^{2+}]_i$ (Fig. 3a). Similar treatment of cells maintained in culture for an additional 2 days (when they express bradykinin receptors) did not affect the mobilization of intracellular Ca^{2+} stores by bradykinin, suggesting that such brief exposure to Ca^{2+}-free medium did not significantly deplete intracellular Ca^{2+} stores (data not shown). Additional evidence that the $[Ca^{2+}]_i$ increase resulting from activation of 5-HT_3 receptors depends on Ca^{2+} influx is provided by the spatial organization of the Ca^{2+} signal. In response to activation of 5-HT_3 receptors, $[Ca^{2+}]_i$ increased initially at the perimeter of the cell, followed by a slower 'in-

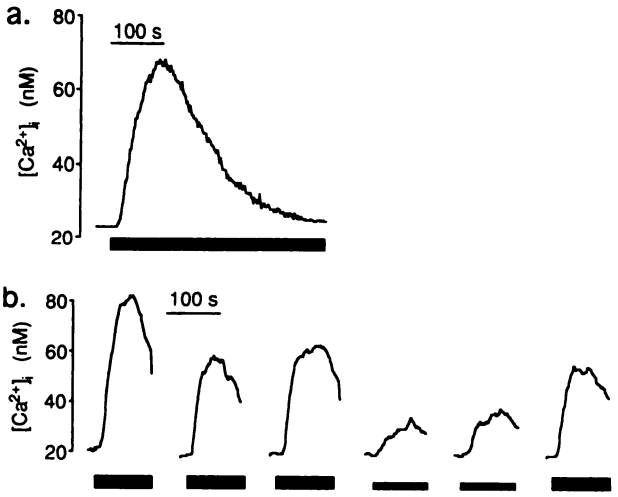

Fig. 1. Activation of 5-HT_3 receptors increases $[Ca^{2+}]_i$ in N1E-115 cells. Calibrated fluorescence recordings from single fura-2-loaded N1E-115 cells, each typical of at least 12 similar records, are shown. a, A maximal concentration of mCPBG (400 nM) was applied for the time indicated by the *solid bar*. b, The initial $[Ca^{2+}]_i$ rise in a naive N1E-115 cell in response to mCPBG was always larger than the response to subsequent challenges with the same concentration of mCPBG.[3] The panel shows this phenomenon and demonstrates that, after the first challenge, repeated and reproducible responses to either maximal (400 nM) (*wide bars*) or submaximal (150 nM) (*narrow bars*) concentrations of mCPBG were obtained. Each period of stimulation was separated by an interval of 7 min.

[3] When the same cells were used to examine the responses of naive cells and of cells that had first been challenged with mCPBG (400 nM), the cells were not significantly different ($p > 0.05$, Student t test) in their sensitivity to mCPBG. Half-maximal increases in $[Ca^{2+}]_i$ occurred with 136 ± 54 nM mCPBG in naive cells and with 210 ± 66 nM mCPBG in previously challenged cells (results from three independent fields, within each of which the responses from 4–8 cells were averaged).

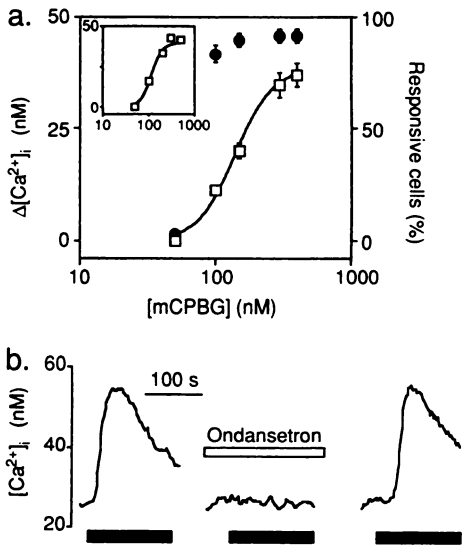

Fig. 2. mCPBG has concentration-dependent effects on $[Ca^{2+}]_i$ in N1E-115 cells. a, The concentration-dependent effects of mCPBG on the increase in $[Ca^{2+}]_i$ in N1E-115 cells are shown (□). Each value represents the mean ± standard error of three independent fields of cells, within each of which the responses from four to eight cells were averaged. *Inset*, typical concentration-effect relationship measured in a single cell. The proportion of cells (three experiments) responding to each concentration of mCPBG is also shown (●), indicating that N1E-115 cells are relatively uniform in their sensitivity to mCPBG. b, The response of a single N1E-115 cell to sequential applications of a maximal concentration of mCPBG (400 nM) (*solid bars*), in the absence or presence of ondansetron (50 nM) (*open bar*), is shown. Similar results were obtained in at least 12 cells from three independent fields.

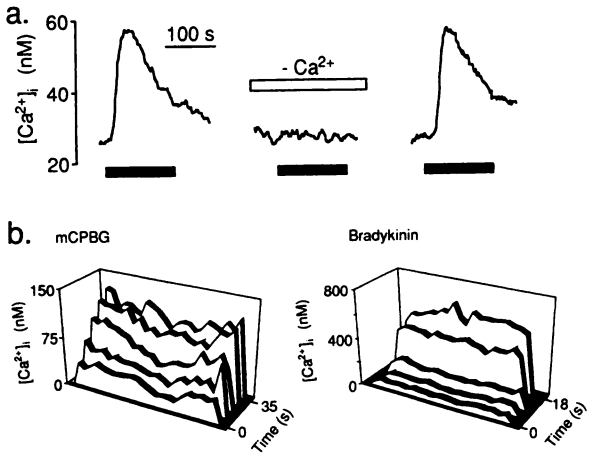

Fig. 3. Activation of 5-HT_3 receptors stimulates Ca^{2+} entry in N1E-115 cells. a, The response of a single N1E-115 cell to sequential applications of a maximal concentration of mCPBG (400 nM) (*solid bars*), in the presence or absence (*open bar*) of extracellular Ca^{2+}, is shown. Similar results were obtained in at least 12 cells from three independent fields. b, Each plot shows the change in $[Ca^{2+}]_i$ with time, along a *line* through the center of a single N1E-115 cell, after stimulation with bradykinin or mCPBG. mCPBG (400 nM) caused $[Ca^{2+}]_i$ to rise initially at the perimeter of the cell (*left*). Bradykinin (50 nM) caused a more uniform rise in $[Ca^{2+}]_i$ (*right*). The cells were maintained in culture for 5 days, to obtain responses to bradykinin and mCPBG in the same cell. Similar results were obtained from four cells.

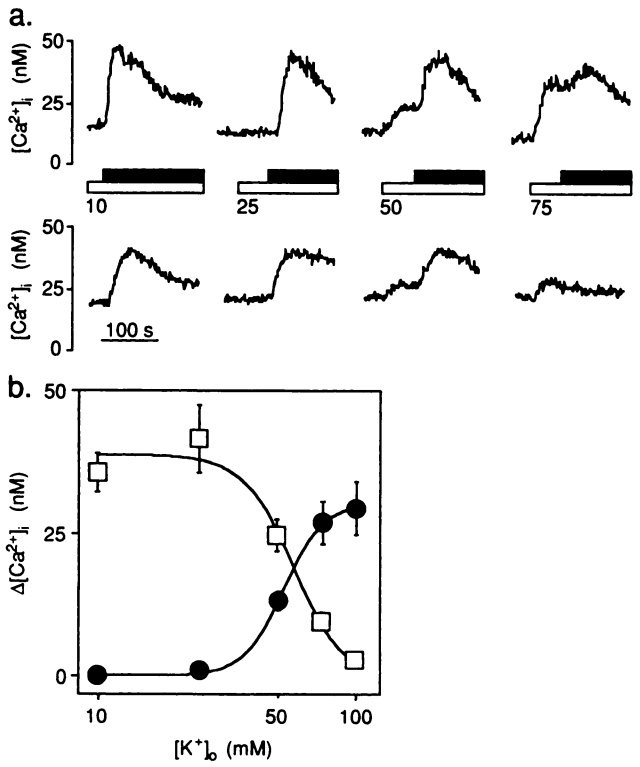

Fig. 4. Increased $[K^+]_o$ causes a rise in $[Ca^{2+}]_i$ and inhibits subsequent responses to mCPBG in N1E-115 cells. a, Traces from two cells show the effects of raising $[K^+]_o$ in Na^+-containing HBM on $[Ca^{2+}]_i$ and subsequent responses to 400 nM mCPBG. The responses of 22 cells from three independent fields of cells were measured and the traces shown represent the two extremes of the responses to raised $[K^+]_o$. Increased $[K^+]_o$ (*open bars*; *numbers below the bars*, $[K^+]_o$, in mM) caused a rise in $[Ca^{2+}]_i$ in all 22 cells tested, although the peak $[Ca^{2+}]_i$ varied between cells. *Upper traces*, 75 mM $[K^+]_o$ and mCPBG (400 nM) (*solid bars*) caused similar rises in $[Ca^{2+}]_i$; *lower traces*, the $[Ca^{2+}]_i$ rise induced by 75 mM $[K^+]_o$ was <50% of that evoked by mCPBG (400 nM). In all cells, raising $[K^+]_o$ inhibited the mCPBG-evoked rise in $[Ca^{2+}]_i$. b, The graph summarizes results from experiments similar to those shown in a. It shows effects of increasing $[K^+]_o$ in Na^+-containing HBM on the rise in $[Ca^{2+}]_i$ (●) and on the $[Ca^{2+}]_i$ increase evoked by subsequent exposure to mCPBG (400 nM) (□). Each value represents the mean ± standard error of three independent fields of cells, within each of which the responses from four to eight cells were averaged.

filling' of the cytoplasm (Fig. 3b); $[Ca^{2+}]_i$ then decreased rather uniformly back to basal levels over approximately 200 sec. This contrasts with the response evoked by bradykinin, which stimulates formation of inositol-1,4,5-trisphosphate and consequent mobilization of intracellular Ca^{2+} stores. Bradykinin caused a more uniform increase of $[Ca^{2+}]_i$ over the entire cell area (Fig. 3b).

We next attempted to establish whether the Ca^{2+} influx that followed activation of 5-HT_3 receptors in N1E-115 cells was a direct consequence of Ca^{2+} passing through the channel of the receptor or an indirect consequence of activation of voltage-gated Ca^{2+} channels. Both antagonists (nimodipine and verapamil, at 1–10 μM) and an agonist [(±)-Bay K 8644, at 1–10 μM] of L-type voltage-gated Ca^{2+} channels inhibited the $[Ca^{2+}]_i$ rise evoked by activation of 5-HT_3 receptors in N1E-115 and transfected HEK cells,[4] suggesting that these drugs, which have been reported to interact with other ligand-gated ion channels (29, 30), may also interact directly with 5-HT_3 receptors. Depolarization of N1E-115 cells by increasing $[K^+]_o$ from 10 to 75 mM, in otherwise normal HBM, caused a concentration-dependent increase in $[Ca^{2+}]_i$; the maximal increase (31 ± 1 nM, three experiments) occurred when $[K^+]_o$ was 75 mM (Fig. 4). In the same cells, a maximal concentration of mCPBG (400 nM) caused $[Ca^{2+}]_i$ to increase by 38 ± 2 nM (three experiments). However, these pooled results do not reflect the variability between cells; in three independent experiments, the maximal rise in $[Ca^{2+}]_i$ evoked by increases in $[K^+]_o$ was <50% of the maximal response to mCPBG in nine of the 22 cells examined (Fig. 4a). Irrespective of the magnitude of the $[Ca^{2+}]_i$ response to increased $[K^+]_o$, the increases in $[Ca^{2+}]_i$ evoked by mCPBG (400 nM) diminished as the $[K^+]_o$ increased (Fig. 4), and they were half-maximally inhibited when $[K^+]_o$ was approximately 50 mM. This inhibitory effect of $[K^+]_o$ could not be overcome by further increasing the concentration of mCPBG to 40 μM (data not shown).

The depolarization of the plasma membrane that follows activation of 5-HT_3 receptors is due mainly to an influx of Na^+. Replacement of extracellular Na^+ with the impermeant cation NMDG (12) therefore reduces the depolarization evoked by mCPBG and would prevent activation of 5-HT_3 receptors from opening voltage-sensitive Ca^{2+} channels. Complete replacement of extracellular Na^+ with NMDG greatly potentiated the increase in $[Ca^{2+}]_i$ evoked by mCPBG; in normal medium a maximal concentration of mCPBG (400 nM) caused $[Ca^{2+}]_i$ to increase by 36 ± 4 nM (three experiments), whereas in Na^+-free medium the same concentration of mCPBG caused a >10-fold greater increase in $[Ca^{2+}]_i$, to 412 ± 56 nM (three experiments) (Fig. 5, a and b). The inhibitory effect of extracellular Na^+ was concentration dependent; half-maximal inhibition occurred when $[Na^+]_o$ was 17 ± 3 mM, and 91 ± 1% of the response was inhibited when $[Na^+]_o$ was 75 mM (Fig. 5b). The substantial increase in the magnitude of the mCPBG-evoked $[Ca^{2+}]_i$ rise in Na^+-free medium was accompanied by a very

[4] A. C. Hargreaves, S. C. R. Lummis, and C. W. Taylor, unpublished observations.

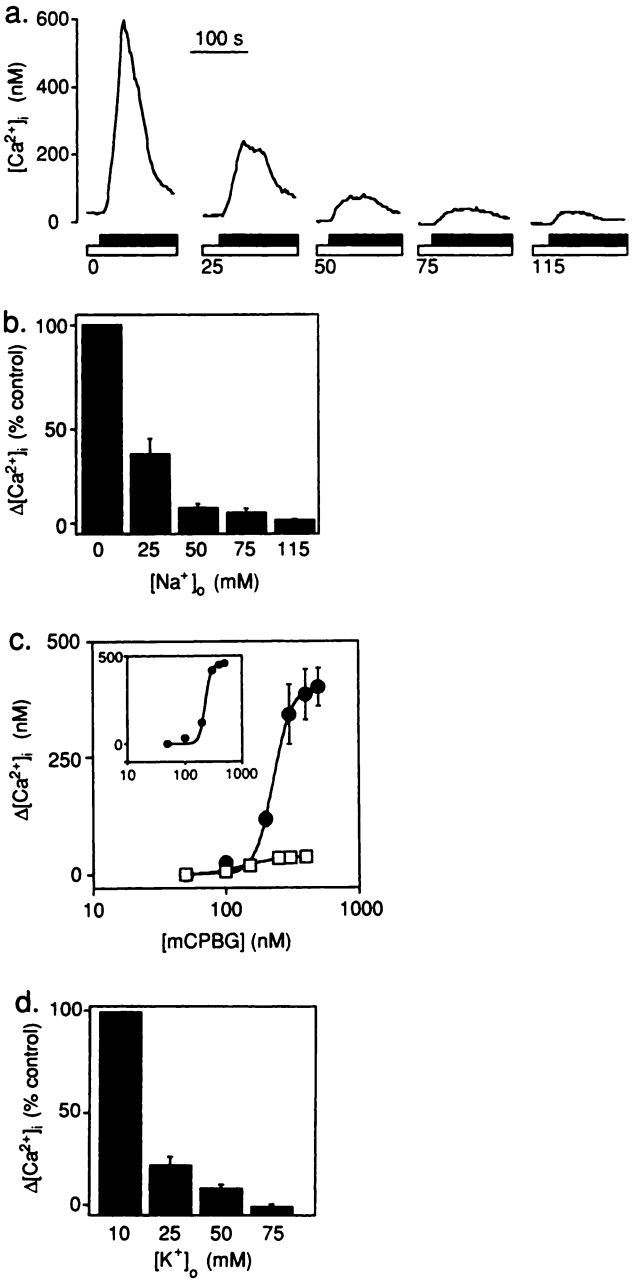

Fig. 5. Extracellular K^+ and Na^+ inhibit the mCPBG-evoked rise in $[Ca^{2+}]_i$ in N1E-115 cells. a, Traces show the response of a single cell stimulated with mCPBG (400 nM) (*solid bars*) in Na^+-free HBM in which NMDG was progressively replaced by the indicated concentrations of extracellular Na^+ (*open bars*; *numbers below the bars*, concentrations, in mM). The traces are representative of four to eight cells from each of three independent fields. b, Results from experiments similar to those shown in a are summarized, showing the inhibitory effects of increasing $[Na^+]_o$ on responses to mCPBG (400 nM). Each value represents the mean ± standard error of three independent fields of cells, within each of which the responses from four to eight cells were averaged. c, The concentration-dependent effects of mCPBG on the increase in $[Ca^{2+}]_i$ in N1E-115 cells in the presence (115 mM) (□) and absence of extracellular Na^+ (●) are shown. Results are shown as mean ± standard error of three independent fields of cells, within each of which the responses from four to eight cells were averaged. *Inset*, typical concentration-effect relationship in a single cell, measured in the absence of extracellular Na^+. d, The effects of increasing $[K^+]_o$ in Na^+-free HBM on the increase in $[Ca^{2+}]_i$ evoked by mCPBG (400 nM) are shown. Each value is the mean ± standard error of three independent fields of cells, within each of which the responses from four to eight cells were averaged.

modest decrease in the sensitivity of the cells to mCPBG; half-maximal effects (EC_{50}) occurred when the mCPBG concentration was 264 ± 34 nM (three experiments) in Na^+-free medium, compared with 131 ± 4 nM (three experiments) in normal medium (Fig. 5c). The enhanced response to mCPBG in Na^+-free medium is unlikely to reflect inhibition of Na^+/Ca^{2+} exchange across the plasma membrane and consequent prolongation of the Ca^{2+} signals, because the increase in $[Ca^{2+}]_i$ evoked by bradykinin (50 nM) was similar in normal medium (448 ± 37 nM, three experiments) and in Na^+-free medium (501 ± 70 nM, three experiments).

In similar experiments but with K^+ replacing the NMDG in Na^+-free medium, the response to mCPBG (400 nM) was attenuated as $[K^+]_o$ increased. Half-maximal inhibition of the mCPBG-evoked $[Ca^{2+}]_i$ rise occurred when $[K^+]_o$ was 13 ± 1 mM, and the response was inhibited by 95 ± 1% when $[K^+]_o$ was 75 mM (Fig. 5d). Both extracellular Na^+ and K^+ therefore inhibit the $[Ca^{2+}]_i$ signals evoked by activation of 5-HT_3 receptors; the slightly greater inhibitory effect of K^+ (95% versus 91% with Na^+) probably reflects a decrease in the electrochemical gradient for Ca^{2+} entry after K^+-evoked depolarization.

HEK 293 cells express neither 5-HT_3 receptors nor voltage-sensitive Ca^{2+} channels (31) (see below); they therefore provide an opportunity to examine the behavior of transfected 5-HT_3 receptors in the absence of any secondary effects from activation of voltage-sensitive Ca^{2+} channels. The basal $[Ca^{2+}]_i$ values were similar in untransfected HEK 293 cells (50 ± 5 nM, nine cells), HEK/5-HT_3A cells (67 ± 3 nM, 14 cells), and HEK/5-HT_3As cells (95 ± 15 nM, 13 cells), and neither untransfected HEK 293 cells nor cells transfected with the vector pRc/CMV responded to mCPBG (≤10 μM). Application of mCPBG to either HEK/5-HT_3A or HEK/5-HT_3As cells caused an increase in $[Ca^{2+}]_i$, the kinetics of which were similar to those of the response seen in N1E-115 cells (Fig. 6a). In Na^+-free medium, the EC_{50} values for the mCPBG-evoked $[Ca^{2+}]_i$ rise in N1E-115 cells (264 ± 34 nM, n_H = 3.3 ± 0.6, three experiments), HEK/5-HT_3A cells (203 ± 24 nM, n_H = 4.1 ± 0.2, three experiments), and HEK/5-HT_3As cells (214 ± 16 nM, n_H = 4.7 ± 0.8, three experiments) were not significantly different (Fig. 6b). The maximal increase in $[Ca^{2+}]_i$ was, however, greater in transfected cells (562 ± 169 nM and 966 ± 129 nM for HEK/5-HT_3A and HEK/5-HT_3As cells, respectively; three experiments) than in N1E-115 cells (412 ± 56 nM, three experiments) (Fig. 6b; Table 1). However, whereas most (92 ± 3%) N1E-115 cells responded to a maximal concentration of mCPBG (400 nM), only 42 ± 8% of HEK/5-HT_3A cells and 36 ± 6% of HEK/5-HT_3As cells were responsive. In HEK/5-HT_3As cells, as in N1E-115 cells, the $[Ca^{2+}]_i$ rise evoked by a maximal concentration of mCPBG (400 nM) was substantially attenuated (by 82 ± 4%, 11 experiments) in medium containing Na^+ (115 mM) (Fig. 7b). In all cells, the response to mCBPG was abolished by removal of extracellular Ca^{2+} or addition of ondansetron (50 nM) (data not shown).

In saturation binding experiments (27) using the selective 5-HT_3 receptor antagonist [^{3}H]granisetron, the maximal numbers of binding sites (B_{max}) and their affinities (K_d) for [^{3}H]granisetron were similar in membranes prepared from N1E-115 cells (B_{max} = 1.22 ± 0.26 pmol/mg of protein, K_d = 0.59 ± 0.20 nM, three experiments), HEK/5-HT_3A cells (B_{max} = 0.62 ± 0.19 pmol/mg of protein, K_d = 0.37 ± 0.14 nM, three experiments),

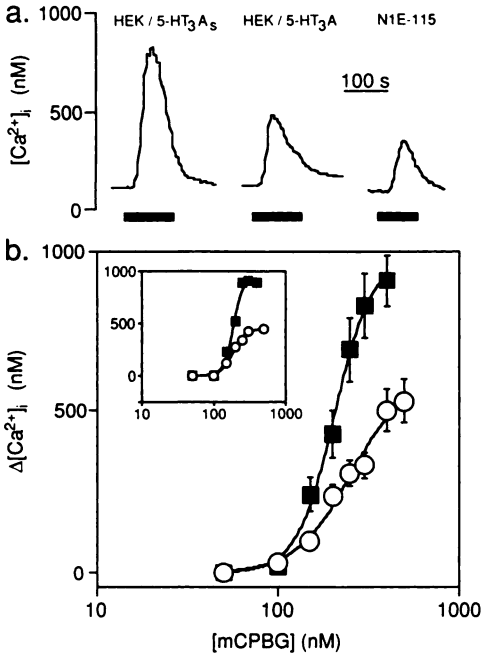

Fig. 6. mCPBG stimulates an increase in $[Ca^{2+}]_i$ in HEK/5-HT_3A and HEK/5-HT_3As cells. a, Traces show the increase in $[Ca^{2+}]_i$ in single HEK/5-HT_3A, HEK/5-HT_3As, and N1E-115 cells stimulated with mCPBG (400 nM) (*solid bars*) in Na^+-free HBM. Results are representative of three to six cells from each of three independent fields. b, The concentration-dependent effects of mCPBG on the increase in $[Ca^{2+}]_i$ in HEK/5-HT_3A (○) and HEK/5-HT_3As cells (■) are shown. Each value is the mean ± standard error of three independent fields of cells, within each of which the responses from three to six cells were averaged. *Inset*, typical concentration-effect relationships measured in two single cells.

TABLE 1

Summary of the effects of mCPBG on $[Ca^{2+}]_i$ in N1E-115, HEK/5-HT_3A, and HEK/5-HT_3As cells

In all cases, $[K^+]_o$ was 10 mM. Each value is the mean ± standard error of three independent fields of cells, within each of which the responses from three to eight cells were averaged.

	EC_{50} for mCBPG	Maximal $[Ca^{2+}]_i$ increase	n_H
	nM	nM	
$[Na^+]_o$ = 115 mM			
N1E-115 cells	131± 21	36± 4	4.3 ± 0.3
$[Na^+]_o$ = 0 mM			
N1E-115 cells	264 ± 34	412 ± 56	3.3 ± 0.6
HEK/5-HT_3A cells	203 ± 24	562 ± 169	4.1 ± 0.2
HEK/5-HT_3As cells	214 ± 16	966 ± 129	4.7 ± 0.8

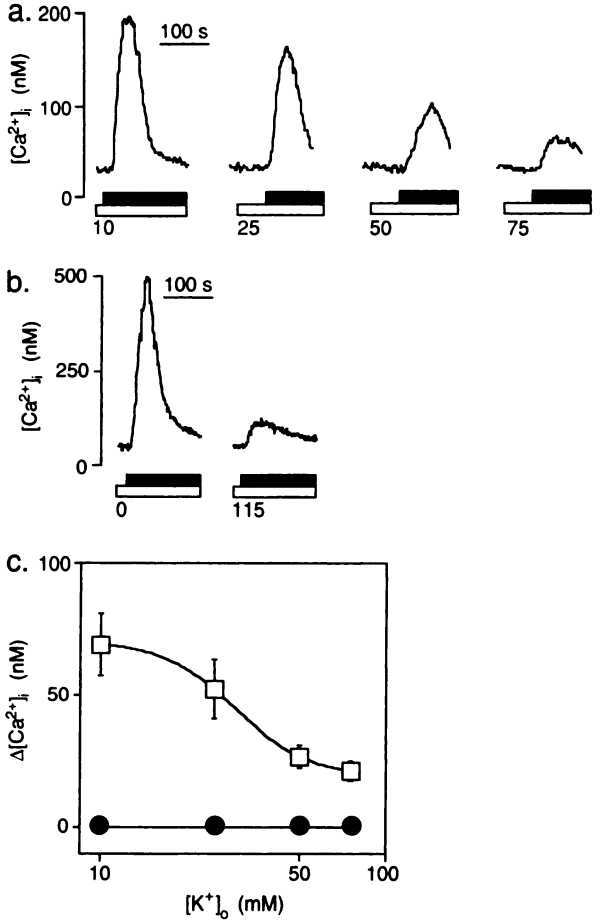

Fig. 7. Extracellular K^+ and Na^+ directly inhibit mCPBG-evoked increases in $[Ca^{2+}]_i$ in HEK/5-HT_3As cells. a, Traces from a single HEK/5-HT_3As cell incubated in Na^+-containing HBM in the presence of increasing $[K^+]_o$ (*open bars*; *numbers below the bars*, $[K^+]_o$, in mM) and subsequently stimulated with mCPBG (400 nM) (*solid bars*) are shown. In 12 cells from three independent fields, increased $[K^+]_o$ never evoked an increase in $[Ca^{2+}]_i$ but consistently inhibited the response to mCPBG. b, Traces from a single HEK/5-HT_3As cell stimulated with a maximal concentration of mCPBG (400 nM) (*solid bars*) in HBM in which Na^+ was replaced by NMDG ($[Na^+]$ = 0) (*left*) or in normal HBM ($[Na^+]$ = 115 mM) (*right*) (*open bars*) are shown. Similar results from 11 cells from three independent fields established that the response to mCPBG was decreased by 82 ± 4% in Na^+-containing medium. c, The effects of increasing $[K^+]_o$ by replacement of $[Na^+]_o$ in HBM on $[Ca^{2+}]_i$ (●) and the rise in $[Ca^{2+}]_i$ evoked by stimulation with mCPBG (400 nM) (□) are shown. Results are means ± standard errors from 12 cells from three independent fields.

or HEK/5-HT_3As cells (B_{max} = 0.84 ± 0.32 pmol/mg of protein, K_d = 0.29 ± 0.10 nM, three experiments).

In three experiments with 13 cells, treatment of HEK/5-HT_3As cells with medium containing increased $[K^+]_o$ (≤75 mM) never evoked a detectable increase in $[Ca^{2+}]_i$ (Fig. 7, a and c), confirming previous results suggesting that HEK 293 cells lack voltage-activated Ca^{2+} channels (31). However, increasing $[K^+]_o$ (10–75 mM) caused a concentration-dependent inhibition of the response to a normally maximal concentration of mCPBG (400 nM). The inhibitory effect of $[K^+]_o$ was half-maximal when $[K^+]_o$ was about 30 mM, and the response was inhibited by 81 ± 3% when $[K^+]_o$ was 75 mM (Fig. 7c). These results demonstrate that, even in the absence of any possible effects on voltage-operated Ca^{2+} channels, increased $[K^+]_o$ inhibits mCPBG-evoked $[Ca^{2+}]_i$ signals.

The $[Ca^{2+}]_i$ signals resulting from activation of either native or cloned 5-HT_3 receptors were steeply dependent on the concentration of mCPBG (Table 1) (n_H = 3.2–4.8). Such a steep concentration-effect relationship could result from cooperative activation of the 5-HT_3 receptor by mCPBG or may reflect amplification of a small Ca^{2+} influx by CICR from intracellular stores, mediated by either inositol trisphosphate (32) or ryanodine receptors (33). Addition of caffeine (10 mM), an activator of most forms of the ryanodine receptor (34), to N1E-115 cells consistently failed to evoke a detectable rise in $[Ca^{2+}]_i$, either alone or in combination with ryanodine. Furthermore, pretreatment (30 min) of N1E-115 cells with HBM containing caffeine (10 mM) and ryanodine (10 μM), to activate ryanodine receptors, and thapsigargin (10 μM), to deplete intracellular stores of Ca^{2+} by inhibiting the Ca^{2+}-ATPases (35), had no significant effect on the concentration-response relationship for mCPBG (EC_{50} = 179 ± 42 nM mCPBG, n_H = 3.4 ± 0.5, E_{max} = 43 ± 12

nM Ca^{2+}, three experiments) (Fig. 8). These results indicate that cooperative responses to mCBPG occur in the absence of functional intracellular Ca^{2+} stores.

Discussion

Activation of 5-HT_3 receptors in N1E-115 cells evokes an increase in $[Ca^{2+}]_i$ (Figs. 1 and 2) that results entirely from Ca^{2+} influx across the plasma membrane, because the response is abolished by removal of extracellular Ca^{2+} (Fig. 3) and is unaffected by prior depletion of intracellular Ca^{2+} stores (Fig. 8). This Ca^{2+} signal could result from Ca^{2+} passing either through the channel of the 5-HT_3 receptor or through voltage-gated Ca^{2+} channels activated by the depolarization that accompanies the flux of monovalent cations through active 5-HT_3 receptors. Because previous studies have not established a consistent pattern for the Ca^{2+} permeability of 5-HT_3 receptors (see the introduction), we have examined the effects on $[Ca^{2+}]_i$ of activation of native and cloned 5-HT_3 receptors under conditions that prevent activation of voltage-gated Ca^{2+} channels.

The most straightforward means of depolarizing the plasma membrane and thereby preventing activation of 5-HT_3 receptors from causing further depolarization and consequent activation of voltage-gated Ca^{2+} channels is to incubate cells in medium containing increased $[K^+]_o$. Increased $[K^+]_o$ caused an increase in $[Ca^{2+}]_i$ in N1E-115 cells and a decrease in the magnitude of the rise in $[Ca^{2+}]_i$ evoked by subsequent application of mCPBG (Fig. 4). However, this inhibition was not due solely to activation of voltage-gated Ca^{2+} channels, because there was no clear correlation between the amplitude of the K^+-evoked $[Ca^{2+}]_i$ rise and the extent of inhibition of the subsequent response to mCPBG (Fig. 4a). Furthermore, in at least 40% of cells the amplitudes of the $[Ca^{2+}]_i$ rises evoked by $[K^+]_o$ levels that almost totally inhibited the response to mCPBG were substantially less than the maximal responses to mCPBG in normal medium. These results, together with evidence that the inhibitory effects of K^+ are mimicked by Na^+ (Fig. 5, a and b) and that they also occur in cells lacking voltage-gated Ca^{2+} channels (see below), indicate that increased $[K^+]_o$ affects 5-HT_3 receptors independently of its ability to activate voltage-gated Ca^{2+} channels.

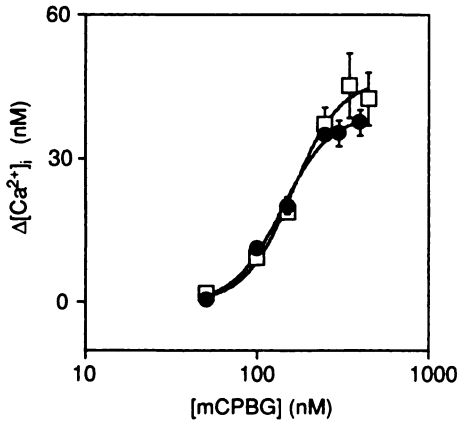

Fig. 8. Intracellular Ca^{2+} stores do not contribute to mCPBG-evoked $[Ca^{2+}]_i$ rise in N1E-115 cells. The concentration-dependent effects of mCPBG on the increase in $[Ca^{2+}]_i$ in N1E-115 cells after pretreatment with caffeine (10 mM), ryanodine (10 μM), and thapsigargin (10 μM) for 30 min (□) or no pretreatment (●) are shown. Results are means ± standard errors of three independent fields, within each of which the responses from four to eight cells were averaged.

Transfection of HEK 293 cells with cloned 5-HT_3 receptor subunits provides an opportunity both to examine the effects of receptor activation on $[Ca^{2+}]_i$ in the absence of any contribution from voltage-gated Ca^{2+} channels and to independently assess the behavior of the two known subtypes of 5-HT_3 receptors. Transfection with either the long (HEK/5-HT_3A) or short (HEK/5-HT_3As) form of the 5-HT_3 receptor led to expression of receptors that both bound [^{3}H]granisetron with appropriate affinity and responded to mCPBG with an increase in $[Ca^{2+}]_i$ (Fig. 6; Table 1). Although the transfected cells and N1E-115 cells expressed similar densities of [^{3}H]granisetron binding sites (about 1 pmol/mg of protein), most N1E-115 cells (92 ± 3%) responded to mCPBG with an increase in $[Ca^{2+}]_i$, whereas only about 40% of transfected cells were responsive. This heterogeneity, which is probably an inevitable feature of transient expression systems, highlights the utility of single-cell Ca^{2+} imaging.

The responses of HEK/5-HT_3A and HEK/5-HT_3As cells to mCPBG were indistinguishable; both responded with an increase in $[Ca^{2+}]_i$ that depended on Ca^{2+} entry, and the two were similarly sensitive to mCPBG (EC_{50} values of 203 ± 24 nM and 214 ± 16 nM, respectively) (Fig. 6b). Because depolarization of HEK 293 cells by increased $[K^+]_o$ failed to evoke an increase in $[Ca^{2+}]_i$ (Fig. 7), confirming earlier reports that these cells lack voltage-gated Ca^{2+} channels (31), we conclude that homomeric forms of the long and short subunits of the 5-HT_3 receptor are similarly Ca^{2+} permeant and that their activation leads to sufficient Ca^{2+} entry to generate a significant increase in $[Ca^{2+}]_i$.

The inhibitory effect of extracellular K^+ on the mCPBG-evoked Ca^{2+} signals in HEK/5-HT_3As cells (Fig. 7) suggests that a similar direct inhibitory effect of elevated $[K^+]_o$ probably occurs in N1E-115 cells and would explain the poor correlation between K^+-evoked $[Ca^{2+}]_i$ increases and the inhibition of subsequent responses to mCPBG (Fig. 4). The Ca^{2+} signals evoked by activation of the native 5-HT_3 receptors of N1E-115 cells or the expressed homopentamer of short subunits were almost totally inhibited when $[K^+]_o$ was 75 mM, and they were half-maximally inhibited when the $[K^+]_o$ of Na^+-free HBM was 30–50 mM (Figs. 5d and 7c).

We have not further investigated the mechanisms underlying the inhibitory effects of extracellular K^+ and the similar effects of Na^+ (Figs. 5, a-c, and 7b). They may result from inhibition of mCPBG binding or they may reflect competition between Ca^{2+} and monovalent ions for passage through the open channel. Because in the absence of extracellular Na^+, modest (low millimolar) (Fig. 5d) increases in $[K^+]_o$ substantially inhibit responses to mCPBG, we considered the possibility that the increases in $[K^+]_o$ that can occur in the local environment of repetitively firing neurons (36) might be sufficient to modulate the behavior of 5-HT_3 receptors. The effects of changes in $[K^+]_o$ were therefore examined by progressive replacement of extracellular Na^+, rather than by addition of K^+ to medium otherwise lacking permeant cations, to better reproduce the *in vivo* situation. Under these conditions, increases in $[K^+]_o$ inhibited the Ca^{2+} signals evoked by mCPBG (Fig. 4), but the inhibition occurred at $[K^+]_o$ values (IC_{50} of about 50 mM) far in excess of those likely to occur physiologically. We conclude that, although extracellular Na^+ and K^+ substantially inhibit the Ca^{2+} entry evoked by activation of 5-HT_3 receptors, the inhibition is likely to be almost maximal (i.e., about 90%

inhibition) under physiological conditions and is unlikely to be further modulated by physiological changes in monovalent cation concentration.

In either the presence or the absence of extracellular Na^+, the concentration-effect relationships for mCPBG-evoked Ca^{2+} signals were steep (n_H = 2–4) in both N1E-115 cells and HEK 293 cells expressing the long or short form of the 5-HT_3 receptor (Figs. 2a and 6b). Such steep concentration-effect relationships could reflect cooperative activation of the 5-HT_3 receptor resulting from multiple binding steps, amplification of the Ca^{2+} influx by CICR once the influx exceeds a critical threshold (33), or our failure to detect the small Ca^{2+} influxes evoked by the lowest concentrations of mCPBG. The latter explanation is unlikely, because the magnitudes of the Ca^{2+} signals evoked by mCPBG varied by >10-fold in Na^+-containing and Na^+-free media (Fig. 5c) and by >2-fold between N1E-115 and HEK/5-HT_3As cells, yet under each condition the concentration-effect relationships for mCPBG-evoked Ca^{2+} signals were steep. Nor is CICR likely to contribute to the concentration-effect relationship, because prior treatment of N1E-115 cells with caffeine and ryanodine to activate ryanodine receptors and thapsigargin to prevent Ca^{2+} loading of intracellular stores had no effect on the subsequent response to mCPBG and, most notably, it had no significant effect on the Hill coefficient describing the concentration-effect relationship (n_H of 4.3 ± 0.3 in control cells and 3.3 ± 0.6 in cells with depleted intracellular Ca^{2+} stores) (Fig. 8). Our results with cultured cells do not preclude the possibility that in neurons that do express CICR, mediated by either ryanodine or inositol-1,4,5-trisphosphate receptors, amplification of the Ca^{2+} influx may still further increase the cooperativity of the response to activation of 5-HT_3 receptors.

We conclude that 5-HT_3 receptors are Ca^{2+} permeant and that the Ca^{2+} influx is sufficient to generate a significant rise in $[Ca^{2+}]_i$; this Ca^{2+} signal may be substantially greater in synapses enriched in 5-HT_3 receptors and in the cytosol immediately beneath the plasma membrane than is evident from measurements averaged over entire cells (37). Furthermore, because the A and As subunits of the 5-HT_3 receptor behave similarly when transfected into HEK 293 cells, we conclude that homo-oligomeric complexes of either subunit form Ca^{2+}-permeant channels. The conflicting results from electrophysiological analyses of Ca^{2+} currents through 5-HT_3 receptors cannot be explained by differences between these two subunits, suggesting that additional subunits that have not yet been structurally identified probably contribute to the diversity of 5-HT_3 receptors.

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