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Primary Structure and Functional Expression of a Novel Non-selective Cation Channel

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A non-selective cation channel is believed to play important roles in varous tissues. A novel complementary DNA encoding non-selective cation channel was isolated from MIN6, a mouse insulin secreting β-cell line. This channel (mNSC1) conducts predominantly monovalent cations in *Xenopus* oocytes and is selective for cations over anions (P_K/P_{Cl} = 10). The current was completely blocked by lanthanum and niflumate. The mNSC1 of 423 amino acids contains a characteristic leucine repeat and unique membrane topology. The messenger RNA of this channel are abundant in the brain, heart, and lung. We may therefore conclude that studies with this channel will provide important information for understanding physiological functions of the excitable cells as well as non-excitable epi[illegible].

Key Words: **cloning; cDNA.**

The advent of patch clamp techniques h[illegible] revealed the frequent occurrence of chann[illegible] types virtu[illegible]y unpredictable from earlier works on m[illegible]oscopic properties of membrane ion conducta[illegible]n-selective cation (NS) channel is such a surprising [illegible]mpl[illegible] ubiquitously found by patch clamp e[illegible]ri[illegible]ents. [illegible] channels have been widely studied i[illegible] vario[illegible] excitable and non-excitable tissues, although [illegible]e relati[illegible]e incidence is quite different from one to the [illegible]er, and physiological roles of NS channels are variably [illegible]eculated (1).

Three types of NS channels are frequently observed (2–8). The first type is activated by intracellular Ca^{2+}, the second one is activated by hydrostatic pressure or stretch, and the third one is unaffected by either Ca^{2+} or hydrostatic pressure. NS channels are quite selective for cations over anions, but do not discriminate appreciably among different monovalent cations, especially Na^+ and K^+ ions in the physiological milieu. NS channels in general exhibit linear current-voltage (I-V) relations under the most physiological conditions, although they are nonlinear under certain conditions. The single channel conductance of these channels range about 10-100 p[illegible]ith th[illegible] common range being 20-40 pS, when[illegible]ey a[illegible] deter[illegible]ined in symmetrical K^+ solutions at [illegible]y[illegible]logic[illegible] ionic strength. NS channels often h[illegible]e lon[illegible] open[illegible]me with a complicated kinetics which [illegible]nnot be [illegible]d to a simple first-order process. Th[illegible]efo[illegible] NS channels, being distinct molecules, have quite d[illegible]rent electrical properties from other c[illegible]ion channels.

To [illegible]cidate t[illegible]e physiological functions and biophysical p[illegible]perties [illegible] non-selective cation channels at the molecu[illegible]level, we have cloned a cDNA encoding the [illegible]ouse non[illegible]selective cation channel (mNSC1). The electrop[illegible]logical properties of mNSC1 channel has [illegible]a[illegible]cteristics of the non-selective cation channel, w[illegible]ch dominantly distributed in the brain, heart and lu[illegible]

EXPERIMENTAL PROCEDURES

Cloning of mNSC1. To identify a source of mRNA suitable to clone a K permeable channel, we isolated poly $(A)^+$ RNA from various tissues including the brain, the heart, and the kidney, and the cell lines, including cultured fetal cardiac myocytes, cultured aortic smooth muscle cells, mesangial cells, MIN6 (insulin-secreting β-cell lines from mouse (9)), SV40 transformed renal proximal tubule cell line, SV40 transformed renal collecting duct cell line, and OK cells (opposum kidney cells). During expression of the current with 50 - 100 ng of mRNA injected *Xenopus* oocytes, a fraction from MIN6 cells (10) revealed K and probably Na permeable currents was found. The library from the mRNA fraction was constructed by a λZapII cDNA construction kit (Stratagene). Before further expression study, the resulting plaques (10^4/pfu) were transferred to a nitrocellulose membrane (Hibond, Pharmacia) and hybridized (50 °C) to a probe coding for R-repeat (GGNATHMGNGTNATHMGNYT) (11). Positive or near positive plaques were again hybridized using dot blot methods. Positive and near positive colonies were isolated, and individual mRNA was transcribed from ApaI-digested DNA using a methylation capping analogue and T3 polymerase (12) to express their function.

Both strands of the mNSC1 cDNA were sequenced using a sequencer (373-S, Applied Bio Instrument). The initiation codon of ATG is contained within a consensus sequence, AGCAUG (13).

Functional expression of mNSC1 in Xenopus oocytes. Mature females of *Xenopus* laevis were purchased from Hamamatsu Animal Co. Ltd. (Shizuoka, Japan). *Xenopus* oocytes (stage V), were collected from the ovary of frogs anaesthetized with 0.1% solution of ethyl-m-aminobenzoate in water.

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After injection of mRNA, oocytes were incubated in a modified Barth solution (10mM KCl, 3 mM $MgCl_2$, 5 mM HEPES, 80 mM NaCl, pH 7.8) at 19 °C, and electrophysiological studies were undertaken 2-4 days later. Oocyte were exposed to collagenase (2mg/ml; Sigma Type I) in the modified Barth solution for 1.5 - 2 h and then defolliculated manually before the electrical measurements. Two-electrode voltage-clamp experiments were carried out with a commercially available amplifier (Nihon Koden CEZ-1250, SET-1201) with microelectrodes which, when filled with 3 M KCl, had resistance of 2-3 MΩ. Oocyte were voltage-clamped at 0 mV and voltage-steps of 1.0 s duration were applied to cells from 10 to −100 mV in 10 mV decrements every 5 s. The experimental chamber (2 ml volume) was perfused continuously with gravity flow at a rate of 2 ml/min. Various bath solutions were connected to the chamber through a multi-channel unit. In each batch of oocyte injected with cRNA, control oocyte injected with RNase-free water and subsequently voltage-clamped to ensure that there were no endogenous ionic currents. If significant endogenous current was seen, then all the oocyte in the batch were discarded. Bath solution contained 10mM KCl, 3 mM $MgCl_2$, 5 mM HEPES and 80 mM NaCl (pH 7.4).

If K-selective channel were expressed, the resting membrane potential (Em) was shifted to hyperporalized. While, when the current from the fraction was expressed, the Em was close to zero from −25 ± 4.2 mV to −3 ± 5.2 (n = 6) with MIN6 mRNA. When the Em was near 0, we changed the bath solution to 90 mM KCl and 5 mM HEPES (pH 7.4). The current was increased suggesting K conductance. We, therefore, decided it as a successful expression of a K/Na permeable cation channel. After isolation of mNSC1 cDNA, some processes were changed to measure current more carefully. To diminish leak current, defolliculation was performed before 2 days of the electrophysiological experiments. Amplifier was changed to Dagan CA-1 and computer system (Axon. ver5) to subtract leak current. If the leak was over 20 nA after subtraction, the electrodes and oocyte were discarded. The stimulation and data storage were controlled by a computer (Compaq Prolinear 40/5) and analyzed with Axon software (ver. 5.52). Bath solution to 90 mM KCl and 5 mM HEPES and then changed to 90 mM K gluconate and 5 mM HEPES. [illegible] current was predominantly expressed, the inward current was decreased by this procedure. In this case, the batch was discarded. When mNSC1 channel was successfully expressed, the currents were usually and apparently not changed by this procedure. Following this checking, the solution was changed as described in the results.

Reagents, niflumic acids, amiloride, nifedipine, dibutylic cAMP, phorbor ester and $GdCl_3$ were dissolved in DMSO or water as appropriate and stored at −20 °C before use. While these reagents were dissolved in bath solutions, $LaCl_3$ was directory added to the chamber to give a final concentration of 1 mM. $LaCl_3$ completely blocked mNSC1 current as well as basal current in water injected control. The addition of $LaCl_3$ was then usually performed in each experiment to check the leak current. If the leak was higher than the pre-experimental currents, the data was not used for analysis.

Northern blots. mRNA was isolated by using the guanidine thiocyanate method with organic extraction. An aliquot of 10 μg of mRNA in each lane was transferred to a nitrocellulose membrane. A 2486 bp EchoRI fragment of mNSC1 was labeled with ^{32}P. Hybridization was done as described (11).

RESULTS

Electrophysiology of mNSC1 currents. A single clone (mNSC1) isolated from MIN6 library, carrying a cDNA of 2.8 kb, gave rise to the current non-specific for cations (Fig.1.a). Since oocytes may express their own non-selective cation channels (8), the following expression studies were carried out with some changes described in the methods. Non-selective cation currents induced by mNSC1 were usually observed in two or three days after the injection of mRNA, but not in one or four days after the injection, suggesting that the expression by exogenous mRNA is transient. Averaged conductance at the voltage of −100 to 10 mV of the expressed was 7.1 ± 1.4 nS/oocyte, the value being significantly high ($p<0.01$) compared to their control 2.9 ± 0.9 nS/oocyte (n = 12).

The current-voltage relation was almost linear between 0 to −80 mV. Relative permeabilities for several cations were examined by the serially exchanging the bathing solution (Fig. 1.b). The mNSC1 channel is permeable to monovalent cations. The sequence of the conductance was $K^+ > Rb^+ > NH_4^+ > Na^+ > Cs^+ >$ tetraethylammonium. N-methyl-D-glucamine was impermeant through this channel. Substitution of Cl with glutamate in KCl bath solution indicated low anion permeability of the mNSC1 channel. The single channel experiments gave P_{Cl}/P_K to be about 0.1 as calculated by Nernst equation, suggesting that mNSC1 channel conducts cations specifically for cations: During inside-out patches with 90 mM KCl in pipette, the bath Cl was substituted to glutamate, where Cl/glutamate (mM/mM) concentration was varied as 90/0, 45/45 and 30/60 without changing symmetrical 90 mM K^+ concentration. The reversal potential was varied as 3 ± 1.2, −7.2 ± 3.2 to −9.6 ± 2.6 mV, respectively (n = 4), suggesting P_{Cl}/P_K as 0.1. Blockade of the currents were tested by using $LaCl_3$, $GdCl_3$, nifedipine (10^{-5} M), amiloride (10^{-5} M) and niflumate (100 μM) (14). $LaCl_3$ and $GdCl_3$ at 1 mM and niflumate blocked the mNSC-induced K^+ currents (Fig.1.c). Addition of dibutylic cAMP (10^{-5} M) for activation of protein kinase A and phorbor ester (10^{-8} M) for protein kinase C failed to stimulate the mNSC1 current in *Xenopus* oocytes (n = 3, data not shown).

To test the permeability to divalent cations, the bathing solution was changed from KCl to $CaCl_2$. The currents were reduced by 90% and undistinguished from those observed in water injected control cells. To investigate the voltage-dependency of this current, the effect of prepulse (−100 - 100 mV step 10) was inserted to the following holding potential (−50 mV). The currents endowed by −50 mV were not affected by the prepulse, suggesting that the mNSC1 induced current was not dependent on the voltage at least in physiologic range.

Primary structure of mNSC1 channel. The cDNA of mNSC1 encodes 2785 nucleotides with poly A tail. The nucleotide sequence of open reading frame is sought to BLAST program and any significant homology was found. The deduced mNSC1 cDNA amino-acid sequence is shown in Fig.2. Homology of the amino acids was sought but there were no homologous alignments to the previous sequence. A hydrophobicity analysis revealed the presence of four probable membrane spanning hydrophobic segments (M1-M4), though the third might not

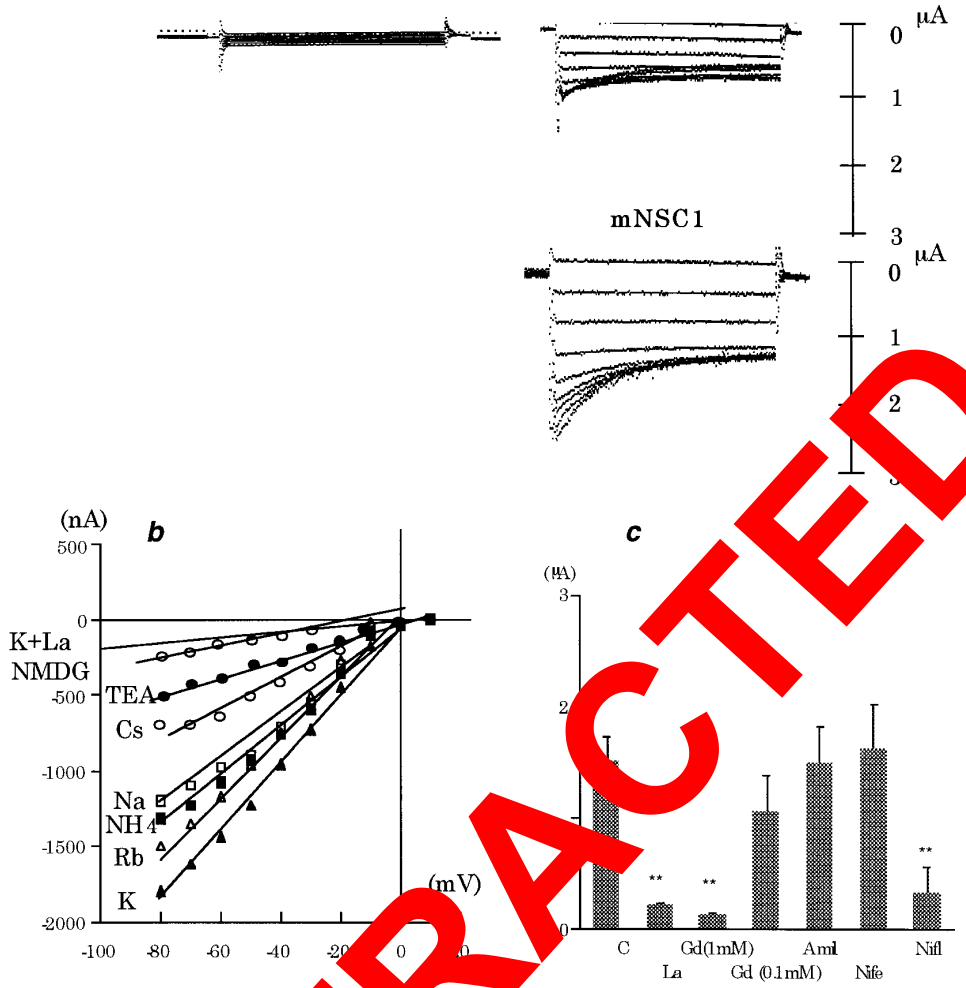

FIG. 1. Non-selective cation currents of mNSC channel essed in *Xenopus* oocyte. (a) Currents records under two-electrode voltage clamp in 90 mM KCl solution from *Xenopus* oo jected wi ater or mRNA transcribed from mNSC1 cDNA (mNSC1). Two electrode voltage clamp was performed. The holding po tia 0 mV, steps of 1.0 s duration +10, −10, −20, −30, −40, −50, −60, −70, and −80 mV are shown. Scale bar 0 indicates zero- rrent bef he holding voltage. Bath solution contained 90 mM KCl or 3 mM $MgCl_2$, 5 mM HEPES (pH 7.4). (b) Normalized whole c lar currents a plotted against membrane voltage during a series of exchanges of bath solution of the given cation of chloride salt (90). Ea point is the mean of six measurements. Bath solution contained 90 mM KCl, RbCl, NH_4Cl, NaCl, CsCl, TEACl, or NMDG Cl wi mM gCl2, 5 mM HEPES (pH 7.4). Average current after an addition of $LaCl_3$ is lined. (c) Current amplitude at the voltage −50 mV was red (c trol) with the KCl solution in *Xenopus* oocytes. The effects of addition of $LaCl_3$, $GdCl_3$ (0.1 and 1 mM), nifedipine (Nif ilori and niflumic acid (Nifl) are plotted (n = 6). (**p<0.01, Anova).

be hydrophobic sufficien r transmembrane domain. Haptad leucine repeats are served in M1 (L52 - L73) and a cluster of charged amino acids, like as probe used for the screening, is also observed (RXXRXRXXR) before M2 (R257-R265) of mNSC1 polypeptides. Transmembrane segments were sought for homology, where the fourth transmembrane segment of *trpl*, a Ca-activated Ca permeable channel is similar to the first transmembrane segment of the mNSC1 (Fig.2b). Interestingly, the aligmnet (40% match) between trpl and mNSC1 is more homologous to that between trpl and Ca channel as described previously (15).

Distribution of mNSC1 channel in mouse tissues. We analyzed by northern blotting the expression of mNSC1 mRNAs in various tissues of the mouse. mRNAs from forebrain, heart, lung, cerebellum, kidney, liver, pancreas, intestine, colon and skeletal muscle were hybridized to mNSC1 cDNA, suggesting that mNSC1 was abundant in forebrain, heart and lung (Fig. 3). mRNAs from testis, spleen and aorta were also hybridized but without signals (data not shown).

DISCUSSION

We have cloned a novel cDNA encoding mouse NS channel. The expression of the channel in *Xenopus* oocytes resulted in generation of non-selective cation current having electrophysiological properties consistent with NS channels.

a.

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     MYVLHSWKSK EGIRFPWNWF NGWEPPCGCW ELNSGFLEEL PDHLTAESSL
M1   QLREGFSGLF VGCFKLIHIL CFLQVAHMLT LKLQEIKELR FSHGLPKPQM
     CEDKDELGRV SLWHMPIILV LGRQRQEDFE ANLTTQMIPG QLVLHSETFL
     NTKEPGDNPL VGNSTYYTNM KAESESPSVT HSRYGCVCYN LVLWGEDRQV
     PESSDKPGYK LANFSSSETF SQRSERVQQW EISSVLLWPL CVHTIKNKEK
M2   WSRFELLIRN YRERCHRTVP SKGRGWSLMF TNGSEDQCFQ IQLKTFVCNL
M3   IVFMCFWKQE SHYIALAGLE LTEIHLPLLS SVLELNCFNC LFCFNVFNFF
M4   YRSHSITQPA LVVYTWSQAL FLVFNFTLRI CFASVYVCVY VDVCTCMCMP
     QRLEEGICWI PWNWSWDSYR HPCG·
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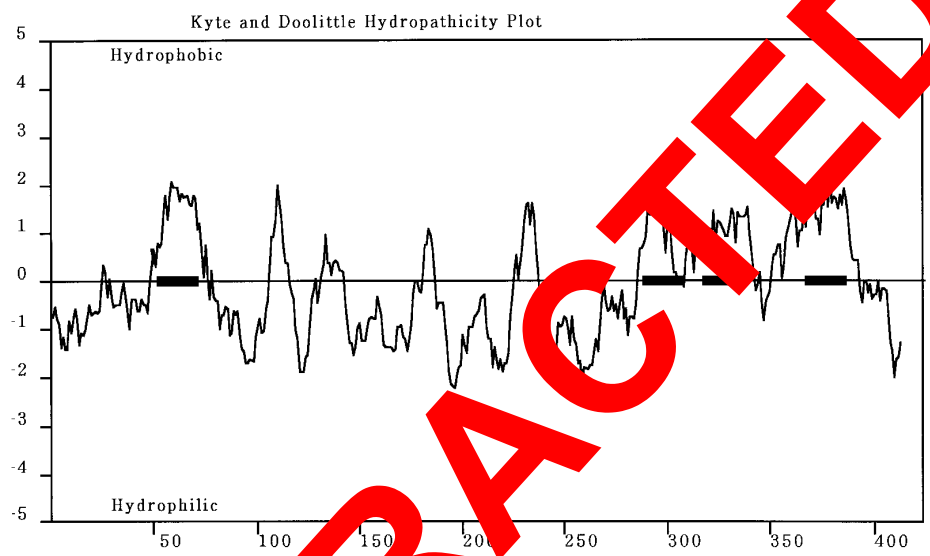

b.

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                    *  **  *     *    **  *  *  *
NSC M          SSLCLLR EG FSGL FVGCF KLIH ILCFL
trp                LIAEGLFAAA NVFSALKLVH -L-F
trpS4              LLSEGAFAAG MVFSYLKLVH I--F
                   AFHPTLVAEGLFAFA NVLSYLRLFF MYTT
Ca channelII         GVSVFRCV RLLRIFKVTR HW
  channelIII         VVKILRVL RVLRPLRAIN RA
  channelIV          ITFFRLF RVMRLVKLLS RG
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FIG. 2. Amino-acid sequences of mNSC1 channel cDNA. (a) Amino-acid sequences of mNSC1 channel are shown. The proposed transmembrane segments, M1-M4, are underlined. Arginine (R) rich domain and leucine repeat discussed in the text are marked in boxes. Hydrophobicity values were calculated under Kyte and Doolittle with a window size of 19 amino-acids. The nucleotide sequence is detected in the GSDB/EMBL/DDBJ/NCBI nucleotide sequence databases with Accession No. D50656. (b) Alignments of the first transmembrane segment of mNSC1, the fourth transmembrane segment of trp/trpl and Ca channel. Similar amino-acids are marked as astrisks.

The mNSC1 channel conducts nonselectively monovalent cations but not anion. Divalent cations might also be permeable, though not detected in the present experiments. Blockade experiments were suggesting the characteristics of this channel. Gd^{3+}, a specific blocker of stretch-activated NS channel at 10 μM (16), did not show any significant blockade, though inhibitory at mM concentration (Fig.1). Amiloride, which was also reported to block certain stretch-activated NS channel (14) did not show blocking effect. In contrast,

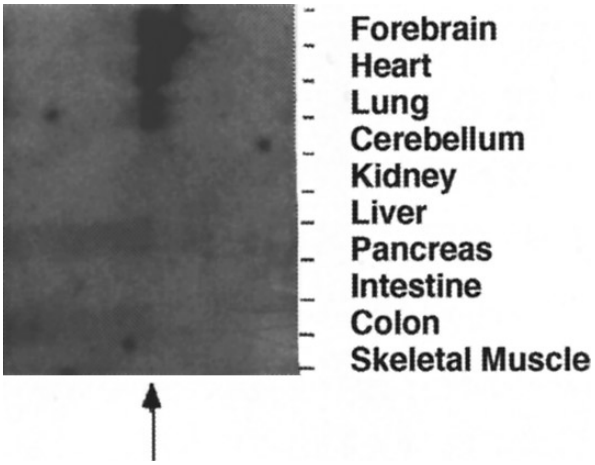

FIG. 3. Localization of mNSC1 channel. Northern blot to detect mNSC1 channel mRNA tissue localization. Forebrain, heart, lung, pancreas, and colon are positive. mRNA was isolated by using the guanidine thiocyanate method with organic extraction. An aliquot of 10 μg of mRNA in each lane was transferred to a nitrocellulose membrane. A 2486 bp EcoRI fragment of mNSC1 was labeled with ^{32}P. Hybridization was done as described previously (11).

niflumic acids (17) is a blocker to a class of Ca-activated NS channel. Therefore, the mNSC1 may be NS_{Ca} reported in pancreatic duct cells and in insulinoma cells (18,19).

mNSC1 channel was not similar to endogenous nonselective cation channels. A stretch activated NS [illegible] observed with patch clamping in *Boltenia villos* (8) [illegible] cyte, which is inactive in cell attached con[illegible]rati[illegible] Endogeneous NH_4 permeability, possibly [illegible]rou[illegible]ion-selective cation channel, is suggested in *[illegible]nopus* o[illegible]te (20), but the permeability is only pa[illegible]a[illegible] blocked [illegible]y $LaCl_3$. Whereas the mNSC1-induced curre[illegible]was completely blocked by $LaCl_3$. Basal Ca^{2+} influx, [illegible]rough non-slective cation channel, is [illegible]o re[illegible]rted in *Xenopus* oocyte (21), while mNSC1 is [illegible] impermeable to Ca^{2+} ion.

The primary structur[illegible] NSC1 [illegible] particular interest with regard to [illegible]n ch[illegible]nel structure and evolution. We propose that [illegible]esses four transmembrane segments in this report, [illegible]it might have less number of the transmembrane segm[illegible]ts. It should be clarified in the future study. Although alignment of amino-acids is novel, haptad leucine repeat in M1 of mNSC1 polypeptides is observed, which is functionally important for gating in *Shaker* K^+ channel (22,23) and widely conserved within voltage-dependent cation channels. The cluster of charged amino acids, considered to be a voltage-sensor, such as the arginine repeat of *Shaker* K^+ channel (RXXRXXRXXR) (24), is also observed (RXXRXRXXR) before M2 of mNSC1 channel. However, this is not involved in transmembrane segment and might not be related to channel function. The four possible transmembrane segments are individually sought with the receptor-operated non-selective cation channels, glutamate, acetylcholine, serotonin and ATP receptors or with cGMP-gated cation channels. All of these are the channels that conduct nonselectively for cations but physiologically for Ca^{2+} channels. We failed to find the similar sequences among these cation permeable channels (22–26) but found a similarity in Ca-activated Ca-permeable channel (15). Since both channel conduct cation activated by Ca, they may root on the same members near to voltage-gated K^+, Na^+, Ca^{2+} channels.

NS_{Ca} channels were observed in a number of epithelia such as salivary gland, lac[illegible]ral g[illegible]nd, thyroid follicular cells, pancreatic duct[illegible]lls, lens [illegible]ithelium, and renal tubule cells. The chan[illegible] were [illegible]lso studied in nonepithelial cells, incl[illegible]ng net[illegible]bl[illegible]oma cells, neutrophil, adipose tiss[illegible] cells, Helix [illegible]euron, mast cells, macrophage, root ga[illegible]lio[illegible] cells, [illegible]nd insulin secreting cell line (27). I[illegible]the t[illegible]ues [illegible]here mNSC1 was detected, NS_{Ca} [illegible]nnels m[illegible]ave various physiologic roles. In pa[illegible]rea[illegible] cells, it may play a role in maintaining the depolar[illegible]d membrane during the Ca-signal tra[illegible]uction. The [illegible]ecretion of insulin of the β-cells is acc[illegible]mpanied [illegible]y a rise in intracellular Ca^{2+}. This rise is [illegible]rovided [illegible]y Ca influx via voltage-dependent Ca channe[illegible]since [illegible]e voltage-dependent Ca channel is ac[illegible]e in depolarized membrane, the activity is evoked [illegible]y [illegible]g of K_{ATP} channel (28) and is possibly sustained [illegible]. NS_{Ca} channel. In cardiac tissue, NS_{Ca} channel has b[illegible]n proposed to underlie the arrythmogenic transient-in[illegible]rd current in ventricular myocytes (14). As sug[illegible]ested (1,14), NS_{Ca} has three roles; maintains depolarization in excitable cells, enhances secretion and transport sodium, which may be clarified in the future by this molecular probe.

We may therefore conclude that the cloned mNSC1 encodes non-selective cation channel with unique alignments and structure, which plays various roles widely in the excitable cells as well as non-excitable cells.

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