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Expression of an mNSC1 in Mammalian Cells

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We have cloned a cDNA inducing a cation-permeable current (mNSC1) from pancreatic β-cells, which shows niflumate-sensitive current in *Xenopus* oocytes. To elucidate the expression in mammalian cells, mNSC1 was expressed in CHO cells. The reversal potential by mNSC1 was shifted toward positive which was significantly reversed by flufenamic acid. Single-channel analysis showed a characteristic of a Ca-activated nonselective cation channel. Therefore, we ma[illegible] clude that mNSC1 expresses a fenamates-sensitiv[illegible] at-ion channel, inducing membrane depolarization [illegible] a mammalian cell.

Key Words: **nonselective cation channe[illegible]MI[illegible] β-ce[illegible] niflumic acid.**

We have recently cloned a n[illegible]el c[illegible]A encoding cation but not anion permeab[illegible] channe[illegible]NSC1) from pancreatic β-cell line (MI[illegible]) by expression cloning [1]. Expression was succes[illegible]lly [illegible]ieved in *Xenopus* oocytes, showing some char[illegible]istics [illegible] nonselective cation channels.

Three types o[illegible]ons[illegible]ective [illegible]tion channels are frequently observ[illegible] [2–[illegible] first type is activated by intracellular Ca^{2+}[illegible]e second one is activated by hydrostatic pressure o[illegible]retch, and the third one is unaffected by either Ca^{2+} or hydrostatic pressure. Nonselective cation channels are not selective for cations and exhibit linear current–voltage (I–V). The single channel conductance of these channels range about 10–100 pS with the most common range being 20–40 pS, when they are determined in symmetrical K^+ solutions at physiological ionic strength. Nonselective cation channels often have long open-time with a complicated kinetics, which cannot be fitted to a simple first-order process. Therefore, nonselective cation channels, being distinct molecules, have quite different electrical properties from oth[illegible]tion c[illegible]nnels. Ca-activated nonselective ca[illegible]n (CA[illegible]ch[illegible]nels were observed in a number of [illegible]helia su[illegible] as salivary ground, lacrimal ground, thy[illegible]d follicular cells, pancreatic duct cells, le[illegible]pitheliu[illegible]and renal tubule cells [2–5, 8]. The [illegible]annels were also studied in nonepithelial cells, including n[illegible]uroblastoma cells, neutrophil, adipose tissue [illegible]ls, He[illegible] neuron, mast cells, macrophage, root ganglion[illegible] and insulin secreting cell line [2]. Although far from specific, CAN channels are blocked by a group [illegible]onsteroidal anti-inflammatory drugs, the fenamates. Fenamates blocked CAN channels among nonselective cation families, but also blocked Ca-activated Cl channels [4, 5].

In the present studies, mNSC1-induced channels were expressed in mammalian cultured cells. The results suggests that mNSC1 encode at least a subunit of CAN channel.

MATERIALS AND METHODS

Functional expression of mNSC1 in Chinese hamster ovary cells (CHO cells) and patch clamp experiments. mNSC1 cDNA was ligated to an expression vector pCMV-SPORT (Liftech, U.S.A.) in *Sal*I–*Not*I site. To search a successful expression, a plasmid expressing green fluorescence protein (pEGFP-N1, Clontech, U.S.A.) was coinjected. CHO cells were thus each injected with the mixture of two plasmids (0.1 mg/ml) by microinjector (Eppendolf, 5243) and micromanipulator (Eppendolf, 5170). After a 2-day incubation, fluorescent signal of the cells was determined (excitation at 490 nm) and then patch clamp was performed in the bright cells. The bath solution contained (in mM) 125 NaCl, 25 $NaHCO_3$, 5 KCl, 1.2 $MgSO_4$, 1 Na_2HPO_4, 0.913 $CaCl_2$ and 3 Hepes. Patch pipette for whole cell is a filtered solution of 20 KCl, 70 K–gluconate, 3 Hepes, 1 EGTA and 20 NaCl (pH 7.2). Maintaining the intracellular calcium higher, the solution was supplied with 1 mM $CaCl_2$ ($[Ca^{2+}]$ 1 μM). For a single channel study, pipette contained 125 NaCl and 3 Hepes. Bath contained 20 KCl, 70 K–gluconate, 3 Hepes, 1 EGTA and 20 NaCl (pH 7.2) with variable $CaCl_2$. The resultant Ca^{2+} concentration was measured using fura-2 fluorescence.

Electrophysiology. Patch clamp recordings were carried out according to the method described in previous paper [9]. Patch clamp was performed at room temperature (23–27°C). Records were sampled at 10 Hz, and a filter (cutoff 1 kHz) was used for analysis.

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TABLE 1

Inhibitions of Reagents on mNSC1-Induced Currents in *Xenopus* Oocytes

% Inhibition	Mean	SD	$*p < 0.01$
La (1 mM)	100		
La (0.1 mM)	19	±15	
Gd (1 mM)	90	±5	*
Gd (0.1 mM)	1	±2	
Amiloride	−2	±11	
Nifedipine	−15	±22	
Niflumate	88	±3	*
Indomethacin	5	±12	
SITS	−21	±25	
4AP (1 mM)	−12	±10	

Reagents and statistics. Fenamates, amiloride, nifedipine, 4-aminopyridine, quinine, 3′5′-dichlorodiphenylamine-2-carboxylic acid (DCDPC) and $GdCl_3$ were purchased from Sigma, Co. (St. Louis, MO) and dissolved in DMSO or water as appropriate and stored at −20°C before use. These reagents were dissolved in bath solutions. $LaCl_3$ was directly added to the chamber to give a final concentration of 1 mM.

Data are expressed as means ± SE and were analyzed by Student's *t* test. A *p* value of <0.01 was considered statistically significant.

RESULTS

Before the experiments with CHO cells, reag[illegible] were tested to block the mNSC1 current[illegible]le 1) Xenopus oocytes [1]. Influences of re[illegible]gents on th[illegible] mNSC1 current at −100 mV are sh[illegible]n in [illegible]. Mean inhibition rate (%) was expre[illegible]d w[illegible]SD. $GdCl_3$ at 1 mM, niflumate and flufenam[illegible]e at 100 [illegible]M exhibited significant blockades. Fe[illegible]m[illegible]s were effective blockers; IC_{50}; 309 μM niflu[illegible]ate, 112 [illegible]M flufenamate.

Expression of mNSC1 [illegible]annel in CHO cells. Coinjection of the interest g[illegible]e wi[illegible]GFP was useful of the experiments with patch [illegible]ping. Intrinsic nonselective cation channe[illegible]CHO [illegible]was observed about <1/20 patches, [illegible]le t[illegible] mNSC1-encoded current was observed 45/45 [illegible]tch[illegible] GFP positive cells. The mNSC1 encoded c[illegible]nel was expressed in mammalian cells; CHO, COS7, a[illegible] HEK cells. However, the latter two exhibited variable channels having a similar conductance, with incidence of about 50% of patches. By contrast, CHO cells showed channels less frequently. Tight seal whole cellular currents were thus measured in CHO cells. When pipette contained nominally free calcium, the currents between control and mNSC1-expressed were not significantly different. While exaggerated currents were observed in mNSC1-expressed cells, no increment of currents was observed in the control CHO cells by pipette containing one μM Ca^{2+}. Current-voltage relation in untransfected cells possessed reversal potential of around −20 mV. In contrast, reversal potential was shifted toward positive in mNSC1-expressed cells and it was repolarized by an addition of 100 μM flufenamate (Fig. 1). Reversal potential was summarized; mNSC1 transfected CHO cells were significantly affected by flufenamate in compared with control, the GFP transfected cells (Fig. 1). Thus mNSC1 at least encoded a fenamates sensitive cation current, which depolarized the membrane potential.

mNSC1 single channel. In inside-out membrane patches of CHO cells, tracings of the single mNSC1 channel in equimolar KCl/[illegible]Cl (pipette/bath) solutions with cell-free membran[illegible]are [illegible]wn in Fig. 2a. In this trace, two channels [illegible]n, and [illegible]e opening was composed of long lasting a[illegible]short [illegible]rsting. Current distribution was w[illegible] fitted [illegible]Gaussian's analysis. The I-V relationsh[illegible]s of [illegible]d-free [illegible]atches in KCl are shown in Fig. 2b. Th[illegible]nduct[illegible]ce of 41.2 ± 6.2 pS. The probabili[illegible] of an [illegible]NS[illegible] channel being open was related t[illegible] voltage ([illegible]g. 2c, $n = 6$), though not remarkable b[illegible]twe[illegible]−60 and +10 mV, physiologic range.

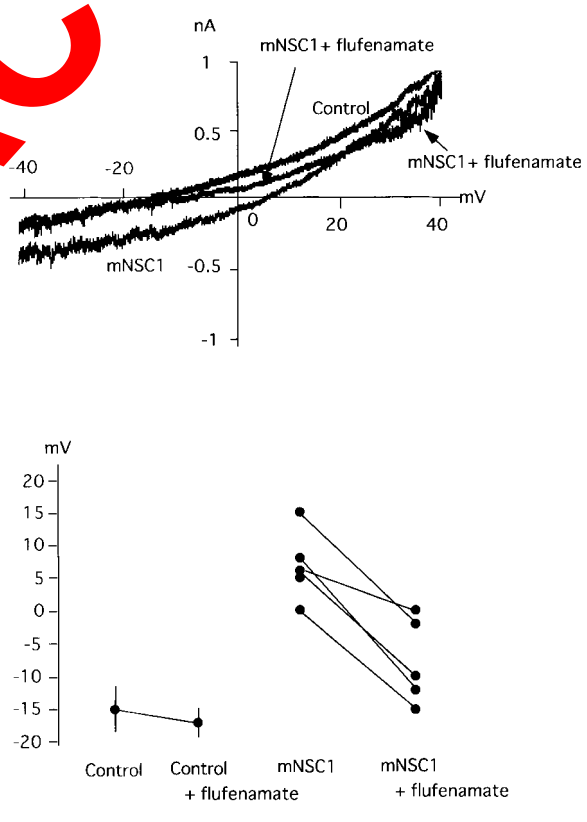

FIG. 1. Whole cell currents of mNSC1-expressed CHO cells. (Upper panel) Representative currents of CHO cells were obtained by tight-sealed whole cell patches by ramp voltage. Control was obtained in a pipet solution of lower Ca^{2+} concentration (100 nM). mNSC1 induced currents in a pipette solution of 1 μM and effect of flufenamic acids are shown. (Lower panel) Reversal potential of control cells with or without 100 μM flufenamic acid are shown on the left. Effect of 100 μM flufenamic acid perfused on the mNSC1 expressed cell is shown on the right, showing a significant decrease ($p < 0.01$).

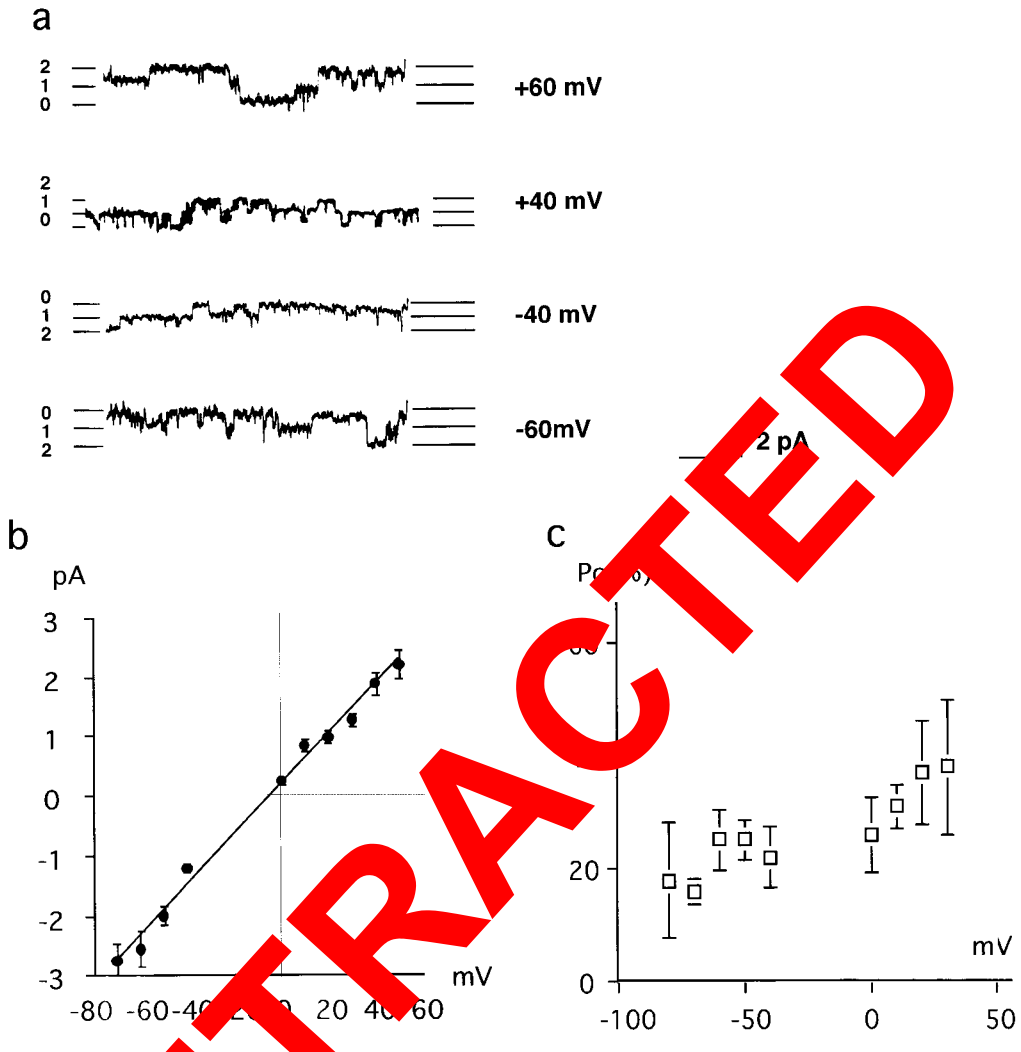

FIG. 2. Single channel analysis of mNSC1 in *Xenopus* oocytes. (a) Representative trace of single nonselective cation channel in inside-out mode in symmetrical KCl solution at the given membrane voltage (negative value of the holding potential). Bars indicate closed (0) and open state (1, 2, . . .) of the channel. (b) Current voltage relation in inside-out mode in symmetrical KCl solution. The lack of plots between −40 to −20 mV is due to the noisy events that are not suitable to the analysis. (c) Open probabilities are plotted against V_m with symmetrical KCl in inside-out mode.

In the present screening, both an endogenous nonselective cation channel and the mNSC1 activity were observed with a single channel analysis. Both possessed similar conductance. To discriminate these channels, flufenamate was added or the activity was observed with an increase in cytosolic Ca^{2+} concentration (Fig. 3). Flufenamate at 10 μM completely blocked the single channel and an increase in cytosolic Ca^{2+} concentration activated the open probability of the mNSC1-induced channel. Whereas, an endogenous channel did not respond to either flufenamate or Ca^{2+}.

DISCUSSION

The previous report for cloning [1] has suggested that mNSC1 induced nonselective cation current in *Xenopus* oocytes, which is inhibited by niflumic acids. These results supposed that mNSC1 might induce a CAN channel. This study is designed to elucidate electrophysiologic characterization of this channel further in mammalian cells.

Characteristics of the mNSC1-induced channel were similar to that of CAN channel, involving kinetics, conductance, blocker and activation by Ca^{2+}. The inhibitory effects of the fenamates on CAN channel have been reported [4, 5] in rat exocrine pancreatic cells [6] the basolateral membrane of the guinea-pig cochlea [10], rat cerebral capillary endothelial cells rat distal colonic crypt cells [7] and mouse mandibular cells [11]. While the nonselective cation channel with similar conductance was found in untreated CHO cells, though not frequently. The endogenous channel was neither

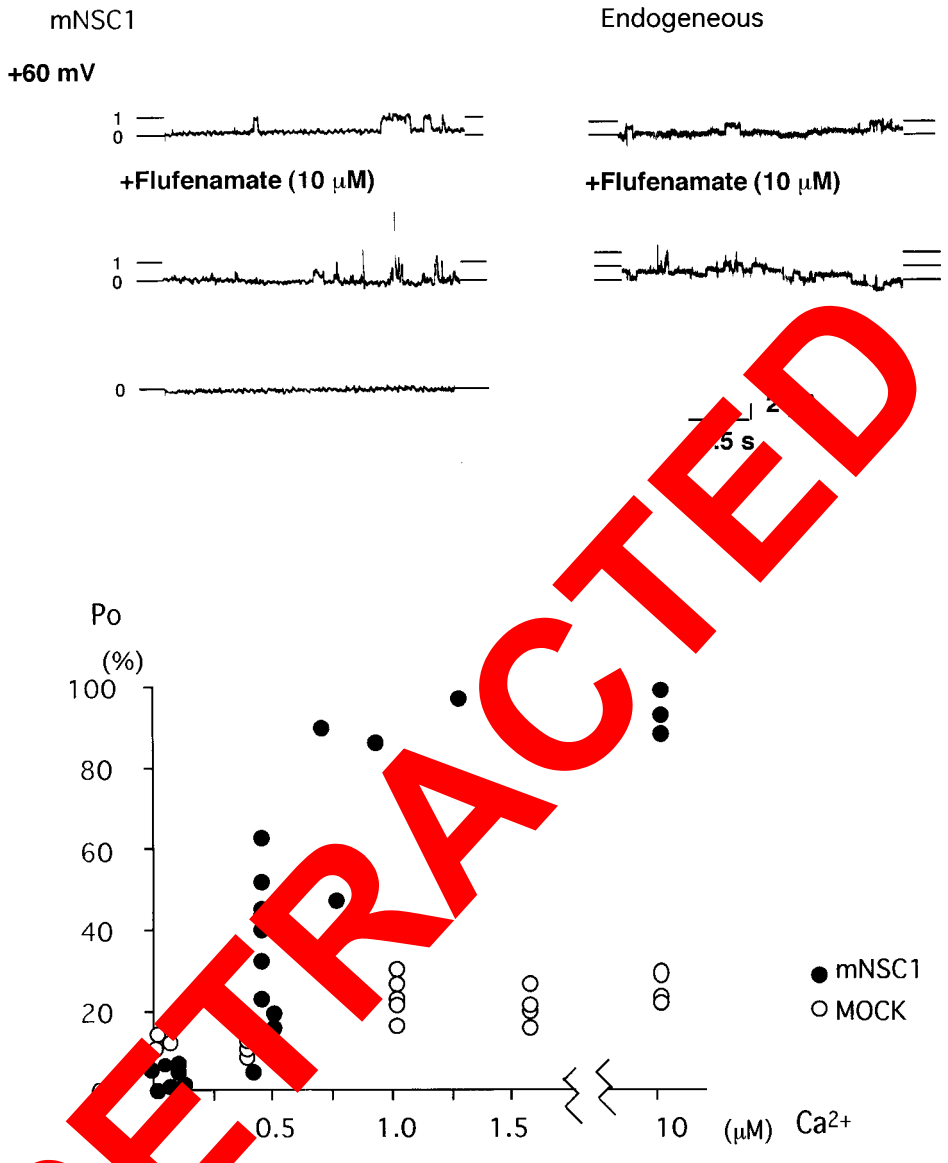

FIG. 3. Single-cha[illegible]lysis [illegible]C1 in CHO cells. Inside-out patches were constructed in CHO cells. (Upper panel) Representative trace of single nons[illegible]tive [illegible]ion cha[illegible]el in inside-out mode in CHO cells at the given membrane voltage (negative value of the holding potential). Bars in[illegible]te cl[illegible]d open state (1) of the channel. Addition of flufenamate rapidly altered the activity (the middle trace in 1 s and the botto[illegible]e in 10 s after addition). Endogenous cation channel at the same voltage is shown on the right. Trace 10 s after addition of flufenamate [illegible]own below. (Lower panel) Open probabilities of the mNSC1 induced (closed circles) and endogenous (open circles) channel are plotted to the [illegible] Ca^{2+} concentration, a value of which was measured by fluorescence with fura-2.

blocked by flufenamate nor activated by cytosolic Ca^{2+}. Thus, though not exactly discriminate them, we concluded that the mNSC1 at least could induce a new CAN channel in CHO cells. We did not neglect the possibility that mNSC1 is a supportive subunit rather than construct an α-subunit of CAN channel molecule.

There is two class of CAN channels reported, Ca-permeable and Ca-impermeable. La^{3+} and high concentration of nifedipine appear to be a blocker of nonselective cation channels [2]. These class of CAN might be a Ca permeable channels different from mNSC1. A Ca-permeable CAN is probably encoded by TRP/TRPL-related protein [12, 13]. While mNSC1 channel has been hard to conduct Ca^{2+} ion, and thereby mNSC1 is involved in CAN channel for monovalent cation. This latter type is reported in pancreatic acinar cells with a similar conductance [6].

CAN channel in CRI-GI insulinoma cells is blocked by 4-aminopyridine and amiloride [2, 8]. Amiloride or 4-aminopyridineis reported as blockers for certain class of CAN channels. CAN channel in lung epithelia is blockable by amiloride [2]. However, we did not find blockage effects of both reagents on the mNSC1 channel, though mRNA of mNSC1 was detected in lung and

in insulin secreting cell line MIN6 [1]. We observed that addition of $LaCl_3$ after 4-aminopyridine did not completely restore the control current. Thus, 4-aminopyridine at mM concentration might be toxic.

Based on the present studies, mNSC1 may encode a CAN channel sensitive to fenamates in several tissues. When activated, mNSC1 induced the depolarization lasting a few seconds, which may play a role in enhancement of Ca^{2+} influx through voltage-dependent channels.

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