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Cerebroprotective properties of SM-20220, a potent Na^+/H^+ exchange inhibitor, in transient cerebral ischemia in rats

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Abstract

The aim of this study was to investigate the contribution of the Na^+/H^+ exchanger to cerebral ischemia using SM-20220 (*N*-(aminoiminomethyl)-1-methyl-1*H*-indole-2-carboxamide methanesulfonate), a newly synthesized compound. In in vitro experiments, we evaluated the inhibitory effect of SM-20220 on the Na^+/H^+ exchanger in cultured neurons and glial cells. The IC_{50} of SM-20220 in neurons and glial cells was 5 nM and 20 nM, respectively. To examine the in vivo effects of SM-20220 on brain injury, we used a transient middle cerebral artery occlusion model in rats. SM-20220 given intravenously 1 h after occlusion significantly reduced the extent of cerebral edema, Na^+ content and infarcted area in a dose-dependent manner. The results of the present study suggest that the Na^+/H^+ exchanger is involved in the aggravation of brain edema and infarction, and its inhibitor may exert protective effects on post-ischemic brain damage.

Keywords: Cerebral ischemia; Na^+/H^+ exchanger; Brain edema; Infarction; SM-20220

1. Introduction

Cerebral ischemia causes intracellular acidosis, energy failure, which leads to neuronal and glial cell death. Recently, several different mechanisms of ischemic brain damage have been proposed (Pulsinelli, 1992; Urenjak and Obrenovitch, 1996). Regulation of intracellular pH (pH_i) and cellular ion homeostasis has received much attention. The Na^+/H^+ exchanger is a ubiquitously expressed plasma membrane protein thought to play an important role in the regulation of cell volume and pH_i (Orlowski and Grinstein, 1997). The driving force for H^+ extrusion is provided by the gradient across the cell membrane. The Na^+/H^+ exchanger is expressed in neurons and glial cells, and in some other cell types (Pizzonia et al., 1996). Ischemia/reperfusion may activate the Na^+/H^+ exchanger and such activation causes an increase of intracellular Na^+ (Na_i^+). Excessive accumulation of Na_i^+ leads to Ca^{2+} overload via the Na^+/Ca^{2+} exchangers in neurons and glial cells (Siesjö, 1992; Matsuda et al., 1997). Cytotoxic edema is the result of an increase in glial cellular water content in association with Na_i^+. Thus, it is conceivable that inhibition of the Na^+/H^+ exchanger during ischemia/reperfusion could ameliorate the ischemic brain damage.

Although the protective effects of the Na^+/H^+ exchanger inhibitors such as amiloride and its analogues have been investigated in simulated ischemic conditions, using cultured neurons (Manev et al., 1990; Vornov et al., 1996), these drugs are not suitable tools because of their low selectivity for the Na^+/H^+ exchanger (Kleyman and Cragoe, 1988). Moreover, very little is known about the effect of selective the Na^+/H^+ exchanger inhibitors on cerebral ischemia in vivo.

In the present study, we characterized a new compound, SM-20220 (*N*-(aminoiminomethyl)-1-methyl-1*H*-indole-2-carboxamide methanesulfonate) (Fig. 1), as the Na^+/H^+ exchanger inhibitor in cultured neurons and glial cells. Furthermore, cerebroprotective effects of SM-20220 were examined in rats subjected to transient focal ischemia.

2. Materials and methods

2.1. Culture preparation

Primary neuronal cell cultures were prepared as previously described (Hatanaka et al., 1990). Cerebral cortices

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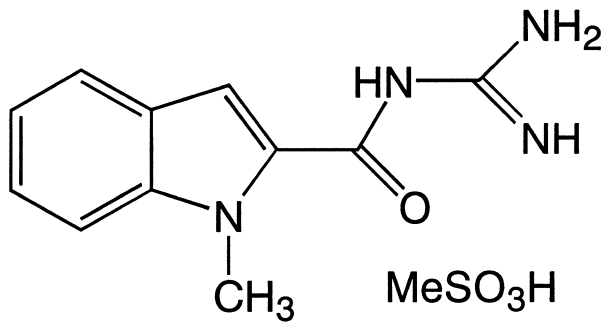

Fig. 1. Chemical structure of SM-20220 (*N*-(aminoiminomethyl)-1-methyl-1*H*-indole-2-carboxamide methanesulfonate).

from Sprague–Dawley rat embryos at 17 days of gestation were dissociated enzymatically at 37°C with 0.3 mg/ml papain in complete-Hank's Balanced Salt Solution, which is a Ca^{2+}, Mg^{2+}-free Hank's Balanced Salt Solution, supplemented with 10 mM HEPES, 1 mM sodium pyruvate and 0.5% glucose. After papain digestion, the supernatant was removed and the tissue was dissociated into single cells mechanically by gentle trituration. Cells were seeded in Minimum Essential Medium supplemented with 2% fetal bovine serum, 5.4 mM glucose, 24 mM $NaHCO_3$ and 10 mM HEPES on poly-D-lysine (Sigma)-coated cover glasses at a density of 1.5×10^5 cells per cm^2 on 35-mm dishes. The cells were incubated for 2 h, following which the medium was removed and 2 ml of Neurobasal Medium, supplemented with 0.5 mM L-glutamine and 2% B27 supplement (Gibco) was added. Cell cultures were incubated for up to 5–8 days.

Glial cells were prepared in a similar way with neuronal cell cultures from cerebral cortices of 1-day-old Sprague–Dawley rats. Cerebral cortices were pooled, minced and incubated at 37°C with 0.3 mg/ml papain and 0.01% DNase I (Takara) in complete-Hank's Balanced Salt Solution. The cells were seeded and plated onto 75-cm^2 flasks at a density of 1 brain per 25 cm^2 in Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum. Secondary cultures were used for the experiments. Neuronal and glial cultures were maintained in 37°C and 5% CO_2 in a humidified incubator. The culture medium was changed twice a week.

2.2. Measurement of intracellular pH

The pH_i of neurons and glial cells was measured using the fluorescent pH indicator dye, 2′, 7′-bis-2-carboxyethyl-5-(6)-carboxyfluorescein (BCECF) (Raley-Susman et al., 1991). The cells were loaded with acetoxymethylester of BCECF (BCECF-AM, Dojin) and the fluorescence intensity of dye was measured with excitation pairs of 450 nm/490 nm and an emission wavelength of 530 nm with a microscope-based microspectrofluometer system (ARGUS-50, Hamamatsu Photonics, Japan). The fluorescence ratio 490 nm/450 nm was calculated. Calibration of pH_i was accomplished using the high-K^+-nigericin method (Thomas et al., 1979). The fluorescence ratio was plotted against pH_i and fitted by linear regression ($r^2 = 0.941$ for neurons, $r^2 = 0.982$ for glial cells).

2.3. The Na^+/H^+ exchanger inhibition in vitro

The Na^+/H^+ exchange activity was estimated using the ammonium pre-pulse technique in which cells were exposed to two pulses of NH_4^+ in HEPES-buffered, bicarbonate-free solutions (standard solution) (Boron and De Weer, 1976; Pizzonia et al., 1996). The cells were initially superfused with standard solution. In order to evaluate the ability of cells for pH_i recovery from acidosis, the NH_4^+ pulse was performed by changing the superfusate to standard solution containing 20 mM NH_4Cl. After 5 min, the superfusate was replaced with standard solution without NH_4Cl, and subsequent pH_i recovery from the acid load was measured. The cells in which pH_i did not return to the initial level were eliminated from further experiments. This first NH_4^+ pulse was always conducted in the absence of SM-20220. After the first NH_4^+ pulse as described above, the second NH_4^+ pulse was performed to evaluate the Na^+/H^+ exchanger inhibitory effect of the drug. The inhibitory effect of SM-20220 was estimated by comparing the rate of pH_i recovery at the second NH_4Cl pulse in the presence and absence of the drug.

2.4. Cerebroprotective effects in vivo

The effects of SM-20220 in cerebral ischemia were estimated in rats. Male Wistar rats weighing 201–243 g were used. All surgical procedures were performed under halothane anesthesia (induction at 4% and maintenance at 1% in 70% N_2O/30% O_2). Core body temperature was maintained at 37°C by a heating pad with a flexible thermocouple, which was inserted 6 cm into the rectum during the surgical procedure. Transient focal ischemia was induced by occluding the left middle cerebral artery using an intraluminal suture technique (Longa et al., 1989). The origin of the left middle cerebral artery was occluded by insertion of a nylon thread, one end of which was pre-coated with silicone resin (0.45 mm in diameter). The suture was inserted 16 mm from the left common carotid artery bifurcation. SM-20220 or vehicle (8% polyethyleneglycol 400) was administered via the penile vein 1 h after middle cerebral artery occlusion. At 2 h after middle cerebral artery occlusion, reperfusion was established by pulling the thread.

To measure brain water and Na^+ content, the rats were decapitated at 4 h after reperfusion. The left hemisphere was sectioned into 4-mm thick slices from 1 mm anterior to 3 mm posterior to the bregma, and the water content was determined by the dry-weight method (Kuribayashi et al., 1994). These hydrated samples were used for measure-

ment of the Na^+ content. Tissue extracts were prepared according to Sparrow and Johnstone (1964), and the Na^+ content was measured by atomic absorption spectroscopy (Z-9000, Hitachi, Japan).

For the measurement of infarct size, the rats were decapitated at 22 h after reperfusion. The cerebrum was coronally sectioned into 2-mm thick slices from 3 mm anterior to 5 mm posterior to the bregma. Five consecutive slices were stained with 2,3,5-triphenyl tetrazolium chloride solution (Longa et al., 1989). Photographs of the slices were then taken. The infarcted area of each slice was quantified with a computerized image analysis system (NIH Image), and was expressed as a percentage of the coronal section of the left hemisphere.

2.5. Statistics

The data were expressed as the means ± S.E.M. The significance of differences was calculated by Welch's test or Williams's multiple comparison, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Steady state pH_i

Steady state pH_i values in neurons and glial cells in this study were 7.18 ± 0.01 and 7.19 ± 0.01, respectively, similar to values reported previously (Shrode and Putnam, 1994; Baxter and Church, 1996).

3.2. Inhibition of the Na^+/H^+ exchanger

Fig. 2 shows the time course of the pH_i recovery in neurons and glial cells. Removal of NH_4Cl resulted in rapid intracellular acidification. The pH_i rapidly returned toward a value similar to that at the start of the experiment in the absence of SM-20220 in both cells. Recovery from the acidification was strongly inhibited by 10^{-7} M SM-20220. SM-20220 concentration dependently inhibited the recovery of pH_i in both cells (Fig. 3). The IC_{50} for inhibition of the recovery by SM-20220 in neurons and glial cells was 5 nM and 20 nM, respectively.

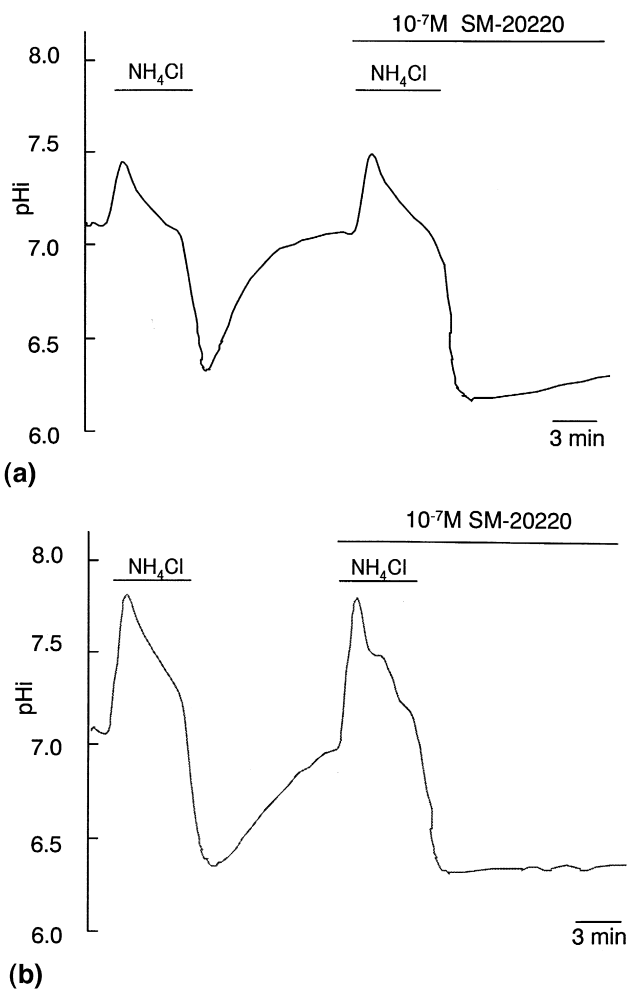

Fig. 2. Effect of SM-20220 on pH_i recovery from acid load in cultured neurons (a) and glial cells (b).

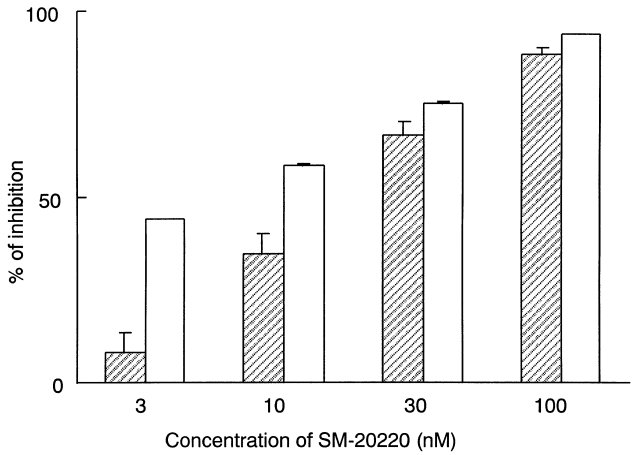

Fig. 3. Inhibition of the Na^+/H^+ exchanger by SM-20220 in cultured neurons (open columns) and glial cells (hatched columns). Columns and vertical bars represent the mean ± S.E.M. of the percentage inhibition of pH_i recovery in 26–28 experiments. The values of S.E.M. in 3 nM and 100 nM of SM-20220 in neurons were 1.9 and 0.7, respectively.

3.3. Cerebral edema and Na^+ content

The cerebral water and Na^+ contents at 4 h after reperfusion are shown in Figs. 4 and 5. In the normal control rats, the water content was 80.0 ± 0.1%. This increased to 83.3 ± 0.3% in the vehicle group. Treatment with SM-20220 significantly reduced the water content to 82.6 ± 0.3% (0.3 mg/kg, $P < 0.05$) and 82.1 ± 0.3% (1.0 mg/kg, $P < 0.01$). Treatment with SM-20220 also showed significant attenuation of the increase in post-ischemic Na^+ contents (normal control: 176.7 ± 10.0 μmol/g dry weight; vehicle: 359.5 ± 18.4 μmol/g dry weight; SM-20220: 0.3 mg/kg, 301.4 ± 19.1 μmol/g dry weight, $P < 0.05$; 1.0 mg/kg, 289.1 ± 22.1 μmol/g dry weight, $P < 0.01$).

3.4. Cerebral infarction

Fig. 6 shows the infarcted area at 22 h after reperfusion. SM-20220 dose-dependently reduced the infarcted area in all five slices. At a dose of 0.3 mg/kg, however, such

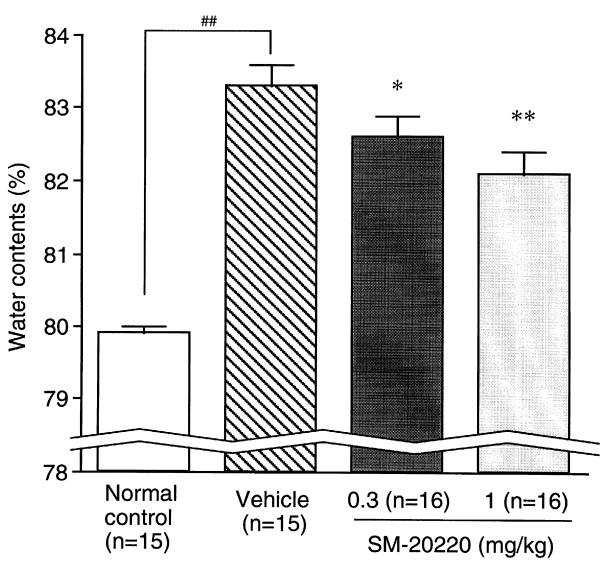

Fig. 4. Effect of SM-20220 on cerebral water content induced by transient focal cerebral ischemia. Columns and vertical bars represent the mean ± S.E.M. $^{\#\#}P < 0.01$ compared with the normal control group (Welch's test). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group (Williams's multiple comparison).

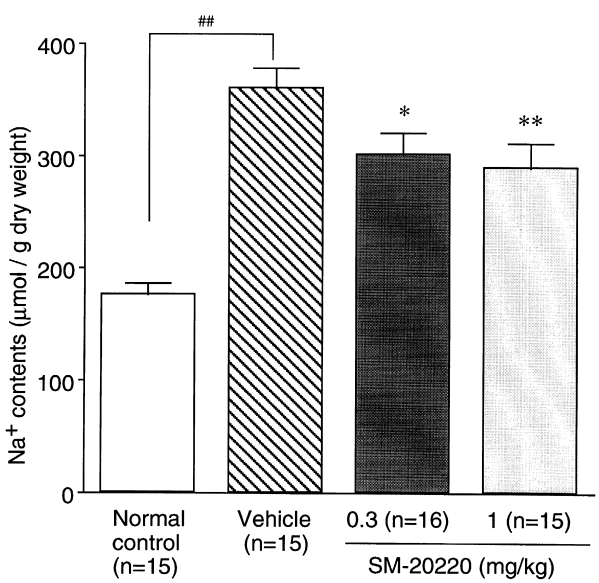

Fig. 5. Effect of SM-20220 on change in cerebral Na^+ content induced by transient focal cerebral ischemia. Columns and vertical bars represent the mean ± S.E.M. $^{\#\#}P < 0.01$ compared with the normal control group (Welch's test). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group (Williams's multiple comparison).

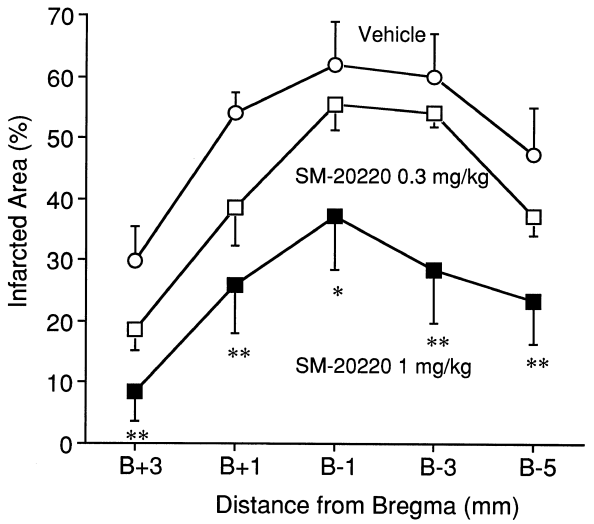

Fig. 6. Effect of SM-20220 on the infarcted area (percentage against left hemisphere) induced by transient focal cerebral ischemia. Points and vertical bars represent the mean ± S.E.M. of five experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group (Williams's multiple comparison).

reduction did not reach statistical significance. A significant difference was noted at 1 mg/kg ($P < 0.05$–0.01).

4. Discussion

The present study provided the first evidence that SM-20220, a potent Na^+/H^+ exchanger inhibitor, ameliorates ischemic damage in vivo. First, we evaluated the in vitro effect of SM-20220 on the Na^+/H^+ exchange activity in cultured rat neurons and glial cells, and demonstrated that the agent markedly inhibited the Na^+/H^+ exchange activities in both cells. Second, we then examined the in vivo effect of the agent to determine whether it could protect against brain damage induced by transient middle cerebral artery occlusion. SM-20220 significantly attenuated the extent of cerebral edema, Na^+ content and infarction in this model.

It is well-established that Ca^{2+} overload is a major mechanism of ischemic cell damage (Pulsinelli, 1992; Siesjö, 1992). Recent studies have indicated that the increase in Na_i^+ initiates several modulatory events, probably triggering cell destruction (e.g., activation of the Na^+/Ca^{2+} exchanger, glutamate release). In fact, Na_i^+ modulating drugs exert a protective action on ischemia-induced neuronal damage (Taylor and Meldrum, 1995). It is generally accepted that Na^+/K^+ ATPase regulates Na_i^+ under normal physiological conditions. However, Na^+/K^+ ATPase is inactivated by low ATP levels combined with an increased inorganic phosphate level, low pH and generation of free radicals in the ischemia/reperfusion period (Van Emous et al., 1998). This in turn increases Na_i^+. In this period, activation of the Na^+/H^+ exchanger can occur as a consequence of acidosis and/or through phosphoinositide hydrolysis, resulting in the production of diacylglycerol which activates protein kinase C which, in turn, stimulates the Na^+/H^+ exchange activity (Frelin et al., 1988). Eventually, a marked increase of Na_i^+ may occur, and this Na^+ overload leads to Ca^{2+} overload. Thus, Ca^{2+} and Na^+ overload may play an important role in ischemic brain injury.

SM-20220 had very little effect as established by a commercially available screening service against another 16 different enzymes, 45 different receptors and various channels including Na^+ channel, K^+ channel, Ca^{2+} channel (L and N type) and glutamate receptor even at a concentration of 1000 and/or 10 000 nM (MDS PanLabs enzyme and radioligand binding assays, data not shown). Based on these findings, the mechanisms of protective effects of SM-20220 in transient focal ischemia may be explained at least, in part, by the inhibition of the Na^+/H^+ exchanger in neurons and glial cells.

Ischemia/reperfusion was associated with two-fold elevation in the Na^+/H^+ exchanger mRNA expression in our preliminary study (data not shown). The exact role of the Na^+/H^+ exchanger in cerebral ischemia, however, is still poorly understood. For example, the Na^+/H^+ exchanger inhibition causes a reduction in platelet-activating factor formation in endothelial cells (Ghigo et al., 1988) and prevention of neutrophil chemotaxis (Simchowitz and Cragoe, 1986). Mounting evidence supports the hypothesis that platelet-activating factor and accumulated neutrophils aggravate ischemic injury (Bielenberg et al., 1992; Kochanek and Hallenbeck, 1992). Considering the variety of physiological and biochemical effects of the Na^+/H^+ exchanger, further studies of the effects of SM-20220 are necessary in order to better define its mechanisms of action.

In conclusion, the present study showed the Na^+/H^+ exchanger inhibitor to be a promising drug for the treatment of ischemic stroke.

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