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Nitrous Oxide Enhances Na^+/Ca^{++} Exchange in the Neuroblastoma Cell Line SK-N-SH[1]

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ABSTRACT

Changes in the concentration of cytosolic free calcium ($[Ca^{++}]_i$) play fundamental roles in the initiation and regulation of many neuronal processes. Altered regulation of $[Ca^{++}]_i$ has been implicated in the action of some anesthetics. We investigated the effects of nitrous oxide (N_2O) on Ca^{++} mobilization and membrane potential in the human neuroblastoma cell line SK-N-SH. $[Ca^{++}]_i$ was monitored by fluorescence spectrophotometry of cells loaded with fura-2 or fluo-3. N_2O reversibly suppressed carbachol-stimulated increases in $[Ca^{++}]_i$. N_2O also inhibited increases in $[Ca^{++}]_i$ induced by calcium ionophore or depolarization suggesting a mechanism involving enhanced efflux or sequestration of cytosolic Ca^{++}. The inhibitory effect of N_2O was attenuated when the transmembrane Na^+ gradient was altered either by suspending cells in nominally Na^+-free buffer or by pretreating cells with ouabain. The inhibitory effect of N_2O was also attenuated by the Na^+/Ca^{++} exchange inhibitor 3,4-dichlorobenzamil. The effects of N_2O on membrane potential were measured fluorimetrically using bis(1,3-dibutylthiobarbituric acid)-trimethine oxonol. In the presence of N_2O, resting membrane potential was hyperpolarized, a condition that would favor Ca^{++} efflux mediated by the electrogenic Na^+/Ca^{++} exchanger. Taken together, these findings indicate that N_2O suppresses carbachol-stimulated increases in $[Ca^{++}]_i$ by enhancing Na^+/Ca^{++} exchange activity. Enhancement of neuronal Na^+/Ca^{++} exchange may contribute to the anesthetic action of N_2O.

Transient increases in $[Ca^{++}]_i$ play fundamental roles in the initiation and regulation of many neuronal processes including neurotransmitter release, excitability and synaptic plasticity (Kennedy 1989, Miller 1991, Kress and Tass, 1993, Bertolino and Rodolfo, 1992, Kostyuk 1992). The multiple roles of Ca^{++} in neurons are reflected in numerous and complex signalling and homeostatic mechanisms. Altered regulation of $[Ca^{++}]_i$ has been implicated in the mechanism of action of some anesthetics (Kress and Tass, 1993, Terrar, 1993). Previous work in this laboratory demonstrated that N_2O inhibits the ADP-stimulated rise in $[Ca^{++}]_i$ and subsequent aggregation of human blood platelets (Philp *et al.*, 1992). Our study investigated the effects of N_2O on $[Ca^{++}]_i$ in the human neuroblastoma cell line SK-N-SH. Phenotypically, these cells share many similarities with neurons of the central nervous system. The SK-N-SH cell line has been well characterized both biochemically and pharmacologically (Fisher *et al.*, 1989, Noronha-Blob *et al.*, 1989).

Materials and Methods

Materials. The acetoxymethyl esters of fura-2 and fluo-3 (fura-2 AM and fluo-3 AM) and bisoxonol were obtained from Molecular Probes Inc. (Eugene, OR). N_2O was obtained from Sure-Arc Welding (London, Ontario, Canada). Carbachol and digitonin were obtained from BDH Chemicals (Toronto, Ontario, Canada). DCB and MIA were obtained from Merck, Sharp & Dohme (Rahway, NJ). All other chemicals were obtained from Sigma Chemical (St. Louis, MO) All culture media and supplements were from Gibco Laboratories (Burlington, Ontario, Canada).

Cell culture. SK-N-SH cells were grown in an α-minimum essential medium supplemented with (5% v/v) fetal bovine serum and 10 μg/ml gentamycin by the methods of Fisher and Heacock (1988). Cultures were incubated at 37°C in humidified air (95:5, air:CO_2). Cells were trypsinized (0.05% w/v) and passaged once weekly (1 into 4) and fed every third day until confluence (~1 wk). Cells from passage 3 to 25 were used for experiments.

Fluorescence measurements of $[Ca^{++}]_i$. Medium was aspirated from the culture flasks and cells were then mechanically resuspended in fresh medium containing the acetoxymethyl ester of fura-2 (2 μM) or fluo-3 (2 μM) and incubated for 30 min at 33°C. The organic anion-transport inhibitor, probenecid (2 mM) was present

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ABBREVIATIONS: N_2O, nitrous oxide; $[Ca^{++}]_i$, cytosolic free calcium concentration; DCB, 3,4-diclorobenzamil; MIA, methylisobutylamiloride; bisoxonol, bis (1,3-dibutylthiobarbituric acid)-trimethine oxonol; Caq, aqueous concentration.

while cells were loaded with fluo-3 and throughout these experiments to inhibit efflux of fluo-3. Cells were then harvested, sedimented (400 × *g* for 5 min) and washed twice in standard buffer (consisting of (in mM), NaCl 142, KCl 5.6, D-glucose 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1, $NaHCO_3$ 3.6, sodium N-hydroxyethylpiperazine-N′2-ethanesulfonic acid 30, adjusted to pH 7.3). Cells were then suspended in fresh buffer at 2×10^6 cells/ml and aliquoted (1 ml) into microcentrifuge tubes and placed on a rotator for 30 to 40 min at room temperature before use.

SK-N-SH cells (2×10^6 cells) were suspended in 2 ml of the indicated buffer, which was constantly stirred and maintained at 37°C. $[Ca^{++}]_i$ was monitored using a Hitachi F-4010 spectrofluorimeter. For fura-2 experiments, excitation and emission wavelengths were 340 and 505 nm, respectively. Slit widths were 10 nm (excitation) and 20 nm (emission). To avoid quenching of the fura-2 dye by contaminating heavy metals, buffer was supplemented with the selective heavy metal chelator, dimethylenetriaminepenta-acetic acid anhydride (0.3 mM). After each experiment, digitonin (250 μM) was added to permeabilize cells and saturate the dye with Ca^{++}, giving maximum fluorescence (F_{max}). Subsequent addition of $MnCl_2$ (10 mM) yielded autofluorescence (F_{auto}). The fluorescence of the free dye was calculated from the system constant $(F_{max}\text{-}F_{auto})/(F_{min}\text{-}F_{auto})$, which was determined as described previously (Rink *et al*. 1983). Apparent $[Ca^{++}]_i$ corresponding to the fluorescence intensity, F, was then determined using the relation $[Ca^{++}]_i = K_d\ (F\text{-}F_{min})/(F_{max}\text{-}F)$ with the K_d for fura-2-Ca^{++} taken as 224 nM (Grynkiewicz *et al*. 1985).

For fluo-3 experiments, excitation and emission wavelengths were 510 and 525 nm respectively. Slit widths were 5 nm (excitation) and 5 nm (emission). Calibration of the fluorescence signal was performed by adding digitonin (250 μM) to lyse the cells and thereby obtain F_{max}. For fluo-3, autofluorescence equals F_{min} and was determined using unloaded cells. Apparent $[Ca^{++}]_i$ corresponding to the fluorescence intensity, F, was then determined using the relation $[Ca^{++}]_i = K_d\ (F\text{-}F_{min})/(F_{max}\text{-}F)$ with the K_d for fluo-3-Ca^{++} taken as 864 nM (Merritt *et al*. 1990).

Measurement of membrane potential. Membrane potential was measured fluorimetrically using bis (1,3-dibutylthiobarbituric acid)-trimethine oxonol (bisoxonol) by the method of Rink *et al*. (1980). The dye undergoes potential-dependent distribution between the cytoplasm and the extracellular medium. Depolarization results in increased fluorescence, and hyperpolarization results in decreased fluorescence. Bisoxonol accurately resolves changes in membrane potential that occur over a time scale of tens of seconds to minutes. Bisoxonol (0.3 μM final) was added to cells (2×10^6 cells/ml) suspended in the indicated buffer and placed in the fluorimeter. The dye was allowed to equilibrate for 5 to 6 min, after which a stable baseline was obtained. Cells were then treated with the indicated compounds. Fluorescence was monitored with excitation at 540 nm (5-nm slit width) and emission at 580 nm (5 nm slit width). Calibration of fluorescence *vs*. membrane potential was obtained by adding gramicidin (0.1 μM, final) to cells suspended in standard buffer in which varying amounts of Na^+ were replaced by the impermeant cation N-methyl-D-glucamine$^+$. Membrane potential was then calculated as membrane potential = 60 log $([Na^+]_0/[Na^+ + K^+]_i)$ assuming that Na^+ and K^+ are equally permeable through gramicidin and $[Na^+ + K^+]_i = 142$ mM.

Experimental protocols. To examine the effects of N_2O on $[Ca^{++}]_i$, 1 ml samples of cells loaded with either fura-2 or fluo-3 were sedimented (400 × *g* for 30 sec) and resuspended in 2 ml of N_2O-equilibrated buffer in a modified acrylic fluorescence cuvette capped with a rubber stopper fitted with an exhaust/pipette access port and a 2 mm (i.d.) polyethylene N_2O delivery line. The cuvette apparatus allowed the application of agonist although maintaining a slight positive pressure of N_2O gas in the space above the cell suspension. Equilibration of the buffer was achieved by bubbling 100% N_2O (2–3 psi) into 40 ml of the indicated buffer for at least 1 hr. Cells were incubated (10 min) with or without N_2O in the buffer and then stimulated with indicated test substance. For experiments conducted in the absence of extracellular Ca^{++}, cells were sedimented (400 × *g* for 30 sec) and resuspended immediately before use in 2 ml of Ca^{++}-free buffer supplemented with EGTA (0.5 mM) to chelate residual Ca^{++}. For experiments conducted in nominally Na^+-free buffer, Na^+ was replaced by N-methyl-D-glucamine$^+$ to maintain isotonicity. To investigate the effects of ouabain, cell cultures were pretreated with ouabain before dye loading. Briefly, culture medium was aspirated from the culture flasks and cells were mechanically resuspended in fresh medium containing ouabain (100 μM) or vehicle and incubated for 12 hr.

Determination of N_20 concentration. N_2O concentration in the equilibrated buffer was determined using a Puritan-Bennett gas monitor for N_2O. To convert between partial pressure in absolute atmospheres (P% atm) and aqueous concentrations (Caq), the method of Franks and Lieb (1993) was used as follows: Caq (mM) = 122 λ (P % atm)/(273 +Tc), where Tc is the buffer temperature (C°) and the Ostwald water/gas partition coefficient (λ) for N_2O is reported to be ≈0.48 (Gabel and Schultz, 1973, Kozam *et al.*, 1970)

Statistical analysis. Statistical differences were assessed using unpaired two-tailed Student's *t* test, or one-way analysis of variance followed by the Bonferonni post test for multiple comparisons. Data are reported as means ± S.E.M., where *n* is the number of samples from at least three independent experiments. For all statistical tests, a $P < .05$ was considered significant.

Results

Fura-2-loaded SK-N-SH cells were suspended in standard buffer and stimulated with carbachol (5 μM) in the absence (fig. 1Ai) or presence (fig. 1Aii) of N_2O. Responses were quantified as the $[Ca^{++}]_i$ at the peak of the Ca^{++} transient induced by carbachol. In the presence of N_2O, the magnitude of the Ca^{++} response to carbachol was significantly reduced (fig. 1B, $P < .02$, $n = 14$). The primary effect N_2O was to blunt the initial phase of the Ca^{++} transient (≈1 min) without substantially altering the shape of the subsequent response (*cf*. traces i and ii in fig. 1A)[4].

The P% atm of N_2O in buffer samples was 33 ± 2% ($n = 3$). From this the Caq of N_2O was calculated to be ≈ 7 mM. The osmolarity and pH of N_2O-equilibrated buffer was not different from that of standard control buffer.

To test whether the effects of N_2O were reversible, cell suspensions were exposed to N_2O which was subsequently removed by degassing. Degassing was aided by creating negative pressure within the fluorimetric cuvette via an attached vacuum line for 15 min. The response of degassed cell suspensions to carbachol (5 μM) was not significantly different than that of controls (cells suspended in standard buffer). In degassed samples, carbachol caused an increase in $[Ca^{++}]_i$ from basal levels of 100 ± 8 nM to peaks of 344 ± 34 nM ($n = 7$); similarly in control samples, carbachol caused an increase in $[Ca^{++}]_i$ from basal levels of 94 ± 10 nM to peaks of 363 ± 20 nM ($n = 10$). We conclude that the effects of N_2O on carbachol-stimulated increase in $[Ca^{++}]_i$ are readily reversible. To further rule out possible artifacts, we examined the response of cells suspended in standard buffer that was equilibrated with air instead of N_2O. In contrast to the inhibitory effect of N_2O shown in figure 1, the response of cells suspended in air-equilibrated buffer was not significantly

[4] In this series of experiments, basal Ca^{++} levels were also reduced by N_2O (fig. 1B). However, in all subsequent experiments N_2O did not significantly alter resting levels of $[Ca^{++}]_i$.

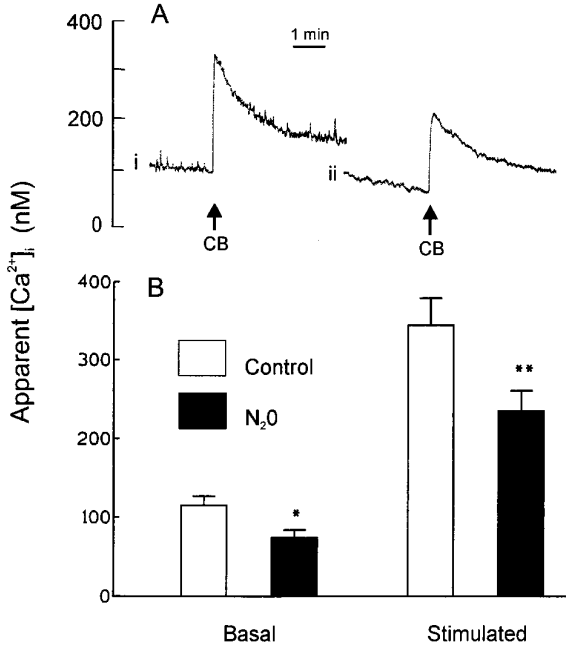


Fig. 1. Effect of N_2O on basal Ca^{++} levels and carbachol-stimulated Ca^{++} response. increases in $[Ca^{++}]_i$. Fura-2-loaded cells were suspended in standard buffer with or without N_2O and $[Ca^{++}]_i$ was monitored by fluorescence spectrophotometry as described in "Materials and Methods." A, tracing (i) illustrates representative control response to the addition of carbachol (CB; 5 μM) at the time indicated. Tracing (ii) illustrates representative response to carbachol (5 μM) at the time indicated for cells suspended in N_2O-equilibrated buffer. The histogram in B summarizes the effects of N_2O on basal and carbachol-stimulated increases in $[Ca^{++}]_i$. Stimulated $[Ca^{++}]_i$ was defined as the peak height of the Ca^{++} transient after the addition of carbachol. Data are presented as means ± S.E.M., $n = 14$. The asterisk indicates values significantly different from basal control ($P < .02$). The double asterisk indicates values significantly different from stimulated control ($P < .02$).

different than that of controls. In air equilibrated samples, carbachol caused an increase in $[Ca^{++}]_i$ from basal levels of 127 ± 19 nM to peaks of 395 ± 43 nM ($n = 4$).

Responses were compared in the absence of extracellular Ca^{++} to examine the effects of N_2O on Ca^{++} release from intracellular stores (fig. 2). N_2O had no effect on carbachol-stimulated increases in $[Ca^{++}]_i$ when cells were suspended in Ca^{++}-free buffer supplemented with 0.5 mM EGTA ($n =$ 10–14). These data suggest that carbachol-stimulated release of Ca^{++} from intracellular stores is unaffected by N_2O.

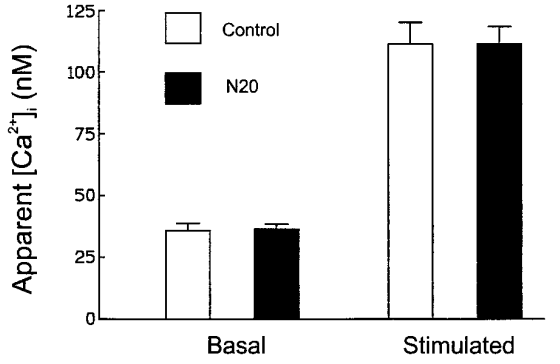


Fig. 2. Effect of N_2O on basal Ca^{++} levels and carbachol-stimulated Ca^{++} response in the absence of extracellular Ca^{++}. Cells were suspended in Ca^{++}-free buffer supplemented with 0.5 mM EGTA with or without N_2O. Changes in $[Ca^{++}]_i$ were monitored as described in "Materials and Methods." Data are presented as the mean ± S.E.M., $n = 11$ to 14.

We next examined the effect of N_2O on the Ca^{++} elevation induced by the Ca^{++} ionophore, ionomycin. First we determined the concentration of ionomycin that induced a transient Ca^{++} response comparable to that induced by 5 μM carbachol (fig. 3). Ionomycin (10^{-9}-10^{-7} M) induced transient elevations of $[Ca^{++}]_i$ in a concentration-dependent manner. An ionomycin concentration of 25 nM was found to produce a $[Ca^{++}]_i$ response similar to that induced by 5 μM carbachol.

N_2O significantly suppressed the increase in $[Ca^{++}]_i$ induced by ionomycin (25 nM) (fig. 4). N_2O inhibited the magnitude of the ionmycin-induced Ca^{++} transient to a similar extent as that induced by carbachol (5 μM) (28 *vs.* 31%, respectively). In contrast, the smaller Ca^{++} transient induced by ionomycin (5 nM) was not significantly affected by N_2O (peak Ca^{++} elevations were 39 ± 3 nM and 45 ± 5 nM above basal levels in the absence and presence of N_2O, $n = 8$).

We also examined the effect of N_2O on the Ca^{++} transient induced by KCI (100 mM). KCI elevates $[Ca^{++}]_i$ by activation of voltage-operated Ca^{++} channels that have been previously characterized in SK-N-SH cells (Noronha-Blob *et al.* 1989). The Ca^{++} transient induced by KCI-induced depolarization was significantly inhibited by N_2O (peak Ca^{++} elevations were 90 ± 9 and 59 ± 8 nM above basal levels in the absence and presence of N_2O, $n = 9$, $P < .05$).

Thus, Ca^{++} elevations induced by carbachol, ionomycin or KCI were all suppressed by N_2O. These findings suggest that the site of action of N_2O is not a Ca^{++} uptake pathway such as voltage or receptor-operated Ca^{++} channels located on either the plasma membrane or endoplasmic reticulum, but rather a Ca^{++} efflux pathway.

To investigate the possible involvement of Na^+/Ca^{++} exchange, we examined the effects of N_2O on the Ca^{++} response to carbachol in the presence and absence of extracellular Na^+ (fig. 5). Both basal levels and carbachol-stimulated increases in $[Ca^{++}]_i$ were elevated when extracellular Na^+ was replaced with N-methyl-D-glucamine$^+$. However, N_2O-induced inhibition of the carbachol-stimulated increase in

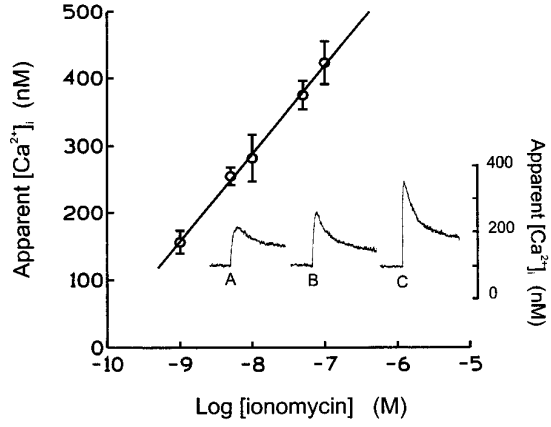


Fig. 3. Ca^{++} responses to ionomycin. $[Ca^{++}]_i$ was monitored as described in "Materials and Methods." Cells were challenged with a single addition of ionomycin at the concentrations indicated. Data are elevations of $[Ca^{++}]_i$ measured at the peak height of the Ca^{++} transient after the addition of ionomycin (means ± S.E.M, $n = 4$–5). The data were fitted by least-squares. Inset illustrates representative responses to ionomycin (A 5 nM, B 50 nM, C 100 nM). From these data, an ionomycin concentration (25 nM) was chosen to yield a response comparable to that produced by 5 μM carbachol.

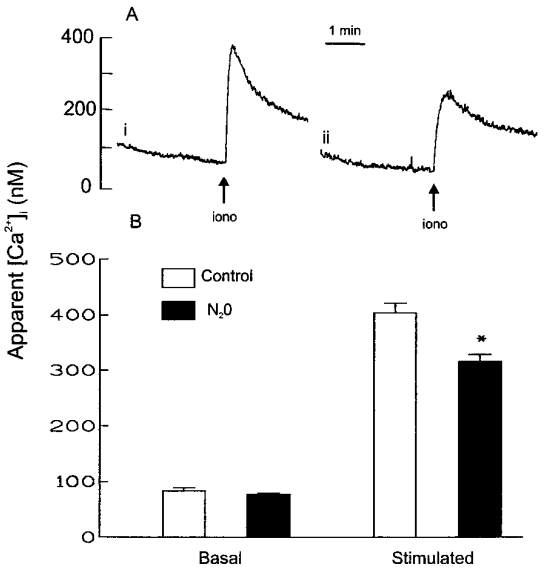


Fig. 4. Effect of N_2O on basal Ca^{++} levels and ionomycin-induced Ca^{++} transients. Fura-2-loaded cells were suspended in standard buffer with or without N_2O and $[Ca^{++}]_i$ was monitored by fluorescence spectrophotometry as described in "Materials and Methods." A, Traces illustrate representative responses to the addition of ionomycin (iono; 25 nM) in the absence (i) or presence (ii) of N_2O. B, Histogram summarizes the effects of N_2O on basal Ca^{++} levels and ionomycin-induced Ca^{++} transients. Data are means ± S.E.M., $n = 10$. The asterisk indicates significant difference from control, $P < .003$.

$[Ca^{++}]_i$ was attenuated when cells were suspended in nominally Na^+-free buffer (fig. 5B, $n = 18–19$).

To investigate further the dependence on Na^+ gradient, responses of cells pretreated with ouabain (100 μM for 12 hr) were compared to those of vehicle-treated controls (fig. 6). Ouabain inhibits the plasma membrane Na^+/K^+-ATPase causing cells to become Na^+ loaded. When cells were pretreated with ouabain, both basal Ca^{++} levels and carbachol-stimulated increases in $[Ca^{++}]_i$ were not significantly different than in vehicle-treated control cells. However, pretreatment with ouabain did attenuate the inhibitory effect of N_2O on the carbachol-stimulated increase in $[Ca^{++}]_i$.

DCB is a selective inhibitor of the plasma membrane Na^+/Ca^{++} exchanger (Kaczorowski *et al.*, 1988). The effects of DCB on $[Ca^{++}]_i$ were measured using fluo-3, because DCB is fluorescent at the wavelengths used for fura-2. DCB (1.45 μM), added 5 min before carbachol, had no significant effect on either basal Ca^{++} levels or carbachol-stimulated increases in $[Ca^{++}]_i$ (fig. 7). However, N_2O-induced inhibition of the carbachol-stimulated increase in $[Ca^{++}]_i$ was significantly attenuated in the presence of DCB, ($P < .01$, $n = 10$). The effect of DCB was most clearly evident when carbachol-induced changes in $[Ca^{++}]_i$ (*i.e.*, peak Ca^{++} elevations above basal levels) were compared (fig. 7C).

MIA is a compound that is structurally related to DCB, however, it is a highly specific Na^+/H^+ exchange inhibitor (Moffat and Karmazyn, 1993). MIA was therefore used to examine the specificity of the DCB effects. The effects of N_2O on carbachol-stimulated increases in $[Ca^{++}]_i$ in the absence and presence of MIA (1 μM) were compared (fig. 8). MIA had no effect on either basal Ca^{++} levels or carbachol-stimulated increases in $[Ca^{++}]_i$. Furthermore, the ability of N_2O to inhibit the Ca^{++} response was unaffected by MIA ($n = 8–9$).

Representative tracings illustrating the effects of N_2O and carbachol (5 μM) on membrane potential are presented in figure 9. The resting membrane potential in control cell suspensions was -34 ± 3 mV ($n = 5$). A significantly more negative membrane potential was observed when cells were suspended in N_2O-equilibrated buffer, -43 ± 4 mV ($P < .003$, $n = 5$). In response to carbachol (5 μM), an increase in fluorescence, which indicates depolarization of the membrane potential, was observed. The depolarization induced by carbachol was significantly less in the presence of N_2O (4 ± 1 mV) compared to controls (7 ± 2 mV, $P < .05$, $n = 5$).

Discussion

The hypothesis that general anesthesia could be mediated by mechanisms dependent on intracellular Ca^{++} was first proposed by Krnjevic in 1974. It is now well known that Ca^{++} plays a fundamental role in the initiation and regulation of many neuronal processes that may be related to anesthesia. Our study examined the effects of N_2O on the mobilization and regulation of $[Ca^{++}]_i$ in the neuroblastoma cell line SK-N-SH. N_2O reversibly suppressed carbachol-stimulated increases in $[Ca^{++}]_i$. Some of the mechanisms by which this suppression could occur include. 1) Reduced Ca^{++} uptake from the extracellular fluid through receptor or voltage operated Ca^{++} channels. 2) Reduced Ca^{++} release from intracellular stores. 3) Enhanced Ca^{++} sequestration by intracellular organelles such as the endoplasmic reticulum and mitochondria. 4) Enhanced Ca^{++} sequestration by Ca^{++}

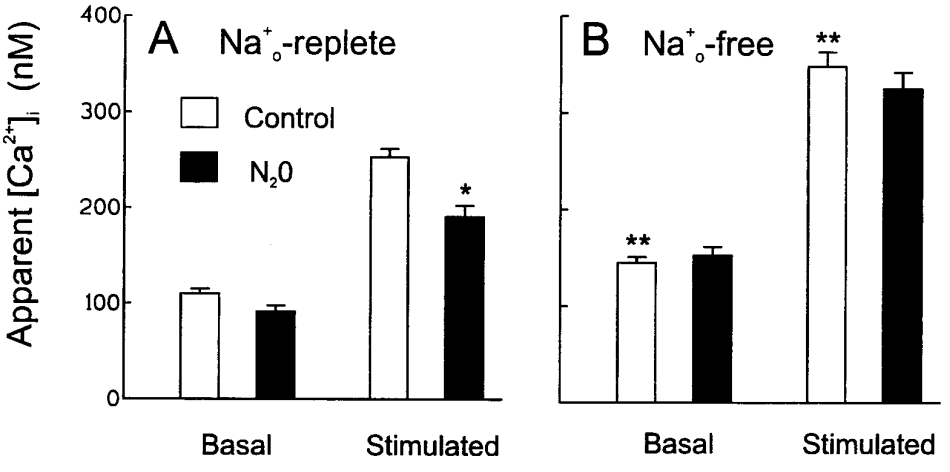


Fig. 5. Effect of N_2O on basal Ca^{++} levels and carbachol-stimulated Ca^{++} response dependence on extracellular Na^+. Fura-2-loaded cells were suspended in standard buffer (Na^+_o-replete) or in nominally Na^+-free buffer in which Na^+ was replaced with N-methyl-D-glucamine$^+$ (Na^+_o-free). Ca^{++} responses to carbachol (5 μM) were monitored in the presence and absence of N_2O. Histograms summarize the effects of N_2O on basal Ca^{++} levels and carbachol-stimulated peak increases in $[Ca^{++}]_i$. Data are means ± S.E.M., $n = 18$. The asterisk indicates value significantly different from control $P < .01$. The double asterisk indicates values significantly different from controls in A ($P < .001$).

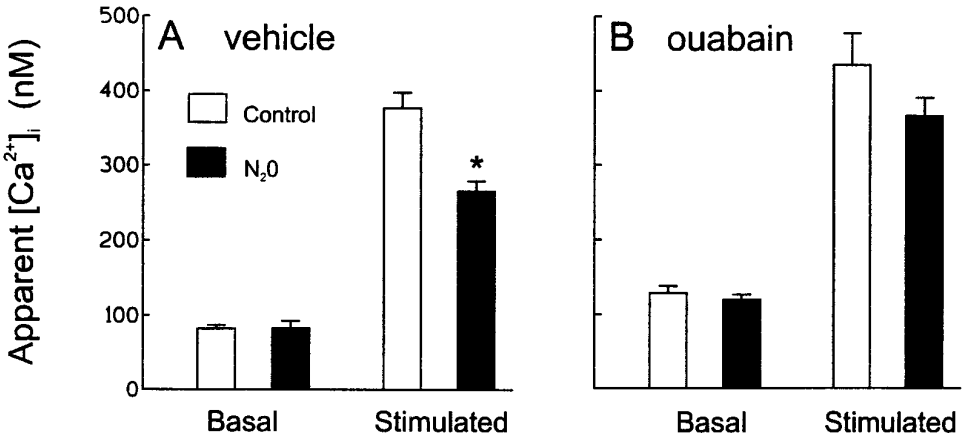


Fig. 6. Effect of ouabain on N_2O-induced inhibition of the carbachol-stimulated Ca^{++} response. Cell cultures were pretreated with ouabain (100 μM, 12 hr) (B) or vehicle (A). Cells were then loaded with fura-2 and suspended in standard buffer with or without ouabain (100 μM). $[Ca^{++}]_i$ responses to carbachol (5 μM) were monitored in the presence and absence of N_2O. Histograms summarize the effects of N_2O on basal Ca^{++} levels and carbachol-stimulated peak increases in $[Ca^{++}]_i$. Data are means ± S.E.M., $n = 8$ to 9. The asterisk indicates value significantly different from control $P < .05$.

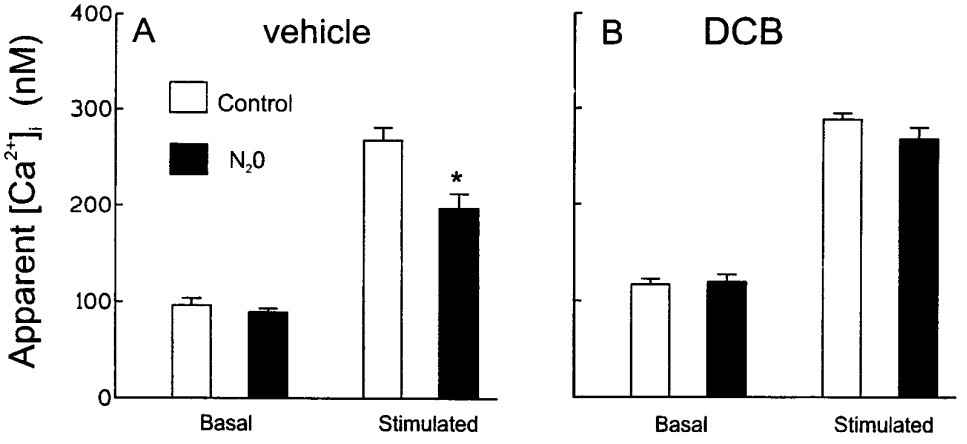
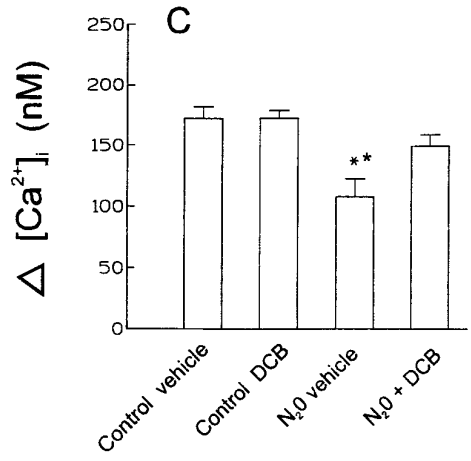


Fig. 7. Effect of DCB on N_2O-induced inhibition of the Ca^{++} response to carbachol. Cells were loaded with fluo-3 and suspended in standard buffer with or without N_2O. Vehicle (A) or DCB (1.45 μM, B) was added 5 min before carbachol (5 μM). Histograms A and B summarize the effects of N_2O on basal Ca^{++} levels and carbachol-stimulated peak increases in $[Ca^{++}]_i$. C illustrates the maximum increase in $[Ca^{++}]_i$ above basal levels in response to carbachol under each of the four experimental conditions. Data are means ± S.E.M., $n = 10$. The asterisk indicates value significantly different from control in A and B ($P < .05$). The double asterisk indicates significant difference from control vehicle in C ($P < .001$).



binding proteins. 5) Enhanced Ca^{++} extrusion via the plasma membrane Ca^{++}-ATPase and/or Na^+/Ca^{++} exchanger.

Ionomycin elevates $[Ca^{++}]_i$ electroneutrally by mediating the exchange H^+ for Ca^{++} with a 2:1 stoichiometry (Woolley *et al.*, 1995). Ionomycin causes an increase in $[Ca^{++}]_i$ without activation of Ca^{++} channels in the plasma membrane or the endoplasmic reticullum. N_2O inhibited the ionomycin-induced increase in $[Ca^{++}]_i$ to the same extent as when cells were stimulated with carbachol or high $[K^+]_o$, suggesting that N_2O enhances sequestration or extrusion of Ca^{++} from the cytosol.

The involvement of Na^+/Ca^{++} exchange was explored under several experimental conditions that affect the inwardly directed transmembrane Na^+ electrochemical gradient. When the Na^+/Ca^{++} exchanger extrudes Ca^{++}, energy is supplied by the Na^+ electrochemical gradient that is maintained by the plasma membrane Na^+/K^+ ATPase (Miller 1991). In our study, when extracellular Na^+ was removed and replaced with the large impermeant cation N-methyl-D-

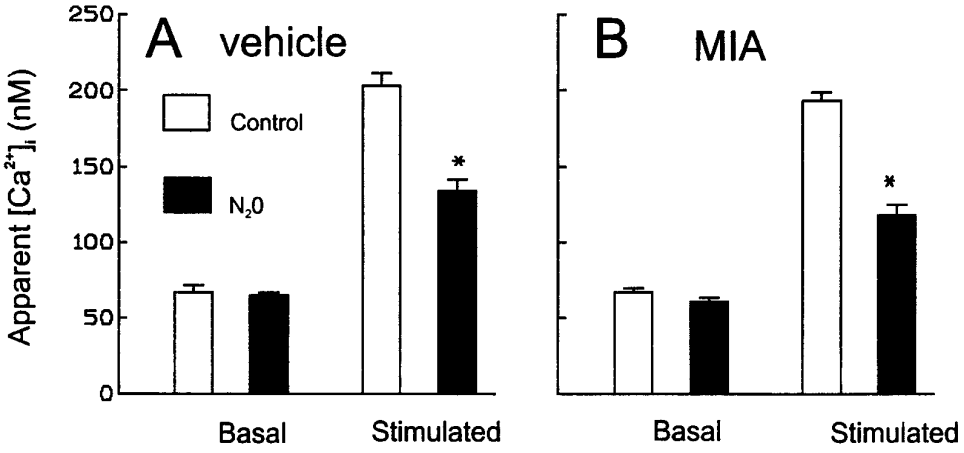


Fig. 8. Effect of MIA on N_2O-induced inhibition of the Ca^{++} response to carbachol. Cells were loaded with fura-2 and suspended in standard buffer with or without N_2O. Vehicle (A) or MIA (1 μM, B) was added 5 min before carbachol (5 μM). Histograms summarize the effects of N_2O on basal Ca^{++} levels and carbachol-stimulated peak increases in $[Ca^{++}]_i$. Data are means ± S.E.M., n = 8 to 9. The asterisk indicates significant difference from appropriate control, $P < .001$.

glucamine$^+$, the inhibitory effects of N_2O were attenuated. Similarly, ouabain blockade of the plasma membrane Na^+/K^+-ATPase which results in Na^+-loading (Blaustein *et al.*, 1991) attenuated the inhibitory effects of N_2O. Taken together, these findings are consistent with the action of N_2O on $[Ca^{++}]_i$ involving a Na^+-dependent Ca^{++} transport mechanism such as Na^+/Ca^{++} exchange.

DCB, a derivative of amiloride, is a potent and selective inhibitor of the Na^+/Ca^{++} exchanger and has been used by a number of investigators to probe the function of the transporter (Nakamura *et al.*, 1992, Phillipson and Nicoll, 1993). In our study, DCB attenuated the effects of N_2O on carbachol-stimulated increases in $[Ca^{++}]_i$. The concentration of DCB used (1.45 μM) was below those (≥40 μM) that have been reported to inhibit K^+ channels, voltage-operated Ca^{++} channels and the Na^+/H^+ exchanger (Kaczorowski *et al.*, 1988). MIA, like DCB, is an amiloride derivative, but is a highly selective Na^+/H^+ exchange inhibitor (Moffat and Karmazyn, 1993). In our study, MIA had no effect on the ability of N_2O to inhibit carbachol-stimulated increases in $[Ca^{++}]_i$, ruling involvement of Na^+/H^+ exchange and supporting Na^+/Ca^{++} exchange as the target for N_2O.

The Na^+/Ca^{++} exchanger has a low affinity, but large transport capacity for Ca^{++}, and is therefore active after large increases in $[Ca^{++}]_i$ (Kress and Tass, 1993). These properties are consistent with the effect of N_2O on the larger Ca^{++} response elicited by 25 nM ionomycin, but not the smaller response elicited by 5 nM ionomycin. They are also in keeping with the observation that N_2O blunts the initial phase of the Ca^{++} transient induced by either carbachol or ionomycin. Similarly, the lack of effect of N_2O on cells suspended in Ca^{++}-free buffer may be due to the modest size of the Ca^{++} transient elicited by carbachol under these conditions.

Na^+/Ca^{++} exchange involves the transmembrane movement of Ca^{++} coupled to the reciprocal movement of Na^+ with a stoichiometry of 3 Na^+ to 1 Ca^{++}, reflecting its electrogenic properties (Carafoli 1987). Therefore, the magnitude of Ca^{++} flux during Na^+/Ca^{++} exchange is determined not only by the relative magnitude of the Na^+ and Ca^{++} gradients but also by membrane potential. The membrane potential of SK-N-SH cells was slightly hyperpolarized by N_2O. Hyperpolarization favours Na^+/Ca^{++} exchange and could contribute to the inhibitory effect of N_2O on carbachol-stimulated increases in $[Ca^{++}]_i$. This possibility is in keeping with the observation that Na^+/Ca^{++} exchange activity during the passage of an action potential in squid axon increased maximally at the end of the action potential when the membrane was hyperpolarized (Blaustein 1988).

Na^+/Ca^{++} exchange has been shown to play a significant role in regulating $[Ca^{++}]_i$ in a variety of cell types including neurons. For example, Na^+/Ca^{++} exchange at mammalian nerve terminals may play an important role in the swift termination of neurotransmitter release. In agreement with this hypothesis, Payza and Russell (1991) demonstrated that extracellular Na^+ inhibits hormone release and stimulates Ca^{++} efflux from rat neurosecretosomes by promoting Na^+/Ca^{++} exchange. Similarly, Thompson (1994) showed that under normal conditions Na^+/Ca^{++} exchange is effective in limiting Ca^{++} accumulation near the membrane in molluscan neurons. It has also been suggested that during firing activity, the rise in intracellular Na^+ produces less efficient Ca^{++} extrusion by the exchanger and thus potentiates intracellular Ca^{++} signals and Ca^{++}-mediated phenomenon such as neurotransmitter release (Kaczorowski *et al.*, 1988). Similarly, Lin and coworkers (1994) using bovine chromaffin cells showed that changes in Na^+/Ca^{++} activity can alter the rate at which elevated $[Ca^{++}]_i$ returns to its basal level and can consequently alter the cellular response to subsequent stimulation.

The effects of other anesthetics on Na^+/Ca^{++} exchange have been examined previously. The local anesthetics procaine and lidocaine promote Ca^{++} efflux via Na^+/Ca^{++} exchange from cultured human glial cells (Kim-Lee *et al.*, 1994). Haworth *et al.* (1989) reported that octanol and pentobarbital inhibit Ca^{++} influx in Na^+-loaded rat myocardial

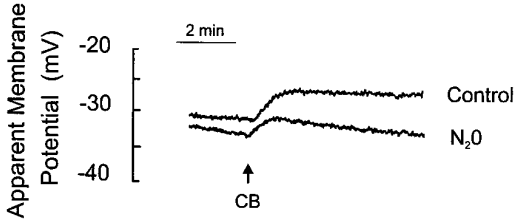


Fig. 9. Effects of N_2O and carbachol on membrane potential. Cells were suspended in standard buffer with or without N_2O and membrane potential was monitored fluorimetrically using bisoxonol as described in "Materials and Methods." Carbachol (CB, 5 μM) was added at the time indicated. Tracings are representative of responses from at least five separate cell preparations.

cells. This same group described the inhibitory effects of the volatile anesthetics halothane, enflurane and isoflurane on Na^+/Ca^{++} exchange activity in rat sarcolemmal vessel preparations. More recently, Baum and Wetzel (1994) have shown that halothane reversibly inhibits the Na^+/Ca^{++} exchange current in neonatal rabbit ventricular myocytes. Thus, it appears that anesthetics, with different chemical and pharmacological profiles are capable of significantly affecting Na^+/Ca^{++} exchange, whether by enhancing or inhibiting its activity.

Our findings indicate that N_2O inhibits carbachol-stimulated increases in $[Ca^{++}]_i$ in SK-N-SH cells by enhancing Na^+/Ca^{++} exchange activity. Perturbations of the membrane lipid environment have been shown to affect Na^+/Ca^{++} exchange. More specifically, it has been shown that anionic lipid components activate Na^+/Ca^{++} exchange and do so more potently, if they also cause disordering of the lipid bilayer (Luciani *et al.*, 1991, Phillipson and Nicoll, 1993). However, the possibility of a direct protein target for N_2O is supported by the findings of Dong *et al.* (1994), who showed using infrared spectroscopy that N_2O interacts with both soluble and membrane-bound proteins directly occupying sites within the interior of these proteins. Whether N_2O acts directly on the Na^+/Ca^{++} exchange protein or accessory proteins or indirectly by perturbing the lipid environment of the exchanger requires further investigation.

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