Inhibition of Na-Ca Exchange by General Anesthetics

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General anesthetics, typically octanol, were found to inhibit the influx of calcium in isolated sodium-loaded adult rat heart cells, using $^{45}$Ca, quin 2, or indo 1. Inhibition by octanol, like inhibition by sodium, was competitive with calcium. Octanol and sodium together inhibited calcium influx synergistically. At physiological levels of extracellular calcium and sodium, the EC$_{50}$ was 177±37 µM for octanol and 48±5 µM for decanol. These values are threefold to fourfold larger than those reported to cause 50% loss of righting reflex in tadpoles, a measure of their anesthetic effectiveness. We conclude that general anesthetics inhibit Na-Ca exchange at the sarcolemma. We suggest that octanol inhibits like sodium, and the synergism stems from the cooperativity of sodium inhibition at the binding and regulatory sites of the exchanger. Insofar as Na-Ca exchange may regulate inotropy, the inhibition of Na-Ca exchange by general anesthetics could contribute to their negative inotropic effect. (Circulation Research 1989;65:1021-1023)

The mechanism of the Na-Ca exchanger and its role in excitation-contraction coupling in the heart has been intensively investigated in recent years (reviews, References 1-3). Several inhibitors of Na-Ca exchange are known though none of them is specific. The list includes competing ions such as manganese, amiloride analogues such as 3',4'-dichlorobenzamil, and cationic amphiphiles such as dodecylamine. Inhibition by the latter had two interesting corollaries: anionic amphiphiles stimulated the rate of Na$^+$-Ca$^{2+}$ exchange, and the effectiveness of either amphiphile was increased as the length of the alkyl chain increased. This suggested a profound influence of the membrane phospholipid environment on the activity of the exchanger. Uncharged amphiphiles, on the other hand, had no effect though some inhibition of Na-Ca exchange activity by neutral general anesthetics has been reported briefly. General anesthetics are known to depress contractility in the myocardium. We report here that general anesthetics can exert a profound inhibition of calcium influx by Na-Ca exchange and that this effect is most apparent under conditions where sodium is competing with calcium for the exchanger. These conditions, which are physiological for cells (high extracellular sodium and calcium), are not usually used when Na-Ca exchange kinetics are measured in vesicles.

Materials and Methods

Cell Isolation

Suspensions of quiescent calcium-tolerant adult heart cells were isolated from female retired breeder rats as previously described.

Experimental Medium

Cells were suspended (16 mg protein/ml) in a medium containing (mM) NaCl 118, KCl 4.8, N$_2$-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 25, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, EGTA 0.25, pyruvic acid 5, glucose 11, and insulin 1 (µM), adjusted to pH 7.4 with NaOH. Suspensions were maintained aerobic by equilibration with air in a shaking incubator at 37° C.

Sodium Loading of Cells

Cells in experimental medium were loaded with sodium by incubation for 30 minutes at 37° C in the presence of 1 mM ouabain. We have previously found that such treatment increases intracellular sodium levels from 17 mM up to 94 mM. A major route for sodium entry under these conditions is Na-H exchange. Sodium entry by calcium channels is less significant since it is only large when magnesium as well as calcium is absent. Cells were put on ice, ruthenium red was added (5 µM, see dilution media below), and experiments began after 15 minutes. Aliquots for dilution were removed over the next 40 minutes. We have not been able to detect any change in the rate of calcium uptake on...
dilution over the course of this time; this observation indicates no further change in the sodium-loaded state of the cells.

**Sodium Depletion of Cells**

Cells in experimental medium were depleted of sodium by washing three times in sodium-free experimental medium. This medium had sodium chloride replaced by choline chloride and was neutralized by tetramethylammonium (TMA) hydroxide. Measurements with $^{22}$Na (not shown) indicated that intracellular sodium was quickly removed under these conditions.

**Dilution Media**

Normal sodium medium: experimental medium containing 1 mM ouabain, 5 µM ruthenium red, and CaCl$_2$ to give the free-calcium levels shown in the figures. Ruthenium red was included to prevent any possible calcium uptake by the mitochondria of damaged cells.

Sodium-free medium: sodium medium with sodium chloride replaced by choline chloride and neutralized by TMA hydroxide.

Intermediate levels of sodium concentration were obtained with mixtures of the above two media.

**Measurement of Cell $^{45}$Ca Content**

Sodium-loaded cells at time zero were added to 4 vol (Figure 1) or 9 vol (Figures 2 and 5) dilution medium containing tritiated water (1 µCi/ml) and $^{45}$Ca (0.1 µCi/ml) at 37°C. Aliquots (0.5 ml) were removed at time intervals and centrifuged through a layer of bromododecane, as described previously.$^{10}$

**Measurement of Intracellular Calcium Levels With Quin 2 and Indo 1**

Cells were labeled with dye during sodium loading by incubation with 30 µM quin 2-AM (the acetoxymethyl ester of quin 2) or 5 µM indo 1-AM. Cells were washed twice in the presence of ouabain and put on ice. Before use, 2.5 ml cells were washed once more by allowing them to settle in the cuvette for 17 minutes on ice and removing 2.3 ml supernatant. The cells were resuspended to 2.5 ml in experimental medium with ouabain at 37°C (approximately 1.6 mg/ml), and the cuvette was placed in a fluorescence spectrophotometer (SPEX Fluorolog, SPEX Industries, Edison, New Jersey). Quin 2 fluorescence was measured on stirred suspensions at 37°C at 339 nm excitation λ and 500 nm emission λ, with a slit width of 4 nm. Indo 1 fluorescence was measured at 310 nm excitation λ and 400 nm emission λ. At the end of the tracing, the corresponding level of intracellular free calcium was calculated from fluorescence values before and after sequential addition of manganese, diethylenetriaminepentaaetic acid, digitonin, and EGTA, as previously described.$^{10}$

**Results**

When sodium-loaded cells were diluted into a sodium-free medium containing 1 mM calcium, a rapid and massive uptake of $^{45}$Ca occurred (Figure 1A), as previously observed.$^{10}$ With the dilution factor of 1:5 the final sodium concentration in this experiment was 28 mM. Note that in this and all subsequent experiments ouabain was present in the dilution medium to ensure continued inactivation of the sodium pump. Under these conditions calcium uptake is strongly favored by Na-Ca exchange because of the reversed sodium gradient. When octanol was included in the sodium-free dilution medium to give a final concentration of 0.8 mM on addition of cells, $^{45}$Ca uptake was inhibited by about 30% (Figure 1A). Sodium-depleted cells, on the other hand, showed very little $^{45}$Ca uptake when diluted into a sodium medium containing 1 mM calcium (Figure 1A), as expected from the opposite sodium gradient, which will strongly disfavor net calcium entry by Na-Ca exchange. Thus, the $^{45}$Ca uptake measured under the latter conditions prob-
ably corresponds to extracellular calcium binding and calcium binding to dead cells. This was confirmed by the observation (not shown) that depletion of cells of both sodium and ATP, by incubation of sodium-depleted cells with rotenone (3 μM) plus p-trifluoromethoxyphenylhydrazone (FCCP) (0.3 μM) for 10 minutes, did not further decrease the uptake of 45Ca when cells were diluted into sodium medium containing 1 mM Ca. We have previously shown10 that such ATP depletion is by itself a very powerful inhibitor of calcium entry by Na-Ca exchange. We may, therefore, justifiably consider the uptake beyond that of sodium-depleted cells (Figure 1A) as being intracellular calcium accumulation. A similar baseline value could be measured directly in sodium-loaded cells by prior ATP depletion plus 1 mM octanol, and this condition was also used in some experiments (Figures 2 and 4).

When sodium-loaded cells were diluted into a normal sodium medium containing 1 mM calcium, a large 45Ca uptake was also observed (Figure 1B) though, as previously10, the uptake was not as rapid as that by cells diluted into a sodium-free medium. In the presence of 0.8 mM octanol, however, 45Ca uptake was inhibited by about 80% under these conditions (Figure 1B).

Since the 45Ca uptake minus that of sodium-depleted cells was near linear for the initial 30 seconds (Figure 1A) and for 1 minute (Figure 1B), this difference, at these times, was used to measure the degree of inhibition of 45Ca uptake as octanol levels were varied. Figure 2 shows that octanol was about four times more potent an inhibitor in the normal sodium medium (140 mM) than in the low sodium medium (14 mM after dilution in this experiment).

The rate of calcium uptake by sodium-loaded cells could in principle be limited by the rate of sequestration of cytosolic calcium by organelles. Any inhibition in the rate of uptake could thus reflect inhibition of uptake by such organelles rather than inhibition of entry at the sarcolemma. To investigate this possibility, we first measured the effect of octanol on the rate of rise of intracellular calcium; we used the calcium-sensitive fluorescent dyes quin 2 and indo 1. Figure 3 shows that the rate of calcium influx into the cytosol of sodium-loaded cells is strongly inhibited by 0.8 mM octanol. The level of intracellular calcium under these conditions after 3½ minutes was 450 nM without octanol and 9 nM with octanol, as measured by quin 2 (Figure 3A). With indo 1 (Figure 3B), the values 48 seconds after calcium addition were 225 nM without octanol and 29 nM with octanol. The values with indo 1 are subject to some uncertainty because a fraction of the cellular indo 1 is not released by digitonin and is probably mitochondrial.10 It should also be borne in mind that quin 2 itself inhibits Na-Ca exchange activity in heart cells.10 However, we may clearly conclude that octanol inhibits calcium uptake at the sarcolemma. Next, we investigated the extent to which inhibition of mitochondrial calcium uptake affects the measured initial rate of cellular calcium uptake and the effect of octanol on mitochondrial calcium uptake. We investigated the former by comparing the rate of cellular calcium uptake by sodium-loaded cells with and without inhibition of mitochondrial calcium uptake by use of oligomycin plus FCCP under conditions like those used in the kinetic measurements (Figures 1, 2, and 5). Although FCCP alone is sufficient to block mitochondrial calcium uptake, it also depletes cellular ATP by inducing mitochondrial ATPase activity. The oligomycin was included to block this ATPase. We
found (Figure 4A) that there was a 37% inhibition of the rate of $^{45}$Ca uptake by oligomycin plus FCCP compared with an almost complete inhibition by octanol. The effect of these agents on mitochondrial calcium uptake was then determined by measuring calcium uptake of cells treated with digitonin to rupture the sarcolemma. The normal sodium medium was used (see "Materials and Methods"), but without ruthenium red, which would block mitochondrial uptake if present. We found a 15% inhibition of calcium uptake by octanol and a 96% inhibition of calcium uptake by oligomycin plus FCCP (Figure 4B). Note the change in scales between panel A and panel B. In the experiment in Figure 4A, ATP levels were also measured 6 minutes after addition of calcium and were 11.9±1.3 nmol/mg in the control cells and 5.2±1.1 nmol/mg in cells with oligomycin plus FCCP. We have previously found that complete ATP depletion causes an almost total inhibition of Na-Ca exchange activity. The reduced rate of calcium uptake with oligomycin plus FCCP could, therefore, be due to a reduced rate of entry across the sarcolemma because of ATP depletion. Either way, even a complete inhibition of mitochondrial calcium uptake (Figure 4B) allows a high initial rate of cellular calcium uptake (Figure 4A). Therefore, it is unlikely that the small inhibition of mitochondrial calcium uptake by octanol (Figure 4B) has any influence on the inhibition by octanol of the initial rate of cellular $^{45}$Ca uptake (Figure 4A).

To determine the nature of the inhibition of calcium influx by octanol, we have investigated the kinetics of calcium uptake. We performed some initial experiments to ensure that the measured uptake rates reflected the true initial rate of calcium influx by Na-Ca exchange. First, we determined that rates of calcium uptake were linear to a good approximation under all conditions used (not shown). Second, since the rate of Na-Ca exchange in vesicles has been shown to be promoted by intravesicular calcium,13 we investigated the effect of calcium loading on measured initial uptake rates. This was accomplished by adding 350 μM Ca to the cells during the final 2 minutes of incubation of concentrated cells with ouabain to give a final free calcium of 100 μM. This resulted in calcium uptake sufficient to cause the cells to round up. However, the initial rate of $^{45}$Ca uptake measured subsequently on dilution of the cells was identical to that of cells not preexposed to calcium over the entire range of uptake rates measured (not shown). This may mean either that there was no activation of Na-Ca exchange by intracellular calcium or that, under these conditions, entering calcium caused the activation so quickly that preexposure to calcium made no difference. We favor the latter interpretation, since we have found that quin 2 itself inhibits calcium influx into sodium-loaded cells.10 This finding suggests that intracellular calcium can have an activating effect. Third, we investigated how verapamil affected the measured rate of $^{45}$Ca uptake. Lambert et al14 found that 2 μM verapamil inhibits the rate of rise of intracellular calcium measured by quin 2 when calcium is given to sodium-loaded cells. These authors concluded on the basis of this observation that calcium influx through calcium channels contributes significantly to calcium uptake under these conditions. We also have found that verapamil inhibits the rate of rise of intracellular calcium measured by quin 2 but that the effect of verapamil on the rate of $^{45}$Ca uptake was considerably less. Here we have checked the effect of verapamil on the synergic inhibition of calcium uptake by octanol and sodium. We found no effect of verapamil (2 μM) on the percent inhibition induced by octanol at various levels of extracellular sodium (Table 1); hence, the synergistic effect of sodium and octanol (Figure 2) cannot be related to calcium channel activity. Verapamil did cause a small inhibition of all rates of $^{45}$Ca uptake, as we previously observed. It is not clear whether this is related to calcium channel inhibition or an effect of verapamil directly on Na-Ca exchange. Fourth, to investigate the effect of octanol on sarcolemmal integrity, we looked for octanol-induced changes in the sucrose-permeable space of the cell pellets. For sodium-loaded cells diluted into a sodium-free medium containing 1 mM calcium, sucrose-permeable spaces after 30 seconds were 38.1±0.7% without octanol.

**FIGURE 4.** Graphs showing effect of inhibition of mitochondrial calcium uptake on the rate of cellular calcium uptake. Panel A: Sodium-loaded cells in normal sodium medium were treated with 4 μM oligomycin (oli) plus 0.67 μM p-trifluoromethoxyphenylhydrazone (•, FCCP), with 0.8 mM octanol (△, oct) or with no addition (○) 1 minute before addition of 1 mM labeled calcium at time zero. Baseline is given by sodium-loaded cells depleted of ATP by addition of 3 μM rotenone plus 0.3 μM FCCP 8 minutes before calcium addition and 0.8 mM octanol 1 minute before calcium addition. Panel B: Normal cells in normal sodium medium but without ruthenium red and containing 1 mM calcium were treated with inhibitors, as in panel A, before addition of digitonin (22 μg/ml) at time 0.
Inhibition of Na-Ca Exchange by Anesthetics

TABLE 1. Lack of Effect of Verapamil on Sodium-Octanol Synergism

<table>
<thead>
<tr>
<th>Final calcium (mM)</th>
<th>Final sodium (mM)</th>
<th>Inhibition of calcium uptake rate by 0.8 mM octanol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>-Verapamil: 41.3±5.2 +Verapamil (2 µM): 42.7±6.6</td>
</tr>
<tr>
<td>1</td>
<td>64</td>
<td>-Verapamil: 61.0±9.3 +Verapamil (2 µM): 66.3±1.2</td>
</tr>
<tr>
<td>1</td>
<td>140</td>
<td>-Verapamil: 83.2±11.6 +Verapamil (2 µM): 81.3±4.3</td>
</tr>
<tr>
<td>0.1</td>
<td>14</td>
<td>-Verapamil: 68.7±6.6 +Verapamil (2 µM): 73.7±4.2</td>
</tr>
</tbody>
</table>

Values shown are mean±SD from three experiments. Initial rates of calcium uptake were measured as in Figure 5. None of the values for percent inhibition by octanol obtained with verapamil was significantly different from those obtained without verapamil. Absolute values for calcium uptake with verapamil were, however, decreased by an average of 12.6% compared with those without verapamil.

*Values obtained using lithium instead of choline as a substitute for sodium.

In these preliminary experiments indicate that the measured 45Ca uptake rates reflect the true kinetics of calcium influx by Na-Ca exchange. Measurements of initial rates of calcium uptake at different calcium concentrations in the absence of octanol and near absence of sodium (14 mM) gave a linear reciprocal plot (Figure 5), showing that uptake could be described by Michaelis-Menten kinetics. This plot gives best fit values of Vmax = 46.4 nmol/mg/min and Km = 0.962 mM, the maximum velocity and dissociation constant, respectively, for calcium uptake under these conditions. When either 64 mM sodium or 0.8 mM octanol was present, the rate of calcium uptake was inhibited competitively: the inhibited rate could be accelerated by higher calcium, and by extrapolation to infinite calcium concentrations, a similar Vmax was obtained. These reciprocal plots were not, however, linear (Figure 5).

Since the conditions of Figure 2 (filled circles) are close to physiological in terms of extracellular sodium and calcium concentrations, we have determined under such conditions the relative potency of some different anesthetics for the purpose of comparison with other known physiological anesthetic effects. Table 2 shows that the potency of the alcohols increases as the chain length increases. Decanol was as potent an inhibitor as dichlorobenzamil. The local anesthetic procaine was a very poor inhibitor, while the barbiturate sodium pentobarbital was an effective inhibitor.

Since cationic amphiphiles were found to be effective inhibitors of Na-Ca exchange in vesicles, we also compared the effect of octylamine with that of octanol. We found that octylamine was only slightly more potent than octanol and also that the same synergism with sodium was evident (Table 2). Some synergism was also observed with the inhibitor dichlorobenzamil.

Discussion

The calcium uptake by cells observed in this study was dependent on intracellular sodium load-
ing and inhibited by extracellular sodium. These properties identify the uptake pathway classically as the Na-Ca exchanger. Since the octanol-induced inhibition of calcium uptake clearly occurred at the sarcolemma (Figure 3), we conclude that general anesthetics inhibit calcium influx by the Na-Ca exchanger. This conclusion is somewhat at variance with that of Philipson, who observed no effect of neutral amphiphiles on Na-Ca exchange in sarcosomal vesicles. He did not report having tried alcohols, however. An inhibition of Na-Ca exchange in sarcosomal vesicles by enfurane has been briefly reported. These authors saw an effect only on the extent of calcium uptake and not on the initial rate, which again is different from our observation. The cause of these differences is not clear to us. One factor that will certainly account for part of the discrepancy is that the potency of the inhibition exerted by general anesthetics was considerably enhanced by extracellular sodium (Figures 1 and 2). In vesicles, Na-Ca exchange is measured in the virtual absence of extravesicular sodium, so this component of inhibition will have been absent. We did observe an inhibition of Na-Ca exchange by octylamine (on dilution of cells into a sodium-free medium) similar to that observed previously with vesicles. The similar effect of sodium on inhibition by octanol and octylamine (Table 2) suggests that the mechanism of inhibition is similar for the alcohol and the amine. The effect of sodium on the potency of these agents suggests that sodium can work synergistically with either. Octylamine was somewhat more potent than octanol (Table 2). It could be, however, that the amine group exerted two conflicting influences: a promotion of inhibition by Na⁺ charge emulation and a decrease in inhibition because of a decrease in membrane solubility by virtue of the charge. In terms of effective membrane concentration, the octylamine could be much more potent than the octanol. The reciprocal plot (Figure 5) shows that the inhibition by octanol is very similar to that exerted by sodium. This is surprising since they are so dissimilar in nature. The synergism observed between sodium and octanol could simply be a reflection of cooperativity of sodium binding to the transport and regulatory sites of the exchanger. Some evidence for such cooperativity already exists from vesicle data: Dixon plots of inhibition of calcium uptake by sodium are linear only when the abscissa is the value of sodium concentration squared. We also find this for uptake by cells, and when the highest (most physiological) sodium concentrations are included, even these plots curve upward (data not shown), signifying even greater cooperativity. In addition, if the reciprocal plot (Figure 5) truly reflects the state of ligand binding to the exchanger, the upward curve in the presence of octanol or sodium implies that not only do these agents compete with calcium but that they also promote the formation of active species with more than one calcium bound. The upward curve is not consistent with a simple pattern of competitive inhibition.

From our data we are encouraged to believe that the measured initial rates of calcium uptake do accurately reflect the initial rate of Na-Ca exchange across the sarcolemma. Although some effect of anesthetics on the rate of sequestration of cytosolic calcium by intracellular organelles is to be expected, such an effect can only influence the rate of calcium entry insofar as the rate of calcium entry is affected by the level of cytosolic calcium. Our ⁴⁵Ca uptake data (Figure 1) indicate that the rate of calcium entry remains linear over a time period when the level of cytosolic free calcium increases considerably from near zero (Figure 3). Thus, during this period, the rate of calcium entry is uninfluenced by the rate of intracellular sequestration. This is borne out, at least with regard to mitochondrial uptake, by the experiments with oligomycin plus FCCP (Figure 4). Since the level of cytosolic free calcium observed in the presence of octanol (Figure 3) was well below values that, in the absence of octanol, were still not influencing rates of calcium uptake, we can safely conclude that the effects of octanol on the rate of cellular calcium uptake were through its sarcolemmal action and were uninfluenced by any effects on intracellular organelles.

It is of interest to compare the kinetics of Na-Ca exchange as measured in vesicles and cells. The assay conditions for vesicles and cells are quite different although both depend on measuring isotopic calcium influx driven by an artificially imposed sodium gradient. The maximum uptake rates measured in vesicles (about 1,800 nmol/min/mg) are 40-fold faster than those measured in cells, perhaps reflecting the similar degree of purification of sarcolemma during isolation, as measured from enrichment of potassium-dependent p-nitrophenyl phosphatase activity. The measured \( K_\text{f} \) for calcium with vesicles is variable, depending on whether or not exchange has been activated by calcium, and ranges from 33 \( \mu \text{M} \) with calcium to about 200 \( \mu \text{M} \) for EGTA-treated vesicles. With cells that were sodium loaded in the presence of EGTA, we found a \( K_\text{f} \) for calcium of 0.962 \( \mu \text{M} \), which was much higher than that found with vesicles, though it should be borne in mind that we had 1.2 \( \mu \text{M} \text{MgCl}_2 \) and 5 \( \mu \text{M} \) ruthenium red present. These ions will also compete with calcium. In spite of these differences between vesicles and cells, there are some noteworthy kinetic similarities: the competitive inhibition of calcium uptake by sodium and the linearity of the reciprocal plot (Figure 5) have also been observed for Na-Ca exchange in vesicles. The nature of the inhibition of Na-Ca exchange by dichlorobenzamil has also been studied in vesicles, and a competition with sodium was observed; inhibition by dichlorobenzamil was reversed by elevation of intravesicular sodium. This is consistent with our observation of synergism (Table 2); in the vesicle experiments, intravesicular sodium was...
by intracellular calcium, it is quite possible that the exchanger undergoes beat-to-beat cycles of activation and inactivation. Calcium entry by calcium channels may activate the exchanger. Blockage of such channels by nifedipine may, therefore, indirectly block calcium influx by Na-Ca exchange. If so, then Na-Ca exchange could contribute to calcium entry even under normal conditions, and blockage of such exchange by anesthetics could contribute to their negative inotropic effect. Such a conclusion is difficult to draw with any certainty, however, since anesthetics have effects on other transport systems and a primary effect is difficult to isolate. Moreover, a mechanism for a positive inotropic effect of anesthetics can also be envisaged: a reduced rate of calcium efflux by Na-Ca exchange would be expected to result in a net gain in average tissue calcium. The inotropic effect that would prevail would depend on whether the exchanger normally carries more calcium into the cell than out or carries more calcium out of the cell than into the cell.

References


**KEY WORDS**

- Na-Ca exchange
- general anesthetics
- heart cells
- sarcoplasmic vesicles
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