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Volatile Anesthetic Effects on Sarcoplasmic Reticulum Ca Content and Sarcolemmal Ca Flux in Isolated Rat Cardiac Cell Suspensions

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Background: **Cardiac cellular Ca metabolism is central to the control of the inotropic state of the heart and is altered in various ways by the volatile anesthetics halothane, enflurane and isoflurane. Specifically, differences among the agents regarding their effect on the uptake and release of Ca from the sarcoplasmic reticulum (SR) have been found, but the nature of such differences is not yet certain. At the sarcolemma, the effects of the anesthetics on the peak Ca current generally are believed to be similar among the three agents, but their impact on other aspects of sarcolemmal Ca transport is less understood. The authors sought to measure the direct action of these agents on SR Ca content and, in the same preparation, to provide a measure of Ca transfer across the sarcolemma during sustained depolarizations.**

Methods: **In stirred suspensions of quiescent rat cardiac cells, the effects were measured of halothane, enflurane, and isoflurane on changes in quin2Ca fluorescence produced by the addition of caffeine (10 mM) and by depolarization with increased extracellular K^+. The peak of the fluorescence response to caffeine, which is due to a sudden release of Ca from the SR into the cytoplasm, was used as an index of SR Ca content. Analysis of the fluorescence increase that occurred after increasing extracellular K^+ from 5 mM to 30 mM in the presence of caffeine provided a measure of net Ca influx across the sarcolemma during sustained depolarizations.**

Results: **The Ca channel blocker nitrendipine maximally inhibited 77% of the initial net Ca influx during 30 mM K^+ depolarization, indicating that most of this influx involves L-type Ca channels. Of the volatile anesthetics, isoflurane (2.6 vol% or 0.57 mM) and enflurane (4.3 vol% or 1.25 mM) inhibited initial net Ca influx during K depolarization significantly more than halothane (1.7 vol% or 0.50 mM), which had no apparent effect. Isoflurane caused no transient change in cytoplasmic Ca concentration and had no effect on the SR Ca content of these quiescent cells. Enflurane (4.3 vol%) caused a significant reduction in SR Ca content.**

Conclusions: **As previously reported, halothane depleted the SR of Ca in quiescent rat cardiac cells, and the present results indicate that enflurane had a similar effect. However, isoflurane did not produce any SR Ca depletion and thus must not significantly alter the balance between SR Ca efflux and uptake in these quiescent cells. The different effects of the three volatile anesthetics on a Ca influx largely carried by L-type Ca channels stand in contrast to the reported findings of similar inhibition of peak L-channel current among the three agents. This result may indicate a differential action (at least in the case of halothane) on peak and steady-state Ca currents. (Key words: Anesthetics, volatile: enflurane; halothane; isoflurane. Calcium channel blockers: nitrendipine. Heart: calcium channels; sarcoplasmic reticulum. Ions: calcium.)**

TO understand the actions of a drug on the heart, often it is important to evaluate its effect on Ca transport at both the sarcoplasmic reticulum (SR) and the sarcolemma (SL). A specific case involves the negative inotropic effect of halothane, enflurane, and isoflurane. Each anesthetic has actions at both the SR and SL, but the relative importance of these effects may differ for each agent, and their priority has not been clearly determined.[1] We present here a method by which endpoints related to both loci of action were measured in the same suspension of cardiac cells. The method is based on the property of high concentrations of caffeine (≥4 mM) to inhibit abruptly the sequestration of Ca by the cardiac SR.[2,3] In isolated rat cardiac cells, appli-

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cation of such concentrations of caffeine produced a transient increase in cytoplasmic Ca, reflecting the release of Ca from the SR.[4] The amplitude of the caffeine-induced cytoplasmic Ca transient (as measured by the increase in quin2 fluorescence) was independent of extracellular Ca and sensitive to ryanodine in a dose-dependent fashion,[4] indicating that this amplitude could be used as a measure of the Ca content of the SR. Furthermore, caffeine inhibited the release of Ca into the cytoplasm induced by the Ca ionophore ionomycin, which renders all membranes permeable to Ca. The small ionomycin-induced cytoplasmic Ca transient remaining in the presence of caffeine was also ryanodine-insensitive.[4] These results indicate that the ionomycin-induced Ca transient in the presence of caffeine or ryanodine represents Ca release from an intracellular compartment unaffected by ryanodine and thus very likely to be a compartment other than the SR. Therefore, caffeine (≥4 mM) appears to obliterate the ability of the SR to sequester any amounts of Ca more than the minimum detectable by their release into the cytoplasm. In the presence of caffeine, therefore, a subsequent depolarization of the cell will open sarcolemmal Ca channels. This action allows net Ca influx across the SL without further detectable release of Ca from the SR. In the absence of caffeine, a depolarization would lead to both Ca influx across the SL and release from the SR. If cytoplasmic Ca is measured during exposure to caffeine and during a subsequent depolarization in the continued presence of caffeine, then a determination of the SR Ca content can be made from the response to caffeine and a measure of the net Ca influx across the SL induced by depolarization can be obtained also. Although similar techniques have been reported,[4,5] we present a systematic analysis of the means to infer membrane properties from such cytoplasmic Ca measurements and the application of such techniques to the actions of the volatile anesthetics.

Methods

Cell Isolation

Suspensions of cardiac cells were obtained as previously described[6] and summarized here. The protocol was approved by the institutional Animal Care and Use Committee. A 2- or 3-month-old Wistar rat was decapitated by guillotine, its thorax opened, and the heart rapidly excised. The decapitation method was chosen not only because it is rapid and reliable but also because it avoided the potential influence of barbiturates or other anesthetics on myocardial Ca metabolism. The aortic root was cannulated, and perfusion of the heart was begun with a nominally Ca-free Earle's salt solution (all in mM: Na 142, K 5.4, Cl 116, HCO_3 26.2, H_2PO_4 1, $MgSO_4$ 0.8; glucose 5.6) at a constant perfusion pressure. After 5 min of this Ca washout, the perfusate was changed to an Earle's salt solution containing collagenase, protease, and Ca 50 μM. The enzyme concentrations were varied to obtain optimum cell yield, and a typical concentration and source were as follows: 1 mg/ml collagenase (Worthington Biochemical, Freehold, NJ), 0.04 mg/ml protease (Sigma, St. Louis, MO).

Perfusion with the enzyme solution proceeded for 14 min and was followed by a 4-min perfusion without enzyme, using an Earle's salt solution containing 100 μM Ca. Perfusion then was discontinued, the atria and great vessels were discarded, and the ventricles were minced in the final perfusion solution. After straining through a coarse mesh, the suspension settled by gravity and was resuspended in Earle's salt solution with 250 μM Ca and subsequently in Earle's with 1 mM Ca. A final resuspension was performed using the following solution: Earle's salts with Ca 1 mM, bovine serum albumin (fraction V) 25 mg/ml, glucose 20 mM, and HEPES 10 mM. The cell suspension then was divided into 2 ml aliquots, placed into capped vials, and maintained with gentle shaking at 37° C. All solutions were bubbled with 95% O_2/5% CO_2 before use. The head space of vials containing the cell suspension was purged periodically with the above gas mixture.

Quin2 Loading

Each aliquot of cells was loaded with quin2 during a 30–35 min incubation with 50 μM quin2 AM (acetoxymethyl ester of quin2, Calbiochem, San Diego, CA) at 37° C. At the end of loading, cells were pelleted with a 40g spin for 30–45 s and resuspended in 2 ml of the final solution described in the preceding paragraph.

Fluorescence Measurement

Once quin2-loaded, the 2-ml aliquot of cell suspension was placed in a cuvette and then into a fluorometer chamber (Ratio Fluorometer-2, Farrand Optical, New York, NY). The chamber was gassed with 95% O_2/5% CO_2 and maintained at 37° C. Settling of cells was prevented through continuous stirring by magnetic disk. Excitation light at 333 nm was provided by a mercury lamp and interference filter. The emitted fluorescence

passed through a short-pass filter with 480-nm cutoff. Additions to the cell suspension in the fluorometer were made by syringe injection without interruption of fluorescence recording.

Caffeine stock was made as a 250 mM aqueous solution, which required warming to achieve dissolution. Nitrendipine was dissolved in polyethylene glycol-400 (PEG) and protected from light. Aqueous solutions of nitrendipine were mixed from the PEG stock just before each experiment and also shielded from light. Addition of PEG to cell suspensions produced no changes in the response to caffeine or depolarization. An additional check was performed by making a second nitrendipine stock using absolute ethanol as the solvent; no difference was found when compared to results with nitrendipine in PEG.

Volatile anesthetics were dissolved in aqueous solution by mixing of liquids in a sealed septum vial before addition to the cell suspension. At least 45 min of continuous agitation was used to ensure complete dissolution of the volatile agents. In situations where relatively large volumes of injectate ($\geq$300 μl) were added to the cell suspension, the injected solution was prewarmed to 37° C. Anesthetic concentrations were determined by sampling representative solutions from the fluorometer cuvette and performing gas chromatography after heptane extraction. Conversion to gas phase concentration used the Krebs' solution/air partition coefficients of 0.75, 0.74, and 0.55 for halothane, enflurane, and isoflurane, respectively.[7] To test the possibility of a direct effect of the volatile anesthetics on quin2Ca fluorescence, anesthetic-containing solution was added, during fluorescence recording, to a cell-free solution containing quin2 and a Ca concentration buffered to near the mid-range of fluorescence. There were no differences in the expected small fluorescence downstep due to dilution between the anesthetic-containing solution and anesthetic-free additions.

Sequence of Solution Addition to Suspensions

In all experiments, the stirred cell suspension was allowed to equilibrate in the fluorometer cuvette for 3 min to allow the temperature and fluorescence to stabilize. Anesthetic agents (in aqueous solution) then were added to the suspension in the cuvette by syringe injection. For control runs, the carrier solution was injected. Once the fluorescence recording stabilized after these additions, caffeine was added to the suspension to a final concentration of 10 mM. This addition evoked a rapid increase in fluorescence as Ca was released from the SR into the cytoplasm. Approximately 3 min after the caffeine injection, KCl was added to the suspension to depolarize the cells and initiate a net Ca influx. The details of the KCl addition are addressed below. Once the fluorescence reached a new steady state, a calibration sequence was performed.

This sequence consisted of additions of 100 μM $MnCl_2$ (to eliminate fluorescence related to extracellular quin2), 5 μM digitonin (to permeabilize the SL and allow the Mn^{2+} to quench cytoplasmic quin2 fluorescence), and 1 mM diethylenetriaminepenta-acetate (to chelate Mn and allow maximum quin2Ca fluorescence). This calibration was not used to convert the fluorescence values to Ca concentrations, but was used to provide a value for maximum intracellular quin2-Ca fluorescence ($quin2_{tot}$), as required by the analysis described below.

Depolarization with Potassium

To initiate a net Ca influx into the cells, depolarization with elevated extracellular K^+ was used. In experiments comparing effects of the volatile anesthetics, an aliquot of 1 M KCl was injected into the suspension to produce a final K^+ concentration of 30 mM. A separate series of experiments was performed to evaluate the response of these cell suspensions to different final concentrations of K^+. In this group of experiments, the solutions added to the cell suspensions were mixtures of KCl and sucrose so that an equal volume of equiosmolar solution was added in each instance. The solution in which the cells were suspended in these experiments also was modified by reducing the Na concentration to 128 mM and Cl to 100 mM, to prevent the calculated osmolarity after the KCl/sucrose addition from exceeding 600 mM.

Data Analysis and Interpretation

All data involving drug additions to a cell suspension were compared to control aliquots of cells from the same suspension. As often as possible, the comparison was made to the average of the controls bracketing the test aliquot in the experimental sequence.

The Ca content of the SR was assessed by measurement of the increase in quin2Ca fluorescence that occurred when caffeine (10 mM) was added to the cell suspension. As previously described,[4,8] this increase was transient, with fluorescence returning to baseline over about 1 min. The peak or maximum fluorescence change was used because this portion of the signal most

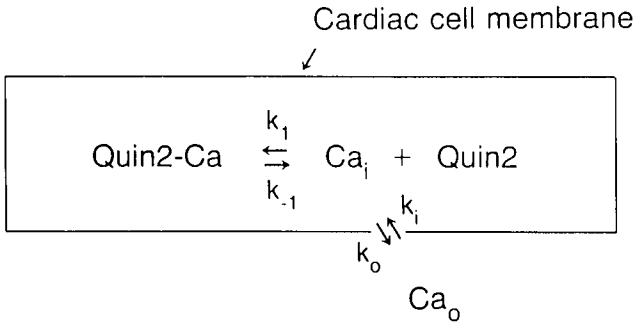

Fig. 1. Compartmental model for analysis of Ca influx during KCl depolarization in the presence of caffeine. Calcium is assumed to have two intracellular compartments: free (Ca_i) and bound to quin2 (quin2-Ca). k_1 and k_{-1} are the rate constants for the association and dissociation of quin2 and Ca; k_i and k_0 are the rate constants for transsarcolemmal Ca transport. Ca_0 represents extracellular calcium.

likely reflects the Ca release from the SR before significant amounts of Ca have been extruded from the cells.

Analysis of the response of cytoplasmic Ca to depolarization (KCl-induced in this case) was directed toward extraction of a quantity closely related to transsarcolemmal Ca influx. Attention to the kinetics of the fluorescence changes thus was required in addition to measurement of the steady-state effects of depolarization. The analysis was based on the model shown in figure 1. This model is a series, two-compartment system for Ca. The quin2-Ca complex (quin2Ca) is the fluorescent moiety, and its concentration can be determined from fluorescence measurement (with certain assumptions discussed in the appendix). The primary assumption for analysis of the model is that the relationship of quin2Ca and Ca_i is governed by their equilibrium relationship. That is, the association and dissociation of quin2Ca must be quite rapid relative to sarcolemmal Ca transport. For quin2 and other Ca-sensitive fluorescent dyes, this assumption is quite likely to be adequate.[9] Also, no intracellular compartments are recognized: The SR is assumed disabled by caffeine and the mitochondrial contribution assumed negligible. Given the above assumptions, a quantity proportional to net Ca influx can be calculated from the quin2 fluorescence changes after addition of K^+. The equations used in this process and their derivation are shown in the appendix.

Cell Heterogeneity

All of our cell suspensions consisted of a heterogeneous cell population. Morphologically intact cells were present along with damaged cells. Fortunately, it appears that the latter cells did not retain significant amounts of quin2. Deliberately Ca-shocked suspensions, which consisted of a high percentage of rounded (damaged) cells, produced fluorescence signals barely greater than autofluorescence when loaded with quin2 in the usual manner. Unintentionally "poor" suspensions likewise produced small fluorescent signals, both in absolute terms as well as in the changes related to caffeine or depolarization. Therefore, it appeared that most of the fluorescence changes that were measured emanated from the intact cells. All data reported originated from suspensions with ≥60% morphologically intact (rod-shaped) cells.

Statistical Methods

All data derived from drug-treated aliquots of cells were reduced to a percent of a control (or bracketing controls) aliquot of cells from the same cell suspension. To determine whether results for a given drug and concentration were significantly different from control, one-sample *t* tests were used, with the null hypothesis defined as 100%. To compare results between the three volatile anesthetics at approximately equipotent concentrations, analysis of variance was used and Fisher's least significant difference test was employed for *post hoc* comparisons. The data presented in table 1 (on the interaction of nitrendipine and isoflurane) were analyzed by repeated measures analysis of variance, with *post hoc* comparisons using Fisher's least significant difference test.

Table 1. Initial Net Calcium Influx in Isolated Heart Cell Suspensions: Effects of Isoflurane and Nitrendipine

Date	Control*	Isoflurane (2.3 vol%)	Nitrendipine (1 μM)	Isoflurane and Nitrendipine
4/18/89	29.1†	24.4	10.4	8.6
1/4/90	45.7	22.7	7.5	5.4
1/5/90	56.5	31.9	8.6	5.4
1/9/90	24.5	20.8	5.6	8.8
1/10/90	48.0	38.7	3.4	10.5
Means ± SD	40.8 ± 13.5	27.7 ± 7.5	7.1 ± 2.7	7.7 ± 2.3‡

* Control values are the means of two aliquots of cells, one run before and one run after the drug-treated aliquots.

† Calcium influx values are expressed in arbitrary fluorescence units per minute and are dependent on suspension density.

‡ Significantly different from isoflurane alone ($P < 0.01$); not different from nitrendipine alone ($P > 0.9$).

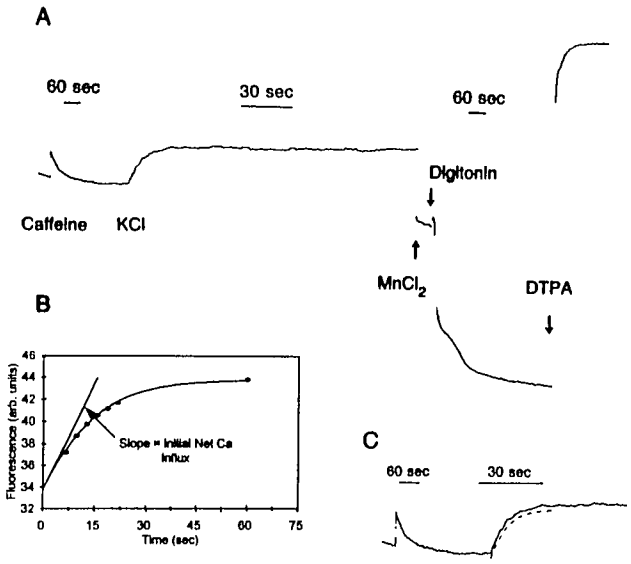

Fig. 2. *(A)* **A quin2-Ca fluorescence tracing of an aliquot of rat heart cells during additions of caffeine (10 mM), KCl (30 mM final K^+ concentration), $MnCl_2$ (0.1 mM), digitonin (5 μM), and diethylenetriaminepentaacetate (DTPA, 1 mM). Chart speed is increased before the KCl addition and decreased just before $MnCl_2$.** *(B)* **Quin2-Ca fluorescence changes immediately after KCl addition. The dots represent points averaged from 49 aliquots of cells. These fluorescence curves were normalized to a constant overall fluorescence change due to KCl before averaging. The smooth curve represents the best fit of the mathematical model to these points. The straight line represents the initial net Ca influx, which is the slope of the fluorescence *versus* time curve at the moment of depolarization with potassium.** *(C)* **A portion of the fluorescence tracing from *A* showing, as a dashed line, the best fit of the model to the fluorescence changes after KCl. The fitted curve is shifted downward because it is otherwise obscured by the data.**

Results

A typical quin2Ca fluorescence tracing during which the cells were first exposed to caffeine (10 mM) and then partially depolarized with K^+ (30 mM) is shown in figure 2A. The fluorescence change after K^+ depolarization was analyzed according to the model described previously. Figures 2B and 2C compare the model to actual data, in both a composite and a typical record. A variety of quantities related to Ca transport may be calculated from the model, but the one which proved the most repeatable was the initial net Ca influx (net Ca influx extrapolated to time zero, *i.e.*, the moment of depolarization). This quantity depends on the rate constants for sarcolemmal Ca transport and on the quin2-Ca concentration before depolarization and that achieved at steady state after depolarization. Considered another way, the initial net Ca influx is a function of the shape, the starting point, and the ending point of the fluorescence curve. It is this dependence on the entirety of the data available that probably accounts for the robustness of the initial net Ca influx compared to other derived quantities.

Several consequences of the model were tested. To confirm that the quantities derived from the model were independent of quin2 loading, the concentration of quin2 AM was varied among aliquots of several cell suspensions. As shown in figure 3, the initial net Ca influx did not depend on dye concentration, in agreement with the analysis model. The analysis model also should have applicability to Ca-sensitive indicators other than quin2 and in situations other than stirred cell suspensions. An example of a K^+-induced depolarization of a single heart cell loaded with the Ca-sensitive dye indo-1 is shown in figure 4. The indo-1 fluorescence ratio changes are fit well by the model and serve to suggest that the results from cell suspensions reflect the responses of single, viable cells.

The effect of depolarization with different concentrations of K^+ was studied in several cell suspensions. As shown in figure 5, initial net Ca influx increases

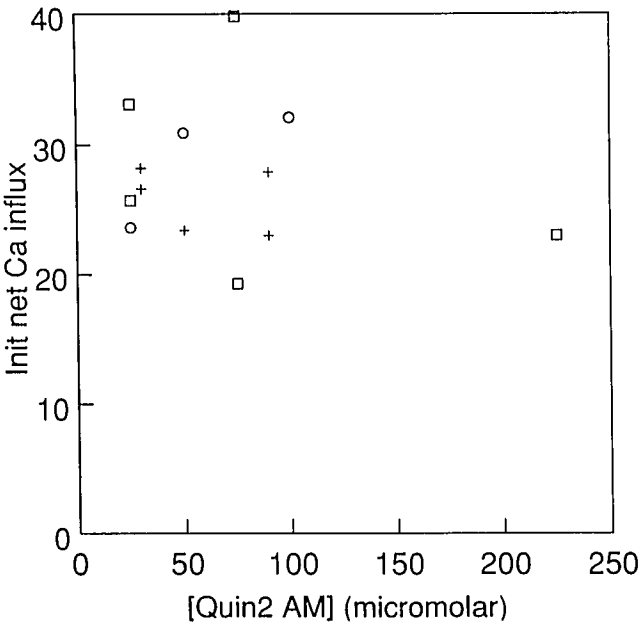

Fig. 3. Initial net Ca influx during 30 mM K^+ depolarization, as a function of quin2 loading condition (various concentrations of quin2 AM). Similar symbols represent different aliquots of the same cell suspension. The initial net Ca influx was calculated according to the model described in the text and is presented in arbitrary fluorescence units per minute.

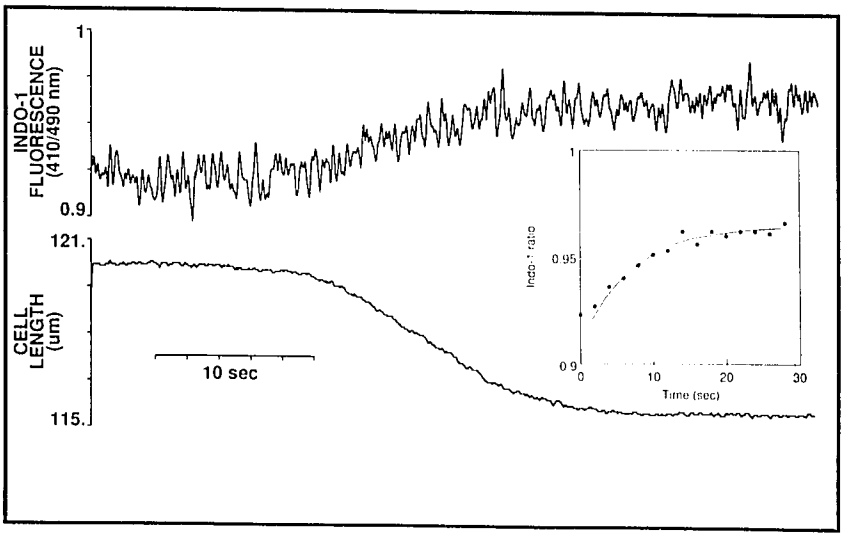

Fig. 4. Simultaneous recording of the indo-1 fluorescence ratio and cell length of a single rat heart cell during a change of superfusion solution from one containing 5 mM K^+ to one with 30 mM K^+. Throughout this recording, the caffeine concentration is 10 mM, and temperature is 22–23° C. Unlike the situation in the cell suspension experiments, this cell is superfused continually with a solution buffered by HEPES 20 mM (adjusted to *p*H 7.4) and devoid of HCO_3^- and albumin. *(Inset)* Solid dots represent time-averaged points from the indo-1 ratio trace. The smooth curve is the best fit of the model to those points.

with increasing K^+ concentration up to a plateau or peak at about 46 mM K^+. The 30 mM K^+ concentration was selected for the anesthetic comparison experiments because it generated a substantial Ca influx while presenting a relatively small osmotic stress to the cells (about a 16% increase in osmolarity).

The portion of net Ca influx during K-induced depolarization that involves voltage-sensitive Ca channels was determined by examining its sensitivity to the Ca channel blocker nitrendipine. A dose-inhibition curve is presented in figure 6. Of the initial net Ca influx in response to a 30 mM K^+ depolarization, 77% was blocked by nitrendipine. The IC_{50} for this process was 63 nM, consistent with nitrendipine binding[10] to and nitrendipine blockade[11] of cardiac Ca channels in situations when the vast majority of the channels are in the resting state.[10] Also observed was a suggestion of a stimulation of Ca influx by nitrendipine at very low concentrations. Such an effect has been reported in other preparations.[11] Nitrendipine had no effect on the fluorescence change in response to caffeine, thus indicating that it has little, if any, effect on the SR Ca content of these quiescent cells.

Halothane (up to 1.7 vol% or 0.50 mM) had no effect on the initial net Ca influx due to 30 mM K^+ depolarization. However, both enflurane (4.3 vol% or 1.25 mM) and isoflurane (2.6 vol% or 0.57 mM) depressed this Ca influx (fig. 7) and had an effect significantly different from that of halothane (1.7 vol% or 0.50 mM). The magnitude of the enflurane effect at its highest concentration indicated that enflurane probably was acting on the nitrendipine-sensitive portion. To determine whether isoflurane's action involved inhibition of L-type Ca channels, a set of experiments was done to compare the effects of isoflurane, nitrendipine (10^{-6} M), and the two drugs together. As shown in table 1, isoflurane caused no further decrease in the presence of nitrendipine. This suggests that isoflurane's effect is on L-type Ca channels, because the effect was not observed when channels were blocked with nitrendipine.

With respect to effects on the caffeine-induced quin2Ca fluorescence transient, differences between the volatile anesthetics were apparent, as shown in figure 8. Isoflurane (up to 2.6 vol% or 0.57 mM) produced no decrease in the Ca release from the SR due to caffeine. Halothane, as previously reported, produces a decrease, and its effect at 1.7 vol% (or 0.50 mM) was significantly different from that of isoflurane (2.6 vol% or 0.57 mM). Enflurane significantly depressed the caffeine-induced response, and its effect at 4.3 vol% (or 1.25 mM) was significantly different from that of isoflurane (2.6 vol% or 0.57 mM).

The addition of halothane-containing solution to quin2-loaded, quiescent cell suspensions has been reported to produce a transient increase in fluorescence.[8] This effect also was observed during the experiments conducted for the present study. In contrast, the addition of isoflurane-containing solution (final isoflurane concentrations up to 2.6 vol% or 0.57 mM) generated no transient changes in the quin2-Ca fluorescence signal. However, the response of the fluorescence signal upon addition of enflurane was difficult to determine with certainty. First, the separation of true changes in quin2-Ca fluorescence from those due to dilution ef-

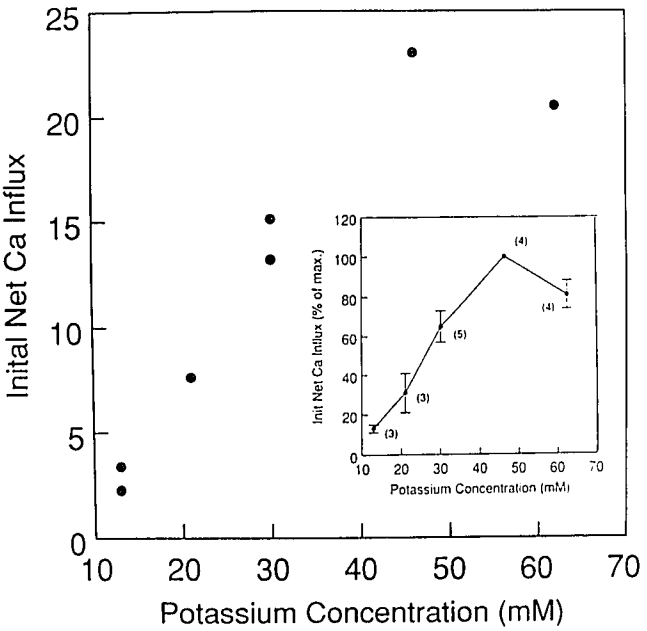

Fig. 5. Dependence of the initial net Ca influx due to K^+ depolarization (as measured in quin2-loaded rat heart cell suspensions) on the concentration of K^+ used to depolarize the suspension. Each point in the main part of the figure was derived from a separate aliquot of a single cell suspension. *(Inset)* Similar plot in which each point represents the mean of data obtained from various aliquots of four different cell suspensions. The data are normalized to a Ca influx of 100 for the 46 mM K^+ depolarization from each cell suspension.

fects and autofluorescence changes produces an inherent uncertainty in the result. Second, the residual transient fluorescent change after most enflurane additions was small, scarcely greater than the noise envelope of the fluorescent signal. Third, the presence of an apparent transient increase in fluorescence after enflurane was variable; that is, it could not always be detected. An example of the responses to additions to the volatile anesthetics is shown in figure 9. The summary of these results is, of necessity, qualitative. Halothane additions produced a clear transient increase in cytoplasmic Ca, as previously reported.[8] Isoflurane does not exhibit any such action. The presence of any transient changes in cytoplasmic Ca after enflurane cannot be determined conclusively from our data.

Discussion

Our results indicate that the three commonly used volatile anesthetics have differences in their ability to reduce the SR Ca content in quiescent cell suspensions. Halothane and enflurane each reduced the SR Ca content, whereas isoflurane (up to a 1.8 MAC concentration) did not exhibit even a trend toward SR Ca content reduction. We conclude that, in this concentration range, isoflurane does not significantly alter the balance of the Ca leak and Ca uptake in isolated rat heart cells. Enflurane and halothane, however, appear to have a direct effect on the cardiac SR that leads to a reduction in SR Ca content in these cells.

Our results do not completely agree with those of Wilde *et al.*, who also measured SR Ca content by following cytoplasmic Ca concentration in quiescent rat heart cells during the application of caffeine.[12,13] They found, as did we, that halothane reduced the SR Ca content. Also in both studies, enflurane caused a reduction in SR Ca content, but Wilde *et al.* found it to occur to a greater extent and at a lower concentration than in our results. The one qualitative departure between their results and ours is that Wilde *et al.* found that isoflurane reduced SR Ca content at relatively high concentrations (≥1.5 MAC), yet we found no effect at 1.8 MAC. Although there are several methodologic differences between their work and ours, an explanation of this difference in results is not readily apparent. In

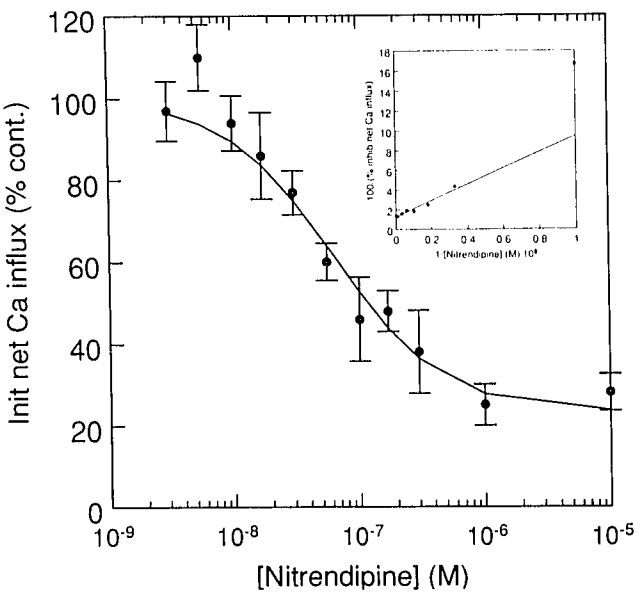

Fig. 6. Dose-inhibition curve for the initial net Ca influx during 30 mM K^+ depolarization in the presence of various concentrations of nitrendipine. Error bars represent SEM. *(Inset)* Linaweaver-Burke plot of dose-inhibition data, which reveals a maximum nitrendipine inhibition of 77% of the initial net Ca influx.

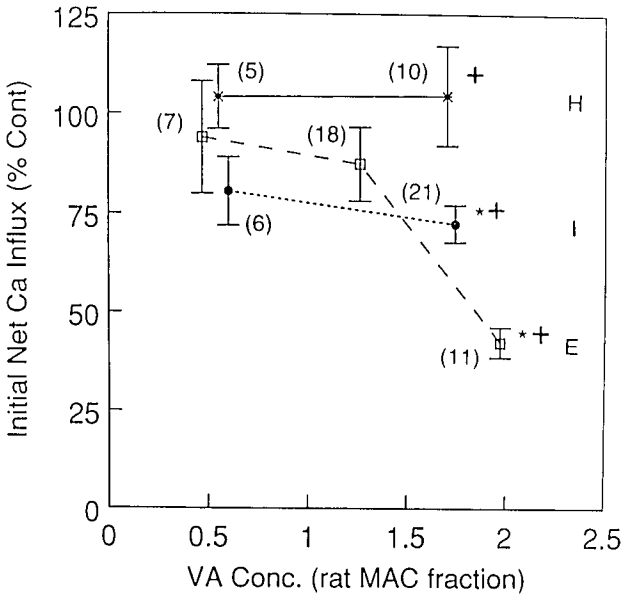

Fig. 7. Effect of halothane (H), enflurane (E), and isoflurane (I) on the initial net Ca influx due to 30 mM K^+ depolarization. All depolarizations were conducted in the presence of 10 mM caffeine. Error bars represent SEM, and the number in parentheses are the Ns for each point. Asterisks (*) show which points are significantly different from control. Crosses (+) indicate points significantly different from the other agents at the comparable concentration.

another study of SR Ca content in a quiescent preparation, Su and Kerrick[14,15] and Su and Bell[16] measured the tension generated by skinned rabbit papillary muscle fibers in response to caffeine as an index of SR Ca net uptake (or content). They found that all three anesthetics had the potential to reduce SR Ca content, but there were striking potency differences between the agents. In their studies, a 40% reduction in caffeine-induced tension was caused by 1.0% halothane or 2.5% enflurane, but a reduction of this magnitude was not achieved by isoflurane at concentrations up to 4.0%.

A number of studies have measured anesthetic effects on the SR in beating cardiac preparations. In most such experiments, a reduction in SR Ca content cannot be interpreted unambiguously as a direct anesthetic action at the SR. A reduction of transsarcolemmal Ca influx (such as through voltage-sensitive Ca channels) could contribute indirectly to a reduction in the SR Ca content. However, comparisons among the anesthetic agents can provide an indication of their relative potency for SR effects, especially because their inhibition of peak Ca current is similar.[17]

Katsuoka and Ohnishi, using isolated, perfused guinea pig hearts that were stimulated to beat, found that halothane and enflurane both reduced SR Ca content, as measured by the left ventricular pressure developed during a caffeine exposure in a Ca-free environment.[18] They did not report results with isoflurane in this preparation. However, in suspensions of rat heart cells that were stimulated to beat, these authors have shown that all three agents reduce total Ca content (and presumably SR Ca content), with enflurane the most potent of the three.[19] Komai and Rusy used potentiated state contractions and rapid cooling contractures in rabbit papillary muscle as an indicator of SR Ca content and concluded that halothane and possibly enflurane reduce the SR Ca content, whereas isoflurane does not.[20–22] To summarize these results from beating preparations, it appears that halothane produces a reduction in SR Ca, that enflurane may produce a comparable effect, and that the action of isoflurane on SR Ca content is inconclusive.

Results from studies of a different nature also correlate with our findings regarding the effect of anesthetics on SR Ca content. Ryanodine binding to isolated cardiac SR vesicles serves as in index of the activity (or open-state probability) of the SR Ca release channel. Two

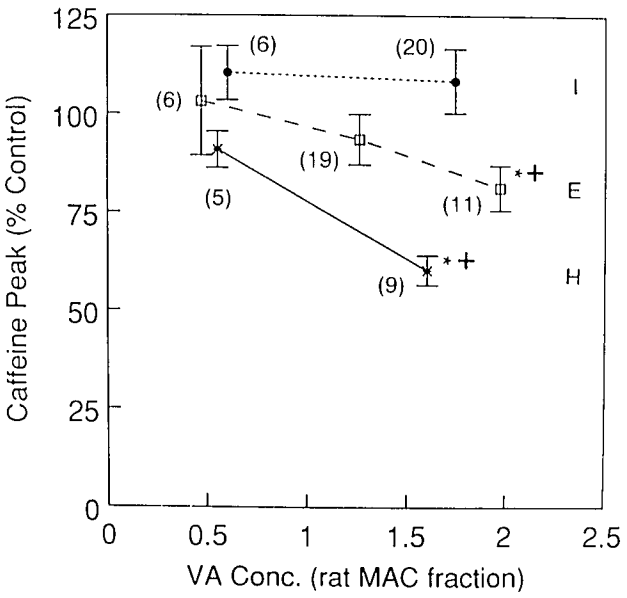

Fig. 8. Effect of halothane (H), enflurane (E), and isoflurane (I) on the height of the quin2-Ca fluorescence response to 10 mM caffeine. The error bars are SEMs, and the Ns for each point are shown in parentheses. *Significantly different from control. +Significantly different from isoflurane at the comparable concentration.

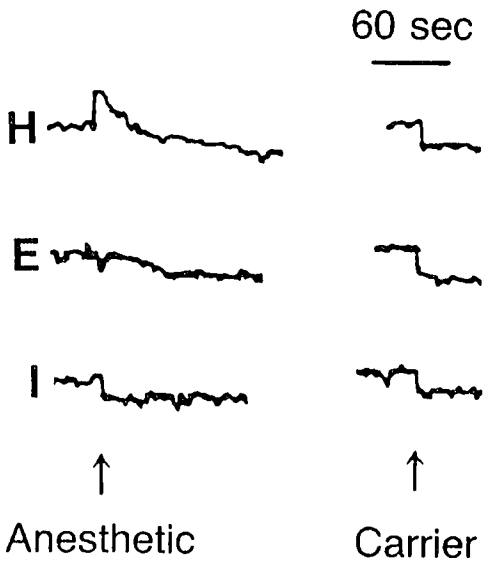

Fig. 9. Quin2 fluorescence tracings of three aliquots of the same cell suspension. At the first arrow, halothane 1.1 vol% (H), enflurane 1.0 vol% (E), or isoflurane 0.9 vol% (I; final concentrations in each case) was added. At the second arrow, an identical volume (100 μl) of carrier solution was added.

groups recently found that halothane increases ryanodine binding to cardiac SR vesicles, whereas isoflurane does not.[23,24] These results suggest that halothane opens the Ca release channel. Such an action would be expected to enhance the leak of Ca from the SR, causing a reduced SR Ca content. These radioligand binding studies in isolated SR thus demonstrate a specific mechanism to explain halothane-induced SR Ca depletion and the lack of such action in the case of isoflurane.

Compared to their actions on SR Ca content, a different spectrum was observed for effects of the volatile anesthetics on the net Ca influx induced by K^+ depolarization. An inhibition of Ca influx, likely involving L-type Ca channels, was found in the presence of isoflurane and enflurane, but not in the case of halothane. These results stand in contrast to a variety of results that show that halothane, as well as isoflurane and enflurane, depressed the second inward (or Ca) current. Specifically, Lynch *et al.*[25,26] and Lynch[27] have shown that these agents depress the upstroke of slow action potentials in partially depolarized cells, an endpoint identified as an index of the magnitude of the slow inward current. More directly, Terrar and Victory[28,29] and Bosnjak *et al.*[17] measured the second inward current in voltage-clamped myocytes and also found inhibition with each agent. It is not clear why halothane did not affect a Ca influx that is 77% nitrendipine-sensitive in our experiments, although it is clearly a Ca-channel inhibitor in others. One possible explanation involves the different time course of the depolarizations. The initial net Ca influx from our K-induced depolarizations is calculated from data obtained over about 30 s of depolarization, whereas the typical voltage clamp pulse lasts 200 ms. Furthermore, most reported measurements of the effect of anesthetics on calcium currents involve the peak current. In prolonged depolarizations such as in our experiments, the steady-state current is the more relevant property, and Ca "window" currents may come into play.[30] Thus, it is possible that the effect of anesthetics on peak Ca currents during short voltage clamp pulses may differ from those during prolonged depolarizations. It also should be noted that most of the work involving voltage clamp measurements and volatile anesthetics has been performed on guinea pig cells, whereas we used rat cells. Although the properties of Ca channels in these two species are similar, the substantial differences between these species in other electrophysiologic properties also may play a role in our results.

The situation of prolonged K-induced depolarization has a specific physiologic relevance. During severe ischemia, potassium accumulates in the extracellular space of cardiac muscle, and cells become partially depolarized.[31] In a variety of preparations, volatile anesthetics have been shown to increase the tolerance of myocardial tissue to ischemia.[32–34] One of the mechanisms proposed for such an effect is an inhibition of Ca channels reducing the ultimate Ca load within the cell. Our results indicate that this explanation may not apply in the case of halothane. It should be noted that our preparation involves a species with somewhat unusual electrophysiologic properties: It does not truly model ischemic myocardium, but only mimics one feature of it. However, the depolarization is the factor that would likely activate Ca channels during ischemia.

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References

1. Rusy BF, Komai H: Anesthetic depression of myocardial contractility: A review of possible mechanisms. ANESTHESIOLOGY 67:745–766, 1987

2. Hess P, Wier WG: Excitation-contraction coupling in cardiac Purkinje fibers: Effects of caffeine on the intracellular [Ca^{2+}] transient,

membrane currents, and contraction. J Gen Physiol 83:417–433, 1984

3. Clusin WT: Do caffeine and metabolic inhibitors increase free calcium in the heart?: Interpretation of conflicting intracellular calcium measurements. J Mol Cell Cardiol 17:213–220, 1985

4. Hansford RG, Lakatta EG: Ryanodine releases calcium from sarcoplasmic reticulum in calcium tolerant rat cardiac myocytes. J Physiol (Lond) 390:453–467, 1987

5. Powell T, Tatham PER, Twist VW: Cytoplasmic free calcium measured by quin2 fluorescence in isolated ventricular myocytes at rest and during potassium-depolarization. Biochem Biophys Res Comm 122:1012–1020, 1984

6. Capogrossi MC, Kort AA, Spurgeon HA, Lakatta EG: Single adult rabbit and rat cardiac myocytes retain the Ca^{2+}- and species-dependent systolic and diastolic properties of intact muscle. J Gen Physiol 88: 589–613, 1986

7. Renzi F, Waud BE: Partition coefficients of volatile anesthetics in Krebs' solution. ANESTHESIOLOGY 47:62–63, 1977

8. Wheeler DM, Rice RT, Hansford RG, Lakatta EG: The effect of halothane on the free intracellular calcium concentration of isolated rat heart cells. ANESTHESIOLOGY 69:578–583, 1988

9. Bayley P, Ahlstrom P, Martin SR, Forsen S: The kinetics of calcium binding to calmodulin: quin2 and ANS stopped-flow fluorescence studies. Biochem Biophys Res Comm 120:185–191, 1984

10. Bean BP: Nitrendipine block of cardiac calcium channels: High-affinity binding to the inactivated state. Proc Natl Acad Sci USA 81:6388–6392, 1984

11. Brown AM, Kunze DL, Yatani A: Dual effects of dihydropyridines on whole cell and unitary calcium currents in single ventricular cells of guinea-pig. J Physiol (Lond) 379:495–514, 1986

12. Wilde DW, Gutta R, Haney MF, Knight PR: Effects of volatile anesthetics on the intracellular Ca^{2+} concentration in cardiac muscle cells, Mechanisms of Anesthetic Action in Skeletal, Cardiac and Smooth Muscle. Edited by Blanck TJJ, Wheeler DM. New York, Plenum, 1991, pp 125–141

13. Wilde DW, Davidson BA, Smith MD, Knight PR: Effects of isoflurane and enflurane on intracellular Ca^{2+} mobilization in isolated cardiac myocytes. ANESTHESIOLOGY 79:73–82, 1993

14. Su JY, Kerrick WGL: Effects of halothane on caffeine-induced tension transients in functionally skinned myocardial fibers. Pflugers Arch 380:29–34, 1979

15. Su JY, Kerrick WGL: Effects of enflurane on functionally skinned myocardial fibers from rabbits. ANESTHESIOLOGY 52:385–389, 1980

16. Su JY, Bell JG: Intracellular mechanism of action of isoflurane and halothane on striated muscle of the rabbit. Anesth Analg 65: 457–462, 1986

17. Bosnjak ZJ, Supan FD, Rusch NJ: The effects of halothane, enflurane, and isoflurane on calcium current in isolated canine ventricular cells. ANESTHESIOLOGY 74:340–345, 1991

18. Katsuoka M, Ohnishi ST: Inhalation anaesthetics decrease calcium content of cardiac sarcoplasmic reticulum. Br J Anaesth 62: 669–673, 1989

19. Katsuoka M, Kobayashi K, Ohnishi ST: Volatile anesthetics decrease calcium content of isolated myocytes. ANESTHESIOLOGY 70: 954–950, 1989

20. Komai H, Redon D, Rusy BF: Effects of isoflurane and halothane on rapid cooling contractures in myocardial tissue. Am J Physiol 257: H1804–H1811, 1989

21. Komai H, Rusy BF: Negative inotropic effects of isoflurane and halothane in rabbit papillary muscles. Anesth Analg 66:29–33, 1987

22. Komai H, Rusy BF: Contribution of the known subcellular effects of anesthetics to their negative inotropic effect in intact myocardium, Mechanisms of Anesthetic Action in Skeletal, Cardiac, and Smooth Muscle. Edited by Blanck TJJ, Wheeler DM. New York, Plenum, 1991, pp 115–123

23. Connelly TJ, Hayek R-E, Rusy BF, Coronado R: Volatile anesthetics selectively alter [^{3}H]ryanodine receptor binding to skeletal and cardiac ryanodine receptors. Biochem Biophys Res Comm 186: 595–600, 1992

24. Lynch C III, Frazer MJ: Ca release channels of cardiac sarcoplasmic reticulum are activated by halothane, but not by isoflurane (abstract). ANESTHESIOLOGY 77:A630, 1992

25. Lynch C III, Vogel S, Sperelakis N: Halothane depression of myocardial slow action potentials. ANESTHESIOLOGY 55:360–368, 1981

26. Lynch C III, Vogel S, Pratila MG, Sperelakis N: Enflurane depression of myocardial slow action potentials. J Pharmacol Exp Ther 222:405–409, 1982

27. Lynch C III: Differential depression of myocardial contractility by halothane and isoflurane *in vitro*. ANESTHESIOLOGY 64:620–631, 1986

28. Terrar DA, Victory JGG: Effects of halothane on membrane currents associated with contraction in single myocytes isolated from guinea-pig ventricle. Br J Pharmacol 94:500–508, 1988

29. Terrar DA, Victory JGG: Isoflurane depresses membrane currents associated with contraction in myocytes isolated from guinea-pig ventricle. ANESTHESIOLOGY 69:742–749, 1988

30. Cohen NM, Lederer WJ: Calcium current in isolated neonatal rat ventricular myocytes. J Physiol (Lond) 391:169–191, 1987

31. Kleber AG: Resting membrane potential, extracellular potassium activity and intracellular sodium activity during acute global ischemia in isolated perfused guinea pig hearts. Circ Res 52:442–450, 1983

32. Freedman BM, Hamm DP, Everson CT, Wechsler AS, Christian CM: Enflurane enhances post ischemic functional recovery in the isolated rat heart. ANESTHESIOLOGY 62:29–33, 1985

33. Davis RF, Sidi A: Effect of isoflurane on the extent of myocardial necrosis and on systemic hemodynamics, regional myocardial blood flow, and regional myocardial metabolism in dogs after coronary artery occlusion. Anesth Analg 69:575–586, 1989

34. Warltier DC, Al-Wathiqui MH, Kampine JP, Schmeling WT: Recovery of contractile function of stunned myocardium in chronically instrumented dogs is enhanced by halothane or isoflurane. ANESTHESIOLOGY 69:552–565, 1988

Appendix

Given the model shown in figure 1 and the assumptions noted in the text, quin2Ca and Ca_i are related as follows:

$$Ca_i = \frac{k_{-1}}{k_1}\frac{quin2Ca}{quin2} \quad (A1)$$

or

$$Ca_i = \frac{k_{-1}}{k_1}\frac{quin2Ca}{quin2_{tot} - quin2Ca}, \quad (A2)$$

where $quin2_{tot}$ is the total cytoplasmic quin2 concentration, and quin2 and quin2Ca are the concentrations of the Ca-free and -bound forms, respectively. By conservation of mass, the following holds:

$$\frac{dCa_i}{dt} + \frac{d(quin2Ca)}{dt} = k_i Ca_0 - k_0 Ca_i. \quad (A3)$$

Taking the time derivative of equation A2 provides an independent expression for dCa_i/dt for substitution into equation A3. The result simplifies to:

$$\frac{d(quin2Ca)}{dt} = k_i Ca_0 - \frac{k_0 k_{-1}}{k_1} \frac{quin2Ca}{quin2_{tot} - quin2Ca}. \quad (A4)$$

Under many circumstances, the sarcolemmal transport rate constants (k_0 and k_i) reasonably may be assumed constant over a defined interval. Of the remaining quantities in equation A4, quin2Ca is then the only variable. Unfortunately, a simple analytical solution for quin2Ca does not result from integration of equation A4. A numerical approach may be used to solve equation A4, and this was done for the results reported here.

For the case of K-induced depolarizations, the quin2Ca fluorescence approaches a constant, steady-state value ($quin2Ca(\infty)$). At steady state, the time derivatives are zero, so equation A4 reduces to:

$$k_i Ca_0 = \frac{k_0 k_{-1}}{k_1} \frac{quin2Ca(\infty)}{quin2_{tot} - quin2Ca(\infty)}. \quad (A5)$$

The above expression can be used to eliminate $k_i Ca_0$ from equation A4 and produce an equation involving just one unknown rate constant (k_{-1}/k_1 is considered known) along with experimentally determinable quantities.

$$\frac{k_0 k_{-1}}{k_1} = \frac{\frac{d(quin2Ca)}{dt}}{\frac{quin2Ca(\infty)}{quin2_{tot} - quin2Ca(\infty)} - \frac{quin2Ca}{quin2_{tot} - quin2Ca}}. \quad (A6)$$

Thus, for any point in the experiment at which k_i and k_0 are constant and quin2Ca and its rate of change and steady-state value can be determined, k_0 can be calculated, and from there, k_i. In practice, this was done at multiple points along the experimental curve and the "best" value of k_0 (and thus k_i) determined.

If the above model is to be applied to actual experiments, fluorescence values must be substituted for the quin2Ca, $quin2_{tot}$, and $quin2Ca(\infty)$ terms. The fluorescence values used must be proportional to the cytoplasmic quin2Ca concentration. Two factors are involved in this proportionality: (1) the geometry and efficiency of the instrument and (2) the total volume of cytoplasm from which the fluorescence is recorded. The first factor is likely to be constant for any one experimental setup. The second will be determined, in the case of cell suspensions, largely by the number of viable cells per unit volume of the suspension. In circumstances in which different aliquots of the same suspension are compared, it becomes reasonable to assume a similar total cytoplasmic volume across various aliquots and compare the results from them directly. Differences in dye loading will not alter the rate constants and other quantities derived from the model.