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Propofol Attenuates Capacitative Calcium Entry in Pulmonary Artery Smooth Muscle Cells

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Background: **Depletion of intracellular Ca^{2+} stores results in capacitative Ca^{2+} entry (CCE) in pulmonary artery smooth muscle cells (PASMCs). The authors aimed to investigate the effects of propofol on CCE and to assess the extent to which protein kinase C (PKC) and tyrosine kinases mediate propofol-induced changes in CCE.**

Methods: **Pulmonary artery smooth muscle cells were cultured from explants of canine intrapulmonary artery. Fura 2–loaded PASMCs were placed in a dish (37°C) on an inverted fluorescence microscope. Intracellular Ca^{2+} concentration was measured using fura 2 in PASMCs using a dual-wavelength spectrofluorometer. Thapsigargin (1 μM), a sarcoplasmic reticulum Ca^{2+}–adenosine triphosphatase inhibitor, was used to deplete intracellular Ca^{2+} stores after removing extracellular Ca^{2+}. CCE was activated when extracellular Ca^{2+} (2.2 mM) was restored.**

Results: **Thapsigargin caused a transient increase in intracellular Ca^{2+} concentration (182 ± 11%). Restoring extracellular calcium (to induce CCE) resulted in a peak (246 ± 12% of baseline) and a sustained (187 ± 7% of baseline) increase in intracellular Ca^{2+} concentration. Propofol (1, 10, 100 μM) attenuated CCE in a dose-dependent manner (peak: 85 ± 3, 70 ± 4, 62 ± 4%; sustained: 94 ± 5, 80 ± 5, 72 ± 5% of control, respectively). Tyrosine kinase inhibition (tyrphostin 23) attenuated CCE (peak: 67 ± 4%; sustained: 74 ± 5% of control), but the propofol-induced decrease in CCE was still apparent after tyrosine kinases inhibition. PKC activation (phorbol 12-myristate 13-acetate) attenuated CCE (peak: 48 ± 1%; sustained: 53 ± 3% of control), whereas PKC inhibition (bisindolylmaleimide) potentiated CCE (peak: 132 ± 11%; sustained: 120 ± 4% of control). Moreover, PKC inhibition abolished the propofol-induced attenuation of CCE.**

Conclusion: **Tyrosine kinases activate and PKC inhibits CCE in PASMCs. Propofol attenuates CCE primarily *via* a PKC-dependent pathway. CCE should be considered a possible cellular target for anesthetic agents that alter vascular smooth muscle tone.**

CAPACITATIVE Ca^{2+} entry involves the influx of Ca^{2+} across the sarcolemma in response to depletion of intracellular Ca^{2+} stores.[1–4] Capacitative Ca^{2+} entry is insensitive to voltage-gated Ca^{2+} channel inhibitors.[2,3] The mechanisms involved in regulating capacitative Ca^{2+} entry are not well understood. We recently demonstrated that capacitative Ca^{2+} entry exists in canine pulmonary artery smooth muscle cells (PASMCs) and serves to refill the sarcoplasmic reticulum Ca^{2+} pool.[5] Moreover, we observed that capacitative Ca^{2+} entry is required to maintain α-agonist–induced oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and is involved in the contractile response to α-adrenoreceptor activation.[5] Because capacitative Ca^{2+} entry is involved in the regulation of $[Ca^{2+}]_i$ and vasomotor tone, it could serve as a cellular target for anesthetic agents that exert vasoactive effects.

The goal of the current study was to investigate the effect of propofol on capacitative Ca^{2+} entry in PASMCs. We tested the hypothesis that propofol would attenuate capacitative Ca^{2+} entry in PASMCs. The rationale for this hypothesis is based on two factors. First, propofol is known to inhibit voltage-gated influx of extracellular Ca^{2+} in vascular smooth muscle.[6–8] Second, we have demonstrated that intravenous anesthetics,[9,10] including supraclinical concentrations of propofol,[9] attenuate phenylephrine-induced $[Ca^{2+}]_i$ oscillations in PASMCs, which require capacitative Ca^{2+} entry. Because a tyrosine kinase is involved in the signal transduction pathway for capacitative Ca^{2+} entry in PASMCs,[5] we also tested the hypothesis that tyrosine kinase inhibition would prevent the effects of propofol on capacitative Ca^{2+} entry. Finally, we investigated the effects of protein kinase C (PKC) activation and inhibition on capacitative Ca^{2+} entry and assessed the extent to which PKC is involved in propofol-induced changes in capacitative Ca^{2+} entry.

Materials and Methods

Animals

Pulmonary arteries were isolated from adult mongrel dogs. The technique of euthanasia was approved by the Cleveland Clinic Institutional Animal Care and Use Committee (Cleveland, OH). All steps were performed aseptically during general anesthesia with intravenous pentobarbital sodium (30 mg/kg) and intravenous fentanyl citrate (20 μg/kg). The dogs were intubated and ventilated, exsanguinated by controlled hemorrhage *via* a femoral artery catheter, and euthanized with electrically induced ventricular fibrillation. A left lateral thoracotomy was performed, and the heart and lungs were removed *en bloc*. The pulmonary arteries were isolated and dissected in a laminar flow hood during sterile conditions.

Cell Culture of Pulmonary Artery Smooth Muscle Cells

Primary cultures of PASMCs were obtained as previously described.[11] Intralobar pulmonary arteries (ID =

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2–4 mm) were carefully dissected and prepared for tissue culture. Explant cultures were prepared according to the method of Campbell and Campbell,[12] with minor modifications. Briefly, the endothelium and adventitia were removed together with the most superficial part of the tunica media. The media was cut into 2-mm^2 pieces and explanted in 25-cm^2 culture dishes nourished by D-MEM/F-12 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum and a 1% antibiotic–antimycotic mixture solution (10,000 units/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin B) and kept in a humidified atmosphere of 5% CO_2:95% air at 37°C. PASMCs began to proliferate from explants after 7 days in culture. Cells were allowed to grow for an additional 7–10 days before being subcultured nonenzymatically to 35-mm glass dishes designed for fluorescence microscopy (ΔT system; Bioptechs Inc., Butler, PA). Cells were used for experimentation within 72 h. Cells from the first and second passage were used for experiments. More than 90% of the cells stained positive for smooth muscle α actin.[11]

Fura 2–Loading Procedure

Pulmonary artery smooth muscle cells were loaded with fura 2 as previously described.[11] Twenty-four hours before experimentation, the culture medium containing 10% fetal bovine serum was replaced with serum-free medium to arrest cell growth, allow for establishment of steady state cellular events independent of cell division, and to prevent a false estimate of $[Ca^{2+}]_i$ resulting from binding of available dye to serum protein in the medium. PASMCs were washed twice in loading buffer, which contained 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 11 mM glucose, 1.8 mM $CaCl_2$, 25 mM HEPES, and 0.2% bovine serum albumin, at pH 7.40 adjusted with NaOH. PASMCs were then incubated in loading buffer containing 2 μM fura 2/AM, the acetoxymethyl ester derivative of fura 2 (Molecular Probes, Eugene, OR), at ambient temperature for 30 min. After the 30-min loading period, the cells were washed twice in loading buffer and incubated at ambient temperature for an additional 20 min before initiating the study. This provided enough time to wash away any extracellular fura 2/AM and for intracellular esterases to cleave fura 2/AM into the active fura 2.

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured as previously described.[11] Culture dishes containing fura 2–loaded PASMCs were placed in a temperature-regulated (37°C) chamber (Bioptechs, Inc.) mounted on the stage of an Olympus IX-70 inverted fluorescence microscope (Olympus America Inc., Lake Success, NY). Fluorescence measurements were obtained from either individual PASMCs or from a cluster (two to three cells) of neighboring cells in a culture monolayer using a dual-wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, Lawrenceville, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The volume of the chamber was 1.5 ml. The cells were superfused continuously at 1 ml/min with Krebs-Ringer buffer, which contained 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_2$, 11 mM glucose, 2.5 mM $CaCl_2$, and 25 mM HEPES, at pH 7.40 adjusted with NaOH. The temperature of all solutions was maintained at 37°C in a water bath. Solution changes were accomplished rapidly by aspirating the buffer in the dish and transiently increasing the flow rate to 10 ml/min. Just before data acquisition, background fluorescence (*i.e.*, fluorescence between cells) was measured and subtracted automatically from the subsequent experimental measurements. Fura 2 fluorescence signals (340, 380, and 340/380 ratio) originating from PASMCs were continuously monitored at a sampling frequency of 25 Hz and were collected using a software package from Photon Technology International.

Experimental Protocols

Capacitative Ca^{2+} entry is triggered by the depletion of intracellular Ca^{2+} stores. Thapsigargin increases $[Ca^{2+}]_i$ *via* irreversible inhibition of sarcoplasmic reticulum Ca^{2+}–adenosine triphosphatase (ATPase).[13] In the absence of extracellular Ca^{2+}, thapsigargin (1 μM) was used to deplete sarcoplasmic reticulum Ca^{2+} stores. With thapsigargin still present, capacitative Ca^{2+} entry was then induced by restoring the extracellular Ca^{2+} concentration (2.2 mM).[5] The effects of propofol (1, 10, and 100 μM), tyrosine kinase inhibition (tyrphostin 23: 100 μM), PKC activation (phorbol 12-myristate 13-acetate: 1 μM) and PKC inhibition (bisindolylmaleimide: 1 μM) on capacitative Ca^{2+} entry were assessed.

Drug Preparation

Propofol, thapsigargin, phorbol 12-myristate 13-acetate (all obtained from Research Biochemical International, Natick, MA), tyrphostin 23 (Calbiochem, La Jolla, CA), and bisindolylmaleimide 1 (Sigma, St. Louis, MO) were all dissolved in dimethyl sulfoxide as stock solutions. Aliquots of each stock solution were diluted 1:1000 in Krebs-Ringer buffer to achieve final concentrations in the bath. Similar dilutions of dimethyl sulfoxide in Krebs-Ringer buffer have no effect on $[Ca^{2+}]_i$. Pure propofol was used to avoid any effects of the intralipid emulsion on the fluorescence signal.

Data Analysis

Peak and sustained increases in $[Ca^{2+}]_i$ were measured in PASMCs when the superfusion solution was switched from a Ca^{2+}-free solution to a solution containing 2.2 mM Ca^{2+}. Peak and sustained fluorescence ratio values were averaged before and after each intervention and are expressed as percent of control. The control response to which all interventions were compared was the first

capacitative Ca^{2+} entry response after thapsigargin pretreatment. This value was set at 100%. Therefore, each cell served as its own control. The "peak" response was calculated as the fluorescence change from baseline to peak fluorescence. The "sustained" response represents the fluorescence values measured 5 min after reintroduction of Ca^{2+} to the buffer. Results are presented as mean ± SEM. Statistical analysis was performed using repeated-measures analysis of variance followed by Bonferroni–Dunn *post hoc* testing. Differences were considered statistically significant at $P < 0.05$.

Results

Capacitative Ca^{2+} Entry in Pulmonary Artery Smooth Muscle Cells

Capacitative Ca^{2+} entry can be triggered by thapsigargin-induced depletion of intracellular Ca^{2+} stores. In the absence of extracellular Ca^{2+}, thapsigargin (1 μM) increased $[Ca^{2+}]_i$ by 182 ± 11%, followed by a return of $[Ca^{2+}]_i$ to baseline values (fig. 1, top). Once the baseline fluorescence signal had stabilized, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was restored (2.2 mM) in the continued presence of thapsigargin (fig. 1, top). Restoring $[Ca^{2+}]_o$ resulted in a rapid peak increase (246 ± 12% of baseline) in $[Ca^{2+}]_i$ and a sustained increase (187 ± 7% of baseline) in $[Ca^{2+}]_i$ (*i.e.*, capacitative Ca^{2+} entry was induced). The sustained increase in $[Ca^{2+}]_i$ returned to baseline when $[Ca^{2+}]_o$ was removed. The reproducibility of inducing capacitative Ca^{2+} entry was assessed by sequentially removing and restoring $[Ca^{2+}]_o$ three consecutive times in the continued presence of thapsigargin. There were no significant differences in the peak or sustained increases in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was restored three consecutive times (fig. 1, bottom).

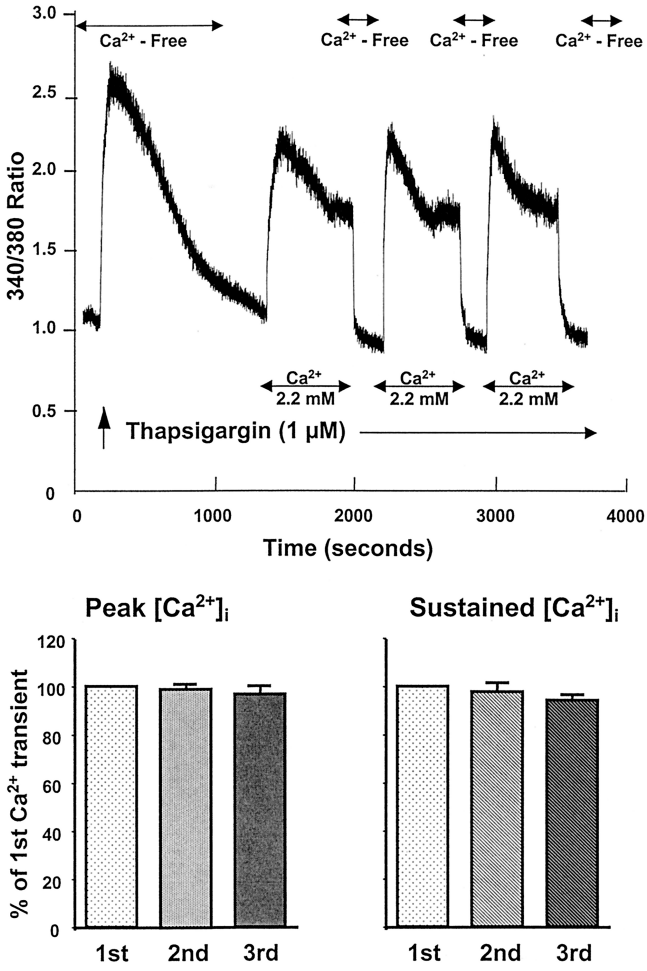

Fig. 1. (*Top*) Representative trace depicting capacitative Ca^{2+} entry after depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin. In the absence of extracellular Ca^{2+} (Ca^{2+} free buffer plus 2 mM EGTA), thapsigargin stimulated a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by releasing Ca^{2+} from sarcoplasmic reticulum Ca^{2+} stores. After $[Ca^{2+}]_i$ had returned to baseline, Ca^{2+} was added back to the buffer, which induced a peak and sustained increase in $[Ca^{2+}]_i$, *i.e.*, capacitative Ca^{2+} entry. $[Ca^{2+}]_i$ returned to baseline values after removing extracellular Ca^{2+}. Extracellular Ca^{2+} was sequentially added and removed three times. In this and all other representative traces, the thapsigargin and capacitative Ca^{2+} entry traces represent consecutive recordings from the same cells. (*Bottom*) Summarized data showing the reproducibility of capacitative Ca^{2+} entry (n = 7 cells).

Effect of Propofol on Capacitative Ca^{2+} Entry

After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative Ca^{2+} entry was compared in the absence or presence of propofol, which was added to the superfusion buffer 5 min before restoring $[Ca^{2+}]_o$ a second time (fig. 2, top). Propofol had no effect on baseline $[Ca^{2+}]_i$ before the addition of $[Ca^{2+}]_o$. Propofol caused dose-dependent decreases in both the peak and sustained increases in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was restored (fig. 2, bottom). After washout of propofol, capacitative Ca^{2+} entry was similar in magnitude to the response measured before propofol administration (fig. 2, top).

Role of Tyrosine Kinases in Propofol-induced Attenuation of Capacitative Ca^{2+} Entry

We previously demonstrated that tyrosine kinases play a role in regulating capacitative Ca^{2+} entry in PASMCs.[5] In the current study, tyrphostin 23 (100 μM) was used to inhibit tyrosine kinases. Tyrosine kinase inhibition attenuated both the peak (67 ± 4% of control) and sustained (75 ± 5% of control) increases in $[Ca^{2+}]_i$ mediated through capacitative Ca^{2+} entry (fig. 3). However, in the presence of tyrosine kinase inhibition, propofol (100 μM) further attenuated the peak (46 ± 4% of control) and sustained (55 ± 2% of control) increases in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was restored (fig. 4).

Role of Protein Kinase C in Propofol-induced Attenuation of Capacitative Ca^{2+} Entry

Protein kinase C has been implicated in the regulation of capacitative Ca^{2+} entry in a variety of cell types.[14–16] However, the extent to which PKC is involved in capac-

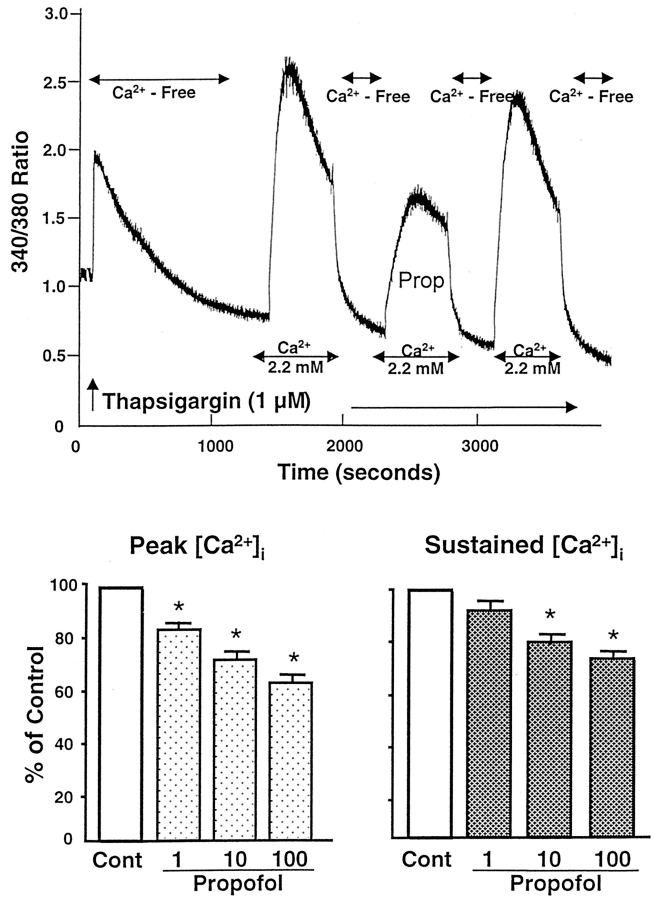

Fig. 2. (*Top*) Representative trace depicting the effect of propofol (100 μM) on capacitative calcium entry. After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative calcium entry was compared in the absence or presence of propofol, which was added to the superfusion buffer 5 min before restoring extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). (*Bottom*) Summarized data showing the dose-dependent inhibitory effects (*$P < 0.05$) of propofol (1, 10, 100 μM) on the peak and sustained increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by capacitative calcium entry compared with control (Cont) (n = 8 cells).

itative Ca^{2+} entry in PASMCs has not been previously investigated. Activation of PKC with phorbol 12-myristate 13-acetate (1 μM) attenuated both the peak (48 ± 1% of control) and sustained (53 ± 3% of control) increases in $[Ca^{2+}]_i$ mediated *via* capacitative Ca^{2+} entry (fig. 5). In contrast, PKC inhibition with bisindolylmaleimide (1 μM) potentiated both the peak (132 ± 11% of control) and sustained (120 ± 4% of control) increases in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was restored (fig. 6). Moreover, in the presence of PKC inhibition, propofol (100 μM) had no effect on capacitative Ca^{2+} entry (fig. 7). Thus, PKC inhibition abolished the propofol-induced attenuation in capacitative Ca^{2+} entry.

Discussion

The main findings of our study are as follows. First, both tyrosine kinases and PKC are involved in the signal transduction pathway for capacitative Ca^{2+} entry in PASMCs. Tyrosine kinases positively regulate capacitative Ca^{2+} entry, whereas PKC negatively regulates capacitative Ca^{2+} entry. Second, propofol causes dose-dependent inhibition of capacitative Ca^{2+} entry in PASMCs. Third, the propofol-induced attenuation of capacitative Ca^{2+} entry is still observed after inhibition of tyrosine kinases. Finally, PKC inhibition abolishes the propofol-induced attenuation in capacitative Ca^{2+} entry.

It is well known that $[Ca^{2+}]_i$ is an important determinant in the regulation of cardiac and smooth muscle contraction. In vascular smooth muscle, agonist-induced increases in $[Ca^{2+}]_i$ primarily occur *via* release of Ca^{2+} from intracellular stores by a 1,4,5 inositol triphosphate–dependent mechanism. In addition, some agonists can trigger Ca^{2+} influx across the sarcolemma, primarily *via* voltage-gated or receptor-operated Ca^{2+} channels. An increase in $[Ca^{2+}]_i$ activates the myosin light chain kinase through a calmodulin-dependent mechanism, re-

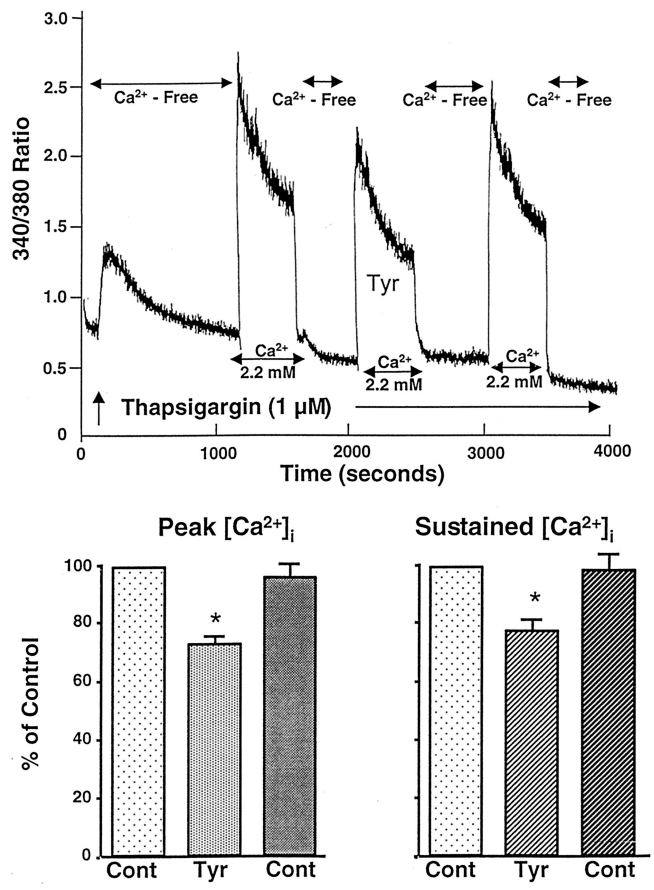

Fig. 3. (*Top*) Representative trace depicting the effect of the tyrosine kinase inhibitor tyrphostin 23 (Tyr; 100 μM) on capacitative calcium entry. After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative calcium entry was compared in the absence or presence of tyrphostin 23, which was added to the superfusion buffer 5 min before restoring extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). (*Bottom*) Summarized data showing the inhibitory effect (*$P < 0.05$) of tyrphostin 23 on capacitative Ca^{2+} entry. This inhibitory effect of tyrphostin 23 was entirely reversible (n = 7 cells).

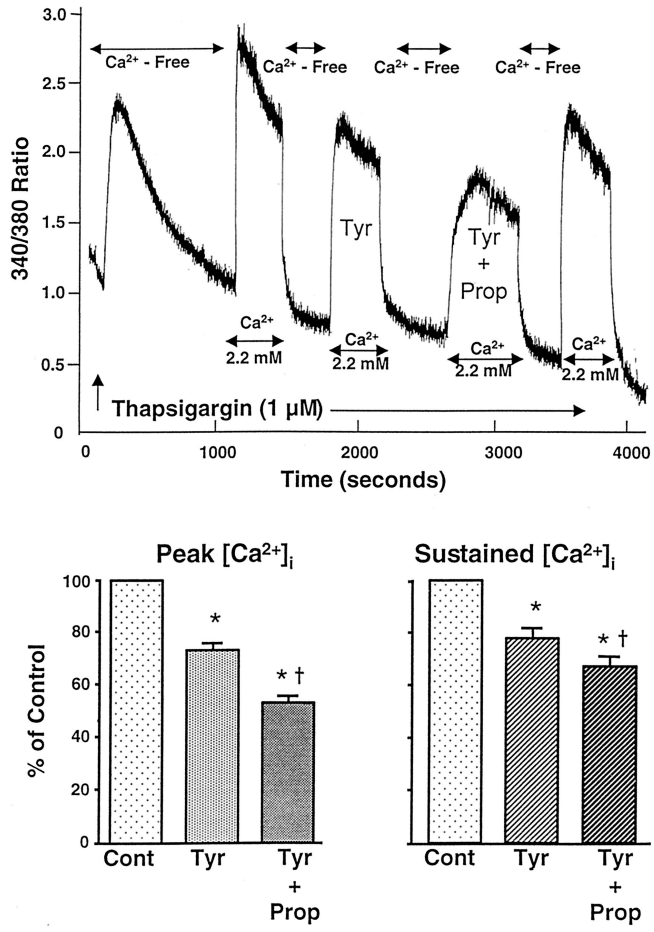

Fig. 4. (*Top*) Representative trace depicting the effect of tyrphostin 23 (Tyr; 100 μM) alone and in combination with propofol (100 μM) on capacitative Ca^{2+} entry. After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative Ca^{2+} entry was compared in the absence or presence of tyrphostin 23 and tyrphostin 23 plus propofol, which were each added to the superfusion buffer 5 min before restoring extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). (*Bottom*) Summarized data showing that, although tyrphostin 23 alone attenuated (*$P < 0.05$) capacitative Ca^{2+} entry, the addition of propofol resulted in a further reduction (†$P < 0.05$) in capacitative Ca^{2+} entry (n = 7 cells).

sulting in phosphorylation of the myosin light chains and initiation of contraction.[17] $[Ca^{2+}]_i$ is ultimately restored either by pumping the Ca^{2+} out of the cell *via* the sarcolemmal Ca^{2+} ATPase or Na^+–Ca^{2+} exchanger, or by resequestering Ca^{2+} into the intracellular store by the sarcoplasmic reticulum Ca^{2+} ATPase.[18,19]

In addition to the aforementioned sarcolemmal ion channels, Ca^{2+} influx can also be controlled by the filling-state of the intracellular Ca^{2+} store. The concept of capacitative Ca^{2+} entry was first postulated by Putney.[1] According to his model, depletion of intracellular Ca^{2+} stores results in activation of a Ca^{2+} influx pathway that is somehow sensitive to the state of filling of intracellular Ca^{2+} stores. Influx of Ca^{2+} *via* capacitative Ca^{2+} entry refills the intracellular Ca^{2+} stores that have been depleted in response to agonist activation. In the current study, thapsigargin was used as a tool to deplete the sarcoplasmic reticulum pool of Ca^{2+} in the absence of extracellular Ca^{2+} and thereby activate capacitative Ca^{2+} entry. Consistent with our previous study,[5] the amplitude of the thapsigargin-induced increase in $[Ca^{2+}]_i$ was variable, which likely reflects differences in the size of the sarcoplasmic reticulum Ca^{2+} store in different cells. The size of the sarcoplasmic reticulum Ca^{2+} store may depend on the cell passage number, the phase of the cell cycle, the length of time in serum-free medium, or whether the response was derived from an individual cell or a cluster of two to three neighboring cells. Restoring $[Ca^{2+}]_o$ stimulated capacitative Ca^{2+} entry, which was typically characterized by both a peak and sustained increase in $[Ca^{2+}]_i$. The rapid peak increase in $[Ca^{2+}]_i$ results from massive influx of Ca^{2+} into the cytosol *via* SK&F 96365–sensitive Ca^{2+} channels that open in response to depletion of sarcoplasmic reticulum Ca^{2+} stores.[5] The sustained or pro-

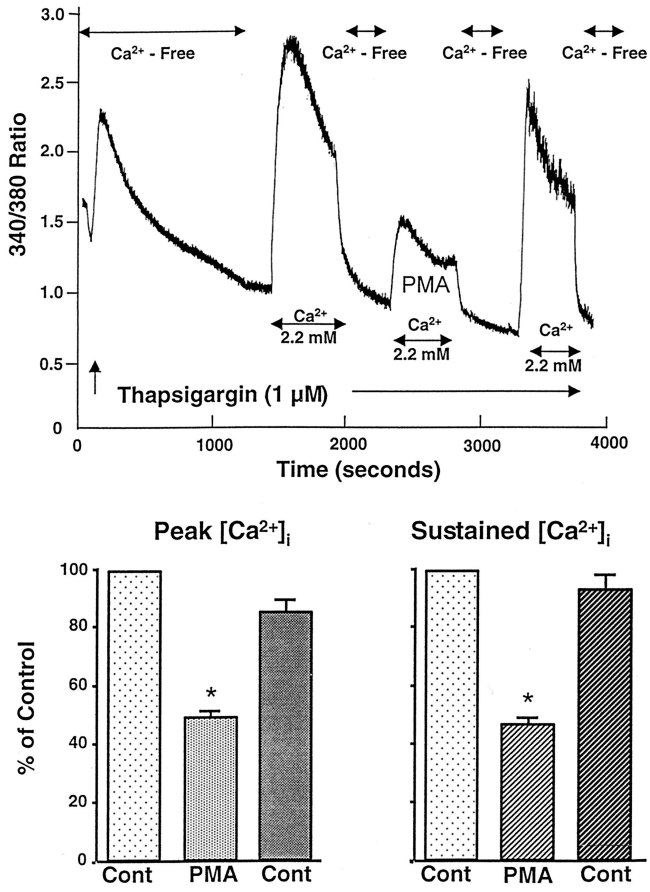

Fig. 5. (*Top*) Representative trace depicting the effect of the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA; 1 μM), on capacitative Ca^{2+} entry. After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative Ca^{2+} entry was compared in the presence and absence of PMA, which was added to the superfusion buffer 5 min before restoring extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). (*Bottom*) Summarized data showing the inhibitory effect (*$P < 0.05$) of phorbol 12-myristate 13-acetate on capacitative Ca^{2+} entry. This effect was reversible (n = 7 cells).

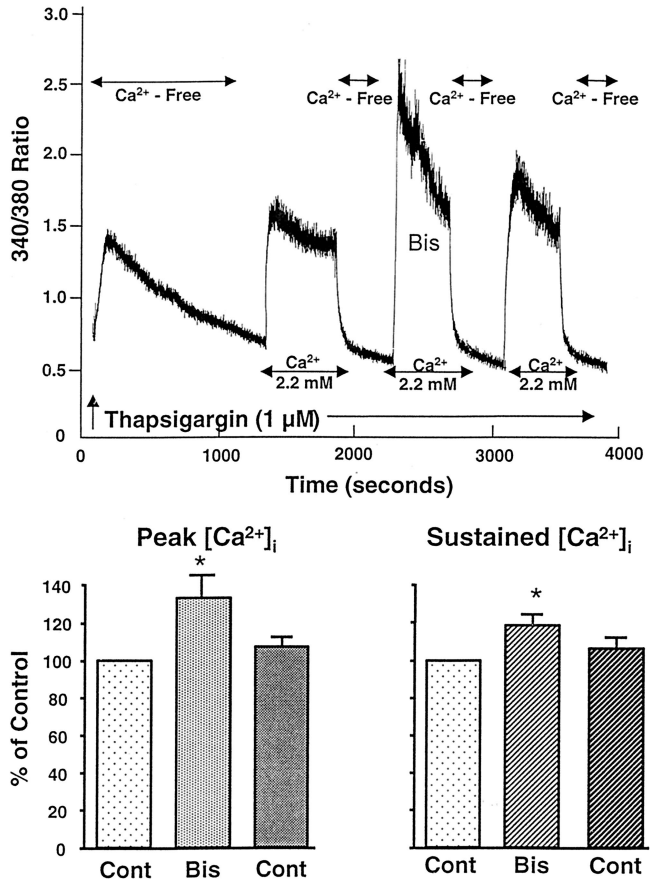

Fig. 6. (*Top*) Representative trace depicting the effect of the protein kinase C inhibitor bisindolylmaleimide (Bis; 1 μM) on capacitative Ca^{2+} entry. After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative Ca^{2+} entry was compared in the presence and absence of bisindolylmaleimide, which was added to the superfusion buffer 5 min before restoring extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). (*Bottom*) Summarized data showing the potentiating effect (*$P < 0.05$) of bisindolylmaleimide on capacitative Ca^{2+} entry. This effect was reversible (n = 7 cells).

longed increase in $[Ca^{2+}]_i$ is more complex. Because thapsigargin is an irreversible inhibitor of the sarcoplasmic reticulum Ca^{2+} ATPase, the sarcoplasmic reticulum is incapable of refilling with Ca^{2+} despite the increased availability of cytosolic free Ca^{2+}. As a result, capacitative Ca^{2+} entry across the sarcolemma is sustained, and a new steady state level of $[Ca^{2+}]_i$ is achieved as other mechanisms regulating Ca^{2+} extrusion (Na^+–Ca^{2+} exchanger and the sarcolemmal Ca^{2+} ATPase) begin to offset the continued influx of Ca^{2+}. If the sarcoplasmic reticulum is allowed to refill, as we have previously demonstrated using a reversible inhibitor of the sarcoplasmic reticulum Ca^{2+} ATPase (cyclopiazonic acid), the response to capacitative Ca^{2+} entry is only transient, and a sustained phase is not evident.[5]

Thapsigargin-induced capacitative Ca^{2+} entry does not involve activation of intracellular second messengers (*e.g.*, 1,4,5 inositol triphosphate). The cellular mechanism that mediates capacitative Ca^{2+} entry has been intensively investigated, although it has yet to be definitively identified. Various models have been postulated to explain how the sarcoplasmic reticulum communicates with the plasma membrane. These models can generally be divided into those that propose the existence of a diffusible factor and those that suggest that the signal is transferred *via* protein phosphorylation and dephosphorylation.[2] Potentially important diffusible second messengers released from the storage organelles include cytochrome P450 metabolites,[20] G proteins,[21] or a low-molecular-weight compound called Ca^{2+} influx factor.[22] Models based on phosphorylation and dephosphorylation interactions suggest that PKC activation either inhibits[14,23] or stimulates[24] capacitative Ca^{2+} entry depending on the cell type, whereas tyrosine kinase or protein kinase A activation are consistently associated with activation of capacitative Ca^{2+} entry.[14,25–27]

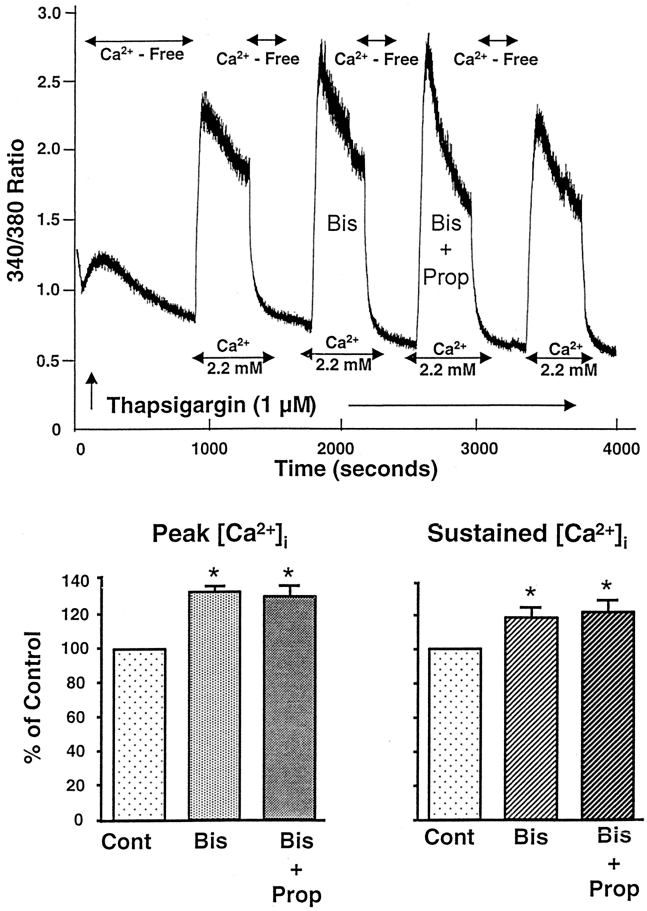

Fig. 7. (*Top*) Representative trace depicting the effect of bisindolylmaleimide (Bis; 1 μM) alone and in combination with propofol (Prop; 100 μM) on capacitative Ca^{2+} entry. After depletion of sarcoplasmic reticulum Ca^{2+} stores with thapsigargin, capacitative Ca^{2+} entry was compared in the absence or presence of bisindolylmaleimide and bisindolylmaleimide plus propofol, which were each added to the superfusion buffer 5 min before restoring extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). (*Bottom*) Summarized data showing that bisindolylmaleimide alone potentiated (*$P < 0.05$) capacitative Ca^{2+} entry. In the presence of bisindolylmaleimide, propofol no longer had an inhibitory effect on capacitative Ca^{2+} entry (n = 7 cells).

Because capacitative Ca^{2+} entry is involved in the regulation of $[Ca^{2+}]_i$ and vasomotor tone in PASMCs,[5] the ion channel associated with capacitative Ca^{2+} entry may serve as a cellular target for propofol. In cultured A10 and rat aortic smooth muscle cells, propofol was reported to inhibit voltage-gated Ca^{2+} channels but to have no effect on capacitative Ca^{2+} entry.[6] In contrast, propofol attenuated capacitative Ca^{2+} entry in cultured aortic smooth muscle cells from normotensive rats,[28] and this effect was even more prominent in hypertensive rats. In our study using canine PASMCs, propofol caused a dose-dependent decrease in capacitative Ca^{2+} entry. Typical free plasma concentrations of propofol range from 1 to 10 μM. Thus, propofol attenuated capacitative Ca^{2+} entry at clinically relevant concentrations. Tyrosine kinase inhibition also attenuated capacitative Ca^{2+} entry. However, in the presence of tyrosine kinase inhibition with tyrphostin 23, propofol further suppressed capacitative Ca^{2+} entry. These results suggest that inhibition of tyrosine kinases is not the primary mechanism for propofol-induced inhibition of capacitative Ca^{2+} entry. In contrast, inhibition of PKC with bisindolylmaleimide resulted in potentiation of capacitative Ca^{2+} entry, whereas activation of PKC with phorbol 12-myristate 13-acetate resulted in attenuation of capacitative Ca^{2+} entry. Moreover, pretreatment with bisindolylmaleimide prevented the propofol-induced attenuation of capacitative Ca^{2+} entry. Taken together, these results suggest that propofol inhibits capacitative Ca^{2+} entry *via* a PKC-dependent mechanism. The cellular target for this propofol-induced, PKC-mediated attenuation of capacitative Ca^{2+} entry remains to be elucidated. The propofol-induced attenuation of the peak response may either directly or indirectly be caused by PKC-dependent inhibition of Ca^{2+} channels that mediate capacitative Ca^{2+} entry, whereas attenuation of the sustained response may involve effects on the Na^+–Ca^{2+} exchanger or the sarcolemmal Ca^{2+} ATPase. The current results are consistent with previous reports that propofol activates purified brain PKC.[29,30] Moreover, we have preliminary data that suggest that propofol increases myofilament Ca^{2+} sensitivity in PASMCs *via* a PKC-dependent mechanism.[31]

In previous studies from our laboratory,[5,11] we demonstrated that α-adrenoreceptor–mediated Ca^{2+} oscillations were not altered by the nonselective, broad-range protein kinase inhibitor staurosporine. In contrast, the Ca^{2+} oscillations were abolished by SK&F 96365, an inhibitor of capacitative calcium entry, and were attenuated by tyrosine kinase inhibition with genestein or tyrphostin. In this study, selective inhibition of PKC potentiated capacitative Ca^{2+} entry, whereas selective inhibition of tyrosine kinases attenuated capacitative Ca^{2+} entry. Because staurosporine effectively blocks both tyrosine kinases and PKC, the lack of effect of staurosporine on the α-adrenoreceptor–mediated Ca^{2+} oscillations is likely a result of offsetting effects on capacitative Ca^{2+} entry, resulting in no net effect on the Ca^{2+} oscillations. Although propofol (1–10 μM) attenuated capacitative Ca^{2+} entry in this study, we previously reported that the propofol-induced attenuation of α-adrenoreceptor–mediated Ca^{2+} oscillations was apparent only at supraclinical concentrations.[9] It should be noted that capacitative Ca^{2+} entry is not the only mechanism regulating α-adrenoreceptor–mediated Ca^{2+} oscillations. Moreover, regulation of capacitative Ca^{2+} entry and Ca^{2+} oscillations has not been entirely elucidated but appears to involve multiple mechanisms. In the current study, we demonstrated opposing actions of tyrosine kinases and PKC activation on capacitative Ca^{2+} entry (CCE). It is possible that propofol alters multiple mechanisms involved in the regulation of the Ca^{2+} oscillations, some of which may offset the effects of the other.

Although our results suggest that the effects of propofol on capacitative Ca^{2+} entry are primarily mediated *via* activation of PKC, alternative interpretations are possible. Propofol alone and tyrphostin alone inhibited capacitative Ca^{2+} entry by approximately 30–35%. In the setting of tyrosine kinase inhibition, propofol further attenuated capacitative Ca^{2+} entry by approximately 20%. Therefore, it could be argued that a portion of the inhibitory effect of propofol on capacitative Ca^{2+} entry may be mediated *via* inhibition of tyrosine kinases. However, this possibility seems unlikely because if the inhibitory effect of propofol on CCE is mediated by a pathway different from PKC, then propofol should continue to exert an inhibitory effect on CCE in the presence of PKC inhibition. Our results indicate that it did not. Given that PKC activation attenuated CCE and PKC inhibition abolished the propofol-induced attenuation in CCE, it seems reasonable to conclude that the effects of propofol are mediated (at least primarily) through PKC activation. This PKC-mediated attenuation of capacitative Ca^{2+} entry induced by propofol does not appear to be a general characteristic of intravenous anesthetics. In preliminary studies,[32] thiopental had no effect on capacitative Ca^{2+} entry, whereas ketamine attenuated capacitative Ca^{2+} entry *via* a mechanism that did not involve PKC. These results indicate that intravenous anesthetics can have differential effects on capacitative Ca^{2+} entry that are mediated by more than one cellular mechanism.

In summary, propofol attenuates capacitative Ca^{2+} entry in PASMCs. This effect is not altered by inhibition of tyrosine kinases but is abolished by inhibition of PKC. Capacitative Ca^{2+} entry should be considered as a possible cellular target for anesthetic agents that alter vascular smooth muscle tone.

References

1. Putney JW Jr: A model for receptor-regulated calcium entry. Cell Calcium 1986; 7:1–12

2. Berridge MJ: Capacitative calcium entry. Biochem J 1995; 312:1–11
3. Penner R, Fasolato C, Hoth M: Calcium influx and its control by calcium release. Neurobiology 1993; 3:368–74
4. Fasolato C, Innocenti B, Pozzan T: Receptor-activated Ca^{2+} influx: How many mechanisms for how many channels? Trends Pharmacol Sci 1994; 15: 77–83
5. Doi S, Damron DS, Horibe M, Murray PA: Capacitative Ca^{2+} entry and tyrosine kinase activation in canine pulmonary arterial smooth muscle cells. Am J Physiol 2000; 278:L118–30
6. Xuan Y-T, Glass P: Propofol regulation of calcium entry pathways in cultured A10 and rat aortic smooth muscle cells. Br J Pharmacol 1996; 117:5–12
7. Chang KSK, Davis RF: Propofol produces endothelium-independent vasodilation and may act as a Ca^{2+} channel blocker. Anesth Analg 1993; 76:24–32
8. Imura N, Shiraishi Y, Katsuya H, Itoh T: Effect of propofol on norepinephrine-induced increases in $[Ca^{2+}]_i$ and force in smooth muscle of the rabbit mesenteric resistance artery. ANESTHESIOLOGY 1998; 88:1566–78
9. Hamada H, Damron DS, Murray PA: Intravenous anesthetics attenuate phenylephrine-induced calcium oscillations in individual pulmonary artery smooth muscle cells. ANESTHESIOLOGY 1997; 87:900–7
10. Hong S-J, Damron DS, Murray PA: Benzodiazepines differentially inhibit phenylephrine-induced calcium oscillations in pulmonary artery smooth muscle cells. ANESTHESIOLOGY 1998; 88:792–9
11. Hamada H, Damron DS, Hong S-J, VanWagoner DR, Murray PA: Phenylephrine-induced Ca^{2+} oscillations in canine pulmonary artery smooth muscle cells. Circ Res 1997; 81:812–23
12. Campbell JH, Campbell GR: Culture techniques and their applications to studies of vascular smooth muscle. Clin Sci 1993; 85:501–13
13. Thastrup O, Cullen PJ, Drbak BK, Hanley MR, Dawson AP: Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci U S A 1990; 87:2466–70
14. Song SK, Choi S-Y, Kim K-T: Opposing effects of protein kinase A and C on capacitative calcium entry into HL-60 promyelocytes. Biochem Pharmacol 1998; 56:561–7
15. Montero M, Garcia-Sancho J, Alvarez J: Transient inhibition by chemotactic peptide of a store-operated Ca^{2+} entry pathway in human neutrophils. J Biol Chem 1993; 268:13055–61
16. Peterson CC, Berridge MJ: The regulation of capcitative calcium entry by calcium and protein kinase C in Xenopus oocytes. J Biol Chem 1994; 269: 32246–53
17. Somlyo AP, Somlyo AV: Signal transduction and regulation in smooth muscle. Nature 1994; 372:231–6
18. van Breemen C, Saida K: Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. Annu Rev Physiol 1989; 51:315–29
19. O'Donnell ME, Owen NE: Regulation of ion pumps and carriers in vascular smooth muscle. Physiol Rev 1994; 74:683–721
20. Alvarez J, Montero M, Garcia-Sancho J: Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx. Biochem J 1991; 274:193–7
21. Peterson CC, Berridge MJ: G-protein regulation of capacitative calcium entry may be mediated by protein kinases A and C in Xenopus oocytes. Biochem J 1995; 307:663–8
22. Randiamampita C, Tsien RY: Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. Nature 1993; 364:809–14
23. Mene P, Pugliese F, Cinotti G: Regulation of capacitative calcium influx in cultured human mesangial cells: Roles of protein kinase C and calmodulin. J Am Soc Nephrol 1996; 7:983–90
24. Bode HP, Göke B: Protein kinase C activates capacitative calcium entry in the insulin secreting cell line RINm5F. FEBS Lett 1994; 339:307–11
25. Takenaka H, Imoto K, Sakano S, Kaneko M, Ohshika H: Involvement of tyrosine kinase in capacitative Ca^{2+} entry pathway in rat glioma C6 cells. Mol Pathway Pharm 1997; 98:127–40
26. Yule DI, Kim ET, Williams JA: Tyrosine kinase inhibitors attenuate "capacitative" Ca^{2+} influx in rat pancreatic acinar cells. Biochem Biophys Res Commun 1994; 202:1697–704
27. Sargeant P, Farndale RW, Sage SO: ADP- and thapsigargin-evoked Ca^{2+} entry and protein-tyrosine phosphorylation are inhibted by the tyrosine kinase inhibitors genistein and methyl-2,5-dihydroxycinnamate in fura-2-loaded human platelets. J Biol Chem 1993; 268:18151–6
28. Samain E, Bouillier H, Marty J, Safar M, Dagher G: The effect of propofol on angiotensin II-induced Ca^{2+} mobilization in aortic smooth muscle cells from normotensive and hypertensive rats. Anesth Analg 2000; 90:546–52
29. Hemmings HC Jr, Adamo AI: Effects of halothane and propofol on purified brain protein kinase C activation. ANESTHESIOLOGY 1994; 81:147–55
30. Hemmings HC Jr, Adamo AI, Hoffman MM: Biochemical characterization of the stimulatory effects of halothane and propofol on purified brain protein kinase C. Anesth Analg 1995; 81:1216–22
31. Tanaka S, Kondo I, Damron DS, Murray PA: Propofol increases myofilament calcium sensitivity via protein kinase C activation (abstract). ANESTHESIOLOGY 2000; 92:A1347
32. Kondo I, Tanaka S, Damron D, Murray PA: Intravenous anesthetics inhibit capacitative calcium entry in pulmonary artery smooth muscle cells (abstract). ANESTHESIOLOGY 2000; 92:A1327