Ca-Mediated and Independent Effects of Arachidonic Acid on Gap Junctions and Ca-Independent Effects of Oleic Acid and Halothane

Ahmed Lazrak, Antonio Peres,* Stefano Giovannardi,* and Camillo Peracchia
Department of Physiology, University of Rochester, Rochester, New York 14642 USA, and *Dipartimento di Fisiologia e Biochimica Generali, Universitá di Milano, Milano, Italy

ABSTRACT In Novikoff hepatoma cell pairs studied by double perforated patch clamp (DPPC), brief (20 s) exposure to 20 μM arachidonic acid (AA) induced a rapid and reversible uncoupling. In pairs studied by double whole-cell clamp (DWCC), uncoupling was completely prevented by effective buffering of Ca_i^{2+} with BAPTA. Similarly, AA (20 s) had no effect on coupling in cells perfused with solutions containing no added Ca^{2+} (SES-no-Ca) and studied by DPPC, suggesting that Ca^{2+} influx plays an important role. Parallel experiments monitoring $[Ca^{2+}]_i$ with fura-2 showed that $[Ca^{2+}]_i$ increases with AA to 0.7–1.5 μM in normal $[Ca^{2+}]_o$, and to ~400 nM in SES-no-Ca solutions. The rate of $[Ca^{2+}]_i$ increase matched that of G_j decrease, but $[Ca^{2+}]_i$ recovery was faster. In cells studied by DWCC with 2 mM BAPTA in the pipette solution and superfused with SES-no-Ca, long exposure (1 min) to 20 μM AA caused a slow and virtually irreversible uncoupling. This result suggests that AA has a dual mechanism of uncoupling: one dominant, fast, reversible, and Ca^{2+}-dependent, the other slow, poorly reversible, and Ca^{2+}-independent. In contrast, uncoupling by oleic acid (OA) or halothane was insensitive to internal buffering with BAPTA, suggesting a Ca^{2+}-independent mechanism only.

INTRODUCTION

Gap junction channels mediate direct cell-to-cell diffusion of ions and small cytoplasmic molecules and are endowed with gates that close in response to cell damage or other phenomena that alter the intracellular ionic composition, resulting in functional cell-to-cell uncoupling (reviewed in Peracchia et al., 1994). Cell uncoupling is generally believed to be primarily a safety mechanism by which cells isolate themselves from damaged neighbors, but recent evidence for gap junction sensitivity to $[Ca^{2+}]_i$ in the physiological range (Peracchia, 1990; Lazrak and Peracchia, 1993) indicates that modulation of coupling can also play an important role in Ca^{2+}-mediated phenomena involving second messengers.

A second messenger known to affect gap junction permeability is arachidonic acid (AA) (Giaume et al., 1989; Spray and Burt, 1990; Fluri et al., 1990; Massey et al., 1992). AA is a fatty acid that diffuses readily across cell membranes and affects directly or through metabolites various cellular functions and membrane currents (Piomelli et al., 1987; Ordway et al., 1991). AA is produced by phospholipase A2 (Parker et al., 1987) after receptor-mediated or independent (hypoxia) stimulation and is metabolized to prostaglandins and thromboxanes by cyclooxygenases, and to leukotrienes and hydroxyeicosatetraenoic acid by lipoxygenases. These compounds, eicosanoids, participate in signal transduction modulation in many cell systems (Axelrod et al., 1988; Smith, 1989; Shimizu and Wolfe, 1990).

The mechanisms by which AA affects cell function are still poorly understood, but in several cases a role for Ca_i^{2+} has been postulated. AA was found to increase $[Ca^{2+}]_i$ in gastric mucosa (Wilkes et al., 1991) and cardiac myocytes (Damron and Bond, 1993), and to cause a Ca_i^{2+}-dependent catecholamine release from chromaffin cells mediated by phosphoinositide metabolism (Negishi et al., 1990). Recently, Mochizuki-oda et al. (1993) reported that external application of AA reversibly activates voltage-insensitive Ca^{2+} channels and increases $[Ca^{2+}]_i$. Other studies have focused on thromboxane A2, a cyclooxygenase breakdown product of AA, as an important element of AA-induced $[Ca^{2+}]_i$ increase (Yamagishi et al., 1992; Rayes et al., 1992; Dorn and Becker, 1993; Kent et al., 1993).

The effects of AA on gap junction channel gating have been interpreted exclusively on the basis of Ca^{2+}-independent mechanisms (Giaume et al., 1989; Fluri et al., 1990; Massey et al., 1992), possibly involving perturbation of the junctional membrane structure (Massey et al., 1992). A direct, Ca^{2+}-independent effect on gap junctions has also been suggested for oleic acid (OA) (Burt et al., 1991) and halothane (Peracchia, 1991).

To understand further the mechanism of arachidonic acid-induced uncoupling, we have studied its effects on both junctional conductance and $[Ca^{2+}]_i$ in Novikoff hepatoma cells, a cell line known to express the rat heart connexin (Cx43, Meyer et al., 1992). The effects of AA have also been compared with those of OA and halothane (a general anesthetic). Our data show that brief (20 s) exposures to 20 μM AA induce significant increase in $[Ca^{2+}]_i$ and reversibly reduce junctional conductance in a Ca^{2+}-dependent manner, whereas long (1 min) exposures to AA are likely to affect gap junctions via both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. In contrast, OA and halothane appear to close

Received for publication 8 April 1994 and in final form 6 June 1994.

Address reprint requests to Dr. Camillo Peracchia, Department of Physiology, University of Rochester, 601 Elmwood Ave, Box 642, Rochester, NY 14642. Tel.: 716-275-1610; Fax: 716-461-3259.

0006-3495/94/09/1052/08 $2.00

gap junction channels in a Ca^{2+}-independent way only. The study also provides a comparison of the intracellular Ca^{2+} buffering efficiency of EGTA and BAPTA. Preliminary data from this study have appeared in abstract form (Lazrak et al., 1994).

MATERIALS AND METHODS

Cell culture

Novikoff hepatoma cells were cultured as previously described (Johnson et al., 1974; Meyer et al., 1992; Lazrak and Peracchia, 1993). Briefly, Novikoff hepatoma cells (line N1-S1, CRL 1604, American Type Culture Collection, Rockville, MD) were grown in suspension in sealed flasks at 37°C in S210 medium (Gibco BRL, Life Technologies, Gaithersburg, MD) supplemented with: $NaHCO_3$ 50 mM, 1% penicillin-streptomycin, 0.1% Pluronic F68 (Sigma Chemical Co., St. Louis, MO), pH 7.2 (NaOH 1 N). Before cell culture renewal, the cells were treated with trypsin for 5–10 min at 37°C (2.5% trypsin-EDTA; Sigma). For best results and yield consistency, the cell suspension was renewed daily.

Electrophysiology

Before each experiment, 20–24 h old cells were suspended in a standard external saline (SES) containing (in mM): NaCl 145, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 2, Glucose 5.5, HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]) 10, pH 7.2 (NaOH, 1 N) at ~22°C; for Ca^{2+}-free experiments, no Ca^{2+} was added to SES (SES-no-Ca). The cell suspension (2 ml) was transferred to a polystyrene dish (LUX 5221, Nunc Inc., Naperville, IL), and the cells were allowed to settle at the bottom for 30 min at 37°C before the dish was mounted on the stage of a Diaphot inverted microscope (Nikon Inc., Garden City, NY). The cells were continuously perfused at 0.5 ml/min (T = ~22°C).

Patch pipettes were made from capillary tubing (Pyrex, Brand Laboratory Glassware, Corning, NY) with a vertical puller (700 B, David Kopf Instruments, Tujunga, CA) and back-filled, for double perforated patch clamp (DPPC, Horn and Marty, 1988) experiments, with a standard internal solution (SIS) containing (in mM): KCl 145, NaCl 6, $MgCl_2$ 0.5, ATP 3, HEPES 10, pH 7.2 (KOH, 1 N), and nystatin (100 μg/ml, Sigma). Nystatin was dissolved in dimethyl sulfoxide (5 mg/ml), stored in frozen aliquots, and diluted to the final concentration just before use. For standard double whole-cell clamp (DWCC, Hamill et al., 1981) experiments, nystatin was omitted and BAPTA was added to the pipette solution to a final concentration of 1 mM. In experiments comparing BAPTA and EGTA as internal Ca^{2+} buffers, different concentrations of BAPTA or EGTA, ranging from 0 to 10 mM were added to the pipette solutions.

Membrane capacitance and series resistances were monitored every 10 min by zeroing, with each patch clamp amplifier, the transient current induced by a voltage pulse applied simultaneously to both cells of the pair (I_j = 0). In perforated patches, after gigaseal formation the series resistances dropped with time as new nystatin pores formed; recording was started only after the series resistances had reached values lower than 20 MΩ. The final series resistances were similar to those obtained with DWCC. Large tip-diameter pipettes (2–3 MΩ) were used to allow fast solution exchange between pipette and cytosol in DWCC and to create a large number of nystatin pores in DPPC. Voltage clamp recording was performed with two EPC-7 amplifiers (EPC-7, List Electronics, distributed by Medical Systems Corp., Greenvale, NY). Membrane capacitance (slow and fast components) and series resistances were monitored and compensated by the amplifiers. Both pulse generation and data acquisition were performed by means of an IBM-AT compatible computer equipped with Pclamp software (Axon Instruments, Foster City, CA) and A/D-D/A interface (Labmaster TL-1, Axon).

For studying the electrical properties of the junctional membrane, the cells were initially voltage-clamped to the same holding potential (V_H = −20 mV), to have zero junctional current flow at rest (I_j = 0 pA). A V_j gradient was created by imposing a voltage step (V_1) to cell 1 while maintaining V_2 at −20 mV, the common holding potential. The negative feedback current (I_2), injected by the clamp amplifier in cell 2 for maintaining V_2 constant, was used for G_j calculation, because it corresponds to the junctional current (I_j), but with opposite sign ($I_j = -I_2$; Fig. 1 *A*); using Ohm's law, $G_j = I_j/V_j$.

Fura-2 loading and Ca^{2+} measurement

Single cells were loaded with the calcium indicator by a 15-min incubation at room temperature in SES containing 2 μM fura2-AM (Sigma). A spectrophotometer was used for double excitation, decoding the emitted light and analog computation of the emission ratio R, as previously described (Peres et al., 1990). Ca^{2+} concentrations were calculated from the emission ratio R, according to Grynkiewicz et al. (1985), using the calibration procedure based on ionophore permeabilization suggested by Williams et al. (1990). However, one must be aware that the determination of absolute $[Ca^{2+}]_i$ values with fura-2 is not definitive (Poenie, 1990; Roe et al., 1990).

Arachidonic acid, oleic acid, and halothane

Arachidonic acid (AA) and Oleic acid (OA; Sigma) were dissolved in ethanol and dimethyl sulfoxide, respectively, and stored in aliquots, at 30 and 100 mM concentrations, at −20°C. They were diluted to a final concentration of 20 μM just before use, and light-protected to prevent decay. They were added to the cell dish via local perfusion, as closely as possible (100–200 μm) to the cell pair under investigation, by means of a polyethylene tubing of 0.24 mm (ID), connected to a micro-injector (Tracor Atlas, INC., Houston, TX). The tubing was replaced before each experiment. Halothane (1.6 mM) was added to SES (0.4 μl/ml) and mixed by vigorously stirring the solution in a sealed glass flask for 3–4 h. Halothane was applied to cell pairs by the general perfusion system. During the experiment, the rate of general perfusion was increased from 0.5 to 1.5 ml/min, for fast solution exchange and elimination of added substances from the bath.

RESULTS

Effects of brief exposure to arachidonic acid on junctional conductance

In Novikoff hepatoma cell pairs studied by double perforated patch clamp (DPPC), a 20 s application of 20 μM AA by local perfusion induced a rapid decrease in junctional current (I_j) and nonjunctional current (I_1) (Fig. 1 *A*, initial G_j = 36.7 nS), reflecting a large drop in junctional conductance (G_j) and virtually complete cell-cell uncoupling (Fig. 1 *B*, initial G_j = 17.24 ± 7.56 nS, mean ± SE, n = 12). In 97% of the cell pairs studied, G_j decreased to 5–10% of the initial values, ranging from 5 to 40 nS, in less than 1 min. The AA-induced uncoupling was always reversible, with G_j recovering by ~70% in 4–5 min.

Efficacy of Ca_i^{2+} buffers in preventing the effects of 20 s exposure to arachidonic acid on junctional conductance

In cell pairs studied by double whole-cell clamp (DWCC), uncoupling by AA (20 μM, 20 s) was inhibited by Ca_i^{2+} buffering. The degree of inhibition depended greatly on the

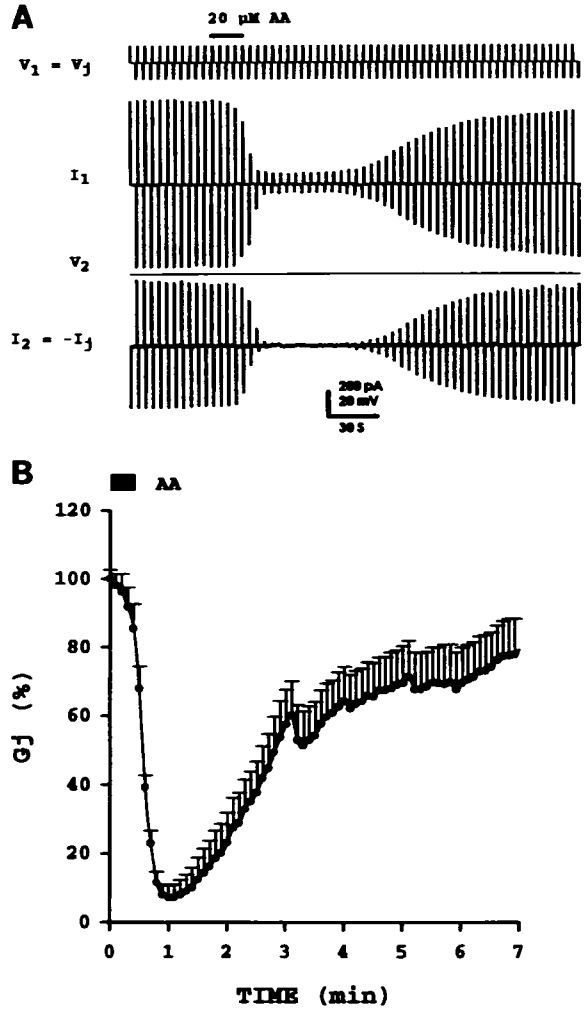

FIGURE 1 Effects of a brief (20 s) exposure to 20 μM AA on Novikoff hepatoma cell pairs studied by DPPC. The currents and voltages recorded from a cell pair before, during, and after AA application are shown in *A*. Note that AA induces a rapid and reversible uncoupling, reflected by the dramatic decrease in both I_1 and I_2 ($I_j = -I_2$, initial G_j = 36.7 nS); voltage pulses of ±20 mV and 100 ms were applied to cell 1 every 5 s. With AA, G_j decreases as average to less than 10% of initial values (*B*, initial G_j = 17.24 ± 7.56 nS; mean ± SE, n = 12).

type of intracellular buffer used: BAPTA, at a concentration as low as 0.1 mM caused about 20% inhibition, and at 1–2 mM virtually abolished the AA effect (Fig. 2 *A*). In contrast, no inhibition was seen with EGTA at 2 mM (Fig. 2 *B*), and even at 10 mM EGTA inhibition was only partial: AA still caused a 20% decrease in G_j (Fig. 2 *C*). The presence of EGTA (5 mM or greater) also caused a longer delay between AA application and drop in G_j.

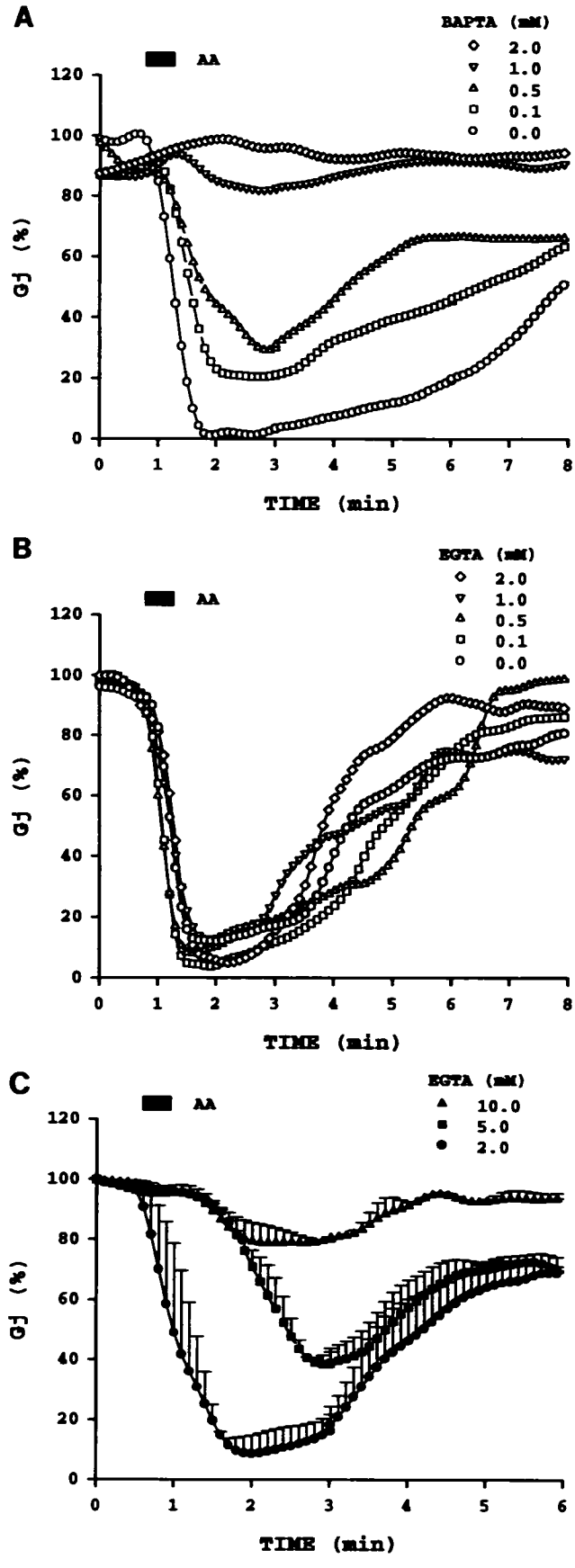

FIGURE 2 The uncoupling effect of AA (20 μM, 20 s) depends on Ca_i^{2+} buffering. Under DWCC conditions, BAPTA in the pipettes causes a 20% inhibition at concentrations as low as 0.1 mM and completely eliminates the uncoupling effects of AA at 1–2 mM concentrations. In contrast, EGTA in the same concentration range is totally ineffective in inhibiting the AA uncoupling effect (*B*). EGTA causes a delay between AA application and G_j decay and inhibits uncoupling by ~40 and ~80% (n = 3) at 5 and 10 mM concentrations, respectively (*C*).

Effects of arachidonic acid on junctional conductance and $[Ca^{2+}]_i$ in the presence and absence of external Ca^{2+}

In cell pairs perfused for at least 10 min with solutions containing no added Ca^{2+} (SES-no-Ca) and studied by DPPC, G_j was not affected by a 20 s exposure to 20 μM AA (Fig. 3 *A*). This suggests that Ca^{2+} influx plays a role in AA-induced uncoupling.

Parallel experiments monitoring the effect of AA on $[Ca^{2+}]_i$, by fura-2 ratio measurement, showed that $[Ca^{2+}]_i$ increases with AA to 1183 ± 490 nM (mean ± SE, n = 5) from resting values of 104 ± 18 nM (mean ± SE, n = 5), in cells bathed in normal $[Ca^{2+}]_o$, and to 439 ± 151 nM (mean ± SE, n = 6) from 101 ± 18 nM (mean ± SE, n = 6), in cells perfused with SES-no-Ca (Fig. 3 *B*). The rate of $[Ca^{2+}]_i$ increase was similar to that of G_j decrease (Fig. 1 *B*), but $[Ca^{2+}]_i$ recovery was generally faster.

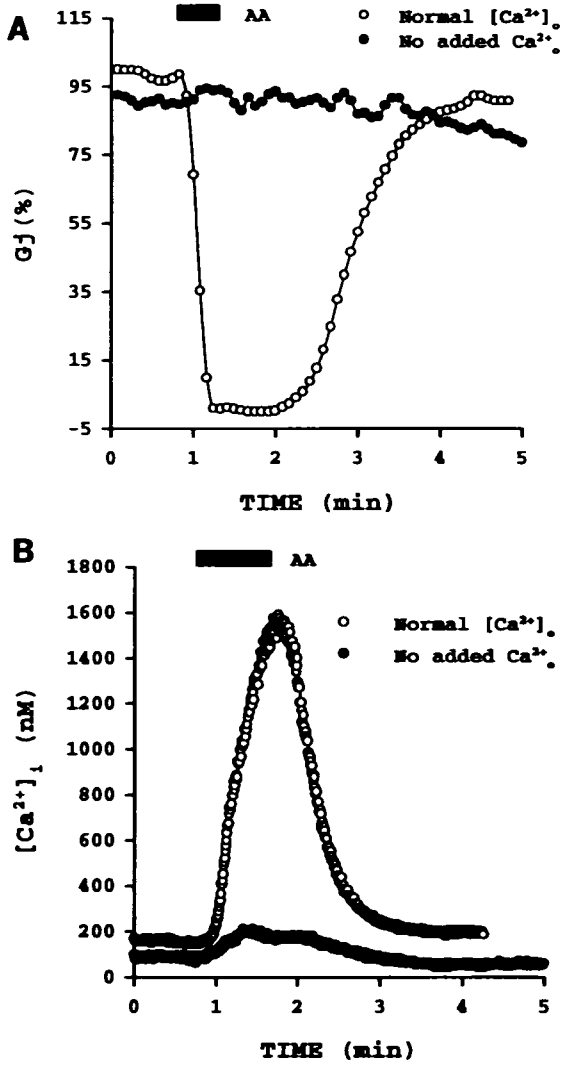

FIGURE 3 Effects of exposures to AA on G_j (*A*) and $[Ca^{2+}]_i$ (*B*) in cell pairs superfused with either normal $[Ca^{2+}]_o$ or SES-no-Ca. AA (20 μM, 20 s) has no effect on G_j in cell pairs perfused with SES-no-Ca (*A*). $[Ca^{2+}]_i$ (*B*) measured, in parallel experiments, in fura-2-loaded cells, increases with AA (20 μM) to 1183 ± 490 nM (mean ± SE, n = 5) from resting values of 104 ± 18 nM (mean ± SE, n = 5), in cells bathed in normal $[Ca^{2+}]_o$, and to 439 ± 151 nM (mean ± SE, n = 6) from 101 ± 18 nM (mean ± SE, n = 6), in cells superfused with SES-no-Ca. With normal $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ rose at a rate similar to that of G_j decrease (Fig. 1 *B*), but $[Ca^{2+}]_i$ recovery was faster.

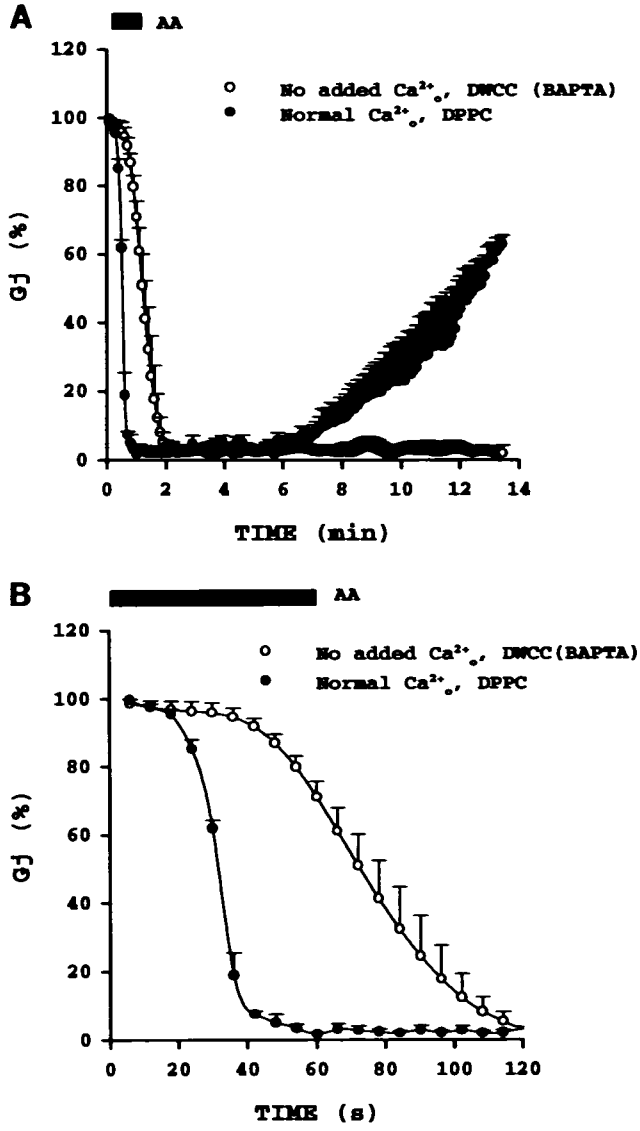

FIGURE 4 Effects of long exposures to AA on G_j. In cell pairs studied by DWCC with 2 mM BAPTA in the pipettes and superfused with SES-no-Ca, a 1-min application of 20 μM AA decreases G_j down to complete uncoupling (*A*, mean ± SE, n = 3). G_j drops at a rate of 1.89% per s (*B*, mean ± SE, n = 3), considerably slower than that (7.14% per s) in the presence of normal Ca_o^{2+} with DPPC (*B*, mean ± SE, n = 4). Recovery is absent in the absence of Ca_o^{2+} with DWCC and BAPTA in the pipettes (*A*), and it is present, but slow, in the presence of Ca_o^{2+} with DPPC (*A*). The presence of nanomolar $[Ca^{2+}]_i$ (DPPC), rather than a normal $[Ca^{2+}]_o$, is necessary for recovery, because recovery also occurs in SES-no-Ca and DWCC recording with $[Ca^{2+}]_i$ buffered to pCa 7 with Ca-BAPTA (data not shown). Note that in the absence of Ca^{2+}, G_j starts decreasing only after the Ca^{2+}-dependent effect has reached its maximum. The initial G_j was 30.47 ± 3.1 nS (mean ± SE, n = 3) and 21.08 ± 3.31 nS (mean ± SE, n = 4) in cells studied by DWCC and by DPPC, respectively.

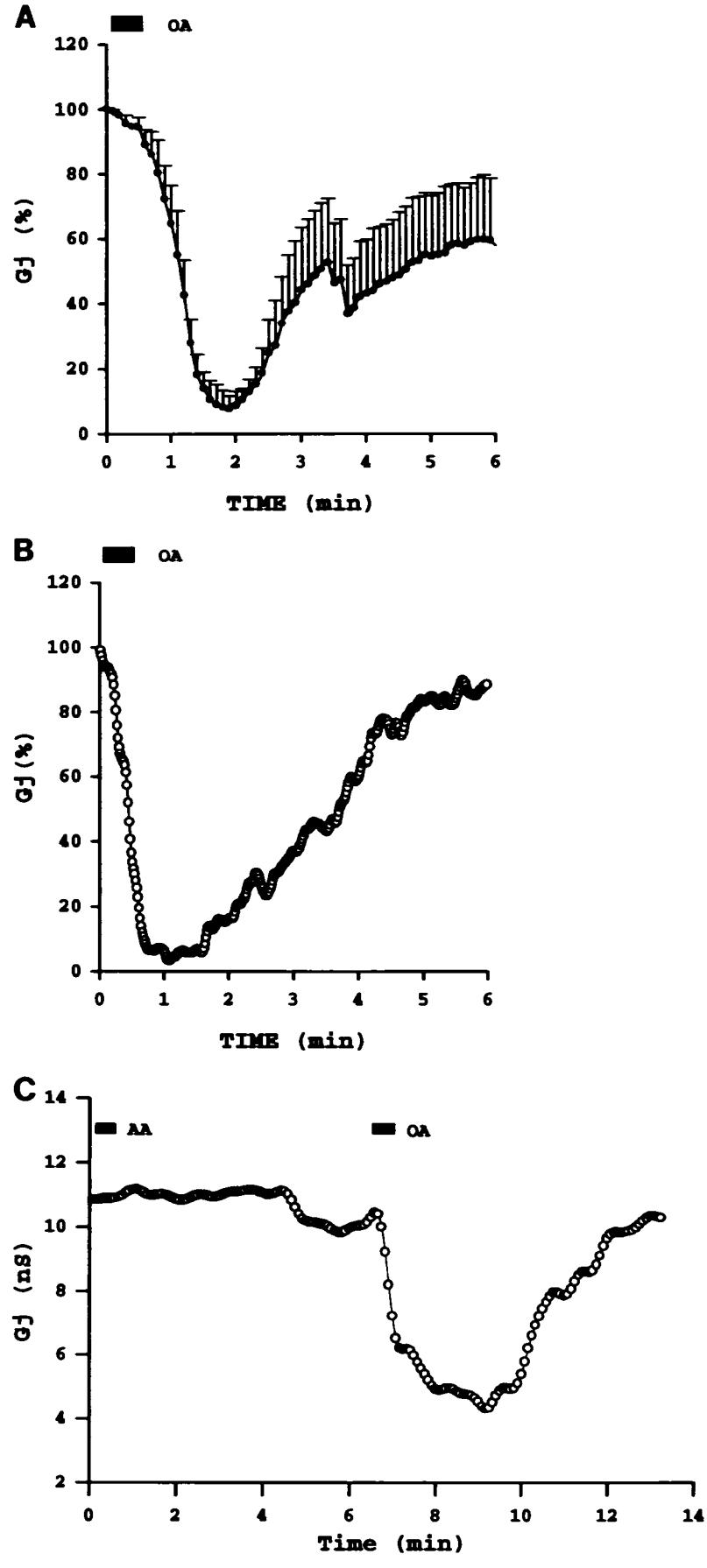

FIGURE 5 In cell pairs studied by DPPC, a 20 s application of 20 μM OA decreases G_j to virtually complete uncoupling (*A*, initial G_j = 17.31 ± 5.74, mean ± SE, n = 6). As with AA, with OA G_j decreases to 5–10% of the initial values, but the uncoupling rate is slightly slower. After OA treatment, G_j recovers by ~60% in 5–6 min. Uncoupling by OA is not inhibited either by internal BAPTA, in cells studied by DWCC (*B*, initial G_j = 16.67 nS), or by perfusing with SES-no-Ca cells studied by DPPC (*C*), a treatment that, in contrast, completely abolishes the effects of short (20 s) exposures to 20 μM AA (*C*; initial G_j = 10.26 nS).

Effects of long exposures to arachidonic acid on junctional conductance

In cell pairs studied by DWCC with 2 mM BAPTA in the pipettes and perfused with SES-no-Ca, a 1 min application of 20 μM AA decreased G_j to virtually complete cell-cell uncoupling (Fig. 4 *A*), suggesting a Ca^{2+}-independent component. However, G_j decreased at a rate of 1.89% per s, which is much slower than that observed with DPPC in normal Ca_o^{2+} (7.14% per s). In addition, the delay between AA application and drop in G_j was much greater (Fig. 4 *B*). Recovery occurred, although very slowly, with normal $[Ca^{2+}]_o$ and DPPC recording, but there was no recovery in the absence of Ca_o^{2+}, in DWCC recording mode, with BAPTA in the pipettes (Fig. 4 *A*). Recovery, however, did occur in SES-no-Ca and DWCC, when $[Ca^{2+}]_i$ was buffered to pCa 7 with Ca-BAPTA in the pipettes (data not shown). The presence of nanomolar $[Ca^{2+}]_i$, rather than a normal $[Ca^{2+}]_o$, seems to be necessary for recovery from AA-induced uncoupling.

Effects of oleic acid and halothane on junctional conductance

In cell pairs studied by DPPC, a 20 s application of 20 μM oleic acid (OA) by local perfusion induced a decrease in G_j that resulted in almost complete cell-cell uncoupling (Fig. 5 *A*). As with AA, OA decreased G_j to 5–10% of the control value (initial G_j = 17.31 ± 5.74 nS, mean ± SE, n = 6), but the uncoupling rate was slightly slower (Fig. 5 *A*). The OA-induced uncoupling was reversible, with G_j recovering by ~60% in 5–6 min. Uncoupling by OA was not inhibited either by intracellular buffering with BAPTA cell pairs studied by DWCC (Fig. 5 *B*) or by perfusing with SES-no-Ca solutions cell pairs studied by DPPC (Fig. 5 *C*), a treatment that completely abolishes the effect of a short exposure to AA in the same cell pair (Fig. 5 *C*).

Similar results were obtained with halothane. A 3 min perfusion with SES containing 1.6 mM halothane caused complete uncoupling in 1–2 min, followed by complete recovery in 4–5 min. Halothane was as effective in cells studied by DPPC (data not shown) as in cells internally buffered for Ca^{2+} with 1 mM BAPTA (Fig. 6); note that, in contrast, BAPTA completely prevented uncoupling by a 20 s exposure to 20 μM AA in the same cell pair (Fig. 6).

DISCUSSION

This study shows that external application of arachidonic acid (AA) increases $[Ca^{2+}]_i$ and reduces gap junctional conductance (G_j). Evidence from experiments testing different internal Ca^{2+} buffers and external solutions containing no added Ca^{2+} (SES-no-Ca) indicates that brief exposure to AA induces Ca^{2+} entry that results in a rapid, reversible, and Ca^{2+}-mediated junctional uncoupling. With long exposure, AA has both a dominant, Ca^{2+}-mediated, effect and, in addition, a less rapid, poorly reversible, and Ca^{2+}-independent effect. In contrast, the uncoupling mechanism of oleic acid (OA) or halothane appears only to be Ca^{2+}-independent.

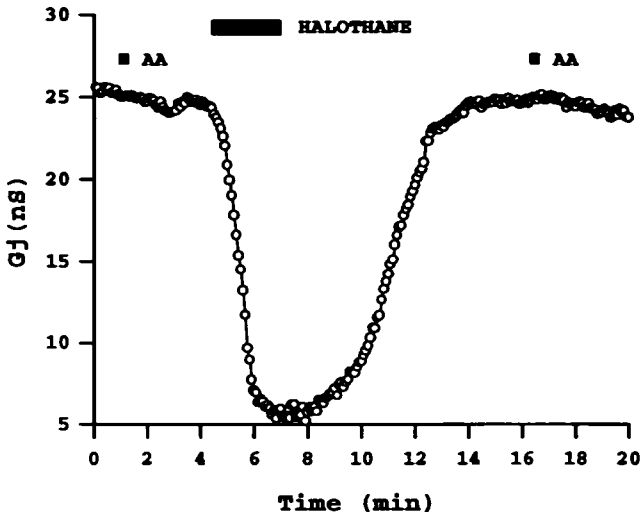

FIGURE 6 Results similar to those obtained with OA (Fig. 5 *B*) are obtained with halothane. A 3 min perfusion with SES containing 1.6 mM halothane causes complete uncoupling in 1–2 min (initial G_j = 25.60 nS), followed by complete recovery in 4–5 min, in cells studied by DWCC with 1 mM BAPTA in the pipettes. In contrast, BAPTA prevents uncoupling by 20 μM AA (20 s) in the same cell pair.

AA increases Ca_i^{2+} to concentrations in the low micromolar range. This is certainly sufficient to cause junctional uncoupling as $[Ca^{2+}]_i$ as low as 500 nM significantly decreased G_j regardless of pH_i (Lazrak and Peracchia, 1993). In the present study, the participation of Ca^{2+} in uncoupling evoked by brief exposures to AA is supported by the exquisite sensitivity of the AA effect to intracellular Ca-buffering and, in particular, to the type of Ca-buffer used. It was not surprising to find BAPTA significantly more effective than EGTA, because EGTA has been shown to be relatively slow in buffering cytosolic Ca^{2+}. Calculations of the gradient of $[Ca^{2+}]$ in the vicinity of a channel pore show that EGTA does not effectively buffer $[Ca^{2+}]$ within macromolecular distances from the pore, whereas BAPTA is quite effective (Marty and Neher, 1985; Adler et al., 1991; Stern, 1992). Our data indicate that BAPTA is at least 10 times more effective than EGTA in buffering $[Ca^{2+}]_i$ in the vicinity of gap junction channels or where crucial steps in gap junction regulatory mechanisms take place.

Data from experiments with SES-no-Ca solutions provide some clues on the mechanism of Ca_i^{2+} increase. The absence of uncoupling in SES-no-Ca solutions clearly indicates that Ca^{2+} entry plays a role. However, a participation of internal Ca^{2+} release cannot be excluded, because an increase in $[Ca^{2+}]_i$, although very small, was also observed with AA in cells perfused with SES-no-Ca solutions. In this study, we have not attempted to determine whether AA itself or one of its metabolites is involved in inducing the increase in $[Ca^{2+}]_i$ and/or in gating junctional channels, although previous gap junction studies suggested that AA alone (Giaume et al., 1989) or both AA and a 5-lipoxygenase metabolite (Massey et al., 1992) affect gap junction conductance.

AA has been shown to increase $[Ca^{2+}]_i$ in parietal cells of gastric mucosa (Wilkes et al., 1991) and in cardiac myocytes

(Damron and Bond, 1993), and was reported to induce a Ca_i^{2+}-dependent catecholamine release from chromaffin cells (Negishi et al., 1990). Mochizuki-Oda et al. (1993) reported that external application of AA reversibly activates voltage-insensitive Ca^{2+} channels and increases $[Ca^{2+}]_i$ primarily by increasing Ca^{2+} entry. In contrast, Damron and Bond (1993) suggested Ca^{2+} release from stores as the major factor in cytosolic Ca^{2+} increase. Although our data seem to agree with the interpretation of Mochizuki-Oda et al. (1993), further work will be needed for testing in detail the participation of Ca^{2+} stores in the AA effect on $[Ca^{2+}]_i$.

Previous reports on the effect of AA on gap junctions interpreted it entirely as a Ca^{2+}-independent phenomenon (Giaume et al., 1989; Fluri et al., 1990; Massey et al., 1992), possibly involving perturbation of the junctional membrane structure (Massey et al., 1992). All of these studies measured G_j by DWCC with EGTA in the patch pipettes, and two of them (Giaume et al., 1989; Massey et al., 1992) measured $[Ca^{2+}]_i$ with fura-2 in parallel experiments. Although our data on the low efficiency of EGTA in buffering Ca_i^{2+} raise questions on the interpretation that Ca^{2+} plays no role in AA induced uncoupling, the absence of $[Ca^{2+}]_i$ increase with AA in rat lacrimal cells (Giaume et al., 1989) and in neonatal cardiac cells (Massey et al., 1992) indicates that differences among cell systems can exist.

Our evidence for both a dominant, Ca^{2+}-mediated and a secondary, Ca^{2+}-independent effect of AA on gap junctions suggests two possible physiological scenarios. With brief exposure to AA, gap junction channels would close quickly, reversibly, and by a Ca^{2+}-dependent mechanism. With long exposure to AA, superimposed on the dominant, Ca^{2+}-dependent effect, there is a slow, Ca^{2+}-independent effect, with slow reversibility, that starts only after the Ca^{2+}-dependent effect has reached its maximum. The Ca^{2+}-independent effect might result from a direct, hydrophobic interaction between AA and connexins or neighboring membrane lipids. This is suggested by the delayed AA effect, likely to reflect the slow incorporation of AA into the hydrophobic membrane compartment. In view of its delayed onset and slow recovery, the Ca^{2+}-independent mechanism might play a greater role in maintaining uncoupling than in triggering the uncoupling process. Interestingly, after long exposures to AA, G_j recovered in cells studied with DPPC, although at a slower rate, but it did not in cells studied by DWCC with BAPTA in the pipettes, indicating that Ca_i^{2+} is also necessary for recovery. It is reasonable to believe that some steps of the AA enzymatic cascade require nanomolar $[Ca^{2+}]_i$, such that in the absence of Ca^{2+} (internal BAPTA), AA can survive longer and exert a sustained effect on gap junction permeability.

A dual mechanism could have important physiological consequences, because it could enable cells to extend the uncoupled state beyond $[Ca^{2+}]_i$ recovery. In cells in which AA does not induce an increase in $[Ca^{2+}]_i$, prolonged exposure to AA would be expected to cause a Ca^{2+}-independent uncoupling only, which is slower and poorly reversible. Rat lacrimal cells (Giaume et al., 1989) and neonatal cardiac cells (Massey et al., 1992) might belong to the latter type.

In contrast to the dual mechanism of AA, OA and halothane appear to act exclusively in a Ca^{2+}-independent way. This observation confirms previous studies on rat cardiac and vascular cells (Burt et al., 1991) and on crayfish axons (Peracchia, 1991).

In conclusion, AA, OA, and halothane cause rapid and reversible electrical uncoupling of Novikoff hepatoma cell pairs. Present evidence indicates that brief exposure to AA uncouples via Ca^{2+}, whereas prolonged exposure has a dual effect on coupling: a slow, poorly reversible, and Ca^{2+}-independent effect superimposed on a dominant, faster, rapidly reversible, and Ca^{2+}-dependent effect. An increase in Ca^{2+} influx appears to be a factor in the action of AA on coupling, but the participation of Ca^{2+} stores cannot be entirely excluded. In contrast, uncoupling by OA or halothane appears to be Ca^{2+}-independent only.

The authors wish to thank Drs. Trevor J. Shuttleworth and Shaun C. Martin for helpful criticism.

This study was supported by National Institutes of Health grant GM20113 to C. Peracchia and the Italian CNR Progetto Bilaterale n. CT92.00730. CT04.115.11537 to A. Peres and C. Peracchia.

REFERENCES

Alder, E. M., G. J. Augustine, S. N. Duffy, and M. P. Charlton. 1991. Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *J. Neurosci.* 11:196–1507.

Axelrod, J., R. M. Burch, and C. L. Jelsema. 1988. Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and metabolites as second messengers. *Trends Neurosci.* 11:117–123.

Burt, J. M., K. D. Massey, and B. N. Minnich. 1991. Uncoupling of cardiac cells by fatty acids: structure-activity relationships. *Am. J. Physiol.* 260: C439–C448.

Damron, D. S., and M. Bond. 1993. Modulation of Ca^{2+} cycling in cardiac myocytes by arachidonic acid. *Circ. Res.* 72:376–386.

Dorn, G. W., and M. W. Beker. 1993. Thromboxane A2 stimulated signal transduction in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* 265: 447–456.

Fluri, G. S., A. Rüdisüli, M. Willi, S. Rohr, and R. Weingart. 1990. Effects of arachidonic acid on the gap junctions of neonatal rat heart cells. *Pflügers Arch.* 417:149–156.

Giaume, C., C. Randriamampita, and A. Trautmann. 1989. Arachidonic acid closes gap junction channels in rat lacrimal glands. *Pflügers Arch.* 413: 273–279.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Hamill, O. P., A Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85–100.

Horn, R., and A. Marty. 1988. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.* 92:145–159.

Johnson, R., M. Hammer, J. Sheridan, and J.-P. Revel. 1974. Gap junction formation between reaggregated Novikoff hepatoma cells. *Proc. Natl. Acad. Sci. USA.* 71:4536–4540.

Kent, K. C., L. J. Collins, F. T. Schwerin, M. K. Raychowdbury, and J. A. Ware. 1993. Identification of functional PGH2/TxA2 receptors on human endothelial cells. *Circ. Res.* 72:958–965.

Lazrak, A., and C. Peracchia. 1993. Gap junction gating sensitivity to physiological internal calcium regardless of pH in Novikoff hepatoma cells. *Biophys. J.* 65:2002–2012.

Lazrak, A., A. Peres, S. Giovannardi, and C. Peracchia. 1994. Junctional uncoupling by ATP and arachidonic acid is Ca_i^{2+}-mediated. *Biophys. J.* 66:149*a*. (Abstr.)

Marty, A., and E. Neher. 1985. Potassium channels in cultured bovine adrenal chromaffin cells. *J. Physiol.* 367:117–141.

Massey, K. D., B. N. Minnich, and J. M. Burt. 1992. Arachidonic acid and lipoxygenase metabolites uncouple neonatal rat cardiac myocyte pairs. *Am. J. Physiol.* 263:494–501.

Meyer, R. A., D. W. Laird, J. P. Revel, and R. G. Johnson. 1992. Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell. Biol.* 119:179–189.

Mochizuki-Oda, N., M. Negishi, K. Mori, and S. Ito. 1993. Arachidonic acid activates cation channels in bovine adrenal chromaffin cells. *J. Neurochem.* 61:1882–1890.

Negishi, M., S. Ito, and O. Hayaishi. 1990. Arachidonic acid stimulates phosphoinositide metabolism and catecholamine release from bovine adrenal chromaffin cells. *Biochem. Biophys. Res. Commun.* 169:773–779.

Ordway, R. W., J. J. Singer, and J. V. Walsh. 1991. Direct regulation of ion channels by fatty acids. *Trends Neurosci.* 14:96–100.

Parker, J., L. W. Daniel, and M. Waite. 1987. Evidence of protein kinase C involvement in phorbol diester-stimulated arachidonic acid release and prostaglandin synthesis. *J. Biol. Chem.* 262:5385–5393.

Peracchia, C. 1990. Increase in gap junction resistance with acidification in crayfish septate axons is closely related to changes in intracellular calcium but not hydrogen ion concentration. *J. Membr. Biol.* 113:75–92.

Peracchia, C. 1991. Effects of the anesthetics heptanol, halothane and isoflurane on gap junction conductance in crayfish septate axons: a calcium- and hydrogen-independent phenomenon potentiated by caffeine and theophylline, and inhibited by 4-aminopyridine. *J. Membr. Biol.* 121:67–78.

Peracchia, C., A. Lazrak, and L. L. Peracchia. 1994. Molecular models of channel interaction and gating in gap junctions. *In* Membrane Channels—Molecular and Cellular Physiology. C. Peracchia, editor. Academic Press, S. Diego, CA. 361–377.

Peres, A., C. Racca, R. Zippel, and E. Sturani. 1990. Cytosolic calcium and membrane conductance in response to PDGF and bradykinin stimulation in single human fibroblasts. *Eur. J. Cell Biol.* 53:290–295.

Piomelli, D., E. Shapiro, S. J. Feinmark, and J. H. Shwartz. 1987. Metabolites of arachidonic acid in the nervous system of Aplysia: possible mediators of synaptic modulation. *J. Neurosci.* 7:3675–3686.

Poenie, M. 1990. Alteration of intracellular Fura-2 fluorescence by viscosity: a simple correction. *Cell Calcium.* 11:85–91.

Rayes, J. L., E. Neva, and M. C. Namorado. 1992. Receptor-mediated effect of a synthetic thromboxane-analogue on cytosolic calcium in isolated proximal tubules. *Prostaglandins.* 44:145–154.

Roe, M. W., J. J. Lemasters, and B. Herman. 1990. Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium.* 11:63–73.

Shimizu T., and L. S. Wolfe. 1990. Arachidonic acid cascade and signal transduction. *J. Neurochem.* 55:1–15.

Smith, W. L. 1989. The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* 259:315–324.

Spray, D. C., and J. M. Burt. 1990. Structure-activity relations of the cardiac gap junction channel. *Am. J. Physiol.* 258:195–205.

Stern, M. D., 1992. Buffering of calcium in the vicinity of a channel pore. *Cell Calcium.* 13:183–192.

Yamagishi, T., T. Yanagisawa, and N. Taira. 1992. Ca^{2+} influx induced by the agonist U46619 is inhibited by hyperpolarization induced by the K^+ channel opener cromakalim in canine coronary artery. *Jpn. J. Pharmacol.* 59:291–299.

Wilkes, J. M., M. Kajimura, D. R. Scott, S. J. Hersey, and G. Sachs. 1991. Muscarinic responses of gastric parietal cells. *J. Membr. Biol.* 122: 97–110.

Williams, D. A., and F. S. Fay. 1990. Intracellular calibration of the fluorescent calcium indicator Fura-2. *Cell Calcium.* 11:75–83.