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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

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Summary. This study has monitored junctional and nonjunctional resistance, $[Ca^{2+}]_i$ and $[H^+]_i$, and the effects of various drugs in crayfish septate axons exposed to neutral anesthetics. The uncoupling efficiency of heptanol and halothane is significantly potentiated by caffeine and theophylline. The modest uncoupling effects of isoflurane, described here for the first time, are also enhanced by caffeine. Heptanol causes a decrease in $[Ca^{2+}]_i$ and $[H^+]_i$ both in the presence and absence of either caffeine or theophylline. A similar but transient effect on $[Ca^{2+}]_i$ is observed with halothane. 4-Aminopyridine strongly inhibits the uncoupling effects of heptanol. The observed decrease in $[Ca^{2+}]_i$ with heptanol and halothane and negative results obtained with different $[Ca^{2+}]_o$, Ca^{2+}-channel blockers (nisoldipine and Cd^{2+}) and ryanodine speak against a Ca^{2+} participation. Negative results obtained with 3-isobutyl-1-methylxanthine, forskolin, CPT-cAMP, 8Br-cGMP, adenosine, phorbol ester and H7, superfused in the presence and absence of caffeine and/or heptanol, indicate that neither the heptanol effects nor their potentiation by caffeine are mediated by cyclic nucleotides, adenosine receptors and kinase C. The data suggest a direct effect of anesthetics, possibly involving both polar and hydrophobic interactions with channel proteins. Xanthines and 4-aminopyridine may participate by influencing polar interactions. The potentiating effect of xanthines on cell-to-cell uncoupling by anesthetics may provide some clues on the nature of cardiac arrhythmias in patients treated with theophylline during halothane anesthesia.

Key Words gap junctions · anesthetics · heptanol · halothane · isoflurane · caffeine · theophylline · intracellular Ca^{2+} · 4-aminopyridine

Introduction

Free exchange of ions and small metabolites among neighboring cells is mediated by gap junction channels. Gap junction permeability can be reduced down to complete cell-to-cell uncoupling by a variety of treatments including cell damage, inhibition of metabolism, acidification, hypoxia, exposure to anesthetics and halomethanes, etc. (reviewed in Loewenstein, 1981; Ramon & Rivera, 1987; Peracchia, 1980, 1987; Spray & Burt, 1990). In most of these treatments, the change in gap junction permeability reportedly involves a Ca^{2+} and/or H^+ effect on gap junction proteins or uncoupling intermediates. In contrast, for neutral anesthetics (alkanols, halothane, etc.) the uncoupling mechanism is still poorly understood and a participation of Ca^{2+} and/or H^+ has not yet been entirely ruled out.

The uncoupling effects of alkanols, first described by Johnston, Simon and Ramon (1980) in crayfish septate axons, were soon confirmed in all vertebrate and invertebrate systems tested (Délèze & Hervé, 1983; Bernardini, Peracchia & Peracchia, 1984; Meda et al., 1986). Johnston and Ramon (1981) proposed an extracellular site of action for alkanols because uncoupling resulted only from their extracellular application to axons internally perfused with Ca^{2+}- and H^+-buffered solutions. This would seem to exclude entirely the involvement of Ca_i^{2+} and H_i^+. However, Veenstra and DeHaan (1988) observed only a partial reduction of junctional conductance with octanol in embryonal cardiac cells dialyzed by patch pipettes strongly buffered for Ca^{2+}, while complete uncoupling was seen only with weak Ca^{2+} buffers. Furthermore, Requena et al. (1985) and Vassort, Whittembury and Mullins (1986) monitored a small increase in $[Ca^{2+}]_i$ with arsenazo III in squid axons treated with octanol.

In contrast, Meda et al. (1986) did not observe changes in $[Ca^{2+}]_i$ with quin-2 in exocrine pancreatic cells uncoupled by heptanol, and alkanols displayed normal uncoupling efficiency in ventricular cell pairs dialyzed with pipette solutions strongly buffered for Ca^{2+} (Niggli et al., 1989; Rüdisüli & Weingart, 1989). Furthermore, alkanols cause modifications of electrical and mechanical parameters that would be hard

to reconcile with an increase in $[Ca^{2+}]_i$ (Wojtczak, 1985; Niggli et al., 1989).

The uncoupling effects of halothane were first described by Hauswirth (1969) in cardiac cells and later confirmed in the same tissue by Wojtczak (1985), White et al. (1985), Burt and Spray (1988) and Niggli et al. (1989). Thus far, the effects of halothane on coupling have not been tested in any other system and possible uncoupling effects of isoflurane have never been tested. No measurement of $[Ca^{2+}]_i$ and $[H^+]_i$, with ion-selective microelectrodes, in cells exposed to neutral anesthetics, has been performed as well.

The present study reports data from simultaneous monitoring of junctional resistance (R_j) and either $[Ca^{2+}]_i$ or $[H^+]_i$ in crayfish septate axons uncoupled with heptanol in the presence and absence of xanthines and provides the first evidence of halothane-induced uncoupling in noncardiac cells, of the ability of isoflurane to reduce coupling, of the capacity of xanthines to potentiate the uncoupling efficiency of heptanol, halothane and isoflurane, and of an inhibitory action of 4-aminopyridine on heptanol-induced uncoupling. The inclusion of xanthines in the protocols was suggested by recent findings of caffeine effects on low pH_i-induced uncoupling (Peracchia, 1990*a*), that confirmed previous evidence for the involvement of Ca_i^{2+} and Ca stores in the mechanism of channel regulation by acidification (Peracchia, 1990*b*).

Materials and Methods

Experimental Preparation

Crayfish (*Procambarus clarkii*), purchased from Carolina Biological Supply (Burlington, NC) were kept in a well-oxygenated aquarium at 22–24°C. The animals were anesthetized by cooling and sacrificed by decapitation. The ventral nerve cord was removed and the sheath covering the dorsal side of the cord was cut away. A segment of the nerve cord comprising three ganglia was cut off and pinned dorsal side up to a plexiglass chamber. Either the third or fourth abdominal ganglion was used. The chamber was continuously perfused at a flow of 1 ml/min with a standard saline solution for crayfish (SES) (Johnston & Ramon, 1981) containing (in mM): NaCl, 205; KCl, 5.4; $CaCl_2$, 13.5 and HEPES, 5 (pH 7.5). The level of the solution in the chamber was maintained constant by continuous suction.

For testing the effects of anesthetics, the axons were superfused with SES containing either 2.8–5.6 mM 1-heptanol (heptanol; Fisher Scientific, Fair Lawn, NJ), 9.5–28.5 mM 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane; Halocarbon Labs, Augusta, SC), or 23.6 mM 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether (isoflurane; Anaquest, Madison, WI), in the presence and absence of either 10–20 mM caffeine (Sigma Chemical, St. Louis, MO), or 10–20 mM theophylline (Sigma). For testing drugs active on Ca stores, 10 μM ryanodine (Calbiochem, La Jolla, CA) was added to either heptanol or heptanol-caffeine solutions. Possible effects of cyclic nucleotides were tested by adding either 500 μM chlorophenyl-thio-cAMP (CPT-cAMP; Boehringer, Mannheim, FRG) or 5 μM forskolin (Sigma) or 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma) or 100–200 μM 8-bromo-cGMP (8Br-cGMP; Sigma) to SES or heptanol solutions.

For testing the possible participation of Ca_o^{2+} and Ca^{2+} channels, either 1–10 μM nisoldipine (Miles Pharmaceuticals, West Haven, CT) or 400–500 μM Cd^{2+} or solutions with high or low $[Ca^{2+}]$ were used in the presence of heptanol. The solutions with high $[Ca^{2+}]$ contained (in mM): NaCl, 188; KCl, 5.4; $CaCl_2$, 27; HEPES, 5 (pH 7.5). The solutions with low $[Ca^{2+}]$ contained (in mM): NaCl, 216; KCl, 5.4; $CaCl_2$, 7; HEPES, 5 (pH 7.5). The possible participation of kinase C was tested by adding either an activator, 162 nM 4β-phorbol-12β-myristate-13α-acetate (TPA, Sigma), or an inhibitor, 100 μM 1-(5-isoquinolinysulfonyl)-2-methylpiperazine (H7; Sigma), of this enzyme to SES or heptanol solutions.

The possible participation of adenosine receptors was tested by adding 1.3–5 mM adenosine (Sigma) to either SES or heptanol solutions. Electrical uncoupling by acidification was obtained by superfusing the axons with a sodium acetate saline solution (Ac) containing (in mM): Na acetate, 205; KCl, 5.4 and $CaCl_2$, 13.5 (pH 6.3). The effects of the K^+-channel blocker 4-aminopyridine (4-AP) were tested by adding 4-AP (Aldrich Chemical, Milwaukee, WI) to SES, heptanol and Ac, and adjusting the pH to either 7.5 (SES and heptanol) or 6.3 (Ac).

Electrical Measurements

Microelectrodes were pulled from borosilicate glass capillaries 1.2 mm (o.d.), 0.68 mm (i.d.) (Kwik fill, WP Instruments, New Haven, CT) and filled with a 2.5 M KCl solution buffered to pH 7 with 20 mM HEPES. The electrodes had an 8–10 MΩ resistance in SES. Four microelectrodes were inserted into a lateral giant axon, two on each side of the septum. The bath was grounded with a silver-silver chloride reference electrode connected to the chamber via an agar-SES bridge.

Hyperpolarizing square current pulses (150 nA, 300 msec) were generated by a Dell System 200 computer coupled to a D/A converter (DT 2801, Data Translation, Marlborough, MA) and a voltage-to-current converter (701 M, WPI). The pulses were injected every 10 sec, alternatively into the posterior (C1) and anterior (C2) axon segment. The resulting electrotonic potentials V_1, V_2 (from current injection in C_1), V_{1^*}, V_{2^*} (from injection in C_2) and the membrane potentials (E_1 and E_2) were recorded with two voltage microelectrodes through a voltage follower (AM-4, Biodine Electronics, Santa Monica, CA). The voltage signals were displayed on a storage oscilloscope and on a chart recorder and were digitized and stored both on the hard disc of the computer and on VCR tape (Peracchia, 1990*b*).

Ion-Sensitive Microelectrodes

$[Ca^{2+}]_i$ and $[H^+]_i$ were measured with ion-sensitive microelectrodes based on neutral carrier sensors. The microelectrodes were prepared as previously described (Peracchia, 1990*b*).

Ca^{2+} microelectrodes used a recently developed calcium cocktail (ETH 129, Schefer et al., 1986) (Fluka Chemical, Ronkonkoma, NY). This cocktail contains the Ca^{2+} ionophore N,N,N1,N1-tetracyclohexyl-3-oxapentanediamide, which forms an ideal coordination sphere of nine oxygen atoms for Ca^{2+}-

uptake (Schefer et al., 1986). Ca microelectrodes prepared with this cocktail have a logarithmic response down to $[Ca^{2-}]$ of 5×10^{-10} M and are virtually insensitive to other ions (Ammann et al., 1987), including H^+ and acetate (Peracchia, 1990*b*). The microelectrodes were backfilled with filtered Ca^{2+} solutions buffered with EGTA (*p*Ca 7; Alvarez-Leefmans, Rink & Tsien, 1981) or citrate (pH 7) and containing 220 mM KCl to match $[K^+]_i$. The Ca^{2+} sensitivity and response time of the microelectrodes were tested as previously described (Peracchia, 1990*b*). The electrode response ranged from 15 to 25 mV per *p*Ca unit. Addition of heptanol to test solutions did not change the magnitude of the microelectrode response. Micoelectrodes typically had a 50% response time of 14 sec.

A proton cocktail (Fluka) containing the neutral carrier tri-*n*-dodecylamine (Amman et al., 1981) was used for pH microelectrodes. The microelectrodes were backfilled with a filtered 2.5 M KCl solution buffered to pH 7 with 20 mM HEPES. The H^+ sensitivity and response time of the microelectrodes were tested as previously described (Peracchia, 1990*b*). The microelectrode response ranged from 50 to 55 mV per pH unit. Microelectrodes typically had a 50% response time of 7.5 sec.

Analysis of Data

Both membrane (Rm_1, Rm_2) and junctional (R_{j1}, R_{j2}) resistances were calculated and plotted on-line on the computer monitor by means of a program written in ASYST language (Adaptable Laboratory Software, Rochester, NY). The resistances were calculated from current (I_1, I_2) and voltage (V_1, V_2, V_{1^*}, V_{2^*}) records using the π-t transform (Bennett, 1966).

The voltage signal detected by the ion-sensitive microelectrodes was passed through the high impedance amplifier, filtered (0.1 Hz) and displayed on the pen recorder after subtraction of membrane potential. At 10-sec intervals, the voltage of the Ca^{2+} microelectrode was also sampled by the computer 100 msec before each current pulse. The voltage signal was digitized, stored unfiltered on the hard disc and plotted on-line after subtraction of the membrane potential (Peracchia, 1990*b*).

Results

Effects of Heptanol on Junctional Resistance

Lateral giant axons had a membrane potential (E_1, E_2) ranging from -80 to -95 mV and were well coupled at the septum. The junctional resistance (R_j), measured soon after impalement with four microelectrodes, was 150 ± 53.7 kΩ (mean ± SD; 28 measurements in 28 axons). Superfusing the axons with 2.8–5.6 mM heptanol caused a small membrane depolarization (Figs. 1*A* and *B* and 3*A*) and increased R_j to 191.3 ± 83% (mean ± SD; 87 measurements in 28 axons) of basal R_j values, averaging 160 ± 56.2 kΩ (mean ± SD; 87 measurements in 28 axons). In some experiments the R_j curve was fairly symmetrical (Fig. 1*C*), but in others the recovery phase was quicker (Fig. 3*B*); in these cases, also the membrane potentials, in addition to the electrotonic potentials, recovered more quickly (Fig. 3*A*). The effects of heptanol varied significantly from axon to axon. An 8-min treatment with 5.6 mM heptanol increased R_j to values ranging from 110 to 413% of basal values. Although in some axons a 6-min treatment with 2.8 mM heptanol increased R_j as much as 629% (from 140 to 880 kΩ) of control values, in others a 46-min treatment with 5.6 mM heptanol increased R_j as little as 383% (from 120 to 460 kΩ) of basal values (Fig. 1*D*). Most often the R_j sensitivity to heptanol was relatively constant within a preparation (Fig. 1*C*), but occasionally R_j sensitivity changed with time (Fig. 2). Therefore, treatments found to change heptanol efficiency were always compared with heptanol controls immediately preceding or following them.

Effects of Caffeine on Changes in Junctional Resistance with Heptanol

Addition of 10–20 mM caffeine to heptanol solutions caused a greater increase in V_1 and V_{2^*}, a greater reduction of V_2 and V_{1^*}, (Figs. 1*A* and 3*A*), and consequently a significant increase in R_j maxima with respect to controls (Figs. 1*C*, 2 and 3*B*). The R_j maxima with heptanol-caffeine were 309.3 ± 265% (mean ± SD; 24 measurements in 13 axons) of those with heptanol alone. The magnitude of increase in R_j maxima was not affected by caffeine pretreatment (Fig. 1*C*, *compare* second and fourth uncoupling events). Similarly, caffeine pretreatments did not change the uncoupling efficiency of heptanol alone (Fig. 2, eighth heptanol treatment). Therefore, caffeine appears to affect the heptanol-induced uncoupling only when it is used in conjunction with heptanol. When caffeine was added to heptanol several minutes after the beginning of heptanol treatment, the rate of increase in R_j rose significantly. Figure 1 (*B* and *D*) shows that a 7-min treatment with heptanol-caffeine following 22 min in heptanol alone virtually doubled the magnitude of R_j increase caused by heptanol alone. In this experiment, addition of caffeine to heptanol enhanced the maximum rate of R_j increase from 14 to 35 kΩ/min (Fig. 1*D*).

Do Calcium and Hydrogen Ions Participate in the Uncoupling Mechanism of Heptanol and Heptanol-Caffeine?

The possible participation of Ca^{2+} and/or H^+ in the effects of heptanol and heptanol-caffeine on R_j was tested by measuring $[Ca^{2+}]_i$ and $[H^+]_i$, and by applying treatments that alter either Ca^{2+} entry or

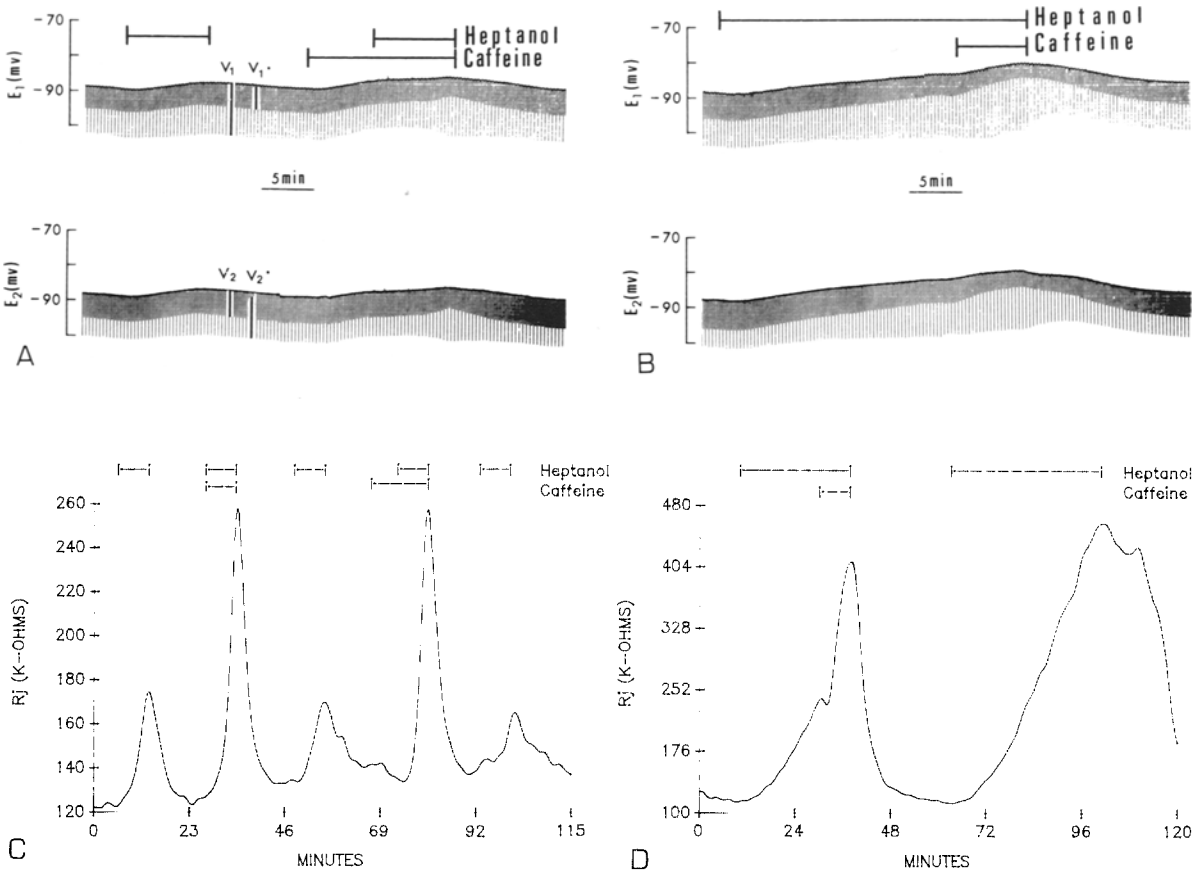

Fig. 1. Time course of changes in electrotronic potentials and R_j in crayfish septate axons uncoupled with heptanol in the presence and absence of 20 mM caffeine. (*A* and *B*) low speed chart recording of membrane and electrotonic potentials in the posterior (upper trace) and anterior (lower trace) axon segment. Hyperpolarizing square current pulses (150 nA, 300 msec) are injected every 10 sec alternatively into the posterior (C_1) and anterior (C_2) axon segment. The resulting electronic potentials V_1 and V_2 (from current injection in C_1), and V_{1^*} and V_{2^*} (from current injection in C_2) as well as the membrane potentials (E_1 and E_2) are displayed on the chart recording and stored on VCR tape and on the computer's hard disc for calculating R_j and R_m. With heptanol, V_1 and V_{2^*} increase and V_{1^*} and V_2 decrease, due to an increase in R_j. With heptanol-caffeine a larger change in electrotonic potentials takes place, indicating a larger increase in R_j. Note that exposure to caffeine alone (*A*), as a pretreatment, causes mild depolarization but does not change the amplitude of the electrotonic potentials; in contrast, addition of caffeine to heptanol during heptanol superfusion (*B*) causes a rapid change in the amplitude of the electrotonic potentials indicative of rapid increase in R_j. (*C*) Computer calculated changes in R_j from the experiment shown in part in *A* (third and fourth heptanol treatments). Note that R_j increases with heptanol-caffeine 2–3 times as much as with heptanol alone. The R_j maxima with heptanol-caffeine are the same both in the presence and absence of caffeine pretreatment (*compare* second and fourth heptanol treatments). (*D*) Computer calculated changes in R_j from the experiment shown in part in *B* (first heptanol treatment). During the first heptanol treatment R_j increases only to 190% of basal values with a 22-min heptanol superfusion; in contrast, upon addition of caffeine R_j increases to 340% of control values in just 7 min. The second heptanol treatment shows that the maximal rate of R_j increase with heptanol is 14 kΩ/min; with heptanol-caffeine (first treatment) the rate more than doubles (35 kΩ/min)

Ca^{2+} release from intracellular stores. With Ca^{2+}-sensitive microelectrodes, $[Ca^{2+}]_i$ was found to decreased slightly with either heptanol or heptanol-caffeine (Fig. 3). In both cases R_j and pCa maxima coincided (Fig. 3*B*), suggesting that both parameters may be influenced by similar factors. With pH-sensitive electrodes, $[H^+]_i$ was also found to decrease with heptanol (Fig. 4), although only transiently. The absence of Ca^{2+} participation in heptanol-induced uncoupling was further supported by the observation that neither different $[Ca^{2+}]_o$ (ranging from 7 to 27 mM) nor blockers of Ca^{2+} entry, such as Cd^{2+} (500 μM) and nisoldipine (10 μM), significantly changed heptanol uncoupling efficiency (*data not shown*).

To biock Ca^{2+} release from internal stores, ryanodine (10 μM) was added to both heptanol and heptanol-caffeine. The R_j maxima with either heptanol-ryanodine (*data not shown*) or heptanol-caffeine-ryanodine (Fig. 5) were not significantly different from R_j maxima with either heptanol or

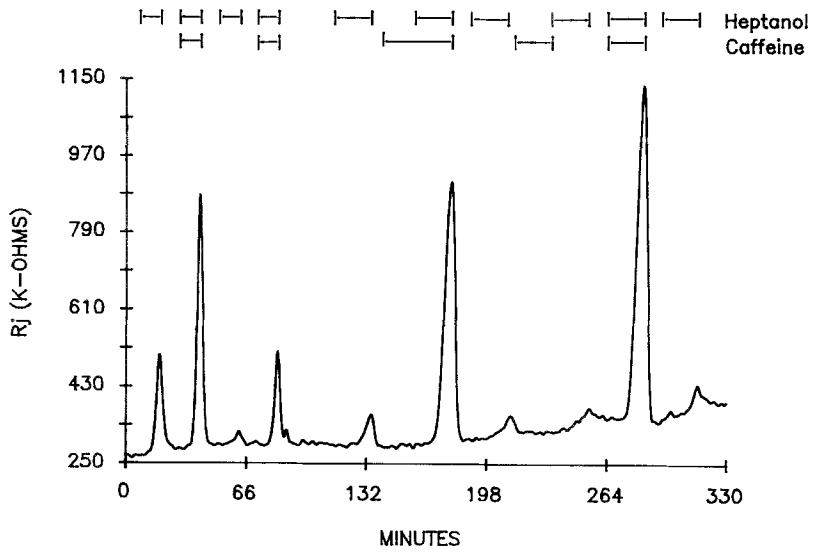

Fig. 2. Time course of R_j changes in crayfish axons uncoupled by heptanol in the presence or absence of caffeine. In this preparation the uncoupling efficiency of heptanol decreases significantly during the first two hours. Note the difference in R_j maxima between first and third uncoupling events, in spite of the same duration of heptanol superfusion (8 min). Similarly, R_j maxima decrease in the presence of caffeine (*compare* second and fourth uncoupling events). After the first two hours an increase in superfusion duration to 15 min is needed to obtain R_j maxima of similar magnitude (*compare* third and fifth uncoupling events). Note that caffeine pretreatment does not change the uncoupling efficiency of both heptanol-caffeine (sixth uncoupling event) and heptanol (eighth uncoupling event)

heptanol-caffeine, respectively, indicating that neither uncoupling treatment involves the participation of caffeine-ryanodine-sensitive Ca^{2+} stores.

Are the Effects of Heptanol and Heptanol-Caffeine on Junctional Resistance Mediated by Kinases?

The possibility that caffeine enhances the effects of heptanol on junctional resistance by increasing the concentration of cyclic nucleotides via inhibition of phosphodiesterases was tested by exposing the axons to other xanthines (theophylline and IBMX), to an activator of adenylate cyclase (forskolin) and to diffusible cAMP and cGMP (CPT-cAMP and 8Br-cGMP).

Addition of 10–20 mM theophylline to heptanol solutions dramatically enhanced the heptanol effects on R_j (Fig. 6*A*). The R_j maxima with heptanol-theophylline were 676 ± 386% (mean ± SD; 4 measurements in 3 axons) of those with heptanol alone. Similarly to caffeine (Fig. 3), theophylline did not significantly modify the effects of heptanol on $[Ca^{2+}]_i$ (Fig. 6*A*). In contrast, additions to heptanol of 1 mM IBMX (Fig. 6*B*), a xanthine 200 times more potent than caffeine in inhibiting phosphodiesterases (Kramer & Wells, 1980), 5 μM forskolin (Fig. 6*C*), 500 μM CPT-cAMP, or 200 μM 8Br-cGMP (*data not shown*) did not have any significant effect on R_j maxima.

To test the possible involvement of kinase C the axons were superfused with heptanol solutions containing either 162 nM TPA or 100 μM H7. Neither the activator (TPA) nor the inhibitor (H7) of kinase C had any appreciable effect on heptanol uncoupling efficiency (*data not shown*). No change in R_j was also observed with TPA alone.

Are the Heptanol Effects on Junctional Resistance Mediated by Adenosine Receptors?

Since caffeine is a powerful inhibitor of adenosine receptors, the effects of adenosine on both R_j and R_j changes with heptanol were tested. Superfusion of 1.3–5 mM adenosine, added to either SES or heptanol solutions, did not significantly change either the basal R_j values or the R_j maxima with heptanol (*data not shown*), indicating that adenosine receptors are unlikely to participate in the effects of heptanol and caffeine on coupling.

Inhibitory Effects of 4-Aminopyridine on Heptanol-Induced Increase in Junctional Resistance

The K^+-channel blocker 4-AP, tested for unrelated reasons, was found to strongly inhibit the heptanol-induced uncoupling (Fig. 7*A*). With heptanol solutions containing 5 mM 4-AP the R_j maxima were 26.2 ± 20% (mean ± SD; 12 measurements in 5 axons) of those with heptanol alone. In contrast, addition of 4-AP (5 mM) to acetate solutions did not alter their uncoupling effects (Fig. 7*B*). 4-AP did not signifi-

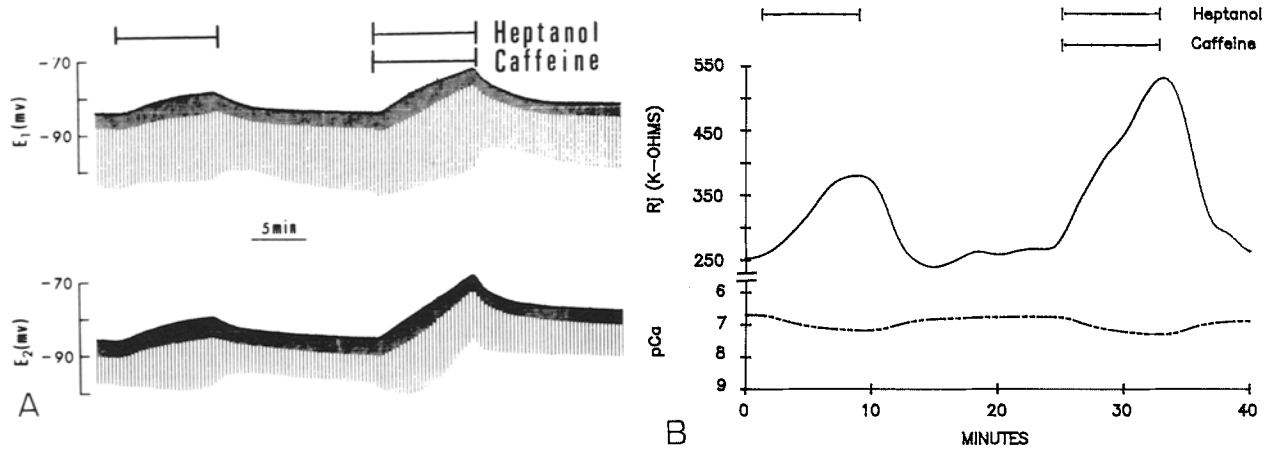

Fig. 3. Time course of changes in electrotonic potentials (*A*) and both R_j and pCa_i (*B*) in crayfish axons uncoupled first with heptanol and then with heptanol-caffeine (20 mM caffeine). Both treatments cause a similar increase in pCa_i, in spite of the fact that the R_j maximum with heptanol-caffeine is twice as high as with heptanol alone. The R_j recovery rate is faster than the onset rate with both heptanol and heptanol-caffeine (*see also* Figs. 1*D* and 6*A*). Note the increased depolarization in the presence of caffeine (*A*)

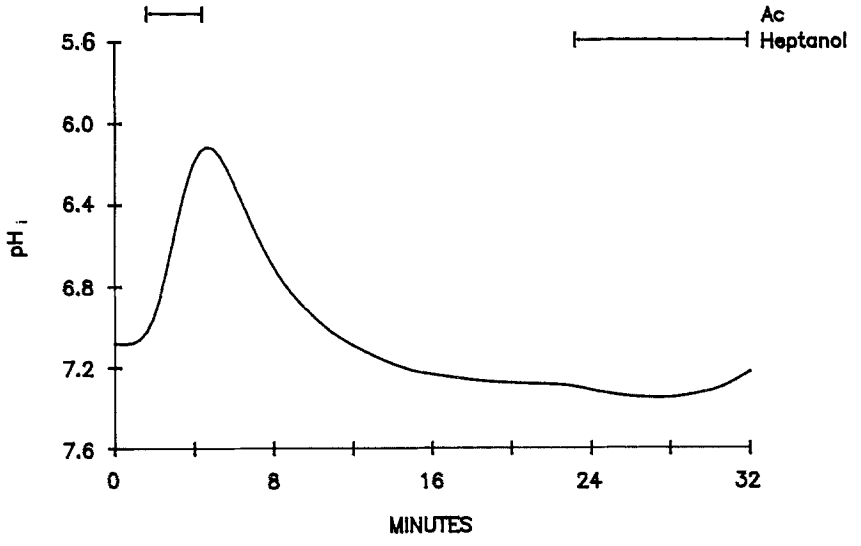

Fig. 4. Time course of pH_i in crayfish axons uncoupled with acetate (*Ac*) and with heptanol. Although both treatments increase R_j, the pH_i changes in opposite directions. With heptanol there is a small and transient increase in pH_i

cantly change the effects of heptanol on $[Ca^{2+}]_i$ (*data not shown*). No effect on R_j was seen with 4-AP alone, superfused in SES for periods as long as 3 min, the only observable change being a 3–4 mV positive deflection in membrane potential, probably the result of K^+-channel blockage.

Effects of Halothane and Isoflurane on Junctional Resistance and Their Potentiation by Caffeine

Halothane (28.5 mM), superfused for 4–10 min, caused a small bimodal change in membrane potential (Fig. 8*A*)and increased R_j to 155.6 ± 56% (mean ± SD; 9 measurements in 4 axons) of basal R_j values averaging 226 ± 73 kΩ (mean ± SD; 9 measurements in 4 axons) (Fig. 8*B* and *C*). Addition of 20 mM caffeine to halothane solutions caused greater increase in V_1 and V_{2^*}, greater reduction of V_2 and V_{1^*}, and more marked depolarization (Fig. 8*A*), reflecting a greater increase in R_j maxima than with halothane alone (Fig. 8*B* and *C*). The R_j maxima with halothane-caffeine were 329 ± 147 (mean ± SD; 8 measurements in 4 axons) of those with halothane alone. As seen with heptanol, caffeine pretreatment did not change the R_j maxima obtained with halothane-caffeine (*data not shown*).

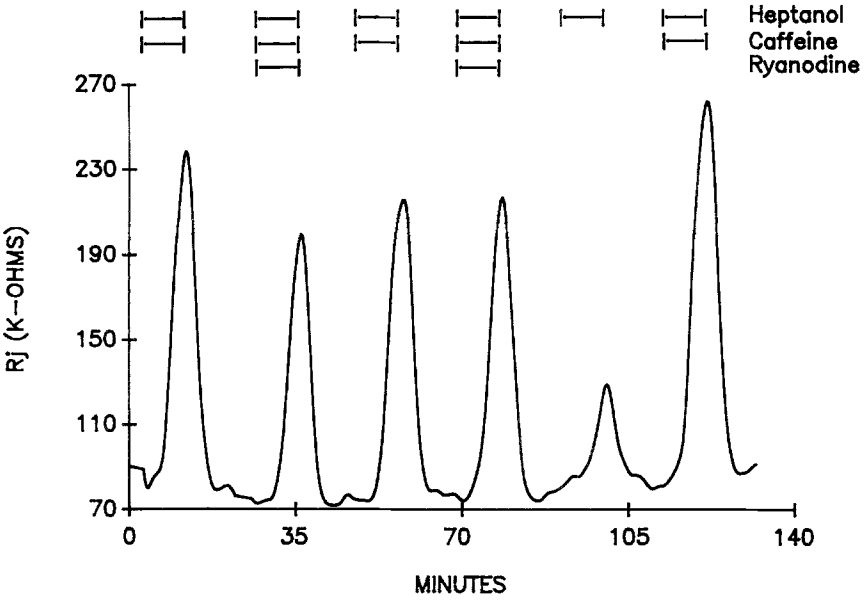

Fig. 5. Time course of changes in R_j in crayfish axons uncoupled with heptanol or with heptanol-caffeine (20 mM caffeine) in the presence and absence of ryanodine (10 μM). The caffeine-induced increase in heptanol uncoupling efficiency (*compare* the fifth uncoupling event with the first, third and sixth) is not altered by the presence of ryanodine (second and fourth uncoupling events)

Similar results were obtained in preliminary experiments with isoflurane. A 10-min superfusion with SES containing 23.6 mM isoflurane caused a 4–5 mV depolarization and increased R_j to approximately 125% of basal values (Fig. 8*C*). Addition of 20 mM caffeine to isoflurane solutions increased the R_j maxima to approximately 170% of R_j maxima with isoflurane alone (Fig. 8*C*).

Effects of Halothane on Intracellular Calcium Concentration

In preliminary experiments with Ca^{2+}-sensitive microelectrodes halothane was found to decrease $[Ca^{2+}]_i$ (Fig. 9). However, while with heptanol $[Ca^{2+}]_i$ remained lower than normal throughout the entire treatment (Figs. 3 and 6*A*), with halothane the decrease in $[Ca^{2+}]_i$ was transient. In Fig. 9, $[Ca^{2+}]_i$ decreased only within the first third of the 9 min of halothane treatment. At the end of the treatment there was a small and transient increase in $[Ca^{2+}]_i$ (Fig. 9).

Discussion

This study has probed the uncoupling mechanism of the neutral anesthetics heptanol, halothane and isoflurane by monitoring R_j, $[Ca^{2+}]_i$, $[H^+]_i$ and the effects of xanthines, different $[Ca^{2+}]_o$, Ca^{2+}-channel blockers, an inhibitor of Ca_i^{2+} release, diffusible cyclic nucleotides, an activator of adenylate cyclase, both an inhibitor and an activator of kinase C, adenosine and a K^+-channel blocker. The data support a Ca^{2+}- and H^+-independent uncoupling mechanism potentiated by caffeine and theophylline and inhibited by 4-AP. The xanthine effects do not appear to be mediated by internal Ca^{2+} release, phosphodiesterase activation and inhibition of adenosine receptors.

The effects of anesthetics on coupling differ in magnitude and time course from those of acidification. The slower rate of R_j increase with anesthetics could result from slow diffusion into the hydrophobic membrane compartment. The smaller R_j maxima could indicate either that fewer channels are affected or that anesthetics affect open channel probability to a lesser extent than acidification. Indeed, anesthetics have been shown to change open channel probability, but not single channel conductance (Burt & Spray, 1988; Veenstra & De Haan, 1988; Rüdisüli & Weingart, 1989).

The observation that the R_j recovery from heptanol is often faster than the onset could suggest that the cytosolic, more rapidly reversible, fraction of heptanol has a larger impact on R_j than the fraction incorporated into membranes. Alternatively, heptanol could be released by hydrophobic domains of channel proteins and/or boundary lipids faster than from other membrane regions.

The effect of caffeine on uncoupling by anesthetics was believed at first to indicate a participation of Ca^{2+} release from stores, as previously shown with acidification (Peracchia, 1990*a*,*b*). However, $[Ca^{2+}]_i$ decreased both with heptanol and with halo-

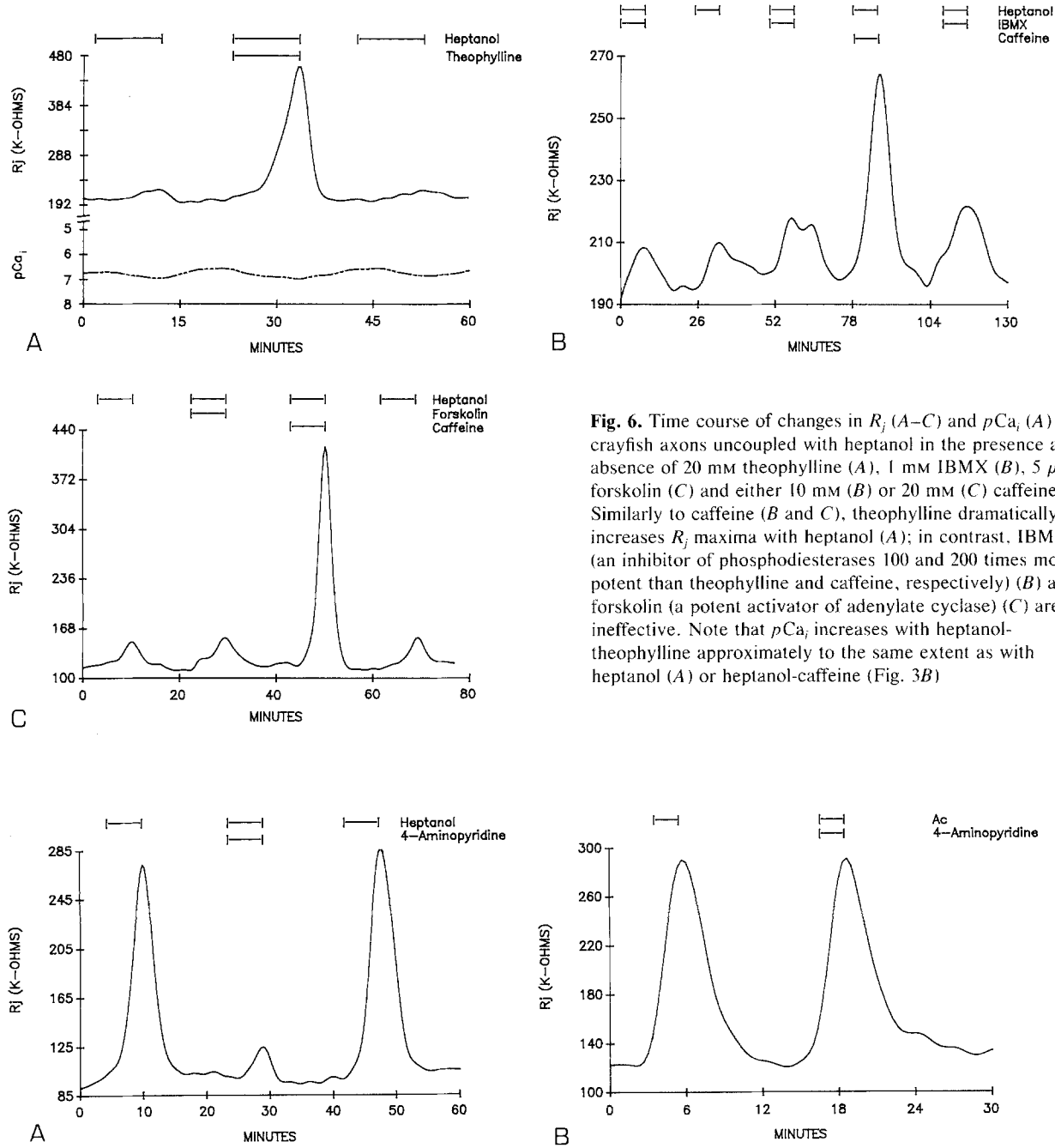

Fig. 6. Time course of changes in R_j (*A–C*) and pCa_i (*A*) in crayfish axons uncoupled with heptanol in the presence and absence of 20 mM theophylline (*A*), 1 mM IBMX (*B*), 5 μM forskolin (*C*) and either 10 mM (*B*) or 20 mM (*C*) caffeine. Similarly to caffeine (*B* and *C*), theophylline dramatically increases R_j maxima with heptanol (*A*); in contrast, IBMX (an inhibitor of phosphodiesterases 100 and 200 times more potent than theophylline and caffeine, respectively) (*B*) and forskolin (a potent activator of adenylate cyclase) (*C*) are ineffective. Note that pCa_i increases with heptanol-theophylline approximately to the same extent as with heptanol (*A*) or heptanol-caffeine (Fig. 3*B*)

Fig. 7. Time course of changes in R_j in crayfish axons uncoupled with heptanol (*A*) or acetate (*B*) in the presence or absence of 5 mM 4-aminopyridine (4-AP). The R_j maximum with heptanol-4AP is much smaller than with heptanol alone (*A*). In contrast, addition of 4-AP to acetate solutions (*Ac*) does not inhibit its uncoupling efficiency (*B*). This indicates that the effect of 4-AP is not aspecific

thane, the former in the presence and absence of either caffeine or theophylline, and Ca^{2+} release was excluded by the inability of either caffeine pretreatment or ryanodine to reduce R_j maxima with heptanol or heptanol-caffeine; in contrast, these treatments reduced the R_j maxima with acidification (Peracchia, 1990*a*). The negative results obtained with high and low Ca^{2+} solutions and with addition of Cd^{2+} or nisoldipine to heptanol also exclude, at least in part, an increase in Ca^{2+} influx.

Earlier studies have produced conflicting results on the involvement of Ca^{2+} in the action of anesthetics on gap junctions. Morphological studies described similar changes in gap junction particle size

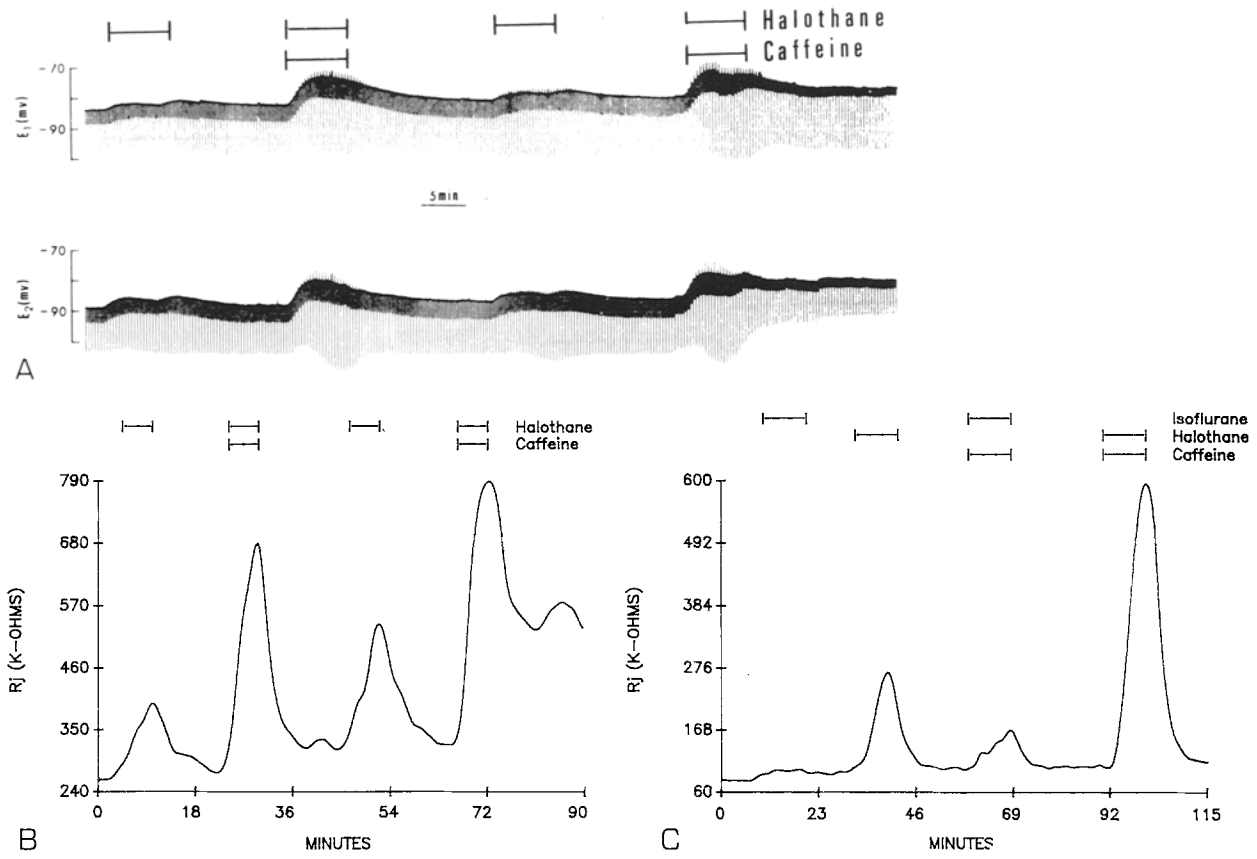

Fig. 8. (*A* and *B*) Time course of changes in electrotonic potentials (*A*) and R_j (*B* and *C*) in crayfish axons uncoupled with halothane in the presence and absence of 20 mM caffeine. With halothane-caffeine the increase in V_1 and V_{2^∞}, and the decrease in V_{1^∞} and V_2 are larger than with halothane alone (*A*), due to a greater increase in R_j maxima (*B*). Note that with caffeine there is a larger depolarization and the biphasic change in membrane potential is less pronounced (*A*). Following the last halothane-caffeine treatment, coupling recovered only partially (*A* and *B*). (*C*) Time course of changes in R_j from another experiment in which the axons were exposed to either isoflurane or halothane in the presence and absence of 20 mM caffeine. Similarly to heptanol and halothane, caffeine has a pronounced effect on the uncoupling efficiency of isoflurane. However, isoflurane has a much weaker effect on R_j than the other two anesthetics

and spacing with heptanol and other uncouplers known to increase $[Ca^{2+}]_i$, in heart (Délèze & Hervé, 1983, 1986) and in pancreas (Bernardini et al., 1984). In a double whole-cell clamp study octanol decreased junctional conductance to 4% of control values with patch-pipette solutions weakly buffered for Ca^{2+} (0.1 mM EGTA), and to 30% with strongly buffered solutions (5 mM EGTA) (Veenstra & De Haan, 1988). A moderate increase in $[Ca^{2+}]_i$ was monitored with the Ca^{2+} indicator arsenazo III in squid axons exposed to alkanols (Requena et al., 1985; Vassort et al., 1986).

In contrast, Meda et al. (1986) reported no significant changes in $[Ca^{2+}]_i$ measured with quin-2, in exocrine pancreas treated with heptanol. Several double whole-cell clamp studies (Burt & Spray, 1988; Somogyi & Kolb, 1988; Niggli et al., 1989, Rüdisüli & Weingart, 1989) reported normal uncoupling efficiency of alkanols and halothane with patch-pipette solutions strongly buffered for Ca^{2+}. The negative inotropic effect of heptanol and halothane on the heart (Lynch et al., 1981; Wojtczak, 1985; Niggli et al., 1989) is also inconsistent with an increase in $[Ca^{2+}]_i$.

Caffeine could potentiate the uncoupling effect of anesthetics by increasing the concentration of cyclic nucleotides, because, aside from affecting Ca^{2+} stores, it inhibits phosphodiesterases (Butcher & Sutherland, 1962). The similar effect of theophylline on heptanol-induced uncoupling could be consistent with this idea, because theophylline is even a more powerful inhibitor of phosphodiesterases (Butcher & Sutherland, 1962). However, IBMX, a xanthine 200 times more powerful than caffeine in inhibiting phosphodiesterases (Kramer & Wells, 1980), forskolin, a stimulator of cAMP synthesis,

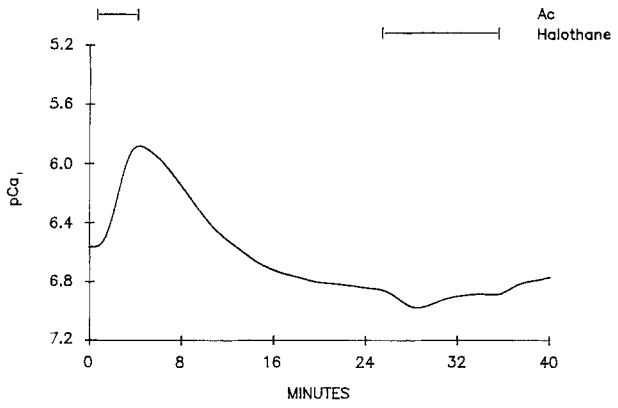

Fig. 9. Time course of changes in pCa_i in crayfish axons superfused with either acetate (*Ac*) or halothane. Although both treatments increase R_j, they have an opposite effect on pCa_i. With halothane there is a transient increase in pCa_i in the first few minutes of treatment and a transient increase upon return to SES

and high concentrations of diffusible cAMP (CPT-cAMP) or cGMP (8Br-cGMP) had no effect on heptanol uncoupling efficiency.

Another effect of xanthines is adenosine receptor inhibition (Daly, Bruns & Snyder, 1981). However, xanthines inhibit these receptors at concentrations 3 to 4 orders of magnitude lower than those that affect coupling, and adenosine, tested at concentrations as high as 5 mM, affected neither basal R_j values nor R_j maxima with heptanol. Other pharmacological effects of xanthines (reviewed in Dews, 1984) seemed totally unrelated to the action of anesthetics and to mechanisms of coupling regulation, and thus worth testing. Therefore, present evidence seems to suggest a novel, possibly direct, pharmacological effect of xanthines on coupling regulation.

The mechanism of 4-AP-induced inhibition of heptanol uncoupling is also unclear. Although its effect may be unrelated to K^+-channel blockage, different K^+-channel blockers and other molecules of the pyridine family will need to be tested.

In view of the fact that Ca^{2+} does not seem to be involved in uncoupling by anesthetics, that a participation of H^+ is excluded by evidence of an increase in pH_i, in agreement with data on squid axons (Vassort et al., 1986), and that the participation of kinase C, another uncoupler (Murray & Fitzgerald, 1979; Yotti, Chang & Trosko, 1979; Gainer & Murray, 1985; Yada, Rose & Loewenstein, 1985), is excluded by negative results with TPA and H7, it seems likely that anesthetics affect gap junction channels directly, as originally proposed by Johnston et al. (1980). In addition to gap junction channels, anesthetics are believed to directly block several other channels, including Na^+ channels (Oxford & Swenson, 1979; Bean, Shrager & Goldstein, 1981; Hirche, 1984; Rodriguez, Villegas & Requena, 1988), K^+ channels (Bean et al., 1981), Ca^{2+} channels (Twombly & Narahashi, 1989) and the ryanodine-sensitive Ca^{2+}-release channel of SR (Fill & Coronado, 1988).

The mechanism of anesthetic-induced channel blockage is still poorly understood. Probably, it involves both a disordering of lipids neighboring channel proteins, resulting in lipid expansion (reviewed in Hydon, Elliot & Hendry, 1984), and a direct interaction with channel proteins (Franks & Lieb, 1982). Since anesthetic potency correlates more with octanol than hydrocarbon partition coefficient, the binding site is likely to be amphiphilic (Frank & Lieb, 1982), possibly involving both hydrophobic interactions and hydrogen bond formation with channel proteins and/or accessory proteins. Xanthines may participate in this scheme by favoring the interaction between anesthetics and gap junction channel proteins. Interestingly, other amphiphilic molecules such as fatty acids are also believed to directly block gap junction channels (Burt, 1989; Giaume, Randriamampita & Trautmann, 1989). Their action has been attributed to both a disordering effect on perichannel lipids and an ionic interaction between the negatively charged carboxyl group and positively charged residues at the cytoplasmic end of gap junction proteins (Burt, 1989). Basic amphiphilic sequences with structures similar to calmodulin-binding sites, recently identified at the base of the carboxy-terminus of liver, heart, and *Xenopus* embryo gap junction proteins, as well as the lens channel protein MIP26 (Peracchia, 1988; Peracchia & Girsch, 1989; C. Peracchia, *unpublished observation*) are good candidates for the site of action of anesthetics and fatty acids. Indeed, there is evidence for both an involvement of calmodulin-like proteins in gap junction channel gating (Peracchia, 1988) and the ability of fatty acids to directly activate calmodulin-dependent enzymes in the absence of calmodulin (Tanaka & Hidaka, 1980; Niggli, Aduryach & Carafoli, 1981).

In conclusion, this study provides evidence for the capacity of heptanol, halothane and isoflurane to uncouple crayfish axons. Their effects on coupling are significantly enhanced by xanthines and do not appear to involve changes in calcium or hydrogen concentrations and activation of kinases. A direct interaction between anesthetics and amphiphilic chains of gap junction proteins seems likely. This mechanism could involve both hydrophobic and polar interactions. The latter could be favored by xanthines and inhibited by 4-aminopyridine.

In connection with evidence for a potentiating effect of caffeine and theophylline on anesthetic-

induced uncoupling, future studies should explore possible detrimental consequences of clinical procedures involving combined treatment with anesthetics and xanthines. In this respect, the known occurrence of cardiac arrhythmias following injection of theophylline in patients subjected to halothane anesthesia may not be coincidental. However, it should be kept in mind that the concentrations of anesthetics and xanthines tested in our study were significantly higher than those clinically used.

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