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ω3 polyunsaturated fatty acid modulates dihydropyridine effects on L-type Ca^{2+} channels, cytosolic Ca^{2+}, and contraction in adult rat cardiac myocytes

(patch-clamp recording/indo-1 fluorescence)

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ABSTRACT **The effect of docosahexaenoic acid (DHA; C22:6) on dihydropyridine (DHP) interaction with L-type Ca^{2+} channel current (I_{Ca}), cytosolic Ca^{2+} (Ca_i), and cell contraction in isolated adult rat cardiac myocytes was studied. The DHP L-type Ca^{2+}-channel blocker nitrendipine (10 nM) reduced peak I_{Ca} (measured by whole-cell voltage clamp from −45 to 0 mV) and reduced the amplitude of the Ca^{2+} transient (measured as the transient in indo-1 fluorescence, 410/490 nm) and the twitch amplitude (measured via photodiode array) during steady-state electrical stimulation (0.5 Hz). The DHP L-type Ca^{2+} channel agonist BAY K 8644 (10 nM) significantly increased I_{Ca}, the amplitude of the Ca_i transient, and contraction. When cells were exposed to DHA (5 μM) simultaneously with either BAY K 8644 or nitrendipine, the drug effects were abolished. Arachidonic acid (C20:4) at 5 μM did not block the inhibitory effects of nitrendipine nor did it prevent the potentiating effects of BAY K 8644. DHA modulation of DHP action could be reversed by cell perfusion with fatty acid-free bovine serum albumin at 1 mg/ml. Neither DHA nor arachidonic acid alone (5 μM) had any apparent effect on the parameters measured. DHA (5 μM) had no influence over β-adrenergic receptor stimulation (isoproterenol, 0.01–1 μM)-induced increases in I_{Ca}, Ca_i, or contraction. The findings that DHA inhibits the effect of DHP agonists and antagonists on Ca^{2+}-channel current but has no effect alone or on β-adrenergic-induced increases in I_{Ca} suggests that DHA specifically binds to Ca^{2+} channels at or near DHP binding sites and interferes with I_{Ca} modulation.**

The types of fatty acids and their interactions with membranes chiefly govern the physicochemical properties and function of membranes (1), yet the mechanisms and extent of influence on function await further definition. Potentially, the release of fatty acids from membrane phospholipids by activated phospholipases may free them to act as highly specific modulators or messengers altering cell function. McLennan *et al.* (2, 3) have shown that dietary lipid modulation of cardiac cell membranes in rats and nonhuman primates alters myocardial substrate vulnerability to arrhythmic stimuli. Increased cardiac membrane incorporation of $n - 3$ polyunsaturated fatty acids, predominantly eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), is associated not only with a lower incidence of ventricular fibrillation but also with increased mean left ventricular ejection fraction, end-diastolic volume, and myocardial energy efficiency (4).

In neonatal rat cardiac myocytes, Hallaq *et al.* (5) have reported that 5 μM EPA or DHA, but not arachidonic acid [AA; C20:4 ($n - 6$)], inhibited free cytosolic Ca^{2+} (Ca_i) overload as induced by ouabain and BAY K 8644 [dihydropyridine (DHP) L-type Ca^{2+}-channel agonist] and blocked the effects of nitrendipine (NITR; DHP L-type Ca^{2+} antagonist).

The present study investigates the effects of acutely administered low concentrations of DHA and AA on L-type Ca^{2+} current (I_{Ca}) in whole-cell voltage clamp experiments and on cytosolic Ca^{2+} transients and contractions in adult indo-1-loaded cardiac myocytes. The potential interaction between acute DHA or AA with cardiac β-adrenoreceptors also was studied using isoproterenol (ISO) in the same experimental protocols.

MATERIALS AND METHODS

Preparation of Isolated Cardiac Myocytes. Cardiac myocytes from 2- to 3-month-old Sprague–Dawley rats were enzymatically dissociated with collagenase type B and protease type XIV as described (6). Single cardiac myocytes were suspended in a series of Hepes buffers (20 mM Hepes/4.9 mM KCl/137 mM NaCl/1.2 mM NaH_2PO_4/1.2 mM $MgSO_4$/15 mM glucose) with increasing Ca^{2+} until 1 mM $CaCl_2$ was reached. For assessment of intracellular Ca^{2+} concentration ($[Ca_i^{2+}]$) cells were batch loaded at 25°C for 10 min in 2 ml of Hepes buffer containing 1 mM indo-1 free acid (7).

Measurement of I_{Ca}. All experiments were conducted at 25°C. Recordings were made using borosilicate glass microelectrodes (1.5–3 MΩ) and an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). The bathing solution consisted of 137 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1 mM $CaCl_2$, 15 mM dextrose, and 20 mM Hepes, with the pH adjusted to 7.4 using NaOH. Whole-cell current recordings were made with the following pipette solution: 100 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM NaCl, 10 mM EGTA, 5 mM MgATP, and 10 mM Hepes, adjusted to pH 7.2 with CsOH.

Junction potentials were compensated to zero with the pipette in the bath. The pipette capacitance was compensated by the fast capacitance compensation circuit of the amplifier

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Abbreviations: EPA, eicosapentaenoic acid; IFTA, indo-1 fluorescence ratio transient amplitude; TA, twitch amplitude; DL, diastolic length; DHA, docosahexaenoic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acid; DHP, dihydropyridine; NITR, nitrendipine; ISO, isoproterenol; BSA, bovine serum albumin; I, current; I_{Ca}, Ca^{2+}-channel current; V, voltage; $[Ca_i^{2+}]$, intracellular Ca^{2+} concentration; Ca_i, cytosolic Ca^{2+}; VS, velocity of shortening; IFR t_{50}, time from electrical stimulus to 50% IFTA; TA t_{50}, time from electrical stimulus to 50% TA.

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after the formation of a gigaseal and before breaking into the cell. After rupturing the cell membrane, myocyte capacitance was determined by applying small hyperpolarizing current pulses, I (≈0.1 nA), and measuring the resultant voltage change, V. The voltage decay followed a monoexponential time course and allowed measurement of the membrane time constant τ_m. Myocyte capacitance was calculated as $C = \tau_m \cdot I/V$.

I_{Ca} was measured with respect to the holding current prior to 0.1-sec voltage command pulses. The experimental protocol began 5 min after breaking into the cell. To inactivate sodium and T-type I_{Ca}, the holding potential was set to −45 mV. An adaptation protocol that consisted of a train of depolarizing impulses from −45 mV to 0 mV at a frequency of 0.5 Hz for 3 min was used. Cells with rapid rundown (>30% decrease in I_{Ca}) during this adaptation period were discarded. Depolarization pulses ranging from −35 to +45 mV in 10-mV increments were applied at 2-sec intervals to plot I–V relationships. The drugs and/or fatty acids were added to the bath solution 1 min from the start of the adaptation protocol, and the time course of effect was observed for 4 min.

Simultaneous Measurements of Ca_i Transients and Cell Length. Cell length and indo-1 fluorescence were measured simultaneously in a cell perfusion chamber on a modified Zeiss microscope as described (8). Rod-shaped, quiescent cells were selected and were superfused with Hepes buffer at 1 ml/min and field stimulated at 0.5 Hz with 3- to 4-msec square wave pulses as described (6). Myocyte length was monitored by a photodiode array (1024 SAQ, Reticon, CA) while excitation by 10-μsec flashes of 350 ± 5 nm light produced Ca^{2+}-bound and Ca^{2+}-free forms of indo-1 fluorescence (391–434 and 477–507 nm; monitored at 410 and 490 nm, respectively; ref. 8).

Myocyte autofluorescence was not measured prior to loading with indo-1 free acid; thus, calibration for free $[Ca_i^{2+}]$ was not possible. The indo-1 fluorescence recordings are therefore referred to as ratio of fluorescence transients. However, interpretation of the present results is not dependent on quantification of $[Ca_i^{2+}]$ because only changes in the magnitude of the $[Ca_i^{2+}]$ transient are of concern. The indo-1 fluorescence ratio (410/490 nm) transient amplitude (IFTA), an index of the relative change in cytosolic Ca^{2+} and Ca_i^{2+} transient amplitude, was taken as the $\text{ratio}_{peak} - \text{ratio}_{diastolic}$. The time from electrical stimulus to 50% IFTA (IFR t_{50}) was measured in milliseconds. Myocyte extent of shortening (twitch amplitude; TA) was expressed as a percentage of resting cell diastolic length (DL). Velocity of shortening (VS) was normalized to respective control DL (VS/DL). Time from electrical stimulus to 50% TA (TA t_{50}) was measured in milliseconds.

Steady-state myocyte contraction and fluorescence dynamics were assessed prior to and with the following treatments: control Hepes buffer, DHA (5 μM), AA (5 μM), NITR (10 nM), BAY K 8644 (10 nM), ISO (0.01–1 μM), NITR plus DHA, NITR plus AA, BAY K 8644 plus DHA, BAY K 8644 plus AA, ISO plus DHA, and ISO plus AA. In some experiments, to reverse fatty acid effects, fatty acid-free free bovine serum albumin (BSA) was also added to Hepes buffer at 1 mg/ml.

Materials. Collagenase type B was purchased from Boehringer Mannheim. Protease type XIV was from Sigma. BAY K 8644 and fatty acid-, nuclease-, and protease-free BSA fraction V were purchased from Calbiochem. Indo-1 pentapotassium salt was purchased from Molecular Probes. ISO HCl was purchased from Winthrop Pharmaceuticals (New York); *cis*-arachidonic acid and *cis*-docosahexaenoic acid were purchased from Cayman Chemicals (Ann Arbor, MI). Nitrendipine was purchased from Miles. All other chemicals were of the purest reagent grade commercially available.

Statistics. All results are presented as the mean ± SEM. Within-group comparisons were made by paired Student's t test, and between group contrasts were made by one-way analysis of variance. The level of significance was considered at $P < 0.05$.

RESULTS

Polyunsaturated Fatty Acid (PUFA) Interaction with NITR or BAY K 8644 at the L-Type I_{Ca}. Fig. 1*A* shows a representative example of NITR (10 nM) inhibition of I_{Ca} in a single myocyte (*Upper*) and the respective I–V relationships (*Lower*). When myocytes were exposed to DHA plus NITR, a significant inhibition of NITR action was observed (Fig. 1*B*). No significant change in I_{Ca} was observed after exposure to 5 μM AA alone (Table 1) or AA plus 10 nM NITR compared to NITR alone (Table 2).

Fig. 2*A* shows the mean relative change in peak I_{Ca} after exposure to DHA alone, DHA plus NITR, or NITR after DHA. DHA alone produced no significant change in I_{Ca}. NITR alone markedly reduced peak I_{Ca} compared to control.

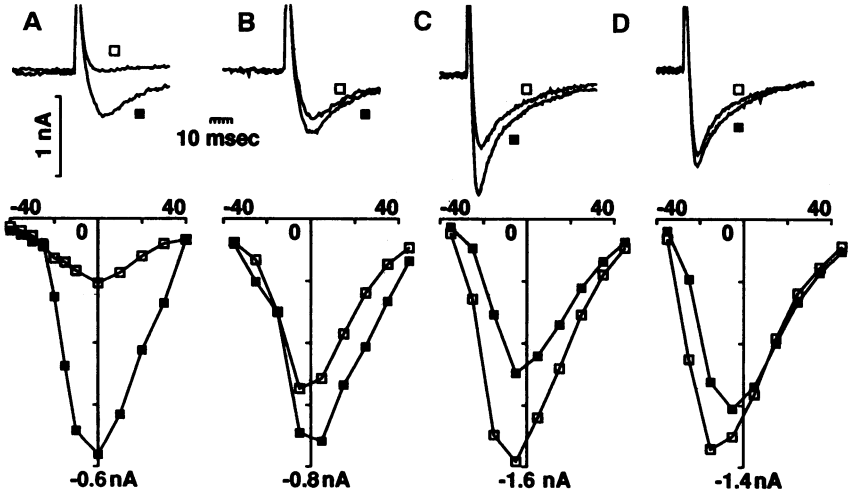

FIG. 1. Effects of 10 nM NITR (*A*), NITR plus 5 μM DHA. (*B*), 10 nM BAY K 8644 (*C*), and BAY K 8644 plus 5 μM DHA (*D*) on I_{Ca} (representative traces) elicited by depolarization to 0 mV from a holding potential of −45 mV. (*Upper*) Effect of drug and DHA (□) on I_{Ca} compared to control (■). (*Lower*) Respective I–V relationship in a single cell. Cell capacitance was as follows: *A*, 118 pF; *B*, 160 pF; *C*, 159 pF; *D*, 145 pF.

Table 1. Steady-state twitch and fluorescence parameters of indo-1 free acid-loaded rat cardiac myocytes

Treatment	DL, μm	TA, %DL	VS/DL	TA t_{50}, ms	$IFR_{diastolic}$	IFTA, % control	IFR t_{50}, ms
Control	103.3 ± 1.8	6.1 ± 0.2	1.2 ± 0.02	353.8 ± 9.99	1.110 ± 0.01	100	240 ± 15
DHA	103.3 ± 3.6	6.5 ± 0.2	1.2 ± 0.03	333.8 ± 8.90	1.116 ± 0.03	99 ± 2.7	231 ± 12
AA	100.7 ± 2.6	6.4 ± 0.2	1.2 ± 0.04	328.1 ± 12.8	1.116 ± 0.01	104 ± 5.6	222 ± 12

Values are means ± SEM (n = 6 per treatment).

DHA inhibited NITR action when both were added simultaneously or if NITR was added after DHA.

Exposure to BAY K 8644 augmented peak I_{Ca} by ≈50% (Figs. 1*C* and 2*A*). DHA plus BAY K 8644 almost completely blocked the effect of BAY K 8644 alone (Figs. 1*D* and 2*A*). No significant change in I_{Ca} occurred after exposure to 5 μM AA alone (Table 1) or AA plus 10 nM BAY K 8644 in comparison to 10 nM BAY K 8644 alone (Table 2).

PUFA Interaction with NITR or BAY K 8644 on Ca^{2+} Transients and Contraction. In myocytes contracting in steady-state 5 μM DHA or 5 μM AA alone, no significant effects to the measures of DL, TA t_{50}, IFTA, or IFR t_{50} were observed (Table 1). NITR at 10 nM reduced TA by more than 50% after a 2-min perfusion (Figs. 2*B* and 3*A*), VS/DL was reduced by ≈70% (Fig. 2*C*), and IFTA was reduced by 35% (Figs. 2*D* and 3*A*). In contrast, simultaneous DHA plus NITR perfusion resulted in almost a complete inhibition of NITR effects on TA, VS/DL, and IFTA (Figs. 2 *B–D* and 3*B*). When myocytes were simultaneously exposed to AA plus NITR, no significant difference, in comparison to NITR alone, was observed in any parameter (Table 2).

Perfusion of the myocytes with BAY K 8644 (10 nM) more than doubled TA, IFTA, and VS/DL (Figs. 2 *B–D* and 3*C*). DHA plus BAY K 8644 almost entirely abolished the increase in the contraction and indo-1 fluorescence parameters evident during exposure to BAY K 8644 alone (Figs. 2 *B–D* and 3*D*). In contrast, AA plus BAY K 8644 had no significant modulatory effect on BAY K 8644-induced stimulation of Ca^{2+} transient and amplitude of contraction (Table 2).

Reversibility of DHA Modulation of DHP Action. To test whether the protective effect of DHA against BAY K 8644 or NITR action was reversible, high-grade, purified fatty acid-free BSA was used to perfuse cells such that "bound" DHA could be removed, leaving the DHP present to act. In this preparation, BSA (1 mg/ml) alone had no effect on I_{Ca}, Ca_i transient, intrinsic autofluorescence, or contraction. Fig. 4*A* shows the influence on cell contraction, DHA alone, DHA plus NITR, and by BSA "washout" of DHA. Myocyte perfusion with BSA resulted in a reduction of TA similar to exposure to NITR alone. Removal of DHA's protective action occurred within 2 min, resulting in inhibition of Ca_i fluorescence transient (data not shown) and contraction amplitude similar to the effect of NITR alone (Figs. 2 and 3*A*).

Fig. 4*B* shows the effect on contraction of DHA alone, DHA plus BAY K 8644, and of BSA washout of DHA. DHA plus BAY K 8644 inhibited the BAY K 8644-induced augmentation of TA. BSA reversed this inhibition, resulting in increased TA similar to perfusion with BAY K 8644 alone.

DHA Modulation of β-Adrenoreceptor Stimulation. The possible interaction between acutely administered DHA or AA with myocardial β-adrenoreceptors was also investigated to test whether these fatty acids modulated the I_{Ca} and cytosolic free Ca^{2+} transients stimulated by non-DHP mechanisms. A concentration of 0.1 μM ISO increased peak I_{Ca}, IFTA, and TA to over 200% of that measured in control myocytes. No significant difference was observed between ISO (0.01–1 μM) plus DHA (5 μM) and ISO alone (0.01–1 μM) for I_{Ca}, Ca_i, or contraction parameters. AA (5 μM) also had no effect on ISO (0.01–1 μM) action (data not shown).

DISCUSSION

The present study investigated the effects of low concentrations of DHA and AA on indices of L-type Ca^{2+}-channel function, including interaction with BAY K 8644 (DHP agonist), NITR (DHP antagonist), and ISO (β-adrenergic receptor agonist), by measuring I_{Ca}, Ca_i transient, and contraction in adult rat isolated cardiac myocytes. No major effects of DHA or AA alone were observed at 5 μM. However, 5 μM DHA inhibited the actions of the Ca^{2+}-channel agonist BAY K 8644 and the Ca^{2+}-channel antagonist NITR. In contrast to DHA, AA had no inhibitory effect on the action of NITR or BAY K 8644. These findings are in agreement with those of Hallaq *et al.* (5), who found that in neonatal rat cardiac myocytes 5 μM EPA or 5 μM DHA prevented the ouabain-induced excessive rise in cytosolic free Ca^{2+} but did not alter the ouabain-induced inhibition of Na^+, K^+-ATPase. Both EPA and DHA were able to prevent the effect of NITR and BAY K 8644, but AA could not prevent these effects. However, both EPA and DHA had no effect when added alone to the neonatal myocytes. Hallaq *et al.* (5) attributed these findings to noncompetitive inhibition of [^{3}H]NITR-specific binding. In addition, the observation that DHA blocks the action of NITR but not the action of the non-DHP Ca^{2+} blockers, verapamil and diltiazem, led to the proposal that the action of DHA may specifically bind to a site at or adjacent to the DHP receptor site of the Ca^{2+}-channel protein.

Direct support for DHA binding to occur at or near the DHP locus rather than in the channel pore comes from the finding that DHA had no effect alone nor did it alter the effects of β-adrenergic receptor stimulation of the channel, which do not involve any interaction with the DHP receptor locus and thus would not be expected to alter β-adrenoreceptor binding to its agonist.

In the present study, Ca^{2+}-channel function modulation by exposure to AA at 5 μM was not apparent. This suggests that

Table 2. Effect of AA interaction with DHPs on steady-state twitch parameters, Ca_i transients, and I_{Ca} of indo-1 free acid-loaded rat cardiac myocytes

Treatment	DL	TA	TA t_{50}	$IFR_{diastolic}$	IFTA	IFR t_{50}	I_{Ca}
NITR	99.7 ± 1.9	48.2 ± 3.4*	95.4 ± 13.4	102.7 ± 1.8	65.3 ± 0.3*	85 ± 13	21 ± 3*
AA + NITR	99.6 ± 4.8	40.6 ± 1.5*	95.8 ± 4.1	102.6 ± 2.7	65.0 ± 1.4*	90 ± 9	30 ± 10*
BAY K 8644	99.5 ± 4.0	186 ± 16.5*	83.8 ± 8.2*	104.0 ± 2.7	152.0 ± 8.1*	93 ± 19	148 ± 11*
AA + BAY K 8644	99.0 ± 2.1	183 ± 34.9*	84.7 ± 4.0*	101.1 ± 1.1	135.9 ± 9.1*	96 ± 6	NA

Values are means ± SEM (n = 6 per treatment) and are expressed as a percentage of the control values. Respective control values did not differ significantly from those shown in Table 1. NA, not available. There was no significant difference between the absence or presence of AA. *Significantly different from control by Student's t test, $P < 0.05$.

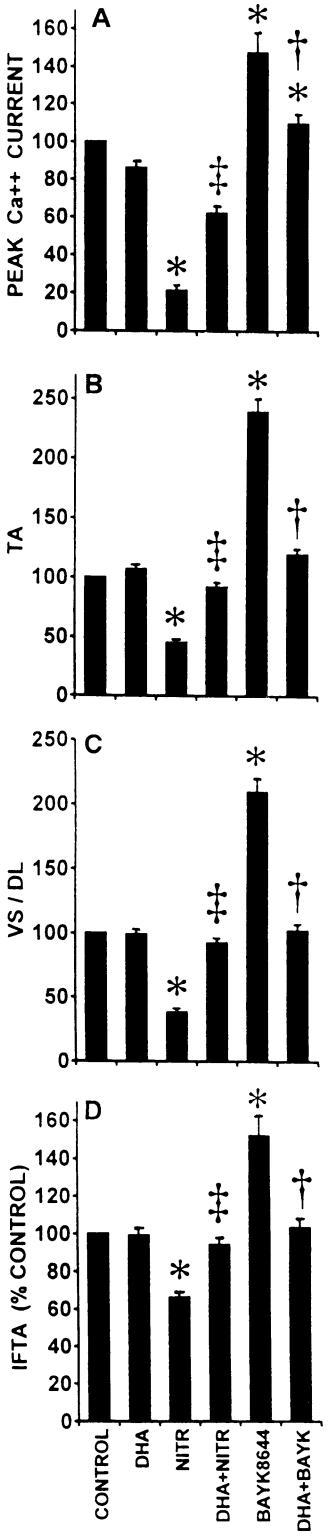

FIG. 2. The effect of NITR (10 nM) and BAY K 8644 (10 nM) and interaction with DHA (5 μM) on peak I_{Ca} (*A*), TA (*B*), VS/DL (*C*), and IFTA (*D*). Mean values ± SEM (n = 6 per treatment) are expressed as a percentage of control values (see Table 1). The average value for control Ca^{2+} current is 0.66 ± 0.23 nA; for control VS/DL the value is 1.2 ± 0.02 ms^{-2}. *, Significantly different from control, $P < 0.05$; ‡, significantly different from NITR, $P < 0.05$; †, significantly different from BAY K 8644, $P < 0.05$.

the action of DHA at the DHP locus of the Ca^{2+} channel was highly specific, possibly as a consequence of the intrinsic structural conformational properties related to the extent of

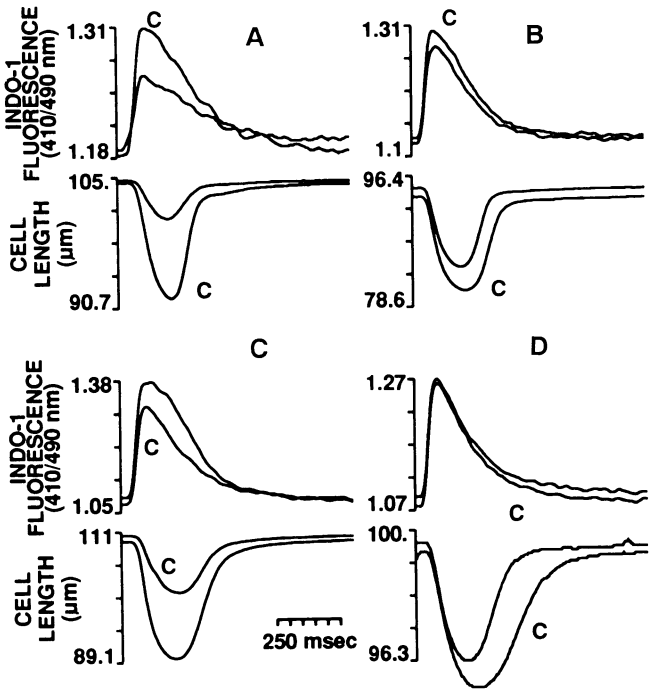

FIG. 3. Representative examples of the effect of DHP and their inhibition by DHA (5 μM) on indo-1 Ca_i transients (410/490 nm) and contraction. Each trace is an average of 4–10 (0.5 Hz) steady-state beats in a single cell. c, control trace. (*A*) NITR (10 nM). (*B*) NITR plus DHA. (*C*) BAY K 8644 (10 nM). (*D*) BAY K 8644 plus DHA.

unsaturation. The number of carbons in the fatty acid backbone determines the capacity for *skew* and *gauche* torsion angles to form in cis-fatty acid isomers (the relative torsional freedom of single bonds, C–C, between or adjacent to double bonds, C=C); thus an increase in carbon chain length facilitates increases in tightly packed van der Waals structures. The highly curved regions of these structures are rich in π-bonds, which permit rapid hydrophobic interactions with the highly curved surfaces of proteins and may also involve hydrogen bonding between such π-bonds and the polar groups of protein structures, such as ion channels and enzymes (9). Such factors may thus play a key role in the differential action of DHA compared to AA at the DHP site of Ca^{2+} channels.

Huang *et al.* (10) reported that AA at 10–50 μM dose-dependently increased voltage-dependent I_{Ca} in guinea pig cardiac myocytes, but 3 μM AA had no significant effect. In contrast, Nakajima and Kurachi (11) observed that ≈16 μM AA decreased the amplitude of I_{Ca} in guinea pig atrial myocytes. We recently observed that 50 μM AA inhibited I_{Ca} by 40% and TA by 25% in rat cardiac myocytes (S.P. and K.B., unpublished data). Damron and Bond (12) showed that exposure to 50 μM AA stimulated a transient increase in cytosolic free Ca^{2+} in suspensions of resting rat cardiac myocytes even after treatment with EGTA but not caffeine. Dettbarn and Pallade (13) observed that 20 and 50 μM AA, but not 5 μM AA, induced Ca^{2+} release from sarcoplasmic reticulum vesicles isolated from cardiac myocytes. The findings of these studies, which used higher concentrations of AA, may be due to a nonspecific influence over Ca^{2+}-channel function because it has been shown that at high concentrations (e.g., 10–50 μM or greater) PUFAs nonspecifically reduce the phase transition of membrane lipids and alter local lipid viscosity (14). Thus the actions of PUFAs at low concentrations likely involve more specific mechanisms. In the present study we have differentiated between the DHA modulation of DHP function and the apparent absence of AA interaction at this site by the use of a low concentration.

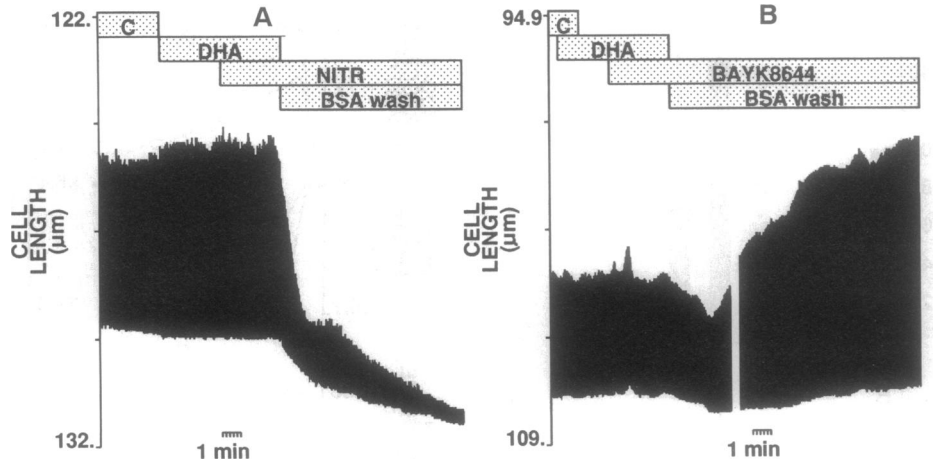

FIG. 4. Representative examples of DHA (5 μM) inhibition of DHP (10 nM) action on myocyte length during steady-state contraction (0.5 Hz) and reversal of DHA protection by fatty acid-free BSA (1 mg/ml). (*A*) Control perfusion (C), DHA, DHA plus NITR, followed by BSA plus NITR. (*B*) Control perfusion, DHA, DHA plus BAY K 8644, followed by BSA plus BAY K 8644.

In conclusion, in addition to previous observations (5), the finding of the present study shows directly that DHA inhibits NITR's blockade of L-type Ca^{2+}-channel influx in addition to inhibiting BAY K 8644-induced potentiation of L-type Ca^{2+}-channel influx. That this property of DHA can be reversed by BSA washout indirectly suggests that DHA acutely binds to a membrane locus associated with the DHP receptor site and it is not irreversible. The acute DHA modulation of DHP function in the present study may be explained by an alteration to a specific protein–lipid and lipid–lipid relationship between L-type Ca^{2+} channels in cardiac membranes. However, the acute effects of DHA may not completely share common mechanisms with the effects of dietary-induced increased incorporation of DHA into cardiac membrane phospholipids as other possible effects were not examined (15). However, the DHA-induced modulation of DHP function described in this study may still be related to the mechanism(s) involved in fish oil protection against ischemia–reperfusion-induced intracellular Ca^{2+} overload associated with cardiac arrhythmias (2, 3).

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