

## Inhibition of cardiac sodium currents in adult rat myocytes by n-3 polyunsaturated fatty acids

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1. The acute effects of n-3 polyunsaturated fatty acids were determined on whole-cell sodium currents recorded in isolated adult rat ventricular myocytes using patch clamp techniques.
2. The n-3 polyunsaturated fatty acids docosahexaenoic acid (22:6, n-3), eicosapentaenoic acid (20:5, n-3) and  $\alpha$ -linolenic acid (18:3, n-3) dose-dependently blocked the whole-cell sodium currents evoked by a voltage step to  $-30$  mV from a holding potential of  $-90$  mV with  $EC_{50}$  values of  $6.0 \pm 1.2$ ,  $16.2 \pm 1.3$  and  $26.6 \pm 1.3$   $\mu$ M, respectively.
3. Docosahexaenoic acid, eicosapentaenoic acid and  $\alpha$ -linolenic acid at  $25$   $\mu$ M shifted the voltage dependence of activation of the sodium current to more positive potentials by  $9.2 \pm 2.0$ ,  $10.1 \pm 1.1$  and  $8.3 \pm 0.9$  mV, respectively, and shifted the voltage dependence of inactivation to more negative potentials by  $22.3 \pm 0.9$ ,  $17.1 \pm 3.7$  and  $20.5 \pm 1.0$  mV, respectively. In addition, the membrane fluidising agent benzyl alcohol (10 mM) shifted the voltage dependence of activation to more positive potentials by  $7.8 \pm 2.5$  mV and shifted the voltage dependence of inactivation to more negative potentials (by  $-24.6 \pm 3.6$  mV).
4. Linoleic acid (18:2, n-6), oleic acid (18:1, n-9) and stearic acid (18:0) were either ineffective or much less potent at blocking the sodium current or changing the voltage dependence of the sodium current compared with the n-3 fatty acids tested.
5. Docosahexaenoic acid, eicosapentaenoic acid,  $\alpha$ -linolenic acid and benzyl alcohol significantly increased sarcolemmal membrane fluidity as measured by fluorescence anisotropy (steady-state,  $r_{ss}$ , values of  $0.199 \pm 0.004$ ,  $0.204 \pm 0.006$ ,  $0.213 \pm 0.005$  and  $0.214 \pm 0.009$ , respectively, compared with  $0.239 \pm 0.002$  for control), whereas stearic, oleic and linoleic acids did not alter fluidity (the  $r_{ss}$  was not significantly different from control).
6. The potency of the n-3 fatty acids docosahexaenoic acid, eicosapentaenoic acid and  $\alpha$ -linolenic acid to block cardiac sodium currents is correlated with their ability to produce an increase in membrane fluidity.

A number of epidemiological studies have shown that the consumption of a diet high in n-3 polyunsaturated fatty acids (PUFAs) can confer protection from coronary heart disease (Kromhout *et al.* 1985; Burr *et al.* 1989; Siscovick *et al.* 1995). In animal studies, dietary PUFAs have a similar protective action, particularly in relation to cardiac arrhythmias (McLennan *et al.* 1988, 1996; Hock *et al.* 1990; Pepe & McLennan, 1996). More recently, it has been reported that some PUFAs can exert powerful anti-arrhythmic actions when applied acutely, as an intravenous infusion in surgically manipulated animals (Billman *et al.* 1994, 1997). Studies using isolated neonatal cultured cardiac myocyte preparations have also demonstrated an anti-arrhythmic effect of acutely added PUFAs (Kang & Leaf, 1994, 1995, 1996).

The electrophysiological mechanism underlying the acute effect of fatty acids, at least in isolated cardiac myocytes,

may involve an increase in the threshold for the generation of the action potential (Kang *et al.* 1995; McMurchie *et al.* 1999), suggesting that PUFAs may mediate their effects by interaction with sodium currents. In this context, n-3 PUFAs have been shown to have potent effects on sodium currents evoked in neurons (Vreugdenhil *et al.* 1996), in HEK293t cells transfected with the alpha subunit of the human cardiac sodium channel (Xiao *et al.* 1998) and in neonatal cardiac myocytes (Xiao *et al.* 1995). However, difficulties arise in the extrapolation of results obtained with cloned channels expressed in transfected cells to adult tissues, since the results are always subject to reservations as to whether channel properties have been modified by the expression system, and whether crucial subunits are missing or modified. Similarly, cultured neonatal cardiac myocytes exhibit important differences from the adult myocardium since many ionic currents are subject to considerable

modulation during development (Wetzel *et al.* 1993; Matsubara *et al.* 1993; Nuss & Marban, 1994; Wang *et al.* 1996; Roden & George, 1997). An additional difficulty with neonatal cells is that sodium channels undergo substantial changes in their properties during development (Sada *et al.* 1995) which include changes in the action of class I anti-arrhythmic agents (Xu *et al.* 1991, 1992). Due to these developmental changes, and of particular concern in the context of arrhythmias, neonatal cardiac myocytes have a fundamentally different electrophysiological profile from adult cells.

We have therefore investigated the effects of a variety of n-3 polyunsaturated fatty acids, differing in their degree of unsaturation and chain length, on sodium currents in isolated adult rat cardiac myocytes using whole-cell patch clamp recording techniques.

The shorthand notation for fatty acid structure is 'a:b, n-c'. The letter 'a' represents the total number of carbon atoms in the fatty acyl chain; 'b', the number of double bonds separated by single methylene groups; and 'n-c', also written as 'omega-c' (or  $\omega$ -c), denotes the number of carbon atoms between the first double bond and the methyl end of the chain. The n-3 fatty acids are those in the linolenic acid family; n-6 fatty acids are in the linoleic acid family.

We have focused in particular on docosahexaenoic acid (DHA) (22:6, n-3), which occurs in relatively high concentrations in cardiac membrane phospholipids. In addition, we have investigated the effects of the n-3 PUFAs on sarcolemmal membrane fluidity in isolated cardiac myocytes using steady-state fluorescence anisotropy to determine whether changes in cell membrane physical properties correlate with the effects of PUFAs on sodium current activity. In order to investigate the idea that sodium current block by fatty acids is related to changes in membrane fluidity, we have also investigated the effects of benzyl alcohol, a known membrane fluidising agent, on both membrane fluidity and sodium currents.

## METHODS

Animals used in these studies were cared for according to the Australian National Health and Medical Council Guidelines for the Care and Use of Animals. All experimental procedures were subject to prior approval by the University of Adelaide and CSIRO Human Nutrition Animal Ethics Committees.

### Isolation of cardiac myocytes

Enzymatic isolation of cardiac myocytes was performed as previously described (Saint *et al.* 1992). Briefly, male Sprague-Dawley rats (300–350 g) were injected with heparin (2000 i.u., I.P.). After 30 min, animals were anaesthetised with pentobarbitone sodium (60 mg kg<sup>-1</sup>) which was injected intraperitoneally. The heart was removed, washed in ice-cold, oxygenated, calcium-free Tyrode solution for 1 min, and then perfused, via an aortic cannula, with the same calcium-free Tyrode solution warmed to 37 °C at a perfusion rate of between 9 and 10 ml min<sup>-1</sup>. This facilitated the removal of blood from both the coronary vasculature and ventricular chambers. The Tyrode solution contained (mM): NaCl, 134; HEPES, 10; KCl, 4; MgCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 11, and was adjusted to pH 7.4 with 1.0 M NaOH. After 5 min of

perfusion the heart was subjected to enzymatic dissociation by perfusion with 25  $\mu$ M calcium Tyrode solution containing collagenase (0.3 mg ml<sup>-1</sup>, Worthington CLS II), protease (0.1 mg ml<sup>-1</sup>, Sigma) and fetal calf serum (1  $\mu$ g ml<sup>-1</sup>). After approximately 25 min when the heart became pale and flaccid, the ventricles were removed, cut into small pieces in fresh 25  $\mu$ M calcium-Tyrode solution and triturated to dissociate the myocytes. Cell suspensions were then allowed to settle at room temperature and the Ca<sup>2+</sup> concentration was increased gradually to 200  $\mu$ M. Finally, cells were resuspended in Tyrode solution containing 1 mM calcium and plated onto glass coverslips. For fluidity measurements, cells were plated onto coverslips coated with 50  $\mu$ g ml<sup>-1</sup> laminin (Sigma). Except for the perfusion of the heart, all the preparation and maintenance of the cells was at room temperature (25 °C).

### Electrophysiological recording of cardiac Na<sup>+</sup> current

Electrodes were prepared from borosilicate glass using a two-stage puller (Narishige Scientific Instruments, Tokyo, Japan) and resistances were typically between 1 and 3 M $\Omega$  when containing the pipette solution. Whole-cell currents were recorded 5 min after achievement of a whole-cell patch clamp configuration. Current recording was performed using an Axopatch 200A amplifier (Axon Instruments). Whole-cell capacitance and series resistance compensation was achieved using the controls on the amplifier; recording was only performed if series resistance compensation of at least 90% could be achieved. Satisfactory voltage control was indicated by the following criteria: (1) the negative limb of the current–voltage curve spanned at least 25 mV; (2) there were no abnormal notches in the current–voltage curve; (3) there was no crossover between recordings at different voltages of the inactivation curve. Whole-cell sodium currents were evoked by voltage steps generated by a computer program which outputs the waveform via a digital to analogue converter connected to the command input of the amplifier. The resulting currents were filtered at 5 kHz and recorded through an analogue to digital converter operating at 20 kHz. Glass coverslips with adhering cardiac myocytes were transferred to a superfusion chamber (mounted on an inverted microscope) containing 0.5 ml of bath solution. Cardiac myocyte currents were recorded 5 min after achieving a whole-cell patch clamp configuration.

### Membrane fluidity measurements

Membrane fluidity was determined by measuring the steady-state fluorescence anisotropy ( $r_{ss}$ ) of the probe *N*-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl)propyl) trimethyl-ammonium *p*-toluenesulphonate (TMAP-DPH) (Molecular Probes, Eugene, OR, USA) according to a modification of a method described previously (de Jonge *et al.* 1996). This probe readily partitions into the cell membrane (Lentz, 1989). Isolated ventricular cardiac myocytes attached to laminin-coated glass coverslips were washed in Tyrode buffer. Cardiac myocytes were then loaded with 1  $\mu$ M TMAP-DPH for 15 min at 37 °C. The coverslips were placed in a glass cuvette containing 20  $\mu$ M test fatty acids or 10 mM benzyl alcohol and  $r_{ss}$  values were measured according to the following formula as described previously (Lentz, 1989):

$$r_{ss} = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})}$$

where  $I_{VV}$  and  $I_{VH}$  represent the fluorescence intensity parallel and perpendicular to the excitation plane (when set vertically), respectively.  $G$  is a correction factor for the difference in the transmission efficiency for vertically and horizontally polarised light, and is calculated by  $I_{HV}/I_{HH}$ , where  $I_{HH}$  represents the fluorescence intensity parallel to the excitation plane when set horizontally. Measurements were obtained using a spectrofluorimeter

(Hitachi 650-10S), provided with vertical and horizontal polarisation filters (Polaroid, Australia). The excitation and emission monochromators were positioned at wavelengths of 350 and 430 nm, respectively, with slit width set to 10 nm for both excitation and emission modes. Readings were corrected for both background fluorescence of TMAP-DPH and light scatter by the cardiac myocyte preparation itself.

### Solutions and fatty acids

The standard external (bath) solution used for sodium current measurements contained (mM): NaCl, 20; *N*-tris-(hydroxy-methyl)-methyl-2-aminoethanesulphonic acid (Tes), 10; KCl, 5; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 2; CoCl<sub>2</sub>, 5; CsCl, 5; glucose, 10; choline chloride, 110; pH adjusted to 7.4 with 5.0 M NaOH. The pipette solution for all experiments contained (mM): CsF, 120; Tes, 10; MgCl<sub>2</sub>, 2; Na<sub>2</sub>-EGTA, 20; CaCl<sub>2</sub>, 2; pH adjusted to 7.4 with 5.0 M KOH. These solutions are designed to block all ionic currents other than sodium currents. In addition, the low extracellular sodium concentration (20 mM) is designed to reduce the peak sodium current and hence minimise series resistance errors in the clamp potential. Fatty acids (Sigma) were dissolved in ethanol at a final concentration of 50 mM containing 0.003% (w/v) butylated-hydroxyanisole and stored at -80 °C.

### Statistical analysis

Appropriate equations were fitted to individual data sets using the algorithm built into the graphics program GraphPad Prism version 2.00 (Graphpad Software Inc., San Diego, CA, USA). Data are presented as means  $\pm$  standard error of the mean (S.E.M.). Significance between means was tested either using the two tailed Student's paired *t* test or one-way ANOVA with Dunnett's multiple comparisons test. The significance level was set at  $P < 0.05$ .

## RESULTS

### Block of voltage-dependent sodium currents in adult rat cardiac myocytes

The average capacitance of cardiac myocytes was  $120.3 \pm 5.2$  pF ( $n = 51$ ). Sodium currents in control cells were

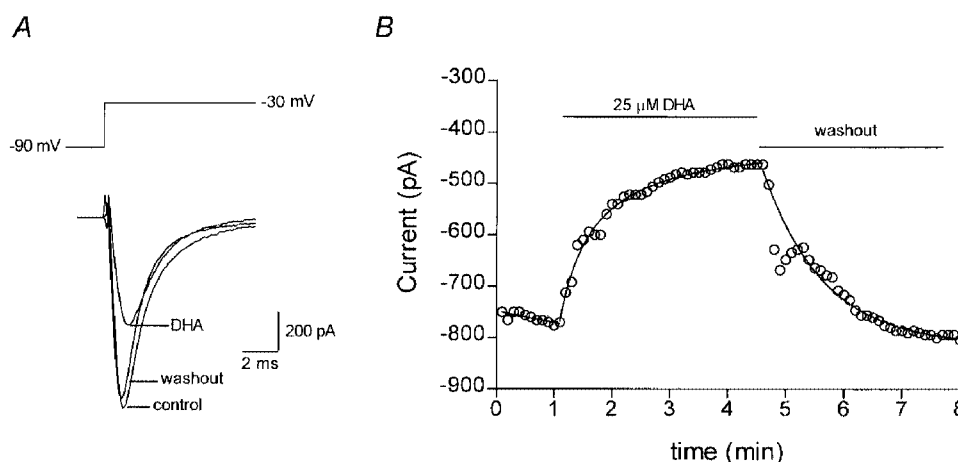
activated at approximately -55 mV and reached a maximum current at approximately -25 mV. The maximum sodium current densities elicited by a voltage step from -90 mV to -30 mV for extracellular Na<sup>+</sup> concentrations of 20 mM and 70 mM were  $8.9 \pm 0.4$  pA pF<sup>-1</sup> ( $n = 51$ ) and  $13.8 \pm 2.9$  pA pF<sup>-1</sup> ( $n = 7$ ), respectively. Figure 1A shows an example of typical sodium currents evoked in a single myocyte by a step in membrane potential from a holding potential of -90 mV to -30 mV, and the effect of 25  $\mu$ M DHA on this current. In this cell, 25  $\mu$ M DHA applied to the extracellular solution blocked the peak current amplitude by 42%, after approximately 4 min of incubation. The time course of this block is shown in Fig. 1B. The block by DHA reached a plateau after approximately 3 min exposure and elimination of the effect of DHA could be achieved by a washout procedure by adding delipidated bovine serum albumin (BSA) to the extracellular solution for 2 min (in the absence of fatty acid).

### Effect of fatty acids on sodium current activation

The effect of fatty acids on the voltage dependence of activation was determined by evoking current-voltage steps to various potentials between -90 mV and +30 mV from a holding potential of -140 mV (as depicted in upper panel of Fig. 2A). Representative sodium currents are shown in the lower panel of Fig. 2A. The maximum amplitude of the currents evoked was plotted against the test potential, as shown in Fig. 2B for control cells and cells in the presence of 25  $\mu$ M DHA. The data points were fitted by the equation:

$$I = (G_{\max}(V - E_{\text{rev}})) / (1 + \exp((V - V_{1/2})/k)), \quad (1)$$

using a least squares fitting algorithm, where  $G_{\max}$  is the maximum conductance,  $V$  is the membrane potential for half-activation of the channels,  $V_{1/2}$  is the test membrane



**Figure 1.** Time course of sodium current inhibition by DHA in an adult rat ventricular myocyte

A, depiction of the voltage step (upper panel) and the current evoked by a step in membrane potential to -30 mV from a holding potential of -90 mV (lower panel) and following incubation in DHA (25  $\mu$ M) and subsequent washout. B, time course of the inhibitory effect of DHA on the peak sodium current recorded from an adult rat ventricular myocyte. Sodium currents were evoked in whole-cell recording mode once every 6 s with 300 ms duration pulses to -30 mV from a holding potential of -90 mV. The horizontal bars indicate the period during which DHA was applied to the cell or the period of washout with a solution containing 1 mg ml<sup>-1</sup> delipidated BSA.

Table 1. Activation parameters from least squares fit of eqn (1)

Structure	$G_{\max}$ (nS)	$V'$ (mV)	$k$ (mV <sup>-1</sup> )	$V_{\text{rev}}$ (mV)
Control	20.5 ± 1.7	-42.9 ± 2.9	2.7 ± 0.5	23.9 ± 1.1
DHA 25 μM (22:6, n-3)	13.4 ± 1.4 †	-33.7 ± 2.1 †	5.0 ± 0.5 †	25.7 ± 2.0
Control	22.2 ± 1.3	-39.2 ± 2.1	3.2 ± 0.4	37.8 ± 2.3
EPA 25 μM (20:5, n-3)	15.6 ± 1.0 †	-29.2 ± 2.0 ‡	4.8 ± 0.4 ‡	39.8 ± 2.2
Control	22.1 ± 1.7	-33.6 ± 2.5	4.1 ± 0.3	31.9 ± 2.2
ALA 25 μM (18:3, n-3)	18.2 ± 1.2 †	-25.3 ± 2.1 ‡	5.3 ± 0.4 †	40.9 ± 3.7*
Control	18.4 ± 2.1	-41.7 ± 1.6	2.9 ± 0.8	27.4 ± 0.4
LA 25 μM (18:2, n-6)	17.4 ± 2.0	-39.9 ± 1.5	2.5 ± 0.9	32.9 ± 2.6
Control	16.6 ± 2.7	-46.6 ± 2.9	2.4 ± 0.5	32.6 ± 1.9
OA 25 μM (18:1, n-9)	16.2 ± 2.5	-46.2 ± 2.3	2.3 ± 0.7	34.1 ± 1.4
Control	17.9 ± 2.2	-47.8 ± 2.8	2.2 ± 0.4	32.2 ± 1.9
SA 25 μM (18:0)	17.9 ± 2.1	-49.0 ± 2.8	1.4 ± 0.2*	30.3 ± 3.0
Control	23.7 ± 0.5	-46.0 ± 1.5	5.1 ± 0.9	10.5 ± 1.4
Benzyl alcohol (10 mM)	7.7 ± 3.0*	-38.2 ± 1.8	4.8 ± 0.3	9.1 ± 1.1

\*  $P < 0.05$ , †  $P < 0.01$ , ‡  $P < 0.001$ , significantly different from control (two-tailed Student's paired  $t$  test).

Abbreviations: DHA, docosahexaenoic acid ( $n = 5$ ); EPA, eicosapentaenoic acid ( $n = 10$ ); ALA,  $\alpha$ -linolenic acid ( $n = 6$ ); LA, linoleic acid ( $n = 5$ ); OA, oleic acid ( $n = 7$ ); SA, stearic acid ( $n = 8$ ). Benzyl alcohol ( $n = 3$ ).

potential,  $E_{\text{rev}}$  is the reversal potential for the current and  $k$  is a slope factor. The control data were best fitted by  $G_{\max} = 19.3$  nS,  $V' = -37.2$  mV,  $k = 4.17$  mV<sup>-1</sup> and  $E_{\text{rev}} = 25.9$  mV. In the presence of 25 μM DHA the maximum conductance,  $G_{\max}$ , was reduced by 50% to 9.6 mV,  $V'$  was -26.2 mV (i.e. shifted to more positive potentials by 11.0 mV),  $k$  was 6.43 mV<sup>-1</sup> and  $E_{\text{rev}}$  was 29.0 mV (not significantly different from control). The means of all the above parameters for the least squares fit of eqn (1) for the

n-3 PUFAs DHA, eicosapentaenoic acid (EPA) and  $\alpha$ -linolenic acid (ALA), all at 25 μM final concentration, are given in Table 1. Following treatment with DHA ( $n = 5$ ), EPA ( $n = 10$ ) or ALA ( $n = 6$ ),  $G_{\max}$  values were significantly lower than controls ( $P < 0.01$ ) and the  $I$ - $V$  relation was significantly shifted to more positive potentials. This shift in membrane potential was similar for all n-3 PUFAs tested ( $9.2 \pm 2.0$ ,  $10.0 \pm 1.1$  and  $8.3 \pm 0.9$  mV for DHA, EPA or ALA-treated cardiac myocytes, respectively). However,

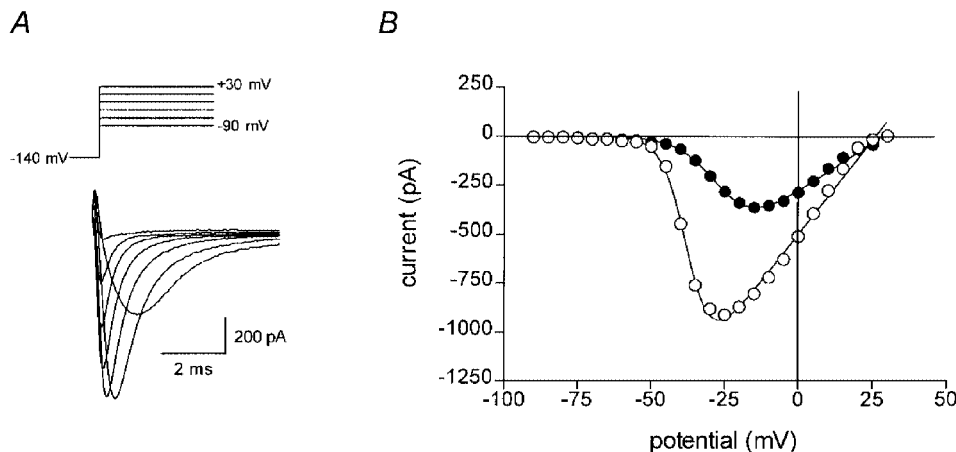


Figure 2. Effect of DHA on the voltage dependence of activation in an adult rat ventricular myocyte

*A*, sodium currents were evoked by voltage steps from a holding potential of -140 mV to various voltages between -90 mV and +30 mV as depicted (upper panel). Plotted below are superimposed currents evoked at -40, -30, -20, -10, 0, 10 and 20 mV. *B*, the peak current amplitude was plotted against the pulse potential. Typical data are shown as points representing control (○) or in the presence of 25 μM DHA (●). The continuous line shows the least squares best fit of eqn (1). The parameters for the best fit in each case were: control,  $G_{\max} = 19.3$  nS,  $V' = -37.2$  mV,  $k = 4.17$  mV<sup>-1</sup> and  $E_{\text{rev}} = 25.9$  mV; and in the presence of 25 μM DHA,  $G_{\max} = 9.6$  nS,  $V' = -26.2$  mV,  $k = 6.43$  mV<sup>-1</sup> and  $E_{\text{rev}} = 29.0$  mV, where  $G_{\max}$  is the maximum conductance,  $V'$  is the voltage at which 50% of the channels are activated,  $k$  is the slope factor for the voltage dependence of activation and  $E_{\text{rev}}$  is the reversal potential.

**Table 2. Inactivation parameters from least squares fit of eqn (2)**

	Structure	$I_{max}$ (pA)	$V'$ (mV)	$k$ (mV <sup>-1</sup> )
Control		-850 ± 92	-74.3 ± 1.5	5.2 ± 0.1
DHA 25 μM	(22:6, n-3)	-544 ± 88*	-96.6 ± 1.3‡	6.5 ± 0.2‡
Control		-1258 ± 107	-69.3 ± 2.1	6.5 ± 0.2
EPA 25 μM	(20:5, n-3)	-816 ± 106†	-86.5 ± 3.5†	7.4 ± 0.3‡
Control		-988 ± 103	-68.4 ± 2.0	5.8 ± 0.3
ALA 25 μM	(18:3, n-3)	-740 ± 83†	-88.9 ± 2.0‡	7.0 ± 0.2‡
Control		-869 ± 105	-71.0 ± 2.8	6.3 ± 0.4
LA 25 μM	(18:2, n-6)	-898 ± 119	-77.3 ± 3.0‡	6.9 ± 0.4
Control		-901 ± 128	-76.0 ± 2.9	6.2 ± 0.3
OA 25 μM	(18:1, n-9)	-902 ± 156	-79.5 ± 2.3*	6.4 ± 0.1
Control		-974 ± 121	-73.7 ± 1.7	6.2 ± 0.2
SA 25 μM	(18:0)	-919 ± 105	-73.6 ± 2.2	6.9 ± 0.4
Control		-716 ± 63	-86.0 ± 1.8	8.4 ± 0.4
Benzyl alcohol (10 mM)		-140 ± 77†	-110.6 ± 3.8*	6.8 ± 1.4

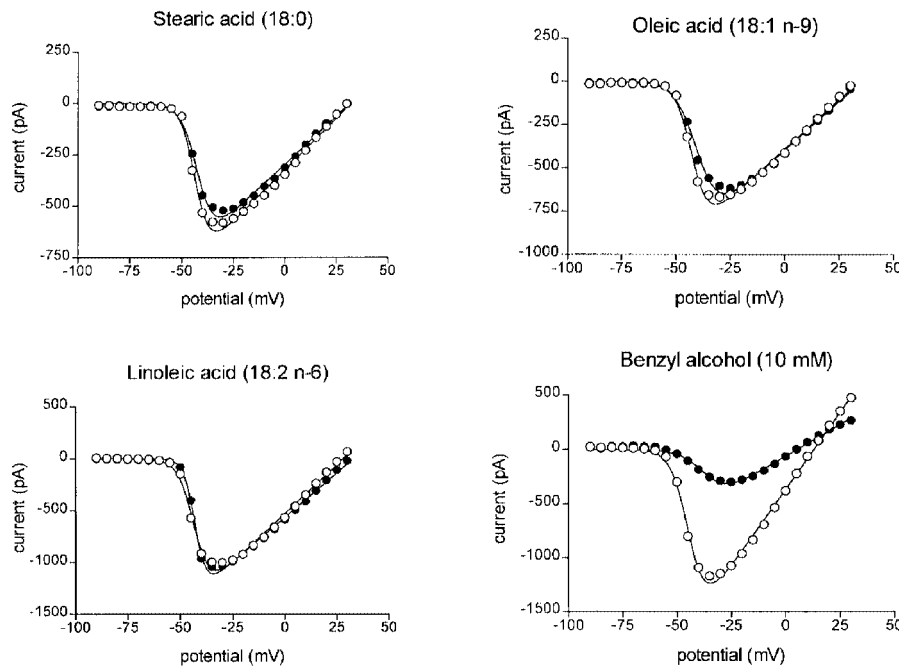
\*  $P < 0.05$ , †  $P < 0.01$ , ‡  $P < 0.001$ , significantly different from control (two-tailed Student's paired  $t$  test). Abbreviations: DHA, docosahexaenoic acid ( $n = 5$ ); EPA, eicosapentaenoic acid ( $n = 10$ ); ALA,  $\alpha$ -linolenic acid ( $n = 6$ ); LA, linoleic acid ( $n = 5$ ); OA, oleic acid ( $n = 7$ ); SA, stearic acid ( $n = 8$ ). Benzyl alcohol ( $n = 3$ ).

DHA was more potent than either EPA or ALA at inhibiting  $G_{max}$  in these cells. Linoleic acid (18:2, n-6), oleic acid (18:1, n-9) and the saturated fatty acid, stearic acid (18:0), did not significantly alter any of the sodium current activation parameters measured (Fig. 3 and Table 1). Benzyl

alcohol at 10 mM ( $n = 3$ ) significantly ( $P < 0.05$ ) reduced the  $G_{max}$  as shown in Table 1 and Fig. 3.

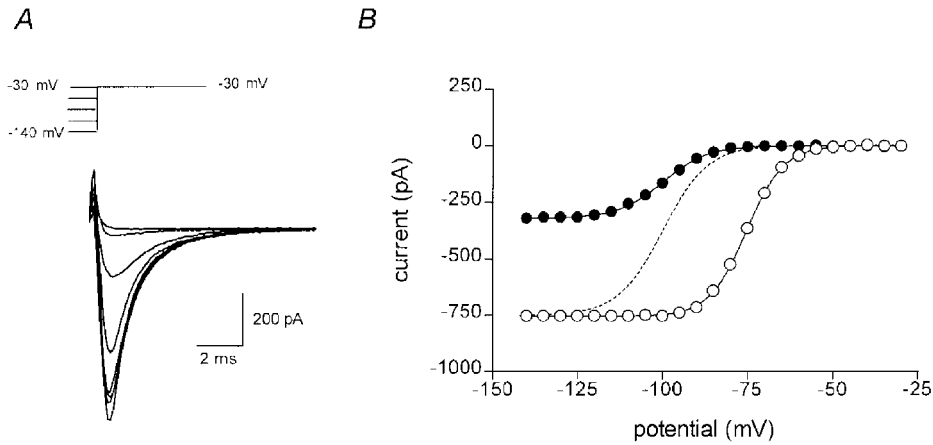
**Effect of fatty acids on sodium current inactivation**

The effect of the fatty acids on the voltage dependence of inactivation of the sodium current was investigated by



**Figure 3. Effect of various fatty acids on the voltage dependence of activation in adult rat ventricular myocytes**

Sodium currents were evoked by voltage steps to various potentials from a holding potential of -140 mV to various voltages between -90 mV and +30 mV as depicted in Fig. 2A. The peak current amplitude was plotted against the pulse potential. Points represent typical control data (○) or in the presence of 25 μM fatty acid or benzyl alcohol (●) as indicated.



**Figure 4. Effect of DHA on the voltage dependence of inactivation in adult rat ventricular myocytes**

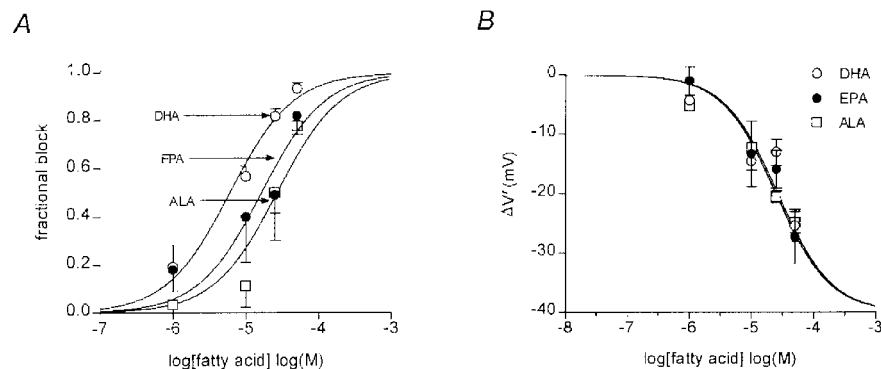
*A*, sodium currents were evoked by voltage steps to  $-30$  mV from various holding potentials, as depicted (upper panel). Plotted below are currents evoked at  $-130$ ,  $-120$ ,  $-110$ ,  $-100$ ,  $-90$ ,  $-80$ ,  $-70$ ,  $-60$  and  $-50$  mV, shown superimposed. *B*, the peak current amplitude is shown plotted against the holding potential. Points represent typical control data (○) or in the presence of  $25 \mu\text{M}$  DHA (●). The continuous line shows the least squares best fit of eqn (2) for control or DHA. The parameters for the best fit in each case were: control,  $I_{\text{max}} = -746$  pA,  $V' = -75.2$  mV and  $k = 5.28$  mV $^{-1}$ ; and in the presence of  $25 \mu\text{M}$  DHA,  $I_{\text{max}} = -320$  pA,  $V' = -99.9$  mV and  $k = 6.60$  mV $^{-1}$ . The dashed line shows the data points for DHA scaled to the same maximum as the control data.

stepping the membrane potential to a test potential of  $-30$  mV from holding potentials which varied between  $-140$  and  $-30$  mV (Fig. 4*A*). The peak amplitude of the evoked current was plotted against the holding potential for both control cells and cells in the presence of  $25 \mu\text{M}$  DHA, and the data points fitted with the Boltzmann equation:

$$I = I_{\text{max}} (1 / (1 + \exp((V - V')/k))), \quad (2)$$

where  $I_{\text{max}}$  is the maximum current,  $V$  is the test membrane potential,  $V'$  is the membrane potential at which half of the channels are inactivated, and  $k$  is a slope factor. In this cell,  $25 \mu\text{M}$  DHA induced a shift to more hyperpolarised

potentials in the voltage dependence of inactivation by  $24.7$  mV and  $I_{\text{max}}$  was reduced from a control value of  $-746$  pA to  $-320$  pA in the presence of DHA. The mean values for the parameters  $I_{\text{max}}$ ,  $V'$  and  $k$ , for the least squares fit of eqn (2) for the n-3 polyunsaturated fatty acids DHA ( $n = 6$ ), EPA ( $n = 10$ ) and ALA ( $n = 6$ ) all at  $25 \mu\text{M}$  final concentration, as well as benzyl alcohol ( $n = 3$ ) at  $10$  mM, are given in Table 2. In addition to the n-3 PUFAs, the following fatty acids were also tested: linoleic acid ( $n = 5$ ), oleic acid ( $n = 7$ ) and the saturated fatty acid, stearic acid ( $n = 8$ ) (Table 2). A significant reduction in  $I_{\text{max}}$  occurred following treatment with either DHA ( $P < 0.05$ ),



**Figure 5. Concentration-dependent block of n-3 polyunsaturated fatty acids in adult rat ventricular myocytes**

*A*, concentration-response curves for the n-3 PUFAs, DHA, EPA and ALA. The degree of block of the sodium current for each cell was measured as a fraction of the control current, to compensate for differences in peak current amplitude due to differences in cell size. The lines show the least squares fit using the Hill equation (eqn (3)), which gave  $\text{EC}_{50}$  values of  $6.0 \pm 1.2 \mu\text{M}$  (mean  $\pm$  s.e.m.,  $n = 5$ ),  $16.2 \pm 1.3 \mu\text{M}$  ( $n = 5$ ) and  $26.6 \pm 1.3 \mu\text{M}$  ( $n = 6$ ) for DHA (○), EPA (●) and ALA (□), respectively. *B*, dose-response relationship for shift in inactivation ( $\Delta V'$ ) and fatty acid concentration.

EPA ( $P < 0.01$ ) or ALA ( $P < 0.01$ ). Benzyl alcohol also significantly reduced  $I_{\max}$  ( $n = 3$ ,  $P < 0.01$ ). A shift of  $V'$  to more hyperpolarised potentials was also observed following treatment with DHA ( $22.3 \pm 0.9$  mV;  $P < 0.001$ ), EPA ( $17.1 \pm 3.7$  mV;  $P < 0.01$ ) or ALA ( $20.5 \pm 1.0$  mV;  $P < 0.001$ ). The fatty acids linoleic acid, oleic acid and stearic acid did not significantly alter  $I_{\max}$ , although addition of linoleic acid and oleic acid was associated with a small shift of  $V'$  to more hyperpolarised potentials ( $6.3 \pm 0.6$  and  $3.5 \pm 1.2$  mV, respectively). Nevertheless, the shift in voltage dependence was considerably less with these three fatty acids than that observed by any of the n-3 PUFAs (Table 2).

#### Concentration dependence of sodium current block by n-3 polyunsaturated fatty acids

When whole-cell sodium currents were evoked from a holding potential of  $-90$  mV (close to the resting potential of the cells *in vivo*), DHA, EPA and ALA produced a concentration-dependent block of peak sodium current amplitude over the concentration range  $1$ – $50$   $\mu\text{M}$ . The degrees of block by DHA, EPA and ALA over this concentration range in several cells are shown in Fig. 5A. The  $\text{EC}_{50}$  for each of the fatty acids was estimated from the least squares fit of the Hill equation:

$$y = 1 / (1 + (K_A/[A])^{n_H}), \quad (3)$$

where  $y$  is the fractional block,  $K_A$  is the apparent affinity constant,  $[A]$  is the concentration of fatty acid and  $n_H$  is the Hill coefficient. The most potent polyunsaturated fatty acid was DHA ( $\text{EC}_{50} = 6.0 \pm 1.2$   $\mu\text{M}$ ,  $n = 5$ ), followed by EPA ( $\text{EC}_{50} = 16.2 \pm 1.3$   $\mu\text{M}$ ,  $n = 5$ ), and ALA ( $\text{EC}_{50} = 26.6 \pm 1.3$   $\mu\text{M}$ ,  $n = 6$ ). The potency for each of the fatty acids DHA, EPA and ALA to shift the voltage dependence of inactivation was also determined. Figure 5B shows the dose–response relationship for the shift in inactivation ( $\Delta V'$ ) plotted against log concentration for each of the fatty acids. In this case there was no obvious difference in potency between DHA, EPA and ALA. The other fatty acids examined, linoleic acid, oleic acid and stearic acid, did not produce a measurable shift in voltage dependence up to concentrations of  $25$   $\mu\text{M}$ .

#### Adult rat cardiac myocyte membrane fluidity

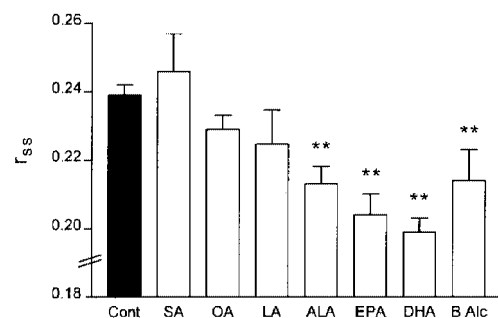
The effect of acute addition of fatty acids on cardiac myocyte membrane fluidity was determined by steady-state fluorescence anisotropy using the probe TMAP-DPH. The fluorescence anisotropy ( $r_{\text{ss}}$ ) following incubation in stearic acid ( $20$   $\mu\text{M}$ ;  $n = 6$ ), oleic acid ( $20$   $\mu\text{M}$ ;  $n = 10$ ) or linoleic acid ( $20$   $\mu\text{M}$ ;  $n = 7$ ) was not significantly different from control (no fatty acid additions,  $n = 23$ , Fig. 6). However,  $r_{\text{ss}}$  was significantly decreased following incubation with ( $20$   $\mu\text{M}$ ) DHA ( $0.199 \pm 0.004$ ,  $n = 21$ ,  $P < 0.01$ ), EPA ( $0.204 \pm 0.006$ ,  $n = 9$ ,  $P < 0.01$ ), or ALA ( $0.213 \pm 0.005$ ,  $n = 11$ ,  $P < 0.01$ ) compared with control ( $0.239 \pm 0.003$ ). These data indicate that cardiac myocyte membrane fluidity was increased following acute addition of DHA, EPA and ALA. In addition, the fluorescence anisotropy of TMAP-DPH was

significantly decreased to  $0.214 \pm 0.009$  (indicative of an increase in membrane fluidity) following addition of the membrane fluidising agent benzyl alcohol as shown in Fig. 6.

## DISCUSSION

Polyunsaturated fatty acids, particularly of the n-3 class, are known to exert an anti-arrhythmic effect in a variety of *in vitro* and *in vivo* preparations (Leifert *et al.* 1999). This may in part be a consequence of an alteration in the electrophysiology of the myocardium, since a reduction in excitability of the myocardium (either in the intact heart or in single cardiac myocytes) is the most often observed effect. While it has been shown that PUFAs can influence a range of ionic currents in cardiac myocytes, for example,  $\text{Ca}^{2+}$  current (Pepe *et al.* 1994) and transient outward  $\text{K}^+$  current (Bogdanov *et al.* 1998), one of the most powerful means of producing a reduction in cell excitability is by block of voltage-dependent sodium channels, which produces an increase in threshold for action potential generation. Indeed, voltage-dependent sodium channel blockade is the principal mechanism of action of class I anti-arrhythmic agents such as lidocaine (lignocaine).

This study shows that acute addition of micromolar concentrations of n-3 PUFAs to adult rat ventricular myocytes rapidly blocks the sodium current and shifts the voltage dependence of inactivation to more hyperpolarised potentials. In the presence of  $25$   $\mu\text{M}$  DHA, the most potent of the n-3 PUFAs tested, the maximum conductance was reduced by 50%, and the voltage dependence of inactivation shifted by about 20 mV. These effects were also apparent for the other n-3 PUFAs tested, EPA and to a lesser extent, ALA. The non n-3 fatty acids stearic, linoleic and oleic acid were without effect on most of the activation and inactivation



**Figure 6.** Effect of fatty acids on adult rat ventricular myocyte sarcolemmal membrane fluidity

Ventricular myocytes plated on coverslips were incubated for 15 min with  $1$   $\mu\text{M}$  TMAP-DPH at  $37$   $^{\circ}\text{C}$ . Cardiac myocytes were then transferred to a glass cuvette containing  $20$   $\mu\text{M}$  fatty acids and the steady-state fluorescence anisotropy of TMAP-DPH was measured (as described in Methods). Data are presented as means  $\pm$  s.e.m. \*\* $P < 0.01$  compared with control. Abbreviations: Cont, control (no additions); SA, stearic acid (18:0); OA, oleic acid (18:1, n-9); LA, linoleic acid (18:2, n-6); ALA,  $\alpha$ -linolenic acid (18:3, n-3); EPA, eicosapentaenoic acid (20:5, n-3); DHA, docosahexaenoic acid (22:6, n-3); B Alc, benzyl alcohol.

parameters associated with the sodium current. The fatty acids presumably exert their effects by dissolving in the cell membrane, since washing with control solution failed to reverse their effects, but perfusion with medium containing delipidated BSA did reverse the effects (presumably by removing membrane-associated PUFAs because of the high affinity of BSA for fatty acids).

The mechanism(s) by which n-3 PUFAs may effect cardiac sodium currents are unclear. The spectrum of changes produced by the n-3 PUFAs (a reduction in maximum current, a slight shift in the voltage dependence of activation, and a substantial shift in the voltage dependence of inactivation), is similar to that observed with certain general anaesthetic agents (Saint, 1998). It has been suggested that the effects of general anaesthetics on ion channel activity may be induced by alterations in membrane physical properties, particularly fluidity (Haydon & Urban, 1983), leading to the hypothesis that fatty acids may similarly affect sodium channels via a change in membrane fluidity. This effect seems plausible if, as has been suggested by others (Tan & Weaver, 1997), it is a change in the physical properties at the interface between the channel protein and the surrounding membrane lipid environment which is responsible for the change in channel properties. Consistent with this notion, the fatty acids increased cardiac myocyte membrane fluidity in this study, as measured by steady-state fluorescence anisotropy, in a similar rank order potency to their effect on the sodium current. The n-3 PUFAs were the most potent inhibitors of the sodium current while fatty acids of other classes were much less potent. Within the n-3 PUFAs examined, DHA was the most potent and ALA the least potent with regards to effects on both membrane fluidity and sodium current block. The results therefore suggest that the n-3 PUFAs induce a block of ventricular myocyte sodium currents which may in part be associated with changes in membrane lipid physical properties, since one would anticipate that protein molecules embedded in the membrane would be influenced by the properties of the membrane lipids which surround them (McMurchie, 1988; McMurchie *et al.* 1997; Tan & Weaver, 1997). This conclusion was supported by the data on the well known membrane fluidising agent benzyl alcohol (Staiman & Seeman, 1975; Sinicrope *et al.* 1992), a molecule unrelated in structure to fatty acids, which increased membrane fluidity and produced similar effects on the sodium currents. Our conclusion is also consistent with results which reported that changing membrane lipid content, and thus membrane fluidity, can modify the function of ion channels such as  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in smooth muscle cells (Bregestovski & Bolotina, 1989).

As noted above, the original impetus for work on the effects of fatty acids in the heart came from epidemiological and dietary studies. Dietary fatty acids are incorporated into the phospholipid structure of the membrane, rather than being free in solution, but nevertheless appear to exert an

anti-arrhythmic effect. A possible mechanism is that the PUFAs incorporated into the membrane phospholipids may provide a pool of n-3 PUFAs that are made available by the action of phospholipases activated in ischaemia or in other circumstances. It is known that the accumulation of free fatty acids which occurs early during myocardial ischaemia depends upon activation of phospholipase  $\text{A}_2$ , and it has been estimated that the free fatty acid concentration following their release from membrane phospholipids may result in a concentration of  $20 \mu\text{M}$  in the rat heart after 1 h of ischaemia (Van der Vusse *et al.* 1997). Hence, the concentrations of PUFAs used in this study ( $1\text{--}50 \mu\text{M}$ ) are similar to those found in the ischaemic myocardium, and the release of fatty acids from the membrane by phospholipases may explain the anti-arrhythmic effects of dietary PUFAs.

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