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Peroxidation of docosahexaenoic acid is responsible for its effects on I_{TO} and I_{SS} in rat ventricular myocytes

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1 Exposure to docosahexaenoïc acid (DHA), a long-chain polyunsaturated fatty acid, is known to block several ionic currents such as the transient outward current I_{TO}. It has also been reported to activate certain potassium channels. It has been suggested that these effects, observed in single-cell experiments, participate in the antiarrhythmic properties of these compounds *in vivo*.

2 DHA is highly prone to peroxidation. To investigate the influence peroxidation may have on the effects of DHA on ion channels, we studied I_{TO} and the steady-state outward current I_{SS} in isolated rat ventricular myocytes under ruptured whole-cell patch-clamp conditions.

3 A measure of 10 μM DHA alone reduced I_{TO}, evoked by a pulse to +70 mV, by 74.8±10.8% ($n=7$) and activated a delayed outward current with kinetic properties different from I_{SS}.

4 When an antioxidant, alpha-tocopherol (1 μM), was added together with DHA, the blockade of I_{TO} was reduced to 38.5±7.7% ($n=8$) and the delayed outward current was not activated. α-Tocopherol alone had no effect on these currents.

5 When an oxidant, hydrogen peroxide (1 μM), was applied together with DHA, the blockade of I_{TO} was almost complete (98.4±1.0%, $n=7$) and a large delayed outward current was activated. A measure of 1 μM hydrogen peroxide alone had no effect on these currents.

6 Measurements of nonperoxidized DHA in experimental solutions confirmed the negative relation between DHA concentration and the effects on the currents.

7 We conclude that rather than DHA itself, it is the peroxidation products of DHA that block I_{TO} and activate a delayed outward current in *in vitro* single-cell experiments. These findings have important implications for the extrapolation of *in vitro* experimental findings to the antiarrhythmic effects of DHA *in vivo* because, *in vivo*, peroxidation of DHA is unlikely to occur.
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Abbreviations: DHA, docosahexaenoic acid; I_{CaL}, L-type calcium current; I_{DHA}, ionic current activated by DHA; $I_{K,AA}$, potassium current activated by arachidonic acid; I_{Na}, fast sodium current; I_{SS}, steady-state current; I_{TO}, transient outward current; PUFA, polyunsaturated fatty acid

Introduction

It is known that n-3 long-chain polyunsaturated fatty acids (PUFAs) have cardioprotective effects (Gulbjarnason & Hallgrimson, 1975; Murnaghan, 1981). In animal studies, it has been shown that dietary PUFAs decrease ventricular fibrillation in rats (McLennan, 1993) and increase ventricular fibrillation threshold in marmosets (McLennan *et al*., 1993). Billman *et al*. (1994, 1999) reported that fatal ventricular arrhythmias induced by coronary ligation could be prevented by a fish oil emulsion containing primarily a mixture of eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA) infused intravenously just prior to the ischaemic stress in conscious exercising dogs. Studies using isolated neonatal rat cardiac myocytes have confirmed a putative antiarrhythmic effect of acutely applied PUFAs (Kang & Leaf, 1994, 1995, 1996). The mechanisms of the antiarrhythmic actions of n-3 PUFAs are not well understood. To explain these effects, two hypotheses have been put forward: a direct effect of n-3 PUFAs on ion channels or a modification of the membrane phospholipid composition, primarily the inositol phosphate fraction, which in turn is responsible for changes in cellular homeostasis. Cardiac myocytes isolated from fish oil-fed pigs have higher levels of n-3 PUFAs in the phospholipid fraction (Nair *et al*., 2000). After α1-adrenergic stimulation, the levels of inositol triphosphate (IP_3) were significantly reduced compared to myocytes isolated from beef tallow-fed pigs. These reduced levels of IP_3 may be responsible for the reduced arrhythmias (Anderson *et al*., 1996). Conversely, Pound *et al*. (2001) suggest it is unlikely that n-3 PUFAs affect the packing of phospholipids within cell membranes. In isolated cardiac myocytes, it has been reported that acutely applied n-3 PUFAs are able to modulate ionic channels activity. n-3 PUFAs induced an inhibition of voltage-gated Na^+ current (Xiao *et al*., 1995; Kang & Leaf, 1996; Leifert *et al*., 1999). It has also been

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demonstrated that n-3 PUFAs suppressed Na^+ current in HEK293t cells transfected with the subunit of the human cardiac Na^+ channel (Xiao *et al*., 1998, 2001). Xiao *et al*. (1997) showed an inhibition of the L-type Ca^{2+} current by n-3 PUFAs. Potassium channels are also sensitive to n-3 PUFAs, the repolarizing transient outward (I_{TO}) and delayed rectifier (I_K) potassium currents of rat ventricular cardiac cells, are inhibited, whereas the inward rectifier (I_{K1}) channel is unaffected (Pound *et al*., 2001). All these authors believe that the antiarrhythmic effects of n-3 PUFAs are associated with the blockade of these channels.

Thus, the effects of n-3 PUFAs on cardiac ion channels have important clinical relevance. However, these compounds are very sensitive to oxidative compounds that can change their pharmacological properties. It is known that the beneficial preventive effects of DHA on breast cancer occurrence and development can be modulated by the presence of a pro- or antioxidant in the diet (Cognault *et al*., 2000). Since DHA is also reported to be cardioprotective, it is of importance to determine which form of n-3 PUFAs is involved in the blockade of ion channels. This knowledge might help evaluate the involvement of ion channels in the cardioprotective effects of n-3 PUFAs. In this study, we acutely applied DHA to isolated rat ventricular myocytes without or with α-tocopherol (Vitamin E, to prevent peroxidation) or hydrogen peroxide (to enhance peroxidation) and evaluated the effects on I_{TO} and I_{SS} in order to test the hypothesis that actions attributed to n-3 PUFAs on cardiac K^+ channels *in vitro* may in fact be related to their products of peroxidation.

Methods

Cell isolation

Male Wistar rats (250–300 g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}), which was injected together with heparin (600 U/100 g^{-1}). The heart was removed, washed in a calcium-free solution and perfused for 5 min on a Langendorff apparatus with the same calcium-free solution warmed to 37°C (composition in mM: NaCl 117, KCl 5.7, $NaHCO_3$ 4.4, KH_2PO_4 1.5, $MgCl_2$ 1.7, HEPES 21, Glucose 11.1, Taurine 20; pH adjusted to 7.15 with NaOH). The heart was then perfused with collagenase-containing solution (collagenase type IV, 1.5 mg ml^{-1}, Worthington) supplemented with $CaCl_2$ (20 μM). After approximately 1 h, the ventricles were removed, placed in fresh enzyme solution and triturated to dissociate the myocytes. Single ventricular myocytes were collected in a low calcium solution containing 0.1% BSA. Cells were stored at 22–24°C.

Electrophysiological studies

Myocytes were transferred to a 1.5 ml perspex chamber containing a Tyrode solution placed on the stage of an inverted microscope (Nikon, Eclipse TE 300). The chamber was continuously perfused at a rate of 1–2 ml min^{-1} with a Tyrode solution (composition in mM: NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES 10, glucose 11.1; pH adjusted to 7.4 with NaOH).

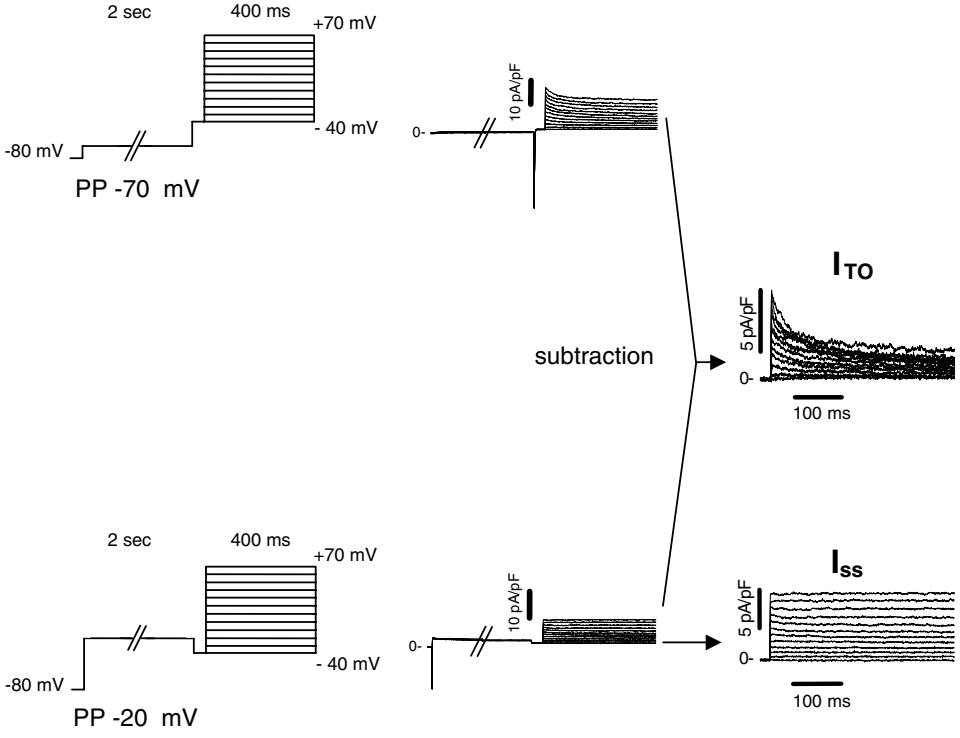

Figure 1 Schematic representation of the voltage clamp design used to study I_{TO} and I_{SS}. Holding potential was set at −80 mV. After a 2 s prepulse to −70 mV (left upper, to evoke I_{TO} and I_{SS}) or to −20 mV (left lower, to evoke I_{SS}), a 40 ms depolarization to −40 mV was used to inactivate I_{Na}. $I-V$ curves were constructed from depolarizing pulses from −40 to +70 mV in 10 mV steps. I_{TO} was obtained by subtracting currents after a prepulse to −20 mV (I_{SS}, lower right) from those after a prepulse to −70 mV (upper right).

Electrodes were prepared from borosilicate glass (Clark Instruments, GC150TF-15, U.K.) using a two-stage puller (Narishige, PC-10, Japan) with resistances typically between 2 and 4 MΩ when filled with a solution containing (in mM) K-aspartate 110, KCl 15, Mg-ATP 5, HEPES 11, EGTA 10; pH adjusted to 7.1 with KOH. Whole-cell voltage-clamp experiments were performed with an Axopatch 200B amplifier (Axon Instruments, La Jolla, U.S.A.). Whole-cell capacitance and series resistance were compensated by 60%. Experiments were performed at 22–24°C.

To measure I_{TO} and I_{SS}, cells were locally superfused via a solution switching device with a Tyrode solution containing 10 μM nifedipine and 1 mM $BaCl_2$ to block, respectively, I_{CaL} and I_{K1}. Holding potential was set at −80 mV. Current–voltage relation (range −40 to +70 mV) were measured with 400 ms clamp steps in 10 mV increments every 10 s that followed a 40 ms clamp step to −40 mV to inactivate I_{Na}. To distinguish I_{TO} from I_{SS}, we used a protocol designed by Himmel *et al.* (1999), 2 s conditioning clamp steps to −70 and −20 mV were used. Both I_{TO} and I_{SS} are activated after a conditioning step to −70 mV, but I_{TO} is inactivated after a conditioning step to −20 mV. Thus, digital subtraction of the two sets of current tracings results in the isolation of currents mainly due to I_{TO}, currents activated from a membrane potential of −20 mV reflecting mainly the activation of I_{SS} (see Figure 1). I_{TO} was taken as the difference between the peak current and the end pulse current, which predominantly represents the fast component of I_{TO}.

Fatty acids solutions

DHA (sodium salt) and α-tocopherol solutions were prepared under nitrogen. DHA was dissolved in ethanol at a concentration of 10 mM and stored under nitrogen at −20°C. α-Tocopherol was dissolved in chloroform at a concentration of 1 mM and stored in the same conditions. The experimental concentration of DHA (10 μM) was obtained by dilution of the stocks in Tyrode solution containing 10 μM nifedipine and 1 mM $BaCl_2$. Either 1 μM α-tocopherol (to prevent peroxidation, Burton & Ingold, 1989) or 1 μM hydrogen peroxide (to enhance peroxidation, Janero *et al.*, 1991) was added as required before adding DHA. Fresh solutions were made every 4 h. All the products were obtained from Sigma (Grenoble, France).

Quantification of DHA in solutions

Unperoxidized DHA concentrations in Tyrode solutions were determined using gas chromatography. To extract lipids from the Tyrode solution, we used a method described by Bligh & Dyer (1959). Lipids were extracted by mixing 1.6 ml experimental solution with 2 ml chloroform, 4 ml methanol and 1.6 ml distilled water. After centrifugation, the supernatant, containing lipids, was mixed with chloroform and water (1 : 1, vol vol^{-1}). It was then centrifuged and the phase containing lipids was filtered on glass wool and sodium sulphate. The solvent was evaporated. Lipids were taken at a concentration of 10 mg ml^{-1} with chloroform and transmethylated in methanol using boron fluoride as a catalyst. The analysis by gas chromatography was performed on a GC Trace apparatus (Thermofinnigan, France) equipped with an on-column injector, a flame ionization detector and a polar capillary column BPCX70 (50 m × 0.32 mm, SGE, France). Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a commercially available mixture of fatty methyl ester (Supelco component fame).

Statistics

Data are expressed as means ± s.e. of n observations. Statistical analysis was performed using a two-way repeated measures ANOVA followed by a Student–Newman–Keuls test when appropriate. In Figure 5, we performed a one-way ANOVA followed by a Dunn's test. A $P<0.05$ was considered significant.

Results

Effects of DHA alone on I_{TO} *and* I_{SS}

Since DHA has been shown to block I_{TO} (Pound *et al.*, 2001) and activate $I_{K,AA}$ (Honoré *et al.*, 1994) at micromolar concentrations, we applied this fatty acid at a concentration of 10 μM and tested its effects on I_{TO} and on remaining currents

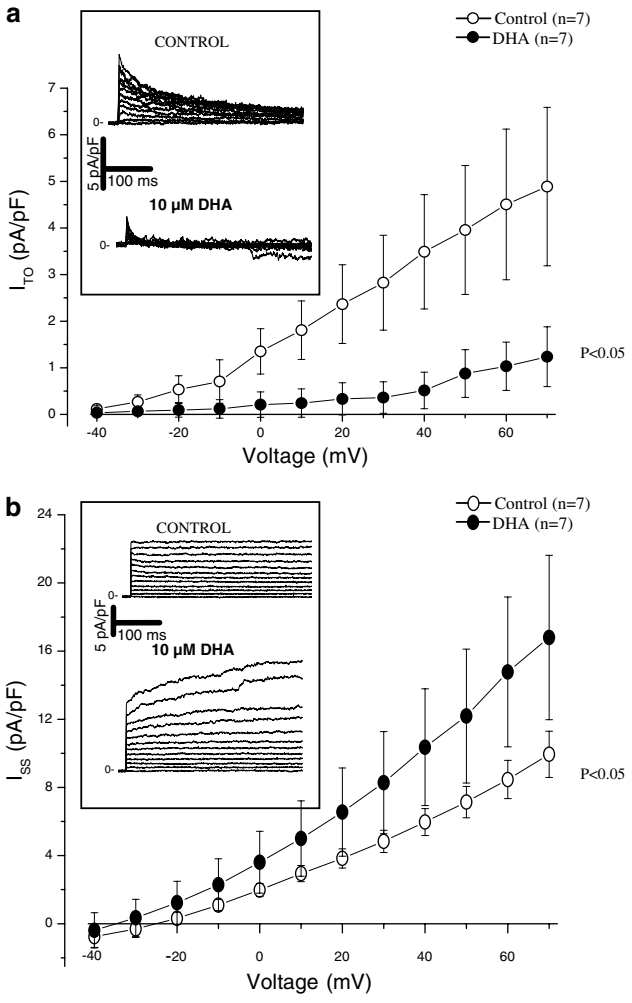

Figure 2 Effects of DHA alone on I_{TO} and I_{SS}. (a), Superfusing cells with a Tyrode solution containing 10 μM DHA induced a significant reduction of I_{TO} at all voltages compared to control Tyrode conditions ($P<0.05$, $n=7$). Inset shows a typical example of the effect of DHA on I_{TO}. (b) While in Tyrode conditions, I_{SS} was the only visible current, 10 μM DHA induced the appearance of a large delayed outwardly rectifying current after a prepulse to −20 mV, I_{DHA} ($P<0.05$, $n=7$). An example of the DHA-activated current is shown in the inset.

after a −20 mV conditioning pulse. In control conditions, the remaining currents are mainly composed of I_{SS} (Himmel *et al*., 1999). The application of DHA lasted 10 min. In accordance with previous observations using such PUFAs on cardiac cells (Kang & Leaf, 1994, 1995, 1996), we observed that the effects appeared after 2–3 min of application and measurements were performed between the 7th to 10th min of application when an apparent steady state was reached.

As shown in Figure 2a, 10 μM DHA induced a large, but incomplete, blockade of I_{TO} (amplitude reduced by 74.8±10.8%, at +70 mV, $n=7$). A current was also activated at all voltages, after a 2 s conditioning pulse to −20 mV (Figure 2b). This conditioning pulse was used to inactivate K^+ currents other than I_{SS}; however, the DHA-activated current is unlikely to be I_{SS} since it activates more slowly than I_{SS}. Thus, we call the current activated in the presence of DHA, I_{DHA}. These effects were weakly reversible, with never more than 20% of recovery after 20 min of washout (data not shown).

Effects of DHA on I_{TO} *and* I_{SS} *in the presence of an antioxidant*

To test the possibility that peroxidation of DHA in solution might influence its effects, we performed the same experiments in the presence of the antioxidant 1 μM α-tocopherol (TOCO). At this concentration, TOCO alone had no effect on I_{TO} (Figure 3a) and I_{SS} (Figure 3b). However, we observed that in the presence of TOCO, the effects of 10 μM DHA on both I_{TO} and I_{DHA} were reduced (Figures 3c and d).

Effects of DHA on I_{TO} *and* I_{SS} *in the presence of an oxidant*

A measure of 1 μM H_2O_2, an oxidizing agent, had no effect on I_{TO} and I_{SS} when applied by itself (Figures 4a and b) but potentiated the effects of DHA on I_{TO} (Figure 4c) and on I_{DHA} (Figure 4d).

Negative relation between unperoxidized DHA and the effects on I_{TO} *and* I_{SS}

Thus, it seems that the effects of DHA on the membrane currents under investigation depended strongly on its peroxidation state. The effects were significantly ($P<0.05$) enhanced when peroxidation was promoted but significantly ($P<0.05$) attenuated by preventing peroxidation (Figures 5a and b). To confirm this analysis, we measured the nonperoxidized form of DHA in the different experimental conditions: 10 μM DHA

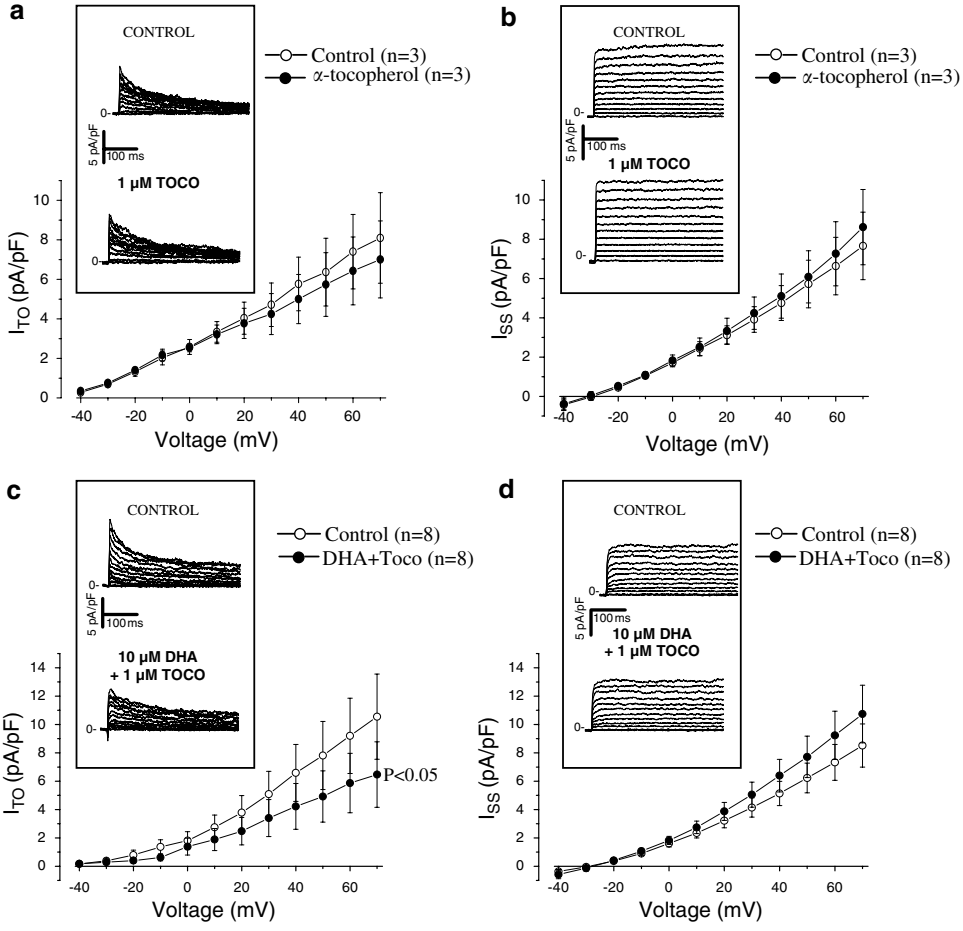

Figure 3 Effects of 1 μM α-tocopherol on I_{TO} and I_{SS} in the presence or absence of 10 μM DHA. (a, b) 1 μM α-tocopherol has no effect on I_{TO} (a) or I_{SS} (b) at any voltage ($P>0.05$, $n=3$) when compared to control conditions. (c) In presence of 1 μM α-tocopherol, 10 μM DHA has still a blocking effect on I_{TO} ($P<0.05$, $n=8$) compared to control conditions but this is less than that seen in the absence of 1 μM α-tocopherol (see Figure 5). (d) In the presence of 1 μM α-tocopherol, the effect of 10 μM DHA on I_{SS} was not significant ($P>0.05$, $n=8$, compared to control conditions).

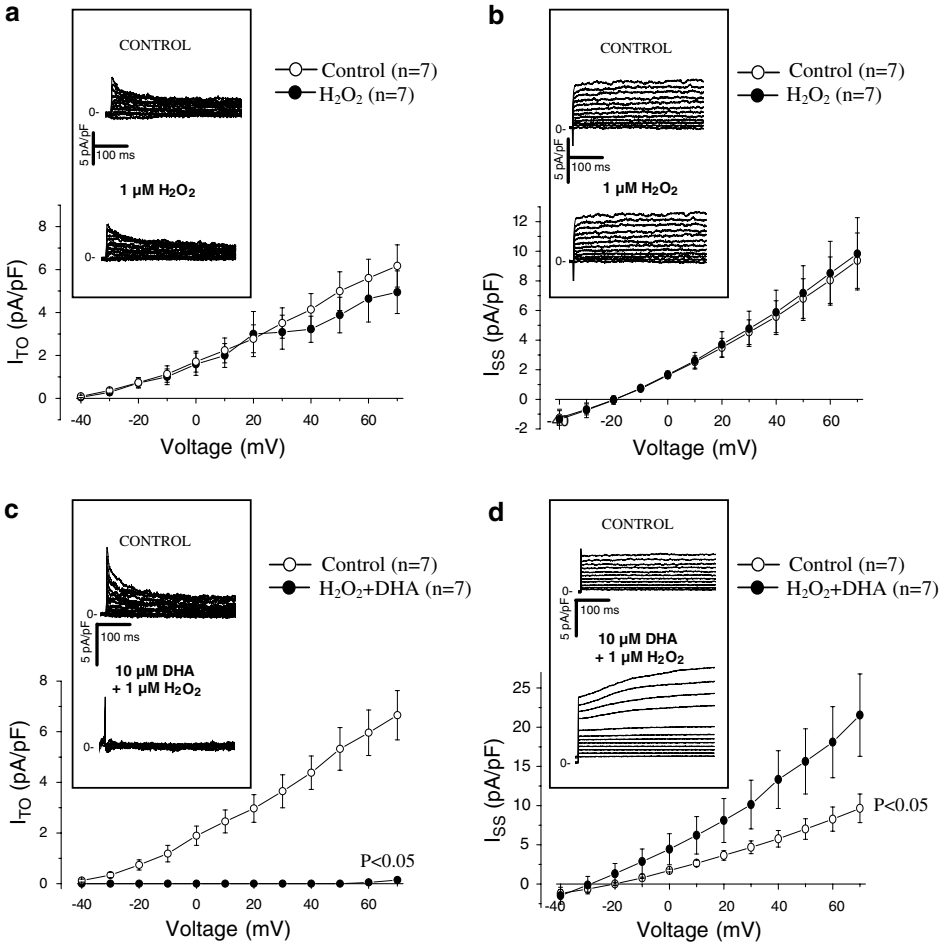

Figure 4 Effects of 1 μM hydrogen peroxide on I_{TO} and I_{SS} in the presence or absence of 10 μM DHA. (a, b) 1 μM hydrogen peroxide has no effect on I_{TO} (a) or I_{SS} (b) at any voltage ($P>0.05$, $n=7$) when compared to control conditions. (c) In the presence of 1 μM hydrogen peroxide, 10 μM DHA fully blocks I_{TO} ($P<0.05$, $n=7$) compared to control conditions. (d) When 1 μM hydrogen peroxide was present, 10 μM DHA induced an increase of I_{SS} ($P<0.05$, $n=7$) when compared to control conditions that was greater than that seen in the absence of hydrogen peroxide (see Figure 5).

alone, with 1 μM α-tocopherol or with 1 μM H_2O_2. As shown in Figure 5c, 1 μM α-tocopherol prevents partially the peroxidation of DHA that occurs when the fatty acid is added alone to the Tyrode solution. A measure of 1 μM H_2O_2 enhances the peroxidation as indicated by the reduced concentration of nonperoxidized DHA. It appears from these measurements that the effects on the currents, I_{TO} and I_{DHA}, are negatively related to the concentration of DHA and that these effects are correlated with the degree of peroxidation of DHA.

Discussion

Our main finding is that the well-known effects of DHA on I_{TO} and I_{SS} are mainly due to a peroxidized product of the PUFA. We chose to focus our work on I_{TO} and I_{SS} since these currents can be studied simultaneously and are differentially affected. The choice of the concentration of DHA (10 μM) is based on the reported EC_{50} for the effects of PUFAs on ion channels that ranges between 1 μM (Xiao *et al*., 1998) and 30 μM (Honoré *et al*., 1994). This concentration was also used to show antiarrhythmic properties of this fatty acid in single cell (Kang & Leaf, 1994, 1995, 1996).

Wallert *et al*. (1991) showed that long-chain fatty acids can activate outward potassium channels. These channels belong to a family of channels (TWIK) that possess two pore-forming regions and four transmembrane domains (Lesage *et al*., 1996). In rat ventricular cells, such a current called I_{TREK-1} has been described (Aimond *et al*., 2000). It is possible that the DHA-sensitive outward current we observed is the same current. Activation of such a current is potentially antiarrhythmogenic since it would stabilize the resting membrane potential and reduce the entry of Ca^{2+} into the cells during the action potential.

α-Tocopherol is a fatty acid that can insert into the membrane and thus can modify channel properties. The concentration (1 μM) of α-tocopherol was therefore chosen to avoid direct effects on the currents, although this may not completely prevent oxidation of DHA. H_2O_2 affects potassium currents at concentrations around 10 μM (Bérubé *et al*., 2001). At 1 μM, H_2O_2 has no effect by itself on the currents but is able to peroxidize sensitive molecules like long-chain PUFAs.

From the present study, it seems that it is a peroxidation product of DHA that affects the properties of the channels we studied. This might explain the observation that blockade of I_{Na} by DHA is not observed when an ethyl ester form of the

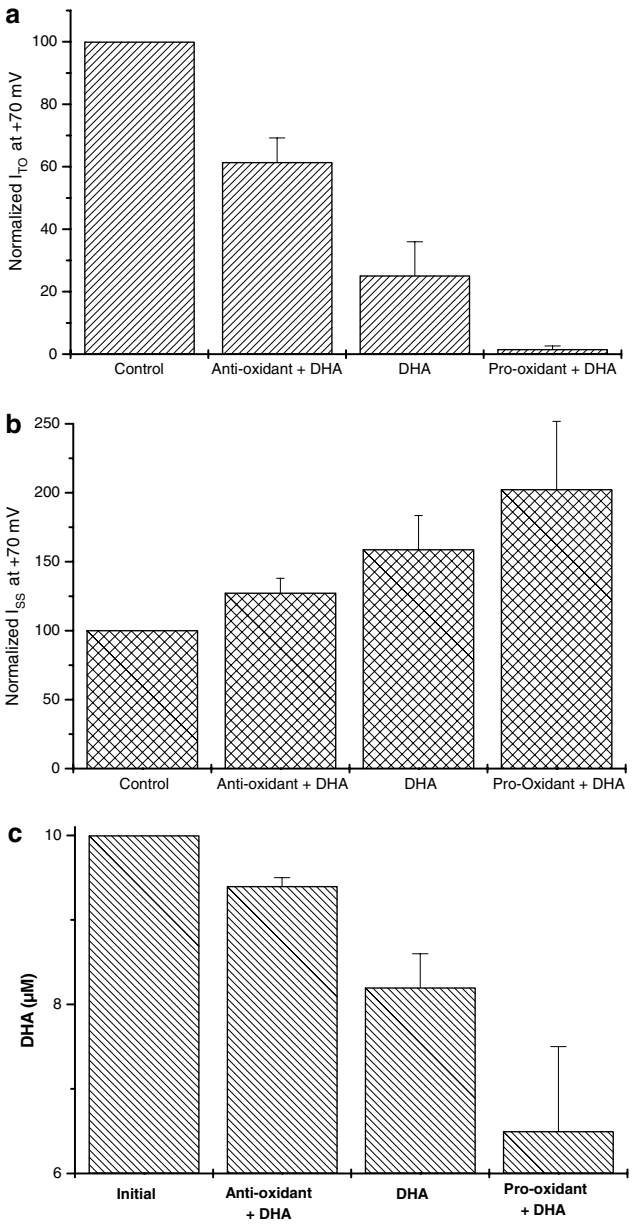

Figure 5 Comparison of the effects of 10 μM DHA alone, in presence of 1 μM H_2O_2 or of 1 μM α-tocopherol on I_{TO} (a) and I_{SS} (b), *n* indicates the number of cells studied in the different conditions. Increasing the oxidative status of the Tyrode is associated with a larger effect of DHA on both currents. All the means are significantly different from each other ($P<0.05$). (c) Concentrations of unperoxidized DHA in the different experimental conditions. Initial: gives the initial concentration of DHA added in the solution, 10 μM; antioxidant + DHA: 1 μM α-tocopherol + 10 μM DHA; DHA: 10 μM alone; pro-oxidant + DHA: 1 μM hydrogen peroxide + 10 μM DHA. The concentrations were determined twice in each of two different sets of solutions.

fatty acid is used instead of a free acid form (Xiao *et al*., 1995). These results were interpreted in terms of a required free carboxyl group to block the channel. However, ethyl ester forms of PUFAs are much less sensitive to peroxidation than free acid forms (Chacon *et al*., 2000). Thus, it is possible that decreased peroxidation is responsible for the lower blockade of I_{Na} when using ethyl ester PUFAs. At present, we cannot say whether it is a specific product of DHA peroxidation that has direct effects on ion channels or if such products destabilize the membrane and the activity of proteins embedded in. The aim of the present study was not to identify the oxidized product of DHA and its mechanism of action but rather to test whether the oxidation state of DHA could affect ionic channel activity. Presently, measurement of lipid peroxidation products is still a matter of debate (Moore & Roberts, 1998). It is known that DHA peroxidation products such as F_4-isoprostane are very different from the precursor, DHA, and thus might have different pharmacological properties (Roberts *et al*., 1998). Also, these compounds can change membrane fluidity (Greco *et al*., 2000). It has been proposed that the blockade of I_{Na} by n-3 PUFA was related to their ability to change the membrane fluidity (Leifert *et al*., 1999). It is thus possible that peroxidation products of DHA change the membrane fluidity and thereby modify the activity of ion channels.

In this study and in others that investigate the effects of PUFAs on ion channels, great care is taken to prepare the PUFAs solution under a nitrogen atmosphere because of its susceptibility to peroxidization. However, when added to experimental solutions the oxidative status of the PUFA is often not controlled. We suggest that at this stage of procedures peroxidation can occur (see Figure 5c). If an antioxidant, like α-tocopherol, is present in the solution before addition of PUFAs, less peroxidation will occur, as indicated by the higher concentration of DHA we measured. Conversely, hydrogen peroxide increased peroxidation.

In conclusion, we show that exposure to DHA results in blockade of I_{TO} and activation of a delayed outward current in rat ventricular myocytes. It seems likely that effects on K^+ channels (and possibly other classes of channels) classically attributed to DHA are more likely due to product(s) of its peroxidation rather than to DHA itself.

Our observations are important since circulating PUFAs, *in vivo*, are unlikely to be peroxidized when absorbed through the diet due to high levels of antioxidant in the circulating system (Abadie *et al*., 1993). Thus, it would appear to be unperoxidized DHA that has antiarrhythmic effects (as suggested by the GISSI-Prevenzione trial, Trial, 1999) and that *in vivo*, the beneficial effects of DHA do not involve direct effect on ionic currents such as I_{TO} and I_{SS}. It is possible that the antiarrhythmic effects of DHA might occur through changes in the metabolism of phosphoinositides (PI) as proposed by Anderson *et al*. (1996) and Nair *et al*. (2000). Indeed, it is known that α1-adrenergic stimulation is important in post-infarction arrhythmias (Anderson *et al*., 1995) and that this stimulation involves the PI pathway. The oxidized state of DHA must be established in animal experiments that study the antiarrhythmic properties of DHA if a correct mechanistic interpretation is to be made and *in vitro* findings extrapolated to the actions of these agents *in vivo*.

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