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Inhibition of gap junctional communication by polyunsaturated fatty acids in WB cells: evidence that connexin 43 is not hyperphosphorylated

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Polyunsaturated fatty acids have attracted much interest due to their wide spectrum of biological activities which include the modulation of gap junctional communication (GJC). Since gap junctions play critical roles in maintaining the functional integrity of organs and tissues, and loss of intercellular communication is associated with a number of pathological conditions, we investigated the effects of the n – 6 and n – 3 series of polyunsaturated fatty acids and their derivatives on GJC in WB cells as determined by the ability of Lucifer Yellow-loaded cells to transfer the dye to neighbouring recipient cells. Studies were also conducted to investigate the possible mechanisms of action of the fatty acids. Treatment of cells with 10 μM arachidonic acid (20:4 n – 6) resulted in a rapid and transient loss of communication competence. The response to 20 μM 20:4 (n – 6) was prolonged (>210 min) but was readily reversible by washing the cells with fatty acid-free bovine serum albumin. Cells which had regained their communication competence responded to further additions of 20:4 (n – 6). The fatty acids, 18:3 (n – 6), 20:5 (n – 3), 22:6 (n – 3) and the 15-hydroxy- and the 15-hydroperoxy-derivatives of 20:4 (n – 6) were also powerful inhibitors of GJC, while 23:4 (n – 6) was a relatively weak inhibitor. The saturated 20 carbon fatty acid, 20:0, and the methyl ester of 20:4 (n – 6) were without effect. This illustrates the importance of unsaturation and the carboxyl group as structural requirements for activity. 20:4 (n – 6)-induced inhibition of dye transfer was not attenuated by pretreating the cells with either phorbol-12-myristate-13-acetate (PMA) or indomethacin, suggesting that regulation of gap junctional permeability by 20:4 (n – 6) in WB cells was neither dependent on PMA-responsive isozymes of protein kinase C nor required the metabolism of the fatty acids by cyclo-oxygenase. However, the effect of 20:4 (n – 6) was antagonized by preincubating WB cells with either nordihydroguaiaretic acid or (±)-isoproterenol and isobutylmethylxanthine. Western blot analysis of connexin 43 (Cx43), the major gap junctional protein expressed in these cells, revealed no detectable changes to the electrophoretic mobility of Cx43 even after 60 min of incubation in the presence of 20:4 (n – 6). As expected, other inhibitors of gap junctional permeability including epidermal growth factor, phorbol ester or lysophosphatidic acid induced a retardation in the mobility of Cx43, indicating an enhancement in the phosphorylation of Cx43 protein. The data indicate that inhibition of GJC by 20:4 (n – 6) has a novel mechanism which does not require phosphorylation of Cx43 but may require its metabolism to eicosanoids. In addition the inhibitory effect of 20:4 (n – 6) can be modulated by an increase in intracellular cAMP concentration which has been reported to enhance cell–cell communication. The data also argue against a non-specific detergent action of the fatty acids.

Introduction

Polyunsaturated fatty acids (PUFAs*) exert a wide range of effects on cells of diverse origins. Various PUFAs have been shown to stimulate secretion (1,2), stimulate the neutrophil oxidative respiratory burst (3,4), alter ion channel permeability (5), affect components of cell signalling (6,7) and inhibit cell migration (8). In addition unsaturated fatty acids such as 16:1 (n – 9), 18:1 (n – 9), 18:3 (n – 3/n – 6) and 20:4 (n – 6) have been reported to be strong inhibitors of cell–cell communication via gap junctions (9).

Gap junctions are aggregates of transmembranous channels between adjacent cells which allow the passage of low molecular weight substances (<1.5 kDa) from one cell to neighbouring cells. The junctions or connexons are made up of a family of connexin molecules arranged as hexamers (10). Gap junctions are found in almost all tissues of vertebrates and they play crucial roles in developmental processes, proliferation and electrical and metabolic coupling in various organs. Loss of gap junctional communication (GJC) has been associated with a number of pathological conditions including cancer (11). While a number of physical mechanisms may exist to account for inhibition of GJC, it has been demonstrated that inhibition of GJC by phorbol-12-myristate-13-acetate (PMA), epidermal growth factor (EGF), lysophosphatidic acid (LPA) and pp60^{src} transformation is tightly linked to the phosphorylation of gap junctional proteins (11–14). Tyrosine kinase and protein kinase C (PKC) pathways, possibly converging on the mitogen-activated protein kinase (MAPK) cascade, have been implicated in the phosphorylation of connexin 43 (Cx43) (14,15) which is found in cardiac and other tissues including WB cells (10). Purified MAPK has been reported to phosphorylate recombinant Cx43 *in vitro* (15).

Although a number of reports have now described the inhibition of GJC by unsaturated fatty acids (C < 20) in cells of different origin (9,16), neither the role of fatty acid structure nor the mechanism of the fatty acid-induced effects have been given much attention. We were particularly interested to know whether (i) the long chain fatty acids of the n – 3 series, 20:5 and 22:6, affected GJC since these fatty acids are metabolized to products which are different from those obtained from 20:4

*Abbreviations: PUFA, polyunsaturated fatty acid; GJC, gap junctional communication; PMA, phorbol-12-myristate-13-acetate; EGF, epidermal growth factor; LPA, lysophosphatidic acid; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; Cx43, connexin 43; IBMX, isobutylmethylxanthine; FA-free BSA, fatty acid-free bovine serum albumin; NDGA, nordihydroguaiaretic acid.

(n – 6). These long-chain n – 3 series of PUFAs have been used in attempts to treat a range of human diseases through diets rich in these fatty acids such as fish oil (17,18 and refs therein); (ii) changes to the PUFA structure of 20:4 (n – 6) such as the introduction of a hydroxy or a hydroperoxy moiety, the conversion of the carboxyl to a methyl ester moeity or the degree of unsaturation affected activity; and (iii) phosphorylation of Cx43 was associated with the fatty acid-induced inhibition of GJC.

Materials and methods

Fatty acids, isobutylmethylxanthine (IBMX), (±)-isoproterenol and Lucifer Yellow were obtained from Sigma, Australia. The methyl ester derivative of arachidonic acid (20:4, n – 6) was prepared by treatment of 20:4 (n – 6) with ethereal diazomethane (8). 15-(*S*)-Hydroperoxy 20:4 (n – 6) was prepared by incubating 20:4 (n – 6) with soyabean lipoxidase (8). Hydroxy 20:4 (n – 6) was prepared by reduction of hydroperoxy 20:4 (n – 6) with sodium borohydride (8). The fatty acids and derivatives were stored in chloroform at –20°C. Aliquots were dried under nitrogen and resuspended in absolute ethanol prior to use. All fatty acids were of >95% purity as determined by TLC and GLC.

Cell culture and incubation

WB F344, a rat liver epithelial cell line with the phenotypic properties of 'oval' cells was obtained from Dr J.W.Grisham (19). Cells were cultured as described (20) and were used 1 day post-confluency. To pretreat with PMA, WB cells were cultured for 26 h in the presence of 300 nM PMA, the last 2 h in the absence of serum. The fatty acids, dissolved in ethanol, were dispersed into the incubation medium by sonication (3×3 s). Control cells received vehicle (0.1% v/v) which did not affect GJC.

Measurement of cell–cell communication

GJC was measured as described previously (14). Briefly, cells were incubated for various lengths of time with the fatty acids, washed with Ca^{2+}- and Mg^{2+}-free PBS (3×2 ml), and incubated with PBS containing Lucifer Yellow. The dye was loaded into cells by rolling a sharp scalpel blade over the cells. After 3 min the cells were washed with PBS (3×2 ml) to remove excess dye and the extent of dye transfer to secondary recipient cells was examined under a Nikon epifluorescence microscope. Fluorescent cells in 4–8 fields were scored and results were expressed as a percentage of the number of control cells which contained detectable fluorescence.

Wash-out of 20:4 (n – 6) with fatty acid-free bovine serum albumin

Cells were treated with the fatty acid for 10 min, the medium removed and the cells were washed (4×2 ml, ~2 min) with PBS containing fatty acid-free bovine serum albumin (1% w/v, FA-free BSA). The cells were then incubated in serum-free medium containing FA-free BSA for the times indicated before assessing the extent of dye transfer.

Immunoblotting

To detect phosphorylation of Cx43, cells in four 10 cm dishes were treated with arachidonic acid, EGF or LPA, alkali-extracted, pooled, and gap junctional-enriched pellets were prepared for Western blotting as described (20). The affinity-purified antibody to amino acids 314–322 of rat heart Cx43 was raised in our laboratory as described (21). Detection of immunoreactive bands was by ECL (Amersham).

Results

As shown in Figure 1 (inset), 20:4 (n – 6) (20 µM) caused a rapid (<1 min) inhibition of dye transfer to recipient cells. Maximal inhibition was apparent after 5–10 min of treatment and this was maintained for at least 210 min (Figure 1). However, inhibition of dye transfer by a lower concentration of 20:4 (n – 6) (10 µM) was transient and the cells rapidly regained communication competence (Figure 2). Readdition of more fatty acid resulted in a second inhibition of dye transfer, which implies that the recovery of communication competence in the presence of 10 µM 20:4 (n – 6) was due to clearance of the fatty acid by metabolism rather than desensitization to 20:4 (n – 6). This is in clear contrast to the effects of EGF, PMA or LPA as cells which had regained communication competence following incubation with one of

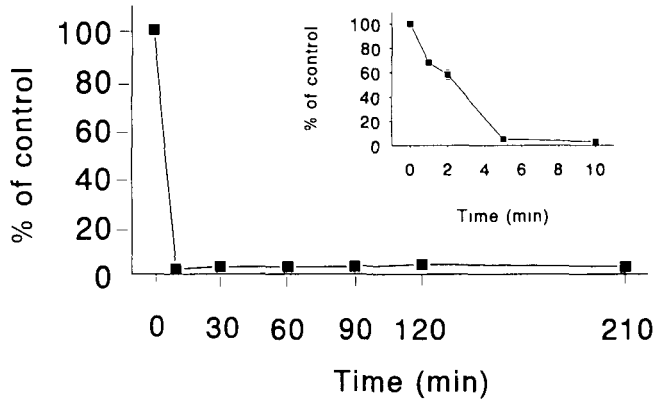

Fig. 1. Inhibition of dye transfer by 20 µM 20:4 (n – 6). WB cells were incubated with 20 µM 20:4 (n – 6) for the times indicated. Cells were washed and the extent of dye transfer was assessed as described in Materials and methods. Results are expressed as percentages of control cells as described in Materials and methods. Data are mean ± SEM of six determinations. Data are representative of two experiments.

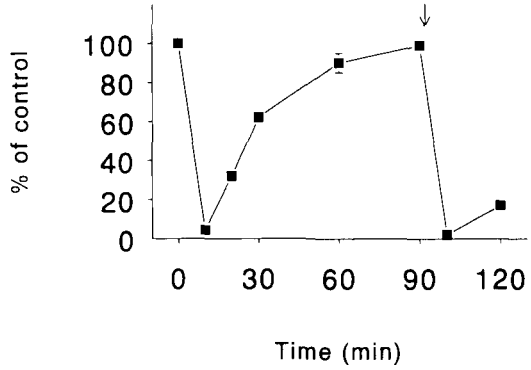

Fig. 2. Inhibition of dye transfer by 10 µM 20:4 (n – 6). Cells were treated and dye transfer was assessed as described in the legend to Figure 1. Arrow indicates the addition of more 20:4 (n – 6) (10 µM). Similar data were obtained from one repeat experiment.

these compounds were unresponsive to a subsequent exposure to the same compound but remained responsive to the other two (14), suggestive of homologous desensitization. When the cells were treated with 20 µM 20:4 (n – 6), removal of the fatty acid by washing with FA-free BSA resulted in a rapid recovery of communication competence (Figure 3). FA-free BSA itself did not affect dye transfer (not shown). Following recovery, communication was rapidly inhibited by a further addition of fatty acid (Figures 2 and 3). A dose–response curve for the inhibition of communication by 20:4 (n – 6) is shown in Figure 4. Inhibition of GJC was clearly detectable at a concentration of 5 µM.

The structure–function relationships of the fatty acids were investigated (Figure 5). These studies revealed that a saturated fatty acid, arachidic acid (20:0), and the methyl ester of 20:4 (n – 6) were without effect. This indicates that unsaturation and the presence of a free carboxyl group are necessary for inhibition of communication. In addition, the 15-hydroxy and 15-hydroperoxy derivatives of 20:4 (n – 6) and the n – 3 series of long-chain PUFAs are also powerful inhibitors of GJC (Figure 5). Increasing the chain length of 20:4 (n – 6) by three carbon atoms to 23:4 (n – 6) resulted in a dramatic loss of biological activity as this compound was only a weak inhibitor of GJC (Figure 5). Thus the order of effectiveness at inhibiting GJC is: 22:6 (n – 3) = 20:4 (n – 6) > 20:5 (n – 3) > 18:3

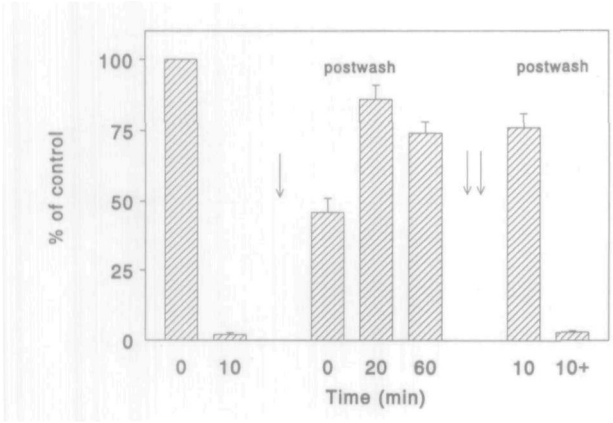

Fig. 3. Effects of the removal of 20:4 (n – 6) by FA-free BSA on dye transfer. Cells were incubated with 20:4 (n – 6) (20 μM) for 10 min. The fatty acid was removed by washing with FA-free BSA (single arrow) as described in Materials and methods. The washed cells were incubated for up to 60 min in medium containing BSA. A second wash with fresh medium was carried out to remove the BSA (double arrows) followed by the addition of more 20:4 (n – 6) (20 μM). The cells were incubated for a further 10 min. The extent of dye transfer to surrounding cells was assessed at the times indicated before the BSA wash, after BSA wash and after removal of BSA. Data are mean ± SEM of six determinations.

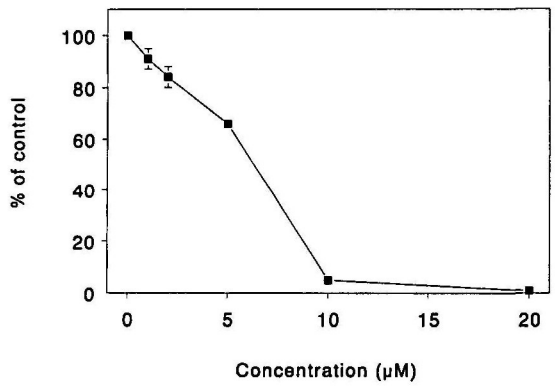

Fig. 4. Effects of increasing concentrations of 20:4 (n – 6) on dye transfer. Cells were incubated with 0–20 μM 20:4 (n – 6) for 10 min. Assessment of the extent of dye transfer and data presentation are as described above. Data are representative of two experiments.

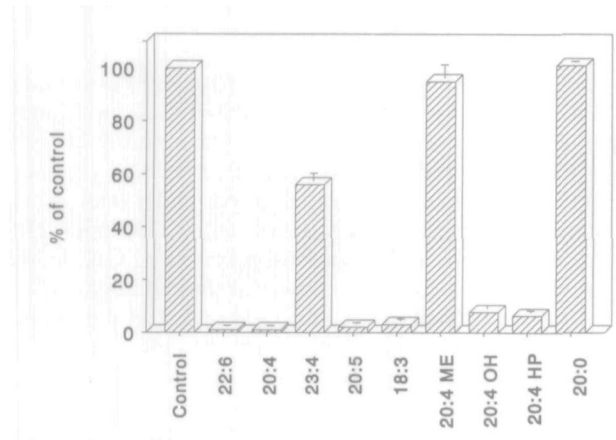

Fig. 5. Effects of various fatty acids (20 μM) on dye transfer. Cells were incubated with the n – 3 series of PUFA (20:5 and 22:6), the n – 6 series of PUFA [20:4, 18:3, the methyl ester of 20:4 (20:4 ME), the hydroxy derivative of 20:4 (20:4 OH) and the hydroperoxy derivative of 20:4 (20:4 HP)] and a saturated fatty acid, 20:0. Assessment of dye transfer and data presentation are as described above. Similar data were obtained in two other experiments.

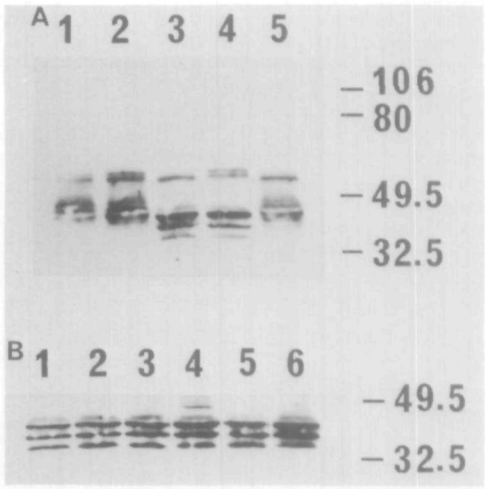

Fig. 6. Effects of 20:4 (n – 6), epidermal growth factor and lysophosphatidic acid on the electrophoretic mobility of Cx43. Cells were incubated for 10 min with 20:4 (20 μM), EGF (10 ng/ml), EGF + 20:4 (n – 6) or LPA (25 μM) (**A**) or for 0–60 min with 20:4 (n – 6) (20 μM) (**B**). Samples of alkali-enriched Cx43 were prepared, proteins separated by SDS–PAGE and immunoblotted as described in Materials and methods. (**A**) **Lane 1**, EGF + 20:4 (n – 6); **lane 2**, EGF; **lane 3**, control; **lane 4**, 20:4 (n – 6); **lane 5**, LPA. (**B**) **Lane 1**, 0 min; **lane 2**, 1 min; **lane 3**, 5 min; **lane 4**, 10 min; **lane 5**, 20 min; **lane 6**, 60 min.

(n – 6) > 15-hydroperoxy-20:4 (n – 6) > 15-hydroxy-20:4 (n – 6) > 23:4 (n – 6).

Previously, we and others have reported that inhibition of GJC by PMA, EGF, LPA or $pp60^{src}$ transformation was accompanied by hyperphosphorylation of Cx43 (12–14,22). This was detected in Western blots as a retardation in the mobility of immunoreactive bands of molecular mass of ~42–47 kDa, which could be reversed by treatment of samples with alkaline phosphatase (14). As shown in Figure 6, incubation of cells with 20 μM 20:4 (n – 6) did not affect the mobility of Cx43 (panels a and b) whereas treatment with either EGF or LPA produced the expected retardation in migration of Cx43 accompanied by a loss of faster migratory bands (panel a, lanes 1, 2 and 5). The presence of 20:4 (n – 6) did not interfere with the phosphorylation of Cx43 induced by EGF (panel a, lane 1).

We have recently observed the translocation of PKC α, ε and δ to a particulate fraction in WB cells incubated with 20:4 (n – 6) (23), consistent with the reports that 20:4 (n – 6) activated PKC (24). In addition, incubation of WB cells with 20:4 (n – 6) led to the appearance of phosphotyrosine-containing proteins (23). To determine whether PKC or tyrosine kinases are involved in 20:4 (n – 6)-induced inhibition of GJC, cells were pretreated with 300 nM PMA to downregulate PMA-responsive PKC isozymes or incubated in the presence of the tyrosine kinase inhibitor, genistein. As shown in Table I, PMA pretreatment did not prevent inhibition of dye transfer by 20:4 (n – 6), whereas the action of PMA on pretreated cells was completely blocked. We have recently shown WB cells to contain α, ε, δ and ζ isoforms of PKC and that pretreatment with PMA for 24 h resulted in a loss of all but the ζ isoform (23). Genistein was ineffective in preventing the effect of 20:4 (n – 6) (Table I), but partially blocked the action of EGF, which binds to a receptor with intrinsic tyrosine kinase activity (25) and has been shown to inhibit dye transfer in WB cells in a PKC-independent manner (12,14).

Elevation of intracellular cAMP concentrations has been

Table I. Effects of PMA pretreatment and the tyrosine kinase inhibitor genistein on dye transfer

Experiment 1	Percentage of Me_2SO control	
Treatment		PMA-pretreated
PMA (100 nM)	2.4±0.3	106±2
Ethanol control	99±2	100±3
20:4 (n – 6) (20 μM)	1±0.1	4±0.1
Experiment 2	**Percentage of control**	
Treatment		+ Genistein (50 μM)
Ethanol control	100	101±8
EGF (10 ng/ml)	12±2	55±4
20:4 (n – 6) (20 μM)	2±0.2	3±0.5

Cells were either pretreated with 300 nM PMA (experiment 1) for 26 h prior to the addition of 20:4 (n – 6) or preincubated with 50 μM genistein (experiment 2) for 3 min prior to the addition of test substances. Dye transfer was assessed as described in Materials and methods. Results are expressed as percentages of controls. Values are mean ± SEM of six determinations.

Table II. Effects of elevation of intracellular cAMP on 20:4 (n – 6)-induced inhibition of dye transfer

Treatment	Dye transfer (% of control)	
		IBMX/isoproterenol
Control	100	123±2
20:4 (n – 6) (20 μM)	2±2	40±3
PMA	2±0.4	9±1

Cells were pretreated with 0.1 mM IBMX and 10 μM (±)-isoproterenol 20 min prior to the addition of either 20:4 (n – 6) or PMA. Dye transfer was assessed as described in Materials and methods. Results are expressed as percentages of controls. Values are mean ± SEM of six determinations.

Table III. Effects of indomethacin and nordihydroguaiaretic acid on dye transfer

Treatment	Percentage of control
Control	100
20:4 (n – 6) (20 μM)	3.7±1
Indomethacin (100 μM)	96±1.4
Indomethacin + 20:4 (n – 6)	3.7±1.4
NDGA (10 μM)	98±3
NDGA + 20:4 (n – 6)	96±3

Cells were preincubated with either indomethacin or NDGA for 3 min prior to the addition of 20:4 (n – 6). After 10 min the extent of dye transfer was assessed. Results are presented as percentage of control. Data are mean ± SEM of six observations. Similar data were obtained in a repeat experiment.

reported to enhance cell–cell communication in unstimulated cells and to antagonize the actions of inhibitors of GJC (26). Table II shows that preincubation of WB cells with (±)-isoproterenol (10 μM) and IBMX (0.1 mM), found to increase intracellular cAMP concentrations (data not shown), significantly attenuated the effect of 20:4, suggesting possible regulation of fatty acid action by intracellular mechanisms.

To determine whether metabolism of 20:4 (n – 6) to an eicosanoid product was required for inhibition, cells were preincubated with indomethacin to inhibit the activity of cyclooxygenase or nordihydroguaiaretic acid (NDGA) to block the activity of lipoxygenase. Preincubation with NDGA but not indomethacin blocked the effect of 20:4 (n – 6) (Table III), suggesting that a metabolite(s) of lipoxygenase might be involved in the fatty acid action. Neither 5-oxo-eicosatetraenoic acid nor leukotriene B4 were candidates since these eicosanoids were unable to block GJC (not shown). NDGA has also been reported to possess antioxidant activity (27). However, oxidation products of 20:4 (n – 6) were unlikely to mediate inhibition of GJC by 20:4 (n – 6) since co-incubation of 20:4 (n – 6) with vitamin E (up to 100 μg/ml), an antioxidant, did not abrogate the effect of the fatty acid (not shown).

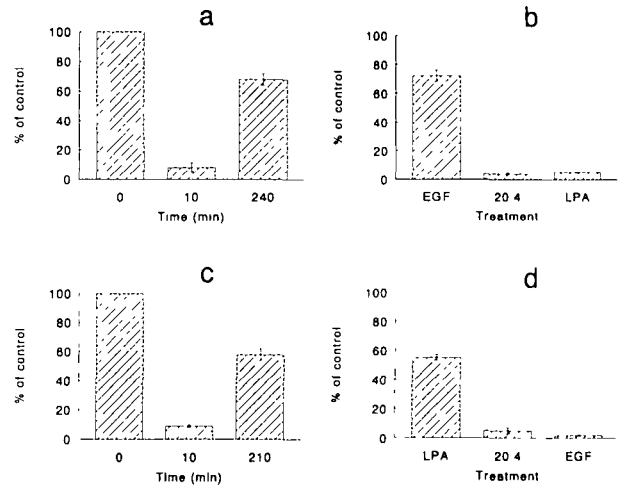

Fig. 7. Effects of pretreatment with epidermal growth factor or lysophosphatidic acid on the inhibition of gap junctional communication by 20:4 (n – 6). Cells were incubated with either EGF (10 ng/ml) for 240 min (**a**) or LPA (25 μM) for 210 min (**c**). Following the return of communication competence at these times (12), EGF- (**b**) or LPA- (**d**) pretreated cells were incubated with EGF (10 ng/ml), LPA (25 μM) or 20:4 (n – 6) (20 μM) for 10 min. Assessment of the extent of dye transfer and data presentation are as described above.

We have previously shown that pretreatment of WB cells with either EGF or LPA rendered the cells refractory to a subsequent challenge with the original agonist following the recovery of communication competence (14; Figure 7a,b). However EGF-pretreated cells remained responsive to LPA and vice versa. In the present experiments cells remained responsive to 20:4 (n – 6) after pretreatment with either EGF (panel b) or LPA (panel d).

Discussion

Previous studies have shown that the n – 6 series of unsaturated fatty acids (16–20 carbon atoms) inhibited GJC in Chinese hamster V79 cells, cardiac myocytes and smooth muscle cells (9,16). Our studies with the long-chain PUFA 20:4 (n – 6) has confirmed this finding in WB cells. Of particular interest was the finding that PUFAs of the n – 3 series, 20:5 and 22:6, were just as effective as 20:4 (n – 6) in inhibiting GJC in WB cells. In addition, we have also observed inhibition of GJC by 22:6 (n – 3) in neonatal rat cardiac myocytes as determined by microinjection of Lucifer Yellow into the cells (manuscript in preparation). This is in contrast to a recent report by Deehan *et al.* (28) who showed that PUFA with >20 carbon atoms were not effective as modulators of GJC. The reason(s) for this discrepancy is unknown but may include differential sensitivity of cell types to fatty acids of >20 carbon atoms. In WB cells, the effect of a 23 carbon fatty acid was less than that of 20:4 (n – 6) (Figure 5).

PUFAs 20:5 and 22:6 have attracted a lot of interest in attempts to treat inflammatory diseases such as arthritis (17)

They have been perceived to act as anti-inflammatory agents by virtue of their ability to compete against 20:4 (n – 6) for the lipoxygenase enzymes, leading to the production of eicosanoids of much lower pro-inflammatory activity. It is evident from our results that 20:4 (n – 6), 20:5 (n – 3) and 22:6 (n – 3) are roughly equipotent at inhibiting GJC. Previously we have demonstrated that 20:5 (n – 3) and 22:6 (n – 3) share similar pro-inflammatory activities with 20:4 (n – 6), which include stimulation of the neutrophil oxygen radical release (4), degranulation, expression of the CR3 receptors and adherance (2,8). All the above findings re-emphasize that the anti-inflammatory properties of the n – 3 series of fatty acids are likely to be a result of the production of eicosanoids with lower pro-inflammatory activity.

It was surprising to find that the 15-hydroxy and 15-hydroperoxy derivatives of 20:4 (n – 6) were also powerful inhibitors of GJC. A number of reports had shown them to possess minimal biological activity in other systems (8). The rearrangement of the double bonds following the addition of the hydroxy or the hydroperoxy group might have rendered them less active than their parent compounds in relation to inhibition of neutrophil migration and activation of the neutrophil NADPH oxidase activity. Clearly the ability of the unsaturated fatty acids to inhibit GJC was unaffected by oxidation by the 15-lipoxygenase. This study, to our knowledge, is not only the first demonstration of comparable activities of the PUFA and their 15-hydroxy and 15-hydroperoxy derivatives, but also the first to report inhibition of GJC by these eicosanoid intermediates. It was clearly evident from our studies that fully saturated fatty acids lacked GJC-inhibitory actions and that a functional free carboxyl group was essential for the inhibition of GJC in WB cells by PUFA. In addition, increasing the chain length from 20 to 23 carbon atoms, while keeping the degree of unsaturation constant, resulted in loss of activity.

20:4 (n – 6) did not exert any obvious deleterious effects on cells since the cells rapidly regained communication competence after removal of the fatty acid with FA-free BSA. The ability of further additions of 20:4 (n – 6) to inhibit dye transfer also suggests that the cells were not desensitized to the fatty acid. Thus the kinetics and the nature of the fatty acid action differed from that reported for PMA, EGF or LPA since cells treated with these agents regained communication competence after ~80 min of treatment (12,14). Upon recovery these cells were refractory to a subsequent challenge with the original test agent but were responsive to others (14). It therefore seems likely that 20:4 (n – 6) initiates its effect on communication through a mechanism different from that associated with PMA, EGF or LPA action. This conclusion is reinforced by the lack of an effect of PUFA on the phosphorylation status of Cx43.

Considerable evidence has accumulated to indicate that GJC can be modulated by hyperphosphorylation of connexin molecules (12,14,22). Many studies have demonstrated that hyperphosphorylated Cx43 exhibits a retarded electrophoretic mobility (12,14). Within the constraint of the resolution power of the gels, treatment of WB cells with 20:4 (n – 6) did not affect the mobility of Cx43, while EGF or LPA did, suggesting that inhibition of GJC by 20:4 (n – 6) was not associated with a change in the phosphorylation status of Cx43. Lack of Cx43 hyperphosphorylation during reduced junctional permeability is not unprecedented. Some inhibitors of GJC, including heptanol and UV, have been found to exert their effects in the absence of Cx43 hyperphosphorylation (29,30). While mobility shift experiments do not directly demonstrate the incorporation of phosphate into a protein, this approach has been widely used to demonstrate alterations in the phosphorylation status, particularly when coupled with the demonstration that shifts can be reversed by phosphatase treatment. Proteins that have been studied in this way include MAPK, phospholipase A_2, PKC and Cx43 (31–34). However, we cannot rule out the possibility that 20:4 (n – 6) induces a low degree of phosphorylation of Cx43 which cannot be detected as a change in mobility in denatured gels and that phosphorylation by some kinases may not lead to mobility shifts (32). In previous studies, we have observed incorporation of $^{32}P_i$ into Cx43 in WB cells following stimulation with EGF (12). However, the increase in label incorporated into Cx43 was found to be very small compared to the background even though EGF induced a readily detectable retardation in the electrophoretic mobility in SDS gels, an effect reversible by alkaline phosphatase (12,14). The relatively high incorporation of $^{32}P_i$ into Cx43 presumably reflects basal phosphorylation of this protein (29). Consequently, in our hands, incorporation of $^{32}P_i$ is not a sensitive index of Cx43 phosphorylation in WB cells.

Although the kinase(s) responsible for phosphorylating Cx43 has not been identified *in vivo*, it has been suggested that MAPK, a serine/threonine kinase, may be one such kinase (14,15). However, we have observed induction of the activities of the 42 and 44 kDa isoforms of MAPK by PUFAs in WB cells (23). Thus, the lack of Cx43 mobility shift observed here appears to be inconsistent with the notion that these isoforms of MAPK are involved in phosphorylating Cx43. It is unlikely that PUFA prevented Cx43 from being phosphorylated by activated MAPK or that PUFA induced the activity of a phosphatase to dephosphorylate phosphorylated Cx43, since co-incubation of EGF with 20:4 (n – 6) did not affect the EGF-induced retardation in the mobility of Cx43. This indicates that the normal flow of signals from the EGF receptor to the kinase(s) culminating in the phosphorylation of Cx43 was not affected by the presence of the PUFA.

20:4 (n – 6) has been reported to induce Ca^{2+} mobilization in a variety of cell types (6), including WB cells (unpublished data). Since Ca^{2+} has been reported to modulate GJC in some cell types (35,36), and the effect of Ca^{2+} is not associated with a change in the electrophoretic mobility of Cx43 (36), it is possible that inhibition of GJC by 20:4 (n – 6) could be mediated through a rise in intracellular Ca^{2+} concentration. However, preliminary experiments demonstrated that removal of extracellular Ca^{2+} did not abrogate the response to 20:4 (n – 6) but converted the prolonged elevation in intracellular Ca^{2+} concentration induced by 20:4 (n – 6) to a transient rise as determined by fura-2 fluorescence (unpublished data). Omission of Ca^{2+} *per se* did not affect dye transfer over the 10 min period of fatty acid treatment. In addition, NDGA did not significantly affect the mobilization of Ca^{2+} by 20:4 (n – 6). These observations suggest that inhibition of GJC by 20:4 (n – 6) was not mediated by a rise in intracellular Ca^{2+}.

The nature of fatty acid interaction with cells is unclear. Some studies have pointed to the existence of a membrane fatty acid binding protein which transports fatty acids across the plasma membrane (37,38). A receptor for 15-hydroxyeicosatetraenoic acid has been claimed to exist on the plasma membrane of PT-18mast/basophil cells (39). Downstream from the interaction with the plasma membrane, there has been very little agreement on the exact mechanisms through which fatty

acids exert their biological activities. Although various PUFAs have been reported to affect components of intracellular signalling (6,7), it is unlikely that such effects at intracellular sites are involved in fatty acid-induced inhibition of GJC since inhibition was rapidly reversed by FA-free BSA and the phosphorylation status of Cx43 was unaffected. The rapid recovery of communication competence after washing cells with FA-free BSA suggested that 20:4 (n – 6) interacted with a site readily accessible BSA and was therefore easily extracted. One is tempted to speculate that the effect of 20:4 (n – 6) was exerted on the external face of the plasma membrane. A recent study on heptanol-induced inhibition of GJC has suggested that the alcohol inhibited GJC by decreasing the fluidity of cholesterol-rich region of the plasma membrane where gap junctional proteins are embedded (39). Such an effect of the fatty acid would be consistent with previous suggestions that fatty acids readily partition into membranes and affect protein–lipid interactions by altering the fluidity of the plasma membrane with consequent functional changes in cells (41). Alternatively, the fatty acid could affect the adhesion molecules that are thought to be important in maintaining functional gap junctions (see refs in 42). Unsaturated fatty acids have been reported to increase the expression of integrin molecules in neutrophil membrane (2). Such an effect could affect the coupling of the connexons, resulting in their inability to form functional channels. However, any inhibition of GJC due to an effect on the expression of adhesion molecules would be delayed and would not be expected to be rapidly reversible. Fluri and co-workers (43) suggested that 20:4 (n – 6) may exert its effect by a direct action on channel proteins

The ability of a rise in intracellular cAMP concentration to antagonize the effect of 20:4 (n – 6) suggests that activation of protein kinase A blocked the fatty acid action. This is consistent with reports that cAMP can counteract the effects of other blockers of GJC (25). The exact mechanisms behind the cAMP effect are unclear but may hold clues to the mechanisms of fatty acid action. Similarly, understanding the mechanisms behind the ability of NDGA to block the action of 20:4 (n – 6) may also shed light on the mode of action of 20:4 (n – 6).

Despite the current uncertainty regarding the mode of fatty acid action, this study suggests that cell–cell communication through gap junctions may be inhibited *in vivo* under conditions where PUFAs can accumulate or are released from the plasma membrane. Such accumulation is seen during inflammation (44), myocardial ischaemia (45) and hypoxia in the brain (46). For example, elevated levels of PUFAs during myocardial ischaemia may inhibit gap junctions, leading to arrhythmia. In studies with cultured neonatal rat cardiac myocytes, we and others have observed that both the n – 6 and n – 3 series of PUFA could inhibit gap junctions (16; manuscript in preparation). This effect of the n – 3 series of PUFAs following an acute exposure, which may be pathogenic, is in clear contrast to the reported cardioprotective effects of fish oils in the diets of experimental animals (47). Diets high in fat content have been associated with the incidence of certain cancers like mammary cancer (48). Numerous studies have shown that tumour promotion by known promoters is closely related to the ability of the promoters to inhibit GJC (25,49) and elevated circulating PUFA levels due to high-fat diets may thus contribute to the growth of these forms of cancer. Further studies are needed to assess the roles that fatty acids play in the pathophysiology of diseases like coronary heart diseases and cancer.

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