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THE EFFECT OF UNSATURATED FATTY ACIDS ON SODIUM NITROPRUSSIDE STIMULATION OF GUANYLATE CYCLASE IN THE HUMAN ASTROCYTOMA CLONE, D384, AND THE HUMAN NEUROBLASTOMA CLONE, NB1-G

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Abstract—Sodium nitroprusside (SNP) stimulates cGMP formation to a greater extent in 20,000 *g* supernatant fractions of the human neuroblastoma clones NB1-G and SH-SY5Y than in the human astrocytoma clone D384. This suggests that these cell lines contain the soluble form of guanylate cyclase. Arachidonic, 8,11,14- and 11,14,17-eicosatrienoic acids inhibit SNP (10^{-4} M)-stimulated cGMP formation more potently than the C_{18} unsaturated fatty acids linolenic and linoleic acids in D384 and NB1-G. In contrast the C_{20} saturated fatty acid, arachidic acid had little effect even at 10^{-4} M concentration. In addition arachidonic and 8,11,14-eicosatrienoic acids inhibited basal guanylate cyclase activity, in NB1-G, over the same concentration range as they inhibited SNP-stimulated cGMP formation. No evidence could be obtained for the stimulation of guanylate cyclase by arachidonic acid in either NB1-G or D384. These results provide further support for suggestions that arachidonic acid or its metabolites may be important regulators of cGMP formation in the nervous system.

A considerable amount of information is available on the regulation of cAMP formation in cell cultures derived from the nervous system (see e.g. Ref. 1). However much less is known about the mechanisms by which cGMP levels are controlled in the nervous system and in particular the part played by glial cells in this process. Guanylate cyclase occurs as membrane bound and soluble forms in most tissues including the nervous system. The membrane bound isoenzyme is probably one form of the ANP-receptor [2]. Thus ANP has been found to stimulate cGMP formation in a number of tissues [3], including glial cell cultures [4–7]. The soluble isoenzyme is a heme protein and is stimulated by nitrovasodilators including sodium nitroprusside [3] which suggests that nitric oxide (NO) is the *in vivo* stimulant. Recent studies have provided direct evidence that cGMP formation in mixed cultures of cerebellar cells [8] and smooth muscle cells [9, 10] is stimulated by nitric oxide (NO). In addition to NO, soluble guanylate cyclase has been reported to be stimulated by arachidonic and other unsaturated fatty acids [3] or by their lipoxygenase products [11]. For example Richelson and his colleagues have provided evidence that the stimulation of cGMP formation in the murine neuroblastoma N1E115 by acetyl choline [11], neurotensin [12], angiotensin II [13] and bradykinin [14] is mediated by arachidonic acid or a lipoxygenase product such as a hydroperoxyeicosanoic acid, which is liberated from phospholipids by activation of phospholipase A2 in response to hormonal induced increases in intracellular calcium. In addition exogeneously applied arachidonic acid and the lipoxygenase products 5-, 12-, and 15-hydroxyeicosatetraenoic acids inhibited carbachol, histamine, bradykinin, neurotensin and thrombin stimulation of cGMP formation in the murine neuroblastoma N1E115 with IC_{50} values of approximately 10 μM [15, 16].

Thus the regulation of cGMP formation in the nervous system is complex with at least two enzyme forms involved which are stimulated to a different extent by ANP, NO, Ca^{2+} and arachidonic acid or its lipoxygenase products. The role of arachidonic acid is particularly confusing as there is little agreement as to whether it is arachidonic acid [17] or its lipoxygenase products [11, 16] which are the active species, and these compounds have been reported to both stimulate basal and inhibit hormonal stimulated cGMP formation [3, 16, 17].

Our previous studies have isolated a clone, D384 from the human astrocytoma G-CCM [18, 19] and shown that cGMP formation is stimulated by ANF [7] and sodium nitroprusside [20], which suggests that this cell line contains both soluble and particulate guanylate cyclase.

The present paper reports that soluble guanylate cyclase of the human astrocytoma clone D384, and the human neuroblastoma NB1-G is inhibited by exogenously applied unsaturated fatty acids, including arachidonic and 8,11,14-eicosatrienoic acids. This agrees with our previous report that sodium nitroprusside stimulation of cGMP formation in intact D384 cells is inhibited by arachidonic acid [20].

MATERIALS AND METHODS

Arachidonic, 8,11,14- and 11,14,17-eicosatrienoic, linolenic, linoleic and arachidic acids were purchased from Sigma Chemical Co. (Poole, U.K.), and dissolved in dimethyl sulphoxide. The unsaturated fatty acids were stored under argon at −70°. The cGMP assay kit was purchased from Amersham International PLC (Amersham, U.K.). All tissue culture

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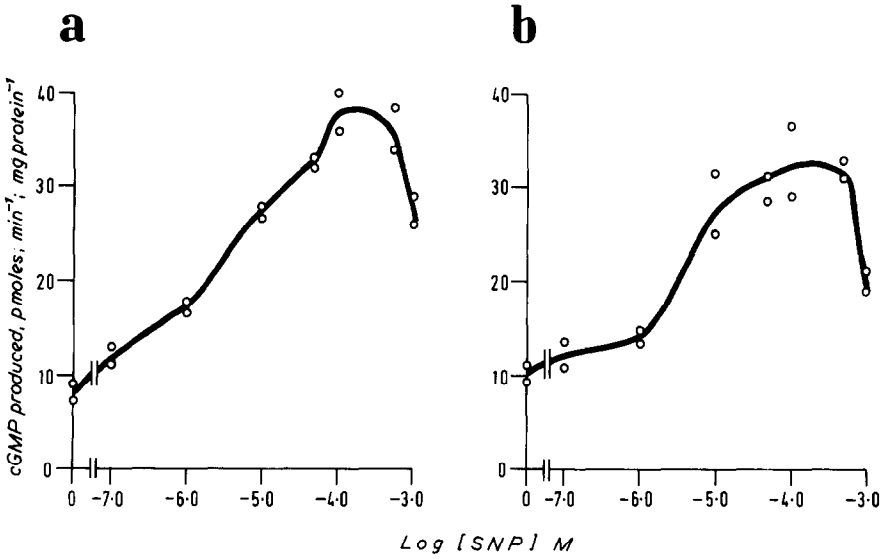

Fig. 1. Stimulation of cGMP formation in (a) initial homogenates and (b) 20,000 *g* supernatant fractions of D384 by SNP. Results represent duplicate values of a single experiment which was carried out three times.

reagents and plastics were purchased from Gibco (Paisley, U.K.). D384 cells were obtained as described previously [18]. Human neuroblastoma NB1-G and SH-SY5Y cells were generously provided by Dr T. Weldon, Belvidere Hospital, Glasgow, and Dr J. L. Biedler, Memorial Sloan-Kettering Cancer Centre, New York, respectively.

Cell culture. Human astrocytoma clone D384 and human neuroblastoma clone NB1-G were subcultured at a density of 2×10^4 cells per mL in 175 cm^3 flasks containing 50 mL of 1:1 Ham's F10/Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 8 mM sodium bicarbonate and supplemented with 10% foetal calf serum. SH-SY5Y was sub-cultured at a density of 5×10^4 cells per mL in 175 cm^3 flasks containing 50 mL Ham's F10/Eagle's minimum essential medium containing 2 mM L-glutamine, 8 mM sodium bicarbonate and supplemented with non-essential amino acids and 10% foetal calf serum. The pH of the medium was adjusted to 7.2 with 1 M NaOH. Cell cultures were maintained at 37° until confluent, 7 days for SH-SY5Y, 8 days for D384, and 10–12 days for NB1-G. The medium was changed for NB1-G cells 2 days before harvesting, and cell lines were harvested for guanylate cyclase assay when confluent.

Formation of cGMP in cell homogenates. Medium was removed from D384, SH-SY5Y and NB1-G confluent monolayers and the cells were harvested by rinsing the monolayers with phosphate buffered saline without calcium and magnesium (20 mL). The cell layers were treated with trypsin (0.25% w/v) in PBS (1 mL) and incubated at 37° for 15 min for D384 and at room temperature for 2–3 min for NB1-G and SH-SY5Y. The trypsinized cell layers were resuspended in PBS (10 mL) and collected by centrifugation at 600 rpm. The PBS was removed and the cell pellet resuspended in homogenization buffer (3 mL) consisting of Tris–HCl (50 mM; pH 7.5), $MgCl_2 \cdot 6H_2O$ (5 mM) and EDTA (0.2 mM). The cell suspension was disrupted by 4×10 sec bursts with a polytron, setting 9, with 30 sec on ice between homogenizations. Where indicated a sample was removed for guanylate cyclase assay in the initial homogenate. The homogenate was centrifuged at 20,000 *g* for 30 min at 4° and guanylate cyclase activity determined in the supernatant, and where relevant the 20,000 *g* pellet was resuspended in 3 mL homogenization buffer.

Guanylate cyclase activity was determined by incubating 75 μL cell fraction D384 or 50 μL cell fraction NB1-G with GTP (0.5 mM), IBMX (0.5 mM) and the concentrations of sodium nitroprusside and fatty acid indicated in the relevant figure, in a final volume of 215 μL Tris–HCl (50 mM; pH 7.5), $MgCl_2 \cdot 6H_2O$ (5 mM) and EDTA (0.2 mM). Incubation was started by addition of cell fraction and carried out at 37° for 10 min. Reaction was terminated by the addition of 1 mL ice-cold ethanol, the solution mixed and the ethanol solution evaporated under a gentle stream of air at 37°. The residue was resuspended in Tris–HCl (0.5 mL; 50 mM; pH 7.2) containing EDTA (4 mM). The cGMP content (diluted where necessary) was determined in 100-μL aliquots following the procedure described in the Amersham kit using [^{3}H]cGMP.

Protein content of the cell fractions was determined [21] in samples diluted in NaOH (0.1 M) containing 2% SDS using bovine serum albumin as standard.

RESULTS

Effect of sodium nitroprusside

Sodium nitroprusside (SNP)-stimulated guanylate cyclase in initial homogenates and 20,000 *g* supernatant prepared from both D384 and NB1-G. Maximal stimulation was obtained with 10^{-4} M SNP for

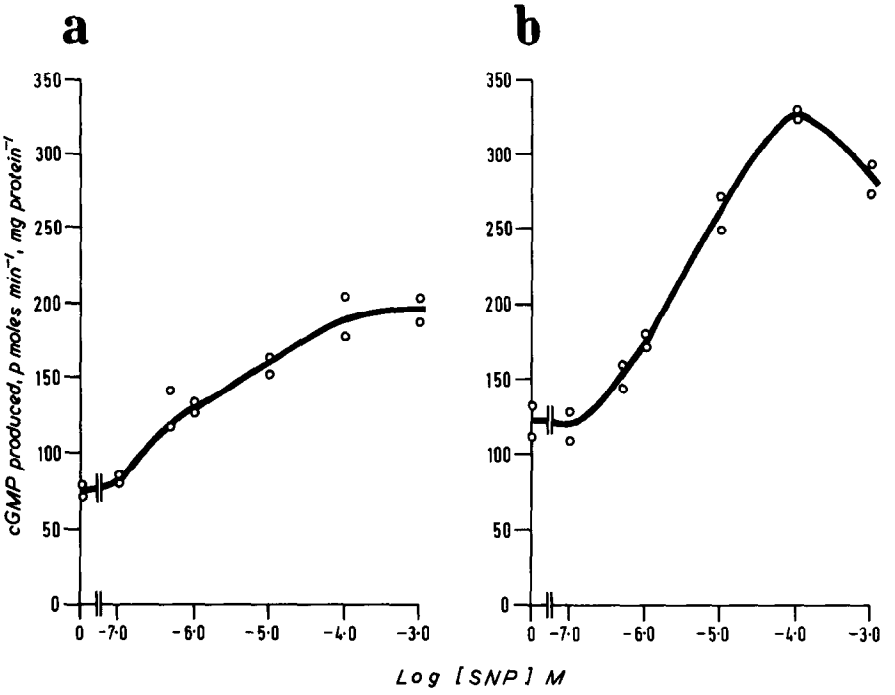

Fig. 2. Stimulation of cGMP formation in (a) initial homogenate and (b) 20,000 *g* supernatant fractions of NB1-G by SNP. Results represent duplicate values of a single experiment which was carried out three times.

Table 1. Activity of guanylate cyclase in cell free preparations of human astrocytoma and neuroblastoma cell lines*

Cell line	Fraction	Guanylate cyclase activity
D384	Initial homogenate	43.6 ± 10.8 (4) A
	20,000 *g* supernatant	42.0 ± 7.7 (12) B
NB1-G	Initial homogenate	158.6 ± 19.5 (4) C
	20,000 g supernatant	260.0 ± 43.4 (9) D
SH-SY5Y	Initial homogenate	66.5 ± 18.4 (3) E
	20,000 *g* supernatant	131.7 ± 25.6 (5) F

* Guanylate cyclase activity was determined in the presence of SNP (10^{-4} M). Activity in each fraction is expressed as pmols cGMP produced per min per mg protein and refers to means ± SE with the number of separate determinations (each carried out in duplicate) in parentheses. C vs A, $P < 0.01$; E vs A, not significant; E vs C, $P < 0.05$; D vs B, $P < 0.01$; F vs B, $P < 0.01$; F vs D, not significant. Statistical comparison made using Student's *t*-test.

both fractions in D384 (Fig. 1) and NB1-G (Fig. 2). Greater activity was observed in the 20,000 *g* supernatant with NB1-G and SH-SY5Y compared with D384 (Table 1). The recovery of guanylate cyclase activity in the 20,000 *g* supernatant represented 50–60% of the activity in the initial homogenate for D384, and 90% for NB1-G. Another difference between D384 and NB1-G guanylate cyclases is that the basal activity is greater in NB1-G. Thus although the total activity is greater in NB1-G than in D384, SNP stimulates guanylate cyclase NB1-G (4.0 ± 1.1 (3)-fold). Furthermore no difference is observed for SNP stimulation of guanylate cyclase in the 20,000 *g* supernatant fraction which is 3.8 ± 0.49 (8)-fold for D384 and 3.5 ± 0.6 (7)-fold for NB1-G. The values refer to mean stimulation in the presence of 10^{-4} M SNP ± SE with the number of separate experiments in parenthesis.

Effect of unsaturated fatty acids

D384. Arachidonic acid (10^{-4} M) inhibits SNP-stimulated cGMP formation in whole D384 cells [20]. The present study shows that 10^{-5} M arachidonic and 8,11,14-eicosatrienoic acids inhibit SNP stimulation of guanylate cyclase activity recovered in the 20,000 *g* supernatant by approximately 40 and 50%, respectively (Fig. 3). In contrast 11,14,17-eicosatrienoic, linolenic, linoleic and arachidic acids were

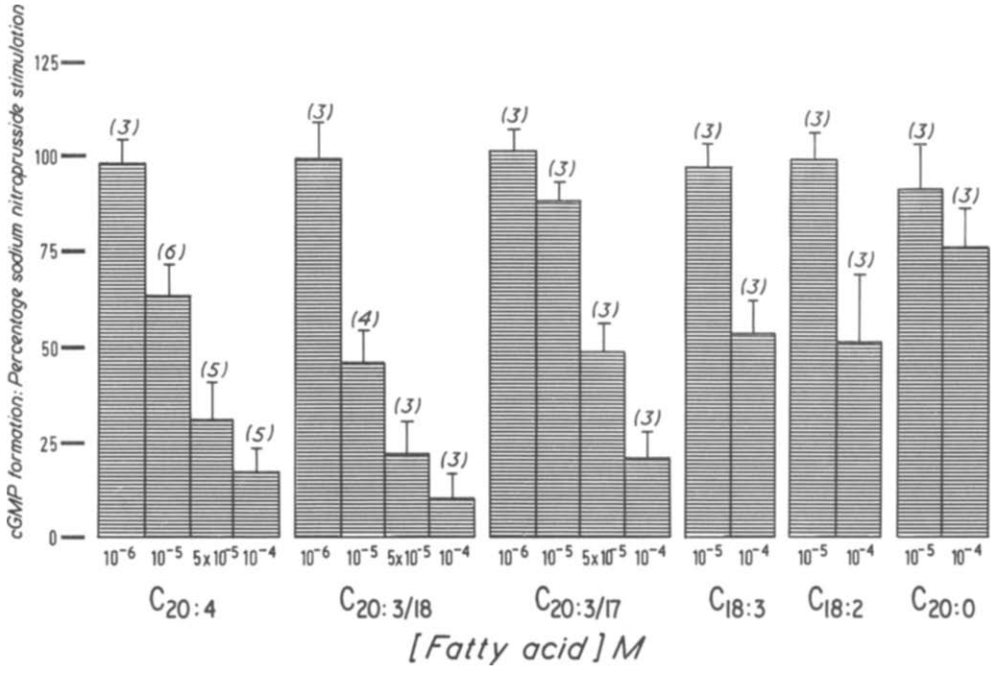

Fig. 3. Inhibition of SNP stimulation of cGMP formation in the 20,000 *g* supernatant fraction of the human astrocytoma clone D384, by arachidonic ($C_{20:4}$), 8,11,14-eicosatrienoic ($C_{20:3/8}$), 11,14-17-eicosatrienoic ($C_{20:3/17}$), linolenic ($C_{18:3}$), linoleic ($C_{18/2}$); and arachidic ($C_{20:0}$) acids. Results represent the means ± SE of the number of determinations shown in parentheses. Data are expressed as a percentage of the stimulation obtained with SNP (10^{-4} M), as indicated in Fig. 1b.

less potent as 10% or less inhibition of SNP-stimulation of guanylate cyclase was observed by 10^{-5} M concentrations of these fatty acids (Fig. 3). Similarly, guanylate cyclase activity is inhibited to a greater extent by 10^{-4} M arachidonic, 8,11,14-, and 11,14,17-eicosatrienoic acids than by the C_{18} linolenic and linoleic acids (Fig. 3). The C_{20} saturated fatty acid (10^{-4} M), arachidic acid, did not appreciably inhibit cGMP formation (Fig. 3). It was not possible to test higher concentrations due to the inhibitory effect of the solvent (DMSO) used to dissolve this compound. In addition it was found that arachidonic and 8,11,14-eicosatrienoic acids inhibit basal guanylate cyclase activity over the same concentration range as they inhibit SNP-stimulated cGMP formation (data not shown).

NB1-G. Arachidonic and 8,11,14-eicosatrienoic acids (5×10^{-5} M) inhibited guanylate cyclase activity in the 20,000 *g* supernatant fraction of NB1-G, by greater than 80% (Fig. 4). In contrast 11,14,17-eicosatrienoic acid appears to be less potent since only about 50% inhibition of SNP-stimulated cyclase was observed at this concentration. The C_{18} acids linolenic and linoleic acids were even less potent since only 40% inhibition was observed with 10^{-4} M concentrations (Fig. 4). In subsequent experiments (data not shown) 10^{-4} M arachidonic and 8,11,14-eicosatrienoic acids inhibited basal guanylate cyclase activity in the absence of SNP over the same concentration range as observed for inhibition of SNP-stimulated guanylate cyclase. The C_{20} saturated fatty acid, arachidic acid (10^{-4} M) inhibited SNP stimulation of NB1-G guanylate cyclase by less than 20% (Fig. 4).

In contrast no stimulation of basal levels of guanylate cyclase by arachidonic acid or the other unsaturated fatty acids over the concentration range 10^{-7} to 10^{-4} M was observed in either D384 or NB1-G.

Effect of arachidonic acid on time course of cGMP formation. The addition of 5×10^{-5} M arachidonic acid in the presence of SNP (10^{-4} M) resulted in an inhibition of cGMP formation without a detectable lag for both D384 (Fig. 5a) and SH-SY5Y (Fig. 5b). Immediate inhibition was observed when arachidonic acid was added either at the beginning of the reaction or during the incubation period (Fig. 5).

DISCUSSION

This study on the effect of unsaturated fatty acids on SNP-stimulated guanylate cyclase in homogenates of the human astrocytoma, D384, confirms our previous report using intact cells [20]. The recovery of 50–60% of SNP-stimulated guanylate cyclase activity in the 20,000 *g* supernatant fraction of D384, together with the previous report [7] that D384 expresses ANP receptors coupled to the stimulation of cGMP formation suggests that this cell line contains both particulate and soluble forms of guanylate cyclase. Thus the clone D384 is similar to the majority of other preparations which express both soluble and particulate forms of this enzyme [3]. It is of interest that previous reports that the rat glioma, C6-2B, contains exclusively the particulate form of the enzyme [22] were not confirmed in subsequent studies [23]. The present study also finds that the cell line NB1-G, isolated from a human neuroblastoma

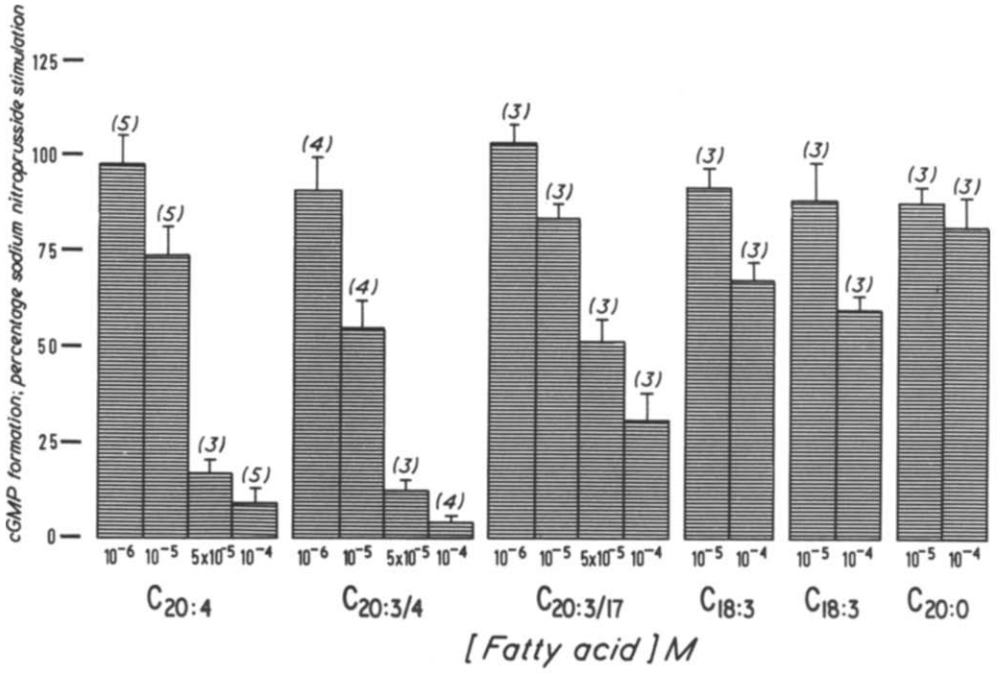

Fig. 4. Inhibition of SNP stimulation of cGMP formation in the 20,000 *g* supernatant fraction of the human neuroblastoma clone NB1-G, by the same fatty acids indicated in the legend to Fig. 3. Results are expressed as in the legend to Fig. 3.

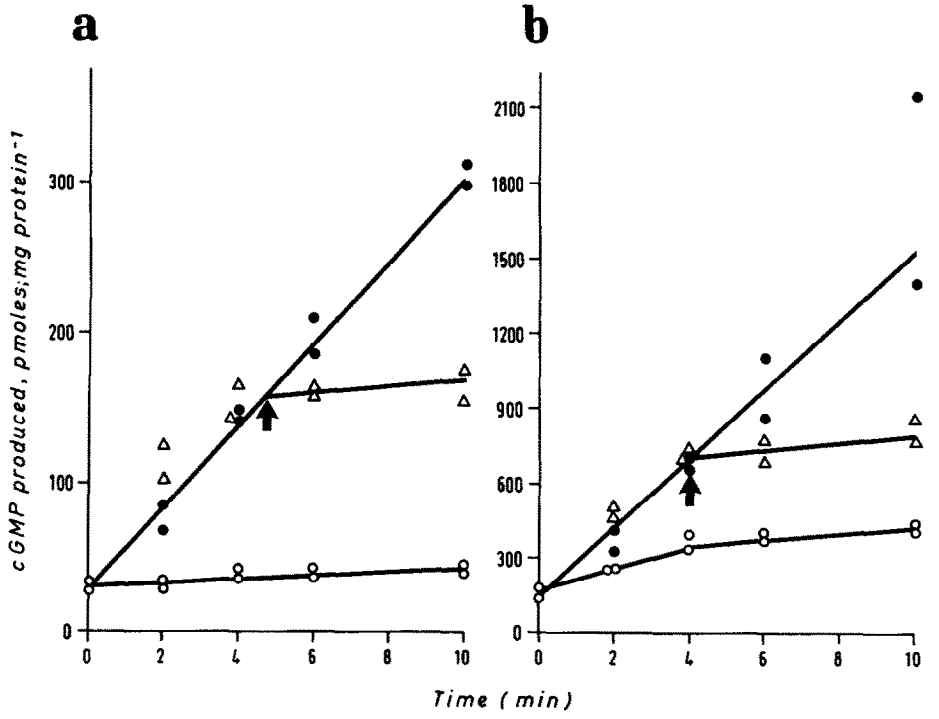

Fig. 5. Effect of arachidonic acid (5×10^{-5} M) on SNP stimulation of cGMP formation in the 20,000 *g* supernatant fraction of D384 (a) and SH-SY5Y (b). Incubations were carried out in the absence of arachidonic acid (●), or with arachidonic acid added at $T = 0$ (○) or 4 min (△). Results represent duplicate values of a single experiment which was carried out three times.

[24] expresses a soluble form of guanylate cyclase, since 80–90% of SNP stimulated activity is recovered in the 20,000 *g* supernatant. Further work is required to establish whether NB1-G also contains a particulate form of the enzyme, stimulated by ANP.

The studies reported in this paper provide support for the hypothesis that unsaturated fatty acids of chain length 18–20 residues can inhibit basal as well as SNP-stimulated soluble guanylate cyclase. This agrees with a previous report [25] which found that arachidonic acid liberated from rat and rabbit platelet membranes inhibited SNP-stimulated cGMP formation. It is not yet established whether the fatty acids themselves or their lipoxygenase products are the active species in the inhibition of soluble guanylate cyclase in these clones derived from the human nervous system. However the occurrence of inhibition without a lag in 20,000 *g* supernatants suggests that the unsaturated fatty acids rather than lipoxygenase products are the active species. Studies using inhibitors of lipoxygenase are in progress to provide further information on the possible role of arachidonic acid metabolites in the inhibition of sodium nitroprusside-stimulated cGMP formation. The data presented in this paper suggest that an important structural requirement for inhibition is the presence of a C_{20} carbon chain with a double bond in the C_8 position. Thus arachidonic and 8,11,14-eicosatrienoic acids are more potent inhibitors (based on degree of inhibition with 10^{-5} M concentrations), than 11,14,17-eicosatrienoic, arachidic, linolenic or linoleic acids. Little difference is apparent between the inhibition caused by these fatty acids in the astrocytoma clone D384 and the neuroblastoma, NB1-G.

It is of interest that the concentration range (1–100 μM) over which arachidonic and 8,11,14-eicosatrienoic acids inhibit SNP stimulation of guanylate cyclase agrees very closely with the concentration over which arachidonic acid inhibits SNP-stimulated soluble guanylate cyclase, purified from bovine lung [17], and arachidonic acid inhibits muscarinic stimulation of cGMP formation in the neuroblastoma N1E 115 [15]. Recent studies have provided evidence that arachidonic acid or more likely its lipoxygenase products, act as modulators of hormonal activity. Thus the inhibitory action of FMRFamide on synaptic transmission between sensory and motor neurones in Aplysia is mediated by lipoxygenase metabolites of arachidonic acid [26]. Furthermore recent electrophysiological studies have provided evidence that the G-protein-gated muscarinic K^+ channel in cardiac cells is activated by lipoxygenase products of arachidonic acid including leukotrienes [27, 28]. Thus evidence is accumulating to suggest that arachidonic acid or its lipoxygenase products can act as second messengers.

The presence of soluble guanylate cyclase in the human astrocytoma clone D384 and neuroblastoma clone NB1-G, suggests that these cell lines will be of value in studies on the regulation of cGMP formation in the human nervous system. In particular they will enable studies to be carried out on the relationship of arachidonic acid and its lipoxygenase products to the NO stimulation of soluble guanylate cyclase.

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