Vol. 136, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1986 Pages 381-389

Activation of protein kinase C inhibits sodium fluoride-induced elevation of human platelet cytosolic free calcium and thromboxane B_2 generation

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Received March 3, 1986

SUMMARY: Addition of NaF to washed platelets produces a dose-dependent and transient elevation of the intracellular free calcium concentration ($[Ca^{++}]_i$), thromboxane B_2 (TxB_2) generation and dense granule release, all of which are significantly inhibited when the extracellular calcium concentration ($[Ca^{++}]_e$) is reduced with EGTA. Inhibition of platelet cyclo-oxygenase by acetylsalicylic acid (ASA) does not affect NaF-induced elevation of $[Ca^{++}]_i$ and dense granule release in the presence of 1 mM $[Ca^{++}]_e$. Pre-incubation of the platelets with the phorbol ester TPA produces a marked inhibition of NaF-induced elevation of $[Ca^{++}]_i$ and TxB_2 generation without affecting dense granule release. Thus, NaF may have more than one site of action. Pretreatment of the platelets with the selective protein kinase C inhibitor H7 prevents TPA induced inhibition of NaF mediated rise in $[Ca^{++}]_i$ and TxB_2 generation. Thus we propose that NaF induced calcium mobilisation is analogous to receptor-operated calcium mobilisation in platelets, as it is readily inhibited by protein kinase C activation or by the reduction of $[Ca^{++}]_e$ and is independent of platelet cyclo-oxygenase activity.

The addition of sodium fluoride (NaF) to platelets induces dense granule release (1,2,3). However, the mechanism of NaF induced platelet activation is unknown. In isolated platelet membranes NaF stimulates both the inhibitory guanine nucleotide binding protein (N_i) and the stimulatory guanine nucleotide binding protein (N_s) regulatory components of adenylate cyclase depending on the concentration of the fluoride ion employed (4). These two components are thought to transduce hormonal signals received at the plasma membrane to the catalytic subunit of adenylate cyclase (5).

Guanine nucleotide dependent proteins (G-Proteins) analogous to those linked to adenylate cyclase are now thought to couple calcium mobilising receptors to phospholipase C activation (6,7). Phospholipase C modulates

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0006-291X/86 $1.50

polyphosphoinositide (PPI) hydrolysis, which leads to the formation of two intracellular secondary messenger molecules, namely the intracellular calcium mobilising agent inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), the endogenous activator of protein kinase C (8). Furthermore, there is evidence that the elevation of intracellular free calcium ($[Ca^{++}]_i$) plays an important role in dense granule release in platelets (for review see 9).

Therefore, the recent observation that NaF causes an elevation of $[Ca^{++}]_i$ in neutrophils (10) led us to re-examine NaF induced human platelet activation.

In this study we have investigated NaF mediated platelet activation by monitoring a coupling event in the stimulus response coupling chain, namely the elevation of $[Ca^{++}]_i$, and by measuring two functional platelet responses, thromboxane B_2 (TxB_2) generation and dense granule release. In addition, in an attempt to locate the site of action of NaF, platelet activation by NaF was examined in the presence of agents which are known to selectively modify receptor mediated platelet activation.

MATERIALS AND METHODS:

Materials. The following chemicals were obtained from the sources indicated: Quin-2-am from Lancaster Synthesis Ltd, UK; [^{14}C]-5-Hydroxy-tryptamine-creatinine-sulphate (5-HT)and [^{3}H]-TxB_2 from Amersham PLC, UK; NaF (purity 99.99%) from Aldrich Chemicals, UK; 12-O-tetradecanoylphorbol-13-acetate (TPA) and reagents for lactate dehydrogenase (LDH) measurement from Sigma Chemicals, UK. Prostacyclin (PGI_2) was kindly provided by Dr B.J.R. Whittle, Wellcome Laboratories, UK. H7 was a generous gift from Prof H. Hidaka, Mie University, Tsu, Japan. TxB_2 and anti-TxB_2 antibody were a kind gift from Dr F. Carey, ICI Laboratories, UK.

Methods. Washed human platelets were prepared by a modified version of the PGI_2 washing method described by Blackwell et al (11). Venous blood was collected by forearm venepuncture from normal healthy volunteers (who denied having taken any medication for the previous 14 days) into 3.2 trisodium citrate (9:1 v/v). PGI_2 (6 nmoles/ml blood) was added and the blood was centrifuged at 314xg for 10 min at room temperature. The platelet rich plasma (PRP) was incubated for 30 min at 37°C with 18 μM quin-2-am alone or with 15 nCi/ml 5-HT when dense granule release was to be determined. After incubation, PGI_2 (1 nmole/ml PRP) was added and the PRP was centrifuged at 1254xg for 15 min. The platelet pellet was resuspended in 15mls of 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5.5 mM glucose and 0.2% BSA (pH 7.4, 37°C). Further PGI_2 (1 nmole/ml buffer) was added and the washing procedure repeated. The final platelet count was adjusted to 2 x 10^8 platelets/ml and 1 mM $CaCl_2$ was added.

Intracellular free calcium was measured using quin-2-am according to the method described by Tsien et al. (12). Fluorescence readings were recorded at 37°C using an Aminco Bowman spectrophotofluorimeter (excitation λ339 nM, emission λ492 nM - 4nM slit width). Calibration of the calcium-quin-2-fluorescence signal was carried out as described previously (12,13).

Agonist and inhibitors of platelet activation were added at the following final concentrations: NaF 3-30 mM, EGTA 2 mM, acetylsalicylic acid (ASA) 0.2 mM, TPA 0.16-16 nM, H7 60 μM.
5-HT or TxB_2 were determined in quin-2 loaded platelets in the fluorimeter cuvette at the same time as measurement of $[Ca^{++}]_i$. At fixed intervals following addition of agonist or vehicle 360μl sub-aliquots were withdrawn from the fluorimeter cuvette and added to ice-cooled tubes containing 40μl of 10% formaldehyde and 100 mM EDTA (for determination of 5-HT) or 40μl of 30 μM indomethacin and 100 mM EDTA (for measurement of TxB_2). The samples were spun down at 12,000g for 2 min. In the supernatant, either 5-HT release was determined according to the method of Holmsen et al (14), or TxB_2 was measured by radioimmunoassay as reported elsewhere (15).
LDH was measured spectrophotometrically as previously described (16).
Statistical analysis was performed by unpaired t-test.

RESULTS AND DISCUSSION: NaF when added in the concentration range 3-30mM to washed human platelets suspended in Hepes buffered tyrode solution containing 1mM Ca^{++} induces a dose related increase in $[Ca^{++}]i$ from a basal value of 94±3 nM (n=25)to 263±8 nM (n=7) as measured using the fluorescent intracellular calcium indicator quin-2 (Figure 1 and 2). As shown in Figure 1 the elevation of $[Ca^{++}]_i$ was preceded by a lag period which was reduced with increasing concentration of NaF. The lag period may reflect the time required for NaF to reach an intracellular site of action, as suggested previously (10). The elevated $[Ca^{++}]_i$ returns towards basal values within 10 min following NaF stimulation (data not shown).

Measurement of dense granule release in the same aliquots of platelets indicates that NaF produces a dose related release of 5-HT (Figure 2). For example, when platelets are incubated with 30mM NaF for 5 min at 37°C, an approximate 50% release of 5-HT is observed. At this time the peak elevation of $[Ca^{++}]_i$ has been attained at all NaF concentrations examined. Longer incubation periods (15 min) result in an enhanced 5-HT release response (69±8% 5-HT release by 30 mM NaF) in agreement with previous findings (17). Furthermore, NaF promotes a dose related increase in TxB_2 production ranging from 2.35±0.16 to 60.5±10.5 pmols/10^8 platelets (Figure 1). This is in contrast to the findings of Murer et al. (3) who reported an absence of cyclo-oxygenase activity as measured by malondialdehyde production. The reason for this difference is not clear.

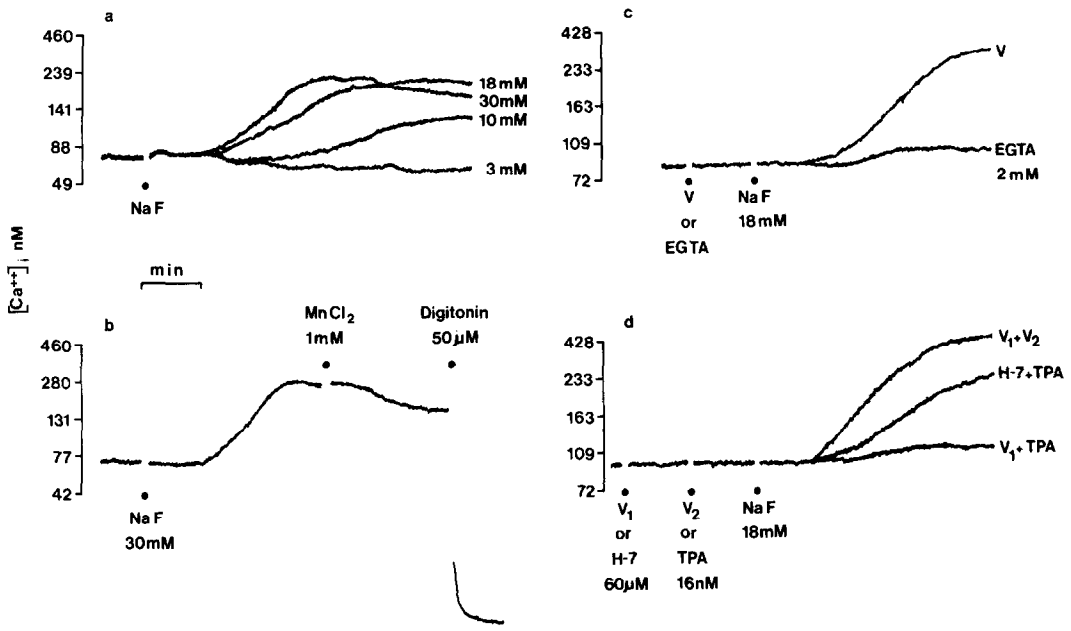
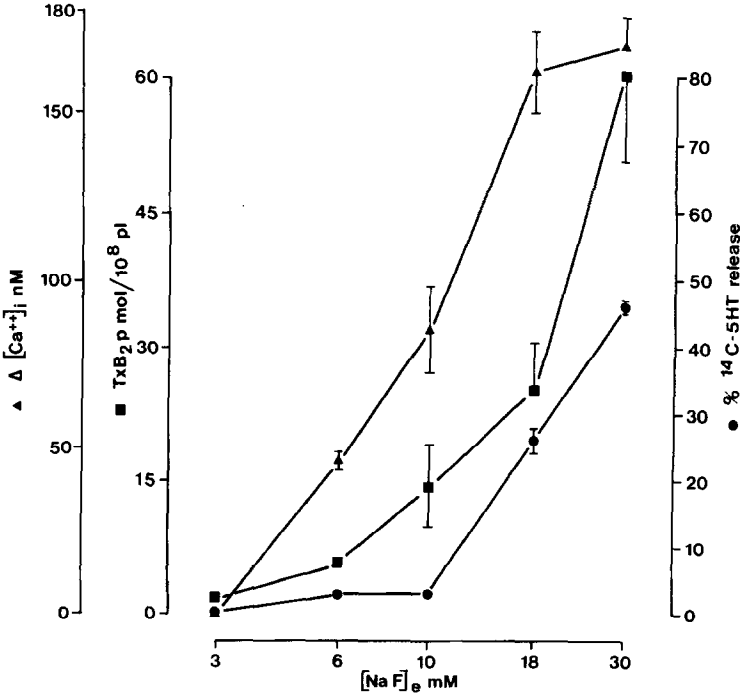


Figure 1. Fluorescent traces obtained by stimulation of quin-2-loaded platelets with (a) NaF in a concentration range of 3 - 30 mM, (b) 30 mM NaF with subsequent additions of 1 mM $MnCl_2$ and 50 µM digitonin, (c) 18 mM NaF following 1 min preincubation with EGTA or vehicle and (d) 18 mM NaF following preincubation with TPA (16 nM) alone or H7 (60 µM) and TPA (16 nM) for 1 min respectively. The non-linear vertical scale is the result of transforming the fluorescent output to platelet $[Ca^{++}]_i$, as described previously (12, 13). The above traces were obtained from one experiment, but are representative of at least 5 experiments.



Addition of sodium chloride (3-30mM) does not produce platelet activation indicating that the effects of NaF are due to the fluoride ion. Manganese chloride ($MnCl_2$), an agent which quenches quin-2 Ca^{++} fluorescence (12,18) when added to NaF stimulated platelets, does not quench the Ca^{++}-quin-2 fluorescence signal indicating that NaF does not induce cell lysis (Figure 1b). Subsequent addition of 50μM digitonin causes cell lysis enabling $MnCl_2$ to produce a dramatic quenching of the fluorescence signal. Furthermore, evidence of the absence of cell lysis was confirmed by the observation that platelet supernatants collected 5 min after NaF stimulation (3-30mM) contain less than 1% of total cell LDH activity.

The above observation with $MnCl_2$ indicates that NaF does not act as an ionophore, as ionophores produce a rapid and irreversible elevation of $[Ca^{++}]_i$ which is quenched immediately by the addition of $MnCl_2$ (18).

In order to further characterize NaF induced platelet activation, the effect of agents known to selectively modify receptor-operated platelet activation was assessed. When the extracellular free calcium concentration $[Ca^{++}]_e$ is reduced to <10nM by the addition of 2mM EGTA 1 min prior to platelet stimulation by 18mM NaF, the elevation of $[Ca^{++}]_i$, the release of 5-HT and the generation of TxB_2 are all markedly inhibited (Table 1). These results suggest that activation of platelets by NaF is very dependent upon $[Ca^{++}]_e$. A similiar relationship between $[Ca^{++}]_e$ and $[Ca^{++}]_i$ has been reported for platelet activation induced by thrombin (19), platelet activating factor (13) and ADP (20). In addition, reduction of both TxB_2 generation (21) and dense granule release (19) has been observed with thrombin stimulated platelets in the absence of $[Ca^{++}]_e$. Thus, both NaF and receptor mediated platelet activation are sensitive to the $[Ca^{++}]_e$.

Figure 2. Dose response curve of NaF-induced peak elevation of $[Ca^{++}]_i$ (▲), 5-HT release (●) and TxB_2 generation (■). $\Delta[Ca^{++}]_i$ nM is the value obtained by subtracting the unstimulated platelet $[Ca^{++}]_i$ from the peak platelet $[Ca^{++}]_i$ produced by the addition of NaF. Each point represents means ± s.e. mean of determinations performed in 4 - 8 experiments. NaF-mediated 5-HT release and TxB_2 generation were measured 5 min after NaF-stimulation.

Table 1

Effect of EGTA, ASA, TPA and H7 on elevation of $[Ca^{++}]_i$, 5-HT release and TxB_2 production induced by 18 mM NaF

Pretreatment	Agent	$\Delta[Ca^{++}]_i$[b] nM	^{14}C-5-HT % release	TxB_2 p mols/10^8 plat
Vehicle	NaF (18 mM)	181 ± 12[a]	23.9 ± 1.2[a]	32 ± 3.7[a]
EGTA (2mM)	"	3.8 ± 2.7*	6.4 ± 1*	1.7 ± 1*
ASA (0.2mM)	"	151 ± 15.5	17.7 ± 11	0.075
TPA (16nM)	"	0.75 ± 0.75*	30.6 ± 5.7	5.55 ± 0.75*
H7(60μM)+TPA(16nM)	"	145 ± 20.5	18 ± 1.5	41.1 ± 7.65

$\Delta[Ca^{++}]_i$[b] nM is the value obtained by subtracting the unstimulated platelet $[Ca^{++}]_i$ from the peak platelet $[Ca^{++}]_i$ produced by the addition of NaF.

Values represent means ± s.e. mean from 4 - 8 different donors
Significance values were calculated by comparing vehicle control group[a] with drug pretreatment groups, * $p < 0.001$ (unpaired t-test)

To determine the effect of cyclo-oxygenase inhibition on NaF induced platelet activation platelets were preincubated for 10 min with 0.2mM ASA. ASA treatment does not have a significant effect on either NaF mediated elevation of $[Ca^{++}]_i$ or dense granule release (Table 1). Therefore, prostaglandin endoperoxide and/or TxA_2 production does not appear to contribute to NaF induced calcium mobilisation or 5-HT release.

Protein kinase C is an enzyme which plays a pivotal role in stimulus response coupling (22) and is thought to be the site of action of the phorbol ester TPA (23). Recent reports suggest that protein kinase C activation operates a negative feed-back system during receptor-operated calcium mobilisation (24,25,26), PPI hydrolysis (24,27,28) and dense granule release (25,26) in platelets. The effect of protein kinase C stimulation on NaF induced platelet activation was investigated by preincubating platelets with TPA in the concentration range 0.16-16nM. Preincubation with TPA at very low concentrations (IC_{50} 0.42nM) inhibits NaF induced elevation of $[Ca^{++}]_i$ but

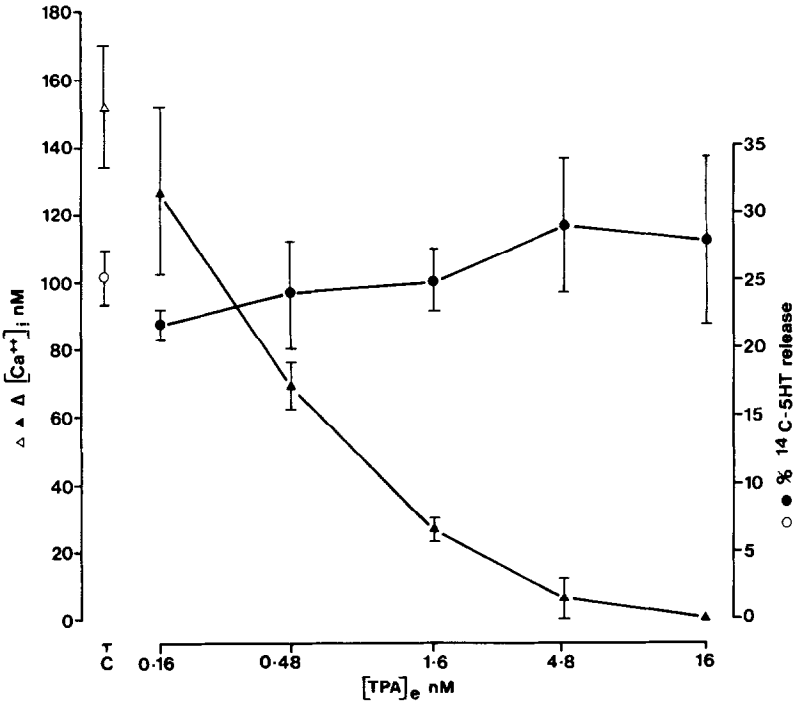


Figure 3. Effect of TPA on NaF-induced peak elevation of $[Ca^{++}]_i$ (▲) and 5-HT release (●). Open symbols (△ , ○) represent the corresponding values obtained in the presence of TPA vehicle. $\Delta[Ca^{++}]_i$ nM is the value obtained by subtracting the unstimulated platelet $[Ca^{++}]_i$ from the peak platelet $[Ca^{++}]_i$ produced by the addition of NaF. Platelets were incubated for 1 min with TPA in a concentration range of 0.16 - 16 nM, followed by the addition of 18 mM NaF. 5-HT release was measured 5 min after NaF-stimulation. Each point represents the mean ± s.e. mean of 4 - 8 determinations.

does not affect 5-HT release (Figure 3). In addition, NaF mediated TxB_2 generation is substantially reduced when platelets are pretreated for 1 min with 16nM TPA (Table 1). The effect of TPA on the elevation of $[Ca^{++}]_i$ and TxB_2 production is prevented by a 1 min pretreatment with the selective protein kinase C inhibitor H7 (29), confirming that TPA exhibits its inhibitory effect via protein kinase C activation (Table 1). Incubation of platelets with H7 alone does not modify NaF induced platelet activation (data not shown). We have recently reported that H7 prevents TPA mediated inhibition of thrombin induced elevation of $[Ca^{++}]_i$ (26). The finding that TPA blocks NaF induced elevation of $[Ca^{++}]_i$ and TxB_2 generation without affecting dense granule release suggests that NaF may have more than one site of action.

Inhibition of NaF induced elevation of $[Ca^{++}]_i$ by EGTA or TPA results in a significant reduction of thromboxane generation. In contrast, when thromboxane production is inhibited by ASA, NaF mediated calcium mobilisation is unaffected. Therefore NaF induced thromboxane synthesis is probably dependent on the elevation of $[Ca^{++}]_i$.

NaF in the concentration range employed in this study stimulates the N_i- rather than the N_s regulatory component of adenylate cyclase in platelet membranes (4). Our data are consistent with these findings as the stimulation of the N_s subunit by NaF would lead to an inhibition of platelet function as a consequence of cyclic AMP elevation (30).

TPA has recently been shown to phosphorylate the α-subunit of the N_i-G protein of adenylate cyclase (31). In platelet membranes this leads to an impaired ability of this component to transduce inhibitory hormonal stimuli (31,32). Thus, our results showing inhibition by TPA of NaF induced elevation of $[Ca^{++}]_i$ and thromboxane generation may be explained by protein kinase C mediated inhibition of the G-protein(s) involved in phospholipase C activation.

A role for G-protein activation in calcium mobilisation and phospholipase C activation has been established using GTP and GTP analogues in permeabilized cell systems including platelets (9). Thus, the elevation of $[Ca^{++}]_i$, the release of 5-HT and the production of TxB_2 could be explained by NaF induced phospholipase C activation. However, platelet phospholipase C activation is thought to be independent of $[Ca^{++}]_e$ (21). Therefore, the inhibition of NaF induced elevation of $[Ca^{++}]_i$ by EGTA is apparently inconsistent with phospholipase C as the site of action of NaF. We are presently investigating this anomaly.

In conclusion we have shown that NaF induces an elevation of $[Ca^{++}]_i$, dense granule release and thromboxane generation which is dependent upon the presence of $[Ca^{++}]_e$. Inhibition of cycloxygenase by ASA is without effect on calcium mobilisation and 5-HT release while the activation of protein kinase C by TPA potently inhibits the rise in $[Ca^{++}]_i$ and thromboxane generation but not dense granule release induced by NaF.

ACKNOWLEDGEMENTS: We are grateful to the British Heart Foundation and Ciba-Geigy Corp (USA) for providing financial support. P. Kyrle is supported by a grant from the Austrian Ministry of Science and Research.

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