Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 98-104

AEQUORIN DETECTS INCREASED CYTOPLASMIC CALCIUM IN PLATELETS STIMULATED WITH PHORBOL ESTER OR DIACYLGLYCEROL

J. Anthony Ware, Peter C. Johnson, Marianne Smith, and Edwin W. Salzman,*

Department of Surgery, Harvard Medical School, Beth Israel Hospital and the Charles A. Dana Research Institute, Boston, MA

Received October 7, 1985

A biochemical pathway to platelet activation involving protein kinase C has been deemed "Ca^{2+}-independent", because the intracellular fluorophore quin2 indicates no rise in cytoplasmic [Ca^{2+}] in platelets stimulated by certain agonists. However, unlike quin2, the Ca^{2+}-sensitive photoprotein aequorin demonstrates a rise in [Ca^{2+}] when platelet aggregation is induced by phorbol ester or diacylglycerol. Aequorin and quin2 appear to report different aspects of Ca^{2+} homeostasis, and the absence of a quin2 signal may not be sufficient to establish that a metabolic pathway is "Ca^{2+}-independent".

Although the activity of protein kinase C requires Ca^{2+} (1), the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (phorbol ester, TPA) or an exogenous diacylglycerol such as 1-oleoyl-2-acetyl glycerol (OAG) can reduce the cytoplasmic ionized Ca^{2+} concentration ([Ca_i^{2+}]) required for protein kinase C activation to that present in unstimulated platelets, ~100nM (1). Furthermore, TPA and OAG appear to stimulate platelet aggregation and secretion without a rise in [Ca_i^{2+}], as they produce no signal with the Ca^{2+}-sensitive fluorophore quin2 (2); their action in respect to activation of protein kinase C has

*To whom correspondence should be addressed at the Department of Surgery, Dana 907, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.

Abbreviations used are: EGTA, ethylene glycol bis (B-aminoethyl ether)-N,N,N',N',-tetraaceticacid, TPA, 12-O-tetradecanoyl phorbol -13-acetate; OAG, 1-oleoyl-2-acetyl-glycerol; [Ca_i^{2+}], cytoplasmic ionized Ca^{2+} concentration; CP/CPK, creatine phosphate/creatine phosphokinase; PGE_1, prostaglandin E_1; ASA, acetyl salicylic acid.

0006-291X/85 $1.50

been termed "Ca^{2+}-independent." However, because quin2 is insensitive to [Ca_i^{2+}] above uM concentration, the level in stimulated cells, and because a millimolar intracellular concentration of quin2 is needed to produce signals that exceed cellular autofluorescence, it is not ideal for indicating small, transient [Ca_i^{2+}] changes in stimulated cells, especially if these should be localized to restricted zones within the cell. These features argue against reliance upon quin2 as the sole Ca^{2+} indicator to designate a pathway to cellular activation as being "Ca^{2+}-independent" (3).

We have measured [Ca_i^{2+}] by incorporation of the Ca^{2+}-sensitive photoprotein aequorin (Mr 20000) into platelets by incubation with ethylene glycol bis (B-amino-ethyl-ether) N,N,N',N'-tetraacetic acid (EGTA) and ATP^{4+} in the cold, and have compared this agent with quin2 (3-5). Aequorin emits blue light in the presence of Ca^{2+}, and its luminescence increases as the 2.5 power of [Ca^{2+}] . Although quin2 more accurately estimates the average basal [Ca_i^{2+}] in unstimulated cells (40-100nM), aequorin is more sensitive to small, transient elevations in [Ca_i^{2+}], is less of an intracellular Ca^{2+} buffer than quin2, being present in nM rather than mM intracellular concentrations, and has a Kd(2-3uM) and range of maximal Ca^{2+} sensitivity(10^{-7}-10^{-5}M) near those of intracellular Ca^{2+}-binding proteins (3). These features allow even local zones of elevated [Ca_i^{2+}] to provide exceptionally bright signals. This study was designed to determine the effect of TPA and OAG on platelet aggregation and [Ca_i^{2+}] as indicated by aequorin.

MATERIALS AND METHODS

Our method for loading aequorin into platelets has been described in detail (3). Briefly, EGTA-washed platelets were incubated at 0° C in a loading solution containing EGTA 10mM, PGE_1 1uM, and ATP 5mM, with aequorin 0.2mg/ml. After 60 minutes, the

platelets were rewashed and incubated in a recovery solution containing EGTA 0.1mM and $MgCl_2$ 10mM without aequorin. Ca^{2+} was added to the cold platelet suspension to raise the final concentration to 1mM; the platelets were allowed to rewarm, gel-filtered through a 9ml bed-volume column of Sepharose 2B preequilibrated with a modified Hepes-Tyrode's buffer containing 1mM Ca^{2+} (3), and finally suspended in the same buffer at a final concentration of $4x10^8$/ml. Aequorin luminescence and aggregation were measured simultaneously in the same sample using a modified Chronolog Lumiaggregometer (5). A parallel sample was used to measure ATP secretion (3). Platelets were loaded with 0.8-1.0mM quin2 as previously described (3); in some experiments, platelets were loaded with both aequorin and a high (~3mM) concentration of quin2 to test the effect of quin2 on aggregation and $[Ca_i^{2+}]$ elevation in aequorin loaded platelets. All experiments were performed at 37°C.

Aequorin was purchased from Dr. John Blinks, Mayo Clinic, Rochester MN. The two preparations of OAG used in this study were kind gifts from Drs. Yasutomi Nishizuka and Susan Rittenhouse. Quin2 was purchased from Amersham; other reagents were the best available grade.

RESULTS AND DISCUSSION

As shown in figure 1, aequorin-loaded platelets indicate a rise in $[Ca_i^{2+}]$ to supramicromolar levels in response to TPA and OAG in media containing either 1mM Ca^{2+} or 2mM EGTA. These signals either coincide with or precede the onset of aggregation. The elevation of $[Ca_i^{2+}]$ is present but diminished when EGTA is added to the media; thus both intracellular mobilization and trans-membrane flux of Ca^{2+} contribute to the aequorin signal produced by either agonist. Both the ADP scavenger system creatine phosphate/creatine phosphokinase (CP/CPK) and aspirin (acetyl salicylic acid) inhibited but did not eliminate aggregation or the $[Ca_i^{2+}]$ response to TPA or OAG (Table1); thus the rise in $[Ca_i^{2+}]$ involves but does not require either secretion of ADP or generation of thromboxane A_2. Prior addition of prostaglandin E_1 (PGE_1) 1uM, which elevates intracellular cyclic 3'5'-AMP, raised to 60ug/ml the concentration of OAG necessary to produce both aggregation and a rise in $[Ca_i^{2+}]$; both $[Ca_i^{2+}]$ elevation and aggregation in response to TPA or OAG were blunted and delayed but not totally inhibited. Addition of PGE_1 at the midpoint of the OAG or TPA-induced aequorin signal caused a prompt fall in $[Ca_i^{2+}]$ and

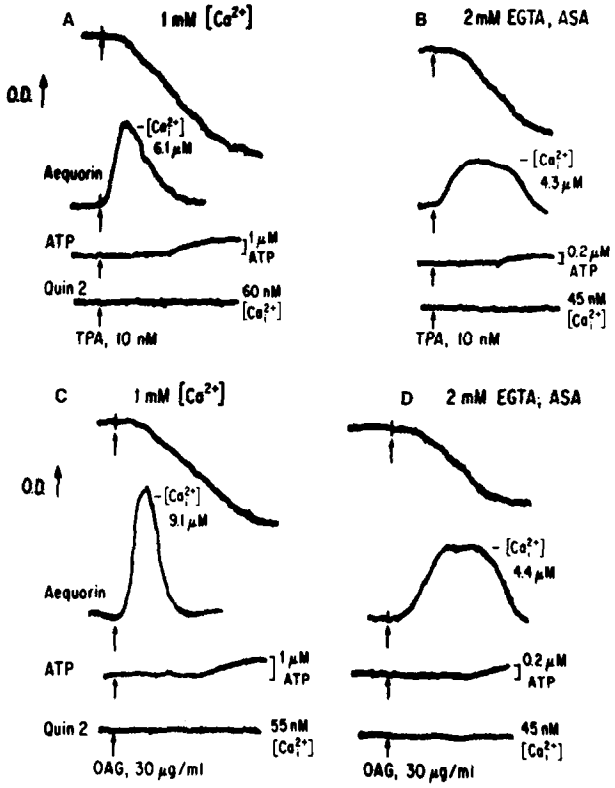

Figure 1: Aggregation, ATP secretion and $[Ca_i^{2+}]$ indicated by quin2 and aequorin in response to TPA and OAG. Purified human fibrinogen (400ug/ml) was added shortly before agonists. Panels A and B demonstrate responses to TPA 10nM of platelets suspended in Hepes-Tyrode's buffer containing 1mM Ca^{2+} (A), and 2mM EGTA plus aspirin (ASA) (B). In each case, an aequorin signal indicating a $[Ca_i^{2+}]$ rise occurs after the agonist is added and either precedes, or occurs coincident with, the first visible platelet functional response. No change in $[Ca_i^{2+}]$ from the basal level was shown by quin2. In panels C and D, similar signals in response to OAG, 30ug/ml. are shown.

aggregation (not shown). In none of these experiments did quin2 indicate a rise in $[Ca_i^{2+}]$ above basal levels. Platelet aggregation by OAG or TPA was greatly enhanced by the addition of fibrinogen; when fibrinogen was omitted, aggregation was markedly inhibited, but the $[Ca_i^{2+}]$ signal indicated by aequorin was unchanged. Therefore, the TPA or OAG-induced rise in $[Ca_i^{2+}]$ was not secondary to platelet aggregation. Furthermore, addition of TPA to unstirred samples of aequorin-loaded platelets increased $[Ca_i^{2+}]$ without aggregation. Resumption of stirring resulted in platelet aggregation without further $[Ca_i^{2+}]$ rise.

Table 1

Aequorin-indicated peak $[Ca_i^{2+}]$ and aggregation in platelets stimulated by TPA, 10nM or OAG, 30ug/ml

Addition	OAG		TPA	
	$[Ca_i^{2+}]$, uM	AGG.SLOPE, mm/min	$[Ca_i^{2+}]$, uM	AGG.SLOPE, mm/min
None	8.0	16	6.1	25
EGTA	4.7	11	4.5	18
ASA	5.9	14	5.8	25
CP/CPK	5.1	3	5.2	9
EGTA/ASA	4.7	9	4.4	15
EGTA/ASA QUIN2	3.1	8	3.8	12
EGTA/ASA -Fg	4.6	0	4.3	4
EGTA/ASA CP/CPK	3.6	1	4.3	3
PGE_1	5.5	13	4.0	16
EGTA/PGE_1	2.8	1	3.1	10

Platelets were prepared as in the text and Ref.5, and suspended in 1mM Ca^{2+} -containing media, with additions as indicated. The following concentrations were used: EGTA, 2mM; CP, 4.4mM; CPK, 20U/ml; aspirin (ASA), 1mM; quin2, 2.9mM (intracellular); PGE_1, 1uM. Purified human fibrinogen 400ug/ml was added to all samples except for (-Fg). Data reported are mean of 3-10 determinations.

The difference in $[Ca_i^{2+}]$ response of the two indicators might be due to their relative calcium chelating power; that is, the $[Ca_i^{2+}]$ registered by aequorin might be buffered by quin2 because of its high intracellular concentration. This possibility was tested by loading quin2 and aequorin into the same platelets (Table 1). In dual-loaded platelets, the peak $[Ca_i^{2+}]$ indicated by aequorin in response to both TPA and OAG was decreased (34%-OAG; 14%-TPA) and delayed (from 85 to 118s-OAG; from 118 to 128s-TPA), as was aggregation, in comparison with platelets loaded with ae-

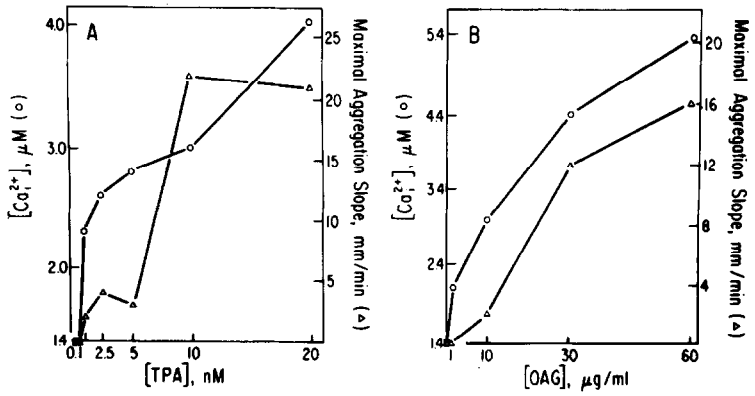

Figure 2: Aggregation (maximum slopes in mm/min) and aequorin-indicated peak $[Ca_i^{2+}]$ after addition of TPA (panel A) and OAG (panel B). Platelets were pre-treated with aspirin and suspended in media containing EGTA 2mM. Aggregation and aequorin luminescence were measured simultaneously in the same sample at 37°C; aliquots of 4 x 10^8 platelets were used.

quorin only. However, addition of quin2 did not totally eliminate the rise in $[Ca_i^{2+}]$ indicated by aequorin with either agonist. No quin2 signal was observed. Thus, aequorin indicates a different aspect of platelet $[Ca_i^{2+}]$ homeostasis from that shown by quin2, rather than merely being more sensitive.

Concentration-response relationships of the TPA and OAG-induced rise in $[Ca_i^{2+}]$ and aggregation are seen in Figure 2. Increased $[Ca_i^{2+}]$ was seen at the concentrations of TPA (1nM) and OAG (1ug/ml) that initiate platelet aggregation. We never saw platelet aggregation by either agonist without an increase in aequorin-indicated $[Ca_i^{2+}]$; this parallels our experience with other agonists, including thrombin, ADP, collagen, epinephrine, and the Ca^{2+}-ionophore A23187 (4).

Clearly, the absence of a quin2 signal may not establish that a metabolic pathway is Ca^{2+}-independent. The intracellular pools of $[Ca_i^{2+}]$ reported by quin2 and aequorin appear to differ, with the aequorin response linked more closely to platelet activation. The source and mechanism of the aequorin-indicated rise in $[Ca_i^{2+}]$ are not established. Although not secondary to aggregation or secretion, a rise in $[Ca_i^{2+}]$ could result from the action of

protein kinase C rather than being required for its activation. However, the timing of the aequorin signal in relation to aggregation, the similarity of the effective agonist concentration ranges, the concentration-dependent nature of both peak [Ca_i^{2+}] and aggregation responses and the inhibition of both by agents that elevate cyclic AMP suggest that an increase in [Ca_i^{2+}] as seen by aequorin is important in TPA and OAG-induced platelet response. There is evidence that local elevations of [Ca_i^{2+}] can occur in other cells and can effectively mediate cell function (6-9). The location of aequorin in the platelet is unknown. It may be fortuitously concentrated in a strategic position to detect [Ca_i^{2+}] rises near the platelet's plasma membrane, which is the proposed site of diacylglycerol-protein kinase C interaction (1) and an important location for agonist-receptor linkage in general.

ACKNOWLEDGEMENTS

This work was supported by NHLBI grants HL25066, HL32677 and HL20079.

REFERENCES

1. Nishizuka, Y. (1984) Nature 308, 693-698.
2. Rink, T.J., Sanchez, A. and Hallam, T.J. (1983) Nature 305, 317-319.
3. Johnson, P.C., Ware, J.A., Cliveden, P.B., Smith, M., Dvorak, A.M. and Salzman, E.W. (1985) J. Biol. Chem. 260, 2069-2076.
4. Ware, J.A., Johnson, P.C., Smith M. and Salzman, E.W. (1985) submitted for publication.
5. Johnson, P.C., Ware, J.A. and Salzman, E.W. (1985) Thromb. Res. in press.
6. Hoffstein, S.T. (1979) J. Immunol. 123, 1395-1402.
7. Rose, B. and Loewenstein, W.R. (1875) Science 190, 1204-1206.
8. Rose, B. and Loewenstein, W.R. (1976) J.Membrane Biol. 87-119.
9. Brown, R.D., Berger, K.D. and Taylor, P. (1984) J.Biol. Chem. 259, 7554-7562.