The role of cytoplasmic free calcium in the responses of quin2-loaded human platelets to vasopressin

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1. Responses to vasopressin were studied in human platelets loaded with the fluorescent Ca^{2+} indicator, quin2. In the presence of 1 mM external Ca^{2+} , vasopressin caused a transient rise in $[\text{Ca}^{2+}]_i$ from the basal level near 100 nM to about 700 nM; peak $[\text{Ca}^{2+}]_i$ was reached in a few seconds and the level then declined towards resting over several minutes. In the absence of external Ca^{2+} there was a much smaller rise of similar time-course, suggesting that vasopressin increases $[\text{Ca}^{2+}]_i$ mainly by stimulated-influx across the plasma membrane but also by partly releasing internal Ca^{2+} . 2. Inhibition of thromboxane A_2 formation somewhat reduced the peak $[\text{Ca}^{2+}]_i$ in the presence of external Ca^{2+} , but had no effect on the response attributed to release of internal Ca^{2+} . 3. With external Ca^{2+} , vasopressin stimulated shape-change, secretion and aggregation. Secretion and aggregation were decreased by about half following blockage of thromboxane production. The ability of vasopressin to induce shape-change and secretion even at near basal $[\text{Ca}^{2+}]_i$ suggests that activators other than Ca^{2+} are involved.

Vasopressin appears to act at two types of membrane receptors. Michell *et al.* (1979) have proposed that the pressor response is mediated by V_1 receptors which cause an increase in $[\text{Ca}^{2+}]_i$ while the antidiuretic effects are mediated by V_2 receptors which stimulate adenylate cyclase and cause an increase in [cyclic AMP] $_i$. It is now clear from many studies, e.g. with isolated hepatocytes (Billah & Michell, 1979; Thomas *et al.*, 1983), that the binding of vasopressin to V_1 receptors also promotes the rapid breakdown of inositol lipids. The increase in $[\text{Ca}^{2+}]_i$ may be partly mediated by inositol trisphosphate causing the discharge of Ca^{2+} sequestered in the endoplasmic reticulum (Joseph *et al.*, 1984; Burgess *et al.*, 1984).

Vasopressin is unlikely to activate platelets through binding to V_2 receptors; cyclic AMP is inhibitory in these cells and vasopressin does not change cyclic AMP levels in human platelets (Haslam & Rosson, 1972). Preliminary experiments with vasopressin antagonists (Thomas *et al.*, 1982) indicate that human platelets have V_1 receptors, which fits in with the report that vasopressin promotes the breakdown of inositol lipids (MacIntyre & Pollock, 1982) and elevates $[\text{Ca}^{2+}]_i$. We report here experiments with human platelets loaded with the fluorescent Ca^{2+} indicator quin2 (Tsien *et al.*, 1982; Rink *et al.*, 1983) which examine the role of $[\text{Ca}^{2+}]_i$ in the response to

vasopressin, the source of activator Ca^{2+} and the role of thromboxane and prostaglandin endoperoxides.

Experimental

Blood was freshly drawn from healthy male donors who denied taking aspirin for the previous 10 days, and mixed with one-sixth volume of acid citrate dextrose (2.5 g of sodium citrate, 1.5 g of citric acid and 2 g of dextrose in 100 ml of water). Platelet-rich plasma was prepared by centrifuging the blood for 5 min at 700 g at room temperature. Platelets were loaded with about 1 mM-quin2 by incubating the platelet-rich plasma with 20 μM -quin2 acetoxymethyl ester at 37°C for 30 min (Hallam *et al.*, 1984). The platelet-rich plasma was then centrifuged at 350 g at room temperature for 20 min. The supernatant was discarded and the cells resuspended in physiological saline {145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 10 mM-glucose and 0.05 unit of hirudin/ml, pH 7.4 at 37°C} at 1.4×10^8 cells·ml⁻¹. The cell suspension was stored at room temperature (20–25°C) in a stoppered plastic tube. The external Ca^{2+} concentration was adjusted by addition of CaCl_2 or $\text{Na}_2\text{H}_2\text{EGTA}$ as required and the cells were equilibrated at 37°C for about 5 min before

use. $[Ca^{2+}]_i$ was estimated from the quin2 fluorescence measured with a Perkin-Elmer MPF 44A spectrophotometer as previously described (Rink *et al.*, 1983; Hallam *et al.*, 1984). The studies were performed in round-bottomed test-tubes held in a purpose-built cuvette holder thermostatted at 37°C. The suspension could be continuously stirred with a magnetic follower. In parallel experiments, secretion of ATP was measured together with shape-change and aggregation in a Chronolog Lumiaggregometer.

Quin2 acetoxymethyl ester was obtained from Lancaster Synthesis (Morecambe, Lancs., U.K.), ionomycin from Squibb, vasopressin, human fibrinogen and hirudin from Sigma, human thrombin from Calbiochem, Chronolume (ATP monitoring reagent) from Coulter Electronics and EGTA from Fluka. Thromboxane B₂ was measured with a radioimmunoassay kit supplied by New England Nuclear.

Results

$[Ca^{2+}]_i$

Addition of vasopressin causes a rapid but transient increase in $[Ca^{2+}]_i$ in the presence of 1 mM external Ca^{2+} (Fig. 1a). In 11 such experiments from six donors addition of a saturating dose of vasopressin, 1 μ M, increased $[Ca^{2+}]_i$ from 127 ± 10 nM (mean $\pm$ S.E.M.) to 736 ± 67 nM. A second application of the same concentration of vasopressin caused no further increase in $[Ca^{2+}]_i$,

although addition of thrombin or A23187 (Fig. 1a) produced an increase in $[Ca^{2+}]_i$, similar to that seen if thrombin or A23187 were added alone, the rise being larger and longer sustained than that to vasopressin.

The experiments shown in Fig. 1 were done without continuous stirring and without added fibrinogen. Under these conditions there was too little cell-cell contact for aggregation to occur. If fibrinogen, 1 mg/ml, was present and the platelets were stirred, conditions normally used for studies of aggregation, then the quin2 fluorescence signal rapidly declined after the peak to below the resting level due to the formation of large aggregates, and the $[Ca^{2+}]_i$ became impossible to monitor (Hallam *et al.*, 1984; Rink *et al.*, 1983). However, the peak $[Ca^{2+}]_i$ was reached before the formation of large aggregates when the cells were continuously stirred in the presence of fibrinogen and was not discernibly different from the peak $[Ca^{2+}]_i$ achieved in unstirred preparations. Therefore, by not allowing aggregation to occur the effect of a second addition of vasopressin or ionomycin on the $[Ca^{2+}]_i$ can be monitored.

Fig. 1(b) shows the effect of vasopressin on $[Ca^{2+}]_i$ in the absence of added Ca^{2+} and with 1 mM-EGTA. Under these conditions the rise in $[Ca^{2+}]_i$ is assumed to be due to release of intracellular calcium. Again vasopressin produces a transient, though much smaller, increase in $[Ca^{2+}]_i$. In seven such experiments $[Ca^{2+}]_i$ increased from 82 ± 5 nM to reach a peak at 135 ± 16 nM. A further addition

Fig. 1. Effect of external Ca^{2+} and cyclo-oxygenase inhibition on $[Ca^{2+}]_i$ responses to vasopressin. The Figure shows the effect of vasopressin on $[Ca^{2+}]_i$ in the presence (a and c) or absence (b and d; 1 mM-EGTA) of external Ca^{2+} ; (c) and (d) show the effect of 100 μ M-aspirin. Vasopressin (VP, 1 μ M), A23187 (50 nM) or thrombin (0.5 unit/ml) were added as indicated. The appropriate calibration scales for $[Ca^{2+}]_i$ are shown at the left side of each fluorescence trace. Gaps in the recording show periods of addition.

of the same dose of vasopressin had no further effect; but thrombin, 0.5 unit/ml, or calcium ionophore, 50 nM-A23187, caused a further increase in $[Ca^{2+}]_i$ similar to that seen under these conditions to those agents alone. The increase in $[Ca^{2+}]_i$ was larger with thrombin or A23187, suggesting that vasopressin can cause only partial discharge of the internal stores of Ca^{2+} .

Stimulation of platelets by most agonists can lead to release of arachidonic acid and consequent formation of prostaglandin endoperoxides and thromboxane A_2 . Vasopressin is no exception. In the presence of 1 mM external Ca^{2+} , 1 mg of fibrinogen/ml and with continuous stirring, 1 μ M-vasopressin caused the production of approx. 470 pg of thromboxane B_2 per 10^8 cells in a 3 min period. This is comparable with the amounts released by platelet-activating factor (T. J. Hallam & T. J. Rink, unpublished work) but considerably less than that produced by thrombin, 1500 pg/ 10^8 cells, or collagen, 1300 pg/ 10^8 cells (Best *et al.*, 1980).

It has been proposed that thromboxane A_2 plays an important part in mobilizing Ca^{2+} , particularly by discharging of Ca^{2+} from internal stores on stimulation by other agonists (Gerrard *et al.*, 1981) and we have reported that arachidonate and stable analogues of thromboxane A_2 can increase $[Ca^{2+}]_i$,

though mainly by stimulated Ca^{2+} influx (Hallam *et al.*, 1983). In order to examine this proposal, we compared responses to vasopressin in the presence or absence of 100 μ M-aspirin (which irreversibly inhibits cyclo-oxygenase and prevents formation of thromboxane A_2 or prostaglandin endoperoxides). The traces in Figs. 1(c) and 1(d) show typical data from these experiments.

In the presence of 1 mM external Ca^{2+} , blockade of cyclo-oxygenase slightly reduced the $[Ca^{2+}]_i$ rise produced by 1 μ M-vasopressin. In nine paired trials, stimulation with 1 μ M-vasopressin in the absence of a cyclo-oxygenase inhibitor resulted in a rise in $[Ca^{2+}]_i$ from 105 ± 5 nM to 736 ± 65 nM. In the presence of 100 μ M-aspirin, $[Ca^{2+}]_i$ rose from 113 ± 4 nM to 535 ± 44 nM.

At very low external Ca^{2+} , where only discharge of Ca^{2+} from internal stores can contribute to the increase in $[Ca^{2+}]_i$, blockade of cyclo-oxygenase had no effect (Figs. 1b and 1d). In seven paired trials, stimulation with 1 μ M-vasopressin resulted in a rise in $[Ca^{2+}]_i$ from 82 ± 7 nM to 135 ± 21 nM in control conditions, while in aspirin-treated cells $[Ca^{2+}]_i$ rose from 81 ± 5 nM to 132 ± 18 nM.

Shape-change, secretion and aggregation

Fig. 2 shows recordings of absorbance and luminescence as a measure of shape-change,

Fig. 2. Effect of external Ca^{2+} and cyclo-oxygenase inhibition on the aggregatory and secretory responses to vasopressin in quin2-loaded human platelets

The Figure shows the simultaneously recorded changes in absorbance and luminescence following stimulation with 1 μ M-vasopressin (VP). Conditions were with 1 mM-external Ca^{2+} (a and c) or 1 mM-EGTA (b and d) and in the absence (a and b) or presence (c and d) of 100 μ M-aspirin.

aggregation and secretion of ATP, from the same batch of quin2-loaded platelets shown in Fig. 1, and under the same conditions (except for the addition of luciferin/luciferase). In the presence of 1 mM external Ca^{2+} , vasopressin, 1 μM , caused shape-change, aggregation and a rapid secretion of ATP. Responses were not discernibly different at doses of 0.1 μM -vasopressin. At approx. 10^{-7}M external Ca^{2+} , in the presence of 1 mM-EGTA, vasopressin still produced shape-change which was almost as rapid as that observed in the presence of 1 mM external Ca^{2+} . There was some secretion, though only 15% of that seen in 1 mM external Ca^{2+} . No aggregation occurred, although this probably reflects a requirement for external Ca^{2+} ions (Phillips & Baughan, 1983).

Inhibition of cyclo-oxygenase had no effect on shape-change but reduced the extent of aggregation and secretion evoked by vasopressin in the presence of 1 mM-external Ca^{2+} (Fig. 2c). In nine trials the peak ATP secretion was reduced from $66 \pm 3\%$ (of that maximally releasable by thrombin) in controls to $31 \pm 2\%$ in cells preincubated with 100 μM -aspirin. In the absence of external Ca^{2+} ions, the small secretory response was not measurably affected by blockade of cyclo-oxygenase, cf. Figs. 2(b) and 2(d). In six paired trials of this kind, ATP secretion in control cells was $9 \pm 2\%$, and in aspirin-treated cells vasopressin caused secretion of $9 \pm 1\%$ of maximal releasable ATP.

Discussion

Vasopressin causes a transient rise in $[\text{Ca}^{2+}]_i$ in quin2-loaded human platelets, supporting the idea that the cells have V_1 receptors. The rise in $[\text{Ca}^{2+}]_i$ is largely dependent on external Ca^{2+} and is only slightly reduced by inhibition of thromboxane production. With very low external Ca^{2+} the response is much reduced. The simplest explanation for this result is that normally most of the $[\text{Ca}^{2+}]_i$ rise results from stimulated Ca^{2+} influx. The residual response in low- Ca^{2+} medium presumably reflects discharge of Ca^{2+} from Ca^{2+} -sequestering organelles. Under these conditions calcium ionophore can produce a larger rise in $[\text{Ca}^{2+}]_i$ than can vasopressin, and after application of Ca^{2+} ionophore vasopressin produces no further effect (results not shown), suggesting that the ionophore releases Ca^{2+} from the same pool accessible to vasopressin. Ca^{2+} ionophore is expected to translocate Ca^{2+} across membranes from regions of high to low $[\text{Ca}^{2+}]_i$ and the occlusion of the responses to ionophore and vasopressin suggests that vasopressin somehow promotes a similar translocation of Ca^{2+} . The mechanisms by which the binding of vasopressin to its receptor stimulates influx or internal release of Ca^{2+} are unknown. We suspect

that Ca^{2+} influx results from the opening of receptor-operated channels; the internal release might be triggered by inositol trisphosphate, as has been shown in pancreas (Streb *et al.*, 1983) and in liver (Joseph *et al.*, 1984), but so far there is no evidence available in platelets. Our results argue against a significant role for thromboxane in the internal release of Ca^{2+} .

The transient nature of the $[\text{Ca}^{2+}]_i$ response in both the presence and the absence of external Ca^{2+} may reflect rapid desensitization of the receptor. A second application of vasopressin was ineffective and rapid desensitization to vasopressin has been found in previous experiments (Hallam *et al.*, 1982). It may be that Ca^{2+} channels open only briefly, allowing a pulse of Ca^{2+} into the cytoplasm which rapidly elevates $[\text{Ca}^{2+}]_i$ and is then pumped out across the plasma membrane and back into the Ca^{2+} -sequestering organelles, returning $[\text{Ca}^{2+}]_i$ towards resting levels. It seems unlikely that the transient nature of the response reflects inactivation of the mechanisms that promote Ca^{2+} flux, since thrombin added after vasopressin elicits a full response. It should be pointed out that the presence of approx. 1 mM intracellular quin2 can reduce the level of $[\text{Ca}^{2+}]_i$ reached after application of the agonist, particularly in the absence of external Ca^{2+} (Pozzan *et al.*, 1982; Tsien *et al.*, 1982), since it adds appreciably to the Ca^{2+} -buffering capacity of the cytoplasm.

The elevation of $[\text{Ca}^{2+}]_i$ evoked by vasopressin is fast enough for it to be a cause of the observed cellular responses. However, as we have found with other agonists (Rink *et al.*, 1982, 1983; Hallam *et al.*, 1984), it is clear that vasopressin activates excitatory pathways other than elevated $[\text{Ca}^{2+}]_i$. In approx. 10^{-7}M - Ca^{2+} medium, where $[\text{Ca}^{2+}]_i$ rises only to 170 nM, vasopressin can still evoke shape-change and secretion; a similar elevation of $[\text{Ca}^{2+}]_i$ produced by Ca^{2+} ionophore is ineffective (Rink *et al.*, 1982; Hallam *et al.*, 1984). These responses cannot be mediated by thromboxane or prostaglandin endoperoxides since they are not blocked by aspirin. The secretory response may be mediated by diacylglycerol formed as a result of stimulated breakdown of inositol lipids (Kaibuchi *et al.*, 1983; Rink *et al.*, 1983). We do not know what triggers the shape-change.

Thromboxane formation does play a part in the response observed in media containing a physiological concentration of Ca^{2+} . The rise in $[\text{Ca}^{2+}]_i$ is somewhat larger when thromboxane formation is permitted, and the extent of secretion and aggregation is reduced by blockade of cyclo-oxygenase. Thromboxane may well reinforce the response by increasing hydrolysis of inositol phospholipids (MacIntyre & Pollock, 1982; Siess *et al.*, 1983) as well as by causing further stimulated Ca^{2+} influx.

Finally, the responses to vasopressin are uncannily like those we have previously seen with platelet activating factor (Hallam *et al.*, 1984). It seems that in liver cells too, so far as has been tested, these two agonists have closely similar effects (cf. Shukla *et al.*, 1983; Buxton *et al.*, 1984; Thomas *et al.*, 1983). Their very different structures make it unlikely that they act at the same receptor site. However, preliminary experiments show some cross-desensitization of the $[Ca^{2+}]_i$ rise evoked by platelet-activating factor and vasopressin, perhaps indicating that they share common activation mechanisms.

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