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Lack of evidence for voltage dependent calcium channels on platelets

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Intracellular calcium was measured in human platelets using the fluorescent calcium indicator Quin 2. A concentration dependent increase was observed with thrombin. Depolarisation induced by high KCl concentrations did not alter $[Ca^{++}]_i$. The calcium agonist Bay K 8644 did not affect resting levels or thrombin stimulated elevation of intracellular calcium. The calcium antagonists diltiazem, verapamil and PN 200-110 did not inhibit the thrombin stimulated elevation in $[Ca^{++}]_i$. Pretreatment of platelets with adenylate cyclase stimulants reduced the rate and magnitude of the maximal $[Ca^{++}]_i$ elevation due to thrombin. In addition, thrombin stimulation of $^{45}Ca^{++}$ influx was insensitive to Bay K 8644, verapamil, diltiazem and PN 200-110. We conclude that functional voltage sensitive calcium channels are not present on human platelets.

The platelet has been extensively studied with respect to agonist induced shape change, aggregation and secretion (1). Changes in intracellular free calcium have been implicated in many of these processes. The importance of calcium as a second messenger has focussed attention on free cytoplasmic calcium concentrations $[Ca^{++}]_i$, intracellular calcium stores and mechanisms controlling influx and extrusion of Ca^{++}. The two major pathways of calcium entry into the cell are potential sensitive channels and receptor operated channels (2). Extrusion of calcium from the cytoplasm is under the control of Ca^{++}ATPase (3), in excitable cells this acts in combination with the Na^{+}/Ca^{++} exchange system (4). Cyclic AMP reduces $[Ca^{++}]_i$ by a mechanism which is thought to involve stimulation of uptake into the intracellular calcium stores (5) or inhibition of calcium mobilization into the cytosol (6). Therefore, platelet activation by most agonists is prevented by increases in cyclic AMP brought about by substances such as prostaglandin E_1, prostacyclin (PGI_2) and forskolin.

A direct method of measuring $[Ca^{++}]_i$, the fluorescent calcium chelator Quin 2, has become available which enables continuous monitoring

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of the calcium signal during activation (7,8). At present, this method appears to be optimal for measuring $[Ca^{++}]_i$ since it can be used in small cells without disruption, has an easily detectable fluorescent signal, is sufficently sensitive and has kinetics fast enough to follow calcium transients which occur during platelet activation (9,10).

Using thrombin as a stimulant, we have examined agents which modify this response. Attention was focussed in particular on a variety of calcium antagonists as well as a recently described calcium agonist, Bay K 8644, which is known to act directly on the potential sensitive calcium channel to increase the inward calcium current (11,12).

MATERIALS AND METHODS

Quin 2 AM, Bay K 8644 and PN 200-110 were prepared by SANDOZ, Basle. Thrombin 63 NIH-U/mg, Roche; Sepharose 2B-CL, Pharmacia. All other reagents were obtained either from Sigma or Fluka and were of the highest purity available.

Samples of whole blood were taken into acid citrate dextrose and centrifuged at 200xg for 20 min at room temperature to obtain platelet rich plasma (PRP). Platelets were separated at room temperature by two different methods: (a) on a Sepharose 2B-CL column (2.5x20cm) which had been preequilibrated with Buffer 1 (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 6 mM glucose, 1 mM $MgSO_4$, 5 mM Hepes and 0.1% bovine serum albumin, pH 7.4). Elution was carried out at a flow rate of 30 ml/h and peak fractions were pooled. (b) By further centrifugation of PRP at 200xg, in the presence of 5 nM PGI_2; this removed remaining erythrocytes, the resulting platelet suspension was centrifuged at 600xg for 20 min at room temperature (13).

For Quin 2 measurements the platelet suspension was incubated for 30 min with 5 µM Quin 2 AM at 37°C in Buffer 1 with 1 mM $CaCl_2$. Excess Quin 2 AM was separated from the platelets by passing the suspension through the Sepharose column. Fluorescence was measured in a Kontron spectrofluorometer SFM 23 with a slit width of 2 nm in a thermostatically controlled cuvette at 37°C. The samples (10^8 cells/ml) were excited at 340 nm and the emitted light read at 490 nm. Baseline (F) and stimulated (F') levels were measured, F_{min} and F_{max} were determined as described by Tsien et al. (8).

For measurement of $^{45}Ca^{++}$ and $^{86}Rb^{+}$ influx, the platelets were resuspended in Buffer 2 (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4 at 37°C) to a final concentration of 10^8 cells/ml. Aliquots (0.5 ml) of the platelet suspension were incubated in triplicate with either 1 µCi $^{45}Ca^{++}$ or 0.5 µCi $^{86}Rb^{+}$ at 37°C. The incubation was terminated by the addition of 5 ml ice cold Buffer 2 containing in addition 5 mM EGTA, followed by rapid filtration through glass fibre filters (Gelman type A/E, 25 mm) and two 5 ml washes. The radioactivity remaining on the filters was determined by liquid scintillation counting.

RESULTS

The resting intracellular concentration of free calcium $[Ca^{++}]_i$ in platelets as assayed by the Quin 2 method is 108 ± 4nM (n=72). A variety of substances examined over the concentration range 10^{-8} - 10^{-5} M, had no effect on resting $[Ca^{++}]_i$; these include epinephrine, norepinephrine, isoprenaline, histamine, PGE_1, forskolin, theophylline. Stimulation with thrombin over the range 0.05 - 0.6 U/ml resulted in a concentration dependent 3-20 fold increase in $[Ca^{++}]_i$ over basal levels. Preincubation of the platelets with PGE_1 (1 μM) or forskolin (1 μM), decreased the rate and magnitude of the maximal response to thrombin. In contrast, preincubation with 1 μM isoprenaline, histamine or 5-HT had no effect on the rate or magnitude of the maximal response. Addition of PGE_1 or forskolin after thrombin stimulation reduced the time required for the stimulated calcium to return to baseline level (Fig. 1a); this was potentiated by theophylline.

Incubation of Quin 2 loaded platelets with the calcium antagonists verapamil, diltiazem, PN 200-110 or with the calcium agonist Bay K 8644 (Fig 1b) over the concentration range 10^{-8} - 10^{-5}M, did not alter the resting calcium level to a significant degree. Depolarisation of the platelets with 50 mM KCl either alone or in combination with Bay K 8644 yielded a negative result. Preincubation with diltiazem or PN 200-110 (10^{-8} - 10^{-5}M) did not inhibit either the rate or magnitude

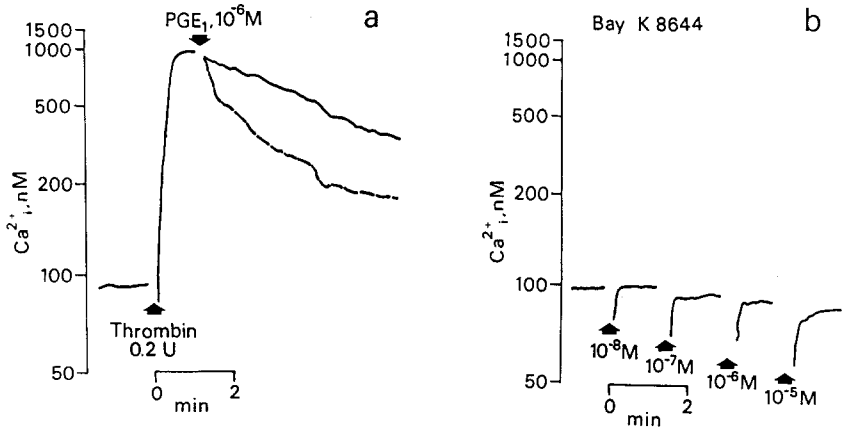

Figure 1: (a) The effect of 1 μM PGE_1 (– – –) on the thrombin (0.2 U/ml) stimulated increase in $[Ca^{2+}]_i$ (——). PGE_1 was added after thrombin as indicated. Basal $[Ca^{2+}]_i$ was 96 nM, thrombin stimulation increased this to 980 nM.

(b) The response of washed platelets to BAY K 8644 (10^{-8} - 10^{-5}M).

of the thrombin stimulated increase in $[Ca^{++}]_i$. At relatively high concentrations (10^{-5}M), verapamil increased the time required to reach the plateau from 40 sec to 3 min without changing the maximal response. Addition of Bay K 8644 (10^{-8} - 10^{-5}) both before and after thrombin stimulation did not have any effect.

The time course of $^{45}Ca^{++}$ uptake into washed platelets was measured. Using thrombin (0.05 - 0.5 U/ml) as a stimulant, an initial rapid uptake was seen (Fig. 2). The increase was concentration dependent over the range 0.05 - 0.5 U/ml. A concentration of 0.2 U/ml was chosen as a sufficient stimulus to give an easily measurable increase in $[Ca^{++}]_i$. Epinephrine (10^{-8} - 10^{-4}M), did not increase $^{45}Ca^{++}$ uptake significantly. The addition of Bay K 8644 or any of the antagonists over the concentration range 10^{-8} - 10^{-5}M, did not in any way effect the unstimulated uptake of $^{45}Ca^{++}$ into the washed platelets (Table 1). As already observed with Quin 2, depolarisation with 50 mM KCl either alone or in combination with calcium channel modulators did not influence $^{45}Ca^{++}$ uptake. When these drugs were added simultaneously with thrombin or 10 min prior to the addition of thrombin, the increase in $[Ca^{++}]_i$ due to thrombin (0.2U/ml) was not affected.

The possible existence of a Na^+/Ca^{++} exchange system was indirectly investigated by blockade of the Na^+/K^+ ATPase. This causes $[Na^+]_i$ to increase and in theory, should cause an increase in Ca^{++} uptake via the Na^+/Ca^{++} exchange mechanism (4). Treatment with 10 µM ouabain (15 min preincubation) resulted in a 41 $\pm$ 6% (n=3) decrease in $^{86}Rb^+$ uptake. However, this did not have any effect on either the resting

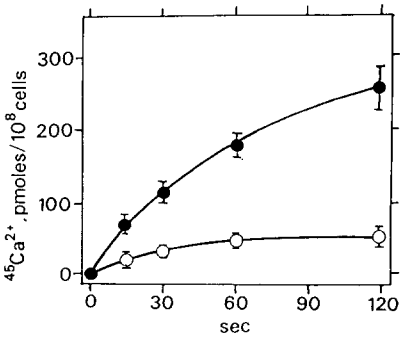

Figure 2: The time course of unstimulated (—○—) and 0.2 U/ml thrombin-stimulated (—●—) $^{45}Ca^{2+}$ influx in washed platelets over 2 min.

Table 1

Effect of calcium antagonists and Bay K 8644 (1 μM) on basal KCl (50 mM) depolarisation and thrombin (0.2 U/ml) stimulation of $^{45}Ca^{2+}$ influx. The incubation time was 2 min and the results are expressed as % of the control values for n≥3. (Basal $^{45}Ca^{2+}$ influx: 56 ± 6 pmoles/10^8 cells, n=12; KCl-stimulated $^{45}Ca^{2+}$ influx: 54 ± 4 pmoles/10^8 cells, n=6; thrombin stimulated $^{45}Ca^{2+}$ influx: 195 ± 16 pmoles/10^8 cells, n=9).

	Basal	KCl	Thrombin
Diltiazem	118 ± 9	104 ± 1	103 ± 2
Verapamil	113 ± 18	106 ± 2	92 ± 6
PN 200-110	123 ± 6	94 ± 2	102 ± 4
Bay K 8644	100 ± 16	93 ± 6	104 ± 8

or thrombin stimulated increase in $[Ca^{++}]_i$ and did not influence $^{45}Ca^{++}$ uptake (Table 2).

DISCUSSION

The main object of this investigation was to identify the mechanisms of calcium entry into the platelet, using a variety of pharmacological tools as probes. We have confirmed that thrombin causes an elevation of $^{45}Ca^{++}$ influx into the cell (14) suggesting an increase in membrane permeability with respect to calcium. It is known that part of the increase in $[Ca^{++}]_i$ is due to release from intracellular stores (6) but the main part is influx of calcium. The results presented here show that calcium influx due to thrombin cannot be blocked by a variety of calcium antagonists, indicating that the influx occurs via a pathway which is different from the potential sensitive calcium channel.

Table 2

The effect of 10^{-5} M ouabain (15 min preincubation) on platelet resting and thrombin stimulated $[Ca^{2+}]_i$ as measured by Quin 2, n=3, and $^{45}Ca^{2+}$ influx (pmoles/10^8 cells/2 min), n=3.

	Resting		Thrombin-stimulated	
	$[Ca^{2+}]_i$ (nM)	$^{45}Ca^{2+}$	$[Ca^{2+}]_i$ (nM)	$^{45}Ca^{2+}$
Control	70 ± 6	61 ± 2	880 ± 80	248 ± 10
Ouabain	70 ± 7	66 ± 2	930 ± 90	276 ± 3

Depolarisation of the platelets with 50 mM KCl, the "classical" method for testing for potential sensitive calcium channels (15,16,17) did not effect either the $[Ca^{++}]_i$ or $^{45}Ca^{++}$ influx (Table 1). Resting $[Ca^{++}]_i$ and unstimulated $^{45}Ca^{++}$ influx were unaffected by calcium antagonists or by the calcium agonist Bay K 8644. It has been reported that calcium channel agonists alone cannot activate calcium channels and that a moderate depolarising stimulus is often required for this type of drug to manifest its potentiating action (18,19,20). However, even with prior depolarisation, Bay K 8644 did not effect either $[Ca^{++}]_i$ or $^{45}Ca^{++}$ influx. A recent report has indicated that CGP 28392, a calcium agonist which is structurally different from Bay K 8644, increases $[Ca^{++}]_i$ in platelets (21).

Our results confirm the observation that $[Ca^{++}]_i$ levels in the platelet are regulated by cyclic AMP (22,23,24). Pretreatment with agents that stimulate adenylate cyclase, prostaglandin E_1 and forskolin, reduce both the rate and magnitude of the maximal response to thrombin stimulation. Addition of these substances after thrombin stimulation causes the elevated $[Ca^{++}]_i$ to rapidly return to the resting level (Fig. 1a). In addition, we have confirmed the observation that activation by epinephrine is independent of calcium influx and intracellular release (14,22,25,26), which however is disputed (27,28) when a different methodological approach is used.

It has been suggested that the increase in $[Na^+]i$ caused by the inhibition of Na^+/K^+ATPase could cause an increase in $[Ca^{++}]_i$ by a decreased activity of the Na^+/Ca^{++} exchange system (29). The increase in $[Ca^{++}]_i$ seen in platelets and other cells in hypertensive patients (30,31) has been explained in part by this hypothesis (29). Our results demonstrate that inhibition of the uptake of $^{86}Rb^+$, indicating Na^+/K^+ATPase blockade, did not influence either $[Ca^{++}]_i$ or $^{45}Ca^{++}$ influx. It appears therefore that a Na^+/Ca^{++} exchange mechanism if present on the human platelet does not play a major role in Ca^{++} homoestasis. In summary, using classical stimulants (high KCl) and a well described platelet stimulant (thrombin) as well as a calcium agonist and antagonists, we have found no evidence for functional potential sensitive calcium channels on platelets.

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