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Cyclic AMP stimulates Ca^{2+}-ATPase-mediated Ca^{2+} extrusion from human platelets

Jonas S. Johansson, Linda E. Nied and Duncan H. Haynes

Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, FL (USA)

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The effect of cAMP on active Ca^{2+} extrusion across the plasma membrane of intact human platelets was studied using quin2, a fluorimetric indicator of free Ca^{2+} in the cytoplasmic compartment ($[Ca^{2+}]_{cyt}$). Elevations of cAMP were achieved by incubation with dibutyryl-cAMP or by forskolin, which was found to selectively elevate cAMP without affecting cGMP levels. Progress curves of Ca^{2+} extrusion from quin2-overloaded platelets were measured. The rate vs. $[Ca^{2+}]_{cyt}$ characteristic was calculated as previously described (Johansson, J.S. and Haynes, D.H. (1988) J. Membr. Biol. 104, 147–163). Forskolin, at a maximally effective concentration of 10 μM, was shown to stimulate Ca^{2+} extrusion by increasing by a factor of 1.6 ± 0.5 the V_m of a saturable component, previously identified with a Ca^{2+}-Mg^{2+}-ATPase located in the plasma membrane. Neither the K_m (80 nM) or Hill coefficient (1.7 ± 0.3) of the Ca^{2+}-ATPase was affected. Forskolin had no effect on the linear, non-saturable component of extrusion (previously identified with a Na^+/Ca^{2+} exchanger) over the $[Ca^{2+}]_{cyt}$ range examined (50–1500 nM). Dibutyryl-cAMP (Bt_2-cAMP, 1 mM) stimulated the Ca^{2+}-Mg^{2+}-ATPase component of Ca^{2+} extrusion by a factor of 2.0 ± 0.6. Separate experiments showed that 10 μM forskolin reduces the resting $[Ca^{2+}]_{cyt}$ from 112 nM to 96 nM. Mathematical analysis showed that this can be accounted for by the above-mentioned increase in V_m of the pump, countered by a 37–74% increase in the rate constant for passive Ca^{2+} leakage across the plasma membrane. The results suggest two mechanisms by which prostacyclin-induced elevation of cAMP inhibits platelet aggregation: (a) lowering of resting $[Ca^{2+}]_{cyt}$ and (b) increasing the rate of Ca^{2+} extrusion after the initial influx or triggered release event.

Introduction

The blood platelet is a Ca^{2+}-activated cell which plays an important role in blood coagulation. Elevation of the Ca^{2+} concentration in its cytoplasm is a necessary and sufficient event in its activation [1,2] which involves a shape change and exocytotic processes leading to aggregation [3–6]. The resting platelet maintains a cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in the 100 nM range [7,8]. This resting $[Ca^{2+}]_{cyt}$ is determined by the balance between passive Ca^{2+} influx and active Ca^{2+} extrusion across the plasma membrane (cf. Ref. 9). The present paper is the first in a series dealing with the effects of cyclic nucleotides on platelet Ca^{2+} handling. It will show that elevated cAMP increases the rate of Ca^{2+} extrusion.

The Ca^{2+} handling systems of the platelet are schematized in Fig. 1. The events in platelet activation and Ca^{2+} movements therein will be described in the companion paper [10]. In the resting state $[Ca^{2+}]_{cyt}$ is

Abbreviations: $[Ca^{2+}]_{cyt}$, the free ('ionized') Ca^{2+} concentration in the cytoplasm; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; quin2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline; quin2/AM, tetraacetoxymethyl ester form of quin2; V and V_m, the velocity and maximal velocity (respectively) of the Ca^{2+}-ATPase extrusion pump located in the plasma membrane; cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; Bt_2-cAMP, N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; CTC, chlorotetracycline; PM, plasma membrane; ROC, receptor-operated channel; K_m, the $[Ca^{2+}]_{cyt}$ giving half-maximal rate of extrusion (V); PGE_1, prostaglandin E_1; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; k_{leak}, rate constant for passive leakage across the PM; k_{linear}, apparent bimolecular rate constant for Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger, equal to its V_m/K_m quotient); X, see Eqn. 4; Fl_{max}, fluorescence of Ca^{2+} complexed form of quin2; Fl_{min}, fluorescence of the uncomplexed form of quin2.

Correspondence: D.H. Haynes, Department of Pharmacology, University of Miami School of Medicine, P.O. Box 016-189, Miami, FL 33101, USA.

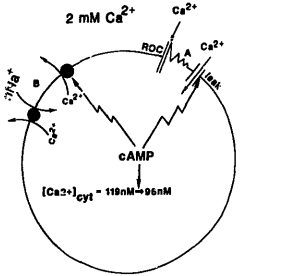

Fig. 1. Schematic representation of the Ca^{2+} influx (A) and extrusion (B) systems determining $[Ca^{2+}]_{cyt}$ in the human platelet platelet at rest. The opened channel represents the passive Ca^{2+} leak. The closed channel represents a receptor-operated channel (ROC). Arrows indicate direction of ion movement. The n value or Na^+/Ca^{2+} stoichiometry of the Na^+/Ca^{2+} exchanger has not yet been determined. The zig-zag arrows indicate loci of stimulation with cAMP (demonstrated in the present study). For the sake of simplicity, the dense tubular system is not represented.

determined by the balance between passive leak and active extrusion processes across the plasma membrane (PM). The platelet PM has a cadmium-sensitive, verapamil-insensitive passive Ca^{2+} permeability (cf. Ref. 11). Passive influx is opposed by an active extrusion system. Evidence supporting the presence of plasmalemmal Ca^{2+}-Mg^{2+}-ATPase [9,12–15] and a Na^+/Ca^{2+} exchanger [9,16–18] activities has been published. In the resting state, $[Ca^{2+}]_{cyt}$ is determined by the balance of the influx and active extrusion pathways [7,9,11]. The dense tubular system is the major internal storage site for Ca^{2+} [7,19]. The dense tubules have a Ca^{2+}-ATPase pump [19,20] enabling the accumulation of Ca^{2+} which is released to the cytoplasm upon stimulation. Through the above mechanisms the dense tubules are capable of influencing $[Ca^{2+}]_{cyt}$ *transiently*. By design of the present study, the dense tubular Ca^{2+} uptake is eliminated by the shunting action of 1.0 μM ionomycin * and by the short duration of exposure to elevated $[Ca^{2+}]_{cyt}$. Thus the activity of the Ca^{2+} extrusion system of the plasma membrane can be studied directly by the rate of decrease of quin2 fluorescence.

* 1.0 μM ionomycin does not contribute to the extrusion process at $[Ca^{2+}]_{cyt}$ values of ≤ 1.5 μM because (cf. Johansson and Haynes, 1988 [9]) the latter is 4 orders of magnitude lower than its K_m for Ca^{2+} (7.7 mM).

Cyclic nucleotides have been advanced as serving important *modulatory* functions in platelets [3,21]. The anti-aggregatory mechanisms of cAMP have received most attention. A physiological stimulus for elevation of cAMP is prostacyclin, which has been shown to decrease platelet aggregation. Prostacyclin is secreted by the capillary endothelium. The effects of elevated platelet cAMP include (a) the inhibition of agonist-induced phospholipase A_2 activation [22], (b) reduction of inositol-lipid hydrolysis [23], (c) decrease in inositol 1,4,5-trisphosphate (IP_3) stimulated Ca^{2+} release [24] and (d) reduction of $[Ca^{2+}]_{cyt}$ elevations after stimulation [25]. The latter are based on behavior of agonist-induced Ca^{2+} transients involving changes in Ca^{2+} influx and dense tubular Ca^{2+} release [26–30]. Elevation of cAMP causes a slight decrease in resting $[Ca^{2+}]_{cyt}$ [31]. The present communication will show that cAMP stimulates the extrusion pump and that this is an important factor underlying the above observations.

In the present study we use a recently-developed method to characterize extrusion of Ca^{2+} across the plasma membrane [9]. The method involves overloading the cytoplasm with quin2 to levels of approx. 3 mmol per liter cell volume (3 mM) such that its Ca^{2+} buffer capacity is much larger than the Ca^{2+} buffer capacity intrinsic to the cytoplasm. Under this quin2-overloaded condition, the quin2 fluorescence is a linear measure of the number of mmol of Ca^{2+} entering or leaving the cytoplasm [9]. The method enables the measurement of the absolute rate of active Ca^{2+} extrusion (via a Ca^{2+}-ATPase pump and a Na^+/Ca^{2+} exchanger) as a function of $[Ca^{2+}]_{cyt}$. The previous study [9] showed that the pump has a V_m of 98 ± 8 μmol Ca^{2+}/liter cell volume/min, a K_m of 80 ± 10 nM, and a Hill coefficient of 1.7 ± 0.3. For $[Ca^{2+}]_{cyt} \leq 400$ nM, the pump makes a much larger contribution to extrusion than does the Na^+/Ca^{2+} exchanger. In the present communication we show that elevated cAMP promotes Ca^{2+} extrusion across the plasma membrane by increasing the maximum rate (V_m) of the Ca^{2+}-ATPase pump.

Part of this work has appeared in abstract form [32].

Materials and Methods

Materials

Forskolin, prostaglandin E_1 (PGE_1), EGTA, Hepes, quin2/AM, quin2, glucose, and dibutyryl-cAMP (Bt_2-cAMP) were purchased from Sigma Chemical Co., St. Louis, MO. Chlorotetracycline (CTC) was obtained from ICN Pharmaceuticals, Cleveland, OH, ionomycin from Calbiochem, La Jolla, CA. Reagents used in the preparation of Tyrode's solution were supplied by Mallinkrodt Inc., Paris, KY. Radioimmunoassay kits for cAMP and cGMP were obtained from Amersham

Corporation, Arlington Heights, IL, and Ecolume[R] scintillation cocktail from ICN Biomedicals, Irvine, CA.

Platelet isolation

Platelets were isolated from whole blood as described previously [11]. The medium for resuspension and for all presented experimentation was a nominally Ca^{2+}- and Mg^{2+}-free Tyrode's solution ($[Ca^{2+}] \approx 1$ μM) of the following composition: 138 mM NaCl/3 mM KCl/10 mM glucose/2 mM $NaHCO_3$/0.4 mM NaH_2PO_4/2.5 mM Hepes with the pH adjusted to 7.35. The platelets lose Ca^{2+} as they approach a new steady state with this low external Ca^{2+} concentration ($[Ca^{2+}]_o$). The cells are therefore referred to as being Ca^{2+}-depleted at the end of the isolation procedure. All experimentation was completed within 4 h of venipuncture.

Fluorometry

All fluorescence measurements were made with a Perkin-Elmer (Model MPF-3L) fluorometer. The wavelengths and filters used, stirring mechanism and precautions taken against possible artifacts have all been described [11]. A platelet concentration of $1.6 \cdot 10^7$ per ml was used for all fluorometric experimentation. This was routinely measured turbidimetrically as an $OD_{600nm} = 0.20$ using a Beckman DB-G grating spectrophotometer and was occasionally verified with a Bright-Line hemacytometer (American Optical).

Use of quin2 to measure the average $[Ca^{2+}]_{cyt}$ and absolute rate of Ca^{2+} extrusion

The theory and methods underlying the use of quin2 for studying Ca^{2+} extrusion from the platelet have been detailed in a previous publication [9]. Platelets are incubated with 20 μM quin2/AM (achieving intracellular dye concentrations of approx. 3 mmol per liter cell volume) to ensure that the fluorophore represents the major cytoplasmic Ca^{2+} buffer (quin2 'overload' condition). In the presence of 2 mM external Ca^{2+} ($[Ca^{2+}]_o$), the addition of 1 μM ionomycin results in rapid flooding of the cytoplasm with Ca^{2+}. Upon saturation of the cytoplasmic dye, EGTA (2.7 mM) is added to reduce $[Ca^{2+}]_o$ to approx. 100 nM. Then active extrusion of Ca^{2+} from the cytoplasm is measured as a function of time. Experimentation with chlorotetracycline fluoresence showed that the internal storage sites are unable to take up Ca^{2+} in the presence of this concentration of ionomycin during the brief exposure to high $[Ca^{2+}]_{cyt}$. Thus the dense tubules do not contribute significantly to the observed decrease in $[Ca^{2+}]_{cyt}$ (cf. Fig. 5 Ref. 9 and Fig. 3 of the present study). When EGTA is used for Ca^{2+} removal, the EGTA stock solution is made slightly alkaline in order to maintain neutral pH after the H^+ liberation accompanying Ca^{2+} complexation.

We have noted that quin2 measures the *average* $[Ca^{2+}]_{cyt}$ in the cytoplasm. In the present and companion papers [10,33], calculations of $[Ca^{2+}]_{cyt}$ in the resting platelets assume that the individual platelets are in steady state. Agonist-induced oscillatory behavior has been described for a number of cell types including fibroblasts, parietal cells, and hepatocytes [34–37]. Recent study on single immobilized platelets showed that serotonin can provoke oscillatory behavior but that ionomycin or aluminum fluoride (putative stimulator of G-proteins) does not [38]. Since the Ca^{2+} manipulations of the present and companion papers [10,39] were accomplished with the use of ionomycin, it is unlikely that oscillatory behavior is evoked. The findings of Nishio et al. (cf. Ref. 38) do suggest that it will be necessary to exercise caution in applying the present methods and steady-state analysis (see below) to agonist-stimulated platelets, or to conditions which open Ca^{2+} channels.

Use of chlorotetracycline to monitor Ca^{2+} uptake by the dense tubules

The principles and methods underlying the use of chlorotetracycline (CTC) to follow real-time Ca^{2+} sequestration by the dense tubules in human platelets have been published [7,11]. Mitochondrial and granule contributions are negligible, and intracellular Mg^{2+} and pH shifts do not play a significant role [7,40].

Cyclic nucleotide radioimmunoassays

Washed sham-loaded and quin2-loaded platelet suspensions at concentrations of $(1.3–1.6) \times 10^8$ cells per ml were placed in plastic cuvettes inside a thermostatically-controlled cell holder at 37°C and stirred continuously at 600 rpm with a teflon-coated magnetic 'flea'. The $[Ca^{2+}]_o$ was set at 2 mM for 5 min and a control sample (0.8 ml) removed and processed for cyclic nucleotide extraction. Forskolin (10 μM) was then added and subsequent 0.8-ml aliquots were removed at 45 s, 15 min and 30 min and processed. Cyclic nucleotides were isolated as described by Loeb and Gear (cf. Ref. 41). Aliquots were added to test tubes containing 0.089 ml of 1 M HCl (final HCl concentration = 0.1 M) at 0°C. The mixture was then put through three cycles of freezing (on solid CO_2) and thawing to disrupt the membranes. This procedure has been described as being effective for halting cyclic nucleotide metabolism and allowing their extraction from the cytoplasm [41]. Cyclic nucleotides were then separated from membranes and proteins by centrifugation at 12000 rpm ($17210 \times g$) for 10 min. The pH of the resulting solution was adjusted to 7.5 ± 0.5 with 1 M NaOH. Samples were stored at −18°C prior to assay.

Curve fitting and statistics

Best fit curves were produced using ASYSTANT (Macmillan Software Company). All data are expressed as the mean ± S.D., except where noted. Statistical analyses using Student's *t*-test were carried out with the aid of EPISTAT (copyright Tracy L. Gustafson).

Results

Forskolin increases the rate of Ca^{2+} extrusion

Forskolin is a diterpene that increases cAMP by direct stimulation of the catalytic subunit of adenylate cyclase (cf. Ref. 42). Fig. 2 shows a typical pair of progress curves for active Ca^{2+} extrusion from quin2-overloaded platelets, comparing the effect of forskolin pretreatment with control. The cytoplasmic quin2 (at 3.6 mmol/liter cell volume) is first saturated with Ca^{2+} by addition of ionomycin in the presence of 2 mM external Ca^{2+}. Next, Ca^{2+} influx is halted by EGTA addition, and the progress curve for active extrusion is measured. The decrease in fluorescence is proportional to the numbers of Ca^{2+} extruded. The non-linear scale on the left side of the figure gives the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) at which the extrusion occurred. Ionomycin does not have sufficient Ca^{2+} affinity to make a contribution to the efflux of Ca^{2+} from the cytoplasm at sub-micromolar concentrations [9]. The reader is referred to a previous paper [9] for other details of the method, its validation and controls. Fig. 2 shows that pretreatment with 10 μM forskolin 1 min prior to the Ca^{2+} and ionomycin increases the rate of extrusion. Experiments using 100 μM forskolin showed no additional increase over that seen with 10 μM (data not shown).

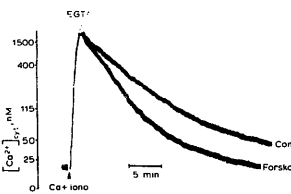

Fig. 2. Standard Ca^{2+} extrusion protocol comparing control and forskolin-treated platelets. Washed platelet suspensions (at a concentration of $1.6 \cdot 10^7$ cells per ml), loaded with 20 μM quin2/AM to give a quin2 concentration of 3.6 mmol per liter cell volume. Forskolin (10 μM) added 1 min prior to the initiation of the trace; 2 mM Ca^{2+} and 1 μM ionomycin were added where indicated. Addition of EGTA (2.7 mM) decreases $[Ca^{2+}]_o$ to approx. 100 nM and allows Ca^{2+} extrusion to occur unopposed by Ca^{2+} influx. The *y* axis is linear with quin2 fluorescence; the non-linear scale indicates the corresponding $[Ca^{2+}]_{cyt}$ values.

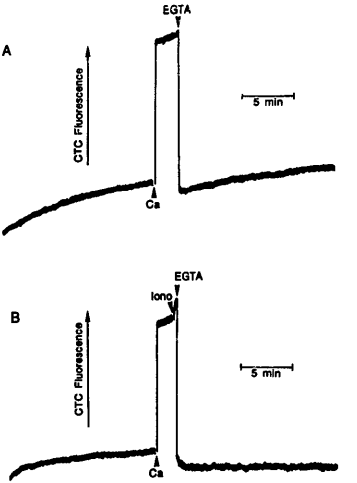

Fig. 3. Typical CTC experiment showing that 1 μM ionomycin eliminates dense tubular Ca^{2+} uptake after removal of external Ca^{2+}. The effect is seen by comparing Panel B with Panel A after EGTA addition. The experiment was conducted in parallel with the experiment of Fig. 2, using the same platelet preparation. Chlorotetracycline (10 μM) was added to Ca^{2+}-depleted washed platelets (at a concentration of $1.6 \cdot 10^7$ per ml) at $t = 0$ and then 2 mM Ca^{2+} together with 10 μM forskolin, followed by 1 μM ionomycin (panel B only) and 2.7 mM EGTA as indicated. The interpretation of the CTC signal has been described at length in previous publications [7,9,11]. Briefly, the slow phase of increase following addition of 10 μM CTC represents the equilibration of the neutral uncomplexed form of the probe between the various platelet compartments. The initial rapid phase of fluorescence increase after addition of 2 mM Ca^{2+} is due mainly to binding of Ca^{2+}-CTC complexes to the external leaflet of the plasma membrane bilayer. The subsequent slow phase of fluorescence increase reflects active Ca^{2+} uptake from the cytoplasm by the dense tubules [7]. The addition of EGTA to complex external Ca^{2+} immediately and completely removes the component due the Ca^{2+}-CTC complexes on the external leaflet and halts further influx of Ca^{2+} to the cytoplasm. In the absence of ionomycin (Panel A), the dense tubules continue to accumulate Ca^{2+} until the Ca^{2+} extrusion pump of the plasma membrane has cleared the cytoplasm. In the presence of ionomycin (Panel B), the dense tubular Ca^{2+} pump is effectively short-circuited and no Ca^{2+} is accumulated during the EGTA phase [9].

Fig. 3 presents a necessary control experiment (cf. Ref. 9) confirming that 1 μM ionomycin was sufficient to shunt the dense tubular Ca^{2+} uptake in forskolin-treated platelets, such that the organelle can not con-

tribute to the process of Ca^{2+} removal monitored in Fig. 2. Dense tubular Ca^{2+} uptake is indicated by the time-resolved increase in chlorotetracycline (CTC) fluorescence after Ca^{2+} addition [7,9,11]. The CTC experiment in Fig. 3 shows that in the presence of 1 μM ionomycin (Trace B), the dense tubules are incapable of accumulating Ca^{2+} after the EGTA addition, whereas in absence of ionomycin (Trace A) they are.

Forskolin effect is due to an increase in V_m of the Ca^{2+}-ATPase pump

We have previously shown how the true kinetics of Ca^{2+} extrusion can be calculated from the rate of decrease in quin2 fluorescence at different $[Ca^{2+}]_{cyt}$ values along the progress curve of Fig. 2 using knowledge of the Ca^{2+} buffer capacity of quin2 relative to the intrinsic cytoplasmic Ca^{2+} binding elements [9]. Fig. 4 shows the calculated absolute rates of Ca^{2+} extrusion from the control and forskolin-treated platelets. The data were fitted with the following function:

$$V = V_m \cdot \frac{[Ca^{2+}]_{cyt}^n}{K_m^n + [Ca^{2+}]_{cyt}^n} + k_{linear} \cdot [Ca^{2+}]_{cyt} \quad (1)$$

which expresses the observed rate of extrusion as the sum of a saturable component (identified with a Ca^{2+}-Mg^{2+}-ATPase) and a linear one (identified with a Na^+/Ca^{2+} exchanger) over the range of $[Ca^{2+}]_{cyt}$ studied (50–1500 nM). The V_m, K_m and n are the maximum velocity of Ca^{2+} transport, Michaelis constant and Hill coefficient, respectively, of the saturable component of extrusion. The rate constant k_{linear} describes the function of the Na^+/Ca^{2+} exchanger, which is poorly resolved in experimentation with quin2. Table I presents values of the kinetic constants for the Ca^{2+} pump and the exchanger in the control and forskolin-treated platelets. Table I shows that forskolin treatment resulted in a 1.6-fold increase in the V_m of the saturable component of the extrusion system. No sig-

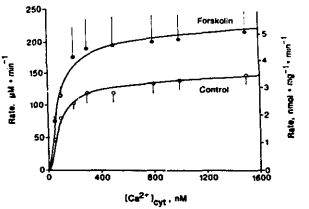

Fig. 4. Effect of forskolin on the rate of Ca^{2+} extrusion vs. $[Ca^{2+}]_{cyt}$ characteristic. Solid circles and open circles are rate data obtained with forskolin-treated (10 μM) and platelets, respectively. Error bars are S.E. The data are the average of four experiments with three preparations. The rates of fluorescence change were measured from the slopes of Fig. 2 (and its repetitions) at the indicated $[Ca^{2+}]_{cyt}$ values. The rates of fluorescence change were converted into absolute numbers of Ca^{2+} removed from the cytoplasm per minute, using the measured intracellular quin2 concentration (3.4 ± 0.3 mmol per liter cell volume). The results contain a small (≤ 25%) correction for the contribution of the intrinsic Ca^{2+} buffer concentration (730 μM, as determined in Johansson and Haynes, Ref. 9). The left-hand ordinate expresses the rates in μmol Ca^{2+} per liter of cell volume per min. The right-hand ordinate expresses the rates in nmol per mg platelet membrane protein per min, calculated as described in Johansson and Haynes (Ref. 9). The $[Ca^{2+}]_{cyt}$ = 1500 nM data are significantly different at $P < 0.04$.

TABLE I

Kinetic constants describing pump- and exchanger-mediated Ca^{2+} extrusion from control and forskolin-treated platelets

Constant	Value		Unit
	control	forskolin-treated	
V_m extrusion pump	123 ± 9 [a]	195 ± 16 [a] (246 ± 74) [c]	μM min^{-1}
	3.0 ± 0.2	4.6 ± 0.4	nmol mg^{-1} min^{-1} [a]
K_m extrusion pump	80	80 (79) [d]	nM
n (Hill coefficient) extrusion pump	1.7 ± 0.3	1.7 ± 0.4	
k_{linear} Na^+/Ca^{2+} exchanger	16 ± 9	16 ± 16	min^{-1} [b]
(V_m/K_m ratio)	(3.9 ± 2.2) · 10^{-4}	(3.9 ± 3.9) · 10^{-4}	liter mg^{-1} min^{-1} [a]

[a] Given as a rate per mg membrane protein (cf. Ref. 9).

[b] Corrected for a small (25%) ionomycin contribution (cf. Ref. 9).

[c] Result for 1 mM Bt_2-cAMP, n = 4. Washed platelet suspensions loaded with 20 μM quin2/AM were diluted to 1.6 · 10^7 cells per ml in plastic cuvettes and treated with 1 mM Bt_2-cAMP for 45 min prior to experimentation. Experiments demonstrated that 1 mM Bt_2-cAMP (a) showed no intrinsic fluorescence at the wavelengths used to monitor quin2 and (b) was without direct effect on the fluorescence of cell-free quin2-Ca^{2+} suspensions.

[d] Calculated using steady-state $[Ca^{2+}]_{cyt,cAMP}$ values for forskolin and Bt_2-cAMP, using Eqn. 6 according to the steady-state method as described in the text.

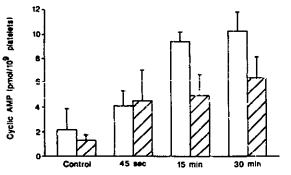

Fig. 5. Changes in platelet cAMP concentrations as a function of time following forskolin (10 μM) addition. Platelets were either sham-loaded (open boxes) or loaded with 20 μM quin2/AM (hatched boxes). Data are presented as the means of three experiments with error bars indicating S.D. The levels of cAMP in both sham-loaded and quin2-loaded platelets are significantly different from levels at 15 and 30 min following forskolin addition. Experiments (not shown) revealed that quin2 present in the platelet lysate following cyclic nucleotide extraction did not interfere with the cAMP radioimmunoassay. The data can be compared with values obtained in the absence (Fig. 3, Refs. 30, 41 and 61) and in the presence [27,63] of the phosphodiesterase inhibitors.

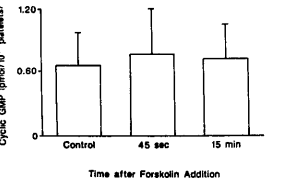

Fig. 6. Lack of effect of forskolin addition on platelet cGMP levels. Washed platelet suspensions ((2.6–3.2)·10^8 cells per ml) not loaded with quin2 were incubated with 2 mM $[Ca^{2+}]_o$. After 5 min, the samples were removed. Forskolin (10 μM) was then added and further samples taken after 45 s and 15 min. Data are presented as the means of four experiments with error bars indicating S.D. There is no significant difference between any of the data sets ($P > 0.60$).

nificant changes in the values for K_m, Hill coefficient or k_{linear} were detected.

Effect of forskolin on cyclic nucleotide levels

Cyclic nucleotide measurements were made to show that the forskolin effect on the Ca^{2+} extrusion pump is due to elevated cAMP. Fig. 5 shows the effect of forskolin (10 μM) on the cAMP concentration in resting platelets at selected time points. Forskolin addition in the presence of 2 mM $[Ca^{2+}]_o$ was found to increase cAMP about 5-fold compared to resting levels both for sham-loaded and quin2-loaded platelets. The concentrations of cAMP at 15 and 30 min are significantly different from their control values ($P < 0.004$ and $P < 0.05$, respectively). The cAMP levels in the quin2-loaded platelets were significantly lower than in sham-loaded platelets.

Fig. 6 shows that forskolin (10 μM) does not affect platelet *cGMP* levels ($P > 0.6$). This observation is important since a companion paper [33] will show that cGMP is also capable of increasing the V_m of the pump. Fig. 7 shows that the Ca^{2+}/ionomycin/EGTA maneuvers used during the efflux protocol (Fig. 2) did not affect cAMP concentrations in sham-loaded platelets.

1 mM Bt_2-cAMP gives comparable increases of the V_m of the Ca^{2+} extrusion pump

The observation that quin2 suppresses resting and forskolin-induced cAMP levels made it desirable to obtain an additional check that 10 μM forskolin had elicited a maximal effect on the Ca^{2+} extrusion pump. Thus the experimentation of Fig. 2 was repeated with preincubation with 1 mM dibutyryl-cAMP (Bt_2-cAMP), a membrane-permeable cAMP analogue. The choice of concentration was based on our finding that 5 or 10 min preincubation with 1 mM Bt_2-cAMP is sufficient to abolish aggregation in platelet-rich plasma in response to 10 μM ADP (cf. Figs. 3–8 of Ref. 43). Pretreatment of platelets with Bt_2-cAMP was found to increase the rate of clearance of Ca^{2+} from the cytoplasm of platelets under the conditions of the standard extrusion protocol. At $[Ca^{2+}]_{cyt} = 400$ nM the Ca^{2+}-ATPase component expresses its V_m and is responsible for the majority (approx. 90%) of Ca^{2+} extrusion (cf. Fig. 13, Ref. 9 and Fig. 4, this paper). At this $[Ca^{2+}]_{cyt}$, 1 mM Bt_2-cAMP was found to increase the rate of Ca^{2+} extrusion by a factor of 2.0 $\pm$0.6 ($n = 4$). This result, which was statistically significant at $P < 0.04$, is entered in Table I.

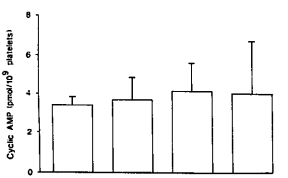

Fig. 7. Lack of effect of Ca^{2+} transient on cAMP concentrations. The experiment was carried out with sham-loaded platelets under conditions chosen to match those of Fig. 2. Washed platelets (1.3·10^8/ml) were exposed to 2 mM Ca^{2+} for 5 min, at which time samples were removed. Then 1 μM ionomycin was added and samples were removed at the indicated times. EGTA (2.7 mM) was added 1 min after the ionomycin. Data are presented as means of four experiments $\pm$S.D.

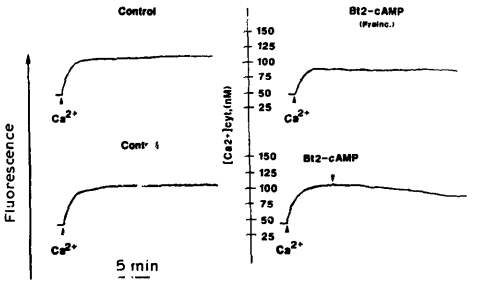

Fig. 8. Effect of Bt_2-cAMP on resting $[Ca^{2+}]_{cyt}$. A platelet sample was loaded with quin2 at an 'indicator concentration' (10 μM quin2/AM) and was suspended in a nominally Ca^{2+}-free Tyrode's solution. Where indicated, 2 mM Ca^{2+} was added to the suspension. In Trace B, the sample was preincubated with 1 mM Bt_2-cAMP 15 min before the Ca^{2+} addition. Trace A is a control. Trace C is the control with the addition of 1 mM Bt_2-cAMP where indicated.

Bt_2-cAMP decreases resting $[Ca^{2+}]_{cyt}$

Fig. 8 shows that preincubation with Bt_2-cAMP lowers the resting $[Ca^{2+}]_{cyt}$, in agreement with previous findings [31]. In the depicted experiment it was lowered from a value of 119 nM to 96 nM. Identical results were obtained for 10 μM forskolin. These results are predicted from the competition between the extrusion process and passive influx (Fig. 1). The $[Ca^{2+}]_{cyt}$ dependence of the rate (V) of the extrusion pump is given by Eqn. 1. We have shown that this is opposed by a cadmium-sensitive, verapamil-insensitive passive leak in the plasma membrane [11]. In the presence of external Ca^{2+}, it opposes the extrusion process to the extent of $k_{leak}\cdot[Ca^{2+}]_o$, where k_{leak} is the rate constant for leakage across the membrane and $[Ca^{2+}]_o$ is the external Ca^{2+} concentration. When the extrusion and influx processes achieve steady state, as in the plateau phase of Fig. 8, we have:

$$k_{leak}\cdot[Ca^{2+}]_o = V_m\cdot\frac{[Ca^{2+}]_{cyt}^n}{K_m^n+[Ca^{2+}]_{cyt}^n} + k_{linear}\cdot[Ca^{2+}]_{cyt} \qquad (2)$$

For $[Ca^{2+}]_{cyt}$ values less than 400 nM we can neglect the term involving k_{linear} (the Na^+/Ca^{2+} exchanger). Substituting 1.7 for n and rearranging gives:

$$\frac{k_{leak}\cdot[Ca^{2+}]_o}{V_m} = \frac{[Ca^{2+}]_{cyt}^{1.7}}{K_m^{1.7}+[Ca^{2+}]_{cyt}^{1.7}} \qquad (3)$$

We define X by

$$X = (k_{leak}\cdot[Ca^{2+}]_o)/V_m \qquad (4)$$

This variable is the rate of Ca^{2+} influx relative to the V_m of the pump. It is also a measure of the pump rate relative to its V_m. By comparing X values for the cAMP-stimulated and control cases with the corresponding V_m values it is possible to determine whether k_{leak} is altered by cAMP. Specifically,

$$k_{leak,cAMP}/k_{leak,control} = (X_{cAMP}/X_{control})\cdot(V_{m,cAMP}/V_{m,control}) \qquad (5)$$

TABLE II

Forskolin- and Bt_2-induced changes in resting $[Ca^{2+}]_{cyt}$ and k_{leak}

Values of X are calculated from Eqns. 3 and 4. Values of $k_{leak}[Ca^{2+}]_o$ were calculated from V_m and Eqn. 4.

Condition	$[Ca^{2+}]_{cyt}$ (nM)	K_m (nM)	V_m (μM/min)	X	$k_{leak}[Ca^{2+}]_o$ (μmol/min)	$k_{leak}/k_{leak,control}$
Control	119	80	123 ± 9	0.663	81.5	(1.0)
Forskolin	96	80	195 ± 16	0.577	112.5	1.37 ± 0.11
Bt_2-cAMP	96	80	246 ± 74	0.577	141.9	1.74 ± 0.52

Table II presents the results of this calculation, showing that k_{leak} is increased 37 ± 11% and 74 ± 52% by forskolin and Bt_2-cAMP, respectively. In the future we will refer to the above system of computation as steady-state analysis of pump vs. leak competition.

Discussion

Cyclic AMP increases the V_m of the Ca^{2+} extrusion pump in the PM

The principal finding of the study is that agents that elevate intracellular cAMP levels stimulate Ca^{2+}-ATPase pump mediated Ca^{2+} extrusion from the platelet. The effect was seen with both forskolin and Bt_2-cAMP. Kinetic analysis shows that the increased extrusion rate is due to an increase in the V_m of a saturable component identified with Ca^{2+}-Mg^{2+}-ATPase activity. The presence of Ca^{2+}-Mg^{2+}-ATPase activity in the plasma membrane of platelets has been demonstrated both cytochemically [12] and by the use of membrane fractionation techniques [13–15,44]. Moreover, Waldmann et al. (Ref. 45) demonstrated cAMP-mediated phosphorylation of a 130 kDa membrane protein in platelets. This molecular weight is typical for Ca^{2+} extrusion pumps located in the plasma membranes [46,47]. Thus our results are fully consistent with a protein kinase A mediated phosphorylation of the pump or a pump-associated protein.

The present results show that elevated cAMP increases the V_m of the Ca^{2+} extrusion pump by a factor of 1.6 (forskolin) to 2.0 (Bt_2-cAMP). No change was observed in the K_m or Hill coefficient of the Ca^{2+}-Mg^{2+}-ATPase. This is very reasonable in terms of our knowledge of Ca^{2+} extrusion pumps in other systems. For example, in the cardiac sarcolemma Ca^{2+}-Mg^{2+}-ATPase, cAMP has been shown to increase the V_m but to have no effect on the K_m [46,48,49]. In bovine cardiac sarcolemma, cAMP-stimulated protein kinase increases the V_m of the Ca^{2+} extrusion pump by a factor of 1.8–2.4 [50]. The K_m of that pump (63 ± 1.7 nM) is close to the observed value for the platelet PM Ca^{2+}-ATPase (80 nM; Ref. 9; present study). However, the bovine cardiac sarcolemmal pump requires calmodulin to achieve such high affinity [49,50]. The experimentation in the present communication does not give any evidence for activation of the platelet PM pump by calmodulin.

Our study did not reveal a cAMP effect on the the linear, non-saturable component of extrusion, identified with Na^+/Ca^{2+}-exchange activity. However, this negative finding should be considered preliminary since the quin2 method does not permit the exchanger to be studied with excellent resolution. More recently we have repeated the Ca^{2+} extrusion protocol using the lower-affinity dye rhod-2, achieving better resolution of the exchanger [18]. That study showed that the exchanger is saturable with respect to $[Ca^{2+}]_{cyt}$, with a K_m of 2.3–6.7 μM and a V_m 1.6–2.7-times that of the extrusion pump. Our preliminary finding of no cAMP effect on the exchanger in the present study is in agreement with results from heart and a number of tissues [51].

cAMP effects on resting $[Ca^{2+}]_{cyt}$

In the resting state the balance between the passive leakage and active extrusion by the Ca^{2+} pump results in an average resting $[Ca^{2+}]_{cyt}$ of 119 nM. The governing equation is

$$[Ca^{2+}]_{cyt} = K_m \cdot \left(\frac{k_{leak} \cdot [Ca^{2+}]_o}{V_m - k_{leak} \cdot [Ca^{2+}]_o} \right)^{1/1.7} \quad (6)$$

which is valid for $k_{leak} \cdot [Ca^{2+}]_o < V_m$ and for the absence of a substantial contribution from the exchanger [9]. The equation summarizes the steady-state analysis made in the previous section (Eqns. 3–5). It predicts that increases in k_{leak} will raise $[Ca^{2+}]_{cyt}$ and increases in V_m will lower it. The active extrusion experimentation (Fig. 2) has shown that elevated cAMP increases the V_m of the Ca^{2+} extrusion pump by a factor of 1.6–2.0. The steady-state analysis also shows that elevation of [cAMP] increases the rate constant of the passive leak by 37 ± 11% to 74 ± 52%. The increased leak does not fully compensate for the increased extrusion, and the resting $[Ca^{2+}]_{cyt}$ is lowered to 96 nM. Eqn. 6 predicts that if k_{leak} had not been increased, $[Ca^{2+}]_{cyt}$ would have been lowered to 52–58 nM. It is possible that lowering $[Ca^{2+}]_{cyt}$ to such an extent would impair platelet activation. It is of interest that elevation of cAMP increases PM Ca^{2+} channel activity in mammalian heart [52] by the mechanism of channel recruitment [53].

Anti-activation effects of cAMP on Ca^{2+} handling

Prostacyclin (PGI_2) is a short-lived eicosanoid which is produced by the vascular endothelium and which functions as an inhibitor of platelet aggregation (cf. Refs. 54 and 55). It is a potent stimulator of platelet adenylate cyclase [56,57]. In the Introduction, we cited studies showing that elevated cAMP inhibits phospholipase A_2 activation, inositol-lipid hydrolysis, IP_3-stimulated Ca^{2+} release and Ca^{2+} transients after stimulation. The cAMP dependent activation of the PM Ca^{2+} pump works against platelet activation in three different ways: Firstly, the lowering of $[Ca^{2+}]_{cyt}$ (from 119 nM to 96 nM) means that an agonist (collagen, ADP or thrombin) must produce a larger influx or release of Ca^{2+} in order to bring $[Ca^{2+}]_{cyt}$ to its threshold level for activation. Thus a higher agonist concentration will be required for activation. Secondly, lowering $[Ca^{2+}]_{cyt}$ will decrease the resting level Ca^{2+}

in the dense tubules which serve as the releasable Ca^{2+} store. However, the companion paper [10] will show that this effect is outweighed by a direct effects of cAMP on the dense tubular pump. Thirdly, an increased V_m of the extrusion pump would also be expected to reduce the size of the $[Ca^{2+}]_{cyt}$ transient after stimulation with an agonist. This is undoubtedly an important factor in the observation that the PGI_2 reduces the amplitude and duration of thrombin-stimulated $[Ca^{2+}]_{cyt}$ transients [58]. Interaction on this level will be considered more closely in the companion studies [10,39].

Possible influence of $[Ca^{2+}]_{cyt}$ on [cAMP]

In the presence of 3.6 mM quin2, the forskolin-induced increase in cAMP was inhibited by 40–50%. This observation is worthy of further study. If the effect proves to be Ca^{2+}-specific, it may be of consequence to the platelet mechanism. Adenylate cyclase is described as being Ca^{2+}-calmodulin stimulated in some tissues but not in others [59]. Calmodulin stimulation of the adenylate cyclase of human platelets has been reported [60]. Interestingly, we observed no change in [cAMP] with the large changes in $[Ca^{2+}]_{cyt}$ induced in the Ca^{2+}/ionomycin/EGTA maneuvers of the experiment of Fig. 2 (cf. Fig. 7). Under this condition $[Ca^{2+}]_{cyt}$ was in the 1.0–1.5 μmol range for $\leqslant$ 1 1/2 min and was returned to the $\leqslant$ 400 nM range within 4–5 min. It would thus appear that our experimentation did not evoke a Ca^{2+}-calmodulin dependent stimulation of adenylate kinase at these concentrations and times of exposure. The quin2 overload condition might work against such an effect. Although the buffering effect of quin2 does not change the average resting $[Ca^{2+}]_{cyt}$ (Table 1, Ref. 9), high quin2 concentrations must be considered capable of suppressing spontaneous and localized Ca^{2+} transients. It would be of interest to test for effects of micromolar $[Ca^{2+}]_{cyt}$ on cAMP concentrations at longer exposure times.

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