Biochimica et Biophysica Acta, 1105 (1992) 29–39

BBAMEM 75545

Stimulation of dense tubular Ca^{2+} uptake in human platelets by cAMP

Jianguo Tao, Jonas S. Johansson and Duncan H. Haynes

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

(Received 6 August 1991)
(Revised manuscript received 25 November 1991)

Key words: Calcium ion accumulation; Calcium ion flux; Calcium pump; ATPase, (Ca^{2+} + Mg^{2+})-; cyclic AMP; Quin2; Chlorotetracycline; (Human platelet)

Elevation of intracellular cAMP is shown to increase the rate (V) and maximal extent of Ca^{2+} uptake by the dense tubules in intact human platelets. Elevation of [cAMP] was accomplished by preincubation with the adenylate cyclase activator forskolin or with dibutyryl-cAMP (Bt_2-cAMP). The free concentration of Ca^{2+} in the dense tubular lumen ($[Ca^{2+}]_{dt}$) was monitored using the fluorescence of chlorotetracycline (CTC) according to protocols developed in this laboratory. The free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was monitored in parallel experiments with quin2. Both $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{dt}$ were analyzed in terms of competition between pump and leak mechanisms in the plasma membrane (PM) and dense tubular membrane (DT). When platelets are incubated in media with approx. 1 μM external Ca^{2+}, $[Ca^{2+}]_{cyt}$ is approx. 50 nM and $[Ca^{2+}]_{dt}$ is very low. When 2 mM external Ca^{2+} is added, $[Ca^{2+}]_{cyt}$ rises to approx. 100 nM and the process of dense tubular Ca^{2+} uptake can be resolved. Forskolin (10 μM) and Bt_2-cAMP increase the rate of dense tubular Ca^{2+} uptake (V) to 2.1 ± 0.60 and 1.70 ± 40 times control values (respectively). The agents also increase the final $[Ca^{2+}]_{dt}$ to 1.70 ± 0.21 and 1.72 ± 0.60 times control values (respectively). Titrations with ionomycin (Iono) showed that the increase was due to an increase in the V_m of the dense tubular Ca^{2+} pump. With [Iono] = 500 nM, $[Ca^{2+}]_{cyt}$ was raised to ≥ 1.0 μM and V_m of the dense tubular pump was elicited. (At [Iono] = 1.0 μM, the final $[Ca^{2+}]_{dt}$ values were degraded 15% due to shunting of Ca^{2+} uptake.) Analysis showed that forskolin (10 μM) and Bt_2-cAMP (1 mM) increase the V_m by a factors of 1.56 ± 40 and 1.56 ± 40, respectively. Analysis showed that neither agent changed the K_m of the pump significantly from its control value of 180 nM. Neither agent changed the rate constant for passive leakage of Ca^{2+} across the DT membrane ($1.7\ min^{-1}$).

Introduction

The platelet is a Ca^{2+}-activated cell which plays an important role in blood coagulation. The present study is the second in a series dealing with the effect of cyclic nucleotides on Ca^{2+} handling by the human platelet. The previous study [1] showed that cyclic AMP (cAMP) increases the rate of Ca^{2+} extrusion across the plasma membrane (PM) by the Ca^{2+}-ATPase pump. The present study reports the effects of cAMP on Ca^{2+} uptake by the dense tubules, the major internal storage site for Ca^{2+} [2–4]. The dense tubules have a Ca^{2+}-ATPase pump [4,5] enabling the accumulation of Ca^{2+}. This Ca^{2+} can be released to the cytoplasm upon stimula-

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; PM, plasma membrane; ROC, receptor-activated channel; $[Ca^{2+}]_{cyt}$, the free ('ionized') Ca^{2+} concentration in the cytoplasm; $[Ca^{2+}]_{dt}$, the free Ca^{2+} concentration in the dense tubular lumen; DT, dense tubules; $[Ca^{2+}]_o$, 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; dibutyryl-cAMP (Bt_2-cAMP), N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; CTC, chlorotetracycline; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Fl_{max}, fluorescence of Ca^{2+} complexed form of quin2; Fl_{min}, fluorescence of uncomplexed form of quin2; A_{fast}, instantaneous CTC fluorescence change after Ca^{2+} addition, A_{slow}, time-resolved CTC fluorescence change after Ca^{2+} addition; R_{CTC}, 'CTC ratio' = A_{slow}/A_{fast}; V and V_m, the velocity and maximal velocity (respectively) of the Ca^{2+}-ATPase pump located in the dense tubular membrane; K_m, the $[Ca^{2+}]_{cyt}$ giving half-maximal rate of extrusion (V); n, Hill coefficient; k_{leak}, rate constant for Ca^{2+} leakage from the dense tubular lumen; k_{Iono}, apparent bimolecular rate constant for ionomycin-catalyzed Ca^{2+} leakage across the dense tubular membrane; $V_{initial}$, cf. Eqns. 3–5; X, ratio of leak rate to V_m of pump.

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

tion. In the agonist-activated state $[Ca^{2+}]_{cyt}$ is rapidly elevated by the contributions of activated channels in the plasma membrane (PM) and in the dense tubular membranes. Studies from this laboratory [6,7] have shown that the levels of resting free cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) and dense tubular Ca^{2+} ($[Ca^{2+}]_{dt}$) are important determinants of the fate of platelets when stimulated with agonists at marginal concentrations.

Cyclic AMP counters platelet activation [8–10]. Aggregometer studies have shown that it inhibits aggregation induced by ADP, epinephrine, collagen and thrombin [11]. As described in the previous study [1], experimentation with fluorimetric indicators has shown that cAMP inhibits Ca^{2+} transients resulting from stimulation with thrombin or platelet activating factor. The observed effects were inhibition of the rise in free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) or increases in the rate of return of $[Ca^{2+}]_{cyt}$ to basal values [12–16]. Phosphodiesterase inhibitors, which elevate cAMP levels, have been shown to reduce the ADP-induced increase in $[Ca^{2+}]_{cyt}$ [17].

The Ca^{2+} transients with the above-mentioned agonists are themselves incompletely understood with respect to proportion of elevation of $[Ca^{2+}]_{cyt}$ arising from influx vs. dense tubular release. Furthermore, the post-activation behavior of the corresponding pumps and channels is not well understood. In principle these phenomena can be systematized as a combination of effects on four systems or processes illustrated in Fig. 1: (A) Ca^{2+} influx, (B) Ca^{2+} extrusion, (C) Ca^{2+} sequestration and (D) Ca^{2+} release. Influx (A) and release (D) can be through passive leakage processes or through receptor-operated channels (ROC). It is recognized that in the resting platelet * the balance between Processes A and B determines $[Ca^{2+}]_{cyt}$ which in turn, affects the rate of the dense tubular pump, which affects $[Ca^{2+}]_{dt}$ (cf. Refs. 6 and 18). Studies with quin2, a fluorescent indicator of $[Ca^{2+}]_{cyt}$, have shown that cAMP decreases the resting $[Ca^{2+}]_{cyt}$ [19,20]. In the companion study [1], we showed that this is due to an increase in Process B, incompletely countered by an increase in Process A.

Studies of cAMP effects on the time course of $[Ca^{2+}]_{cyt}$ during activation using quin2 have been interpreted as decreases in both Ca^{2+} influx (Process A) and in the release of Ca^{2+} from the dense tubules (Process D, Refs. 14 and 21). Thompson and Scrutton [22] showed that elevated cAMP reduced the dense tubular component of the Ca^{2+} transient in response to thrombin [22]. However, the studies did not differentiate between effects on Processes C and D. Cyclic AMP has also been shown to stimulate the return of $[Ca^{2+}]_{cyt}$ to basal levels following agonist-induced elevations [14,15]. In principle, that effect could result from changes in any one or combination of the four processes.

The present communication reports direct monitoring of cAMP effects on dense tubular Ca^{2+} handling in the resting state. Owen and LeBreton [23] reported that under resting conditions, PGE_1 and PGI_2 increased what they termed 'Ca^{2+} binding' indicated by the fluorescence signal of chlorotetracycline (CTC). It is now known [3,18,24,25] that the CTC signal in platelets increases with increasing free concentration in the dense tubular lumen ($[Ca^{2+}]_{dt}$), suggesting that these prostaglandin effects were increased dense tubular uptake (Process C). However, a $^{45}Ca^{2+}$ study of intact platelets [26] reported that 50 nM PGI_2 has no effect on the size of either of two internal pools of

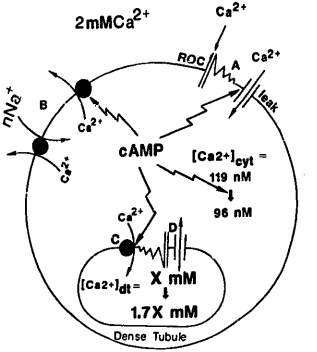

Fig. 1. Ca^{2+} handling systems of the platelet considered in the present study. Stimulatory effects of cAMP (denoted by zig-zag arrow) on the extrusion pump and passive leak in the plasma membrane were demonstrated previously [1]. Stimulatory effects on the dense tubular pump are shown in the present study. ROC denotes receptor-activated channel. Processes A–D are described in the text.

* In this paper we will refer to the 'resting platelet' as an unstimulated platelet bathed in 2 mM Ca^{2+} with time-invariant values of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{dt}$. As we have noted earlier (Methods, Ref. 20) studies of single cells [79] indicate that this is correct for resting platelets and for platelets treated with ionomycin. The same study showed that oscillatory behavior could be provoked by serotonin. We believe that it will be necessary to exercise caution in extending the present methods and steady-state analysis to agonist-stimulated platelets, or conditions which open Ca^{2+} channels.

Ca^{2+}. On the other hand, studies with *isolated* dense tubular vesicles have shown that dense tubular Ca^{2+} uptake is promoted by cAMP [27,28]. Considering the above, it would be useful to establish the effect of cAMP on $[Ca^{2+}]_{dt}$ in the resting state, and to distinguish the direct effects of Processes (C) and (D) from the indirect effects of Process (A) (and (B)).

In this communication, we report the effect of elevated cAMP on dense tubular Ca^{2+} uptake measured *in situ* using chlorotetracycline (CTC), a Ca^{2+} sensitive fluorescent probe which reports the free Ca^{2+} concentration in the dense tubular lumen $[Ca^{2+}]_{dt}$ [29,30]. We use this method in conjunction with measurements of the free Ca^{2+} concentration in the cytoplasm ($[Ca^{2+}]_{cyt}$) using quin2 [31,32] and using ionomycin as a tool to manipulate the latter quantity. We show that cAMP increases the V_m of the dense tubular pump.

Materials and Methods

Forskolin, EGTA, Hepes, quin2, glucose and dibutyryl-cAMP (Bt2-cAMP) were purchased from Sigma Chemical Co, St. Louis, MO. Chlorotetracycline (CTC) was obtained from ICN Pharmaceutical, Cleveland, OH. Ionomycin from Calbiochem, La Jolla, CA. The reagents used in the preparation of Tyrode's solution and Triton X-100 were supplied by Mallinkrodt Inc., Paris, KY.

Platelet isolation

Blood was drawn from normal donors into anticoagulant citrate dextrose. Washed platelets were prepared as described previously [24]. Cells were resuspended and experiments were performed in a normally Ca^{2+}- and Mg^{2+}-free Tyrode's solution of the following composition: 138 mM NaCl/3 mM KCl/10 mM glucose/2 mM $NaHCO_3$/2.5 mM Hepes with the pH adjusted to 7.35. After isolation and storage at this low external Ca^{2+} concentration ($[Ca^{2+}]_0$), the platelets slowly lose cytoplasmic and dense tubular Ca^{2+} as they approach a new steady-state governed pump vs. leak rates. The cells have $[Ca^{2+}]_{cyt}$ of approx. 50 nM, have minimal dense tubular Ca^{2+}, and are therefore referred to as being Ca^{2+}-depleted. When EGTA was added to remove Ca^{2+}, the EGTA was made slightly alkaline to avoid acidification due to the liberated H^+.

Fluorometry

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 occasionally verified with a Bright-line hemacytometer (American Optical). All fluorescence measurements were made with a Perkin-Elmer (Model MPF-3L).

Measurement of dense tubular calcium with CTC

A number of publications from this laboratory [24,25,29,33–34] have described how chlorotetracycline (CTC) responds to Ca^{2+} uptake and functions as a measure of the free Ca^{2+} concentration in the dense tubular lumen ($[Ca^{2+}]_{dt}$). The publications describe numerous controls which have been carried out to ensure the specificity of the measured signal for dense tubular Ca^{2+}. Changes in intracellular Mg^{2+} concentration and pH do not play a significant role under our experimental conditions [29,35]. Mitochondria and storage granules make negligible contributions to CTC fluorescence for short times ($\leq$ 15 min) and sub-micromolar $[Ca^{2+}]_{cyt}$ values. Under conditions where the above does not pertain, mitochondrial uptake can be blocked with rotenone and oligomycin [36,37].

For the Reader's convenience we repeat the following information concerning quantitation of rates and extents of dense tubular uptake using the CTC technique: Rapid phases of fluorescence increase observed after addition of Ca^{2+} (cf. Figs. 2 and 3) are the result of formation of aqueous Ca^{2+}-CTC complexes and binding of these to the external surface of the plasma membrane (PM). The amplitude of the fast phase (A_{fast}) reflects the surface area of plasma membrane exposed for the external medium. The slow (time-resolved) process of fluorescence increase observed after Ca^{2+} addition is the result of the analogous processes occurring in the dense tubular lumen as the result of Ca^{2+} accumulation. The amplitude of the slow phase (A_{slow}) is proportional to the free Ca^{2+} concentration within the dense tubules ($[Ca^{2+}]_{dt}$). The ratio of the slow phase to fast phase amplitudes is taken as R_{CTC}, the 'CTC ratio' (cf. Ref. 18). The rate of dense tubular uptake (V) is similarly normalized ($V = (dF/dt)/A_{fast}$). We will refer to the normalized data as 'CTC units'.

By the strength of the analogy with sarcoplasmic reticulum and electron-microscopic observations that the dense tubules are electron-dense, it is likely that they contain proteins with relatively high capacity and low affinity Ca^{2+} binding similar to calsequestrin of skeletal muscle [38]. The presence of this Ca^{2+} binding capacity does not affect our deductions about the cAMP dependence of the rate (V) or R_{CTC} values since these are measured at the same $[Ca^{2+}]_{dt}$ values. It is our intention to relate the CTC units to mmol Ca^{2+} per liter dense tubular volume (or to μmol Ca^{2+} per liter cell volume) in a future study.

Statistics

All data were expressed as the mean $\pm$ S.D., except where noted. Statistical analysis using Student's t-test were carried out with the aid of EPISTAT (copyright Tracy L. Gustafson).

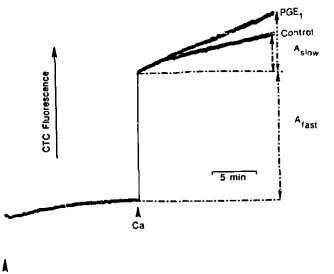

Fig. 2. Typical experiment showing the effect of PGE_1 on the rate of dense tubular Ca^{2+} uptake in intact platelets as monitored with CTC fluorescence. The experimentation used the standard protocol of Jy and Haynes (Refs. 24 and 29). Washed Ca^{2+}-depleted platelets were introduced into the cuvette and 10 μM CTC was added. Calcium (2 mM) was added where indicated. In one experiment prostaglandin (1 μM) added 1 min prior to the Ca^{2+}. The other experiment serves as the control. Quantitation of $[Ca^{2+}]_{dt}$ is by means of the 'CTC ratio' (R_{CTC}) which is calculated as A_{slow}/A_{fast} as illustrated in the figure. Initial rates calculated for the active Ca^{2+} uptake process are also normalized to A_{fast}, as described in Methods.

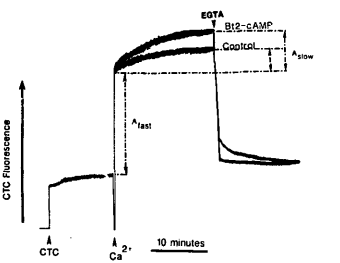

Fig. 3. Typical experiments showing the effect of Bt_2-cAMP on the rate of dense tubular Ca^{2+} uptake in resting platelets as monitored with CTC fluorescence. Experimentation using the standard protocol of Jy and Haynes (Refs. 24 and 29). The platelets were preincubated for 15 min with 4 μM rotenone and 4 μg/ml oligomycin to eliminate the possibility of mitochondrial uptake. Bt_2-cAMP (1 mM) added 15 min prior to Ca^{2+} (2 mM). Control experiments showed that Bt_2-cAMP did not perturb CTC fluorescence. After 15 min external Ca^{2+} was removed by adding 2.7 mM EGTA.

Results

Fig. 2 shows that 1 μM PGE_1 increases the rate of Ca^{2+} uptake by the dense tubules in intact platelets. This agent has been shown to stimulate adenylate cyclase activity [10] (cf. Ref. 39) and has been shown to inhibit thrombin-induced aggregation with an IC_{50} of 0.5–1.0 μM [16]. Further experimentation with 5 μM PGE_1 (not shown) gave equal degrees of stimulation of initial rate indicating that this agent had been titrated to maximal effect. Fig. 3 shows that 1 mM Bt_2-cAMP, preincubated for 15 min, has the same effect. The dense tubular Ca^{2+}-ATPase pump accumulates Ca^{2+} at a faster initial rate. Initial rates of Ca^{2+} uptake ($V_{initial}$, CTC units/min) were measured for the first 10 s of the progress curve of the slow phase of CTC fluorescence increase. The maximal extents of uptake were measured at 15 min and were quantitated as the 'CTC ratio' (R_{CTC}) as described in the Methods and illustrated in Fig. 3. Table I shows the results of Bt_2-cAMP pretreatment for six repetitions of the experiment of Fig. 3. The $V_{initial}$ is elevated by a factor of 1.70 ± 0.40 and the R_{CTC} is elevated by a factor of 1.72 ± 0.69. The experimentation was repeated with 10 μM forskolin, an activator of adenylate cyclase. Comparable elevations of $V_{initial}$ and maximal Ca^{2+} uptake were observed (Table I).

These increases in dense tubular Ca^{2+} uptake occur despite the fact that cAMP has lowered $[Ca^{2+}]_{cyt}$,

TABLE I

Elevation of rate and extent of Ca^{2+} uptake by cAMP

cAMP was elevated in Ca^{2+}-depleted platelets by 15 min preincubation in the cuvette with 1 mM Bt_2-cAMP or 10 μM forskolin. CTC (10 μM), 4 μM rotenone and 4 μg/ml oligomycin were also present during the preincubation. Then 2 mM Ca^{2+} was added to initiate dense tubular uptake which was monitored by CTC fluorescence. Where indicated, 500 nM ionomycin was added simultaneously. The initial rate ($V_{initial}$) and CTC ratios (R_{CTC}) were determined. The table presents the ratio of these quantities for the treated vs. control cases. The presented values are means for 5–8 paired experiments ± S.D.

[Iono] (nM)	Bt_2-cAMP-treated		Forskolin-treated	
	V_{cAMP}/V_{cont}	$R_{CTC,cAMP}/R_{CTC,cont}$	V_{cAMP}/V_{cont}	$R_{CTC,cAMP}/R_{CTC,cont}$
0	1.70 ± 0.40	1.72 ± 0.60	2.10 ± 0.60	1.70 ± 0.21
500	1.42 ± 0.20 [a]	1.17 ± 0.14	1.56 ± 0.40 [a]	1.17 ± 0.40

[a] Identical to $V_{m,cAMP}/V_{m,cont}$ ratio.

making Ca^{2+} less available to the pump [1]. In theory, these effects could be due to an increased V_m, a decreased K_m or a combination of both. Below, we show that the effect is due solely to elevation of V_m.

Determination of the V_m of the dense tubular Ca^{2+} pump by ionomycin titration

The companion paper [1] showed how the steady-state $[Ca^{2+}]_{cyt}$ can be mathematically modelled as the result of passive influx and the active extrusion processes (cf. Eqns. 2 and 6, Ref. 1). The concept can be similarly applied to the dense tubular uptake, in which the rate of dense tubular uptake is modelled (cf. Fig. 1) in terms of the sum of dense tubular Ca^{2+}-ATPase pump action (Process C) and passive leaks (Process D). The following equation will be used:

$$\frac{d[Ca^{2+}]_{dt}}{dt} = V_{m,dt}\frac{[Ca^{2+}]_{cyt}^{1.4}}{\left(K_m^{1.4}+[Ca^{2+}]_{cyt}^{1.4}\right)} - k_{leak}[Ca^{2+}]_{dt} - k_{iono}[Iono]\cdot[Ca^{2+}]_{dt} \quad (1)$$

The rate is expressed in CTC units, the Ca^{2+} concentrations are free Ca^{2+} concentrations, the subscript dt refers to the dense tubules and the exponent is the experimentally-determined Hill coefficient of the dense tubular pump [25]. The constants k_{leak} and k_{iono}* are rate constants for leakage of *dense tubular* Ca^{2+} via leakage pathways intrinsic to the dense tubules, and via ionomycin, respectively. In a study with digitonin-permeabilized platelets, we showed that the initial rate of Ca^{2+} uptake by the dense tubular pump has the $[Ca^{2+}]_{cyt}$ dependence given in the first term of Eqn. 1, with a K_m of 180 ± 5 nM. The same study showed that the maximal Ca^{2+} uptake at steady state has a similar dependence, which was fitted by the $K_m = 160 \pm 5$ nM and a Hill coefficient of 1.50 ± 0.05. This was in line with the maximal Ca^{2+} uptake being the result of the first two terms in Eqn. 1, active uptake vs. passive leakage (cf. Refs. 6 and 25).

The value of V_m was determined by repeating the experiment of Fig. 3 at saturating $[Ca^{2+}]_{cyt}$ levels produced by titrating with ionomycin in the presence of 2 mM external Ca^{2+}. Ionomycin has the dual effect of raising $[Ca^{2+}]_{cyt}$ and increasing Ca^{2+} leakage from the dense tubules. The first effect, which increases the first term in Eqn. 1, occurs at low ionomycin concentrations. The second effect, which results from increases in the third term in the equation, requires higher

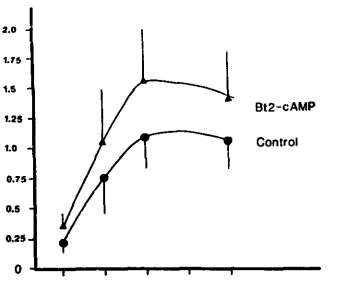

Fig. 4. Effect of ionomycin on initial rate of dense tubular Ca^{2+} uptake measured by CTC fluorescence in the presence and absence of Bt_2-cAMP. The initial rate is given as $V = dFl/dt/A_{fast}$. Platelets were incubated with 10 μM CTC, 4 μM rotenone and 4 μg/ml oligomycin. After 15 min ionomycin was added at the indicated concentration simultaneously with 2 mM Ca^{2+} and the initial rates were determined over the first 10 s of Ca^{2+} uptake. Inhibitors (cf. legend, Fig. 4) were used to ensure that mitochondrial uptake did not contribute at the high $[Ca^{2+}]_{cyt}$ values obtained. The presented data are the average (±S.D.) of 6–8 experiments. Parallel experiments with quin2 (not shown) gave the $[Ca^{2+}]_{cyt}$ values cited in Results.

ionomycin concentrations. Fig. 4 shows that the initial rate of Ca^{2+} uptake ($V_{initial}$) increases with increasing ionomycin concentration, reaching maximal values. Control experiments with quin2 showed that 250 nM ionomycin results in $[Ca^{2+}]_{cyt}$ of 800 nM and that 500 nM ionomycin results in $[Ca^{2+}]_{cyt}$ of ⩾ 1000 nM. A $[Ca^{2+}]_{cyt}$ in this range will saturate the dense tubular Ca^{2+} uptake system to 89–91%, given the K_m of 180 nM [24,25].

Reference to Eqn. 1 shows that the above-described behavior is the result of ionomycin effects on $[Ca^{2+}]_{cyt}$ in the first term of the equation. During the initial phase of the uptake, $[Ca^{2+}]_{dt}$ is very low and the second term does not influence $d[Ca^{2+}]_{dt}/dt$. The observation of a plateau in Fig. 4 for ionomycin concentrations between 500 nM and 1000 nM indicates that the third term is also without influence in the initial phases of the reaction.

cAMP increases the V_m of the dense tubular pump

The values of $V_{initial}$ in the plateau of Fig. 4 are measures of the V_m of the dense tubular pump. Fig. 4 shows that Bt_2-cAMP increases the V_m by a factor of 1.42. We repeated the above experimentation preincubating the platelets for 15 min with 10 μM forskolin

* The ionophore actually shows saturation kinetics with a K_m of 7.7 mM [30] but the present simplification of a first-power dependence does not introduce error into our calculations.

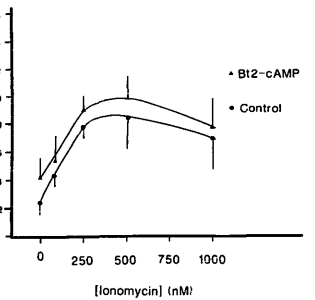

Fig. 5. Effect of ionomycin on maximal extent of dense tubular Ca^{2+} uptake measured by CTC fluorescence in the presence and absence of Bt_2-cAMP. The maximal $[Ca^{2+}]_{dt}$ in steady state is proportional to the 'CTC ratio' (R_{CTC}) which is the ratio of the amplitude of the slow phase to that of the instantaneous phase (A_{slow}/A_{fast}; cf. Fig. 2) following Ca^{2+} additon to platelets (cf. Ref. 18). The data are from the same experiments as in Fig. 4.

rather than Bt_2-cAMP. Forskolin increases the cAMP concentration in platelets by activating adenylate cyclase [40,41]. Forskolin was tested and found to increase the V_m by a factor of 1.56 ± 0.40. These results are presented in Table I.

Fig. 5 shows that the maximal extent of Ca^{2+} uptake in the steady-state ($[Ca^{2+}]_{dt,max}$) measured by the CTC ratio (R_{CTC}) also displays a biphasic dependence on the ionomycin concentration. The descending phase is the result of partial short-circuit of the pump by ionomycin at high concentrations. This behavior is predicted by Eqn. 1. The figure shows that Bt_2-cAMP also increases R_{CTC} ($[Ca^{2+}]_{dt,max}$) by a factor of 1.17 ± 0.14. Experimentation with forskolin gave a 1.17 ± 0.40-fold increase. These data are also listed in Table I. The maximal values of R_{CTC} are determined by competition between uptake and passive leakage. The decrease in maximal extent at the highest ionomycin concentration is indicative of a small contribution of the third term of Eqn. 1 which will be quantitatively assessed in a subsequent section.

cAMP does not affect the K_m of the dense tubular pump

The data on maximal extent of Ca^{2+} uptake in the *absence* of ionomycin are sensitive to both V_m, K_m and $[Ca^{2+}]_{cyt}$. The initial rates of uptake are measured immediately after addition of 2 mM Ca^{2+} to Ca^{2+}-depleted platelets, and thus measure Ca^{2+} uptake at $[Ca^{2+}]_{cyt} \approx 50$ nM. Table I shows that these rates are increased by factors of 1.70 and 2.1 by Bt_2-cAMP and forskolin, respectively. The calculations below will show that these data set an upper limit on how much the K_m value could be changed. We note that the initial rate measurement was made within 10 s of Ca^{2+} addition. Thus $[Ca^{2+}]_{dt}$ is low and the second term of Eqn. 1 does not contribute. For the dense tubular pump operating unopposed by leakage we have:

$$V = V_m \frac{[Ca^{2+}]_{cyt}^{1.4}}{K_m^{1.4} + [Ca^{2+}]_{cyt}^{1.4}} \tag{2}$$

We will define the quantity X by

$$X = \frac{V}{V_m} = \frac{[Ca^{2+}]_{cyt}^{1.4}}{K_m^{1.4} + [Ca^{2+}]_{cyt}^{1.4}} \tag{3}$$

The quantity X is defined as the degree of saturation of the pump with cytoplasmic Ca^{2+}. Defining the initial value of $d[Ca^{2+}]_{dt}/dt$ as $V_{initial}$, and using the subscripts 'cAMP' and 'control' to refer to values obtained with and without elevation of cAMP concentration, and constructing ratios based on Eqn. 3 we get:

$$\frac{V_{initial,cAMP}}{V_{initial,control}} \cdot \frac{V_{m,control}}{V_{m,cAMP}} \cdot X_{initial,control} = X_{initial,cAMP} \tag{4}$$

Substituting the $V_{initial}$ and V_m data from Table I, and calculating $X_{initial,control}$ from $[Ca^{2+}]_{cyt} = 50$ nM and $K_{m,control} = 180$ nM gives $X_{initial,cAMP} = 0.170$. From this we can readily calculate the K_m using

$$K_m = [Ca^{2+}]_{cyt} \cdot ((1 - X)/X)^{1/1.4} \tag{5}$$

which is obtained from rearrangement of Eqn. 3. Using Eqn. 5 under the approximation that $[Ca^{2+}]_{cyt}$ is constant during the approx. 10 s required for the initial rate measurement, we obtain $K_{m,cAMP} = 155$ nM. For forskolin we obtain $K_m = 140$ nM. These represent only a 14% or 22% decrease from the control value, a difference which is probably insignificant. The values are listed in Table II.

Use of 500 nM ionomycin does not seriously degrade the maximal extent of uptake

We will show that the 500 nM ionomycin used to elicit the V_m did not degrade the performance of the pump when measured at steady-state maximal uptake (R_{CTC} values). Using the subscript SS to denote the steady-state condition, and rearranging Eqn. 1, we obtain:

$$\frac{[Ca^{2+}]_{dt,SS} \cdot (k_{leak} + k_{iono}[Iono])}{V_m} = \frac{[Ca^{2+}]_{cyt}^{1.4}}{K_m^{1.4} + [Ca^{2+}]_{cyt}^{1.4}} \tag{6}$$

TABLE II

Effect of Bt_2-cAMP and forskolin-treatment on kinetic values (K_m, V_m) of the dense tubular pump and rate constant (k_{leak}) for Ca^{2+} leak across the dense tubular membrane

$[Ca^{2+}]_{cyt}$ values are for the 'resting' state in the presence of 2 mM Ca^{2+} [1]. The V_m values (CTC unit/min) are calculated from the initial rate at [Iono] = 0.5 μM as described in the text. The K_m values are calculated as described in the text. $[Ca^{2+}]_{dt}$ is the measured R_{CTC} value (Fig. 4). X is the degree of saturation of the pump in steady state, calculated from Eqn. 3 using steady state $[Ca^{2+}]_{dt}$ and K_m value. $R_{CTC,max}$ (CTC units) is the experimental value for 0.5 μM ionomycin or the theoretical value (R_{CTC}/X) for no ionomycin. k_{leak+1}/V_m stands for $(k_{leak} + k_{iono}[Iono])/V_m$ (min^{-1}) which is identical to X/R_{CTC} (Eqn. 6). k_{leak+1}/V_m (CTC units^{-1}) was calculated from k_{leak}/V_m, using $k_{iono} = 0.34 k_{leak}$ (μM^{-1}). Finally, k_{leak} (min^{-1}) was calculated from the preceding column using the appropriate V_m value.

State	[Iono] (μM)	$[Ca^{2+}]_{cyt}$ (nM)	K_m (nM)	V_m (CTC units)	$[Ca^{2+}]_{dt}$	X	$R_{CTC,max}$	$\frac{k_{leak+1}}{V_m}$	$\frac{k_{leak}}{V_m}$	k_{leak} (min^{-1})
Control	0	119	180	1.14	0.24	0.359	0.67	–	1.50	1.71
Control	0.5	≥ 1000	180	1.14	0.85	1.00	0.85	1.17	1.00	1.14
Bt2-cAMP	0	96	155	1.62	0.41	0.338	1.21	–	0.82	1.33
Bt2-cAMP	0.5	≥ 1000	155	1.62	1.01	1.00	1.01	0.99	0.84	1.36
Forskolin	0	96	140	1.78	0.41	0.370	1.11	–	0.90	1.60
Forskolin	0.5	≥ 1000	140	1.78	1.01	1.00	1.11	0.90	0.77	1.37

At the 800–1000 nM $[Ca^{2+}]_{cyt}$ values elicited by ≥ 500 nM ionomycin, both sides of the equation equal 1.0. From Fig. 5 we note that $[Ca^{2+}]_{dt,SS}$ declines 15% from its maximal value when [Iono] is raised from 500 nM to 1,000 nM. Thus $k_{iono} \cdot 0.5\ \mu M = ((100/85) - 1) \cdot k_{leak}$ $= 0.17 \cdot k_{leak}$, or $k_{iono} = 0.34\ (\mu M^{-1}) \cdot k_{leak}$. The maximal values of $[Ca^{2+}]_{dt}$ in the control and cAMP-treated cases are affected equally at 500 nM ionomycin. This small correction will be applied in further calculations of the kinetics in steady state (Table II).

Marginal influence of cAMP on k_{leak}

We can also make use of the data of Table I and Eqn. 5 to show that k_{leak} is not substantially changed by cAMP. Constructing a ratio from Eqn. 5 we obtain:

$$\frac{[Ca^{2+}]_{dt,cAMP}}{[Ca^{2+}]_{dt,control}} \cdot \frac{k_{leak,cAMP}}{k_{leak,control}} \cdot \frac{V_{m,control}}{V_{m,cAMP}} = \frac{X_{cAMP}}{X_{control}} \qquad (7)$$

We note that the first quotient in Eqn. 7 is the quotient of the R_{CTC} values, that the third quotient is the inverse of the ratio of the V_m values, and that the X values are known from the $[Ca^{2+}]_{cyt}$ and K_m values. For the Bt_2-cAMP treated case in the absence of ionomycin, these values are 1.72, 1/1.42 and 0.338/0.359, respectively. This gives $k_{leak,cAMP}/k_{leak,control} = 0.78$, or a 22% decrease. A similar calculation for the forskolin-treated case gives $k_{leak,cAMP}/k_{leak,control} = 0.94$, or a 6% decrease. These decreases, which we judge insignificant, pertain to k_{leak} values assessed at normal resting $[Ca^{2+}]_{cyt}$ values of 96 nM or 119 nM and low levels of $[Ca^{2+}]_{dt}$.

We have calculated *absolute* values of k_{leak} using Eqns. 3 and 6 and data in the presence and absence of 0.5 μM ionomycin. The values of k_{leak} are presented in Table II. The range of variation is only ± 15% of the mean value, with no systematic difference between control, Bt_2-cAMP and forskolin.

Rate of loss of dense tubular Ca^{2+} after removal of external Ca^{2+}

Fig. 3 also shows the progress curve for loss of dense tubular Ca^{2+} after removal of external Ca^{2+} using EGTA. The curves show that dense tubular Ca^{2+} is lost over a 10 min period in both the control and

TABLE III

Comparison of experimental and predicted rates of Ca^{2+} loss from the dense tubules one minute after Ca^{2+} removal

Experimental		Calculated			
State	$-d[Ca^{2+}]_{dt}/dt$ measured (CTC units/min)	$[Ca^{2+}]_{cyt,\,-1\ min}$ [b]	DT pump rate (CTC units/min)	$k_{leak} \cdot [Ca^{2+}]_{dt}$ (CTC units/min)	$-d[Ca^{2+}]_{dt}/dt$ calculated (CTC units/min)
Control	0.139 ± 0.017	87 nM	0.300	−0.410	−0.110
Forskolin	0.275 ± 1.07	51 nM	0.280	−0.540	−0.260

[a] Calculated from the extrusion pump kinetic data and cytoplasmic buffer capacity data given by Johansson and Haynes, (Ref. 30). From these values, $d[Ca^{2+}]_{cyt}/dt$ was −32 nM/min for the control case and −45 nM/min for the forskolin-treated case.

Bt_2-cAMP-treated case. Similar results were seen with the forskolin treatment. Table III presents the initial rates of Ca^{2+} loss ($-d[Ca^{2+}]_{dt}/dt$ in CTC units) determined for the first minute of the process averaged over 6 experiments. The average rates are higher in the forskolin-treated case. The additional columns present calculations showing that this result is predicted by Eqn. 1 using the pump and leak parameters already determined. The second column of data shows the $[Ca^{2+}]_{cyt}$ values at $t = 1$ min calculated with a knowledge of the properties of the extrusion pump located in the PM. In the forskolin-treated case $[Ca^{2+}]_{cyt}$ is lower because the V_m of the PM extrusion pump is elevated. The third column shows that this results in lower rates of transport by the dense tubular pump, despite the higher V_m for the forskolin-treated case. The next column shows that the rate of leakage is larger in the forskolin-treated case. This is due to the higher resting $[Ca^{2+}]_{dt}$, since the k_{leak} value is actually slightly smaller. The final column estimates the *net* rate of decrease in $[Ca^{2+}]_{dt}$ as the difference between the pump rate and the leakage rate. We note that the values are only a fraction of the actual leakage rate. We further note that the calculated $-d[Ca^{2+}]_{dt}/dt$ values are close to their experimental values, and that larger values are calculated for the forskolin-treated case than for control. Qualitative agreement between theory and experiment for this rather complex phenomenon can be taken as further validation of the model. It is noted that the above model is amenable to computer simulation.

Discussion

The present study has shown that the kinetic characteristics of the Ca^{2+}-accumulating ATPase pump of the dense tubules can be determined *in situ*. The study has shown that the V_m of the dense tubular pump is increased 1.42–1.56-fold by elevation of cAMP. The study also corroborates our earlier measurement of $K_m = 180$ nM and showed it to be essentially unchanged by cAMP. As noted earlier [25], this K_m value is in good agreement with values for isolated dense tubules [4]. It is important to note that the K_m of the dense tubular pump is twice as large as that of the plasma membrane pump. This has important implications for the behavior of platelets at rest and after activation. At rest with $[Ca^{2+}]_{cyt} = 116$ nM, the plasma membrane pump is working at approx. 63% of its V_m [30] while the dense tubular pump is working at only 36% of its V_m (cf. X values, Table II). Thus the dense tubular pump has a greater reserve. Similarly, in the resting cell, the dense tubular Ca^{2+} uptakes reach only 28–40% of their maximal achievable values (cf. $[Ca^{2+}]_{dt}$ values, Table II). It is important to understand these differences in terms of the Ca^{2+} requirements of the resting and activated states.

Our studies have shown that the dense tubular pool fills up more slowly than the cytoplasmic pool, after introduction of Ca^{2+}-depleted platelets into a Ca^{2+}-containing medium. Also, the dense tubular pool is depleted more slowly than the cytoplasmic pool when external Ca^{2+} is removed by EGTA. Our initial calculations presented in Table III indicate that these phenomena can be explained by the differential equations describing the dense tubular pump and leak, together with the PM extrusion pump. The value of k_{leak} is not changed appreciably by cAMP. The actual k_{leak} values for the dense tubules (1.7 min^{-1}) are in reasonable agreement with rates of leakage observed in isolated dense tubules [42]. We believe that the progress curve for the CTC fluorescence increase accompanying Ca^{2+} uptake by the dense tubule can be modelled in this way after determination of the contributions of low-affinity Ca^{2+} binding in the dense tubular lumen. It should also be possible to calibrate CTC units in terms of μmol Ca^{2+} per liter cytoplasmic volume.

cAMP increases $[Ca^{2+}]_{dt}$ despite its lowering of $[Ca^{2+}]_{cyt}$

Cyclic AMP stimulates the extrusion pump in the plasma membrane, decreasing $[Ca^{2+}]_{cyt}$ [1]. On the other hand, cAMP increases the rate of the dense tubular pump which accumulates Ca^{2+}. The actions of the two pumps can be regarded as competition between Ca^{2+} extrusion and Ca^{2+} accumulation. Since cAMP stimulates both pumps, mere qualitative reasoning is inadequate to predict whether $[Ca^{2+}]_{cyt}$ should be lowered or raised with cAMP treatment. Our experiments show that Bt_2-cAMP and forskolin increase resting $[Ca^{2+}]_{dt}$ by a factor of 1.7. Our model predicts this result from the $[Ca^{2+}]_{cyt}$ effect and V_m effect. Our model also shows how the cAMP effect can be understood in terms of the mathematics of pump vs. leak for the dense tubular and plasma membrane systems.

Biochemical basis for cAMP-induced increase in the V_m of the dense tubular pump

Our *in situ* study has shown that Bt_2-cAMP and forskolin increase the V_m of the dense tubular pump by factors of 1.42 ± 0.20 and 1.56 ± 0.40, respectively. This corroborates previous studies showing that cAMP promotes Ca^{2+} transport in isolated vesicles of dense tubular origin [27,28,43]. The study of Kaser-Glanzmann et al. [27] showed a 2.6-fold stimulation of ATP-driven, oxalate-supported $^{45}Ca^{2+}$ uptake with 2 μM cAMP in vesicles derived from platelet membranes. Adunyah and Dean [4,28] demonstrated Ca^{2+} uptake in isolated internal platelet membrane vesicles and showed a 2-fold increase in Ca^{2+} uptake by stimulation with cAMP-dependent kinase. They further characterized this as a V_m effect [43], finding no change in

K_m. Two more recent studies must be noted as germane to this: White et al. [44] showed that the catalytic subunit of cAMP-dependent protein kinase doubles the rate of Ca^{2+} uptake into platelet microsomes. However, the authors expressed some reservations since they did not observe the expected behavior in experiments designed to inhibit the catalytic subunit. O'Rourke et al. [45] working in a similar system, did not find stimulation of Ca^{2+} uptake with the catalytic subunit. The above-cited study notwithstanding, our *in situ* results are in excellent agreement with the results of Adunyah et al. [43] in isolated dense tubules.

Cyclic AMP-dependent protein kinase has been shown to phosphorylate membrane proteins of 240, 130, 50, 42 and 22 kDa [46] (see also Ref. 47). The 22 kDa protein phosphorylation was described by Kaser-Glanzmann et al. [27] and Fox et al. [48] in dense tubular membranes. The above-cited studies of Adunyah and Dean [28] and Adunyah et al. [43] showed a close correlation between the cAMP-dependent kinase dependent phosphorylation of this protein and enhanced Ca^{2+} transport. They also showed that the protein undergoes a calmodulin-dependent phosphorylation which is associated with a smaller stimulation of transport [28,43]. The 22 kDa protein has physicochemical similarities to phospholamban in skeletal muscle sarcoplasmic reticulum [49]. However, it affects its target differently than phospholamban. In the dense tubule, the V_m of the pump is increased; in the cardiac sarcoplasmic reticulum the K_m of the pump is decreased [50,51]. Also antibodies raised against canine phospholamban did not crossreact with the human platelet 22 kDa protein [43].

Pro-activation vs. anti-activation effects of cAMP mediated through Ca^{2+}

Prostacyclin (PGI_2), a short-lived eicosanoid produced by the vascular endothelium, functions as an inhibitor of platelet aggregation (cf. Refs. 52 and 53). It is a potent stimulator of platelet adenylate cyclase [54,55]. It is generally accepted that prostacyclin liberated from vascular endothelial cells serves as a short-range signal to inhibit platelet activation. This prostacyclin-mediated effect has recently been demonstrated in an *in vitro* system in which endothelial cells raised the cAMP level in platelets and inhibited aggregation [56]. The prostacyclin-sensitive adenylate cyclase is thought to exist in basal, activated and desensitized states [57]. It is in competition with a phosphodiesterase which is also considered to exist in two states of activation [57] and which is subject to modulation [58,59].

As discussed in the Introduction, cAMP is considered to be anti-aggregatory in platelets (cf. Ref. 60 for review). Effects of cAMP on reactions prior to Ca^{2+} mobilization are inhibitory, and can be considered primary effects. The inhibition of inositol-lipid hydrolysis [61–63] can be considered an anti-activation locus of cAMP. The reaction products inositol trisphosphate and 1,2-diacylglycerol play important roles in platelet activation [64,65]. Inositol trisphosphate has been shown to release Ca^{2+} from dense tubules in isolated preparations [66] and in permeabilized platelets [25]. Diacylglycerol is thought to sensitize the platelet by a protein-kinase C dependent mechanism.

Another important locus of anti-activation effects of cAMP is its inhibition of agonist-induced phospholipase A_2 activation [67], leading to decreased release of arachidonic acid. The latter leads to the formation of thromboxane A_2 [68] which, in turn, contributes to Ca^{2+} mobilization.

As previously mentioned, cAMP causes the phosphorylation of a number of platelet proteins (22, 24, 30, 39, 50, 60 and 250 kDa; Ref. 47). Identification of these proteins and their functional roles is an area of active investigation. Cyclic AMP-dependent phosphorylation of glycoprotein Ib beta [69] inhibits collagen-induced polymerization of actin a step involved in activation) [70]. Also, cAMP increases the rate of loss of fibrinogen binding capacity after stimulation with platelet activating factor (PAF) [71].

Many of the above mechanisms may contribute to the observed effects of cAMP to reduce the Ca^{2+} transient after stimulation with agonists. The present study and the companion study [1] identify two important loci for cAMP effects on activation: The PM Ca^{2+} pump and the dense tubular Ca^{2+} pump. As will be shown below, the effect on the PM pump is anti-activation; the effect on the dense tubular pump is either mildly pro-activation or difficult to evaluate. Both of these effects must be considered in terms of the behavior of the platelet before and after the moment of activation with agonist.

The cAMP-induced increases the V_m of the extrusion pump is clearly anti-activation. It increases the rate of Ca^{2+} extrusion before and after challenge with agonist. The reduction of resting $[Ca^{2+}]_{cyt}$ [1] works against activation, since a larger amount of Ca^{2+} must be introduced into the cytoplasm by triggered influx or dense tubular release to obtain the critical degree of Ca^{2+}-occupation and $[Ca^{2+}]_{cyt}$. After agonist challenge, the increased rate of Ca^{2+} extrusion will help to reduce $[Ca^{2+}]_{cyt}$ to non-activating levels.

In contrast, the effect on the dense tubular Ca^{2+} pump is pro-activation. The V_m of the dense tubular pump is increased to such an extent that it out-weights the effect of reduction of $[Ca^{2+}]_{cyt}$. Thus cAMP raises the resting $[Ca^{2+}]_{dt}$ from 0.24 to 0.41 CTC units (Table II, Fig. 5); there is more dense tubular Ca^{2+} to release at the instant of stimulation. However, studies cited in the Introduction showed that cAMP reduces the amount of dense tubular Ca^{2+} *released* by agonist

stimulation, as deduced from the elevation of $[Ca^{2+}]_{cyt}$ measured in the absence of external Ca^{2+} (absence of Ca^{2+} influx). Coupling this information with our observation of elevated $[Ca^{2+}]_{dt}$ suggests that cAMP down-regulates the Ca^{2+} release channel in the dense tubular membrane. A study of IP_3-induced Ca^{2+} release in saponin-permeablized platelets suggests that this is the case [72].

Considering the cAMP effect on the dense tubular pump from the standpoint of dense tubular behavior immediately after stimulation with agonist, the effect should be anti-activation. The rate of removal by dense tubular sequestration is increased. Indeed, the dense tubular pump, which has a K_m of 180 nM, works most efficiently at $[Ca^{2+}]_{cyt}$ values in the platelet-activating range ($\geq$ 400 nM, cf. Ref. 25). However, if the accumulated Ca^{2+} were later released in an unsynchronized, pulsatile manner, extra Ca^{2+} accumulation would not counter activation. As will be shown in the third paper in this series [20], *cGMP* increases the rate of Ca^{2+} extrusion and does *not* increase dense tubular Ca^{2+} sequestration. Quantitative differences in the anti-activation effects of these two cyclic nucleotides is thus worthy of detailed study.

Comparison with cardiac and skeletal sarcoplasmic reticulum pumps

We now have sufficient information on the characteristics of the dense tubular Ca^{2+}-ATPase pump to allow comparison with the comparable pump in other excitable cells. The rabbit skeletal sarcoplasmic reticulum (SR) and the previously-discussed canine cardiac SR are the best-studied examples of Ca^{2+}-accumulating organelles and their Ca^{2+}-ATPase pumps. The human platelet dense tubular pump shares some features of both. The dense tubular pump shares with the cardiac SR the above-mentioned cAMP and calmodulin-activation [43]. More importantly, the cAMP activation of the dense tubular pump results in an increase in the V_m with no change in K_m. In cardiac SR, cAMP activation results in only small changes in V_m and an appreciable decrease in the K_m [51,73]. Furthermore, the K_m of the dense tubular pump is much lower than that of the cardiac SR pump. As noted above the K_m of the dense tubular pump is 180 nM in essentially all states. For the cardiac SR the K_m is 942 nM for the basal state, 617 nM for the cAMP-activated state, 526 nM for the calmodulin-activated state, and 417 nM for the dually-activated state [51]. In terms of its K_m, the dense tubular pump is closer to the rabbit skeletal SR pump, which has a K_m of 73 nM [74] (cf. Table III, Ref. 75 for tabulation).

Recent molecular genetic investigations of the skeletal SR enzyme have identified amino acid residues critical to the Ca^{2+} affinity of the rabbit skeletal and cardiac SR [76]. Sequences deduced for the sarcolemmal and plasma membrane pumps show significant homology [77,78] (cf. Table II, Ref. 75). It will be a significant challenge to understand subtle changes in Ca^{2+} affinity and its regulation in terms of the amino acid sequence.

Acknowledgements

This work was supported by USPHS HL 38228, HL 07188 and the Florida Heart Association. We thank Mr. William G. Watzek for expert assistance, Dr. William Dean for criticism and discussion, and Dr. Peter Valant for a critical reading of the manuscript.

References

1 Johansson, J.S., Nied, L.E. and Haynes, D.H. (1992) Biochim. Biophys. Acta 1105, 19–28
2 Menashi, S., Weintraub, H. and Crawford, N. (1981) J. Biol. Chem. 256, 4095–4101.
3 Jy, W. and Haynes, D.H. (1984) Circ. Res. 55, 595–608.
4 Adunyah, S.E. and Dean, W.L. (1986) Biochim. Biophys. Acta 261, 3122–3127.
5 Dean, W.L. (1984) Biol. Chem. 259, 7343–7348.
6 Jy, W. and Haynes, D.H. (1987) Biochim. Biophys. Acta 929, 88–102.
7 Shanbaky, N.M., Ahn, Y.S., Jy, W.Y. and Haynes, D.H. (1987) Thromb. Haemostas. 57, 1–10.
8 Marcus, A.J and Zucker, M.B. (1965) Physiology of Blood Platelets, p. 53, Grune and Stratton, New York.
9 Abdulla, Y.H. (1969) J. Atheroscler. Res. 9, 171–177.
10 Salzman, E.W. and Levine, R. (1971) J. Clin. Invest. 50, 131–141.
11 Mills, D.C.B. and Smith, T.B. (1971) Biochem. J. 121, 185–196.
12 Feinstein, M.B., Egan, J.J. and White, J. (1983) Biochem. Biophys. Res. Commun. 113, 594–604.
13 Yamansi, J., Kawahara, Y. and Fukuzaki, H. (1983) Thromb. Res. 32, 183–188.
14 Zavoico, G.B. and Feinstein, M.B. (1984) Biochem. Biophys. Res. Commun. 120, 579–585.
15 MacIntyre, D.E., Bushfield, M. and Shaw, A.M. (1985) FEBS Lett. 188, 383–388.
16 Feinstein, M.M., Zavoico, G.B. and Halenda, S.P. (1985) in The Platelets: Physiology and Pharmacology (Longenecker, G.L., ed.), pp. 237–269, Academic Press, New York.
17 Lanza, F., Beretz, A., Stieerle, A., Corre, G. and Cazenave, J.P. (1987) Thromb. Res. 45, 477–484.
18 Jy, W., Ahn, Y.S., Shanbaky, N., Fernandez, L.F., Harrington, W.J. and Haynes, D.H. (1987) Circ. Res. 60, 346–355.
19 Rink, T.J. and Smith, S.W. (1983) J. Physiol. (London) 338, 66P-67P.
20 Johansson, J.S. and Haynes, D.H. (1992) Biochim. Biophys. Acta 1105, 40–50.
21 Sage, S.O. and Rink, R.J. (1985) FEBS Lett. 188, 135–140.
22 Thompson, N.T. and Scrutton, M.C. (1985) Eur. J. Biochem. 147, 421–427.
23 Owen, N.E. and Le Breton, G.C. (1981) Am. J. Physiol. 241, H613–H619.
24 Jy, W. and Haynes, D.H. (1987) Biochm. Biophys. Acta 929, 88–102.
25 Jy, W. and Haynes, D.H. (1988) Biochem. Biophys. Acta 944, 374–382.
26 Brass, L.F. (1984) J. Biol. Chem. 259, 12563–12570.
27 Kaser-Glanzmann, R., Jakabova, M., George, J.N. and Luscher, E.F. (1977) Biochim. Biophys. Acta 466, 429–440.

28 Adunyah, S.E. and Dean, M.L. (1987) Biochim. Biophys. Acta 930, 401–409.
29 Jy, W. and Haynes, D.H. (1984) Circ. Res. 55, 595–608.
30 Johansson, J.S. and Haynes, D.H. (1988) J. Membr. Biol. 104, 147–163.
31 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell Biol. 94, 325–334.
32 Le Peuch, C.C., Dimerstein, R.J., Roth, L.J. and Feinberg, H. (1976) Biochem. Biophys. Res. Commun. 71, 362–370.
33 Millman, M.S., Caswell, A.H. and Haynes, D.H. (1980) Membr. Biochem. 3, 291–315.
34 Dixon, D., Brandt, N. and Haynes, D.H. (1984) J. Biol. Chem. 259, 13737–13741.
35 Valant, P.A. and Haynes, D.H. (1989) Biophys. J. 55, 338a.
36 Nicholls, D.G. (1978) Biochem. J. 176, 463–474.
37 Scott, I.D. and Nicholls, D.G.(1980) Biochem. J. 186, 21–33.
38 MacLennan, D.H. and Wong, P.T.S. (1971) Proc. Natl. Acad. Sci. USA 68, 1231–1235.
39 Halushka, P.V., Mais, D.E., Mayeux, P.R. and Morinelli, T.A. (1989) Annu. Rev. Pharm. Tox. 10, 213–239.
40 Seamon, K.B. and Daly, J.W.J. (1981) Cyclic Nucleotide Res. 7, 201.
41 Grega, D.S. and Macdonald, R.L. (1987) J. Neurosci. 7, 700–707.
42 Enouf, J., Bredoux, R. and Lévy-Toledano, S. (1984) Biochim. Biophys. Acta 772, 251–258.
43 Adunyah, S.E., Jones, L.R. and Dean, W.L. (1988) Biochim. Biophys. Acta 941, 63–70.
44 White, G.C. II, Barton, D.W., White, T.E. and Fischer, T.H. (1989) Thromb. Res. 56, 575–581.
45 O'Rourke, F., Zavoico, G.G. and Feinstein, M.B. (1989) Biochem. J. 257, 715–721.
46 Waldmann, R., Bauer, S., Gobel, C., Hofmann, F., Jakobs, K.H. and Walter, U. (1986) Eur. J. Biochem. 158, 203–210.
47 Siess, W. and Lapetina, E.G. (1990) Biochem. J. 271, 815–819.
48 Fox, J.E.B., Say, A.K. and Haslam, R.J. (1979) Biochem. J. 184, 651–661.
49 Fischer, T.H. and White, G.C. (1987) Biochem. Biophys. Res. Commun. 149, 700–706.
50 Tada, M., Ohmiro, F., Yamada, M. and Abe, H. (1979) J. Biol. Chem. 254, 319–326.
51 Kranias, E.G. (1985) Biochim. Biophys. Acta 844, 193–199.
52 Vane, J.R., Bunting, S. and Moncada, S. (1982) in International Review of Experimental Pathology (Richter, G.W. and Epstein, M.A., eds.), pp. 162–207, Academic Press, New York.
53 Oliva, D. and Nicosia, S. (1987) Pharmacol. Res. Commun. 19, 735–765.
54 Tateson, J.E., Moncada, S. and Vane, J.R. (1977) Prostaglandins 13, 389–397.
55 Moncada, S. and Vane, J.R. (1978) Br. Med. Bull. 34, 129–135.
56 Alheid, U., Reichwehr, I. and Forstermann, U. (1989) Eur. J. Pharmacol. 164, 103–110.
57 Ashby, B. (1989) Mol. Pharmacol. 36, 866–873.
58 Mogimori, K., Kajikawa, N., Nishio, S. and Yajima, M. (1989) Prostaglandins 37, 205–212.
59 Kahn, N.N. and Sinha,A.K. (1989) Biochim. Biophys. Acta 984, 113–118.
60 Feinstein, M.B., Zavoico, G.B. and Halenda, S.P. (1985) in Platelets, Physiology and Pharmacology (Longenecker, G.L., ed.), pp. 237–269, Academic Press, New York.
61 Rittenhouse, S.E. (1983) Proc. Natl. Acad. Sci. USA 80, 5417–5420.
62 Lazarowski, E.R. and Lapetina, E.G. (1989) Biochem. Biophys. Res. Commun. 158, 440–444.
63 Yada, Y., Nagao, S., Okano, Y. and Nozawa, Y. (1989) FEBS Lett. 242, 368–372.
64 Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705–12708.
65 Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1982) Blood 60, 1247–1250.
66 Adunyah, S.E. and Dean, W.L. (1985) Biochem. Biophys. Res. Commun. 128, 1274–1280.
67 Gerrard, J.M., Peller, J.D., Krick, T.P. and White, J.G. (1977) Prostaglandins 14, 39–50.
68 Samuelsson, B., Folco, G., Granstrom, E., Kindahl, H. and Malmsten, C. (1978) Adv. Prostaglandin Thromb. Res. 4, 1–25.
69 Wardell, M.R., Reynolds, C.C., Berndt, M.C., Wallace, R.W. and Fox, J.E. (1989) J. Biol. Chem. 264, 15656–15661.
70 Fox, J.E. and Berndt, M.C. (1989) J. Biol. Chem. 264, 9520–9526.
71 Van Willigen, G. and Akkerman, J.W. (1991) Biochem. J. 273, 115–120.
72 Tohmatsu, T., Nishida, A., Nagao,S., Nakashima, S. and Nozawa, Y. (1989) Biochim. Biophys. Acta 1013, 190–193.
73 Tada, M., Ohmiro, F., Yamada, M. and Abe, H. (1979) J. Biol. Chem. 254, 319–326.
74 Haynes, D.H. and Mandveno, A. (1983) J. Membr. Biol. 74, 25–40.
75 Dixon, D.A. and Haynes, D.H. (1990) Biochim. Biophys. Acta 1029, 274–284.
76 Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478.
77 Shull, G.E. and Greeb, J. (1988) J. Biol. Chem. 263, 8646–8657.
78 Gre[illegible], J. and Shull, G.E. (1989) J. Biol. Chem. 264, 18569–18576.
79 Nishio, H., Ikegami, Y. and Segawa, T. (1991) Cell Calcium 12, 177–184.