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Cyclic GMP increases the rate of the calcium extrusion pump in intact human platelets but has no direct effect on the dense tubular calcium accumulation system

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Sodium nitroprusside (SNP) and other agents that elevate cGMP levels are known to inhibit the aggregation of human platelets. Published data suggest that cGMP attenuation of agonist-induced Ca^{2+} transients is involved in this effect. The present study shows that elevation of cGMP increases the rate of the Ca^{2+} extrusion pump located in the plasma membrane (PM) but does not have a direct effect on the Ca^{2+} accumulating pump of the dense tubules (DT). The study verifies that SNP can specifically elevate the cGMP level in the platelet. The kinetics of the Ca^{2+} extrusion system were studied in situ in platelets overloaded with the cytoplasmic Ca^{2+} indicator quin2 according to a published protocol developed in this laboratory. Elevation of cGMP by means of (10 μM) SNP increased the V_m of the Ca^{2+}-ATPase pump by 63%, without affecting its K_m (66–80 nM) or Hill coefficient (1.6–1.8). Dibutyryl-cGMP (Bt_2-cGMP), preincubated for 45 min at 1 mM, increased the V_m by a factor of 2.2 ± 0.4. The experiments did not give any indication that SNP or Bt_2-cGMP change the rate of the Na^+/Ca^{2+} exchanger which makes a minor contribution to Ca^{2+} extrusion in the studied $[Ca^{2+}]_{cyt}$ range. The rate constant for passive leakage of Ca^{2+} across the PM was increased by 32 ± 4% by SNP and 90 ± 34% by Bt_2-cGMP. The net result is that the free Ca^{2+} in the cytoplasm ($[Ca^{2+}]_{cyt}$) at 'rest' is lowered from control values of 112 nM to 89 nM or 80 nM, respectively. The kinetics of Ca^{2+} uptake by the dense tubules were determined in situ using the fluorescence of chlorotetracycline (CTC) according to protocols developed in this laboratory. Analysis showed that SNP and Bt_2-cGMP had no effect on the V_m or K_m of the dense tubular pump, and did not affect the rate constant for passive leakage. The agents did decrease resting $[Ca^{2+}]_{dt}$ by 25% or 30%, respectively, but this result can be explained purely in terms of the reduced $[Ca^{2+}]_{cyt}$. The effects of cGMP (vs. cAMP) on the PM and DT pumps are closely correlated with reported effects of cGMP/cAMP induced phosphorylation of a protein of the molecular weight of the PM pump and a 22 kDa activator of the DT pump. Cyclic AMP increases the rate of both the PM and the DT pumps, whereas cGMP increases the rate of the PM pump only. In combination, treatment with maximally-effective doses of Bt_2-cGMP and Bt_2-cAMP had no greater effect on the PM pump than did either agent alone.

Abbreviations: SNP, sodium nitroprusside; PM, plasma membrane; ROC, receptor-operated channel; 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; K_m, the $[Ca^{2+}]_{cyt}$ giving half-maximal rate of extrusion (V); n, Hill coefficient; dibutyryl-cGMP (Bt_2-cGMP), N^2,2′-O-dibutyrylguanosine 3′:5′-cyclic monophosphate; dibutyryl-cAMP (Bt_2-cAMP), N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; $[Ca^{2+}]_{cyt}$, the free ('ionized') Ca^{2+} concentration in the cytoplasm; $[Ca^{2+}]_{ex}$, the free Ca^{2+} concentration in the dense tubular lumen; DT, dense tubules; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; CTC, chlorotetracycline; IC_{50}, concentration for 50% inhibition; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; F_{max}, fluorescence of Ca^{2+} complexed form of quin2; F_{min}, fluorescence of uncomplexed form of quin2; 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); SR, sarcoplasmic reticulum; EDRF, endothelium-dependent relaxing factor; SL, sacrolemma; X, ratio of leak rate to V_m of pump; R_{CTC}, CTC ratio (proportional to $[Ca^{2+}]_{dt}$), ratio of CTC fluorescence amplitude of time-resolved Ca^{2+} uptake phase to 'instantaneous' amplitude.

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Introduction

The present study is the third in a series describing the effects of cyclic nucleotides on the Ca^{2+} handling by the human platelet. The previous two studies [1,2] dealt with the effects of cAMP*, showing that it increases the V_m of the Ca^{2+} extrusion pump in the plasma membrane (PM), increases the rate of passive Ca^{2+} leakage across the PM, and increases the V_m of the Ca^{2+} accumulating pump in the dense tubule. The effect of elevated cGMP levels on platelet function is less well characterized. The present study reports similar investigations with respect to cGMP. Our methods use the fluorescent indicator quin2 as a measure of the free Ca^{2+} concentration in the platelet cytoplasm ($[Ca^{2+}]_{cyt}$) and chlorotetracycline (CTC) as a measure of the free Ca^{2+} concentration in the dense tubular lumen ($[Ca^{2+}]_{dt}$).

It has been hypothesized that cGMP might act as a negative feedback inhibitor of agonist-induced platelet activation [3] as had previously been proposed to be the case in smooth muscle [4]. Additional evidence for this mechanism is found in *in vitro* studies of platelets added to vascular endothelial cells [5], in studies of human platelets transiting rabbit hearts [6] and *in vivo* studies in brain microvasculature [7]. Human platelets contain guanylate cyclase activity [8] and cGMP-dependent protein kinase activity [9]. The former can be stimulated with a variety of nitro compounds, including sodium nitroprusside (SNP). This has been demonstrated in both intact platelets [10,11] and in purified enzyme preparations [12,13]. Sodium nitroprusside is reported to selectively elevate platelet cGMP levels [10,11], although some controversy exists since recent studies [14,15] have reported that SNP elevated both cGMP and cAMP. The present study uses both SNP and the membrane-permeable dibutyryl-cGMP (Bt_2-cGMP) to elevate cGMP and probe its effects on Ca^{2+} extrusion and sequestration.

Sodium nitroprusside inhibits platelet aggregation [13,16,17]. It inhibits a large number of platelet reactions, including myosin light chain phosphorylation and serotonin release [18], diacylglyceride formation and 40 kDa protein phosphorylation [19], the elevation of $[Ca^{2+}]_{cyt}$ in response to PAF [10], and aggregation in response to ADP [11,13] with IC_{50}'s of 0.09–100 μM. Moreover, cGMP analogues have been shown to decrease Ca^{2+}-dependent arachidonic acid liberation after collagen stimulation [20] and thrombin [21].

Three studies have focussed on SNP effects of Ca^{2+} mobilization: In the first study, the agent has been shown to decrease the production of 1,2-diacylglycerol [19], which is believed to be a corequisite with Ca^{2+} for signaling the exocytotic release reaction [22,23]. In the second study, SNP was shown to decrease the thrombin-induced elevation of $[Ca^{2+}]_{cyt}$ [18]. The third study

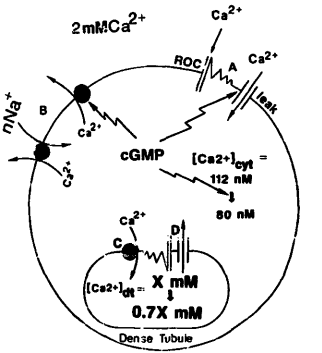

Fig. 1. Ca^{2+} handling systems of the platelet considered in the present study. The four processes considered are: (A) Ca^{2+} influx, (B) Ca^{2+} extrusion, (C) dense tubular Ca^{2+} uptake and (D) Ca^{2+} efflux from the dense tubule. These processes are carried out by receptor operated channels (ROC) and passive leak (A), the Ca^{2+}-ATPase extrusion pump and the Na^+/Ca^{2+} exchanger (B). The Ca^{2+}-accumulating ATPase pump of the dense tubule (C), and passive leakage and ROC's in the dense tubular membrane. The Na^+/Ca^{2+} stoichiometry of the exchanger has not yet been determined in platelets, and is thus designated *n*. Stimulatory effects of cGMP (denoted by zig-zag arrow) on the extrusion pump, and the passive leak in the plasma membrane are demonstrated in the present study.

[10] showed that SNP can increase the rate of clearance of Ca^{2+} from the cytoplasm of platelets stimulated with platelet activating factor (PAF). As discussed earlier [2,25], this type of result can be explained as any one of a number of possible alterations of Ca^{2+} pump and Ca^{2+} permeability of the plasma membrane (PM) and dense tubules. The relationship between these pumps and permeabilities is illustrated in Fig. 1. The present study will show that elevated cGMP increases the rates of Ca^{2+} *extrusion* but does not directly affect the rate of uptake by the dense tubular system.

A previous study from this laboratory [26] has described a protocol whereby the kinetics of the Ca^{2+} extrusion system in the plasma membrane (PM) can be characterized *in situ*. The method involves overloading the platelet with the fluorescent Ca^{2+} indicator quin2, increasing the free concentration of Ca^{2+} in the cytoplasm ($[Ca^{2+}]_{cyt}$) using ionomycin, removing external Ca^{2+} and recording the progress curve for active extrusion. Application of this method showed that cAMP increases the V_m of the extrusion pump by a factor of

1.6–2.0 [25]. Another method developed in this laboratory [26,27] makes use of the fluorescent indicator chlorotetracycline (CTC) to monitor the free Ca^{2+} concentration in the dense tubules ($[Ca^{2+}]_{dt}$) in the intact platelet. Application of this method showed that cAMP increases the V_m of the dense tubular pump by a factor of 1.42–1.56 [2]. That study showed that although stimulation of the extrusion pump lowered $[Ca^{2+}]_{cyt}$, the stimulation of the dense tubular pump is able to overcome this effect and increase the resting $[Ca^{2+}]_{dt}$ by 70–72%. The present study applies these methods to test the effects of *cGMP* on these two pumps and their interplay in controlling $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{dt}$.

Materials and Methods

Materials

Sodium nitroprusside was purchased from Sigma Chemical Co., St. Louis, MO. Monensin was a gift from Dr. B.C. Pressman. Sources for all other chemicals, reagents, assay kits, ionophores and inhibitors were as identified previously [2,24].

Preparation of platelet-rich plasma and washed platelet suspensions

Platelet isolation is as described previously [27]. The platelets were resuspended in a nominally Ca^{2+}- and Mg^{2+}-free Tyrode's solution ($[Ca^{2+}]$ approx. 1 μM) of the following composition (in mM): 138 NaCl/3 KCl/10 glucose/2 $NaHCO_3$/0.4 NaH_2PO_4/2.5 Hepes with the pH adjusted to 7.35. For fluorescence experiments, platelet suspensions were divided into two parts, one of which was loaded with quin2, the other being used for CTC studies. All experiments were completed within 4 hrs. of venipuncture.

Fluorometry, protocols and quantitation

Quin2 experimentation was carried out as described previously [24,25]. Platelets were incubated with 5 μM quin2/AM for experiments on the resting $[Ca^{2+}]_{cyt}$. The degree of quin2 loading was 0.82 ± 0.23 mmol per liter cell volume ('indicator' condition for quin2). For quantitative measurement of the kinetics of Ca^{2+} extrusion [24] the platelets were deliberately overloaded by incubation with 20 μM quin2/AM, reaching quin2 concentrations of 2.42 ± 0.50 mmol per liter cell volume ('overload' condition for quin2). All experiments were repeated at least five times. It was found that 1 mM Bt_2-cGMP produced a fluorescence artifact at the wavelengths used for quin2 experimentation. This was shown to be the result of a Stokes' shift. In the worst case (low degree of quin2 loading resulting from preincubation with 5 μM quin2/AM), 1 mM Bt_2-cGMP increased the apparent fluorescence by $32 \pm 5\%$ (mean $\pm$ S.D., $n = 4$). However, it did not alter the absolute range ($F_{max} - F_{min}$) of the quin2 fluorescence signal.

Measurement of Ca^{2+} uptake by the chlorotetracycline (CTC) technique has been previously described [2,25,27]. Protocols for measuring the V_m and K_m of the dense tubular pump were carried out as described for our recent experimentation with cAMP [2]. The logic of our protocols and methods of calculation are as described previously [2,24].

Radioimmunoassay determinations of cGMP and cAMP

1-ml aliquots of washed platelets ($(1.5–3.2) \cdot 10^8$ per ml) in acrylic cuvettes (Sarstedt) were placed in a thermostatically controlled cell holder at 37°C. External Ca^{2+} was set at 2 mM. Mixing was accomplished with a teflon magnetic 'flea' (600 rpm). For experiments evaluating the concentration of cGMP as a function of time after ionomycin or SNP addition, 3.5 ml of washed platelet suspension was placed in a cuvette and samples (0.8 ml) sequentially removed at the set time points. Quenching and assays were carried out as described previously [24]. Parallel aliquots were treated with SNP or forskolin for varying lengths of time and processed as above. Samples were stored at −18°C prior to assay.

Determination of sodium nitroprusside concentration giving maximal effect

As will be described in the next section, treatment with sodium nitroprusside (SNP) gave rapid changes in [cGMP], and was thus the best means of elevating cGMP and studying its effects on Ca^{2+} handling. Since its reported IC_{50} values show considerable variation as a function of laboratory, and of reaction studied (cf. Introduction), it was necessary to determine the concentration for maximal effect. This was based on the concentration profile for inhibition of aggregation induced by 10 μM ADP. This concentration of ADP was chosen since it has been shown to result in maximal first-phase aggregation kinetics [28]. Fig. 2 presents a dose-response curve for SNP-induced inhibition of ADP-triggered platelet aggregation. Half-maximal inhibition of ADP-induced aggregation was achieved with 1.3 ± 0.2 μM SNP. We chose 10 μM for the SNP concentration in subsequent studies on Ca^{2+} transport. This gave 98% inhibition of aggregation under the conditions of Fig. 2.

Results

Sodium nitroprusside selectively elevates platelet cGMP

Loeb and Gear [29] found that SNP (1 μM) elevated both cGMP and cAMP in human platelets soon after addition (time points < 60 s) while after 10 min only cGMP remained elevated. We therefore examined cyclic nucleotide levels in response to SNP (10 μM) at

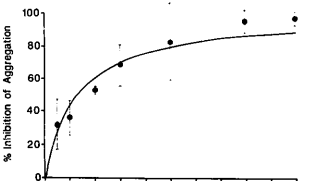

Fig. 2. Concentration dependence of inhibition of platelet aggregation by SNP. Aggregation experiments were carried out as described by Shanbaky et al. [28]. Diluted platelet-rich plasma (PRP), incubated with SNP or vehicle (Tyrode's solution) for 1 min was treated with 10 μM ADP and the extent of aggregation after 2 min (measured as % change in the transmission of 940 nm light) was recorded. The data are the mean of four experiments with error bars representing ± S.D. The solid line is the best fit described by the equation: % Inhibition = 100% · [SNP]/(1.3 μM + [SNP]).

45 s and 10 min after addition. Fig. 3 shows that SNP elevates cGMP in our preparations. Sodium nitroprusside (10 μM) raised cGMP levels 6.8-fold over control levels. The values are in reasonable agreement with the range of published values: 0.5–5 pmol cGMP per 10^9 control platelets and 7.5–26 pmol cGMP per 10^9 platelets treated with 10 μM SNP [10,11,13,29]. We found that the cyclic nucleotide remains at the elevated 45-s value for 10 min without significant change. The data show that less elevation is observed in quin2-overloaded platelets than in sham-loaded platelets. This

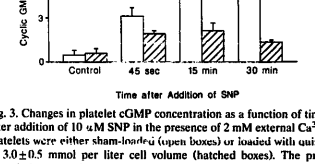

Fig. 3. Changes in platelet cGMP concentration as a function of time after addition of 10 μM SNP in the presence of 2 mM external Ca^{2+}. Platelets were either sham-loaded (open boxes) or loaded with quin2 at 3.0 ± 0.5 mmol per liter cell volume (hatched boxes). The presented data are the average of five experiments with error bars representing ± S.D. The levels of cGMP in both sham-loaded and quin2-loaded platelets were significantly different from control levels at all three time points following SNP treatment (Student's t-test, $P < 0.01$).

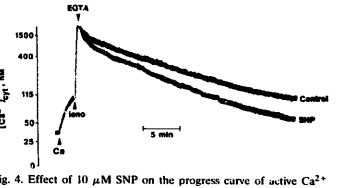

Fig. 4. Effect of 10 μM SNP on the progress curve of active Ca^{2+} extrusion from quin2-overloaded platelets into a low $[Ca^{2+}]_o$ medium. The experiments were carried out according to the protocol described previously [24,25], with platelets at $1.6 \cdot 10^7$ per ml and quin2 loading at 2.7 mmol per liter cell volume. At the high degree of quin2 loading the fluorescence decrease during the course of the extrusion process is a linear measure of the numbers of μmol of Ca^{2+} extruded per liter cell volume. A control experiment with chlorotetracycline (CTC) was carried out as described previously [24,25] to verify that the ionomycin (1 μM) effectively short-circuited dense tubular uptake.

will be discussed in a later section. Control experiments showed that 10 μM SNP did not significantly elevate cAMP levels. Under the conditions of Fig. 3 the cAMP concentration remained at its control value of 2.2 ± 1.8 pmol per 10^9 platelets (not shown). This is corroborated by the finding that SNP reduces dense tubular Ca^{2+} levels (see below) whereas cAMP elevates them [2].

SNP increases the rate of the Ca^{2+} extrusion system

Fig. 4 shows that treatment with 10 μM SNP increases the rate of active Ca^{2+} extrusion from the platelet over the 30 min time course of the process.

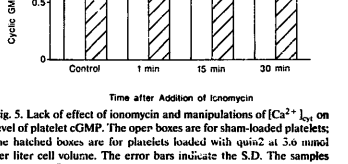

Fig. 5. Lack of effect of ionomycin and manipulations of $[Ca^{2+}]_{cyt}$ on level of platelet cGMP. The open boxes are for sham-loaded platelets; the hatched boxes are for platelets loaded with quin2 at 3.0 mmol per liter cell volume. The error bars indicate the S.D. The samples $((1.5–1.8) \cdot 10^8$ platelets per ml) were treated exactly as in the protocol of Fig. 3. Control: Platelets exposed to 2 mM external Ca^{2+} for 5 min. 1 min: Platelets 1 min after addition of ionomycin sampled immediately after EGTA addition. 15 min and 30 min: Platelets treated as above and sampled at 15 or 30 min.

TABLE I

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

Constant		Value		Unit
		control	SNP-treated	
V_m	extrusion pump (PM)	70.7 ± 3.2	109.5 ± 2.4	$\mu M\ min^{-1}$
		1.7 ± 0.1	2.6 ± 0.1	nmol $mg^{-1}\ min^{-1}$ [a]
K_m	extrusion pump (PM)	66.4 ± 4.5	69.0 ± 2.4	nM
n	(Hill coefficient) extrusion pump	1.8 ± 0.2	1.6 ± 0.1	
k_{linear}	Na^+/Ca^{2+} exchanger (V_m/K_m ratio)	19.6 ± 2.2	21.7 ± 1.5	min^{-1} [b]
		$(4.8 \pm 0.5) \cdot 10^{-4}$	$(5.3 \pm 0.3) \cdot 10^{-4}$	liter $mg^{-1}\ min^{-1}$ [a]

[a] Rate expressed per mg membrane protein.
[b] Corrected for a small (25%) ionomycin contribution (cf. Ref. 25).

A similar result was observed using 1 mM Bt_2-cGMP preincubated for 45 min (see below). Fig. 5 shows that the ionophore addition and manipulation of $[Ca^{2+}]_{cyt}$ in the above protocol do not alter the cGMP concentration. This is in agreement with the finding that the ionophore A23187 does not affect cGMP levels in rat platelets [30].

The extrusion experiment of Fig. 4 was repeated seven times with seven preparations and kinetics were determined as described previously [24,25]. Fig. 6 presents the rates of the extrusion system as a function of $[Ca^{2+}]_{cyt}$ for the SNP-treated and control conditions. As has been shown previously [24,25], the rate vs. $[Ca^{2+}]_{cyt}$ characteristic is composed of two contributions, a saturable one due to the Ca^{2+}-ATPase in the PM which makes its full contribution for $[Ca^{2+}]_{cyt} \leq 400$ nM and a linear one due to the Na^+/Ca^{2+} exchanger which makes its largest contribution for $[Ca^{2+}]_{cyt} > 400$ nM. The figure shows that SNP has little effect on the linear component but has a pronounced effect on the saturable component. As described previously [24,25], the data were fitted with the following equation:

$$V = V_m \cdot \frac{[Ca^{2+}]_{cyt}^{1.7}}{K_m^{1.7} + [Ca^{2+}]_{cyt}^{1.7}} + k_{linear} \cdot [Ca^{2+}]_{cyt} \quad (1)$$

where V is the rate of Ca^{2+} extrusion, V_m is the maximal rate of the Ca^{2+} pump, K_m is its Michaelis constant and 1.7 is its Hill coefficient. The constant k_{linear} describes the contribution of the Na^+/Ca^{2+} exchanger which is not well resolved using quin2. A more recent study using the lower-affinity indicator rhod2 shows its contribution to also be saturable with a K_m between 2.3 and 6.7 μM [31].

Fig. 6 shows that SNP increases the maximal rate of the Ca^{2+} pump (V_m) by 63% but has no effect on the K_m (approx. 69 nM). There was no discernible effect on the Na^+/Ca^{2+} exchanger (k_{linear}). The values for the best-fit constants are given in Table I.

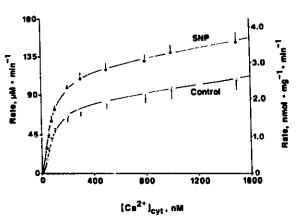

Fig. 6. Effect of 10 μM SNP on the rate of Ca^{2+} extrusion vs. $[Ca^{2+}]_{cyt}$ characteristic. Data are the average (± S.E.) of seven experiments with five preparations, calculated as described previously [24,25]. The left-hand ordinate expresses the rates in μmol Ca^{2+} per liter cell volume per min. The right-hand ordinate expresses the rates in nmol Ca^{2+} per mg platelet protein per min.

Effects of dibutyryl-cGMP and added dibutyryl-cAMP on extrusion pump

The effect of SNP to increase the V_m of the extrusion pump was confirmed by experimentation with

TABLE II

Effect of Bt_2-cAMP and Bt_2-cGMP on the V_m of the Ca^{2+} extrusion pump in the plasma membrane

Platelet suspensions were incubated with 1 mM of each cyclic nucleotide analog for 45 min prior to running the efflux experiments. The maximum transport rates for the cyclic nucleotide-treated samples are reported as ratios relative to the control rates (average ± S.D., $n = 3$ or 4).

Agent	V_m ratio: agent/control
Bt_2-cGMP	2.2 ± 0.4
Bt_2-cAMP	2.0 ± 0.6 [a]
Bt_2-cAMP + Bt_2-cGMP	2.3 ± 0.3

[a] Data from Ref. 24

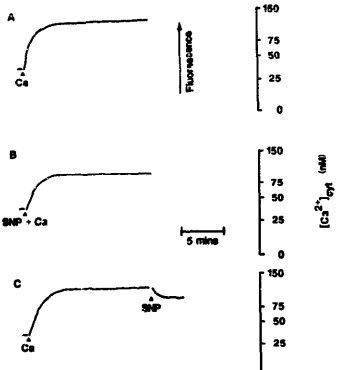

Fig. 7. Effect of SNP on $[Ca^{2+}]_{cyt}$ in resting platelets. A platelet sample with an 'indicator' concentration of quin2 (0.82 mmol per liter cell volume) was suspended in a nominally Ca^{2+}-free Tyrodes's solution and 2 mM Ca^{2+} was added where indicated. Trace A: Control. Trace B: 10 μM SNP added concurrently with Ca^{2+}. Trace C: 10 μM SNP added 15 min after Ca^{2+}.

dibutyryl-cGMP (Bt_2-cGMP), preincubated for 45 min at 1 mM. Table II shows Bt_2-cGMP increases the V_m by a factor of 2.2. The experimentation gave no evidence of any change in K_m. The table also shows that addition of Bt_2-cAMP together with Bt_2-cGMP does not elevate V_m above its cGMP-stimulated value.

SNP and Bt_2-cAMP decrease resting $[Ca^{2+}]_{cyt}$

Since SNP increases the V_m of the extrusion system, it is expected that it would decrease the steady-state $[Ca^{2+}]_{cyt}$ level in resting platelets. Fig. 7 shows that this is indeed the case. Identical $[Ca^{2+}]_{cyt}$ values are achieved when SNP is added before external Ca^{2+} or when SNP is added after steady state is achieved in untreated platelets. Fig. 7 shows that the effect of SNP is rapid. Depression of steady-state $[Ca^{2+}]_{cyt}$ was also observed for 1 mM Bt_2-cGMP. The experimentation of Fig. 7 was carried out in parallel on at least six preparations. Table II summarizes the data. The reduction of $[Ca^{2+}]_{cyt}$ was 21% for SNP and 20% for Bt_2cGMP.

Evidence for increase in k_{leak} of the plasma membrane

We performed calculations to determine whether reduction in $[Ca^{2+}]_{cyt}$ could be explained completely by the increase in V_m. The calculations made use of the method of analysis developed in the previous paper for cAMP (Eqns. 2–5, Johansson et al. [1]). The calculations used experimental data on V_m, K_m (from the extrusion experiment) and $[Ca^{2+}]_{cyt}$ from the steady-state experiment to calculate the rate constant for Ca^{2+} influx (k_{leak}). The calculations, summarized in Table III, show that SNP and Bt_2-cGMP increase k_{leak} $32 \pm 4\%$ and $90 \pm 34\%$, respectively.

SNP causes the resting dense tubular Ca^{2+} ($[Ca^{2+}]_{dt}$) to be decreased

Fig. 8 shows that SNP reduces dense tubular Ca^{2+} uptake measured by CTC fluorescence Table IV summarizes the results obtained in a number of repetitions of this experiment with SNP and Bt_2-cGMP. The table shows that these agents decrease the resting concentration of Ca^{2+} in the dense tubules ($[Ca^{2+}]_{dt}$) by 24–26%. Following the method of analysis described in a previous paper [2], experimentation was carried out to determine whether there was a direct effect on the dense tubular pump.

cGMP does not increase the V_m of the dense tubular pump

Fig. 9 presents initial rate data from the ionomycin challenge test, described previously [2]. At 1 μM ionomycin $[Ca^{2+}]_{cyt}$ exceeds 800 nM and the dense tubular pump is saturated. Fig. 9 shows that the pump velocities measured at elevated $[Ca^{2+}]_{cyt}$ in the presence and absence of 10 μM SNP are identical within the experi-

TABLE III

Sodium nitroprusside- and Bt_2-cGMP-induced changes in resting $[Ca^{2+}]_{cyt}$. Kinetics of the plasma membrane extrusion pump and k_{leak}

Values of X ($= k_{leak}[Ca^{2+}]_o / V_m$) are calculated from Eqns. 3 and 4 of Johansson et al. [24]. Values of $k_{leak}[Ca^{2+}]_o$ were calculated from V_m and Eqn. 4 of Johansson et al. [24]. Values are given as mean ± S.D. with $6 \leq n \leq 15$.

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	112	66.4	70.7 ± 3.2	0.708	50.0	(1.0)
SNP	89	69.0	109 ± 2.4	0.606	66.0	1.32 ± 0.04
Control [a]	100	69.0	73.0 ± 3.2	0.653	47.7	(1.0)
Bt_2-cGMP	80	80.0	161 ± 29	0.562	90.5	1.90 ± 0.34

[a] Control for Bt_2-cGMP.

TABLE IV

Rate and extent of Ca^{2+} uptake by the dense tubules in the presence and absence of cGMP

cGMP was elevated in Ca^{2+}-depleted platelets by preincubation in the cuvette with 10 μM SNP (15 min) or 1 mM Bt_2-cGMP (45 min). CTC (10 μM), 4 μM rotenone and 4 μg/ml oligomycin were also present during the preincubation (15 min). 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)	SNP-treated		Bt_2-cGMP-treated	
	V_{cGMP}/V_{cont}	$R_{CTC,cGMP}/R_{CTC,cont}$	V_{cGMP}/V_{cont}	$R_{CTC,cGMP}/R_{CTC,cont}$
0	0.92 ± 0.16	0.80 ± 0.20	0.92 ± 0.14	0.70 ± 0.21
500	0.97 ± 0.22 [a]	1.00 ± 0.19	0.97 ± 0.25 [a]	1.00 ± 0.24

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

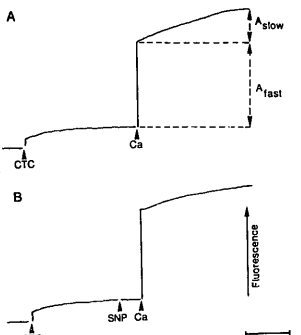

Fig. 8. Effect of SNP on the resting Ca^{2+} level in the dense tubules indicated by CTC fluorescence. Curve A is a control. Curve B is SNP-treated. The experiment was carried out as described previously [2,66]. The platelet concentration was $1.6 \cdot 10^7$ per ml. Concentrations of added agents were 10 μM CTC, 10 μM SNP and 2 mM Ca^{2+}. The CTC ratio (R_{CTC}) is the ratio of the amplitudes of the indicated slow and fast phases.

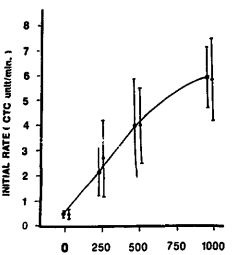

Fig. 9. Effect of ionomycin on the initial rate of Ca^{2+} uptake by the dense tubules in the presence (triangles) and absence (dots) of 10 μM SNP. The procedure is identical to that described by Tao et al. (Ref. 2). Calcium-depleted platelets were incubated with 10 μM CTC, 4 μM rotenone and 4 μg/ml oligomycin for 15 min. Calcium (2 mM) and ionomycin (indicated concentration) were added simultaneously and the initial rates of the slow phase of CTC fluorescence increase were determined. Parallel experiments with quin2 showed that 500 nM ionomycin elevates $[Ca^{2+}]_{cyt}$ to 800–1000 nM.

mental error. This demonstrates that the V_m of the dense tubular pump is not affected by SNP. Fig. 10 shows the absence of a SNP effect on the maximal Ca^{2+} uptake in the presence of ionomycin.

cGMP does not change the K_m of the dense tubular pump

As described above, SNP and Bt_2-cGMP decrease the value of $[Ca^{2+}]_{dt}$ (R_{CTC}) observed in steady state in the absence of ionomycin. This is expected from the lowering of steady-state $[Ca^{2+}]_{dt}$. A previous study [2] showed how information on the K_m of the dense

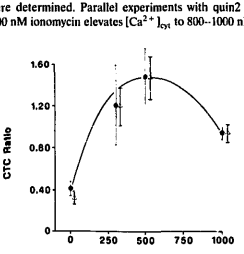

Fig. 10. Effect of ionomycin on the maximal dense tubular Ca^{2+} uptake (CTC ratio) in the presence (triangles) and absence (dots) of 10 μM SNP. The data are from the same experiments as in Fig. 9.

TABLE V

Effect of SNP and Bt_2-cGMP on kinetics of dense tubular Ca^{2+} pump and leak

$[Ca^{2+}]_{cyt}$ values are for the 'resting' state in the presence of 2 mM Ca^{2+}. The V_m values 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 (cf. Figs. 2 and 3, Ref. 2). X is the degree of saturation of the pump in steady state, calculated from Eqn. 3 of Tao et al. [2] using the steady-state $[Ca^{2+}]_{dt}$ and K_c 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+I}/V_m stands for $(k_{leak} + k_{Iono}[Iono])/V_m$ (min^{-1}), which is identical to X/R_{CTC} (Eqn. 6 of Ref. 2). When 0.5 μM ionomycin was present, k_{leak}/V_m (CTC units^{-1}) was calculated, 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. ± values are standard deviations with $6 \leq n \leq 15$.

State	[Iono] (μM)	$[Ca^{2+}]_{cyt}$ (nM)	K_m (nM)	V_m (CTC units)	$[Ca^{2+}]_{dt}$	X	$R_{CTC,max}$	$\frac{k_{leak+I}}{V_m}$	$\frac{k_{leak}}{V_m}$	k_{leak} (CTC units)
Control	0	112 ± 15	180	1.55	0.40 ± 0.07	0.340	1.17	–	0.85	1.31
Control	0.5	⩾ 800	180	1.55	1.51 ± 0.07	1.00	1.51	0.66	0.56	0.87
SNP	0	89 ± 12	193	1.51	0.32 ± 0.08	0.252	1.26	–	0.78	1.18
SNP	0.5	⩾ 800	193	1.51	1.51 ± 0.08	1.00	1.51	0.66	0.56	0.84
Bt_2-cGMP	0	80 ± 8	193	1.51	0.28 ± 0.07	0.255	1.24	–	0.80	1.21
Bt_2-cGMP	0.5	⩾ 800	193	1.51	1.51 ± 0.08	1.00	1.51	0.66	0.56	0.85

tubular pump can be obtained from comparison of initial rates of Ca^{2+} uptake into the dense tubules in the absence of ionomycin. Table IV presents data on the effects of SNP and Bt_2-cGMP on the initial rate ($V = d[Ca^{2+}]_{dt}/dt$) and maximal Ca^{2+} uptake (R_{CTC}) obtained in the absence and presence of 500 nM ionomycin (Figs. 9 and 10). These data were used to determine if the K_m value had changed with cGMP, by the procedure outlined in the previous study (Eqns. 2–5 and Table II of Ref. 2). The results are given in Table V. Briefly, Table V shows little effect of SNP or Bt_2-cGMP on the initial rate of dense tubular uptake in the absence of ionomycin. A K_m of 193 nM was calculated for the SNP- and Bt_2-cGMP-treated cases, a result which is not significantly different from the control value of 180 nM (Table V).

cGMP does not change the k_{leak} of the dense tubular membrane

Table V presents further calculations made to determine whether cGMP had affected the rate of passive Ca^{2+} leak across the dense tubular membrane. The calculations were made as described previously for cAMP (Eqn. 7, Table II, Ref. 2). Table V shows no significant effect of SNP or Bt_2-cGMP on k_{leak}.

TABLE VI

Comparison of cyclic nucleotide effects on pump kinetics and protein phosphorylation

Cyclic nucleotide	Plasma membrane Ca^{2+} pump		Dense tubular Ca^{2+} pump	
	increased V_m	130 kDa phosph.	increased V_m	22 kDa phosph.
cGMP	+	+	0	0
cAMP	+	+	+	+

[a] Data from Ref. 48. They also reported phosphorylation of 100 kDa protein with same pattern as 22 kDa protein.

Discussion

The principal finding of the present study is that cGMP decreases $[Ca^{2+}]_{cyt}$ by increasing the V_m of the Ca^{2+} extrusion pump, but has no direct effect on the dense tubular pump. This is in contrast to cAMP which has been shown to stimulate both [2–4]. As will be shown below, this correlates well with published reports on protein phosphorylation. The next-most important observation is that the cGMP-induced decrease in $[Ca^{2+}]_{cyt}$ results in a decrease in $[Ca^{2+}]_{dt}$. This serves as a basis for the strong anti-aggregatory effects of cGMP.

The pattern of cGMP / cAMP effects (vs. non-effects) on the V_m values of the plasma membrane and dense tubular Ca^{2+} pump correlates with the pattern of cGMP / cAMP-induced protein phosphorylation

The cGMP results of the present study, combined with the cAMP results of the two companion studies [2,24], represent a pattern of activation which correlates perfectly with data on cGMP- and cAMP-stimulated phosphorylation of proteins of correct molecular weight to be pumps or their modulators. The correlation is summarized in Table VI and is explained below. As we have noted earlier [24], Ca^{2+}-Mg^{2+}-ATPase activity has been demonstrated in the platelet plasma membrane using both cytochemical [32], and membrane fractionation techniques [33–36]. Waldmann et al. (Ref. 9) demonstrated that a 130 kDa platelet protein is phosphorylated by both cAMP- and cGMP-dependent protein kinases. The molecular weight is correct for the protein to be the Ca^{2+} extrusion pump of the PM. Although the molecular weight of the

platelet PM Ca^{2+}-Mg^{2+}-ATPase has not been published, the related PM pumps in erythrocytes [37], heart [38], synaptic vesicles [39], and skeletal muscle [40] all have molecular weights in the 125–150 kDa range. An interesting finding of the present study is that the activation effects of cGMP and cAMP are neither additive nor synergistic, suggesting that the phosphorylation may be on the same site(s).

The molecular weight of the platelet dense tubular Ca^{2+} pump is approx. 100 kDa [41] or 105 kDa [34]. This is in line with the general finding that the Ca^{2+}-Mg^{2+}-ATPases of the internal membranes have lower molecular weights than those of the PM (cf. above cited references and Refs. 38 and 42). Furthermore the dense tubular Ca^{2+}-Mg^{2+}-ATPase isolated from human platelets by Dean [41] was found to be antigenically similar, but not identical, to that of sarcoplasmic reticulum (SR). The analogy between the DT and SR is close with respect to the effects of regulatory phosphorylation. As noted in the earlier study [2], the cardiac SR pump is stimulated by cAMP-dependent phosphorylation of a 22 kDa protein named phospholamban [43,44] while the dense tubular pump is stimulated by cAMP-dependent phosphorylation of a 22 kDa protein [45–47]. The cAMP/cGMP phosphorylation pattern for this protein correlates perfectly with our findings on the V_m of the dense tubular pump (Table VI): Elevation of cGMP does not cause phosphorylation of the 22 kDa protein [48] and does not raise the V_m of the DT pump (present study). Elevation of cAMP does cause phosphorylation of the 22 kDa protein [48] and does raise the V_m of the DT pump [2].

cGMP also increases k_{leak} for the plasma membrane

The increase in V_m of the extrusion pump is partially compensated by an increase in the rate constant (k_{leak}) for Ca^{2+} leakage across the plasma membrane. A similar observation was made for cAMP [2]. We believe that leak is as tightly regulated as the pump against which it competes. By analogy to the mammalian heart, the leak is probably a Ca^{2+} channel whose activity is controlled by regulatory phosphorylation [2,49,50]. If cGMP had not increased k_{leak}, the resting $[Ca^{2+}]_{cyt}$ would have been 41–60 nM (cf. Eqn. 6, Ref. 24) and resting $[Ca^{2+}]_{dt}$ would have been very low. It is possible that such a large change would be overly inhibitory to activation.

Effects of cGMP on Ca^{2+} are clearly anti-aggregatory

The present findings are in keeping with the above-cited reports that elevated cGMP is anti-aggregatory, and indeed provide a sufficient explanation of these effects. Cyclic GMP lowers the resting $[Ca^{2+}]_{cyt}$ before the platelet is stimulated. As is the case for cAMP [24], cGMP increases the V_m of the extrusion pump and lowers resting $[Ca^{2+}]_{cyt}$. This results in lowering the initial degree of saturation of the intrinsic cytoplasmic buffers and Ca^{2+} transduction 'machinery'. This means that more Ca^{2+} must be introduced into the cytoplasm to activate the platelet, and that a correspondingly higher agonist concentration will be required for activation. In contrast to cAMP, cGMP does not stimulate the dense tubular Ca^{2+} pump. Without such compensation the resting $[Ca^{2+}]_{dt}$ is reduced 30%. Platelets with elevated cGMP have less stored Ca^{2+} and will thus release less Ca^{2+} to the cytoplasm for a given strength of signal to the dense tubular ROC.

The above effects raise the requirement for the number of receptor operated channels to be opened (mean open time) to reach threshold values of $[Ca^{2+}]_{cyt}$. This would have the effect of increasing the size of the initial stimulus required to commit the platelets to aggregation.

Cyclic GMP also exerts anti-aggregatory effects *after* stimulation.

It would seem that the signal to elevate cGMP can be associated with agonists as well as the antagonist, endothelial-dependent relaxing factor (EDRF; NO). Nimpf and associates [50] have shown that the platelet agonists thrombin, collagen and ADP all produce increases in platelet cGMP levels. This constitutes a negative feedback pathway (cf. Ref. 3). This is of interest since Ca^{2+} elevation, alone, does not affect cGMP levels. This was observed with ionomycin in the present study and in with A23187 in rat platelets [30].

Whether produced by EDRF or by agonist, elevation of cGMP will inhibit events which are known to follow Ca^{2+} mobilization. These include inhibition of myosin light chain phosphorylation and serotonin release [52], inhibition of diacylglyceride formation and inhibition of 40 kDa protein phosphorylation [53]. Furthermore, cGMP decreases the duration of the Ca^{2+} signal as has been demonstrated for activation with thrombin [52] and PAF [10]. The present results suggest that cGMP accomplishes this by increasing Ca^{2+} extrusion. The present finding of increased V_m of the extrusion pump is sufficient to explain the above-mentioned effect on the Ca^{2+} transient. We believe that the behavior of the Ca^{2+} channels in the PM and DT (Processes A and D of Fig. 1) after activation are worthy of further study. We also believe that differential effects of cAMP and cGMP on dense tubular uptake after stimulation are worthy of further study. The present results suggest that cGMP can not give increased dense tubular sequestration after activation whereas cAMP will. Thus cGMP may be a better 'Ca^{2+} antagonist' than cAMP.

It is of interest to consider combined effects of cGMP and cAMP. The present study of the Ca^{2+} extrusion pump shows that when one cyclic nucleotide is present at maximally-effective concentration, the second nucleotide does not produce an additional ef-

fect. There is some evidence of synergism between the two cyclic nucleotides in rabbit platelets [54]. Also, a cGMP-stimulated cGMP/cAMP phosphodiesterase has been isolated from human platelets [55], suggesting that there may be circumstances under which elevations of cGMP concentration can reduce elevations in cAMP concentration.

Caution: Quin2 makes SNP less effective in elevating [cGMP]

It is necessary to discuss an important methodological point: quin2 decreases the SNP-induced increase in [cGMP] relative to sham-loaded platelets (Fig. 3). Although the present experimentation showed that [cGMP] was sufficiently elevated to produce maximal effects on the extrusion pump, this could represent a pitfall to experimentation relying solely on SNP to effect changes in [cGMP]. The nature of this inhibitory effect is not clear since guanylate cyclase is not described as being a Ca^{2+}-regulated enzyme [62]. It is possible that the effect is related to the proposed copper (Cu^{2+}) requirement of the soluble form of guanylate cyclase [64]. Although the affinity of quin2 for Cu^{2+} has not been published, the quin2 analogue EGTA has a formation constant for Cu^{2+} which is 7 orders of magnitude greater than that for Ca^{2+} [65].

Comparison with cGMP effects in other excitable cell types

In cardiac muscle, there is evidence for cGMP effects on the sarcolemmal (SL) pump. Church and Sen [56] reported that cGMP decreased Ca^{2+} uptake in canine cardiac sarcolemma vesicles. They found both a decrease in the V_m and an increase in the K_m. In porcine cardiac sarcoplasmic reticulum (SR), cGMP has been reported to decrease the K_m for Ca^{2+} uptake from 0.4 μM to 0.2 μM [57].

Cyclic GMP clearly stimulates the Ca^{2+} pump in the SL of smooth muscle. This has been demonstrated in studies of SL Ca^{2+}-Mg^{2+}-ATPase activity in porcine smooth muscle [58–60]. It is also suggested in a study of Ca^{2+} extrusion from quin2 loaded rat smooth muscle cells [61]. One study [62] reported that cGMP enhances Ca^{2+} sequestration by the sarcoplasmic reticulum in saponin-permeabilized rat vascular smooth muscle about 20%, although we consider this result difficult to evaluate.

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