J. Membrane Biol. 35, 75–93 (1977)

Mechanism of Ca^{2+}-Dependent Selective Rapid K^+-Transport Induced by Propranolol in Red Cells

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Received 14 June 1976; revised 28 September 1976, 13 December 1976

Summary. Passive Ca^{2+} influx is gradually enhanced by 0.5 to 5mM propranolol in fresh and phosphate ester-depleted human red cells. In fresh cells the active Ca^{2+} efflux tends to counteract Ca^{2+} uptake. Membrane hyperpolarization, induced by the K^+ transport that accompanies Ca^{2+} uptake, further enhances the rate of Ca^2 uptake. The dissociated, positively charged form of propranolol seems to be crucial in the increase of passive Ca^{2+} influx caused by the drug. The effect can be attributed to the release of structural Ca^{2+} from the membrane (lipids).

The release of structural Ca^{2+} promotes the formation of the selectively K^+-permeable membrane structure as well. The transitions of lipid structure responsible for the opening of the passive Ca^{2+} and K^+ pathways, however, are not identical. The opening of the K^+ pathways is prevented by certain highly lipid-soluble substances (chlorobutanol, heptanol, oligomycin, etc.), whereas the formation of the Ca^{2+} pathways is unaffected. Passive K^+ transport is inhibited by high propranolol concentrations (more intensively at alkaline pH), whereas Ca^{2+} transport is promoted. A further difference between the passive K^+ and Ca^{2+} pathways is that SH-proteins also seem to be involved in the formation of the K^+ pathways, whereas they do not play a specific role in the opening of the passive Ca^{2+} channels. The additional Ca^{2+} binding that triggers the formation of the K^+ pathways also seems to occur in the protein area of the inner membrane surface.

It was Manninen *et al.*, who first found that propranolol and pronethalol induced Ca^{2+}-dependent, rapid K^+ transport without disturbing the energy metabolism of human red cells (Ekman, Manninen & Salminen, 1969; Manninen, 1970). Studying the mechanism of this propranolol action, Porzig (1975) claimed that about 20% of membrane-bound Ca^{2+} was liberated by this compound into the cell interior. In preliminary reports we described propranolol elicited enhanced Ca^{2+} uptake in red cells and Ca^{2+} release from red cell membrane (Szász & Gárdos, 1974; Gárdos, Szász & Sarkadi, 1975). Recently we have introduced more sensitive and reliable techniques for studying the propranolol-

induced changes of Ca^{2+} metabolism that lead to the altered K^+ transport of red cells. In this paper the results of this detailed analysis are presented.

Materials and Methods

Chemicals: dl-propranolol (Sigma Chemical Company, St. Louis, Mo.), chlorobutanol (Richter, Budapest), oligomycin (SERVA Feinbiochemica, Heidelberg), iodoacetate (Fluka AG, Buchs), Na bisulfite and inosine (Reanal, Budapest) were of reagent grade. Dipyridamole (Persantin) was a gift of Prof. B. Deuticke; A23187 was kindly provided by Ely Lilly and Co. (Indianapolis, Ind.).

K^+ and Na^+ were determined with an EEL flame photometer.

^{42}K (specific activity = 0.159 C/g K^+) and ^{86}Rb (0.23 C/g Rb^+) were counted in a Beckman Biogamma spectrometer, whereas ^{45}Ca (2.15 C/g Ca^{2+}) in an Intertechnique SL-30 liquid scintillation spectrometer.

Freshly drawn defibrinated human blood was used. Red cells were either washed three times with physiological saline or were exhaustively depleted of phosphate esters by a 4-hr pretreatment at 37 °C with 2.5 mM iodoacetate + 15 mM bisulfite – for 2,3-diphosphoglycerate (DPG) depletion – followed by a 2-hr incubation with 2.5 mM iodoacetate + 10 mM inosine – for ATP depletion – in KCl medium. Cells were freed of the liberated phosphate by thorough washing in isotonic KCl and the phosphate ester regeneration was prevented by adding 10 mM inosine and 2.5 mM iodoacetate. Cellular ATP level was determined by the luciferine-luciferase method of Kimmich, Randles and Brand (1975) using the enzyme preparation of SERVA Feinbiochemica. 2,3-DPG was measured with the aid of the Calbiochem 2,3-DPG kit.

Cells were loaded with Ca^{2+} by treatment with ionophore A23187 and with ^{42}K or ^{86}Rb by making use of the counterflow induced by membrane hyperpolarization during Ca^{2+}-induced K^+ efflux, as described earlier (Sarkadi, Szász & Gárdos, 1976).

Red cell membranes for Ca^{2+}-binding studies were prepared according to Davis and Vincenzi (1971) in a 20 mOsm NaCl medium containing 1 mM EDTA buffered to pH 7.4 with Tris. Before use membranes were washed free of EDTA with 15 mM Tris-HCl buffer of the required pH.

For (Ca + Mg)-ATPase determinations red cell membranes were prepared according to Wolf as modified by Schatzmann (1973).

Membrane lipids were extracted according to Nayler (1966) and phospholipid-P was determined by the method of Allen as modified by Rhodes (1955).

Ca^{2+} influx was studied by the following technique: Washed red cells preincubated at 37 °C for 5 min were added to a similarly preincubated medium containing ^{45}Ca (10 μC/ml) buffered by Tris-HCl (pH 7.4). The haematocrit value was 25%. Immediately after mixing and subsequently at appropriate time intervals 0.2 ml samples were withdrawn and added to 0.2 ml of ice-cold 0.1 M ethyleneglycerol-bis(β-aminoethyl ether)-N,N′-tetraacetate (EGTA). The samples were vigorously shaken and layered upon 7 ml of ice-cold 0.7 M sucrose in 0.16 M KCl. After rapid centrifugation the medium remained on top of the sucrose cushion, whereas the cells formed a compact pellet at the bottom of the tube. The supernatant was discarded, the pellet rinsed with physiological saline and haemolyzed with 0.1% saponin. The haemoglobin content of the haemolysates was measured and the radioactivity of their TCA extracts counted. Cell Ca^{2+} was expressed in mmoles/liter related to the original cell volume calculated from the haemoglobin values.

Ca^{2+} pump activity was measured in intact cells as described before (Sarkadi, Szász & Gárdos, 1976).

Tracer ^{42}K or ^{86}Rb efflux from preloaded cells was followed in a suspension of 5% haematocrit. Samples were centrifuged at $10,000 \times g$ for 1 min and aliquots of the supernatant were counted.

Membrane Ca-ATPase activity was determined according to Schatzmann (1973) in the presence of 10^{-5} M Ca^{2+} at pH 7.0.

The amount of Ca^{2+} in the isolated red cell membrane (later referred to as "structurally bound" or "structural" Ca^{2+}) was not measured. Binding studies were focused on the so-called "additional Ca^{2+} binding". This Ca^{2+} binding capacity of isolated red cell membranes increases proportionally to Ca^{2+} concentration up to 20 mM Ca^{2+} in hypotonic media and up to 80 mM Ca^{2+} in isotonic media. Part of this additionally bound Ca^{2+} is "tightly" bound, inasmuch as it can not be removed by three washings in Ca^{2+}-free media. The maximum capacity for "tight" additional binding has been reported to be 60 nmoles/mg protein in isotonic and 120 nmoles/mg protein in hypotonic media (Sato & Fujii, 1974).

Additional Ca^{2+} binding was measured in 1 ml total volume with 40 μM Ca^{2+} with $^{45}Ca^{2+}$ in a Tris-HCl-buffered medium (ionic strength = 0.025) and 2.5 mg of membrane protein. Total additional Ca^{2+} binding was determined according to the supernatant technique of Kwant and Seeman (1969) by using ^{14}C-inulin for volume correction. The membranes were then washed three times as described by Forstner and Manery (1971), the protein content of samples was determined according to Lowry, Rosebrough, Farr and Randall (1951), the membranes were solubilized by concentrated formic acid (final concentration: 25%) and their radioactivities were counted.

Loosely" bound Ca^{2+} (i.e. Ca^{2+} that could be eliminated by three washings in Ca^{2+}-free media) was a derived value (total-tight).

For the assay of lipid Ca^{2+} binding (exchange) Nayler's (1966) technique was adopted, but for the phase ratios the original description of Folch, Lees and Stanley (1957) was followed to avoid interfacial lipid precipitation. A mixture of 0.2 ml of ^{45}Ca-Ringer's (0.8–1 μC/ml) + 0.05 ml of additives in distilled water was vigorously shaken with 1 ml of lipid extract (lipid-P: 10–15 μg/ml) in chloroform-methanol (2:1). After phase separation and centrifuging the radioactivity of the lower phase was counted. Practically no Ca^{2+} was found in the lower phase if lipids were omitted. Propranolol distribution between the two phases was determined based on its light absorption at 280 nm.

Results

Effect of Propranolol on Ca^{2+} Transport

Propranolol in 0.5–5 mM concentrations transiently increased cell Ca^{2+} of fresh red cells fed with glucose or inosine. As shown on Fig. 1. both effect and duration increased with dose.

Propranolol is known to increase passive K^+ permeability to a level surpassing the charge-conducting Cl^- permeability of the red cell membrane. This shift in relative permeabilities is considered to hyperpolarize the membrane (Glynn & Warner, 1972; Hoffman & Knauf, 1973; Lassen, Pape & Vestergaard-Bogind, 1973), which results in a greatly facilitated influx of the permeable positive cations (K^+, Rb^+) (Glynn & Warner, 1972; Gárdos *et al.*, 1975). Conceivably, Ca^{2+} influx is also facilitated

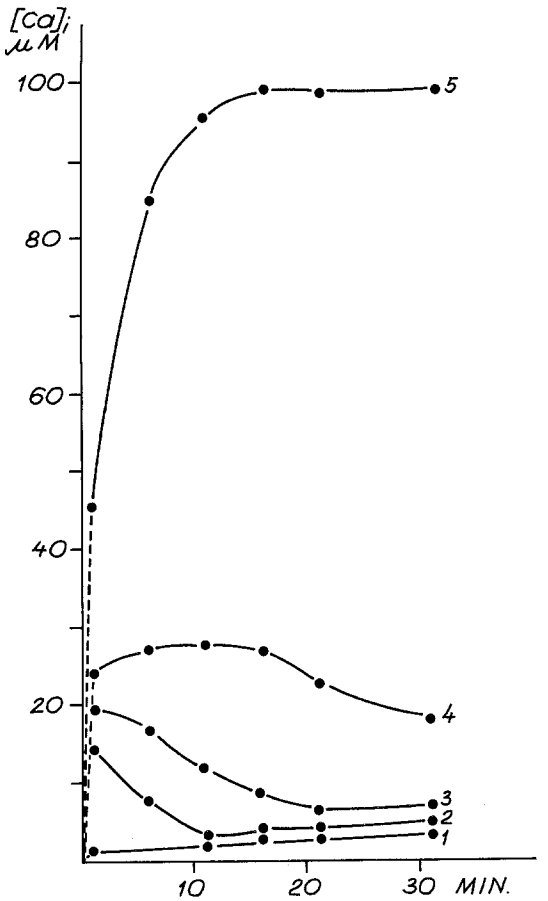

Fig. 1. Effect of propranolol on the Ca^{2+} uptake of fresh washed human red cells. Haematocrit: 25%. Medium was made up of isotonic solutions. Final concentrations in the suspensions (mM): 2.5 $CaCl_2$, 12 Tris-HCl (pH 7.4), 5 glucose, 100 NaCl. Temp: 37 °C. Propranolol stock solution was adjusted to pH 7.4 and diluted with isotonic NaCl. (*1*) Control; (*2*) 0.5 mM propranolol; (*3*) 1 mM propranolol; (*4*) 2.5 mM propranolol; (*5*) 5 mM propranolol. After centrifuging the samples through the sucrose cushion, less than 0.02–0.03% of the medium was trapped in the pellet. The amount of trapped fluid did not change during the incubation. In the control cells ^{45}Ca was taken up corresponding to a Ca^{2+} uptake of 2–4 μmoles/liter of cells/hr. The rate of Ca^{2+} influx induced by a certain propranolol concentration and the time needed for equilibration varied to some extent with different blood specimens (compare curve 3 of this Figure and curve 4 in Fig. 2). In some cases incipient haemolysis occurred at 2.5–5 mM propranolol concentration. One of 12 similar experiments

by the hyperpolarization accompanying rapid K^+ efflux. Experiments were devised for differentiating between the direct and the hyperpolarization-mediated indirect effect of propranolol on Ca^{2+} uptake. To eliminate hyperpolarization, the propranolol-induced K^+ efflux was prevented

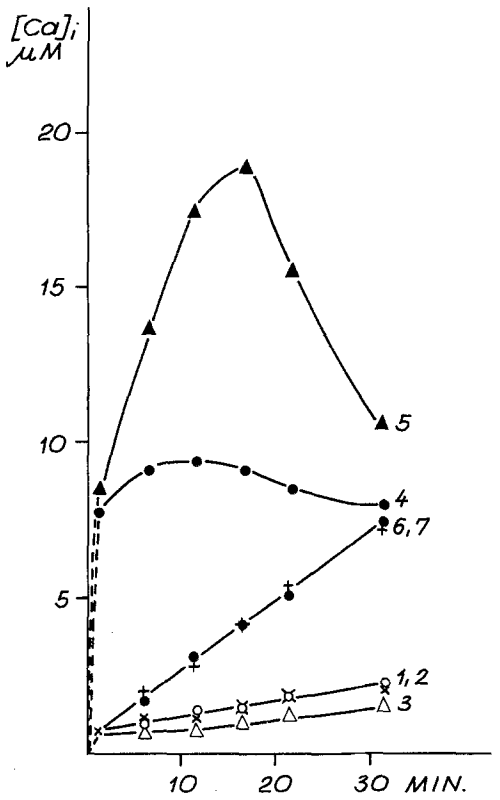

Fig. 2. The role of membrane hyperpolarization in the Ca^{2+} uptake induced by 1 mM propranolol. Haematocrit: 25%. 2.5 mM $CaCl_2$, 5 mM glucose and 12 mM Tris-HCl (pH 7.4) were present in each suspension. Tubes *1–6* contained 100 mM NaCl, whereas tube *7* contained 100 mM KCl. Temp: 37 °C. (*1*) Control; (*2*) 3 mM chlorobutanol; (*3*) 0.2 mM dipyridamole; (*4*) 1 mM propranolol; (*5*) 1 mM propranolol + 0.2 mM dipyridamole; (*6*) 1 mM propranolol + 3 mM chlorobutanol; (*7*) 1 mM propranolol in KCl medium. One of four similar experiments

by a KCl medium or was inhibited by chlorobutanol in NaCl medium (for inhibitory effect *see* Fig. 11). To enhance the propranolol-induced hyperpolarization dipyridamole, a potent inhibitor of the charge conducting Cl^- transport (Hoffman & Knauf, 1973) was applied in NaCl medium. The results obtained are demonstrated in Fig. 2. In the systems where K^+ efflux was inhibited or prevented (lines 6, 7), propranolol still increased Ca^{2+} influx (related to lines 1, 2), but its initial striking effect found in NaCl medium (line 4) disappeared. Dipyridamole, while reducing slightly normal Ca^{2+} uptake (line 3), increased the initial rate and extent but not the duration of propranolol-induced Ca^{2+} uptake in NaCl medium (line 5).

Thus propranolol was found to increase Ca^{2+} influx both directly and indirectly. Its direct effect cannot be attributed to inhibition of the Ca^{2+} pump. It was shown in Fig. 1 that cells pumped out Ca^{2+} in the presence of 0.5–1 mM propranolol. The Ca^{2+} pump function, however, was also tested directly with intact red cells loaded with 1–3 mM Ca^{2+} (Fig. 3). At less than 1 mM concentrations propranolol did not affect Ca^{2+} efflux into Ca^{2+} free KCl media, but at greater concentrations propranolol inhibited this Ca^{2+} efflux. 0.2 mM $LaCl_3$, however, was a more effective inhibitor than 5 mM propranolol. In KCl media with 0–5 mM Ca^{2+}, Ca^{2+} efflux was constant (Sarkadi, Szász & Gárdos, 1976; Sarkadi, Szász, Gerlóczy & Gárdos, 1977), but propranolol inhibition of efflux appeared increased on increasing external Ca^{2+} due to the enhanced influx of Ca^{2+}.

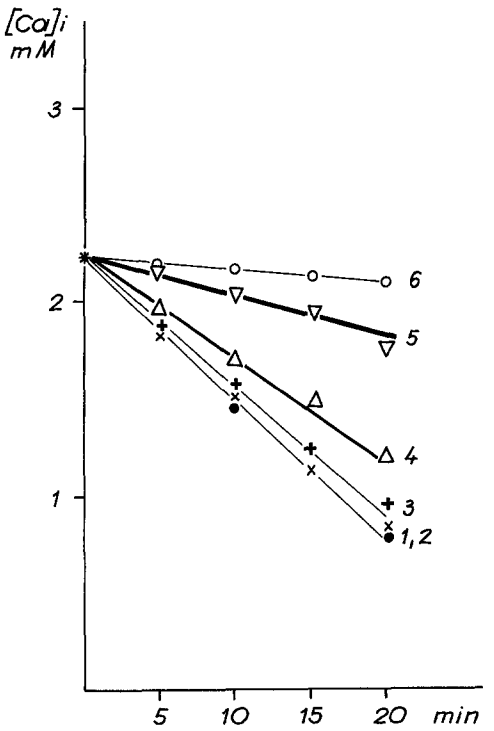

Fig. 3. The effect of propranolol on the Ca^{2+} pump activity of ^{45}Ca-preloaded fresh human red cells. Intracellular [Ca^{2+}]: 2.25 mM. Haematocrit: 20%. Final concentrations in the suspensions: 130 mM KCl, 5 mM glucose, 12 mM Tris-HCl (pH 7.4). Temp: 37 °C. Propranolol stock solution was adjusted to pH 7.4 and diluted with isotonic KCl. (*1*) 1 mM EGTA; (*2*) 1 mM EGTA + 0.5 mM propranolol; (*3*) 1 mM EGTA + 1 mM propranolol; (*4*) 1 mM EGTA + 2.5 mM propranolol; (*5*) 1 mM EGTA + 5 mM propranolol; (*6*) 0.2 mM $LaCl_3$. One of three similar experiments

(Ca + Mg)-ATPase activity of isolated red cell membranes was not inhibited by low concentrations ($\leqq 1$ mM) of propranolol, in good agreement with the findings on intact red cells (Fig. 3). At higher concentrations (Ca + Mg)-ATPase was inhibited and half maximal inhibition was obtained with 2.8 mM propranolol.

Phosphate ester depletion greatly increases the rate of Ca^{2+} entry, but this effect diminishes over 90 min (Fig. 4). Propranolol enhances Ca^{2+} uptake in these cells with an unchanged time course, except at

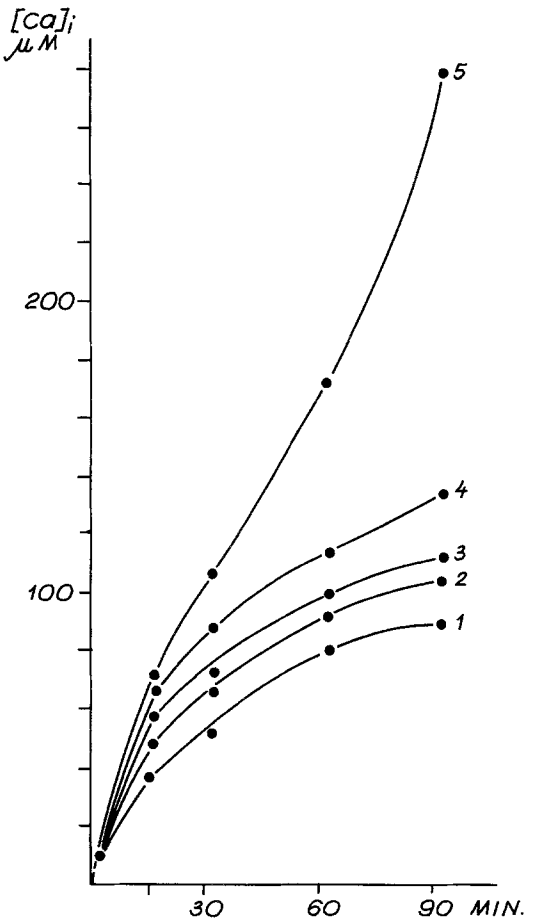

Fig. 4. Effect of propranolol on the Ca^{2+} uptake of exhaustively phosphate ester-depleted cells. Haematocrit: 25%. Final concentrations in the suspensions: 2.5 mM $CaCl_2$, 12 mM Tris-HCl (pH 7.4), 10 mM inosine, 2.5 mM iodoacetate, 95 mM NaCl. Temp: 37 °C. For propranolol concentrations *see* Fig. 1. During incubation with 5 mM propranolol usually haemolysis developed. The 2,3-DPG content was immeasurably low, ATP < 1 μM. One of six similar experiments

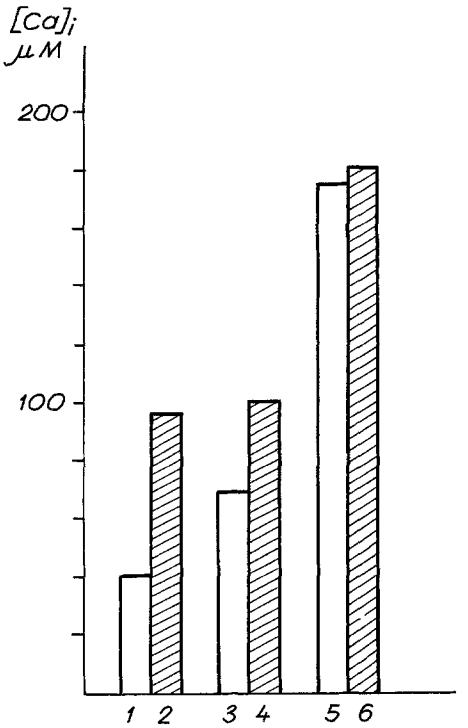

Fig. 5. Effect of 1 mM propranolol on the Ca^{2+} uptake of exhaustively phosphate ester-depleted red cells at various pH values. Haematocrit: 25%. Final concentrations in the suspensions: 2.5 mM $CaCl_2$, 55 mM KCl. The pH was adjusted in suspensions *1–2* to pH 6 with 50 mM acetate-NaOH, in suspensions *3–4* to pH 7.4 in suspensions *5–6* to pH 8.6 with 50 mM Tris-HCl buffer. Incubation time: 60 min; temp: 37 °C. The pH of suspensions did not change throughout the incubation. Open columns: controls; hatched columns: 1 mM propranolol. One of three similar experiments

5 mM where the effect is increased at times greater than 30 min. No recovery from initial influxes of Ca^{2+} can be seen.

Propranolol is a monovalent cation at pH values below its pK (9.45) (Manninen, 1970). As shown in Fig. 5 in phosphate ester-depleted cells the Ca^{2+} influx was increased with pH while the incremental effect of propranolol was increased as pH decreased. The increase in relative propranolol effect at low pH is not due to increased rate of K^+ loss and hyperpolarization (*see* Fig. 9 for data).

Effect of Propranolol on Ca^{2+} Binding

The positively charged form of propranolol is expected to compete with Ca^{2+} for binding sites. We studied the effect of propranolol on

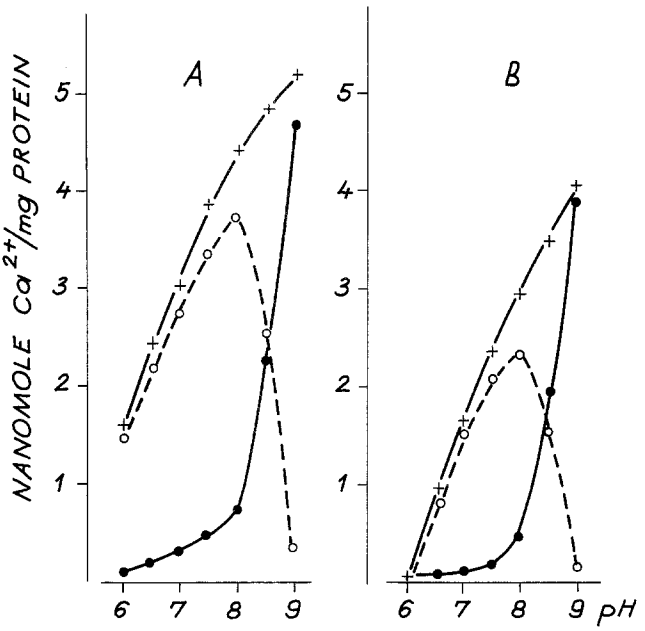

Fig. 6. Ca^{2+} binding of red cell membranes ± 0.5 mM propranolol at various pH values. pH was adjusted with 15 mM Tris-HCl buffers. ×—× total additional, ○—○ "tight" additional, ○—○ "loose" additional Ca^{2+} binding. *A* = Control, *B* = 0.5 mM propranolol. For details *see* Materials and Methods. One of five similar experiments

the "additional" Ca^{2+} binding of isolated red cell membranes, by differentiating between the "tightly" and "loosely" bound Ca^{2+} (for definitions *see* Materials and Methods). As demonstrated in Fig. 6*A*, the elevation of pH increased the total additional calcium bound to the membrane and increased the per cent of this additional calcium which could not be removed in three washings, i.e. was "tightly" bound. The per cent reduction of binding by 0.5 mM propranolol (Fig. 6*B*) was greater at low pH than in the neutral and alkaline range. Fig. 7 shows that at pH 7.4 increasing concentrations of propranolol released mainly the "loosely" bound Ca^{2+}, but "tight" binding was also affected.

Extracted membrane lipids bind (i.e. take over from the water phase) increasing amounts of Ca^{2+} as pH is elevated; the molar ratio of "trapped" Ca:phospholipid-P was found to increase from 0.007 to 0.03 over the pH range 6 to 9. 0.8 mM propranolol in the lipid phase reduced Ca^{2+} binding by 60% at pH 6, but only by 15% at pH 9. In Fig. 7 the data demonstrating the effect of increasing propranolol concentrations on the Ca^{2+} binding of isolated membrane lipids at pH 7.4 are included. The Ca^{2+}-releasing effect of propranolol on red cell membranes

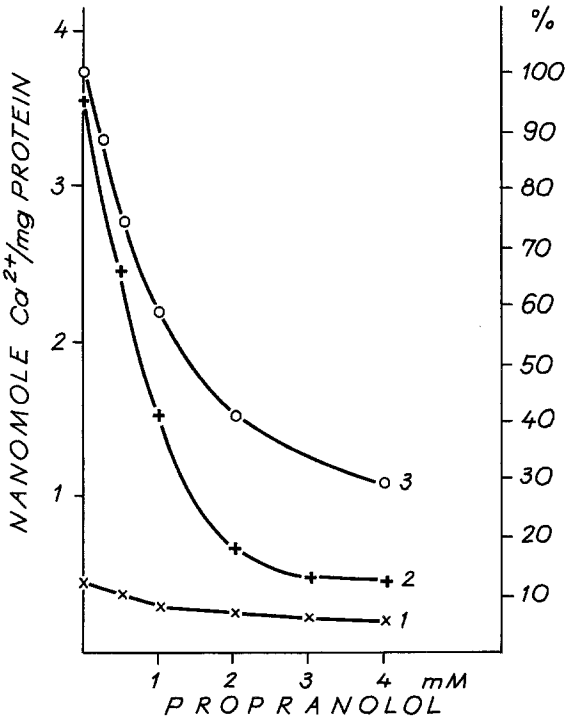

Fig. 7. Effect of increasing propranolol concentrations on the Ca^{2+} binding of red cell membranes and membrane lipids at pH 7.4. Membrane Ca^{2+} binding is related to membrane protein (left ordinate), lipid Ca^{2+} binding to the propranolol-free control aken as 100%. (7.6 nmoles of Ca^{2+} was taken over by 0.38 μmole lipid-P in the control.) (*1*) "Tight" Ca^{2+} binding to red cell membranes; (*2*) "Loose" Ca^{2+} binding to red cell membranes; (*3*) Ca^{2+} binding to isolated red cell lipids. For details *see* Materials and Methods

and membrane lipids is unaffected by oligomycin, chlorobutanol and related compounds (heptanol, *p*-chlorophenol, thymol).

All these experiments provide evidence that propranolol interferes with the Ca^{2+} binding of the membrane and of its components. We tried to find conditions where this effect could be demonstrated in intact cells undisturbed by the interference of propranolol with the Ca^{2+} pump activity. This was attained (*a*) in cells that have taken up a few μM Ca^{2+} during preincubation with tracer Ca^{2+} for 3 hr at 37 °C and (*b*) in the terminal phase of Ca^{2+} pumping in cells loaded with Ca^{2+} by the ionophore method (Fig. 8). Low concentrations of propranolol (0.25–1 mM) increasing passive Ca^{2+} and K^+ permeability but practically unaffecting active Ca^{2+} efflux (*cf.* Fig. 3) were used. Cells contained only a few μM Ca^{2+}, as normally, when the great majority of Ca^{2+} is associated with the membrane. These low concentrations of proprano-

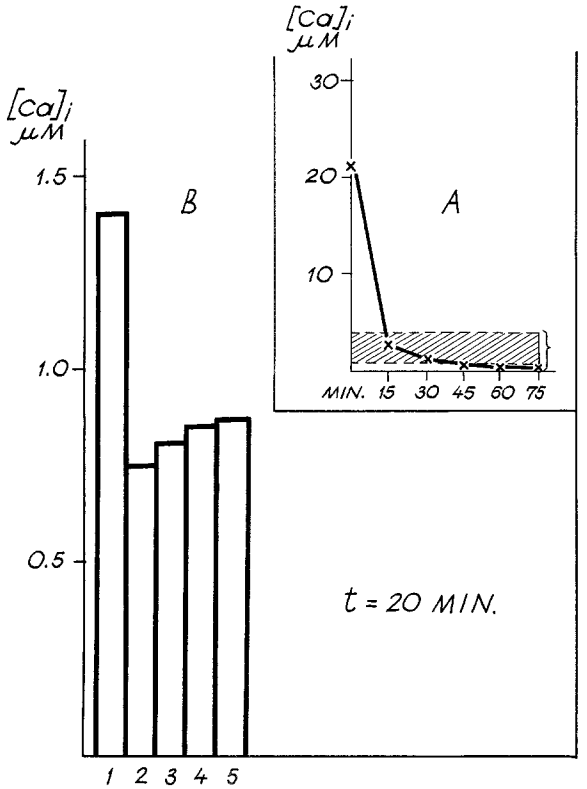

Fig. 8. Effect of propranolol on the Ca^{2+} content of red cells in the final phase of pumping out Ca^{2+} after ^{45}Ca-preloading by the ionophore method. (*A*) Time course of Ca^{2+} pumping. The range in which the effect of propranolol on the residual cell Ca^{2+} content can be reliably studied is hatched. (*B*) Residual Ca^{2+} content of red cells in the presence of various propranolol concentrations after 20-min incubation at 37 °C. Haematocrit: 25%. Final concentrations in the suspensions: 2 mM EGTA, 12 mM Tris-HCl (pH 7.4), 5 mM glucose, 100 mM KCl. Propranolol stock solutions was adjusted to pH 7.4 and diluted with isotonic KCl. (*1*) Control; (*2*) 0.25 mM propranolol; (*3*) 0.5 mM propranolol; (*4*) 0.75 mM propranolol; (*5*) 1 mM propranolol. Cells of samples (0.6 ml) were separated by the sucrose cushion method, and the ^{45}Ca radioactivity of the TCA extracts of their haemolysates was counted. One of four similar experiments. The effect of propranolol compared with the control proved to be highly significant by Student's *t*-test ($p < 0.001$). Intracellular Ca level tended to increase with increasing propranolol concentrations in the range examined (p between 0.05 and 0.02)

lol decreased the residual ^{45}Ca content of the cells in an almost dose-independent manner.

Effect of Propranolol on Ca^{2+}-Dependent K^+ Transport

Ca^{2+}-dependent K^+ transport is induced if intracellular free Ca^{2+} reacts with the inner surface of the membrane (Blum & Hoffman, 1972).

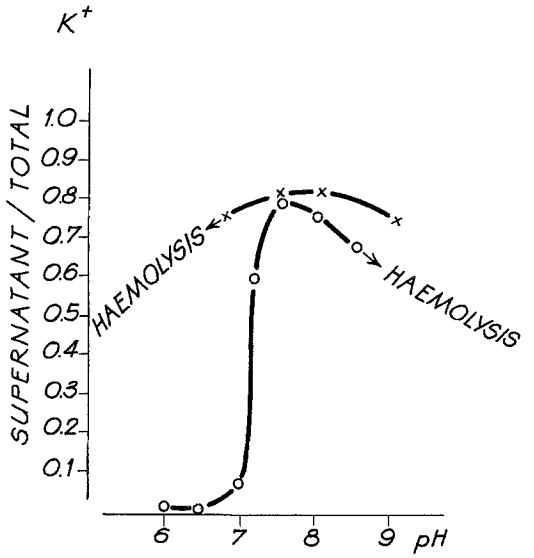

Fig. 9. K^+ distribution in red cell suspensions after 30-min incubation with Ca^{2+} at various pH values. $[Ca^{2+}]=2.5$ mM. Haematocrit: 25%; temp: 37 °C. The pH of suspensions was adjusted with 50 mM acetate-NaOH, or Tris-HCl buffers in a NaCl medium. ×—× exhaustively phosphate ester-depleted cells; ○—○ fresh red cells+0.5 mM propranolol. One of three similar experiments

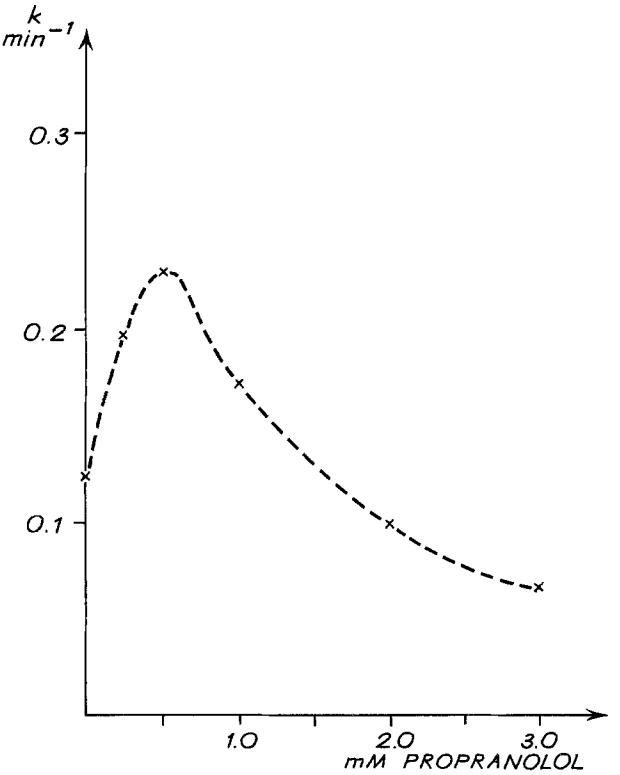

Fig. 10. Effect of propranolol concentration on the rate constant of ^{42}K efflux from Ca^{2+}-loaded red cells. Intracellular $[Ca^{2+}]$: 2.5 mM. Haematocrit: 5%; temp: 37 °C. Medium: 120 mM KCl, 30 mM Tris-HCl (pH 7.4). Initial rates were calculated from the first 5 min

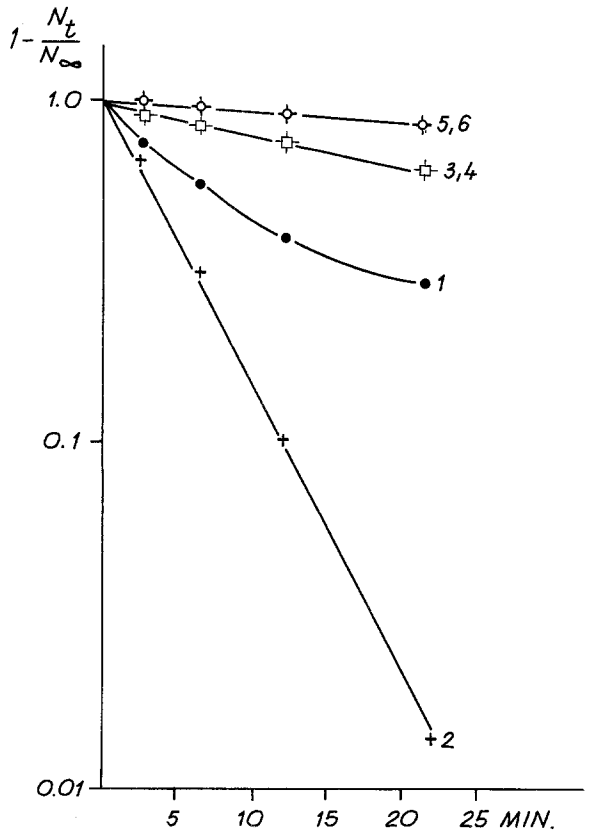

Fig. 11. ^{42}K efflux from Ca^{2+}-loaded red cells. Intracellular $[Ca^{2+}]$: 3.2 mM. Haematocrit: 5%; temp: 37 °C. Medium: 120 mM KCl, 30 mM Tris-HCl (pH 7.4). (*1*) Control; (*2*) 0.5 mM propranolol; (*3*) 10 μg/ml oligomycin; (*4*) 10 μg/ml oligomycin + 0.5 mM propranolol; (*5*) 3 mM chlorobutanol; (*6*) 3 mM chlorobutanol + 0.5 mM propranolol. Oligomycin was dissolved in absolute ethanol; other drugs in isotonic KCl. One of six similar experiments

Fig. 9 shows that in phosphate ester-depleted cells the *pH dependence* of selective K^+ transport is small, whereas in propranolol-treated fresh cells it is pronounced. As seen above (Fig. 6), when the pH is lowered to 6 the number of additional Ca^{2+} binding sites on the membrane decreases (Fig. 6*A*), and the per cent of additional Ca^{2+} released from the membrane by propranolol is augmented (Fig. 6*B*). These effects seem to manifest themselves also in the fact that at this pH propranolol does not induce Ca^{2+}-dependent K^+ transport, although Ca^{2+} influx is enhanced (as shown in Fig. 5). In the alkaline region the cell membrane is damaged by propranolol and above pH 9 haemolysis ensues.

Fig. 10 demonstrates the *effect of propranolol concentration* upon the rate constant of K^+ efflux determined on cells loaded with Ca^{2+} and

^{42}K (or ^{86}Rb), at pH 7.4 in KCl medium, i.e. under exchange conditions with the tracer efflux technique. The selective K^+ permeability of Ca^{2+}-enriched cells is high. This is further enhanced by propranolol up to about 1–1.5 mM concentration (with a maximum at 0.5 mM). The rate constant is diminished by higher propranolol concentrations.

The K^+ efflux from Ca^{2+}-loaded red cells seems to consist of two components. As shown in Fig. 11, low propranolol concentrations make the rapid component predominant. Propranolol, however, cannot suspend the inhibition of K^+ transport caused by substituted alcohols (like chlorobutanol), long-chain alcohols, substituted phenols, and oligomycin. Propranolol concentrations higher than 2 mM inhibit the selective K^+ transport.

Discussion

I. Membrane Effects of Propranolol Influencing Ca^{2+} Transport

The study of Ca^{2+} influx by the washing technique of the cells is laborious and unreliable. During washings the risk of pumping out Ca^{2+} persists and the elimination of adsorbed Ca^{2+} from the cell surface is not ensured. This is especially disturbing if the effect of a drug on Ca^{2+} influx is studied that also influences the binding of Ca^{2+} to the membrane, like propranolol. This is why we applied a treatment with ice-cold EGTA, which removed adsorbed Ca^{2+}, while the Ca^{2+} pump was practically at a standstill. Subsequently, cells were rapidly centrifuged through an ice-cold sucrose-cushion. The Ca^{2+} uptake in the control fed cells, as calculated from the tracer influx, can be attributed to an exchange between intracellular-membrane, and medium-Ca^{2+}. With the technique described we succeeded in detecting the rapid effect of propranolol on Ca^{2+} influx, followed by the pumping out of Ca^{2+} from fresh cells. This biphasic effect of propranolol on cell calcium levels is very similar to the recent findings of Ferreira and Lew (1976), who reported that on addition of Ca^{2+} + A23187, intracellular Ca^{2+} level rapidly increased, then Ca^{2+} pumping started until steady state was achieved. They explain this phenomenon by the initial transitory unequal distribution of A23187 which produces high Ca^{2+} concentration in a fraction of the cells. The same may occur in the case of propranolol addition.

In the enhancement of Ca^{2+} influx the propranolol-induced release of "barrier calcium in the membrane" (in the sense as expounded by Manery, 1966) very probably plays an important role. In view of the

lipid solubility of propranolol [its octanol-buffer partition coefficient is 6.47 at pH 7.0 (Manninen, 1970)], this might occur largely in the lipid region. [The red cell membrane contains about 25 μmoles/liter of cell Ca^{2+}, 34% in the lipid and 66% in the protein fraction (Sato & Fujii, 1974)]. According to our experiments propranolol definitely displaced Ca^{2+} from the membrane lipids. As to the binding and incorporation of propranolol to intact red cells the work of Lovrien, Tisel and Pesheck (1975) is worth mentioning. They revealed that propranolol binding exhibited cooperativity, i.e. the initial binding was followed by a much increased binding later probably due to the exposure of more sites as binding proceeded. Ca^{2+}-displacement may contribute to the cooperative binding of propranolol. The incorporation of cationic anesthetics, like propranolol, into the red cell membrane was studied by Seeman (1972) and Sheetz and Singer (1974). They concluded that the transport of the uncharged molecule was preferred, but that it reacquired a proton at physiological intracellular pH and incorporated preferentially into the phosphatidylserine-rich inner lipid layer, inducing membrane structure changes as a consequence. These data all agree with the expectation that membrane lipids are attacked first by propranolol.

In addition to its direct membrane effects, we found propranolol to facilitate Ca^{2+} uptake also indirectly, by enhancing the K^+ permeability of red cells. The Ca^{2+}-dependent rapid K^+ movement, namely is accompanied by hyperpolarization of the membrane, as shown by the computation of Glynn and Warner (1972) based on ^{42}K uptake of propranolol-treated fresh cells, by ^{42}K influx experiments of Hoffman and Knauf (1973) on ATP-depleted cells, and by the direct membrane potential measurements of Lassen, Pape and Vestergaard-Bogind (1973) on *Amphiuma* red cells. Hyperpolarization facilitates the uptake of permeable cations. Our results with propranolol-treated fresh cells give an insight into the qualitative effects of the inferred hyperpolarization on Ca^{2+} influx (Fig. 2). The comparison of the Ca^{2+} influx data in NaCl versus KCl media with our previous results concerning K^+ movements in these media (Gárdos, Szász & Sarkadi, 1975) indicate that it is hyperpolarization and not the actual K^+ efflux which increases Ca^{2+} influx into propranolol-treated cells in NaCl medium. The quantitative estimation of the extent of hyperpolarization from data on the incremented Ca^{2+} influx is much more problematic than calculated based on K^+ flux experiments. Hence, membrane potential has been estimated from K^+ flux data. In propranolol-treated fresh cells a K^+ permeability of $2.4 \pm 0.26 \times 10^{-7}$ cm/sec and a net Cl^- permeability of 1.63 ± 0.12

$\times 10^{-8}$ cm/sec have been obtained (Schubert & Sarkadi, 1977). These permeability ratios correspond to a membrane potential of about -60 mV, whereas the physiologic value in red cells is -10 mV (Glynn & Warner, 1972).

In brief, our results suggested that propranolol increases Ca^{2+} influx in concentration ranges where it does not interfere yet with the Ca^{2+} pump activity: 1) by displacing Ca^{2+} from the membrane lipids, and 2) by inducing membrane hyperpolarization.

II. Membrane Effects of Propranolol Influencing Selective Rapid K^+ Transport

A. Effects on Ca^{2+} binding. A fraction of Ca^{2+} entering the cell in the presence of propranolol interacts with certain Ca^{2+} receptors at the inner surface of the membrane. This interaction, which is responsible for the "gating" of the K^+-selective pathways in the sense of Lew and Beaugé (1977), represents an additional Ca^{2+} binding (for definition *see* Materials and Methods). The binding site is of low affinity (Lew & Ferreira, 1976), the Ca^{2+} membrane interaction "loose", inasmuch as the rapid K^+ transport can be abolished by washing the cells, by diluting the medium or by adding nonpenetrating Ca^{2+} chelators, like EDTA or EGTA (Gárdos, 1958; Szász, Teitel & Gárdos, 1970). Scatchard analyses of additional total and "tight" Ca^{2+} binding (for definition *see* Materials and Methods) to red cell membranes have been made (Long & Mouat, 1971; Sato & Fujii, 1974). In the present investigation into the effect of propranolol on "loose" Ca^{2+} binding, however, the indirect determination of the loosely bound Ca^{2+} and the irregular Scatchard plot of propranolol binding (Lovrien *et al.*, 1975) would make a Scatchard analysis too complex and not directly pertinent.

The tracer techniques applied for determining additional Ca^{2+} binding conceivably involve also an exchange with the "structural" Ca^{2+} of the membranes. That is the possible reason for the discrepancy between data obtained by tracer techniques and atomic absorption spectrophotometry. Namely, according to the former technique 79% of the "tight" fraction of additionally bound Ca^{2+} is attached to proteins, 16% to lipids and 5% is detected in the aqueous phase (Forstner & Manery, 1971), whereas by the latter method 95% is bound to proteins and only 5% to lipids (Sato & Fujii, 1974). Both techniques indicate, however, that the most probable candidates for "tight" additional Ca^{2+} binding are proteins. Bulk of additional Ca^{2+} binding becomes "tight" at pH

values near 9 (Fig. 6). The Ca^{2+} membrane interactions, however, responsible for inducing K^+ efflux from P-ester-depleted cells remain "loose" even in this alkaline region (*unpublished results*). Thus, the Ca^{2+} binding sites involved in K^+ transport cannot be studied directly. Indirect evidence suggests, however, that proteins are involved in this Ca^{2+} membrane interaction: (*a*) Propranolol at low concentrations, which decrease Ca^{2+} binding to membrane lipids, do not inhibit the rapid K^+ transport; (*b*) SH-reagents (ethacrynic acid, mersalyl, NEM) strongly inhibit the selective K^+ transport (Gárdos *et al.*, 1975), without reducing Ca^{2+} influx and "loose" Ca^{2+} binding (*unpublished results*).

The propranolol-induced release of loosely bound Ca^{2+} from the membrane increases steadily on increasing drug concentration. Thus the specific (presumably protein) binding sites responsible for "gating" the K^+ pathways also become affected at higher propranolol concentrations. This is supposed to be one of the factors resulting in the inhibition of the rapid K^+ transport by higher doses of propranolol. (*See also* Discussion Section II.B.).

B. Effects on the formation of the K^+-permeable membrane structure. The propranolol induced release of Ca^{2+} that fulfilled a "stiffening and tightening" function in the membrane (Manery, 1966) may also facilitate the "gating" of the K^+ pathways. This facilitation could result in the opening of the K^+ pathways even in cells containing "subthreshold" Ca^{2+} concentration. Thus low concentrations of propranolol diminish the resistance of the original membrane structure against the transformation and promote selective K^+ movement (*see* Fig. 10 and Fig. 11, line 2). Higher concentrations of propranolol inhibit selective K^+ movement (*see* Fig. 10) (*a*) by interfering with the Ca^{2+} binding of sites involved in the "gating" process (as mentioned in Section II.A. of the Discussion), and (*b*) by being incorporated into the membrane and perturbing thereby the structure required for the formation of K^+ transport pathways. At alkaline pH, when the ratio of the uncharged, more diffusible propranolol base is higher and the membrane-buffer partition coefficient markedly increases (Seeman, 1972; Sheetz & Singer, 1974) this trend appears at lower propranolol concentrations.

In a similar manner, other highly lipid-soluble substances: such as long-chain alcohols (heptanol) or substituted alcohols (chlorobutanol), phenols (thymol, *p*-chlorophenol) and oligomycin also markedly hinder the formation or maintenance of the K^+-permeable structure. Their effects can be traced back to the perturbation of the lipid structure too.

In the case of heptanol this has been demonstrated by electric resistance (Gutknecht & Tosteson, 1970) and ESR studies (Paterson, Butler, Huang, Labelle, Smith & Schneider, 1972). In contrast to the K^+ permeability, passive Ca^{2+} permeability is not affected by this lipid perturbation.

The conclusion can be drawn that the action of low propranolol concentrations eliciting rapid K^+ transport should be divided into at least two components: (1) Increase of intracellular Ca^{2+} concentration (*see* Discussion Section I.) and (2) Promotion of the formation of the K^+ permeable structure (*see* Discussion Section II.B.).

The inhibition of the selective K^+ transport by higher propranolol concentrations also has a complex nature. The two factors suggested are: (1) Interference with the Ca^{2+} binding of protein sites (*see* Discussion Section II.A.) and (2) Perturbation of the lipid structure (*see* Discussion Section II.B.).

Thanks are due to Drs. A. Egyed and A. Schubert for helpful discussion and to Mrs. E. Irmai and Mrs. S. Andrási for skilled and precise technical assistance. We are indebted to Dr. R.L. Hamill (Ely Lilly and Co., Indianapolis, Ind.), and to Prof. B. Deuticke for providing A23187 and dipyridamole, respectively.

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