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Stereospecific modulation of the calcium channel in human erythrocytes by cholesterol and its oxidized derivatives

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To study the effect of cholesterol and its pathophysiologically important oxidized derivatives (OSC) on the calcium entry channel, the human red blood cell was used as a model system. The calcium ejecting adenosinetriphosphatase (ATPase) was inhibited by vanadate. The cells were loaded with OSC at concentrations between 1.25×10^{-5} and 25×10^{-5} mol/l. 22-Hydroxycholesterol, cholestan-3β,5α,6β-triol, 5α-cholestan-3β-ol,3β,5α-dihydroxycholestan-6-one and 3β-hydroxy-5α-cholestan-7-one stimulated $^{45}Ca^{2+}$ influx by up to almost 90%, whereas 25-hydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol and 7-oxocholesterol inhibited influx by up to 75%. Both stimulation and inhibition were dependent on the amount of OSC incorporated into the membrane. More than 90% of the total modification of calcium influx by OSC was accounted for by an influence on the nitrendipine-inhibitable part of influx. Enrichment of cholesterol in the membrane greatly stimulated, and cholesterol depletion inhibited, Ca^{2+} influx. These results demonstrate that cholesterol and its oxidized derivatives are able to modulate the calcium channel in human red blood cells in a highly stereospecific manner.

Whereas the key role of cholesterol in the pathogenesis of atherosclerosis is uncontested, the mechanism of primary injury to the endothelium remains unclear. Cholesterol has been known for a long time to undergo spontaneous oxidation in air at room temperature (Smith *et al.*, 1967) and it was therefore tempting to speculate that autoxidation products may be the compounds responsible for endothelial injury (for review see Yachnin *et al.*,1979; Anon., 1980). This hypothesis was greatly strengthened by experiments showing that oxidized sterol compounds (OSC; see Fig. 1) cause severe damage to the endothelium when injected into rabbits (Imai *et al.*, 1980) and by the demonstration that OSC occur naturally in food (Taylor *et al.*, 1979) and in human atherosclerotic plaques (Brooks *et al.*, 1971). Furthermore, OSC have a large variety of other effects (Yachnin *et al.*, 1979), the most important of which is inhibition of cholesterol synthesis by some of these compounds (Kandutsch & Chen, 1975; Tanaka *et al.*, 1983; Schroepfer, 1981). This inhibition is thought to involve either reduced synthesis or enhanced degradation of the key enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (Tanaka *et al.*, 1983; Schroepfer, 1981). The chain of events leading to the decrease in this enzyme remains, however, largely obscure.

Because in recent years the universal role of calcium as a second messenger has been recognized (Rasmussen, 1981), it seemed tempting to investigate whether an influence of cholesterol and OSC on the calcium influx channel represents a common denominator for the wide spectrum of effects of these compounds.

Unfortunately, in most cell types calcium influx through the channel can only be measured with great uncertainty due to interference by calcium sequestering intracellular systems and by the two plasma membrane calcium-ejecting systems, the Na^+/Ca^{2+} exchanger and the calcium-specific ATPase.

Abbreviations and trivial names used (see also Fig. 1): ATPase, adenosine triphosphatase; OSC, oxidized sterol compounds; cholesterol, 5-cholesten-3β-ol; 7β-OH-cholesterol, 5-cholestene-3β, 7β-diol; 20α-OH-cholesterol, 5-cholestene-3β,20α-diol; 25-OH-cholesterol, 5-cholestene-3β,25-diol; 7-oxocholesterol, 3β-hydroxy-5-cholesten-7-one; 22*S*-OH-cholesterol, 5-cholestene-3β,22*S*-diol; cholestanol, 5α-cholestan-3β-ol; 6-oxocholestanol, 3β-hydroxy-5α-cholestan-6-one; 7-oxocholestanol, 3β-hydroxy-5α-cholestan-7-one; cholestantriol, cholestan-3β,5α,6β-triol.

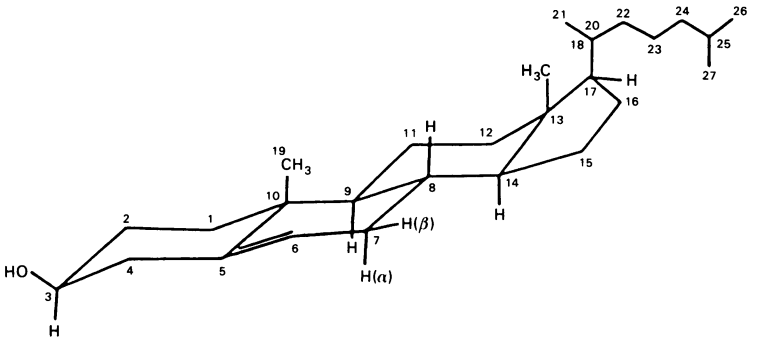

Fig. 1. *Structure of 5-cholesten-3β-ol (cholesterol)*
The oxidized derivatives used in this study are substituted at positions 7, 20, 22 and 25. Derivatives of cholestane lack the double bond at position 5.

The human red blood cell, however, does not present these problems, because it only possesses a calcium influx channel and a calcium-ejecting ATPase (Varecka & Carafoli, 1982). Recently, a simple method has been developed to quantify calcium fluxes through the channel reliably by measuring $^{45}Ca^{2+}$ influx into red blood cells whose Ca^{2+}-ejecting system, the Ca^{2+}/Mg^{2+}-ATPase, is inhibited by vanadate (Varecka & Carafoli, 1982). Because of these advantages the human red blood cell was used in the present study.

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Materials and methods

Materials

20α-OH-cholesterol, 22*S*-OH-cholesterol and cholestanol were purchased from Sigma. Pure cholesterol was from Merck. All other OSC were from Steraloids, Wilton, NH, U.S.A.

OSC and cholesterol were checked for purity by t.l.c. (see below) prior to use and all showed a single spot except for 6-oxocholestanol, which showed a minor second spot. Dipalmitoylphosphatidylcholine was from Fluka, Switzerland. Sodium orthovanadate was from EGA-Chemie, Steinheim, Germany. $^{45}CaCl_2$ was obtained from Amersham International and had a specific radioactivity of 25mCi/mg (0.94GBq/mg).

Nitrendipine (Bay e5009), 0.02% in poly(ethyleneglycol)/ethanol/0.9% saline (3:3:14), was a kind gift from Dr. Aman of Bayer Schweiz. It was kept and handled at 4° in the dark. All other reagents were of the highest purity available.

Buffers

The influx buffer contained 100mM-NaCl, 5mM-KCl, 0.5mM-$MgCl_2$, 5mM-glucose and 50mM-Hepes (sodium salt), pH7.2.

For influx buffer containing albumin 0.25% bovine serum albumin was added. Vanadate was stored frozen as a 0.5M-stock solution in 0.5M-Hepes (sodium salt), pH7.2.

Liposomes

Cholesterol/phosphatidylcholine liposomes were produced according to Cooper *et al.* (1975). Dipalmitoylphosphatidylcholine (40mg) and various amounts of cholesterol (14–125mg) were suspended in 10ml of Hank's balanced salt solution and sonicated continuously under N_2 at 45°C for 50min in a Heat Systems-Ultrasonics W220 sonifier with a standard tip at 70W.

The mixture was centrifuged at 21000*g* for 30min in a Sorvall SS34 rotor to remove metal particles and large lipid aggregates. The final cholesterol/phosphatidylcholine ratio was determined enzymically using a Boehringer kit for cholesterol and a Wako (Osaka, Japan) phospholipids B kit for phospholipids. The absolute lipid concentrations and the cholesterol/phospholipid ratios obtained with this method showed less than 10% deviation from previously published results using acid hydrolysis and inorganic phosphate determination (Cooper *et al.*, 1975, 1978).

Loading with OSC and cholesterol

Heparinized blood (40–50ml) was taken from healthy donors and processed immediately. It was washed three times in influx buffer containing albumin and each time the buffy coat was removed carefully. Loading with OSC was essentially carried out as described previously (Streuli *et al.*, 1981). In short, a final suspension of 1ml of packed red blood cells in 19ml of influx buffer containing bovine serum albumin (without Ca^{2+}) was made and 200μl of OSC in ethanol (final concentration 1.25×10^{-5}–25×10^{-5}M) was added with stirring. Controls contained the same amount of ethanol (1%). After a 1h incubation at 37°C the cells were washed twice in influx buffer containing albumin,

once in influx buffer and 1 vol. of packed cells was resuspended in 2 vol. of influx buffer, yielding a packed cell volume of 25–28%. Incubations with cholesterol/phosphatidylcholine liposomes were carried out for 14 h at 37°C under gentle shaking in the presence of pencillin (50000 units/litre). Under these conditions, no visible haemolysis occurred.

Lipid analysis

The final cholesterol/phosphatidylcholine ratio in the membrane was assessed after lipid extraction (Rose & Oklander, 1965) and resuspension of the lipids in ethanol. Membrane cholesterol and phosphatidylcholine content were then determined with the same kits as for liposomes (see above) except that 1% ethanol (final concentration) was present for complete dissolution of the extract. This amount of ethanol did not interfere with the enzyme activities of the coupled enzyme assay in the kit. The final cholesterol/phosphatidylcholine ratio in the membrane (after 14h incubation) deviated less than 15% from that in the liposomes.

After loading with OSC a 0.5ml sample of packed red blood cells was haemolysed in 2ml of water and frozen for the determination of sterol content. OSC extraction, saponification of the phospholipids and t.l.c. were performed as described previously (Hsu *et al.*, 1980). After charring, the amount of sterol was quantified on a Zeiss PMQ II/4MIII t.l.c. scanner. Recovery of added [^{14}C]cholesterol was between 65 and 70%.

$^{45}Ca^{2+}$-influx assay

Ca^{2+}-influx was measured as previously described (Varecka & Carafoli, 1982). In short, a suspension (measured packed cell volume 25–28%) of sterol-loaded red blood cells was preincubated at 25°C for 15min with 0.5mM-sodium orthovanadate in order to inhibit completely the Ca^{2+}/Mg^{2+}-ATPase. $^{45}CaCl_2$ was then added to a final concentration of 2.25mM ($2{\cdot}5 \times 10^6$ c.p.m./ml). In the experiments using nitrendipine, solutions were handled under sodium light and incubated in light-absorbing plastic tubes. Nitrendipine was added after the 15min preincubation with vanadate, and after a further 5min $^{45}Ca^{2+}$ was added.

Unless stated otherwise, after 60min (at 25°C) 0.5ml aliquots of the suspension were withdrawn and washed twice in 50ml of influx buffer with 0.5mM-vanadate (but without Ca^{2+}). The final pellet was precipitated with 1.5ml of 10% (w/v) trichloroacetic acid containing 20mM-$LaCl_3$ to displace all $^{45}Ca^{2+}$ from the precipitate and then counted in a Packard TriCarb A 300 scintillation counter with 35% efficiency. Packed cell volume. and cell number were determined for each sample and all flux calculations were made using the exact individual packed cell volume.

In 20 independent controls, $^{45}Ca^{2+}$-influx was 6.1 ± 1.2 μmol/h per litre of packed cells. This was about 4–5 times lower than previously described by others (Varecka & Carafoli, 1982). The difference results from the use of fresh instead of outdated blood bank blood (Varecka & Carafoli, 1982) as was confirmed by experiments following calcium influx during ageing of red blood cells.

Intracellular ATP content was measured by using a coupled enzyme assay (Sigma no. 336-UV) after precipitation of the proteins with 6% trichloroacetic acid.

Results

Inhibition of influx by nitrendipine

Nitrendipine was used to define the fraction of the total calcium influx flowing in through the channel.

Fig. 2 shows that nitrendipine blocked calcium influx by almost 70%. The K_i was approx. 2.5 μM. Maximal inhibition was observed at concentrations exceeding 10 μM. In all further experiments the nitrendipine-inhibitable fraction of calcium influx was therefore defined as the portion of total influx inhibited by 15 μM-nitrendipine.

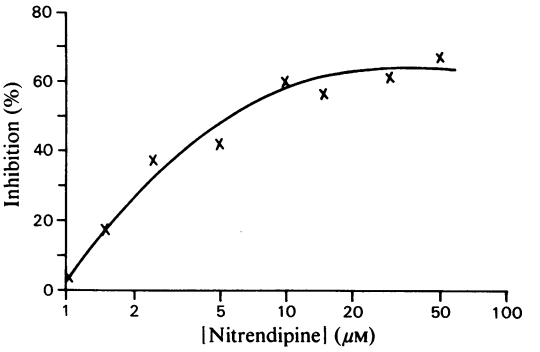

Fig. 2. *Inhibition of calcium influx by nitrendipine*
Red blood cells (0.5ml at a packed cell volume of 27%) were incubated in influx buffer (without calcium) for 15min with 0.5mM-vanadate to inhibit the calcium-ejecting ATPase. Nitrendipine was then added and after 5min influx was initiated by adding $^{45}CaCl_2$ to a final concentration of 2.25mM (2.5×10^6 c.p.m./ml). After 60min at 25°C, the cells were washed twice, precipitated, and $^{45}Ca^{2+}$ in the supernatant was counted. Inhibition refers to controls without nitrendipine. Radioactivity associated with the cells in the absence of vanadate was subtracted. Each point represents the mean of at least two independent experiments done in replicate.

Modulation of calcium influx by oxidized sterols

All oxidized sterol compounds were tested for their influence on both total $^{45}Ca^{2+}$ influx and its nitrendipine-inhibitable fraction.

The results for total influx are shown in Table 1. Two principal classes of OSC could be distinguished. The first class of OSC comprised 22*S*-OH-cholesterol, cholesterol, cholestan-3β,5α,6β-triol, 3β,5α-dihydroxycholestan-6-one and 3β-hydroxy-5α-cholestan-7-one. These compounds stimulated total Ca^{2+} influx. Stimulation was dependent both on the concentration of OSC at preincubation and on the amount incorporated into the membrane (Table 1). The nitrendipine-inhibitable fraction (Table 2) was stimulated to an extent exceeding the total stimulation (Table 1) by 2–3-fold. The fraction which was not inhibited by nitrendipine (30% of total influx; see Fig. 2) showed only minor stimulation. This was demonstrated by the fact that nitrendipine decreased influx into OSC-loaded cells to almost the same values as in the controls. Almost all of the total stimulation could therefore be accounted for by stimulation of the nitrendipine-inhibitable fraction. When the stimulation per 10μg of OSC incorporated in the membranes of 0.5ml of packed cells was calculated, there was a clear hierarchy in stimulatory power. Cholestan-3β,5α,6β-triol stimulated best (12%/10μg), 7-oxocholestanol least (6.2%/10μg) with cholestanol, 22*S*-OH-cholesterol and 3β,5α-dihydroxycholestan-6-one being intermediate.

The second class of OSC comprised those with an inhibitory action on calcium influx, i.e. 25-OH-cholesterol, 20α-OH-cholesterol, 7β-OH-cholesterol and 7-oxocholesterol. Inhibition was dependent on the concentration of OSC at preincubation and in the membrane (Table 1). Again, most of the inhibition of total influx was accounted for by inhibition of the nitrendipine-sensitive fraction (Table 2). An exception was 7β-OH-cholesterol, where both the nitrendipine-sensitive and -insensitive fraction were inhibited by about 50% at 5×10^{-5} μM-7β-OH-cholesterol. In this second class of OSC, too, specific inhibition (per 10μg of incorporated OSC) showed a clear hierarchy in the order 7-oxocholesterol (−17%/10μg), 25-OH-cholesterol (−12.8%/10μg), 7β-OH-cholesterol (−7.4%/10μg) and 20α-OH-cholesterol (−4.2%/10μm).

Modulation by cholesterol

The influence of cholesterol is depicted in Table 3. Liposomes with a ratio cholesterol/dipalmitoylphosphatidylcholine (mol/mol) of 0.8 served as

Table 1. *Modulation of total calcium influx by oxidized sterol derivatives*

Red blood cells at a packed cell volume of 5% were incubated in influx buffer containing albumin for 60min at 37°C with the different sterols dissolved in ethanol (final ethanol concentration 1%), washed twice in the same buffer and once in influx buffer and made to a suspension of 27% packed cell volume. Calcium influx was then performed as described in Fig. 2. Controls were cells preincubated in 1% ethanol. Incorporation of the sterols into the membrane was determined by t.l.c. after lipid extraction and saponification of phospholipids. Values ± S.E.M. for four independent experiments done in replicate ($P < 0.05$ for all values compared with controls in Student's *t*-test, except for 7β-OH-cholesterol at 0.25×10^{-5} M).

Compound	$10^5 \times$ Concentration of OSC at preincubation (M)	Sterol incorporated (μg/0.5ml of packed cells)	Stimulation of influx (%)	Inhibition of influx (%)
22*S*-OH-cholesterol	1.25	18±3	20±5	–
	5.0	125±10	86±9	–
	25.0	617±72	160±22	–
5α-Cholestan-3β-ol	5.0	–	40±9	–
Cholestan-3β,5α, 6β-triol	1.25	22±2	47±7	–
	5.0	74±12	89±15	–
3β,5α-Dihydroxy-cholestan-6-one	5.0	–	21±4	–
3β-Hydroxy-5α-cholestan-7-one	5.0	45±8	28±3	–
Ethanol (control)	–	–	–	–
25-OH-cholesterol	1.25	26±2	–	27±5
	5.0	75±16	–	75±4
	25.0	233±33	–	58±10
20α-OH-cholesterol	5.0	86±7	–	32±3
7β-OH-cholesterol	0.25	–	–	12±2
	1.25	32±4	–	57±7
	5.0	67±6	–	55±6
	25.0	170±30	–	80±9
7-Oxocholesterol	5.0	20±7	–	34±5

Table 2. *Modulation of the nitrendipine-sensitive part of calcium influx by oxidized sterols*

Cells were preincubated with the sterols as described in Table 1. Influx was measured in the absence and presence of 15 μM-nitrendipine. The difference was referred to as the nitrendipine-sensitive part of the influx. Final concentrations of sterol at preincubation was 5×10^{-5} mol/litre (0.3 μg/10^7 cells). % Change refers to the nitrendipine-sensitive influx of the controls preincubated in 1% ethanol without sterol. Values are means ± S.E.M. for four independent experiments.

Compound	Stimulation of influx through the channel (%)	Inhibition of influx through the channel (%)
22*S*-OH-cholesterol	208 ± 21	-
Cholestan-3β,5α,6β-triol	220 ± 25	-
3β-Hydroxy-5α-cholestan-7-one	101 ± 12	-
Ethanol (control)	-	-
25-OH-cholesterol	-	85 ± 6
20α-OH-cholesterol	-	64 ± 9
7β-OH-cholesterol	-	49 ± 9
7-Oxocholesterol	-	18 ± 5

Table 3. *Effect of cholesterol on calcium influx*

Red blood cells (packed cell volume 10%) were incubated at 37°C overnight with cholesterol/dipalmitoylphosphatidylcholine liposomes at the indicated molar ratio of the lipids as described in Cooper *et al.* (1975). After washing twice in influx buffer containing albumin and once in influx buffer, calcium influx was measured in the presence and absence of 15 μM-nitrendipine as described in Fig. 2 and Table 2. Values in parentheses are % change from control.

Cholesterol/dipalmitoylphosphatidylcholine ratio (mol/mol)	Calcium influx (μmol/h per litre of packed cells)	
	Total	Nitrendipine-inhibitable
0.6	6.6 ± 1.1 (−18%)	2.9 ± 0.3 (−10%)
0.8 (control)	8.0 ± 1.7 (−)	3.2 ± 0.4 (−)
2.3	31.0 ± 7.3 (+288%)	18.0 ± 3.2 (+463%)

control. Enrichment of cholesterol in the membrane to a ratio of 2.3 stimulated total calcium influx by +288%; the nitrendipine-sensitive fraction was stimulated by +463%. Cholesterol depletion of the membrane to a ratio of 0.6 (cholesterol/dipalmitoylphosphatidylcholine) was inhibitory (−18% and −10% for the nitrendipine-insensitive and -sensitive fractions, respectively). Further depletion of cholesterol caused haemolysis and made influx measurements impossible.

Kinetic measurements

Fig. 3 shows that inhibition or stimulation of calcium influx by OSC occurred to the same extent at all time points up to 90 min. Influx in the controls was linear up to 240 min. This is in some contrast to Varecka & Carafoli (1982), where a slight slowdown of influx could be observed after 90 min. This, however, was seen at 4–5 times higher influx rates than observed by us due to the use of outdated blood bank blood in Varecka & Carafoli (1982). Furthermore, this linearity is a good indication that calcium ejection via the pump was negligible.

Control experiments

A series of control experiments were performed in order to determine whether OSC and cholesterol exerted their effects indirectly. Since cells depleted of potassium showed decreased calcium influx in this system (Varecka & Carafoli, 1982), the intracellular potassium content was measured both immediately and after 60 min influx. Table 4 shows that incubation with the various substances did not significantly change intracellular potassium content when compared with controls. This also excluded significant activation of the membrane K^+ channel by Ca^{2+} during influx. No cell shrinking occurred as determined by packed cell volume measurement and cell counting (results not shown). Furthermore, all cells were energized to the same extent by ATP (about 1.7 mM). Since the anion channel may be influenced by cholesterol (Schubert & Boss, 1982), we checked whether the compounds had an effect on the penetration of vanadate through the anion channel, thereby preventing the complete inhibition of the Ca^{2+}/Mg^{2+}-ATPase. Table 4 shows that this is not the case,

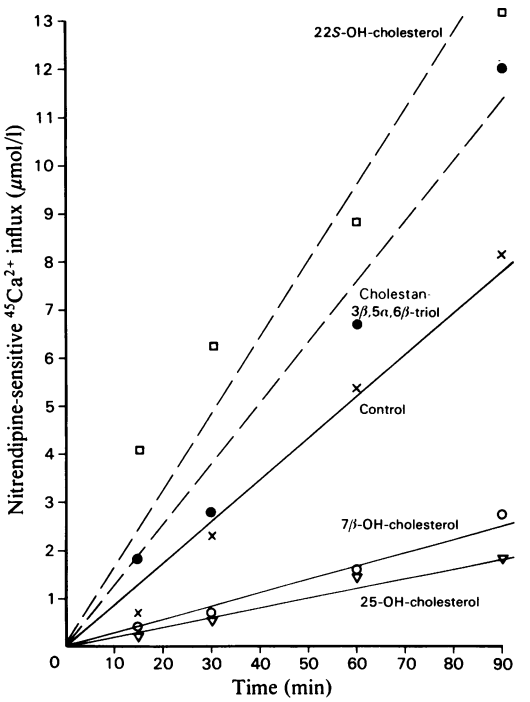

Fig. 3. *Kinetics of calcium influx through the calcium channel*

Cells were preincubated with the indicated sterols (or ethanol in controls) as in Table 1. Influx was carried out for different times in the presence and absence of 15 μM-nitrendipine. The difference between these two values is referred to as nitrendipine-sensitive influx. □, 22*S*-OH-cholesterol; ●, cholestan-3β,5α,6β-triol; ○, 7-OH-cholesterol; ▽,25-OH-cholesterol; ×, controls. Each point represents the mean of at least two independent experiments done in replicate.

since there were no significant differences in intracellular vanadate concentration (about 200 μmol/litre of packed cells). Inhibition of the Ca^{2+}/Mg^{2+}-ATPase was most probably complete since its K_i for vanadate is about 0.6 μM (Niggli *et al.*, 1981) and because of the linearity of influx with time (see above).

Discussion

Two principal conclusions can be derived from our data.

1. Nitrendipine, a dihydropyridine derivative, is a potent inhibitor of calcium influx in the red cell membrane at concentrations similar to those described by others for heart and smooth muscle cells (Lee & Tsien, 1983). This further strongly supports the hypothesis that human red blood cells possess a calcium channel with properties very similar to those of the receptor-mediated calcium gate in smooth muscle cells, heart and brain (Reuter, 1983; Ranganathan *et al.*, 1982).

2. Cholesterol and some of its physiologically and medically important oxidized derivatives are potent and highly stereospecific modulators of the calcium channel in human red blood cells.

Two important questions arise. First, what is the mechanism of interaction between cholesterol or OSC and the channel proteins? Secondly, what is the physiological and pathological significance of these findings?

As to the first question, an influence on the phase transition temperature of the erythrocyte membrane may be an explanation, much in the same way as has recently been described for artificial mixtures of dipalmitoylphosphatidylcholine

Table 4. *Controls to exclude indirect effects of the sterols on calcium influx*

Red blood cells were incubated with the sterols as described in Table 1. Calcium influx was performed as in Fig. 2, except that $^{45}Ca^{2+}$ was replaced by $^{40}Ca^{2+}$. Intracellular potassium was measured immediately after addition of calcium and after 60 min influx, ATP after 60 min influx and vandate after washing the cells twice in influx buffer containing 0.5 mM of the anion-channel inhibitor 4-acetamide-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) to prevent vanadate from leaking out during the washing procedure. Vanadate was measured by atomic absorption. mM is here defined as mmol/litre of packed cells. Values are means ± S.E.M. for four independent experiments.

Sterol	Intracellular K^+ (mM) at time: 0	60 min	Intracellular ATP (mM)	Intracellular vanadate (mM)
Control (buffer)	71.1 ± 5.9	65.1 ± 7.3	1.6 ± 0.3	0.21 ± 0.04
Control (1% ethanol)	76.7 ± 9.0	64.4 ± 6.8	1.8 ± 0.4	0.21 ± 0.03
22*S*-OH-cholesterol	75.3 ± 6.0	67.6 ± 3.3	1.6 ± 0.2	0.21 ± 0.02
Cholestan-3β,5α,6β-triol	72.1 ± 8.3	58.8 ± 9.7	1.4 ± 0.1	0.20 ± 0.01
3β-Hydroxy-5α-cholestan-7-one	73.9 ± 7.1	63.1 ± 4.8	1.6 ± 0.2	0.21 ± 0.03
25-OH-cholesterol	60.2 ± 10.9	56.0 ± 8.9	1.7 ± 0.5	0.22 ± 0.03
20α-OH-cholesterol	73.3 ± 6.7	63.7 ± 5.5	1.9 ± 0.2	0.20 ± 0.01
7β-OH-cholesterol	71.8 ± 5.7	73.5 ± 7.8	2.1 ± 0.6	0.21 ± 0.01
7-Oxocholesterol	76.1 ± 8.0	64.8 ± 5.0	1.8 ± 0.4	0.18 ± 0.02

and OSC (Egli *et al.*, 1984). In the human erythrocyte membrane, however, the phase transition temperature is around −20°C (Gottlieb & Eanes, 1974) and therefore it seems unlikely that this played a major role in our system. Rather, the boundary lipids of the calcium channel protein may be replaced by OSC, thereby folding or unfolding the channel in a highly stereospecific manner depending on the structure of the sterol. Since our interest was centred on pathophysiologically important OSC, an exact listing of the structural requirements for inhibition and stimulation of calcium influx cannot be derived from our data.

As to the second question concerning the physiological and pathological significance of our findings, it should first be noted that the concentrations of OSC we use for incorporation are in the same range (1–20 μM) as those employed by others for experiments with red blood cells (Yachnin *et al.*, 1979; Streuli *et al.*, 1981). For inhibition of cholesterol synthesis in cultured cells concentrations around 1 μg of OSC/10^7 cells are commonly used (Kandutsch & Chen, 1975, 1977; Tanaka *et al.*, 1983). We used 0.3 μM of OSC/10^7 cells (20 μg/6 × 10^8 cells in 1 ml of suspension at loading with OSC) in our standard assay (5×10^{-5} M-OSC at incubation), i.e. a concentration well below the range for cultured cells. Clearly, concentrations of the highly hydrophobic OSC have to be standardized to the amount of hydrophobic cell membrane they distribute in.

The most obvious implication of our results relates to the regulation of cholesterol biosynthesis. 25-OH-cholesterol, 7-oxocholesterol and 7β-OH-cholesterol are extremely potent inhibitors of cholesterol synthesis (Kandutsch & Chen, 1975, 1977; Tanaka *et al.*, 1983). The mechanism of inhibition has been the subject of some debate, but 25-OH-cholesterol has been shown to enhance the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase thereby reducing the enzyme concentration in the cell (Tanaka *et al.*, 1983). 25-OH-cholesterol, 7-oxocholesterol and 7β-OH-cholesterol are at the same time the most potent inhibitors of calcium influx in our system.

It is therefore intriguing to speculate that either phosphorylation or degradation of 3-hydroxy-3-methylglutaryl-CoA reductase is linked to the inhibition of calcium influx, possibly in a cascade involving several regulatory steps (Rasmussen, 1981). Clearly, however, the red blood cell can only be regarded as a model system in this respect and in the future the question needs to be addressed in a system where cholesterol synthesis and the calcium channel can be studied concomitantly.

Besides pointing towards possible mechanism for the inhibition of cholesterol synthesis in nucleated cells by OSC, a second prediction from our findings is that an altered cholesterol content of the cell membrane may modify calcium influx in systems other than the RBC. This would be of great pathophysiological significance, since the tissue content of cholesterol has been demonstrated to be related to plasma lipoproteins (Miller, 1979) and, for example, to be enhanced in patients with myocardial infarction (Fröberg, 1973). Intriguingly, first evidence in patients from this laboratory suggests that there is a strong relationship between calcium influx in our influx system and high-density lipoprotein cholesterol (M. Stimpel *et al.*, unpublished work).

The modulation of the calcium channel by cholesterol and its oxidized derivatives may play an important role in the regulation of cholesterol synthesis by OSC and in the pathogenesis of atherosclerotic lesions.

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