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Effect of anti-inflammatory drugs on the binding of calcium to cellular membranes in various human and guinea-pig tissues

B. J. NORTHOVER

Department of Pharmacology, School of Pharmacy, City of Leicester Polytechnic, Leicester LE1 9BH

Summary

1. Cellular membranes were obtained by centrifugation of homogenates of various human and guinea-pig tissues. These membranes took up added calcium (Ca) when incubated at 25° C for 10 min in histidine buffer at pH 7·4. The uptake of Ca was increased by the presence of adenosine triphosphate (ATP).

2. Evidence is presented that the membrane fragments which took up Ca under these conditions were derived from the endoplasmic reticulum.

3. The ATP-dependent uptake of Ca by membranes derived from guinea-pig aortic or gastric smooth muscle or from human umbilical arterial muscle was inhibited by indomethacin in a concentration-dependent manner.

4. The ATP-dependent uptake of Ca by membranes derived from heart, brain, skeletal muscle or liver of the guinea-pig was not inhibited by indomethacin.

5. Endothelial cells were obtained from the lining of the human umbilical vein. Cellular membranes were prepared from the endothelial cells by homogenization and centrifugation. The endothelial membranes took up added Ca in the presence of added ATP and this uptake was inhibited by indomethacin and also by members of a group of pharmacologically-related drugs.

Introduction

Several tissues isolated from the guinea-pig and bathed in physiological electrolyte solution take up calcium (Ca) and sodium (Na) ions in response to electrical stimulation. Addition of indomethacin to the bathing fluid inhibited the electrically-induced uptake of Ca and Na both by aorta and by gastric smooth muscle, but failed to alter the fluxes of these elements in brain, myocardium or skeletal muscle (Northover, 1971, 1972). Since some of the tissue stores of Ca are bound to cell membranes (Bianchi, 1968) it was of interest to examine whether indomethacin reduces the binding of Ca to cellular and subcellular membranes and whether the drug is more effective on membranes derived from smooth muscle cells than those derived from other tissues of the guinea-pig. It has been suggested that the ability of indomethacin to reduce the uptake of Ca by damaged tissues may be related to its anti-inflammatory activity (Northover, 1972). It was of interest, therefore, to determine whether other nonsteroidal anti-inflammatory drugs inhibit the binding of Ca to cell membranes. Moreover, since anti-inflammatory drugs probably owe at least part of their therapeutic effect to an action on vascular endothelium, it

was of interest to determine whether human vascular endothelial membranes behave in a similar way to membranes derived from smooth muscle of the guinea-pig.

Methods

Guinea-pig tissues. Gastric smooth muscle, aorta, liver, cardiac muscle and skeletal muscle were obtained from freshly killed guinea-pigs as described earlier (Northover, 1971, 1972).

Human tissues. Umbilical cords were placed as soon as possible after delivery in a plastic bag and stored for up to 24 h in a vacuum flask containing melting ice. During delivery most of the mothers received 150 mg pethidine hydrochloride and 50 mg promethazine hydrochloride by intramuscular injection. In addition, an inhalation of 50% N_2O plus 50% O_2 (Entonox, British Oxygen Company) was usually available to the mother. Only cords which were derived from normal babies delivered at full-term were collected. Some cords were opened longitudinally and the two arteries removed. Other cords provided endothelium, which was obtained by tryptic digestion of the umbilical vein. The technique of tryptic digestion is based upon the work of Maruyama (1963) and of Fryer, Birnbaum & Luttrell (1966) and provides a suspension of endothelial cells suitable for tissue culture since most of the cells collected are viable. The placental end of the vein was cannulated and the vessel flushed free of blood with 200–300 ml of 0·9% w/v NaCl solution (saline). The foetal end of the cord was then ligated and the vein filled via the cannula with 20–40 ml (depending upon the length of cord) of a freshly prepared saline solution containing 0·2% trypsin (Sigma). The trypsin, which was derived from bovine pancreas, was twice crystallized, dialysed and then lyophilized. The enzyme contained 10,000 BAEE units of activity/mg, where a BAEE unit is the activity which produces a change of optical density of 0·001 per min at 253 nm using *N*-benzoyl-L-arginine ethyl ester as substrate at pH 7·6 and at 25° C, with a reaction volume of 3·2 ml and a light path of 1 cm.

The cord containing trypsin was incubated in saline at 37° C for 40 min after which the vein was drained and the contents cooled rapidly to 4° C. The suspension of cells was centrifuged at 5,000 *g* for 10 min at 4° C and the pellet washed free of trypsin by resuspending it in 50 volumes of saline and recentrifuging a total of 3 times. The cells had a normal appearance under the microscope, which agrees with the findings of Maruyama (1963). For each experiment 3–4 m of cord were required, which yielded about 9 mg (dry weight) of endothelium/m of cord.

Preparation of tissue homogenates

Tissues to be fractionated by differential centrifugation were suspended at 4° C in 10 volumes of a solution containing (mM): sucrose (B.D.H., Analar Grade) 250; 1,2-bis-2-aminoethoxyethane-*NNN N* -tetraacetic acid (Hopkins & Williams) 0·1, adjusted to pH 7·4 with potassium hydroxide.

When no fractionation was performed the tissue was suspended at 4° C in 30 volumes of histidine buffer solution containing (mM): KCl 45; $KHCO_3$ 30; DL-histidine monohydrochloride 2·5 adjusted to pH 7·4 with potassium hydroxide.

In all instances the tissues were homogenized at 4° C in an all-glass Griffith's tube (Type AG, supplied by Baird & Tatlock).

Centrifugation of tissue homogenates

Fractionation of tissue homogenates was performed by centrifuging at 0–4° C in an M.S.E. Superspeed 50 machine with a No. 2410 angle-head rotor and polypropylene tubes. Centrifugation was performed in 3 stages:

Stage 1, 1,000 *g* for 10 min, sediment discarded.

Stage 2, supernatant from stage 1 at 15,000 *g* for 10 minutes.

Stage 3, supernatant from stage 2 at 100,000 *g* for 60 minutes.

The sediments from stages 2 and 3 are referred to as fractions I and II respectively.

Unfractionated tissue membranes were prepared by centrifuging tissue homogenates for 30 min at 100,000 *g* in a Beckman L2-65B machine at 0–1° C with a type 65 angle-head rotor and polyallomer tubes. The supernatant was discarded.

Measurement of cytochrome c oxidase

Contamination of fraction II with mitochondrial fragments was measured by using the fact that cytochrome *c* oxidase in present in the mitochondrial membranes of smooth muscle but not elsewhere in the cell (Carsten, 1969; Verity & Bevan, 1969). Cytochrome *c* oxidase activity was measured by the method of Cooperstein & Lazarow (1951). Cytochrome *c* (B.D.H., 90–100% pure) was oxidized at room temperature in a Unicam SP 500 series 2 spectrophotometer.

Measurement of binding of Ca to membranes

The membrane preparation was suspended in histidine buffer, briefly rehomogenized in a Griffith's tube and the suspension diluted to a final protein concentration of 0·4–0·9 mg/ml, as measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Except where otherwise stated, adenosine triphosphate (ATP) and $MgCl_2$ were added to the suspension in a final concentration of 2 mM and the mixture incubated at 25° C for 10 min, at the end of which time any drug to be tested was added to the mixture. The reaction mixture was incubated with the drug for a further 5 min and then $CaCl_2$ was added to a final concentration of 0·5 mM. After incubation in the presence of $CaCl_2$ for 10 min the suspension was cooled in melting ice and centrifuged at 0–1° C at 100,000 *g* for 15 minutes. The supernatant was decanted and retained for the estimation of inorganic orthophosphate (P_i). The membrane pellet was transferred with a spatula to a tared crucible and dried to constant weight at 100° C. Ashing of the pellet and the subsequent analysis of the ash for metallic elements were performed as described previously (Northover, 1972). Mineral contents are expressed as μg atoms/g of membrane protein.

The concentration of P_i in the supernatant is a measure of the ATP-ase activity of the membranes. Estimation of P_i was performed by a molybdate method according to the technique of Leloir & Cardini (1957) using a Hilger Spekker absorptiometer with Ilford No. 7 filters. Preliminary experiments revealed an absence of P_i from the incubation medium prior to the addition of membranes and also from the medium which had been incubated without membranes.

Distinction between mitochondrial and sarcoplasmic reticular binding of Ca

Azide has been found to inhibit binding of Ca by mitochondrial but not by sarcoplasmic reticular membranes from smooth muscle (Carsten, 1969; Batra &

Daniel, 1971). Phosphate, on the other hand, has been reported to promote the binding of Ca by mitochondrial but not by sarcoplasmic reticular membranes from smooth muscle (Batra & Daniel, 1971). Succinate has been reported by Sulakhe & Dhalla (1971) to enhance the binding of Ca by mitochondria but it does not appear to have been tested on fragmented sarcoplasmic reticulum. In a variety of tissues oxalate increases the uptake of Ca into vesicles derived from the fragmented endoplasmic reticulum but not into mitochondria (Hasselbach, 1964). In smooth muscle, however, Batra & Daniel (1971) found no effect of oxalate on the binding of Ca to sarcoplasmic reticular fragments.

Results

Guinea-pig gastric smooth muscle membranes

Membrane fractions I and II both contained cytochrome *c* oxidase activity (97 and 34 units/mg membrane protein respectively). Attempts to remove mitochondrial contamination of fraction II were unsuccessful.

Fraction II rapidly took up Ca when it was incubated in histidine buffer with $MgCl_2$ and ATP. Equilibrium was established within 5 min and unfortunately the need to centrifuge the reaction mixture at the end of incubation precluded the measurement of Ca binding at earlier times. The extent of binding of Ca at equilibrium (measured after 10 min of incubation) was inhibited by indomethacin in a concentration-dependent manner (Table 1). Phenylacetic acid, which is related to indomethacin chemically but which is devoid of anti-inflammatory activity (Northover, 1964; Durant, Smith, Spickett & Szarvasi, 1965), failed to alter the binding of Ca to the membranes (Table 1). In the absence of added ATP, fraction II bound less Ca than in its presence. Moreover, the small amount of Ca bound in the absence of ATP was not influenced by the presence of indomethacin (Table 1). Addition of Na azide or of K phosphate plus Na succinate had only a slight effect on the binding of Ca to fractions I or II and failed to modify the inhibitory action of indomethacin (Table 2). Addition of K oxalate to the reaction mixture, on the

TABLE 1. *Effect of indomethacin on the uptake of Ca by fraction II membranes from various tissues of the guinea-pig*

Drug			Content of Ca μg atoms/g protein ± S.E.		Uptake of Ca, μg atoms/g protein	Uptake of Ca as % of control
Name	Concentration mM	Added ATP mM	Without added Ca	With Ca 0·5 mM		
				Stomach		
Control	—	0	6·2±0·6	9·5±0·9‡	3·3	100
Indomethacin	0·5	0	6·4±0·4	10·1±1·1‡	3·7	112
Control	—	2	6·0±0·7	20·2±1·3*	14·2	100
Indomethacin	0·25	2	5·8±0·6	16·1±1·0‡	10·3	73
Indomethacin	0·5	2	5·9±0·5	9·8±0·8‡	3·9	27
Phenylacetate	2·0	2	5·9±0·9	19·0±1·2	13·1	92
				Aorta		
Control	—	2	6·3±0·5	18·5±1·5π	12·2	100
Indomethacin	0·1	2	6·5±0·4	15·7±1·1	9·2	75
Indomethacin	0·25	2	6·1±0·4	10·4±0·8†	4·3	35

A significant difference (Student's *t* test, $P<0·05$) between the value marked * and the values marked ‡, and also between the value marked π and the value marked †.

other hand, greatly increased the binding of Ca by both fractions and rendered the uptake insensitive to indomethacin (Table 2).

TABLE 2. *Effect of indomethacin on Ca-uptake by membranes of fractions I and II from guinea-pig stomach and human umbilical artery*

Concentration of added reagent (mM)				Content of Ca μg atoms/g protein ± S.E.			Uptake of Ca, μg atoms/g protein	Uptake of Ca as % of control
Indomethacin	Azide	Succinate+ phosphate	Oxalate	Without added Ca	With Ca 0·5 mM			
Guinea-pig stomach								
Fraction I								
Control	0	0	0	2·9±0·5	11·2±0·9‡	*	8·3	100
0·5	0	0	0	3·9±0·4	6·4±0·5	*	3·4	42
Control	1·0	0	0	3·1±0·4	10·7±1·0	*	7·6	100
0·5	1·0	0	0	3·3±0·6	6·9±0·3	*	3·6	47
Control	0	3+3	0	2·7±0·3	11·4±0·9	*	8·8	100
0·5	0	3+3	0	2·5±0·5	7·1±0·5	*	4·6	52
Control	0	0	5	2·6±0·2	37·2±1·8‡		34·6	100
0·5	0	0	5	2·8±0·4	36·3±2·1		33·5	97
Fraction II								
Control	0	0	0	6·0±0·7	20·2±1·3†	*	14·2	100
0·5	0	0	0	5·8±0·5	9·8±0·8	*	3·9	27
Control	1·0	0	0	6·5±0·8	19·4±1·0	*	12·9	100
0·5	1·0	0	0	5·7±0·6	10·0±0·7	*	4·3	33
Control	0	3+3	0	6·4±0·4	21·0±1·5	*	14·6	100
0·5	0	3+3	0	6·6±0·8	11·5±0·9	*	4·9	34
Control	0	0	5	5·8±0·6	54·1±3·0†		48·3	100
0·5	0	0	5	5·6±0·7	58·3±3·6		52·7	109
Human umbilical artery								
Fraction I								
Control	0	0	0	4·2±0·6	9·1±1·2	*	4·9	100
0·5	0	0	0	4·6±0·5	5·7±0·3	*	1·1	22
Control	1·0	0	0	4·5±0·3	8·8±0·8	*	4·3	100
0·5	1·0	0	0	3·9±0·7	5·4±0·5	*	1·5	35
Fraction II								
Control	0	0	0	5·6±0·8	8·6±0·9	*	3·0	100
0·5	0	0	0	5·5±0·7	5·9±0·6	*	0·4	13
Control	1·0	0	0	5·9±1·1	9·0±1·1	*	2·1	100
0·5	1·0	0	0	5·7±0·6	6·2±0·5	*	0·5	16

A significant difference exists between the members of a pair of values marked *, † or ‡ (Student's t test, $P<0·05$).

TABLE 3. *ATP-ase activities of membrane preparations measured over* 10 *min, expressed as* μmol P_i/*mg protein* + S.E.

Mg (mM)	Ca (mM)	Without indomethacin: Measured ATP-ase	Without indomethacin: Calculated Mg-dependent ATP-ase	Without indomethacin: Calculated Ca-dependent ATP-ase	With indomethacin: Measured ATP-ase	With indomethacin: Calculated Mg-dependent ATP-ase	With indomethacin: Calculated Ca-dependent ATP-ase
Guinea-pig gastric muscle, fraction II							
0	0	0·2	6·5±0·2*	1·4±0·1	0·3	7·6±0·3*	1·1±0·2
2	0	6·7			7·9		
2	0·5	8·1			9·0		
Human umbilical venous endothelium, unfractionated							
0	0	0·6	6·2±0·4†	1·2±0·2	0·5	7·8±0·5†	1·1±0·3
2	0	6·8			8·3		
2	0·5	8·0			9·4		

A significant difference exists (Student's t test, $P<0·05$) between the members of a pair of values marked * or †.

Membranes of fraction II which had been incubated in histidine buffer with $MgCl_2$, $CaCl_2$ and ATP contained, in addition to Ca, substantial amounts of Na (236 μg atoms/g membrane protein) and of Mg (78 μg atoms/g membrane protein). The content of these two elements, however, was not changed significantly by indomethacin (0·5 mM).

ATP-ase activity

The membranes of fraction II hydrolysed ATP during incubation in histidine buffer. Hydrolysis was accelerated by the presence of Mg (2 mM) and even more by the combined presence of Mg (2 mM) and Ca (0·5 mM). Indomethacin, in a concentration (0·5 mM) which reduced the binding of Ca (Table 1), produced a small increase in Mg-dependent ATP-ase activity but was without effect on either Mg-independent or Ca-dependent ATP-ase activities (Table 3).

Membranes from other guinea-pig tissues

Aortic membrane fractions I and II both contained cytochrome *c* oxidase activity (116 and 21 units/mg membrane protein respectively), and attempts to remove the mitochondrial contaminants from fraction II failed.

Membranes of fraction II derived from the aorta took up added Ca when incubated in histidine buffer with Mg and ATP. This binding was inhibited by indomethacin in a concentration-dependent manner (Table 1). Indomethacin inhibited the uptake of Ca by the aortic fraction II in lower concentrations than were required to produce this effect in fraction II derived from gastric smooth muscle (Table 1).

Fraction II membranes derived from the liver, heart, brain and skeletal muscle also bound Ca but this was not influenced by indomethacin in a final concentration of 0·5 mM.

Human umbilical arterial muscle membranes

Both fractions I and II derived from arterial muscle took up added Ca when incubated in histidine buffer with Mg and ATP. Indomethacin reduced the binding of Ca by both fractions to a similar degree and this effect was still evident in the presence of Na azide (Table 2). Unfortunately the purest preparations of fraction II which have been obtained still retain some cytochrome *c* oxidase activity, indicating contamination with mitochondrial fragments. Fraction I displayed 52 units of activity/mg membrane protein and fraction II 9 units of activity/mg membrane protein.

Human umbilical venous endothelial membranes

Unfractionated membranes derived from endothelial cells took up added Ca when incubated in histidine buffer with Mg and ATP. This uptake was inhibited by several nonsteroidal anti-inflammatory drugs in a concentration-dependent manner, as shown in Figure 1.

The unfractionated membranes also displayed ATP-ase activity, most of which was Mg-dependent and a little of which was Ca-dependent. It can be seen from Table 3 that indomethacin slightly increased the Mg-dependent activity but not the

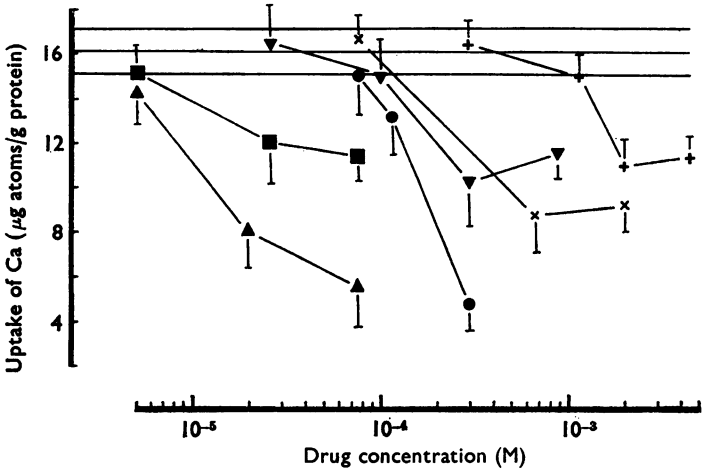

FIG. 1. Effect of anti-inflammatory drugs on the binding of Ca by unfractionated membranes derived from human umbilical venous endothelial cells. The 3 horizontal lines (mean ±one S.E.) towards the top of the graph represent the uptake of Ca by drug-free controls. +——Aspirin, X——Phenylbutazone, ▼—— Fenclozic acid, ●——Indomethacin, ■——Meclofenamic acid and ▲——Flufenamic acid. Vertical bars represent one S.E. The number of observations at each point was between 6 and 10.

Mg-independent or Ca-dependent ATP-ase activities. In these respects the human endothelial cell membranes resemble those derived from smooth muscle.

Discussion

The present investigation has shown that membranes derived from several cell types bind Ca in the presence of Mg and ATP. This binding is inhibited by indomethacin in some tissues but not in others. The ability of indomethacin to reduce Ca-uptake by electrically-stimulated smooth muscle, which was demonstrated previously (Northover, 1971, 1972), is probably due, therefore, to reduced uptake of Ca by the cellular membranes. Evidence in support of this suggestion may be summarized as follows. First, both processes are inhibited by indomethacin but not by the chemically-related substance phenylacetic acid. Secondly, the concentrations of indomethacin required to inhibit the Ca-uptake by electrically-stimulated aorta or stomach muscle are similar to the concentrations required to inhibit Ca-uptake by fractions I and II derived from them, the aorta and membranes derived from it being more sensitive than the stomach wall and membranes derived from it. Finally, indomethacin failed to alter Ca-uptake by electrically-stimulated skeletal muscle, myocardium or brain, and also failed to alter Ca-uptake by fraction II membranes derived from these tissues.

The Ca-uptake by smooth muscle membranes has been shown in the present work to be largely ATP-dependent and this agrees with the findings of Carsten (1969) and of Batra & Daniel (1971). These workers also showed, however, that ATP-dependence is a feature of Ca-uptake by both mitochondria and sarcoplasmic reticulum of smooth muscle. Other tests were required, therefore, to determine which type of sub-cellular membrane was responsible for the binding of Ca under the conditions of the present experiments. Carsten (1969) and Batra & Daniel (1971) reported that uptake of Ca by smooth muscle mitochondria was inhibited by Na azide. In the present experiments Na azide had very little effect on the

Ca-uptake by either fractions I or II of the smooth muscle homogenate and was without effect on the inhibitory action of indomethacin. It seems likely, therefore, that the uptake of Ca which is inhibited by indomethacin under the conditions of the present experiments is by sarcoplasmic reticulum rather than by mitochondria. Phosphate and succinate are known to enhance the uptake of Ca by mitochondria (Sulakhe & Dhalla, 1971). In the present experiments the presence of phosphate and succinate during incubation did not enhance Ca-uptake by either fractions I or II. This constitutes further evidence, therefore, that mitochondrial uptake of Ca did not participate significantly under the conditions of the present experiments.

Oxalate is known to potentiate Ca-uptake by sarcoplasmic reticulum derived from many types of muscle (Hasselbach, 1964), although exceptions have been reported (Batra & Daniel, 1971). In the present experiments the presence of oxalate greatly increased Ca-uptake by fractions I and II from gastric smooth muscle, but the enhanced uptake of Ca was no longer inhibited by indomethacin. Other workers have reported that drugs may alter the uptake of Ca more readily in the absence than in the presence of oxalate (Entmen, Cook & Bressler, 1969). It should be noted that oxalate is unlikely to participate in the uptake of Ca by sarcoplasmic reticulum under most physiological conditions (Katz & Repke, 1967; Besch & Schwartz, 1971).

Human vascular endothelial cell membranes resemble those from smooth muscle in displaying Ca-binding which is susceptible to the inhibitory action of indomethacin. This is another example, therefore, of the close similarity between these two types of cells (Becker, 1970; Northover & Northover, 1970). The present work also reveals that several chemically-unrelated nonsteroidal anti-inflammatory agents share with indomethacin the ability to reduce Ca-binding to the membranes of endothelial cells. This lends support to the suggestion that the ability of drugs of this group to reduce the uptake of Ca is related to their anti-inflammatory action. Experiments are at present being undertaken to determine whether Ca accumulates in vascular endothelial cells *in situ* during inflammation and whether anti-inflammatory drugs alter this process.

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