Correction. In the article "Classification of methanogenic bacteria by 16S ribosomal RNA classification" by G. E. Fox, L. J. Magrum, W. E. Balch, R. S. Wolfe, and C. R. Woese, which appeared in the October 1977 issue of Proc. Natl. Acad. Sci. USA 74, 4537–4541, undetected printer's errors occurred in the first column of Table 2 (superior dots were omitted in some sequences). The correct Table 2 appears below.

Table 2. Post-transcriptionally modified sequences and likely counterparts

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Occurrence in methanogens</th>
<th>Occurrence in typical bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>AACCUG</td>
<td>+ + - -</td>
<td>30%</td>
</tr>
<tr>
<td>AAUCUG</td>
<td>- - + +</td>
<td>None</td>
</tr>
<tr>
<td>AAG</td>
<td>- - + -</td>
<td>55%</td>
</tr>
<tr>
<td>UUAACAG</td>
<td>+ + - -</td>
<td>None</td>
</tr>
<tr>
<td>UAACAAG</td>
<td>- - + +</td>
<td>None</td>
</tr>
<tr>
<td>UGACAU</td>
<td>- - + -</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>AUNCAG</td>
<td>+ + - -</td>
<td>None</td>
</tr>
<tr>
<td>ACNAAG</td>
<td>- - + +</td>
<td>None</td>
</tr>
<tr>
<td>AXGCAAG</td>
<td>- - + -</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>NCCG</td>
<td>+ + - -</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>C(C,C)G</td>
<td>- - - +</td>
<td>None</td>
</tr>
<tr>
<td>NCCG</td>
<td>- - - +</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>CCCCG</td>
<td>- - - +</td>
<td>&gt;95%</td>
</tr>
</tbody>
</table>

Post-transcriptionally modified sequences in methanogens and their likely counterparts in the bacteria that have been examined. In group 1, A is N-6-dMe (21), identified by electrophoretic mobilities of A and AA and by total resistance to U2 nuclease. In group 2, U is partially resistant to pancreatic nuclease, the first A when modified is still U2 nuclease sensitive; the second A is N-6-dMe. In group 3 is resistant to pancreatic nuclease but is electrophoretically U-like. X stands for U or A. In group 4, N and N' are not cleaved by endonucleases; NC and N'C are electrophoretically distinguishable. C is cleaved by pancreatic nuclease and has C-like electrophoretic properties. In group 5, C (21, 22) is not cleaved by pancreatic nuclease and is readily deaminated by NH₂OH.

Correction. In the article "Ionophores stimulate prostaglandin and thromboxane biosynthesis" by Howard R. Knapp, Oswald Oelz, L. Jackson Roberts, Brian J. Sweetman, John A. Oates, and Peter W. Reed, which appeared in the October 1977 issue of Proc. Natl. Acad. Sci. USA 74, 4251–4255, the ordinate and abcissa labels of Fig. 4 were omitted. The corrected Fig. 4 is reprinted below.

Fig. 4. Prostaglandin release by rat renal papilla minces. Minces of renomedullary papillae were prepared and incubated (80–100 mg wet weight) and prostaglandins were measured by gas chromatography/mass spectrometry. Each value is the mean of two experiments, which gave similar results. (Upper) Complete buffer, containing 2.5 mM Ca²⁺; (Lower) calcium-free buffer (about 30 μM Ca²⁺ provided by washout from tissue). A23187 was added after 120 min of incubation in 2.5 μl of ethanol.
Ionophores stimulate prostaglandin and thromboxane biosynthesis*  
(calcium/platelets/renal medulla/A23187/X537A)

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ABSTRACT  The role of calcium in triggering prostaglandin and thromboxane synthesis was studied in several systems with ionophores of different ion specificities. Divalent cationophore A23187 stimulated prostaglandin and thromboxane biosynthesis by washed human platelets in a concentration-dependent manner (0.3–9 μM). A23187 also induces an antimycin A-insensitive burst in oxygen utilization which is partially blocked by 5 mM aspirin or 10 μM indomethacin. Under our conditions, A23187 (up to 10 μM) does not appear to damage platelet membranes since it does not cause appreciable loss of lactate dehydrogenase or β-glucoronidase. Mono- and divalent cationophore X537A also stimulates platelet thromboxane B2 production and oxygen utilization, but monovalent cationophores nigericin, menmosin A, A204, and valinomycin have no effect. The synthesis of prostaglandins E2, D2, and F2α by rat renal medulla minced by 1 and 5 μM A23187 without changes in tissue ATP content, lactate output, or K+ efflux. X537A, menmosin A, and nigericin (all 5 μM) stimulate both prostaglandin output and K+ efflux from renal medulla, while 5 μM valinomycin or A204 has no effect on either. None of the ionophores stimulates renomedullary prostaglandin production if calcium is omitted from the incubation medium. A23187 also stimulates prostaglandin production by human lymphoma cells, rat stomach and trachea preparations, and guinea pig polymorphonuclear leukocytes. These observations suggest a major role for Ca2+ in stimulating prostaglandin and thromboxane biosynthesis and also indicate that prostaglandin-released thromboxane release may partially mediate some of the previously described effects of ionophores on cells and tissues.

Prostaglandins (PG) and thromboxanes are produced in various proportions by nearly every mammalian cell type investigated, with the exception of the erythrocyte (1). While numerous specific (2) and non-specific (3) stimuli have been described that increase prostaglandin and thromboxane release in many systems, there is little information available about the mechanism(s) of action of these stimuli. The rate-limiting step in the biosynthesis of prostaglandins and thromboxanes is currently thought to be liberation of their precursor, arachidonic acid, by phospholipases from tissue lipids (4, 5). Since many phospholipases are calcium-dependent (6, 7), we have studied the effect of altering intracellular calcium concentrations on prostaglandin and thromboxane biosynthesis. To do this, we have compared the effects of calcium-transporting ionophores with effects of ionophores specific for monovalent cations on production of prostaglandins and thromboxane B2 (TXB2) in two very different biological systems. In the present report, we demonstrate that A23187, and certain other ionophores, stimulate the formation of oxygenated products of arachidonic acid by platelets and renal medulla.

METHODS

Platelet Studies. Blood was obtained from healthy male volunteers who had not taken any drugs for at least 10 days. Washed platelets were prepared as described (8) and suspended in Krebs–Henseleit buffer without calcium. For thromboxane and prostaglandin studies, incubations were run in an aggregometer at 37°C and terminated by addition of −70°C acetone (see below). Oxygen uptake was measured with a Gilson Oxigraph K-IC. Lactate dehydrogenase was assayed with Sigma kit 340-UV and β-glucuronidase as described (9). Protein was measured with a biuret method. Ionophores (4 mM stock solution) were added in ethanol, and controls received an equal volume of ethanol.

Renal Medulla Studies. Renal medulla incubations were carried out as described by Danon et al. (10). The buffer was changed every 30 min for six incubation periods and analyzed for prostaglandins either by gas chromatography/mass spectrometry or by bioassay with the rat fundus (10). Ionophores (or an equal volume of ethanol) were added after 120 min of incubation. ATP levels at the end of the sixth incubation period were measured (11) in supernatants of tissue that had been rapidly homogenized in ice-cold 6% perchloric acid, neutralized with 30% KOH, and centrifuged. Potassium and calcium were measured by atomic absorption spectroscopy, and lactate was assayed enzymatically (12).

Determination of Prostaglandins and TXB2 by Gas Chromatography/Mass Spectrometry. The incubation medium or platelet suspension was added to three volumes of −70°C acetone containing the appropriate deuterated internal standards: [2H5]TXB2, [2H5]PGD2, [2H5]PGE2, or [2H5]PGF2α. Trinitiated prostaglandins and TXB2 were used as tracers during purification. After removal of denatured proteins by centrifugation and two washings with petroleum ether (boiling point 35–60°C), prostaglandins and thromboxanes were extracted into chloroform at pH 3.2. The prostaglandins and TXB2 were purified and separated by high pressure liquid chromatography on a μ-Porasil column (Waters Assoc.) as described (13). Derivatized compounds were measured by combined gas chromatography/mass spectrometry, using selected ion monitoring under conditions described previously (14–16).

RESULTS

Effects of ionophores on platelets

The effects of A23187 on platelets have been studied at length, and it has been shown that this ionophore can mobilize the platelets’ intracellular calcium stores (17). During platelet

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Abbreviations: PG, prostaglandin; TXB2, thromboxane B2.

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aggregation, the products of arachidonic acid oxygenation include a large proportion of thromboxanes and relatively small amounts of primary prostaglandins (18). Fig. 1 shows that A23187 (0.3-9 μM) induces a concentration-dependent production of TxB2 by washed platelets suspended in calcium-free buffer. PGD2 formation is also stimulated by the ionophore, but to a lesser extent. Higher concentrations of A23187 (above 10 μM) induce a smaller stimulation of TxB2 and PGD2 production, either due to effects of A23187 or the increasing amounts of ethanol added.

Since 10 μM A23187 appeared optimal to stimulate TxB2 production under these conditions, we chose this concentration to compare effects of A23187 with effects of other ionophores. This concentration of A23187 did not result in platelet damage as assessed by release of a cytoplasmic marker enzyme, lactate dehydrogenase, or an enzyme contained in α granules, β-glucuronidase, during the 2-min incubations. As shown in Fig. 2, ionophores able to form lipophilic complexes with calcium and transport this cation across membranes, i.e., A23187 and X537A (19-21), stimulated TxB2 production by platelets, whereas ionophores capable only of monovalent cation transport, i.e., nigericin, monensin A, and valinomycin (21), were without effect. Similarly, of the ionophores tested, only A23187 and X537A provoked platelet aggregation (17). The magnitude of TxB2 production and degree of aggregation induced by the calcium ionophores were similar to the responses produced by a supraoptimal concentration of thrombin (5 units/ml). The large variation in thrombin-stimulated TxB2 production by platelets from different individuals has been reported previously (22) and is also true for ionophore-stimulated TxB2 production.

Addition of thrombin to platelets results in a brief burst in oxygen utilization, which is partially sensitive to antimycin A (23) and markedly inhibited by aspirin or eicosatetraynoic acid (24, 25). In Fig. 3, oxygen electrode trace A shows that antimycin A completely inhibits the basal respiratory of platelets and markedly diminishes the respiratory response to thrombin, but has little effect on the burst in oxygen utilization produced by A23187. This observation suggests that while the respiratory response produced by thrombin depends on electron transport and presumably ATP synthesis, A23187 stimulation does not and is not due to uncoupling of oxidative phosphorylation (19), since it occurs in the presence of antimycin A. Trace B (Fig. 3) shows that an antimycin A-insensitive burst in oxygen utilization is produced only by ionophores capable of transporting calcium, stimulating TxB2 production, and causing platelet aggregation. The data shown in trace C (Fig. 3) suggest that the extra oxygen uptake stimulated by A23187 is partially associated with oxygenation of arachidonic acid by platelet cyclooxygenase, since both aspirin and indomethacin markedly reduce this response. The complete inhibition of A23187-induced, antimycin A-insensitive oxygen consumption by 50 μM indomethacin is not observed with all platelet preparations and may be due to a nonspecific inhibitory effect (26).

Effects of ionophores on renal medulla

To determine if the ability of calcium ionophores to stimulate arachidonic acid conversion by cyclooxygenase was a more generalized phenomenon, we examined the effects of ionophores on prostaglandin production by minces of rat renal medulla. This tissue seemed particularly suitable, since it had been used previously to study specific hormonal effects on

![Fig. 1](image1.png)

**Fig. 1.** Stimulation of TxB2 and PGD2 production by washed platelets incubated with increasing concentrations of A23187. TxB2 (○) or PGD2 (□) were measured after a 2-min incubation of platelets (4.1 mg of protein or about 2.3 x 10^6 platelets per ml) with A23187.

![Fig. 2](image2.png)

**Fig. 2.** TxB2 production by washed platelets incubated for 2 min with thrombin (THR, 5 units/ml) or ionophores (10 μM): A23187 (A23), X537A (X53), nigericin (NIG), monensin A (MON), or valinomycin (VAL). (CON, control.) Each symbol represents the average of duplicate values obtained with the platelets from an individual donor (4.3-5.2 mg of protein or about 2.4 to 2.9 x 10^6 platelets per ml). Bar graphs show the mean ± SEM; n = 3-5 different donors.
prostaglandin production (10) and the major oxygenation products of arachidonic acid produced by this preparation (PGE₂, PGD₂, and PGF₂α) are not metabolized further (27). Fig. 4 shows that after an initial high rate of prostaglandin production, probably caused by trauma associated with tissue preparation (3), a low, stable rate of release is reached after 90–120 min of incubation. If A23187 is added at this time, there is a stimulation in production of all three prostaglandins, with about a 4-fold increase in PGE₂ and PGD₂ output and a 2-fold increase in PGF₂α output (Fig. 4 upper). When calcium is omitted from the buffer, washout of calcium from the tissue results in a concentration of approximately 30 μM during the last two incubation periods, and A23187 is unable to stimulate prostaglandin production (Fig. 4 lower). If calcium chloride (2.5 mM) is added back with the ionophore, however, the ability of A23187 to stimulate prostaglandin synthesis is restored fully. When the incubation medium contains the usual calcium concentration (2.5 mM) but magnesium is omitted, stimulation of prostaglandin release by 5 μM A23187 is doubled.⁹ Since A23187 binds magnesium nearly as well as calcium, the effects of this competition are consistent with the suggestion that stimulation of prostaglandin biosynthesis by A23187 is mediated by the inward transport of calcium.

Table 1 compares the effects of various ionophores on renomedullary prostaglandin production. None of the ionophores stimulates prostaglandin production in the absence of added calcium. Since hypertonic medium stimulates prostaglandin release by the rat renal medulla (28) to an equal extent whether or not calcium is included in the medium,⁹ it can be inferred that the calcium-free incubation does not impair the potential activity of the cyclooxygenase or phospholipases. A23187 appears to be the most effective ionophore and is the only one to produce a large stimulation of prostaglandin release at 1 μM concentration. In contrast to the observations with platelets, however, two ionophores that are specific for monovalent cations, monensin A and nigericin, stimulate prostaglandin output in a calcium-dependent manner, while two other monovalent cationophores, A204 and valinomycin, have no effect on prostaglandin production.

⁹ H. R. Knapp, unpublished observations.
Table 1. Stimulation of renomedullary prostaglandin output by ionophores

<table>
<thead>
<tr>
<th>Ca²⁺, mM</th>
<th>Ionophore, μM</th>
<th>A23187</th>
<th>X537A</th>
<th>Monensin A</th>
<th>Nigericin</th>
<th>Valinomycin</th>
<th>A204</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>1</td>
<td>3.70 ± 0.84†</td>
<td>1.19 ± 0.18</td>
<td>1.68 ± 0.30‡</td>
<td>1.24 ± 0.04</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2.5</td>
<td>3</td>
<td>3.99 ± 0.57†</td>
<td>2.37 ± 0.50‡</td>
<td>3.25 ± 0.47§</td>
<td>2.30 ± 0.26‡</td>
<td>1.02 ± 0.16</td>
<td>0.85 ± 0.01</td>
<td>0.92 ± 0.06</td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>0.94 ± 0.04</td>
<td>0.75 ± 0.03</td>
<td>1.10 ± 0.10</td>
<td>0.91 ± 0.02</td>
<td>0.82 ± 0.02</td>
<td>0.82 ± 0.07</td>
<td>0.93 ± 0.05</td>
</tr>
</tbody>
</table>

* PGE₂ equivalents bioassayed with the rat fundus. Data are expressed as the ratio of PGE released during the 30 min after ionophore addition (120- to 150-min period) to PGE released during the preceding 30 min (90–120 min), which is considered baseline. Values are the mean ± SEM. Incubations performed without added calcium contained approximately 30 μM Ca⁺⁺ provided by tissue washout.
† Value differs from control at P < 0.05; ‡ P < 0.01; § P < 0.005; ¶ P < 0.001.

The above mentioned monovalent cationophores do not transport calcium directly to any appreciable extent (21). Alterations in intracellular monovalent cation concentrations, particularly sodium, may produce effects that result from increased cytoplasmic calcium (29). To assess whether monovalent cationophores were causing monovalent cation movements in our system, we have examined their ability to produce efflux of potassium from the renal medulla during incubations in potassium-free buffer. Both monovalent cationophores that stimulate prostaglandin output, nigericin and monensin A, stimulate potassium efflux from the tissue, 3.1- and 1.6-fold over control, respectively, during the 30 min after addition. A204 and valinomycin, which do not stimulate prostaglandin output, have no effect on potassium efflux. X537A, which transports a wide range of cations (21), stimulates potassium efflux 1.7-fold. This suggests that X537A may be stimulating prostaglandin output by virtue of either or both monovalent and calcium ionophore capacity. A23187 has no effect on potassium efflux, indicating that, in this preparation, it does not adversely affect cell membrane integrity or cause potassium release secondary to changes in intracellular calcium as in erythrocytes (20).

A23187 lowers ATP levels in some cells (20, 30). Environmental changes that lower tissue ATP levels, such as hypoxia, often stimulate prostaglandin release (3). To be assured that A23187 is not disrupting renomedullary energy metabolism, we have measured ATP levels and lactate release in control tissues and preparations exposed to 5 μM A23187. There is no significant difference between the two groups in either ATP content (control: 13.8 ± 2.7; plus A23187: 16.0 ± 0.3 μmol/g of protein; mean ± SEM) or lactate release (control: 22.4 ± 1.4; plus A23187: 21.9 ± 0.8 μmol/g wet weight per 30 min; mean ± SEM).

**Effects of A23187 on other tissues**

The generality of our findings is further demonstrated by data obtained with several other systems. Rat stomach and trachea minces incubated in a similar fashion to the kidney papillae (see Methods) increase their release of PGE-like activity 5.3 ± 1.4- and 12.9 ± 2.6-fold, respectively, when 5 μM A23187 is added to the medium (mean ± SEM, n = 4, P < 0.01 for both groups). We have also found, in preliminary experiments, that A23187 stimulates prostaglandin and/or thromboxane biosynthesis by guinea pig polymorphonuclear leukocytes and human lymphoma cells.

**DISCUSSION**

These studies have demonstrated that calcium may play a central role in controlling prostaglandin and thromboxane biosynthesis. Phospholipases are believed to catalyze the rate-limiting step in prostaglandin biosynthesis in most tissues and many phospholipases have been shown to be calcium dependent (6, 7). A reasonable explanation for our observations is that calcium ionophores increase the concentration of cytoplasmic calcium which then stimulates phospholipase activity. This would result in increased free arachidonic acid, which would be expected to be converted rapidly to prostaglandins, thromboxanes, or other products. In platelets, A23187 is able to mobilize internal calcium stores (17) and, thereby, presumably, increase cytoplasmic calcium. While our studies were in progress, Pickett et al. reported that 10 μM A23187 liberates arachidonic acid from platelet phospholipids and proposed that the ionophore activates phospholipase A₂ in a calcium-dependent manner (31).

Our results with the renal medulla suggest that calcium plays a pivotal role in prostaglandin production by this tissue also. In addition, our data demonstrate a possible role for monovalent cations in regulating prostaglandin synthesis in this tissue. It has been suggested (21, 29) that increased intracellular sodium can produce an increase in cytoplasmic calcium. Inhibition of the myocardial (Na⁺ + K⁺)-ATPase by cardiac glycosides is thought to result in a positive inotropic effect by somehow increasing cytoplasmic calcium secondary to increased cytoplasmic sodium concentrations (32). Preliminary experiments indicate that 0.1–1 mM ouabain potentiates the stimulation of renomedullary prostaglandin release induced by either 5 μM monensin A or 5 μM nigericin. Whether potassium efflux, sodium influx, or both, are involved in mediating the calcium-dependent stimulation of prostaglandin biosynthesis by monovalent cationophores has not been resolved.

One final point may be made as a result of data in the present studies. A23187 and X537A have been used widely to probe the role of calcium in diverse processes. Our data indicate that stimulation of thromboxane or prostaglandin biosynthesis by ionophores, particularly those which transport calcium, may be a generalized phenomenon and suggest that these products could, in fact, be mediating some of the observed effects of ionophores on cells, tissues, and organs. In addition, ionophores may be powerful tools with which to study bioregulation of prostaglandin and thromboxane synthesis in addition to other calcium-dependent processes.

We thank Jill M. Blackmore and Ruth Oelz for their expert technical assistance and Gerald Shulman for performing the lactate assays. Antimycin A and X537A were generous gifts from Dr. Henry A. Lardy; monensin A, nigericin, A204, and A23187 were generously provided by Dr. Robert Hamill, Eli Lilly and Co. J.A.O. is the Joe and Morris Werthan Professor of Investigative Medicine. P.W.R. is an Established Investigator of the American Heart Association. This study was supported by National Institutes of Health Grant GM-15431 and American Heart Association Grant 74-717.

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