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Intracellular calcium and adenosine 3′,5′-cyclic monophosphate as mediators of potassium-induced aldosterone secretion

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We compared the action of K^+ on aldosterone secretion from isolated bovine adrenal glomerulosa cells with that of ionophore A23187. Addition of either 50nM-A23187 or 8mM-K^+ to perifused cells induces a similar initial aldosterone-secretory responses, and a similar sustained increases in Ca^{2+} entry. However, K^+-induced secretion is more sustained than is A23187-induced secretion, even though each agonist appears to act by increasing Ca^{2+} entry into the cells. When [^{3}H]inositol-labelled cells are stimulated by 8mM-K^+, a small decrease in phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is observed. This decrease is not accompanied by an increase in inositol trisphosphate (InsP_3) concentration. Also, if [^{3}H]arachidonic acid-labelled cells are exposed to 8mM-K^+, there is no increase in [^{3}H]diacylglycerol production. When [^{3}H]inositol-labelled cells are stimulated by 50nM-A23187, a small decrease in PtdIns(4,5)P_2 is observed. This decrease is not accompanied by an increase in InsP_3. The cyclic AMP content of K^+-treated cells was approximately twice that in A23187-treated cells. If cells are perifused simultaneously with 50nM-forskolin and 50nM-A23187, the initial aldosterone-secretory response is similar to that induced by A23187 alone, and the response is sustained rather than transient, and is similar to that seen during perifusion of cells with 8mM-K^+. This dose of forskolin (50nM) causes an elevation of cyclic AMP concentration in A23187-treated cells, to a value similar to that in K^+-treated cells. These results indicate that, in K^+-treated cells, a rise in cyclic AMP content serves as a positive sensitivity modulator of the Ca^{2+} message, and plays a key role in mediating the sustained aldosterone-secretory response.

Small increases in extracellular K^+ concentration act as a physiological stimulator of aldosterone secretion from adrenal glomerulosa cells (Saruta *et al.*, 1972: Fraser *et al.*, 1979). Many studies have shown that the action of K^+ is dependent on Ca^{2+} (Fakunding *et al.*, 1979; Fakunding & Catt, 1980; Foster *et al.*, 1981; Schiffrin *et al.*, 1981), and it is now considered that K^+ acts to regulate aldosterone secretion solely through the Ca^{2+} messenger system. One of the interesting characteristic of the glomerulosa-cell response is that these cells are peculiarly sensitive to extracellular K^+, so that relatively low concentrations of K^+ (6–12mM) cause depolarization of the plasma membrane (Foster *et al.*, 1982). This leads to an opening of voltage-dependent Ca^{2+} channels, resulting in an increase in $[Ca^{2+}]_c$, which has been shown by using Quin 2 (Capponi *et al.*, 1984; Braley *et al.*, 1984). We have shown that the source of mobilized Ca^{2+} in K^+-stimulated glomerulosa cells is exclusively the extracellular pool (Kojima *et al.*, 1984*a*, 1985*a*). Thus influx of Ca^{2+} via a voltage-dependent channel is the initial event in K^+ action. Nonetheless, the exact mechanism of K^+ action is not clear. When the time course of aldosterone secretion is studied in a perifusion system, the effect of K^+ is sustained (Kojima *et al.*, 1985*a*). In comparison, the action of low doses of ionophore A23187 is transient (Kojima *et al.*, 1983, 1984*b*), even though

Abbreviations used: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; $[Ca^{2+}]_c$, free Ca^{2+} concentration in the cell cytosol; Ins$P$, inositol monophosphate; InsP_2 inositol bisphosphate; InsP_3, inositol triphosphate; KRBG, Krebs–Ringer bicarbonate buffer containing glucose (Kojima *et al.*, 1983).

A23187 is also thought to act primarily by enhancing Ca^{2+} entry into glomerulosa cells. One of the reasons for the transient stimulation of aldosterone secretion by A23187 is thought to be due to a Ca^{2+}-calmodulin-dependent activation of the Ca^{2+} pump in plasma membrane by an elevation of $[Ca^{2+}]_c$ (Scharff *et al.*, 1983). In other systems where Ca^{2+} is thought to act as the major intracellular messenger, agonist-induced increases in $[Ca^{2+}]_c$ are only transient (Morgan & Morgan, 1984; Snowdowne & Borle, 1984). Thus one would predict that another unidentified messenger is involved in K^+ action, in order for this agonist to induce a sustained aldosterone-secretory response. Three possibilities have been considered: (*a*) K^+ as well as angiotensin II (Farese *et al.*, 1984; Kojima *et al.*, 1984*b*) activates polyphosphoinositide hydrolysis and diacylglycerol production and thereby activates the C-kinase branch of the Ca^{2+} messenger system (Rasmussen & Barrett, 1984); (*b*) K^+ increases the cyclic AMP content of the adrenal glomerulosa cell (Tait *et al.*, 1974), and cyclic AMP acts as a positive sensitivity modulator of Ca^{2+}-dependent reactions (Rasmussen & Waisman, 1982); or (*c*) the two agents have different effects on plasma-membrane Ca^{2+} influx. The present studies were performed to clarify the possible mechanism of gain control in K^+ action on glomerulosa cells.

Materials and methods

The angiotensin II, [1-Sar,8-Ala]angiotensin II, A23187, PtdIns4*P*, PtdIns(4,5)P_2 and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). Forskolin was obtained from Calbiochem (La Jolla, CA, U.S.A.). The *myo*-[2-^{3}H]inositol (15.8Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (86.6Ci/mmol) and radioimmunoassay kit for cyclic AMP were obtained from New England Nuclear (Boston, MA, U.S.A.).

Isolation and incubation of adrenal glomerulosa cells

Calf adrenal glands were obtained at a local slaughterhouse. Dispersed adrenal glomerulosa cells were prepared by collagenase digestion as described previously (Kojima *et al.*, 1983). Cells were suspended in Krebs–Ringer bicarbonate buffer containing 3.5mM-K^+, 1.25mM-Ca^{2+}, 5.5mM-glucose and 0.2% bovine serum albumin (KRBG buffer). Static incubations were done as described previously (Kojima *et al.*, 1984*a*).

Perifusion of adrenal glomerulosa cells

Perifusion was performed in a four-channel flow-through chamber in a Lucite (perspex) block (Foster & Rasmussen, 1983; Kojima *et al.*, 1983, 1985*a*); 10^7 cells suspended in Sephadex G-15 were applied to each chamber and perifused at flow rate of 0.4ml/min. Cells were perifused for 30min before addition of stimulant, and samples for aldosterone measurement were taken every 4min. A23187 and forskolin were dissolved in ethanol; the final concentration of ethanol was less than 0.3%, and perifusion with this concentration alone did not affect aldosterone production. Aldosterone was measured by radioimmunoassay, with anti-aldosterone antiserum provided by the National Hormone and Pituitary Program as described previously (Kojima *et al.*, 1985*a*).

Measurement of phosphoinositide turnover

Phosphoinositide turnover was measured by using [^{3}H]inositol-labelled glomerulosa cells as described previously (Kojima *et al.*, 1984*b*). In short, cells were labelled in KRGB buffer containing 10μCi of [^{3}H]inositol/ml and 0.1nM-angiotensin II for 2h. At the end of the labelling period, 10nM-[1-Sar,8-Ala]angiotensin II was added to block angiotensin II action. Cells were then washed three times with KRBG buffer containing 10mM-inositol and suspended in KRBG buffer containing 10mM-inositol and 50mM-LiCl. LiCl at 50mM effectively blocks inositol phosphate monophosphatases, without affecting PtdIns(4,5)P_2 hydrolysis (Kojima *et al.*, 1984*b*). To maintain the iso-osmoticity of of the buffer, NaCl was replaced by LiCl. The cell suspension (4×10^6 cells in 0.8ml) was then exposed to 8mM-K^+ or 50nM-A23187 for the indicated time and reaction was stopped by adding 1.5ml of chloroform/methanol (1:2, v/v). Then 0.5ml of chloroform and 0.5ml of 2.4M-HCl were added. Phosphoinositides were extracted, authentic PtdIns4*P* and PtdIns(4,5)P_2 (50μg each) added, and the mixtures separated on a silica gel H plate impregnated with potassium oxalate, with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) as solvent (Shaikh & Palmer, 1977). Lipids were detected by staining with iodine vapour. Spots corresponding to PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 were scraped off the plate, and the radioactivity of each was counted by liquid-scintillation spectrophotometry. Water-soluble inositol phosphates were separated by anion-exchange column chromatography as described by Berridge *et al.* (1983). Diacylglycerol production was measured with [^{3}H]arachidonic acid-labelled cells (Kojima *et al.*, 1984*b*). Neutral lipids were separated by on silica gel G plates, with benzene/chloroform/methanol (16:3:1, by vol.) as solvent (Banschbach *et al.*, 1974).

Measurement of intracellular cyclic AMP

Cells were incubated for the indicated time without phosphodiesterase inhibitor. At the end of incubation, 0.5mM-3-isobutyl-1-methylxanthine

was added. Intracellular cyclic AMP was measured by the method of Podesta *et al.* (1979), by using a radioimmunoassay kit for cyclic AMP. Statistical significance was assessed by Student's *t* test.

Measurement of Ca^{2+} influx

This was done by the method described by Mauger *et al.* (1984). Briefly, the experiment was started by adding 500 μl of a cell suspension containing 2×10^6 cells to a tube containing trace amounts of $^{45}CaCl_2$ (5μCi). The medium was either KRBG buffer containing 2% bovine serum albumin or one containing 50nM-A23187 or 8mM-K^+, and stimulators were added simultaneously with the $^{45}CaCl_2$ or 20min before $^{45}CaCl_2$ addition. Cells and media were prewarmed at 37°C and incubated at this temperature. Samples (100μl) were taken at 15, 45, 75 and 105s. The samples were immediately diluted into 4ml of an ice-cold washing solution containing 144mM-NaCl, 5mM-$CaCl_2$ and 5mM-Tris/HCl, pH7.4. The mixture was filtered through a Whatman GF/C glass-fibre filter, and washed with 3×4ml of the same ice-cold washing solution. The radioactivity retained on the filter was counted in a liquid-scintillation spectrophotometer after addition of an appropriate scintillation fluid. Ca^{2+} influx was calculated from the slope of the particular regression line.

Results

Comparison of the time courses of A23187- and K^+-induced aldosterone secretion

The time courses of change in aldosterone secretory rate after addition of K^+ or A23187 were compared by using the perifusion system (Fig. 1). When 50nM-A23187 was added at zero time, the aldosterone secretion rate increased after an 8min interval (including 2min for the dead space of the perifusion system); it increased rapidly and reached a peak value at 16min. The A23187-induced aldosterone secretion was only transient. The secretion rate decreased quickly after the initial peak to a steady-state rate by 40min that was only slightly higher than the basal rate (Fig. 1*a*). When glomerulosa cells were stimulated by 8mM-K^+, the initial aldosterone secretory response was similar to that after 50nM-A23187, and the peak value observed at 16min was almost identical (Fig. 1*b*). The secretion rate then declined slowly. At 40min the secretion rate was about 80% of the peak value. Thus K^+-induced aldosterone secretion was more sustained than that induced by 50nM-A23187.

Comparison of the effects of A23187 and K^+ on Ca^{2+} influx

The effects of 50nM-A23187 and of 8mM-K^+ on Ca^{2+} influx were studied. The basal influx rate was 0.64 ± 0.09nmol/min per mg of protein and was stable over a 20min period. If either 50nM-A23187 or 8mM-K^+ was added along with $^{45}CaCl_2$, there was an immediate increase in influx rate, to 2.70 ± 0.26 and 2.42 ± 0.22nmol/min per mg respectively. Furthermore, the increase in influx rate induced by each agent was maintained for at least 20min; that by 50nM-A23187 was slightly greater than that by 8mM-K^+.

Effect of K^+ on polyphosphoinositide metabolism

In previous work (Kojima *et al.*, 1983, 1984*b*) we had shown that activation of the C-kinase pathway

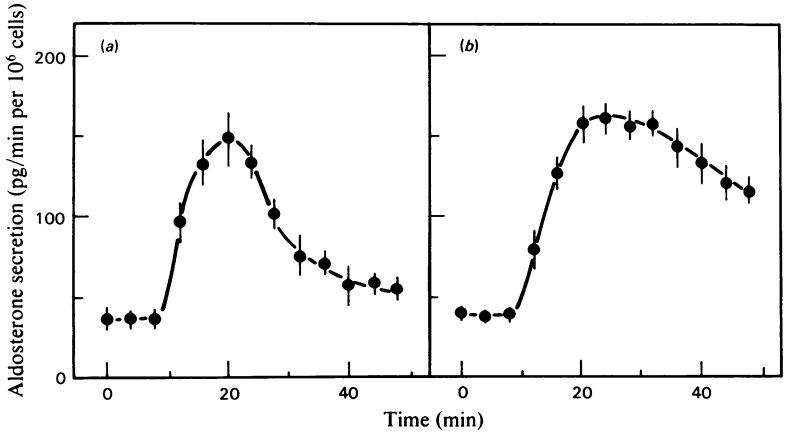

Fig. 1. *Comparison of the time courses of A23187- and K^+-stimulated aldosterone secretion*
Perifusion was performed as described in the Materials and methods section. Cells were perifused with either 50nM-A23187-(*a*) or 8mM-K^+ (*b*) from 0 to 48min. Each point is the mean ± S.E.M. for three experiments from two different cell preparations.

is responsible for the sustained phase of angiotensin II-mediated aldosterone secretion. Diacylglycerol generated by the breakdown of PtdIns(4,5)P_2 acts as a sensitivity modulator of C-kinase (Nishizuka, 1984; Rasmussen & Barrett, 1984). To assess whether or not C-kinase is involved in K^+-mediated aldosterone secretion, the effect of 8mM-K^+ on polyphosphoinositide metabolism was studied by using [^{3}H]inositol-prelabelled cells. As shown in Fig. 2, 8mM-K^+ caused a small but significant decrease ($P<0.05$) in radioactivity in the PtdIns(4,5)P_2 fraction observed at 20 and 45s. In contrast, radioactivity in neither PtdIns4P nor PtdIns changed in this time period. The water-soluble inositol phosphates were also measured to see if this decrease in PtdIns(4,5)P_2 was caused by the activation of a phospholipase C specific to PtdIns(4,5)P_2. As shown in Fig. 3, [^{3}H]InsP_3 did not change after stimulation with K^+. Also, neither the [^{3}H]InsP_2[^{3}H]Ins$P$ contents changed significantly. These results are in contrast with the effect of angiotensin II, caused a rapid 2-fold increase in InsP_3 and a 3-fold increase in InsP_2 within 10s (Kojima *et al.*, 1984*b*). This increase was sustained for at least 30min. To confirm that the decrease in PtdIns(4,5)P_2 was not due to phospholipase C-catalysed breakdown, diacylglycerol production was measured by using [^{3}H]-arachidonic acid-prelabelled glomerulosa cells. As shown in Table 1, radioactivity in diacylglycerol

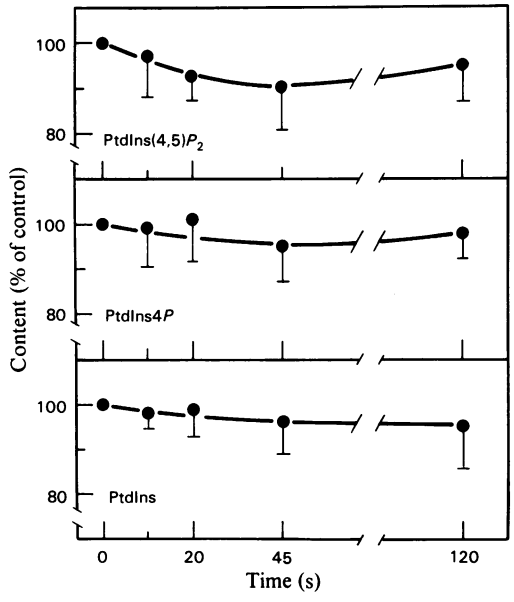

Fig. 2. *Time course of changes in the content of* [^{3}*H*]*-inositol-labelled phosphoinositides after* K^+ *stimulation*
Cells were prelabelled with [^{3}H]inositol as described in the Materials and methods section. A cell suspension containing 4×10^6 cells was incubated with 8mM-K^+ in the presence of 50mM-Li^+ for each time point. Results are the means ± S.E.M. for four to five experments from three different cell preparations each done in duplicate. Values are expressed as percentages of an unstimulated control incubated for each time. At the start of incubation, the radioactivity in each phospholipid (c.p.m.) was: PtdIns, 13524±847; PtdIns4P, 561±34; PtdIns(4,5)P_2, 124±24.

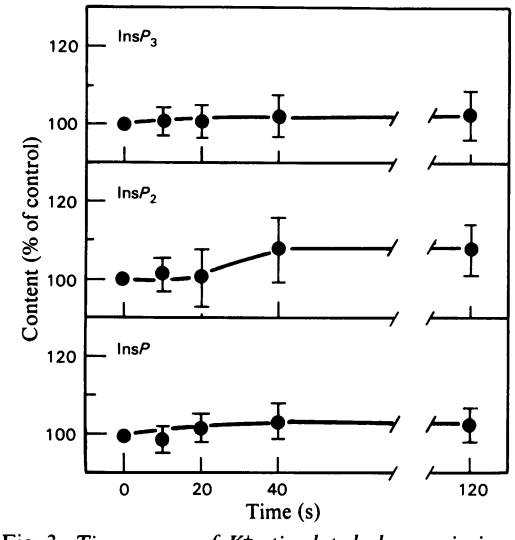

Fig. 3. *Time course of* K^+*-stimulated changes in inositol phosphates*
Cells were prelabelled and stimulated as described in the legend for Fig. 2. Water-soluble inositol phosphates were separated as described in the Materials and methods section. Results are means±S.E.M. for four to five experiments from three separate cell preparations each done in duplicate. Values are expressed as percentages of an unstimulated control. At the start of incubation, the radioactivity in each inositol phosphate (c.p.m.) was: Ins$P$, 2100±96; InsP_2, 174±21; InsP_3, 382±36.

Table 1. *Effect of* K^+ *on* [^{3}*H*]*arachidonic acid-labelled diacylglycerol production*

Cells were labelled with [^{3}H]arachidonic acid for 120min. A cell suspension containing 2×10^6 cells was incubated with 8mM-K^+ for the indicated time, and [^{3}H]arachidonic acid-labelled diacylglycerol was determined as described in the Materials and methods section. Results are means±S.E.M. for four experiments from four different cell preparations, each done in duplicate.

Time after K^+ addition (s)	Radioactivity (c.p.m./10^6 cells)
0	784±46
20	767±51
60	796±64
600	827±101

did not change after stimulation with 8mM-K^+. In a previous study (Kojima *et al.*, 1984*b*) we showed that A23187 (500nM) causes a small, but insignificant, decrease in PtdIns(4,5)P_2 in ^{32}P-labelled glomerulosa cells. The effects of A23187 treatment on phosphoinositide mechanism was further studied by using [^{3}H]inositol-labelled cells. As shown in Table 2, A23187 caused a small but significant ($P<0.05$) decrease in radioactivity in PtdIns(4,5)P_2. Radioactivity in PtdIns4P did not change significantly. The amounts of the water-soluble inositol phosphates were also measured. As shown in Table 2, [^{3}H]InsP_3, [^{3}H]InsP_2 and [^{3}H]Ins$P$ contents did not change significantly. Under identical conditions, angiotensin II addition causes significant increases in the content of each (Kojima *et al.*, 1984*b*). Thus the A23187-induced small decrease in PtdIns(4,5)P_2 was not accompanied by an increase in the amount of any inositol phosphate.

Effect of K^+ and A23187 on intracellular cyclic AMP

Tait and colleagues reported that incubation of glomerulosa cells in high concentrations of K^+ (8–12mM) results in an increase in the cyclic AMP content of glomerulosa cells (Tait *et al.*, 1974; Albano *et al.*, 1974). They found that 8mM-K^+ produced a maximal stimulatory effect. Others have reported that K^+ does not alter the cyclic AMP content in glomerulosa cells (Douglas *et al.*, 1978; Fujita *et al.*, 1979). To measure changes in the cyclic AMP content in stimulated cells, we incubated cells without phosphodiesterase inhibitor for various times. Then isobutylmethylxanthine was added at the end of incubation to prevent cyclic AMP breakdown, the tissue was extracted immediately, and intracellular cyclic AMP content determined by radioimmunoassay. Fig. 4 illustrates the time course of changes in intracellular cyclic AMP. Incubation of cells with 8mM-K^+ led to a slight increase in the cyclic AMP content of the cells at 10 and 30min. On the other hand, addition of 50nM-A23187, which induced a similar initial increase in aldosterone secretory rate, decreased cyclic AMP in the cells at 10 and 30min. To confirm the effect of K^+ on cyclic AMP, intracellular cyclic AMP content after 20min incubation was measured in different sets of experiments. The results are shown in Table 3. The cyclic AMP content was significantly ($P<0.05$) decreased in cells incubated with 50nM-A23187. In K^+-treated cells, the cyclic AMP content was slightly higher than that in control cells, but the difference was not statistically significant. However, compared with A23187-treated cells, K^+-treated cells had a significantly ($P<0.01$) higher content of cyclic AMP.

To determine whether the difference in cyclic AMP content between K^+-treated and A23187-treated cells was sufficient to account for the

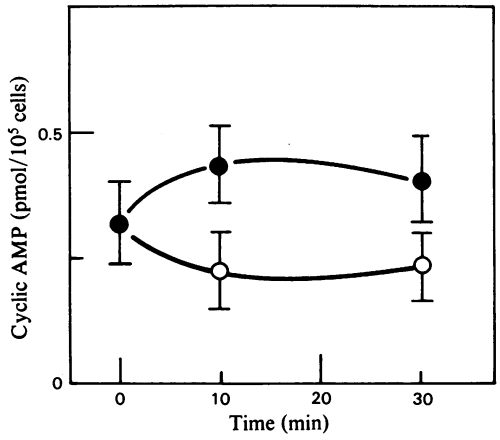

Fig. 4. *Time course of changes in intracellular cyclic AMP content of adrenal glomerulosa cells after treatment with K^+ or A23187*

A cell suspension containing 1×10^6 cells was incubated with either 50nM-A23187 (○) or 8mM-K^+ (●) for the indicated times without isobutylmethylxanthine. Intracellular cyclic AMP was determined as described in the Materials and methods section. Results are the means±S.E.M. for three determinations.

Table 2. *Time course of A23187-stimulated changes in [^{3}H]inositol-labelled phosphoinositides and inositol phosphates*

Cells were prelabelled with [^{3}H]inositol and stimulated by 50nM-A23187 at zero time. Results are means±S.E.M. for three experiments from three separate cell preparations each done in duplicate. Values are expressed as percentages of unstimulated controls. At the start of incubation, the radioactivity (c.p.m.) in each phospholipid or inositol phosphate was PtdIns, 11964±763; PtdIns4P, 541±49; PtdIns(4, 5)P_2, 139±28; Ins$P$, 2017±106; InsP_2, 191±38; InsP_3, 358±41. *P <0.05 versus unstimulated control.

Time after A23187 addition (s)	Phosphoinositides (%)			Inositol phosphates (%)		
	PtdIns(4,5)P_2	PtdIns4P	PtdIns	InsP_3	InsP_2	Ins$P$
20	93.0±3.4*	101.1±3.1	99.3±4.1	98.6±5.3	102.1±4.1	100.3±3.1
40	92.1±4.1*	98.7±5.1	102.1±4.8	99.6±3.1	102.5±4.8	101.1±4.1
120	96.8±5.9	99.4±4.9	101.0±5.3	102.1±4.6	100.4±4.3	102.4±3.9

Table 3. *Changes in the intracellular cyclic AMP content in adrenal glomerulosa cells incubated with either A23187 or K^+*

A cell suspension containing 10^6 cells was incubated with vehicle (ethanol), 50nM-A23187 or 8mM-K^+ for 20min. Intracellular cyclic AMP content was determined as described in the Materials and methods section. Results are the means ± S.E.M. for five independent experiments from five different cell preparations each done in triplicate.

Addition	Intracellular cyclic AMP (pmol/10^5 cells)
0.2% Ethanol	0.43 ± 0.08
50nM-A23187	0.28 ± 0.11
0.2% Ethanol + 8mM-K^+	0.49 ± 0.04

Table 4. *Comparison of effects of K^+ and A23187 plus forskolin on intracellular cyclic AMP content of adrenal glomerulosa cells*

Samples of a cell suspension containing 10^6 cells were incubated with 50nM-A23187 and the given concentration of forskolin for 20min. Intracellular AMP was determined and compared with that in cells treated with 8mM-K^+. Results are means ± S.E.M. for five determinations.

Addition	Intracellular cyclic AMP (pmol/10^5 cells)
0.2% Ethanol	0.42 ± 0.05
50nM-A23187	0.28 ± 0.07
50nM-A23187 + 10nM-forskolin	0.30 ± 0.05
50nM-A23187 + 50nM-forskolin	0.46 ± 0.08
50nM-A23187 + 100nM-forskolin	0.61 ± 0.13
0.2% Ethanol + 8mM-K^+	0.47 ± 0.03

difference between the time course of K^+-stimulated and A23187-stimulated aldosterone production (Fig. 1), forskolin, an activator of adenylate cyclase (Seamon *et al.*, 1981), was employed along with A23187. We have shown that forskolin increases both intracellular cyclic AMP and aldosterone production in a dose-dependent manner (Kojima *et al.*, 1985*b*). In A23187-treated cells, forskolin produced a dose-dependent increase in intracellular cyclic AMP content (Table 4). The concentration of forskolin that led to an increase in cyclic content in A23187-treated cells to a value similar to that in K^+-treated cells was 50nM (Table 4).

When glomerulosa cells were perifused with a combination of A23187 (50nM) and this concentration of forskolin (50nM), the aldosterone-secretory response was altered. As shown in Fig. 5, the basal rates and the initial response were similar to those seen after A23187 alone, but, after reaching a peak value at 24min, the response was sustained in the

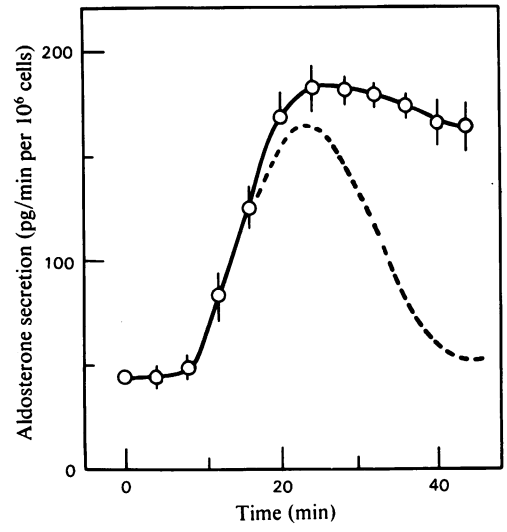

Fig. 5. *Time course of change in aldosterone secretion rate from adrenal glomerulosa cells perifused with combined A23187 and forskolin*

Perifusion was performed as described in the Materials and methods section. Cells were stimulated by either 50nM-A23187 alone (----) or 50nM-A23187 plus 50nM-forskolin (○) from 0 to 40min. Results are means ± S.E.M. for three experiments from three cell preparations.

presence of forskolin, but not in its absence. The secretory response induced by the perifusion with A23187 plus forskolin was similar to that induced by perifusion with 8mM-K^+ (cf. Fig. 5 with Fig. 1*b*). It is noteworthy that perifusion with this low dose of forskolin (50nM) alone caused very little change in aldosterone-secretory rate, but higher doses induced a significant increase in this rate (Kojima *et al.*, 1985*b*).

Discussion

The present studies were undertaken in an effort to determine why the time course of aldosterone production from adrenal glomerulosa cells after low doses of A23187 differs from that after exposure of cells to 8mM-K^+ even though both agents are thought to act by increasing Ca^{2+} uptake into these cells. The results shown in Fig. 1 illustrate this difference. Perifusion of isolated glomerulosa cells with A23187 induces a transient aldosterone-secretory response, whereas perifusion with 8mM-K^+ induces a sustained secretory response.

There are at least three possible reasons why perifusion with those two different agents might lead to a different secretory pattern: (1) K^+ but not A23187 induces the activation of the C-kinase

branch of the Ca^{2+} messenger system; (2) K^+ but not A23187 increases the cyclic AMP content of these cells; or (3) the two agents do not induce the same sustained increase in Ca^{2+} influx.

The last of these possibilities was examined by comparing the effects of 50nM-A23187 and 8mM-K^+ on the initial rate of Ca^{2+} influx into glomerulosa cells. Addition of 50nM-A23187 led to an immediate and sustained (20min) increase in Ca^{2+} influx. The basal value was 0.64 ± 0.09 and the A23187-induced rate was 2.70 ± 0.26nmol/min per mg of protein. Similarly, addition of 8mM-K^+ led to a similar sustained increase in Ca^{2+} influx, 2.42 ± 0.22nmol/min per mg of protein. The rate of Ca^{2+} influx observed after A23187 addition was slightly greater than that after K^+ addition. It seems unlikely that difference in Ca^{2+}-influx rate in response to the two agents explains the difference in the patterns of aldosterone secretion seen after their addition (Fig. 1).

We have previously presented data in support of the hypothesis that the C-kinase pathway is responsible for the sustained phase of angiotensin-mediated aldosterone secretion (Kojima *et al.*, 1984*b*). Since adrenal glomerulosa cells are rich in C-kinase (Kojima *et al.*, 1984*b*) and diacylglycerol generated by PtdIns(4,5)P_2 breakdown acts as a sensitivity modulator of the C-kinase (Kishimoto *et al.*, 1980), the effect of K^+ on phosphoinositide metabolism was studied. Our previous results (Kojima *et al.*, 1984*b*) had shown that A23187 does not activate the C-kinase branch of the Ca^{2+}-messenger system. The results shown in Fig. 2 indicate that 8mM-K^+ causes a small decrease in the pool of labelled PtdIns(4,5)P_2. However, this decrease is not accompanied by an increase in either InsP_3 (Fig. 3) or diacylglycerol (Table 1). Thus the K^+-induced change in PtdIns4,5P_2 is not due to an activation of the phospholipase C specific to PtdIns4,5P_2. We have made similar observations in A23187-stimulated glomerulosa cells (Table 2) and corticotropin-stimulated glomerulosa cells (Kojima *et al.*, 1985*b*). A possible explanation for this decrease in PtdIns(4,5)P_2 is that the rise in $[Ca^{2+}]_c$ induced by A23187, corticotropin or 8mM-K^+ activates a monophosphatase which catalyses the conversion of PtdIns(4,5)P_2 into PtdIns4P, as suggested by Litosch *et al.* (1984). Alternatively, a rise in $[Ca^{2+}]_c$ may lead to the activation of phospholipase A_2, as suggested by the results of Rittenhouse & Horne (1984). In any case, as shown in Table 1, the amount of labelled diacylglycerol does not change after stimulation by K^+ as it does after stimulation with angiotensin II (Kojima *et al.*, 1984*b*). Therefore it seems unlikely that C-kinase is involved in mediating the sustained phase of K^+-mediated aldosterone secretion.

There have been controversial data regarding the effect of increased extracellular K^+ on the cyclic AMP content of adrenal glomerulosa cells (Saruta *et al.*, 1972; Tait *et al.*, 1974; Albano *et al.*, 1974; Douglas *et al.*, 1978; Fujita *et al.*, 1979). One reason for the controversy may be a difference in experimental protocols, particularly the time point at which measurements are made, and whether or not phosphodiesterase inhibitors are employed. To correlate the content of cyclic AMP with its function, we thought it best to measure the amount of cyclic AMP in the cells incubated in the absence of phosphodiesterase inhibitors. The results shown in Fig. 4 and Table 3 suggest that the elevation of $[Ca^{2+}]_c$ induced by A23187 decreases the intracellular content of cyclic AMP presumably because of an activation of a Ca^{2+}-calmodulin-dependent phosphodiesterase, which is known to exist in glomerulosa cells (Koletsky *et al.*, 1983). Compared with A23187-treated cells, K^+-treated cells have a significantly higher intracellular content of cyclic AMP (Fig. 4 and Table 3). Since K^+ as well as A23187 cause an increase in $[Ca^{2+}]_c$, and hence should also cause the cyclic AMP content to fall, the fact that the intracellular cyclic AMP content of K^+-treated cells is higher than that of A23187-treated cells suggests that K^+ activates adenylate cyclase. Our results are similar to those reported by Saruta *et al.* (1972) and Tait *et al.* (1974).

If the difference in cyclic AMP content between A23187-treated and K^+-treated cells is linked in some way to the different secretory patterns shown in Fig. 1, then raising the cyclic AMP concentration to the same value in A23187-treated cells as that observed in K^+-treated cells should alter the A23187-induced secretory pattern. As shown in Fig. 5, the combination of 50nM-A23187 and 50nM-forskolin induces a higher and more prolonged stimulation of aldosterone secretion than with A23187 alone. This observation indicates that a small increase in cyclic AMP content alters the Ca^{2+}-sensitivity of response elements to Ca^{2+} in this tissue. Since glomerulosa cells treated with combined forskolin and A23187 have a similar content of cyclic AMP as do K^+-treated cells (Table 4), it seems likely that a small increase in cyclic AMP content plays a role in the sustained phase of K^+-mediated aldosterone secretion. A similar synergistic effect of forskolin on the behaviour of the Ca^{2+}-messenger system has been shown to operate in the response of adrenal glomerulosa cells to corticotropin (Kojima *et al.*, 1985*b*), in exocrine (Heisler, 1983) and endocrine pancreatic cells (Zawalich *et al.*, 1983), and in cultured pituitary cells (Delbeke *et al.*, 1984).

A comparison of the present results with our previously published results (Kojima *et al.*, 1984*b*) reveals that the transient stimulatory effect of the

ionophore A23187 on aldosterone-production rate from isolated adrenal glomerulosa cells can be converted into a sustained secretory response by either an activator of the C-kinase pathway, such as 12-*O*-tetradecanoylphorbol-13-acetate or 1-oleoyl-2-acetylglycerol, or an activator of adenylate cyclase, such as forskolin. In the concentrations employed, neither 12-*O*-tetradecanoylphorbol-13-acetate nor forskolin itself can induce a similar secretory response. Both 12-*O*-tetradecanoylphorbol-13-acetate and forskolin act synergistically with A23187 to produce a sustained response. These results imply that there are two pathways by which the actions of messenger Ca^{2+} can be enhanced in the glomerulosa cells: one, by the activation of the C-kinase branch of the Ca^{2+}-messenger system; and the other by the positive sensitivity modulation of events in the calmodulin branch of the Ca^{2+}-messenger system by an increase in cyclic AMP concentration.

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