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EFFECTS OF THE POTASSIUM CHANNEL OPENERS CROMAKALIM AND PINACIDIL ON CATECHOLAMINE SECRETION AND CALCIUM MOBILIZATION IN CULTURED BOVINE ADRENAL CHROMAFFIN CELLS

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Abstract—The effects of two K^+ channel openers, cromakalim and pinacidil, on voltage-dependent and receptor-mediated catecholamine secretion and Ca^{2+} mobilization in bovine adrenal chromaffin cells were studied to determine the role of membrane K^+ channels in the regulation of a Ca^{2+}-dependent secretory process. Both cromakalim and pinacidil stimulated the efflux of ^{86}Rb (used to monitor K^+ permeability) from preloaded cells. Cromakalim and pinacidil did not affect the catecholamine secretion induced by excessive depolarization with 56 mM K^+, but inhibited that induced by moderate depolarization with 31 mM K^+ in a concentration-dependent manner (1 μM–100 μM). The 31 mM K^+-induced $^{45}Ca^{2+}$ influx and increase in intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ were also inhibited by these agents at similar concentrations to those for inhibition of catecholamine secretion. Cromakalim and pinacidil inhibited catecholamine secretion, $^{45}Ca^{2+}$ influx and increase in $[Ca^{2+}]i$ induced by stimulation of nicotinic acetylcholine (ACh) receptors with carbamylcholine. Furthermore, both cromakalim and pinacidil inhibited the increase in $[Ca^{2+}]i$ induced by carbamylcholine in the absence of extracellular Ca^{2+}, which is thought to be mediated by muscarinic ACh receptors. On the other hand, they did not affect catecholamine secretion induced by Bay-K 8644, Ba^{2+}, A23187, histamine or bradykinin. These results indicate that the K^+ channel openers, cromakalim and pinacidil, selectively inhibit catecholamine secretion induced by moderate depolarization or by nicotinic ACh receptor stimulation by inhibiting Ca^{2+} influx and increase in $[Ca^{2+}]i$. Furthermore, the results suggest that these K^+ channel openers–sensitive membrane K^+ channels are involved in the regulation of catecholamine secretion mainly indirectly through effects on the voltage-dependent membrane Ca^{2+} channels.

Key words: K^+ channel openers; catecholamine secretion; Ca^{2+} mobilization; adrenal chromaffin cells

Membrane K^+ channels in various cells are known to be responsible for controlling the membrane potential and excitability of cells [1–3]. The activation (opening) of these channels causes hyperpolarization, and conversely, their inhibition (closing) causes depolarization of the cell membrane. Electrophysiological studies using voltage- and patch-clamp techniques have shown the presence of many different types of K^+ channels, which are sensitive to neurotransmitters, hormones, toxins and drugs [2–6].

Recently, a new class of drugs, named K^+ channel openers, which have vasorelaxant, antihypertensive and myocardial protective effects have been reported [6]. These drugs have also been shown to affect the nervous system and modify neurotransmission [7–9].

In the present study, we studied the effects of two K^+ channel openers, cromakalim and pinacidil, on voltage-dependent and receptor-mediated catecholamine secretion and Ca^{2+}-mobilization in cultured bovine adrenal chromaffin cells, which are thought to be a model of catecholamine-containing neurones [10–13]. This study provided evidence for the role of membrane K^+ channels in regulation of the Ca^{2+} dependent secretory process and suggested additional pharmacological effects of cromakalim and pinacidil besides those on K^+ channels.

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‡ Abbreviations: $[Ca^{2+}]i$, intracellular free Ca^{2+} concentration; THI, trihydroxyindole; ACh, acetylcholine; BSS, balanced salt solution.

MATERIALS AND METHODS

Primary cell culture. Isolated bovine adrenal chromaffin cells were prepared by sequential digestion of adrenal medullary slices with collagenase (0.5%) and were maintained as monolayers on 24-well cluster plates (Costar, Cambridge, MA, U.S.A.) at a density of 5×10^5 cells/well, or on 35 mm dishes (Falcon) at a density of 2×10^6 cells/dish, for 3–4 days [14, 15]. Cultured chromaffin cells were washed once with 1 mL of BSS‡ (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 20 mM HEPES/NaOH, pH 7.4) before use in experiments.

Measurement of $^{86}Rb^+$ *efflux.* The cells (2×10^6 cells) were incubated in 2 mL of BSS containing $^{86}RbCl$ (1.0 μCi/mL) at 37° for 60 min to load $^{86}Rb^+$ into the cells. Then, the medium was discarded, and the cells were washed three times with 1 mL of ice-

cold BSS. After washout of the extracellular $^{86}Rb^+$, the cells were incubated at 37° for various periods in 1 mL of BSS with or without drugs. After incubation, the medium was withdrawn and the cells were lysed by adding 1 mL of 1% Triton-X100. The radioactivity in the medium and the cell lysate was determined by Cerenkov counting.

Secretion of catecholamines. The cells (5×10^5 cells) were pre-incubated at 37° for 10 min in 250 μL of BSS with or without drugs, and then catecholamine secretion was induced by various secretagogues in the absence or presence of drugs. High-K^+ solutions (14–56 mM KCl) had the same composition as BSS except that NaCl was replaced by an equimolar amount of KCl. After stimulation with secretagogues for 10 to 40 min, the medium was withdrawn, and the cells were lysed by adding 250 μL of 10% acetic acid, followed by freeze-thawing. Catecholamine in the medium and the cell lysate was assayed fluorometrically by the THI method. The amount of catecholamine secreted from the cells was expressed as a percentage of the total cellular catecholamine.

Measurement of Ca^{2+} influx. The cells (5×10^5 cells) were pre-incubated at 37° for 10 min in 250 μL of BSS with or without drugs, and then stimulated with high K^+ or carbamylcholine in BSS containing 3.0 μCi $^{45}CaCl_2$ for 10 min. The medium was then discarded and the cells were washed four times with 1 mL of ice-cold Ca^{2+}-free BSS. Intracellular $^{45}Ca^{2+}$ was extracted with 200 μL of 1% Triton X-100 and measured in a liquid scintillation counter.

Measurement of $[Ca^{2+}]i$. To estimate the change in $[Ca^{2+}]i$, we used a fluorescence microscope (Optiphoto; Nikon), a photon counter (545A; NF; Hiroshima, Japan) and a filter (Nihon Shinkuukougaku; Osaka, Japan). For this experiment, the cells were maintained on a cover glass for 3–4 days, and preloaded with the Ca^{2+} indicator fura-2 [16] by incubation with 5 μM fura-2 AM at 37° for 20 min in BSS. The cells were then placed in a bath (volume: 0.5 mL) on the stage of the fluorescence microscope, and BSS was superfused continuously over the cells at a rate of 0.8 mL/min. The fluorescent Ca^{2+} indicator dye was alternately excited at 340 nm and 380 nm with filtered light and the ratio of the two excitation fluorescent signals was calibrated to estimate $[Ca^{2+}]i$ as described previously [16]. Experiments were done on single cells.

Materials. $^{86}RbCl$ amd $^{45}CaCl_2$ were purchased from Amersham Corp. (Tokyo, Japan). The following reagents were used: cromakalim (Beecham Lab., London, U.K.), pinacidil (Shionogi Pharmaceutical Co. Japan), A23187 (Boehringer-Mannheim, Germany), carbamylcholine (Sigma Chemical Co., St Louis, MO, U.S.A.), histamine, bradykinin, fura-2 AM, nifedipine (Wako, Japan). Other chemicals used were commercial products of reagent grade.

Statistical methods. All experiments were performed in triplicate and each measurement was repeated at least three times. Data are shown as means ± SD of 3–4 experiments. Statistical analysis was carried out by Student's *t*-test.

RESULTS

Effects of cromakalim and pinacidil on ^{86}Rb efflux from preloaded cells

^{86}Rb was used as a monitor to assess membrane K^+ permeability [17, 18]. As shown in Fig. 1(a), cromakalim (100 μM) and pinacidil (100 μM) stimulated ^{86}Rb efflux from the cells to similar extents, the efflux being rapid in the first 5 min. During the first 5 min, both cromakalim and pinacidil increased ^{86}Rb efflux to about 10% from a basal level of about

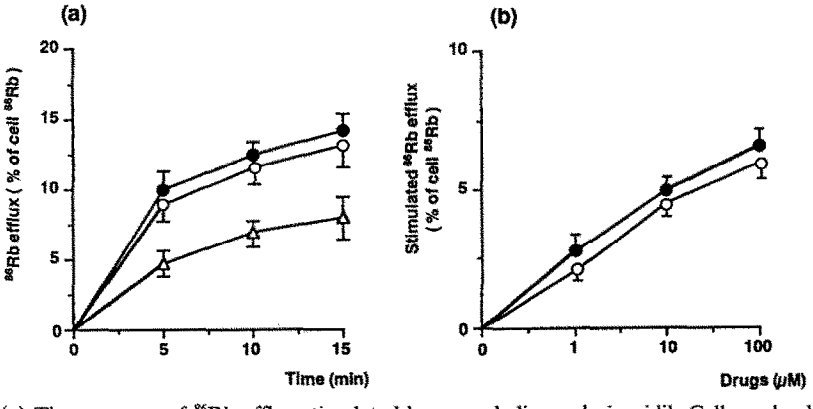

Fig. 1. (a) Time courses of ^{86}Rb efflux stimulated by cromakalim and pinacidil. Cells preloaded with ^{86}Rb were incubated for various periods in the absence (△) or presence of cromakalim (100 μM, ●) or pinacidil (100 μM, ○) as described in Materials and Methods. The results are shown as percentage efflux of the total ^{86}Rb initially present in the cells. Points and bars are means ± SD for 3–4 experiments. (b) Concentration–response curves of cromakalim and pinacidil for the stimulation of ^{86}Rb efflux. Cells preloaded with ^{86}Rb were incubated for 10 min with various concentrations of cromakalim (●) or pinacidil (○) as described in Materials and Methods. Basal ^{86}Rb efflux without drugs was subtracted from the data. Points and bars are means ± SD for 3–4 experiments.

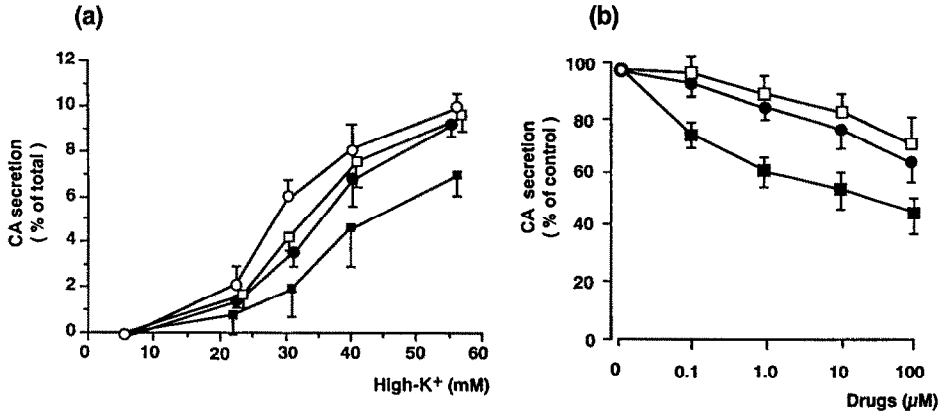

Fig. 2. (a) Effects of cromakalim, pinacidil and nifedipine on catecholamine secretion induced by various concentrations of KCl. Cells were stimulated with various concentration of KCl for 10 min in the absence (○) or presence of cromakalim (100 μM,●), pinacidil (100 μM, □) or nifedipine (100 μM, ■) as described in Materials and Methods. Catecholamine (CA) secretion is shown as a percentage of the total cellular CA. Points and bars are means ± SD for 3–4 experiments. (b) Concentration–response curves of cromakalim, pinacidil and nifedipine for inhibition of catecholamine secretion induced by 31 mM K^+. Cells were stimulated with 31 mM K^+ for 10 min in the absence or presence of various concentrations of cromakalim (●), pinacidil (□) or nifedipine (■) as described in Materials and Methods. Catecholamine (CA) secretion induced by 31 mM K^+ was 6.2 ± 0.8% of the total cellular CA content and was expressed as 100%. Points and bars show 31 mM K^+-induced CA secretion in the presence of various concentrations of drugs as means ± SD for 3–4 experiments.

5% of the total cellular ^{86}Rb. Figure 1(b) shows the concentration-dependent curves of the effects of cromakalim and pinacidil on ^{86}Rb efflux from the cells in 10 min. Both cromakalim and pinacidil had stimulatory effects at concentrations of 1 μM or more and at 100 μM, caused an increase of about 7% in ^{86}Rb efflux.

Effects of cromakalim and pinacidil on catecholamine secretion induced by depolarization by high concentrations of K^+

Figure 2(a) shows the effects of cromakalim (100 μM) and pinacidil (100 μM) on catecholamine secretion induced by stimulation with various concentrations of K^+. Both cromakalim and pinacidil significantly inhibited catecholamine secretion induced by stimulation with 31 mM K^+, but not that induced by stimulation with 56 mM K^+. The effect of nifedipine, a Ca^{2+} channel blocker, was also studied for comparison. Nifedipine (100 μM) inhibited catecholamine secretion induced by 31 mM K^+ and also by 56 mM K^+, and its inhibitory effect was greater than those of the K^+ channel openers. Figure 2(b) shows the concentration-dependent curves of cromakalim, pinacidil and nifedipine for inhibition of catecholamine secretion induced by stimulation with 31 mM K^+. Inhibitory effects of both cromakalim and pinacidil were observed at concentrations of 1 μM or more and at 100 μM, they caused approximately 30–40% inhibition. The inhibitory effects of both drugs were less than that of nifedipine.

Effects of cromakalim and pinacidil on $^{45}Ca^{2+}$ *influx and increase in* $[Ca^{2+}]i$ *induced by stimulation with 31 mM* K^+

To assess the inhibitory effects of the two K^+ channel openers on catecholamine secretion induced by stimulation with 31 mM K^+, we examined their effects on $^{45}Ca^{2+}$ influx and increase in $[Ca^{2+}]i$ induced by 31 mM K^+, which are essential for the initiation of catecholamine secretion. As shown in Fig. 3(a), $^{45}Ca^{2+}$ influx induced by 31 mM K^+ was inhibited by both cromakalim and pinacidil in a concentration-dependent manner. The concentration–inhibition curve was similar to that for inhibition of catecholamine secretion. The inhibitory effects of these K^+ channel openers on $^{45}Ca^{2+}$ influx were also less than that of nifedipine. Figure 3(b) shows the effects of two K^+ channel openers on 31 mM K^+-induced increase in $[Ca^{2+}]i$, measured using the Ca^{2+} indicator fura-2. When individual chromaffin cells were stimulated with 31 mM K^+, $[Ca^{2+}]i$ rapidly increased and then slowly decreased during 5 min. Both cromakalim and pinacidil slightly increased $[Ca^{2+}]i$, but inhibited the increase in $[Ca^{2+}]i$ induced by 31 mM K^+.

Effects of cromakalim and pinacidil on catecholamine secretion induced by carbamylcholine

Figure 4(a) shows the effects of cromakalim (100 μM) and pinacidil (100 μM) on catecholamine secretion induced by various concentrations of carbamylcholine. Both drugs inhibited catecholamine secretion induced by carbamylcholine. Their inhibitory effects were not overcome by increasing the concentration of carbamylcholine. Nifedipine (100 μM) also strongly inhibited catecholamine secretion induced by various concentrations of carbamylcholine. Figure 4(b) shows the concentration–response curves for the inhibitory effects of both cromakalim and pinacidil on catecholamine secretion induced by carbamylcholine (100 μM).

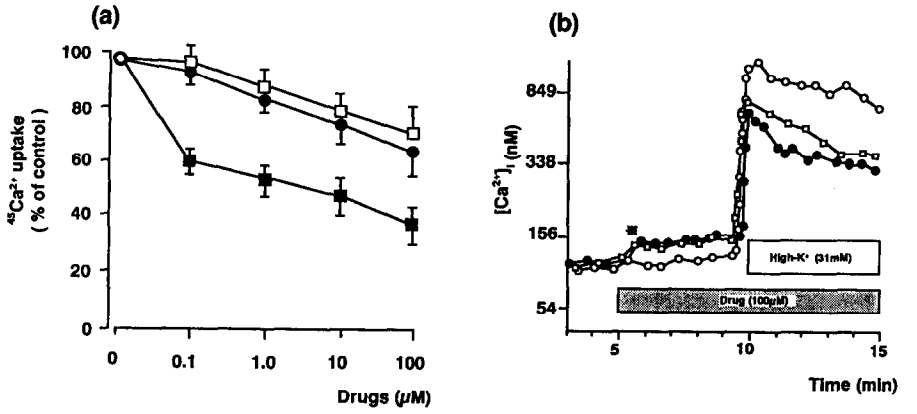

Fig. 3. (a) Concentration–response curves of cromakalim, pinacidil and nifedipine for inhibition of $^{45}Ca^{2+}$ influx induced by 31 mM K^+. Cells were stimulated for 10 min with 31 mM K^+ containing $^{45}CaCl_2$ in the absence or presence of various concentrations of cromakalim (●), pinacidil (□) or nifedipine (■) as described in Materials and Methods. $^{45}Ca^{2+}$ influx induced by 31 mM K^+ was 0.61 ± 0.4 nmoles/well and was expressed as 100%. Points and bars show 31 mM K^+-induced $^{45}Ca^{2+}$ influx in the presence of various concentrations of drugs as means ± SD for 3–4 experiments. (b) Inhibitory effects of cromakalim and pinacidil on the increase in $[Ca^{2+}]i$ induced by 31 mM K^+. Cells, which were maintained on cover glasses and preloaded with the Ca^{2+} indicator fura-2, were stimulated with 31 mM K^+ in the absence (○) or presence of cromakalim (100 μM, ●) or pinacidil (100 μM, □) as described in Materials and Methods. Drugs and 31 mM K^+ were applied as shown by the horizontal bars. Measurements of $[Ca^{2+}]i$ were done on single cells.

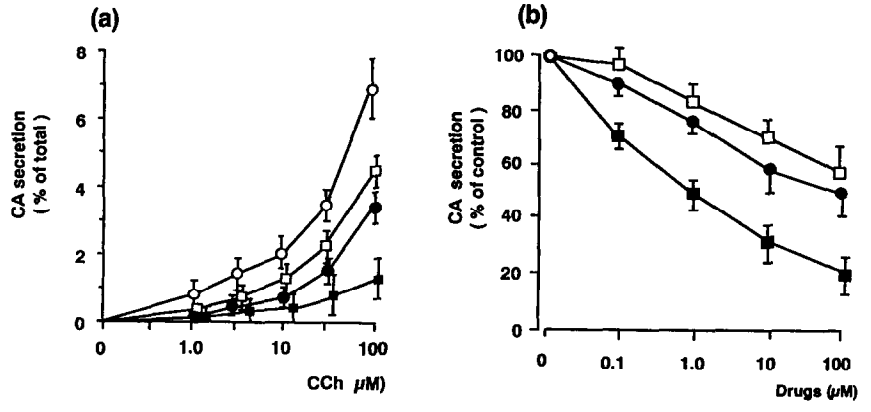

Fig. 4. (a) Effects of cromakalim, pinacidil and nifedipine on catecholamine secretion induced by various concentrations of carbamylcholine. Cells were stimulated with various concentrations of carbamylcholine for 10 min in the absence (○) or presence of cromakalim (100 μM, ●), pinacidil (100 μM, □) or nifedipine (100 μM, ■) as described in Materials and Methods. Catecholamine (CA) secretion is shown as a percentage of the total CA in the cells. Points and bars are means ± SD for 3–4 experiments. (b) Concentration–response curves of cromakalim, pinacidil and nifedipine for the inhibition of catecholamine secretion induced by carbamylcholine. Cells were stimulated with carbamylcholine (100 μM) in the absence or presence of various concentrations of cromakalim (●), pinacidil (□) or nifedipine (■) as described in Materials and Methods. Catecholamine (CA) secretion induced by carbamylcholine (100 μM) was 6.8 ± 0.8% of the total cellular CA content and was expressed as 100%. Points and bars show carbamylcholine-induced CA secretion in the presence of various concentrations of drugs as means ± SD for 3–4 experiments.

Both compounds were inhibitory at concentrations of 1 μM or more and caused 30–40% inhibition at 100 μM. The inhibitory effects of these K^+ channel openers were less than that of nifedipine.

Effects of cromakalim and pinacidil on $^{45}Ca^{2+}$ influx and increase in $[Ca^{2+}]i$ induced by carbamylcholine

In bovine adrenal chromaffin cells, stimulation of nicotinic ACh receptors, but not muscarinic

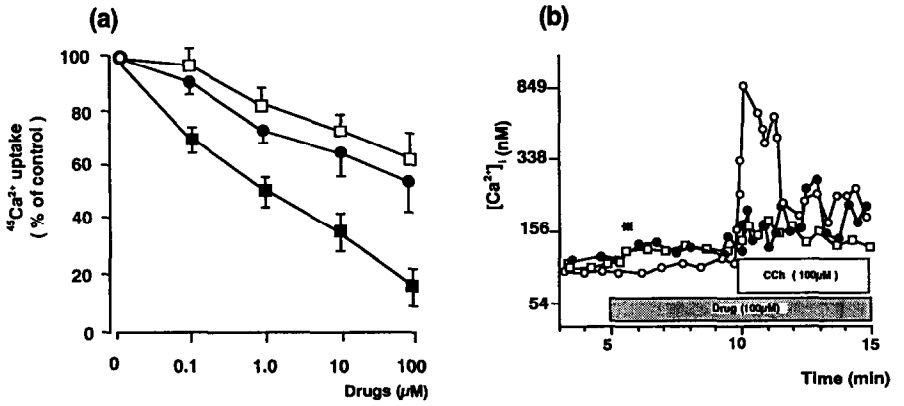

Fig. 5. (a) Concentration–response curves of cromakalim, pinacidil and nifedipine for the inhibition of $^{45}Ca^{2+}$ influx induced by carbamylcholine. Cells were stimulated for 10 min with carbamylcholine in BSS containing $^{45}CaCl_2$ in the absence or presence of various concentrations of cromakalim (●), pinacidil (□) or nifedipine (■) as described in Materials and Methods. $^{45}Ca^{2+}$ influx induced by carbamylcholine (100 μM) was 0.78 ± 0.6 nmoles/well and was expressed as 100%. Points and bars show carbamylcholine-induced $^{45}Ca^{2+}$ influx in the presence of various concentrations of drugs as means ± SD for 3–4 experiments. (b) Inhibitory effects of cromakalim and pinacidil on increase in $[Ca^{2+}]i$ induced by carbamylcholine. Cells, which were maintained on cover glasses and preloaded with the Ca^{2+} indicator fura-2, were stimulated with carbamylcholine (100 μM) in the absence (○) or presence of cromakalim (100 μM, ●) or pinacidil (100 μM, □) as described in Materials and Methods. Drugs and carbamylcholine (CCh) were applied as shown by the horizontal bars. Measurements of $[Ca^{2+}]i$ were done on single cells.

ACh receptors causes catecholamine secretion by increasing Ca^{2+} influx mainly through voltage-dependent Ca^{2+} channels [19]. Therefore, the effects of the K^+ channel openers on Ca^{2+} influx and increase in $[Ca^{2+}]i$ induced by carbamylcholine were examined. As shown in Fig. 5(a), both cromakalim and pinacidil inhibited $^{45}Ca^{2+}$ influx induced by carbamylcholine in a concentration-dependent manner, the concentration–inhibition curves being similar to those for inhibition of catecholamine secretion. Inhibitory effect of nifedipine on $^{45}Ca^{2+}$ influx was also more than those of K^+ channel openers. Figure 5(b) shows the effect of the K^+ channel openers (100 μM) on increase in $[Ca^{2+}]i$ induced by stimulation with carbamylcholine. Carbamylcholine increased $[Ca^{2+}]i$ rapidly and transiently, and this increase was significantly inhibited by both cromakalim (100 μM) and pinacidil (100 μM).

On the other hand, as shown in Fig. 6, both K^+ channel openers (100 μM) also inhibited the increase in $[Ca^{2+}]i$ induced by carbamylcholine in the absence of extracellular Ca^{2+}, which is thought to be mediated by muscarinic ACh receptors. The inhibitory effect of cromakalim was more than that of pinacidil. Although in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is not responsible for the secretion of catecholamine, it is interesting that cromakalim and pinacidil have inhibitory effects on Ca^{2+} mobilization from intracellular pools in response to muscarinic ACh receptor stimulation.

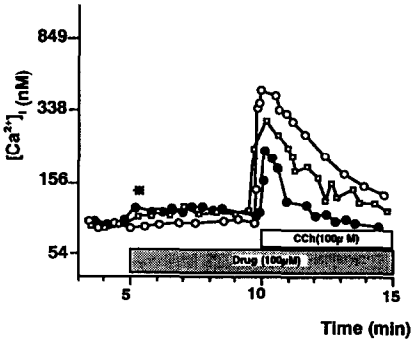

Fig. 6. Effects of cromakalim and pinacidil on increase in $[Ca^{2+}]i$ caused by carbamylcholine in the absence of extracellular Ca^{2+}. Cells, which were maintained on cover glasses and preloaded with the Ca^{2+} indicator fura-2, were stimulated with carbamylcholine (100 μM) in Ca^{2+}-free BSS in the absence (○) or presence of cromakalim (100 μM, ●) or pinacidil (100 μM, ○) as described in Materials and Methods. Drugs and carbamylcholine (CCh) were applied as shown by the horizontal bars. Measurements of $[Ca^{2+}]i$ were done on single cells.

Effects of cromakalim and pinacidil on catecholamine secretion induced by Bay-K 8644, Ba^{2+} *and* A23187

The effects of cromakalim and pinacidil on catecholamine secretion induced by Bay-K 8644, an opener of L-type voltage-dependent Ca^{2+} channels [20], and also that induced by Ba^{2+}, which is known to penetrate into the cells through voltage-dependent Ca^{2+} channels and stimulate catecholamine secretion [21, 22], were examined. As shown in Table 1, neither cromakalim nor pinacidil affected catecholamine secretion induced by these secretagogues. Furthermore, these drugs did not affect cate-

Table 1. Effects of cromakalim and pinacidil on catecholamine secretion induced by various secretagogues

	Cromakalim	CA secretion (% of total)	Pinacidil	CA secretion (% of total)
Bay-K 8644	(−)	8.4 ± 0.8	(−)	8.3 ± 0.7
	(+)	9.9 ± 1.1	(+)	9.3 ± 0.6
Ba^{2+}	(−)	33.5 ± 2.6	(−)	32.5 ± 3.6
	(+)	30.6 ± 1.0	(+)	31.2 ± 2.6
A 23187	(−)	8.7 ± 0.8	(−)	8.5 ± 1.2
	(+)	9.5 ± 1.4	(+)	8.9 ± 1.3

Cells were stimulated with Bay-K 8644 (100 μM), Ba^{2+} (500 μM) or A23187 (10 μM) for 30 min in the absence or presence of K^+ channel openers (100 μM) as described in Materials and Methods. Catecholamine (CA) secretion is shown as a percentage of the total cellular CA content.

Values are means ± SD for 3–4 experiments.

Table 2. Effects of cromakalim and pinacidil on catecholamine secretion induced by histamine or bradykinin

	Cromakalim	CA secretion (% of total)	Pinacidil	CA secretion (% of total)
Histamine	(−)	8.2 ± 1.4	(−)	8.3 ± 0.8
	(+)	7.5 ± 1.3	(+)	8.0 ± 1.4
Bradykinin	(−)	2.4 ± 0.8	(−)	2.8 ± 1.2
	(+)	3.2 ± 0.4	(+)	2.6 ± 0.7

Cells were stimulated with histamine (100 μM) or bradykinin (20 μM) for 40 min in the absence or presence of K^+ channel openers (100 μM) as described in Materials and Methods. Catecholamine (CA) secretion is shown as a percentage of the total cellular CA content.

Values are means ± SD for 3–4 experiments.

cholamine secretion induced by the Ca^{2+} ionophore A23187.

Effects of cromakalim and pinacidil on catecholamine secretion induced by histamine and bradykinin

Histamine [23] and bradykinin [24] are reported to stimulate catecholamine secretion from cultured bovine adrenal chromaffin cells by increasing Ca^{2+} influx largely through voltage-independent pathway and that the time courses and magnitudes of their effects are quite different from those of the effects of high K^+ or carbamylcholine. Therefore, the effects of cromakalim and pinacidil on catecholamine secretions induced by histamine and bradykinin were studied. As shown in Table 2, neither K^+ channel openers affected the catecholamine secretions induced by histamine and bradykinin.

DISCUSSION

The purposes of this study were to examine the effects of the K^+ channel openers cromakalim and pinacidil on voltage-dependent and receptor-mediated catecholamine secretion and Ca^{2+} mobilization in cultured bovine adrenal chromaffin cells, and to determine how much membrane K^+ channels are involved in the regulation of the Ca^{2+}-dependent secretory process. Since cultured bovine adrenal chromaffin cells are thought to be a neuronal model [10–13], the results obtained here provide indications of a role for membrane K^+ channels in regulation of neurotransmitter release in the nervous system, and also of additional pharmacological effects of cromakalim and pinacidil apart from those on K^+ channels.

In an experiment using ^{86}Rb as a monitor of K^+ permeability [17, 18], we found that the K^+ channel openers cromakalim (1 μM–100 μM) and pinacidil (1 μM–100 μM) stimulated ^{86}Rb efflux from the preloaded cultured bovine adrenal chromaffin cells to similar extents. This finding indicates that these K^+ channel openers affected membrane K^+ channels, resulting in an increase in K^+ efflux from these cells. Electrophysiological studies using patch-clamp techniques, have shown that many different kind of K^+ channels are present in various cells [1–3]. In adrenal chromaffin cells, voltage- and Ca^{2+}-dependent K^+ channels have been studied [25–28]. It has been demonstrated in other cells that the sites of action of K^+ channel openers are ATP-sensitive K^+ channels, which are inhibited by the antidiabetic drug glibenclamide [3, 29]. However, in the cultured bovine adrenal chromaffin cells used here, it was uncertain which type of K^+ channels were affected by cromakalim and pinacidil, since glibenclamide did not have a clear inhibitory effect on cromakalim- or pinacidil-stimulated ^{86}Rb efflux (data not shown).

Cromakalim and pinacidil did not affect catecholamine secretion induced by excessive depolarization with 56 mM K^+, but inhibited the catecholamine secretion induced by moderate depolarization with 31 mM K^+ in a concentration-dependent manner (1 μM–100 μM). These drugs also inhibited $^{45}Ca^{2+}$ influx and increase in $[Ca^{2+}]i$ induced by 31 mM K^+ and the concentration–response curve was similar to that for inhibition of catecholamine secretion. These inhibitory effects of the K^+ channel openers were less than those of the Ca^{2+} channel blocker nifedipine, which inhibited catecholamine secretion and $^{45}Ca^{2+}$ influx induced by both 56 mM K^+ and 31 mM K^+. The findings that cromakalim and pinacidil inhibited catecholamine secretion and $^{45}Ca^{2+}$ influx induced by moderate depolarization, but not that induced by excessive depolarization, suggests that in contrast to Ca^{2+} channel blockers [30], which have direct actions on voltage-dependent Ca^{2+} channels, those K^+ channel openers inhibit the voltage-dependent Ca^{2+} channels indirectly through opening of K^+ channels.

Furthermore, both cromakalim and pinacidil inhibited catecholamine secretion, $^{45}Ca^{2+}$ influx and increase in $[Ca^{2+}]i$ induced by stimulation of ACh receptors with carbamylcholine. These inhibitory effects of the drugs were not overcome by increasing the concentration of carbamylcholine, suggesting that the cromakalim and pinacidil did not compete with carbamylcholine at the ACh receptors. In bovine adrenal chromaffin cells, stimulation of nicotinic, but not muscarinic ACh receptors is known to cause catecholamine secretion by increasing Ca^{2+} influx largely through voltage-dependent Ca^{2+} channels [11, 12]. Therefore, it seems that K^+ channel openers inhibit catecholamine secretion induced by carbamylcholine by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors with carbamylcholine.

We also found that cromakalim and pinacidil inhibited the increase in $[Ca^{2+}]i$ induced by carbamylcholine in the absence of extracellular Ca^{2+}. This indicates that the K^+ channel openers have an inhibitory effect on the release of Ca^{2+} from intracellular pools induced by stimulation of muscarinic ACh receptors, which is not responsible for the secretion of catecholamine. In adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide metabolism (the so-called PI response), resulting in the formation of inositol 1,4,5-triphosphate, which induces the mobilization of Ca^{2+} from intracellular pools [31, 32]. However, at present, it is uncertain whether the inhibitory effect of K^+ channel openers on Ca^{2+} movement from intracellular pools is due to their direct effect on the PI response or an indirect effect as a result of the membrane hyperpolarization induced by opening of K^+ channels. Anyway, it is of interest that cromakalim and pinacidil inhibit not only influx of Ca^{2+} through voltage-dependent Ca^{2+} channel, but also mobilization of Ca^{2+} from intracellular pools induced by ACh receptors stimulation.

Cromakalim and pinacidil did not affect the secretion of catecholamine from the cells induced by Bay-K 8644 [20] or Ba^{2+} [21, 22], suggesting that they did not inhibit influx of Ca^{2+} induced by an opener of L-type voltage-sensitive Ca^{2+} channels such as Bay-K 8644, or influx of Ba^{2+}, which is thought to pass through voltage-sensitive Ca^{2+} channels and stimulate catecholamine secretion. Cromakalim and pinacidil also did not affect catecholamine secretion induced by the Ca^{2+} ionophore A23187, indicating that they themselves or the opening of membrane K^+ channels (hyperpolarization of the cells) did not interfere with the intracellular Ca^{2+} dependent secretory mechanism.

Furthermore, cromakalim and pinacidil did not affect the catecholamine secretion induced by histamine or bradykinin, both of which are reported to stimulate the secretion by increasing Ca^{2+} entry mainly through voltage-independent membrane channels [23, 24]. These results indicated that the K^+ channel openers did not affect Ca^{2+} influx through the voltage-independent pathway and therefore, that their inhibitory effect was restricted to Ca^{2+} influx through voltage-dependent channels activated by mild depolarization or nicotinic ACh receptor stimulation.

In conclusion, the present study using cultured bovine adrenal chromaffin cells demonstrated that the K^+ channel openers, cromakalim and pinacidil, increase the permeability of membrane K^+ channels, resulting in inhibition of Ca^{2+} influx through voltage-dependent membrane channels induced by moderate depolarization or nicotinic ACh receptor stimulation and thus inhibition of catecholamine secretion. Furthermore, results showed that cromakalim and pinacidil also inhibited Ca^{2+} mobilization from intracellular pools induced by muscarinic ACh receptor stimulation. These effects may contribute, in part, to the hypotensive effects of these K^+ channel openers, through inhibition of catecholamine secretion from adrenal medullary chromaffin cells and consequent reduction of the catecholamine level in the circulation.

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REFERENCES

1. Petersen OH and Maruyama Y, Calcium-activated potassium channels and their role in secretion. *Nature* **307**: 693–696, 1984.
2. Cook NS, The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol Sci* **9**: 21–28, 1988.
3. Watson S and Abbott A, Receptor Nomenclature Supplement. *Trends Pharmacol Sci* (Suppl); 31–33, 1991.
4. Uceda G, Artalejo AR, Lopez MG, Abad F, Neher E and Garcia AG, Ca^{2+}-activated K^+ channels modulate muscarinic secretion in cat chromaffin cells. *J Physiol* **454**: 213–230, 1992.
5. Attwood MR, Jones PS, Kay PB, Paciorek PM and Redshaw S, The design of a novel class of potassium

channel activating drugs, 2-(2,2-dimethylbenzopyran-4-yl)-pyridine-1-oxides. *Life Sci* **48**: 803–810, 1991.
6. Edwards G and Weston AH, Structure-activity relationships of K^+ channel openers. *Trends Pharmacol Sci* **11**: 417–422, 1990.
7. Petersen OH, New roles for ATP-sensitive K^+ channels in controlling blood flow and neurotransmitter secretion. *NIPS* **5**: 226, 1990.
8. Nakashima M, Li Y, Seki N and Kuriyama H, Pinacidil inhibits neuromuscular transmission indirectly in the guinea-pig and rabbit mesenteric arteries. *Br J Pharmacol* **101**: 581–586, 1990.
9. Soares-da-Silva P and Fernandes MH, Inhibition by the putative potassium channel opener pinacidil of the electrically-evoked release of endogenous dopamine and noradrenaline in the rat vas deferens. *Naunyn–Schmiedeberg's Arch Pharmacol* **342**: 415–421, 1990.
10. Douglas WW, Stimulus–secretion coupling: the concept and clues from chromaffin and other cells. *Br J Pharmacol* **34**: 451–474, 1968.
11. Burgoyne RD, Mechanisms of secretion from adrenal chromaffin cells. *Biochim Biophys Acta* **779**: 201–216, 1984.
12. Oka M, Isosaki M and Yanagihara N, Isolated bovine adrenal medullary cells: studies on regulation of catecholamine synthesis and release. In: *Catecholamines: Basic and Clinical Frontiers* (Eds. Usdin E, Kopin IJ and Brachas J), pp. 70–72. Pergamon Press, Oxford, 1979.
13. Livett BG, Adrenal medullary chromaffin cells *in vitro*. *Physiol Rev* **64**: 1103–1161, 1984.
14. Nakanishi A, Yoshizumi M, Hamano S, Morita K and Oka M, Myosin light-chain kinase inhibitor, 1-(5-chlornaphthalene - 1 - sulfonyl) - 1H - hexahydro - 1,4 - diazepine (ML-9), inhibits catecholamine secretion from adrenal chromaffin cells by inhibiting Ca^{2+} uptake into the cells. *Biochem Pharmacol* **38**: 2615–2619, 1989.
15. Yoshizumi M, Houchi H, Ishimura Y, Masuda Y, Morita K and Oka M, Mechanism of palytoxin-induced Na^+ influx into cultured bovine adrenal chromaffin cells: possible involvement of Na^+/H^+ exchange system. *Neurosci Lett* **130**: 103–106, 1991.
16. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
17. Wada A, Kobayashi H, Arita M, Yanagihara N and Izumi F, Potassium channels in cultured bovine adrenal chromaffin cells: effects of high K, veratridine and carbachol on ^{86}Rubidium efflux. *Neuroscience* **22**: 1085–1092, 1987.
18. Karlsson S and Ahren B, Effects of tacrine on insulin secretion and $^{86}Rb^+$ and $^{45}Ca^{2+}$ efflux from rat pancreatic islets. *J Pharmacol Exp Ther* **263**: 494–498, 1992.
19. Misbahuddin M and Oka M, Muscarinic stimulation of guinea pig adrenal chromaffin cells stimulates catecholamine secretion without significant increase in Ca^{2+} uptake. *Neurosci Lett* **87**: 266–270, 1988.
20. Garcia AG, Sala F, Reig JA, Viniegra S, Frias J, Fonteriz R and Gandia L, Dihydropyridine BAY-K-8644 activates chromaffin cell calcium channels. *Nature* **309**: 69–75, 1984.
21. TerBush DR and Holz RW, Barium and calcium stimulate secretion from digitonin-permeabilized bovine adrenal chromaffin cells by similar pathways. *J Neurochem* **58**: 680–687, 1992.
22. Heldman E, Levine M, Raveh L and Pollard HB, Barium ions enter chromaffin cells via voltage-dependent calcium channels and induce secretion by a mechanism independent of calcium. *J Biol Chem* **264**: 7914–7920, 1989.
23. Bunn SJ and Boyd TL, Characterization of histamine-induced catecholamine secretion from bovine adrenal chromaffin cells. *J Neurochem* **58**: 1602–1610, 1992.
24. Owen PJ, Plevin R and Boarder MR, Characterization of bradykinin-stimulated release of noradrenaline from cultured bovine adrenal chromaffin cells. *J Pharmacol Exp Ther* **248**: 1231–1236, 1989.
25. Marty A, Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* **291**: 497–500, 1981.
26. Marty A and Neher E, Potassium channels in cultured bovine adrenal chromaffin cells. *J Physiol* **367**: 117–141, 1985.
27. Artalejo AR, Garcia AG and Neher E, Small-conductance Ca^{2+}-activated K^+ channels in bovine chromaffin cells. *Pflugers Arch* **423**: 97–103, 1993.
28. Neely A and Lingle CJ, Two components of calcium-activated potassium current in rat adrenal chromaffin cells. *J Physiol* **453**: 97–131, 1992.
29. Schmid-Antomarchi H, Amoroso S, Fosset M and Lazdunski M, K^+ channel openers activate brain sulfonylurea-sensitive K^+ channels and block neurosecretion. *Proc Natl Acad Sci USA* **87**: 3489–3492, 1990.
30. Cena V, Nicolas GP, Sanchez-Garcia P, Kirpekar SM and Garcia AG, Pharmacological dissection of receptor-associated and voltage-sensitive ionic channels involved in catecholamine release. *Neuroscience* **10**: 1455–1462, 1983.
31. Cheek TR, O'Sullivan AJ, Moreton RB, Berridge MJ and Burgoyne RD, Spatial localization of the stimulus-induced rise in cytosolic Ca^{2+} in bovine adrenal chromaffin cells: Distinct nicotinic and muscarinic patterns. *FEBS Lett* **247**: 429–434, 1989.
32. Challiss RAJ, Jones JA, Owen PJ and Boarder MR, Changes in inositol 1,4,5-triphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulations in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J Neurochem* **56**: 1083–1086, 1991.