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EFFECTS OF K^+ CHANNEL AGONISTS CROMAKALIM AND PINACIDIL ON RAT BASILAR ARTERY SMOOTH MUSCLE CELLS ARE MEDIATED BY CA^{++}-ACTIVATED K^+ CHANNELS

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Whole-cell and cell-free inside-out patch-clamp recording techniques were used to examine the actions of potassium channel openers pinacidil and cromakalim in enzymatically isolated smooth muscle cells of rat basilar artery. Delayed rectifier and calcium-dependent potassium currents were identified from the whole-cell recordings. Only the calcium-dependent potassium current was increased by cromakalim and pinacidil. Recordings from inside-out membrane patches revealed a large conductance voltage- and calcium-dependent potassium channel, which was blocked by charybdotoxin but unaffected by ATP < 10 mM. Cromakalim and pinacidil increased the open probability of this channel. On the basis of these results, we suggest that such drugs, acting on cerebral arterial smooth muscle cell potassium channels, may be of some benefit in the treatment of cerebral vasospasm following subarachnoid hemorrhage.

Pinacidil and cromakalim are vasorelaxant agents directly acting through an activation of potassium channels in cardiac myocytes (5, 12, 16, 17) and smooth muscle cells from the aorta (4, 6, 15), mesenteric artery (14, 20, 24) and veins (1, 2, 11). To our knowledge, this is the first report of the effects of these drugs on potassium currents in single smooth muscle cells of cerebral arteries. This study was undertaken to assess the potential for the use of these drugs in the treatment or prophylaxis of cerebral vasospasm after subarachnoid hemorrhage. Although pinacidil has been shown to be less effective at relaxing canine cerebral arteries than coronary, renal and mesenteric arteries in recordings of isometric tension (23), other studies have been encouraging. Nicorandil was found to reverse canine cerebral vasospasm (8) and cromakalim improves rabbit brain blood flow (3, 10).

This paper describes the specific ionic current and channel of rat basilar artery smooth muscle cells affected by cromakalim and pinacidil.

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MATERIALS AND METHODS

Isolation of rat basilar artery vascular smooth muscle cells. Cells were obtained from the basilar artery of Sprague-Dawley rats using mechanical dissociation, collagenase (Type II), elastase and hyaluronidase as described previously (21). Cells were plated on glass cover slips and stored at 4°C in saline containing $CaCl_2$ (0.8 mM) and essentially fatty-acid-free bovine serum albumin (2g/l).

Whole-cell patch-clamp technique. Whole-cell currents were recorded using standard techniques (7) and an Axopatch 1C patch clamp amplifier (Axon Instruments). The patch pipettes had tip resistances of 1-4 MΩ and seal resistances of over 1 GΩ were obtained routinely. Series resistance compensation was not employed. Under whole-cell patch-clamp conditions, the cells had input resistances of 5-10 GΩ and capacitances of 20-30 pF. The membrane potential and current signals were stored on a laboratory computer. Leakage conductance was determined by applying small, hyperpolarizing voltage steps. None of the records shown was leakage corrected. The normal bath solution for the whole-cell recordings was (mM): NaCl 130, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8 (for recording the calcium-activated potassium current) or 0 (for recording the delayed rectifier potassium current), HEPES 10, glucose 5.2 and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with (mM): KCl 139, $MgCl_2$ 0.5, $CaCl_2$ 0.1 (for the calcium-dependent potassium current) or 0 (for the delayed rectifier potassium current), EGTA 0.09 (for the calcium-dependent potassium current) or EDTA 10 (for the delayed rectifier potassium current), HEPES 10, glucose 10 and the pH was adjusted to 7.4 with KOH. Bath solution changes were made with a syringe pump (Sage model 351) modified for simultaneous withdrawal and injection. All experiments were done at room temperature (19-22°C).

Single channel recording. Single channel current recordings were conducted at room temperature in the inside-out configuration (15). The bath (intracellular) solution had the following composition (mM): KCl 140, $CaCl_2$ 0.008, $MgCl_2$ 0.5, HEPES 10, EGTA 0.09, glucose 10 and the pH was adjusted to 7.4 with KOH. The pipette medium (extracellular solution) contained (mM): KCl 140, $CaCl_2$ 0.1, $MgCl_2$ 0.5, HEPES 10, glucose 10, pH 7.4 with KOH. Single channel recordings were made with the Axopatch 1C amplifier and stored digitally on video tape (Neurocorder model 384 digitizer and Sony SL-700 VCR). On playback, data was redigitized at 10 kHz with a DT-2841-F (Data Translation) analog-digital converter on an AT-compatible computer. Amplitude histograms were made from contiguous segments of data 5 min long. Channel openings were detected as crossings of 50% or 150% thresholds between histogram peaks.

Separation of currents. With the low calcium pipette and bath solutions, a whole-cell current of a few hundred pA was obtained. This current activated at potentials above -30 mV with sigmoidal onset kinetics, showed no inactivation over hundreds of ms, and was blocked by procaine (1 mM) or strychnine (0.5 mM). These are characteristics of the delayed rectifier current in many other preparations. In the high calcium solutions, the whole-cell current was usually several nA, activated between -50 and -30 mV, displayed less-sigmoidal kinetics and was blocked by TEA^+ (1 mM) but not by procaine or strychnine. A charybdotoxin-sensitive, voltage- and calcium-dependent 220 pS potassium channel was found in cell-free membrane patches and is believed to account for the large current found in the whole-cell recordings. These are properties of the calcium-activated potassium current.

A small inwardly rectifying potassium conductance is visible in some records. Because of the variable nature of its appearance, the effect of potassium channel agonists on this component was not studied.

Drugs. Drugs were pinacidil (Leo) and cromakalim (Beecham). All other drugs and the agents used in cell preparation were purchased from Sigma. Stock solutions of cromakalim and pinacidil (10 mM) were made up in 95% ethanol. Control experiments established that the vehicle was not responsible for changes in potassium currents that were observed.

RESULTS

Agonists and the delayed rectifier current. Neither pinacidil (100-250 μM; n = 7; Fig 1A) nor cromakalim (50-150 μM; n = 4; Fig 1B) exerted any effect on the amplitude or the steady-state activation of the delayed rectifier current.

Agonists and the calcium-activated potassium current. Application of pinacidil (100-200 μM; n = 5; Fig 1C) or cromakalim (100 μM; n = 4; Fig 1D) to the external solution increased the amplitude of the outward potassium current in the high calcium solutions. In cells exposed to pinacidil, the increase was 50-400% at +60 mV and the increases were reversed upon washout in each of these experiments. Cromakalim (100 μM) roughly doubled the peak current; washout was

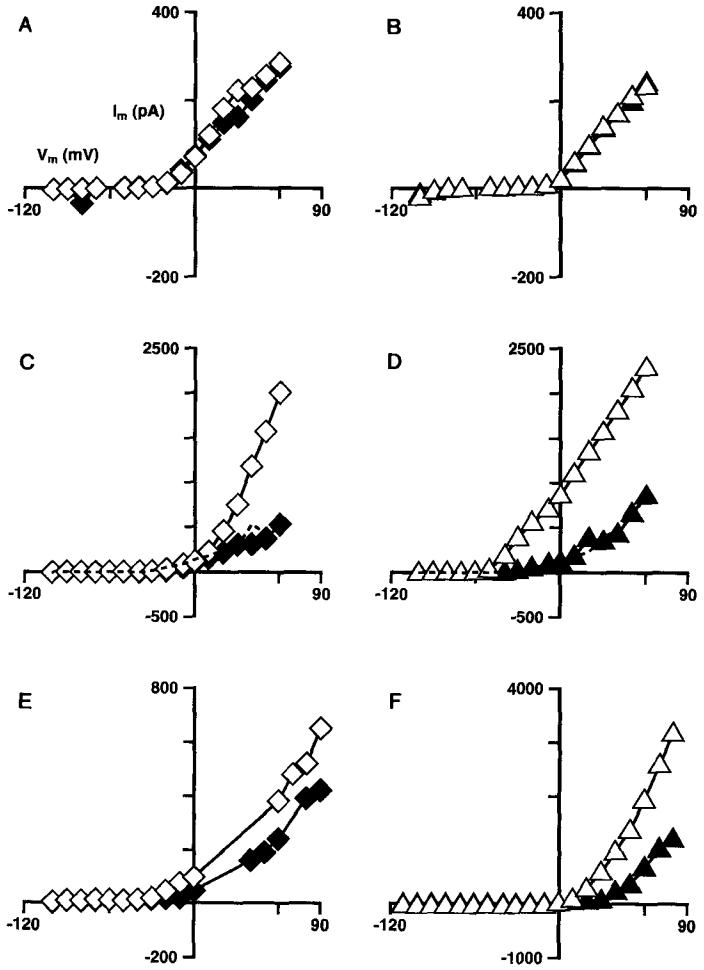

Fig 1. Peak current-voltage plots from whole-cell recordings of rat basilar artery smooth muscle cells. Filled symbols are control curves. ◇ = pinacidil, △ = cromakalim, dashed line = washout. **A, B.** Low Ca^{2+} pipette solution. **A.** [pinacidil] = 200 μM. **B.** [cromakalim] = 100 μM. **C, D.** High Ca^{2+} pipette solution. **C.** [pinacidil] = 200 μM. **D.** [cromakalim] = 100 μM. **E, F.** High Ca^{2+} pipette solution + procaine 1 mM. **E.** [pinacidil] = 100 μM. **F.** [cromakalim] = 100 μM.

successful in two experiments, unsuccessful in one and unattainable because of loss of seal integrity in a fourth.

Potassium channel agonists and procaine. To exclude contamination of the calcium-activated potassium current by the delayed rectifier, 1 mM procaine was used in the bath solution in 3 additional experiments. Pinacidil (100 μM; n = 1; Fig 1E) and cromakalim (100-200 μM; n = 2; Fig 1F) produced increased currents as before. In all 3 of these cells, there was a reversal of effects upon washout.

Single channel recordings. Cromakalim or pinacidil might increase the calcium-activated potassium current by producing a rise in intracellular calcium which was somehow uncompensated by the EGTA in the pipette solution. To control for this possibility, current recordings were made from cell-free membrane patches. In inside-out membrane patches, calcium-activated potassium currents were easily identified by their large conductance, high sensitivity to the cytoplasmic calcium concentration and voltage-dependence. At potentials positive to 0 mV, calcium-activated potassium currents were recorded as downward deflections. The slope of the current-voltage plot was 220 pS and channel open probability was steeply voltage-dependent.

All such patches contained multiple potassium channels. The probability of more than one channel opening simultaneously was reduced by using a low free calcium concentration (estimated to be 1.5 nM) and by holding the patch at about +60 mV. In general, the number of channels in a given patch could not be determined with certainty, so experiments were done in which each patch served as its own control. The average number of channels open was determined from amplitude histograms (Fig 2A, B) based on 5 min data sampled at 10 kHz. This value was expressed as P_x (reflecting the uncertainty about the number of channels in a given patch); a value of P_x of 1% indicates that an average of 0.01 channels were open throughout the 5 minute sample period.

Pinacidil (n = 4; Fig 2A, C) and cromakalim (n = 3; Fig 2B, D) increased the channel open-state probability in a dose-dependent manner. These drugs did not alter channel conductance nor its reversal potential. The increase produced by 200 μM pinacidil was from 2-fold to 5-fold. The largest increases of P_x were produced with cromakalim at 250-300 μM: 1.7- to 7.9-fold. At lower concentrations of 50-200 μM, cromakalim exerted no significant effects.

DISCUSSION

A few previous studies have been directed at the actions of potassium channel openers on intact cerebral vessels, using intracellular recording (8), microsphere measurements of cerebral blood flow (10), or isometric tension recording (23). To our knowledge, the present study is the first report of ionic currents affected by cromakalim or pinacidil in smooth muscle cells of cerebral arteries.

The delayed rectifier and calcium-dependent potassium currents were identified from whole-cell recordings of isolated rat basilar artery smooth muscle cells. Inward currents in these cells were much smaller than outward currents and were suppressed by recording in a low calcium medium.

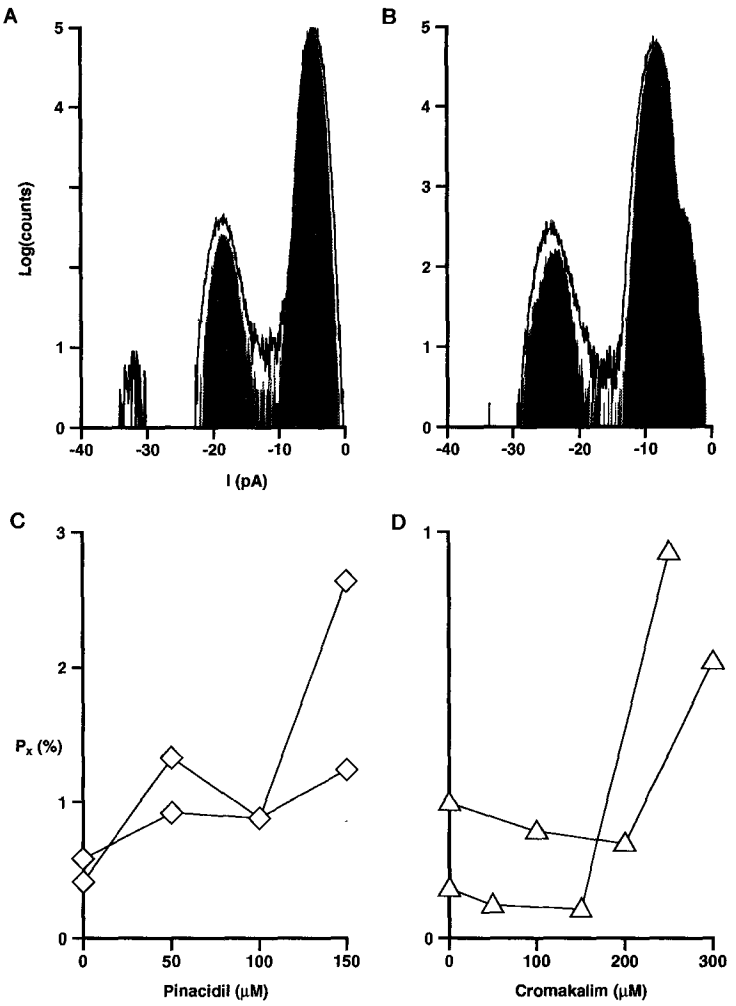

Fig 2. Large conductance K_{Ca} channels studied in inside-out membrane patches. **A, B.** Histograms from $3x10^6$ current samples at 10 kHz. Filled area is control; unfilled area is response to pinacidil (**A**, 200 μM) or cromakalim (**B**, 300 μM). **C, D.** Open percentage dose-response curves from patches exposed to pinacidil (**C**, n = 2) or cromakalim (**D**, n = 2).

Delayed rectifier and calcium-activated potassium currents were separated from one another pharmacologically and by controlling the intracellular calcium concentration. The delayed rectifier current, recorded in a bath containing no added calcium and with a pipette containing EDTA and no added calcium, was typically a few hundred pA in maximum amplitude. Procaine and strychnine were effective blockers of this current. With 1.8 mM calcium in the bath and 90 μM EGTA + 100 μM calcium in the pipette, an outward current of up to several nA could be elicited. This current was insensitive to procaine and strychnine, but was blocked by TEA in the bath. All recordings obtained with cell-free inside-out membrane patches contained multiple potassium channels with a conductance of about 220 pS in symmetrical potassium. These typical maxi-K channels probably underlie most of the calcium-activated potassium conductance detected in the whole-cell recordings.

The low concentration of procaine we used (1 mM) selectively blocked the delayed rectifier current without affecting the calcium-dependent current or the agonist effects of cromakalim and pinacidil. At higher concentration, procaine has been shown to affect relaxation produced by cromakalim and pinacidil (19, 25), but this may be the result of procaine block of calcium-activated potassium current at the higher concentration (data not shown).

Neither the pipette solution used in whole-cell recordings nor the bath solution used in recordings from inside-out membrane patches contained nucleotide triphosphates. The time-and voltage-independent current obtained under these conditions was small, from which we conclude that these cells possess little or no classic ATP-dependent potassium conductance.

Recordings from cell-free membrane patches confirmed that the effects of these drugs on the calcium-activated potassium current were direct and did not require changes in the intracellular calcium concentration. These studies also established that the affected channel type was the large conductance calcium-activated potassium channel. In symmetrical potassium, calcium-activated potassium channels were identified by their large conductance, sensitivity to membrane potential and sensitivity to internal calcium concentration (13, 18, 22). In all such recordings, there were more than one channel per patch. Consequently, without knowing how many channels, these open percentages could not be converted to single channel open probability. However, since each patch was used as its own control, we were still able to show that the drugs increased the channel open probability. Pinacidil and cromakalim showed consistent dose-related increases of channel open percentages.

The results from this study of whole cell recording and single channel recordings support the hypothesis that large conductance calcium-activated potassium channels were activated by pinacidil and cromakalim in rat basilar artery.

The relatively high concentrations used in this study were comparable to those used in other patch-clamping studies on cardiac myocytes (5, 9, 16). They are, however, generally higher than concentrations employed with other vascular smooth muscle. As shown previously (26), this does not necessarily mean that the drugs are less effective at relaxing cerebral vessels.

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REFERENCES

1. Beech, D.J. and Bolton, T.B. (1989) *Br. J. Pharmacol.* **98:** 851-864.
2. Beech, D.J. and Bolton, T.B. (1989) *J. Physiol. Lond.* **412:** 397-414.

3. Cook, N.S. and Hof, R.P. (1988) Br. J. Pharmacol. **93:** 121-131.
4. Economos, D., Peyrow, M., Escande, D. and Bkaily, G. (1990) *Biophys. J.* **57:** 508a.
5. Escande, D., Thuringer, D., Leguern, S. and Cavero, I. (1988) *Biochem. Biophys. Res. Commun.* **154:** 620-625.
6. Gelband, C.H., Lodge, N.J. and van Breemen, C. (1989) *Eur. J. Pharmacol.* **167:** 201-210.
7. Hamill, O.P. Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) *Pflüg. Arch.* **391:** 85-100.
8. Harder, D.R., Dernbach, P. and Waters, A. (1987) *J. Clin. Invest.* **80:** 875-880.
9. Hiraoka, M. and Fan, Z. (1990) *J. Pharmacol. Exp. Ther.* **250:** 278-285.
10. Hof, R.P., Quast, U., Cook, N.S. and Blarer, S. (1988) *Circ. Res.* **62:** 679-686.
11. Hu, S.L., Kim, H.S. Okolie, P. and Weiss, G.B. (1990) *J. Pharmacol. Exp. Ther.* **253:** 771-777.
12. Iijima, T. and Taira, N. (1987) *Eur. J. Pharmacol.* **14:** 139-141.
13. Inoue, R., Kitamura, K. and Kuriyama, H. (1985) *Pflüg. Arch.* **405:** 173-179.
14. Klöckner, U. Trieschmann, U. and Isenberg, G. (1989) *Drug Res.* **39:** 120-126.
15. Kusano, K. Barros, F., Katz, G., Garcia, M., Kaczorowski, G. and Reuben, J.P. (1987) *Biophys. J.* **51:** 55a.
16. Osterrieder, W. (1988) *Naunyn-Schmied. Arch. Pharmacol.* **37:** 93-97.
17. Sanguinetti, M.C., Scott, A.L., Zingaro, G.J. and Siegl, P.K.S. (1988) *Proc. Natl. Acad. Sci. USA* **85:** 8360-8364.
18. Singer, J.J. and Walsh, J.V., Jr. (1987) *Pflüg. Arch.* **408:** 98-111.
19. Southerton, J.S. and Weston, A.H. (1987) *J. Physiol.* **391:** 77P.
20. Standen, N.B., Quayle, J.M., Davies, N.W., Brayden, J.E., Huang, Y. and Nelson, M.T. (1989) *Science* **245:** 177-180.
21. Steele, J., Stockbridge, N., Maljkovic, G. and Weir, B. (1991) *Circ. Res.* **68:** 416-423.
22. Stuenkel, E.L. (1989) *Am. J. Physiol.* **257:** H760-H769.
23. Toda, N., Nakajima, S., Miyazaki, M. and Ueda. M. (1985) *J. Cardiovasc. Pharmacol.* **7:** 1118-1126.
24. Trieschmann, U., Pichlmaier, M., Klöckner, U. and Isenberg, G. (1988) *Pflüg. Arch.* **411:** R199.
25. Wilson, C. (1987) *Br. J. Pharmacol.* **91:** 401P.
26. Zhang, H., Stockbridge, N., Weir, B., Vollrath, B. and Cook, D. *Gen. Pharmacol.* (in press).