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BOMBESIN ACTIVATES LARGE-CONDUCTANCE CHLORIDE CHANNELS IN SWISS 3T3 FIBROBLASTS

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Using the patch-clamp technique (cell-attached patches), we found that bombesin, a Ca-mobilizing peptide mitogen, activates large-conductance Cl channels in Swiss 3T3 fibroblasts. The channel activation required a lag period of about 50 s and was equally observed whether bombesin was applied to the patch-pipette or to the bath. A23187 (10^{-6} M) in the bath induced the similar currents with almost identical current-voltage relationship as bombesin: their slope conductances were 292 +/- 15 (bombesin) and 318 +/- 42 (A23187) pS. In inside-out patches, the induced channels were selective to Cl over gluconate (11:1). These observations strongly suggest that in Swiss 3T3 fibroblasts bombesin activates the Cl channels through a mechanism involving an increase in the intracellular free Ca concentration.

Bombesin and the related brain-gut peptides exert potent mitogenic effects on Swiss 3T3 fibroblasts, a well characterized model for studying cell growth and proliferation (1). We have shown that in these cells bombesin acts as a typical Ca mobilizing hormone, i.e. it stimulates phospholipase C and induces intracellular Ca mobilization via the second messenger inositol-1,4,5-trisphosphate (2). It is quite possible that some of the effects of bombesin is mediated through an increase in the intracellular free Ca concentration. In fact, a calcium ionophore A23187 mimics a number of effects of bombesin, including stimulation of monovalent ion fluxes across the plasma membrane (3). In the present study, we explored a possibility whether bombesin regulates ion channels in Swiss 3T3 cells. Using the patch-clamp technique (cell-attached patches) we have found that bombesin activates large conductance Cl channels. This activation is suggested to be mediated through mobilization of Ca in the cells.

METHODS

Swiss-mouse 3T3 fibroblasts were maintained in a subconfluent state in Dulbecco's modified Eagle's medium (DMEM)

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containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2-95% air at 37 °C. Cells were seeded at 2 x 10^4/ml in 35 mm Corning culture dishes 2 days before experiments, and made quiescent in serum-free media overnight as described (2). Cells were briefly exposed to 0.1% trypsin (Sigma) and were restored in DMEM containing 0.2% bovine serum albumin (Sigma) for 2-6 hours until use. Experiments were performed at 32-34 °C and at room temperature (24-26 °C). The cell-attached, inside-out and outside-out configurations of the patch-clamp technique (4) were used for single-channel recordings. The pipette solution contained (in mM): 145 KCl (or NaCl), 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES. (pH was adjusted to 7.4 by either KOH or NaOH). The bath solution contained (in mM): 140 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, pH 7.4 by NaOH. When Cl was replaced with gluconate, off-set potential produced by Cl asymmetry between pipette and bath was canceled by a patch-clamp amplifier (Nihon-Kohden CEZ2200, Tokyo). Liquid junction potentials were corrected as previously described (5). When bombesin was applied to the bath solution, a beveled perfusion pipette was used.

Patch-pipettes were made from hematocrit tubes (Terumo, Tokyo) that had been pulled and coated by Sylgard 184 according to the standard methods (4). Seal resistances were typically 10-50 GOhm. Single-channel currents were recorded on VCR tape following pulse code modulator (Sony PCM 501 ES). Analysis of the records was performed with the aid of a computer (NEC PC9801 VM2). Data were low pass filtered at 1 KHz and sampled at 2 or 5 KHz into computer memory with a A/D converter (Canopus Analog-ProII, Kobe, Japan). Permeability ratio for Cl over gluconate ($P_{Cl}/P_{gluconate}$) was calculated from the following equations (6):

$$E_{rev1}=(RT/F)\ln((P_{Cl}[Cl]_b+P_{gluconate}[gluconate]_b)/P_{Cl}[Cl]_p) \quad (1)$$

$$E_{rev2}=(RT/F)\ln(P_{Cl}[Cl]_b/(P_{Cl}[Cl]_p+P_{gluconate}[gluconate]_p)) \quad (2)$$

where E_{rev1} and E_{rev2} are the zero current potentials, subscript p refers to pipette and b to bath solutions. R,T, and F have their usual meanings. Values are expressed as means +/- S.E.

RESULTS

Large-conductance outward currents were induced by mitogenic concentrations of bombesin (10^{-10} to 10^{-8} M)(1) in the cell-attached condition. Bombesin (10^{-8} M) in the patch-pipette transiently activated previously quiescent channels after a lag period of 40.8 +/- 16.9 s (mean +/- S.E., n=5)(Fig. 1a). As shown in the lower trace of Fig. 1a (in an expanded time scale), the large-conductance single channel currents were often preceded by and admixed with various sizes of smaller channel currents. These smaller channel currents were always accompanied by the large-conductance channels, and were much less frequently observed after activation of the large-conductance channels. Thus, it is most likely that the smaller currents represent subconductance states, i.e. substates of a large-conductance single channel between fully open and fully closed states (7,8). Figure 1b shows that application of bombesin (5 x 10^{-9} M) to the bath also

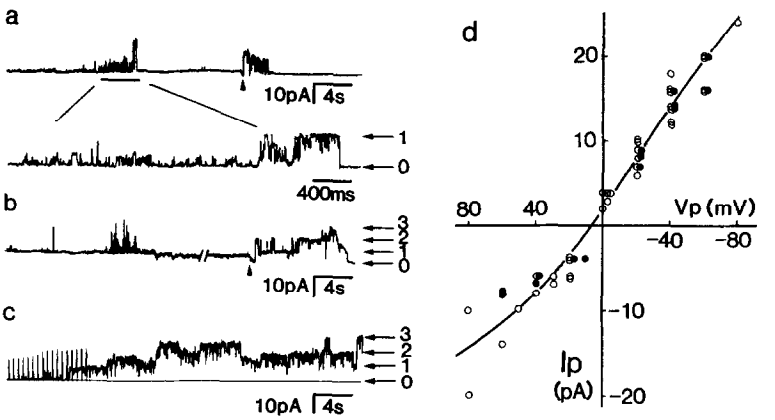

Figure 1. Activation of single channels by bombesin and A23187 in cell-attached patches. Single channel openings are seen as upward steps. Numbers of open channels in the cell-attached patches are indicated at right. a: bombesin (10^{-8}) in the pipette (P) induced transient outward currents at a time of 60 s (Vp=-40 mV). The channel reopened in response to a change in Vp (from -40 to -20 mV, arrow head). Note that smaller currents of various sizes (subconductance states) are also observed, especially before the maximal open-state (lower trace). b: Bombesin (5 x 10^{-9} M) in the bath (B) activated transient burst-opening at a time of 30 s (Vp=-60 mV). The channel reopened in response to a change in Vp (from -40 to -26 mV, arrow head). Break in the middle was 16 s. c: Activation of the outward currents by A23187 (1 μM) at a time of 44 s. 100 ms pulses were given from a holding potential of -20 mV to -40 mV in the beginning of this trace. d: Current/voltage relationships of the channels activated by bombesin (open circles) or A23187 (filled circles).

induced similar outward currents with a similar time course: the lag period was 48 +/- 18 sec (n=4), which is comparable to that observed when bombesin was applied in the patch-pipette. In both conditions, the bombesin-induced channels showed a voltage-dependent activation (arrow heads in Figs. 1a and 1b). Totally, activation of the channel currents by bombesin either in the pipette or in the bath was observed in 9 of 21 cell-attached patches (43 %) at 32-34 °C and 4 of 37 (11 %) at 24-26 °C, indicating that the effect of bombesin on the channel activation is temperature-dependent. However, the mean number of single-channels per patch-membrane was not different between the two conditions (2 +/- 0.3 at 32-34 °C and 3 +/- 1.4 at 24-26 °C). It should be emphasized that the large outward currents were never observed in the cell-attached patch clamp condition in the absence of bombesin (n>100), unless extra high-voltages (more negative than -100 mV) were continuously applied to the patch-membrane.

Since bombesin causes Ca mobilization in Swiss 3T3 cells (2), it is conceivable that an increase in the intracellular free

Ca concentration may be involved in bombesin-induced channel activation. To test this possibility, we examined whether a Ca ionophore A23187 activates the same channel. After the formation of giga-seal A23187 (1 uM) was applied to the bath in the cell-attached condition (Fig. 1c). A23187 induced similar outward currents as induced by bombesin after a lag period of 56 +/- 6 s (n=3). Moreover, single-channel currents induced by bombesin and A23187 displayed superimposable I-V curve that intercepted the X-axis at Vp=8 mV (Fig. 1d): their slope conductances between Vp=0 and -60 mV were 292 +/- 15 pS (bombesin, n=12) and 318 +/- 42 pS (A23187, n=3). These results strongly suggest that the currents induced by bombesin and A23187 are conducted through the same channel, and support the view that activation of the channels by bombesin is mediated through an increase in the intracellular free Ca concentration.

Electrophysiological properties of the large-conductance channels were studied in detail in cell-free, excised membrane patches. It is notable that these channels were spontaneously activated after membrane-excision and that their electrophysiological properties were the same whether cells were pretreated with bombesin or not (see DISCUSSION). Figure 2a shows single channel currents recorded in a symmetrical Cl concentration of 151 mM after jumps to a number of membrane potentials from 0 mV. Single channel currents were outward

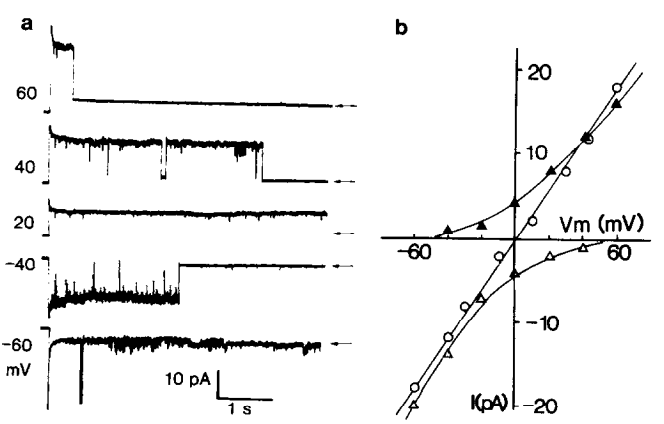

Figure 2. Channel activity in inside-out patches. a: Voltage-dependent closing after stepping from 0 to -60, -40, +20, +40, and +60 mV. Arrows indicate zero current level. b: I-V relationships of the currents obtained when the major anions in the patch-pipette/bath are Cl/Cl (open circles), gluconate/Cl (open triangles), and Cl/gluconate (filled triangles). Each symbol represents mean values of 5-16 patches. The lines were drawn by eye.

(upward) for positive potentials and inward (downward) for negative potentials. The channels were open immediately after the voltage steps in each trace and then closed. The voltage-dependent inactivation occurred more quickly at more polarized potentials. The channel activity was restored when the membrane potential was returned to zero mV. Interestingly, in the cell-free, inside-out patch condition the channels once activated stayed active in the absence of Ca with 2 mM EGTA on a cytoplasmic side of the patch-membrane. By plotting single-channel current amplitude against membrane potentials (Fig. 2b, open circles), conductance of the channels in excised membrane patches was determined to be 312 +/- 7 pS (n=16). This value is comparable to those of the single channel currents induced by either bombesin or A23187 in the cell-attached condition. The reversal potential was very close to 0 mV, as would be expected for symmetrical solutions (151 mM Cl on both sides of patch-membrane). The single channel conductance and the reversal potential were barely affected when Na in the patch-pipette was replaced with K or choline. When Cl was unilaterally replaced with gluconate on either cytoplasmic or pipette side, the I-V curves displayed rectification (open and filled triangles in Fig. 2b). Extrapolated zero current potentials were E_{rev1}=-48 mV for filled triangles and E_{rev2}=+52 mV for open triangles. These results indicate that the channels are selective for Cl over cations (Na, K, and choline) and gluconate. The calculated permeability ratios ($P_{Cl}/P_{gluconate}$, see METHODS) were 11.2 (E_{rev1}=-48 mV) and 10.5 (E_{rev2}=+52 mV). Thus the channel activated by bombesin is a large conductance Cl channel with $P_{Cl}/P_{gluconate}$ of about 11.

DISCUSSION

The present study demonstrates for the first time that bombesin, a Ca-mobilizing peptide growth factor, induces large-conductance Cl channels in Swiss 3T3 fibroblasts. In the cell-attached condition the channels were equally activated whether bombesin was applied to the patch-pipette (Fig. 1a) or to the bath (Fig. 1b), indicating that bombesin-induced channel activation is mediated through diffusible intracellular messenger molecules. This effect of bombesin was readily mimicked by a Ca ionophore A23187, i.e. A23187 activated the channels with an identical I-V relationship in a similar time course (Figs. 1c,d). These observations are consistent with the notion that

bombesin activates the large-conductance Cl channels through a mechanism involving an increase in the intracellular free Ca concentration. It is not known at present, however, whether Ca acts directly on the channel molecules or indirectly through some other mechanisms, which may involve Ca-dependent protein phosphorylation (9). The lag period of several ten seconds before channel activation by either bombesin or A23187 may favor the latter mechanism, since bombesin-induced production of inositol-1,4,5-trisphosphate and the resulting Ca mobilization are maximal within 10 s (2) and an increase in the intracellular Ca by A23187 is probably completed within several seconds.

The large conductance Cl channels have been described in such diverse cell types as cultured kidney cells (7), frog toe muscle (8), rat (10) and chicken (11) myotubes, rat Schwann cells (12), rat histamine secreting cells (13), and human fibroblasts (14). However, activation of the Cl channels has scarcely been reported under physiologically relevant conditions. These channels show several characteristics in common: large single channel conductance (300-460 pS) with various levels of subconductance states, a bell-shaped voltage dependence, activation under high voltages or cell-free condition, and Ca-independent channel activation after membrane-excision. The channels activated by bombesin in the present study demonstrate all of these characteristics. The paradoxical Ca-independent channel activation in cell-free, but not in intact cell preparations, may suggest the presence of a regulatory component in the cytosolic fraction, and further supports the view that Ca does not directly regulate the channel activation.

During the final stage of the preparation of this manuscript, Schwiebert et al. (15) have reported that large-conductance Cl channels in rabbit kidney cortical collecting duct cells in culture are regulated by a pertussis toxin-sensitive GTP binding protein in excised membrane patches. It is possible that there exist subtypes of the large-conductance Cl channels with distinct regulatory mechanisms.

Physiological significance of the present observation remains elusive. However, the present study, together with a recent observation that platelet-derived growth factor activates nonselective cation channels in mouse fibroblasts (L cell line)(16), raises a possibility that growth factors may exert specific modulatory effects on some type of ion channels, thereby regulating transmembrane ion traffics that are crucial for the

cell cycle traverse to DNA synthesis and cell division. Further studies are required to evaluate this possibility.

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REFERENCES

1. Rozengurt, E., and Sinnett-Smith, J. (1983) Proc. Natl. Acad. Sci. U.S.A. 80: 2936-2940.
2. Takuwa, N., Takuwa, Y., Bollag, W.E., Rasmussen, H. (1987) J. Biol. Chem. 262: 182-188.
3. Rozengurt, E. (1986) Science 234: 161-166.
4. Hammill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. (1981) Pfluegers Arch. 391: 85-100.
5. Kawahara, K., Hunter, M., and Giebisch, G.(1987) Am. J. Physiol. 253: F488-F494.
6. Hille, B. (1984) Ionic Channels of Excitable Membranes. p.230. Sinauer, Sunderland, Massachusetts.
7. Kolb, H.A., Brown, C.D.A., and Murer, H. (1985) Pfluegers Arch. 403: 262-265.
8. Woll, K.H., Leibowitz, M.D., Neumcke, B., and Hille, B. (1987) Pfluegers Arch. 410: 632-640.
9. Levitan, I.B. (1985) J. Membr. Biol. 87: 177-190.
10. Blatz, A.L., and Magleby, K.L. (1983) Biophys. J. 43: 237-241.
11. Schwarze, W., and Kolb, H.-A. (1984) Pfluegers Arch. 402: 281-291.
12. Gray, P.T.A., Bevan, S., Ritchie, J.M.R. (1984) Proc. R. Soc. Lond. B. 221:395-409.
13. Lindau, M., and Fernandez, J.M. (1986) J.Gen.Physiol. 88: 349-368.
14. Nobile, M., Galietta, L.J.V. (1988) Biochem. Biophys. Res. Com. 154: 719-726.
15. Schwiebert, E.M., Light, D.B., Fejes-Toth, G., Naray-Fejes-Toth, A., Stanton, B.A. J. Biol. Chem. (1990) 265: 7725-7728.
16. Frace, A.M., and Gargus, J.J. (1989) Proc.Natl.Acad.Sci. U.S.A. 86: 2511-2515.