Properties of the Apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) Channel in PC12 Pheochromocytoma Cells Which Hyper-produce the Apamin Receptor

(Received for publication, February 18, 1986)

Heidy Schmid-Antomarchi, Michel Hugues, and Michel Lazdunski

From the Centre de Biochimie du Centre National de la Recherche Scientifique, Parc Valrose, 06034 Nice Cedex, France

Undifferentiated PC12 cell produce high levels of apamin receptors (measured with \(^{125}\text{I}\)-apamin) after 7 days in culture. These levels are at least 50 times higher than those found in other cellular types which are also known to have apamin receptors and apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) channels in their membranes. Treatment of undifferentiated PC12 cells with nerve growth factor maintains these cells in a state having a low level (10 times less after 7 days of culture) of apamin receptors. Ca\(^{2+}\) injection into PC12 cells with the calcium ionophore A23187 has been used to monitor the activity of the Ca\(^{2+}\)-activated K\(^+\) channel following \(^{86}\text{Rb}\) efflux. A large component of this Ca\(^{2+}\)-activated \(^{86}\text{Rb}\) efflux is inhibited by apamin. Half-maximum inhibition by apamin of both \(^{86}\text{Rb}\) efflux and \(^{125}\text{I}\)-apamin binding was observed at 240 pM apamin. Another component of \(^{86}\text{Rb}\) efflux is due to another type of Ca\(^{2+}\)-activated K\(^+\) channel which is resistant to apamin and sensitive to tetrodylammonium.

The Ca\(^{2+}\) channel activator Bay K8644 also triggers an apamin-sensitive Ca\(^{2+}\)-dependent \(^{86}\text{Rb}\) efflux. Bay K8644 has been used to analyze the internal Ca\(^{2+}\) concentration dependence of the apamin-sensitive channel activity. Under normal conditions, the internal Ca\(^{2+}\) concentration is 109 ± 17 nM, and the apamin-sensitive channel is not activated. The channel is fully activated at an internal Ca\(^{2+}\) concentration of 320 ± 20 nM.

The concentration of potassium ions in cells relative to the extracellular space is normally maintained by K\(^+\) entry systems which include the Na\(^+\)/K\(^+\)-ATPase and the Na\(^+\)/K\(^+\)/Cl\(^-\) co-transport and by K\(^+\) efflux systems including K\(^+\) channels. Among these channels, those whose activity is regulated by intracellular calcium ions (1–4) are particularly important because they provide a link between metabolism of cytoplasmic calcium and membrane potential (2, 3, 5). The presence of Ca\(^{2+}\)-activated K\(^+\) channels has already been demonstrated in a large variety of tissues (6) and individual cells may contain more than one type of Ca\(^{2+}\)-activated K\(^+\) channels (3, 5).

Apamin, a bee venom polypeptide toxin of 18 amino acids (7) blocks one class of these Ca\(^{2+}\)-activated K\(^+\) channels in neuroblastoma cells (8), smooth muscle (9–11), skeletal muscle (12), and hepatocytes (10, 13, 14). \(^{125}\text{I}\)-Apamin was used as a biochemical marker of the apamin-sensitive Ca\(^{2+}\)-de-
concentrations were measured according to Hartree (23) using bovine serum albumin (BSA) as a standard. Homogenates of PC12 cells were prepared at various times of culture from 3 to 8 days with a Potter homogenizer (900 rpm, five strokes). The incubation medium for binding experiments consisted of a 20 mM Tris-Cl buffer at pH 7.5 containing 0.5 mg/ml bovine serum albumin and 5.4 mM KCl.

Cellulose acetate filters (Millipore EGWP, 0.2-μm pore size) used in binding experiments were incubated in 10 mM Tris-Cl, pH 7.5, and 0.1% BSA. Radioactivity bound to filters was measured with an Intertechnique CG 4000 γ counter.

In equilibrium binding experiments, homogenates of PC12 cells (0.5 mg/ml) were incubated with increasing concentrations of 125I-apamin for 60 min at 0 °C. Duplicate aliquots (0.4 ml) were then filtered and the bound radioactivity was measured as described above. Nonspecific binding was determined in parallel experiments in the presence of an excess of unlabeled apamin (1 μM).

In competition experiments between 125I-apamin and unlabeled apamin, PC12 cell homogenates (0.5 mg/ml) were incubated for 60 min at 0 °C with a fixed concentration of 125I-apamin (40 pm) and increasing concentrations of unlabeled apamin. The amount of labeled apamin that remained bound to PC12 cell homogenates in the presence of unlabeled apamin was estimated as described above.

**86Rb** Efflux Measurements—All efflux experiments were carried out at 37 °C in 24-well tissue culture plates. Cells were labeled with 86Rb (1 μCi/ml) 24 h before the beginning of efflux experiments. Fifteen min before the efflux experiments, the calcium ionophore A23187 (10 μM), ouabain (100 μM) to inhibit the (Na+,K+)ATPase, and the different drugs acting either on Ca2+-activated K+ channels or on Ca2+ channels were added to the medium at the desired concentration. After 15 min, the medium was aspirated and 86Rb efflux was initiated by incubating the cells in a medium containing 2 mM CaCl2, 25 mM Hepes/Tris at pH 7.4, 135 mM NaCl, 5 mM KCl, 0.8 mM MgSO4, 5 mM glucose, 0.1 mg/ml BSA, 0.1 mM ouabain, 10 μM of the calcium ionophore A23187 (efflux medium), and the different drugs active on Ca2+ and Ca2+-activated K+ channels at the desired concentrations. After various times, the cells were washed three times with 2 ml of washing medium (100 mM MgCl2) (the washing procedure took less than 10 s). After the third wash, 2 ml of 0.1 n NaOH were added to each well and the radioactivity remaining in the cell was counted in a Kontron MR300 counter. Initial rates of 86Rb efflux were routinely measured after 30 s. [3H]Leucine was used to monitor the number of cells in each well and the efflux data were corrected as function of the [3H]leucine (0.2 CI/ml) counts.

Quin 2/AM Measurements of Intracellular Calcium Concentration—The Quin 2/AM loading procedure was similar to that already described by Pozzan et al. (24) for PC12 cells. Briefly, PC12 slides were incubated at 37 °C during 60 min in the efflux medium containing Quin 2/AM (1 μM) (control experiments), with or without Bay K8644 (30 nM). Bay K8644 was added to the incubation medium 15 min before the beginning of measurements. After 1 h, PC12 slides were washed using the same medium and were directly used for measurements of cellular Ca2+ concentrations as previously described (24, 25). The slides were placed in a thermostated cell at 37 °C containing the efflux medium and experiments were performed with a Perkin-Elmer LS5 luminescence spectrometer using excitation and emission wavelengths of 339 ± 2.5 and 492 ± 10 nm, respectively.

**RESULTS**

**Binding Experiments on PC12 Cells Using 125I-Apamin**—Fig. 1 (main panel) shows the results of equilibrium binding experiments in which increasing concentrations of 125I-apamin were added to a fixed amount of a homogenate (0.5 mg/ml) coming from PC12 cells at 7 days of culture. The nonspecific binding component is small as compared to the specific one. The linearity of the corresponding Scatchard plot (Fig. 1, inset) demonstrates that 125I-apamin binds to a single class of noninteracting sites. Linear Scatchard plots have also been obtained at all times of culture and after NGF-induced differentiation. Fig. 2 indicates values of the dissociation constant Kd and of the maximum binding capacity Bmax at different times in culture. Bmax was maximum at 7 days of culture when it reached a value of 600 fmol/mg of protein. At 8 days of culture, the Bmax value decreased to 150 fmol/mg of protein.

The dissociation constant, Kd, remained constant around a mean value of 350 pm at all stages of culture. Exposure of PC12 cells to NGF prevented the increase in number of 125I-apamin receptor sites (Fig. 2).

**86Rb** Efflux Experiments—The inset of Fig. 3 shows the time course of 86Rb efflux in control PC12 cells (i) in the presence of the Ca2+ ionophore A23187 which permits the injection of Ca2+ into cells and triggers Ca2+-dependent K+ efflux and (ii) in the presence of both A23187 and apamin. Apamin clearly inhibits one large component of the Ca2+-dependent K+ efflux.

The dose-response curve for apamin inhibition of 86Rb efflux triggered by Ca2+ injection with A23187 is presented in the main panel of Fig. 3. The concentration of apamin required
Apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) Channel in PC12 Cells

FIG. 3. Effect of apamin on Ca\(^{2+}\)-activated \(^{86}\)Rb\(^+\) efflux and on \(^{125}\)I-apamin binding. Inset, time course of \(^{86}\)Rb\(^+\) efflux in cells treated with the Ca\(^{2+}\) ionophore A23187. PC12 cells were cultured and preincubated as described under “Experimental Procedures.” \(^{86}\)Rb\(^+\) efflux was measured from the amount of \(^{86}\)Rb\(^+\) remaining in the cells. Control in normal efflux medium without A23187, □; in efflux medium supplemented with A23187, C; and in efflux medium with A23187 and apamin (100 nM), Δ.

Main panel, ○, competition between \(^{125}\)I-apamin and unlabeled apamin to PC12 cells homogenate; Δ, inhibition of apamin-sensitive \(^{86}\)Rb\(^+\) efflux by increasing concentrations of apamin. This experiment was performed in efflux medium containing 20 mM TEA and increasing concentrations of apamin.

FIG. 5. Effect of Bay K8644 on \(^{86}\)Rb\(^+\) efflux and internal Ca\(^{2+}\) concentration dependence of apamin-sensitive Rb\(^+\) efflux. Inset: \(^{86}\)Rb\(^+\) efflux induced by A23187 (10 μM) in the presence of TEA (20 mM) used to inhibit TEA-sensitive Ca\(^{2+}\)-activated K\(^+\) channels; 2, \(^{86}\)Rb\(^+\) efflux induced by Bay K8644 (30 nM) in the presence of TEA (20 mM); 3, same as 2, but in the presence of nitrendipine (100 nM); 4, \(^{86}\)Rb\(^+\) efflux induced by Bay K8644 (or A23187) in the presence of both TEA (20 mM) and apamin (100 nM). Main panel, dependence of the apamin-sensitive \(^{86}\)Rb\(^+\) efflux induced by Bay K8644, in the presence of TEA (20 mM), as a function of the intracellular calcium concentration measured using Quin 2/AM.

FIG. 4. Effect of inhibitors of Ca\(^{2+}\)-activated K\(^+\) channels on Ca\(^{2+}\)-dependent \(^{86}\)Rb\(^+\) efflux from PC12 cell. 1, maximum \(^{86}\)Rb\(^+\) efflux measured in efflux medium containing A23187; 2, per cent of \(^{86}\)Rb\(^+\) efflux inhibited by apamin (100 nM); 3, per cent of \(^{86}\)Rb\(^+\) efflux inhibited by TEA (20 mM); 4, per cent of \(^{86}\)Rb\(^+\) efflux inhibited by a mixture of TEA (20 mM) and apamin (100 nM). A concentration of 10 mM apamin gave the same results as 100 nM apamin (not shown).

Different intracellular concentrations of Ca\(^{2+}\) were obtained by using a fixed concentration of Bay K8644 (30 nM) and variable external Ca\(^{2+}\) concentrations between 1 and 16 mM. Increasing external Ca\(^{2+}\) concentrations increased internal Ca\(^{2+}\) concentrations and, in turn, \(^{86}\)Rb\(^+\) efflux through apamin-sensitive Ca\(^{2+}\)-activated channels. The relationship between the activity of the apamin-sensitive channel measured by \(^{86}\)Rb\(^+\) efflux and the internal Ca\(^{2+}\) concentration is presented in the main panel of Fig. 5. The apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) channel is not active at 109 mM internal Ca\(^{2+}\) and fully active at 320 mM internal Ca\(^{2+}\).

DISCUSSION

Properties of PC12 cells are similar to those of adrenal chromaffin cells (30). However, as with chromaffin tissue under appropriate culture conditions, PC12 cells can be differentiated with nerve growth factor to become sympathetic neuroleike cells (20, 31). \(^{125}\)I-Apamin binding experiments have shown that PC12 cells possess a receptor for the neurotoxin apamin. The presence of such a receptor is usually associated with the presence of apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) channels (4). The originality of PC12 cells among

to produce half-maximal inhibition (K\(_{D50}\)) of \(^{86}\)Rb\(^+\) efflux was 240 pM.

This dose-response curve was compared in the same figure to that obtained for apamin inhibition of \(^{125}\)I-apamin binding.

The two curves are nearly superimposable.

It is clear from Fig. 4 that apamin only inhibits one of the components of Ca\(^{2+}\)-activated K\(^+\) channels, another component is inhibited by tetraethylammonium (TEA) as it has been previously observed in other systems (1-6).

Ca\(^{2+}\) Concentrations At Which the Apamin-sensitive Ca\(^{2+}\)-

activated K\(^+\) Channel Is Triggered—Bay K8644 is an activator of dihydropyridine-sensitive Ca\(^{2+}\) channels (26, 27). It activates voltage-sensitive Ca\(^{2+}\) channels in PC12 cells (28, 29) and Ca\(^{2+}\) flux studies carried out in this laboratory have indicated a maximal effect for the drug at a concentration of 30 nM (not shown). The internal Ca\(^{2+}\) concentration in the absence of Bay K8644 was found to be 109 ± 17 nM. Addition of 30 nM Bay K8644 in the presence of an external Ca\(^{2+}\) concentration of 2 mM raised the internal level of Ca\(^{2+}\) to 210 ± 40 nM. Because of its effect on [Ca\(^{2+}\)]. Bay K8644 induced a component of \(^{86}\)Rb\(^+\) efflux which was inhibited by nitrendipine (100 nM), a well known Ca\(^{2+}\) channel inhibitor (Fig. 5, inset).

Different intracellular concentrations of Ca\(^{2+}\) were obtained by using a fixed concentration of Bay K8644 (30 nM) and variable external Ca\(^{2+}\) concentrations between 1 and 16 mM. Increasing external Ca\(^{2+}\) concentrations increased internal Ca\(^{2+}\) concentrations and, in turn, \(^{86}\)Rb\(^+\) efflux through apamin-sensitive Ca\(^{2+}\)-activated channels. The relationship between the activity of the apamin-sensitive channel measured by \(^{86}\)Rb\(^+\) efflux and the internal Ca\(^{2+}\) concentration is presented in the main panel of Fig. 5. The apamin-sensitive Ca\(^{2+}\)-activated K\(^+\) channel is not active at 109 nM internal Ca\(^{2+}\) and fully active at 320 nM internal Ca\(^{2+}\).
Apamin-sensitive Ca$^{2+}$-activated K$^+$ Channel in PC12 Cells

### Table I

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Animals</th>
<th>$B_{\text{max}}$</th>
<th>$K_D$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblasts (N1E 115)</td>
<td>Mouse</td>
<td>12</td>
<td>22$^a$</td>
<td>17</td>
</tr>
<tr>
<td>Skeletal muscle (primary culture)</td>
<td>Rat</td>
<td>3.5</td>
<td>60$^b$</td>
<td>12</td>
</tr>
<tr>
<td>Hepatocyte (primary culture)</td>
<td>Guinea pig</td>
<td>0.99</td>
<td>350$^a$</td>
<td>14</td>
</tr>
<tr>
<td>Neuron (primary culture)</td>
<td>Rat</td>
<td>7-8</td>
<td>98$^c$</td>
<td>18</td>
</tr>
<tr>
<td>Pheochromocytoma cell (after 7 days of culture)</td>
<td>Rat</td>
<td>800</td>
<td>350$^a$</td>
<td>This work</td>
</tr>
<tr>
<td>NGF untreated cell</td>
<td></td>
<td>60</td>
<td>350$^a$</td>
<td>This work</td>
</tr>
<tr>
<td>NGF-treated cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Binding medium was: 20 mM Tris, 5.4 mM KCl, and 0.5 mg/ml BSA adjusted to pH 7.5.
$^b$ Binding medium was: Eagle's minimal essential medium (Wellcome) supplemented with 2% BSA and 10% newborn calf serum.
$^c$ Binding medium was: 25 mM Hepes, 10 mM glucose, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, and 0.25 mg/ml BSA adjusted to pH 7.2.

other cell types containing an apamin receptor is that they overproduce the apamin receptor. Table I shows that levels of apamin receptor are at least 50 times higher in undifferentiated PC12 cells than in other cells.

Treatment with NGF drastically affects the level of apamin receptors in PC12 cells. NGF-treated cells have about 10 times less apamin receptor after 7 days in culture than untreated cells (Fig. 2). Therefore, NGF, which ultimately transforms undifferentiated PC12 cells into neurone-like cells, prevents the synthesis and/or the incorporation of apamin receptors into the cell membrane (Fig. 2). This situation bears some similarity with that encountered in mammalian skeletal muscle cells which express apamin receptors and apamin-sensitive Ca$^{2+}$-activated K$^+$ channel when they are noninnervated, i.e. not fully differentiated, and which cease to express these membrane entities at the final stage of differentiation when muscle fibers have undergone innervation (32). NGF seems to control in a very different way the expression of the different voltage-dependent ionic channels in PC12 cells. Whereas NGF prevents the incorporation of high densities of apamin-sensitive Ca$^{2+}$-activated K$^+$ channels, it provokes an increase by severalfold of the density of Na$^+$ channels (33). Very recent data suggest that functional dihydropyridine-sensitive Ca$^{2+}$ channels may be in lower amounts in the differentiated state of PC12 cells than before NGF treatment (28).

Because of their high amount of apamin receptors, undifferentiated PC12 cells have been found to be very useful in studying apamin-sensitive Ca$^{2+}$-activated K$^+$ channels using 86Rb$^+$ flux studies. The dose-response curve for the inhibition by apamin of the apamin-sensitive component of Ca$^{2+}$-dependent 86Rb$^+$ efflux is nearly identical with the dose-response curve for the inhibition by unlabeled apamin of the binding of 125I-apamin to its receptor site. There is a very close agreement between receptor binding studies and studies of the physiological properties of the apamin-sensitive Ca$^{2+}$-activated K$^+$ channel. The half-maximal effect of apamin on 125I-apamin binding and on 86Rb$^+$ efflux is observed at $K_{d50} = 240$ nM.

The apamin-sensitive Ca$^{2+}$-activated K$^+$ channel is not the only type of Ca$^{2+}$-activated K$^+$ channel present in undifferentiated PC12 cells. TEA-sensitive Ca$^{2+}$-activated K$^+$ channels are also present in these cells (Fig. 4), although they contribute less to Ca$^{2+}$-dependent 86Rb$^+$ efflux (Fig. 4) than apamin-sensitive K$^+$ channels. Electrophysiological techniques have revealed that TEA-sensitive Ca$^{2+}$-activated K$^+$ channels are present in many cellular types (2). In chromaffin cells, they have a large conductance of 180 picosiemens in symmetrical isotonic K$^+$ solution (34) when analyzed by the patch-clamp technique in the inside-out configuration. The whole cell patch-clamp technique has previously indicated the co-existence of apamin-sensitive Ca$^{2+}$-activated K$^+$ channels and of TEA-sensitive Ca$^{2+}$-activated K$^+$ channels in rat skeletal muscle cells in culture (5). In these muscle cells, the two pharmacologically distinct types of channels have different physiological roles.

In normal physiological conditions, the activity of Ca$^{2+}$-dependent K$^+$ channels is triggered by an increase of the internal Ca$^{2+}$ concentration due to the opening of Ca$^{2+}$ channels which are present in the plasma membrane and/or to the release of Ca$^{2+}$ from internal stores. It has been shown in this work that the voltage-dependent Ca$^{2+}$ channel activator Bay K8644 can be used instead of the Ca$^{2+}$ ionophore A23187 to activate an apamin-sensitive 86Rb$^+$ efflux. This activation is linked to a measurable increase of [Ca$^{2+}$], and is abolished by the Ca$^{2+}$ channel inhibitor nitrendipine. The internal Ca$^{2+}$ concentration can be modulated in the presence of Bay K8644 by changing the external Ca$^{2+}$ concentration. This procedure has offered a mean to determine for the first time the internal Ca$^{2+}$ concentration dependence of the apamin-sensitive Ca$^{2+}$-activated K$^+$ channel. This channel is essentially inactive, or has an activity which is too low to be detected, in normal concentrations of internal Ca$^{2+}$ in standard culture conditions, i.e. 109 ± 17 nM. Full activation of the apamin-sensitive K$^+$ channel is observed near 320 nM. The resting membrane potential under these conditions is near −55 ± 5 mV. These results indicate that the apamin-sensitive channel is activated at very low [Ca$^{2+}$], and that the response of the channel activity to variations of [Ca$^{2+}$] is very cooperative. Whereas very cooperative responses with respect to [Ca$^{2+}$], have also been previously observed for the TEA-sensitive Ca$^{2+}$-activated K$^+$ channel (35), this pharmacologically distinct type of channel responds to variations of internal Ca$^{2+}$ at much higher values of [Ca$^{2+}$]. For example, at a membrane potential of −50 mV in skeletal muscle cells, the TEA-sensitive channels are half-activated at [Ca$^{2+}$], higher than 100 μM (35). This different sensitivity to [Ca$^{2+}$], of apamin-sensitive and TEA-sensitive channels had already been suggested for skeletal muscle cells in culture (5).
Second, they will probably be very useful for patch-clamp analyses of the biophysical properties of this channel at the single channel level. Third, they will be useful in studying mechanisms by which the expression of the apamin-sensitive Ca"^2+-activated K^+ channel is controlled by NGF.

Acknowledgments—We are grateful to Dr. G. Franckowiak (Bayer A.G., FRG) for the generous gift of Bay K8644, and to Dr. I. Greene and Prof. P. Brachet for the PC12 cell line. We thank N. Boyer, M. T. Ravier, and C. Rouliais-Bettelheim for expert technical assistance and Dr. G. Romey for fruitful discussions.

REFERENCES